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Stem Cell Biology with Respect to Carcinogenesis Aspects of Radiological Protection

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CONTENTS

ABSTRACT

Stem Cell Biology with Respect to Carcinogenesis Aspects of Radiological Protection

ICRP Publication 1XX

Approved by the Commission in Month 201Y

Abstract- This report provides a review of stem cells/progenitor cells and their responses to ionising radiation in relation to issues relevant to stochastic effects of radiation which form a major part of the ICRP system of radiation protection. Current information on stem cell characteristics, maintenance and renewal, evolution with age, location in stem cell "niches", radiosensitivity to acute and protracted exposures, is presented in a series of substantial reviews as Annexes concerning haematopoietic tissue, mammary gland, thyroid, digestive tract, lung, skin and bone. This foundation of knowledge of stem cells is used in the main text of the report to provide a biological basis to issues such as the linear-no-threshold (LNT) model, cancer risk among tissues, dose-rate effects and changes in the risk of radiation carcinogenesis by age at exposure and attained age.

Knowledge of the biology and associated radiation biology of stem cells and progenitor cells is more developed in tissues which renew fairly rapidly, such as haematopoietic tissue, intestinal mucosa, and epidermis, although all the tissues considered here possess stem cell populations. Important features of stem cell maintenance, renewal and response are the microenvironmental signals operating in the niche residence which also has a well-defined spatial location identified in some tissues. The identity of the target cell for carcinogenesis continues to point to the more-primitive stem cell population which is mostly quiescent and hence able to accumulate the protracted sequence of mutations necessary to result in malignancy. There is some potential for daughter progenitor cells also to be target cells in particular cases, e.g. in haematopoietic tissue. Several biological processes could contribute in protecting stem cells from mutation accumulation: rapid induced death of injured stem cells, and retention of the DNA parental template strand during divisions in some tissue systems, so that mutations are passed to the daughter differentiating cells and not retained in the parental cell. Also, stem cell competition, whereby undamaged stem cells outcompete damaged stem cells for residence in the niche during long protracted irradiations.

The aforementioned processes may contribute to the carcinogenic radiation risk values among tissues, and may help explain why a rapidly-replicating tissue such as small intestine is free of such risk. They may also provide mechanistic understanding which relates to the dose and dose-rate effectiveness factor (DDREF) currently used in radiation protection guidelines. It is noteworthy that DNA repair operates mainly within a few days after irradiation while stem cell competition is a slow process requiring weeks and/or many months depending on the tissue type. These distinctions are likely to be of importance when considering the differences in the conclusions of human epidemiological studies of chronic radiation scenarios, where some organs and tissues demonstrate no risk up to and above a few

hundred mSv, while others demonstrate a risk at moderate doses comparable to those for acute radiation exposures.

Keywords: Stem cells; Normal tissues; Radiation sensitivity; Chronic radiation; Carcinogenesis; Radiation risk

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PREFACE

The present report was prepared by a task group of ICRP Committee 1, under the following terms of reference: (a) to review stem cell biology and radiobiology with reference to mechanisms of radiation carcinogenesis; (b) to compare the response of stem and associated cells in different tissues with the respective risks of cancer, and elucidate the likely roles of stem cells, progenitor cells, and the stem-cell niche; and (c) to use knowledge of stem-cell/progenitor-cell biology, radiation responses and carcinogenic risks from homogeneous acute exposures, in a discussion of the guiding principles for the projection of stochastic risks for short-range-radiation and chronic-irradiation scenarios.

In its recommendations and various reports, the ICRP has made some judgements and assumptions about the location and radiation response of the target cells responsible for carcinogenesis in various tissues. In most cases the target cells are considered to be the tissue stem cells, and in some cases also their daughter progenitor cells. The renewal and radiation response of these cells change with age and are governed by signals from their "niche" residence. The fundamental evidence for stem cells as target cells has been increasing in recent years. This evidence contributes to the understanding of the biological basis for carcinogensis, and helps to support modelling of human responses. It was considered that a report on this subject of target/stem cells would be topical and valuable in order to put all the target cell evidence for carcinogenic radiation risk in different tissues into a common framework and perspective for the first time.

In order to address stem cell knowledge with respect to particular issues of continuing importance to the ICRP, such as the linear-no-threshold (LNT) model, dose rate effects, location of target cells, tissue risk factors, and age dependent sensitivity to radiation, it was necessary to review evidence for different organ systems. This was undertaken by compiling a series of Annexes as separate reviews using a common template of topics, for each of a chosen series of seven organ systems with different characteristics. The Annexes (and authors) comprise: Annex A. Haematopoietic tissues: role played by stem cells and lineage-committed progenitors in radiation-induced leukaemia (T.M. Seed); Annex B. Mammary gland stem cells (M.H. Barcellos-Hoff); Annex C. Thyroid stem cells (K. Suzuki and S. Yamashita); Annex D. Digestive tract stem cells (J.H. Hendry); Annex E. Lung stem cells (J.W. Shay, M.D. Story, and P. Jacob); Annex F: Skin stem cells and radiation carcinogenesis (M.T. Martin); and Annex G: Bone stem cells (J.D. Harrison and R.K. Globus). Information on both humans and experimental animal systems was reviewed, and projections were made of the possible role of various stem cell processes in cancer risk.

The membership of the Task Group was as follows:

O. Niwa (Chair)	J.H. Hendry	M.T. Martin
M.H. Barcellos-Hoff	T.M. Seed	K. Suzuki

The corresponding members were:

J.D. Harrison	J.W. Shay	P. Jacob
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Main Commission critical reviewers were:

J.D. Boice Jr Jai-Ki Lee

Many helpful comments were received in particular from R. Cox and C. S. Potten (deceased 3 August 2012), Committee 1 members S. Bouffler, D. Laurier, A.J. Sigurdson, M. Tirmarche, R. Wakeford, W. Doerr, as well as C. Land, N. Nakamura, A. Noda, D. Preston, J. Preston, and R. Shore.

The membership of Committee 1 during the period of preparation of this report was:

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EXECUTIVE SUMMARY

(a) The Commission issued new recommendations for a System of Radiological Protection in *Publication 103* (ICRP, 2007). Stochastic effects of radiation, which are cancer and heritable effects, were also considered. Previously, the Commission reviewed various aspects of cancer induction from radiation, for example Skin Cancer Risk in *Publication 59* (ICRP, 1992), Genetic Susceptibility to Cancer in *Publication 79* (ICRP, 1998), and Low-dose Extrapolation of Radiation-related Cancer Risk in *Publication 99* (ICRP, 2005). More recently the Commission also reviewed Lung Cancer Risk from Radon in *Publication 115* (ICRP, 2010) and the Threshold Doses for Tissue Reactions (Deterministic effects) in *Publication 118* (ICRP, 2012).

(b) Cancers arise as a result of mutational changes in single target cells, and an aspect of fundamental importance is the nature and characteristics of the target cells responsible for cancer induction. Previously it has been assumed that in most cases the target cells are the "stem cells" in the tissue in question, without further elaboration. In recent years there has been an enormous increase in knowledge about the lineages of stem cells, daughter progenitor cells and the differentiated functional cells. Knowledge has accumulated also about the regulatory signals in different tissues and how the tissues respond to injury. Hence it was considered appropriate at this time to review those aspects of the field of stem cells and their regulatory processes, which are pertinent to radiation carcinogenesis models. However, there are no recent comprehensive reviews of carcinogenic mechanisms after exposure to ionising radiation, regarding stem cells as target cells in different tissues and including age effects.

(c) The main text of the report consists of Chapters 1-3. These are followed by Annexes A-G which consist of detailed reviews on stem cells in the haematopoietic system, mammary gland, thyroid, digestive tract, lung, skin and bone. These particular tissues were chosen on the basis of differing tissue risk coefficients and weighting factors (w_T), and the fact that information on the stem cell systems for haematopoiesis, intestine, and skin is well developed, and less so for mammary gland, thyroid, lung and bone.

(d) Chapter 1 describes the objectives of the report and lists main questions in relation to radiation carcinogenesis models and stem cell biology: What are the target cells for carcinogenesis, and where are they located? Does the LNT model fit with considerations of stem-cell-based radiation carcinogenesis, and if so, how? Is the current DDREF value supported by information concerning stem-cell-based radiation carcinogenesis? What are the mechanisms related to the stem cell response, and do these mechanisms help to explain the tissue differences in the sensitivity to radiation carcinogenesis? What could be an underlying mechanism related to stem cells for the age-dependent sensitivity to radiation carcinogenesis, and hence radiation risk?

(e) Chapter 2 gives the general features of tissue stem cells, including cell division and differentiation in adult tissues, functional identification and isolation of stem cells, their radiosensitivity and DNA damage response, ageing and exhaustion aspects of tissue stem cells, and the very important stem-cell "niche" residence where they are maintained and regulated (see Fig. 2.1.). It has become clear that in the tissues with long lineages, there is a hierarchical spectrum of stem-cell stages with differing characteristics of quiescence (dormancy), renewal, radiosensitivity, propensity to apoptosis, and replication-mediated mutation rates.

(f) Two biological mechanisms specific to stem cells have been described which may play a role in cancer incidences from protracted compared to acute exposures. The first is the

immortal-DNA-strand hypothesis, whereby the parental DNA template is retained during stem-cell divisions so that their mutational load is kept low, and this would act to protect against carcinogenesis. There is evidence in support of this mechanism in small intestinal crypts, mammary epithelium, some muscle satellite cells and progenitor cells, and some central nervous system (CNS) cells. Supportive evidence was also inferred from studies of tongue epithelium, and it could be the mechanism behind DNA-label retention in epidermis but probably not the hair follicle bulge region. However, the mechanism has been found not to apply in haematopoietic stem cells (HSCs). Hence although there is evidence for it in various tissues, it apparently does not apply universally. Another important feature described recently is the concept of competition between normal and radiation-injured stem cells for residence in the niche. New studies suggest that the niche, i.e. the signal-originating microenvironment, which controls stem cell behaviour, may be susceptible to damage. Niche integrity provides another biological mechanism for keeping low the mutation load from protracted and chronic radiation exposures, which again is tissue-dependent.

(g) Chapter 3 discusses the role of tissue stem cells in radiation carcinogenesis, and those aspects which are pertinent to key issues like the LNT model, DDREF, and age-related effects. Concerning LNT, the dose responses for incidence and mortality from all solid cancers among atomic bomb survivors in general do not show a significant deviation from linearity (notable exceptions are skin and bone, and several other organs and tissues which do not show significant increases in radiation risk such as the testes, pancreas and uterine cervix). Some animal data tend to support the LNT model, although again there are several exceptions. The features of dose-dependent non-targeted and epigenetic effects, and adaptation, demonstrated in various cellular systems, may play a role in the radiation responses, but it is not yet clear how to incorporate these phenomena into a protection framework.

(h) The excess risk of cancer relates to carcinogenesis of target cells. Carcinogenesis depends primarily on three mechanistic factors: (1) the number and sensitivity of target cells to radiation induced mutation; (2) the retention of mutated target cells in a tissue; and (3) the population size of target cells with a sufficient number of predisposing mutations. The excess absolute risk (EAR, the additional risk above the baseline) function is obtained by subtracting the baseline risk of an unexposed population from the overall risk of the exposed population, and therefore it reflects the radiation-induced cancers in an exposed population which are directly related to the number of predisposed target cells. On the other hand, the excess relative risk (ERR, the proportional increase in risk above baseline) function is already normalised for the background risk and therefore reflects the sensitivity of a tissue to radiation carcinogenesis for which the sensitivity of target cells to mutagenesis and their retention in the tissue are contributing factors.

(i) The numerical value(s) of DDREF has been under much discussion recently. Calculation-based evaluation of DDREF uses a linear-quadratic (LQ) model which assumes a similarity of the low dose effect and the low dose rate effect. However, from the information reviewed in the present report, it is clear that the factors DEF (dose effectiveness factor) and DREF (dose rate effectiveness factor) are conceptually different at the biological level. The former applies for low acute doses, and the latter applies for low protracted doses where additional exposure-duration processes may modify target/stem cell responses, e.g. DNA repair kinetics, template-DNA retention, and stem-cell/niche competition processes. It is noteworthy that DNA repair operates mainly within a few days after irradiation while stem cell competition is rather a slow process requiring weeks and/or months depending on the tissue type. In this respect, the two mechanisms operate at different dose rates. Also, low chronic radiation exposure of humans is often concomitant with, or subsequent to, exposure to other carcinogenic agents, which may also affect stem cell systems. This may influence the

background rate of cancer in a given population and affect not only projections of dose-response relationships from acute to chronic exposure scenarios but also comparisons of experimental animal data with human epidemiological studies. These distinctions suggest that more emphasis should be given to epidemiological data, which arise from chronic radiation scenarios, rather than relying on risk projections to lower protracted doses from acute higher-dose scenarios.

(j) The concept of stem cell competition is useful in understanding the behaviour of radiation exposed stem cells. Interestingly, lymphocytes from *in utero* exposed atomic bomb survivors and *in-utero*-exposed mice largely lacked chromosome aberrations after moderate doses of radiation, suggesting a possible competition-mediated elimination of aberrant haematopoietic stem cells. In contrast, stem cell competition is likely to be less stringent during childhood growth, when the stem-cell/niche units increase in number to cope with the increase in the tissue volume during childhood growth. Such behaviour of irradiated stem cells might have relevance to the age-dependent sensitivity to radiation carcinogenesis.

(k) In the Annexes, the role of stem cells is discussed in detail for a number of tissues. Annex A describes the extensive knowledge of the three haematopoietic cell hierarchies (erythroid, granuloid, and lymphoid), and their dependent stem cells. In mammary gland epithelium there is much evidence for the presence of a very small stem cell population (Annex B), and various markers are now available for those cells. The sensitivity to radiation carcinogenesis may be influenced by the irradiated microenvironment, in which specific signals are induced that affect stem cell regulation. In thyroid epithelium (Annex C) there is evidence for the presence of a stem-cell-type lineage, but the niche location and signalling pattern in the thyroid follicles are not yet described. The target cells for colonic tumours (Annex D) are considered to be the crypt stem cells, and the potential inclusion of daughter progenitor cells as target cells for radiation is not yet resolved. Genetically-controlled differences in susceptibility to stem-cell death in small versus large intestine may play a role in the lack of radiation-induced tumours in the small intestine. In the lung (Annex E), multipotent cells exist in the bronchiolar-alveolar duct junction zone (a location of the niches) in mice, and the sensitivity to radiation carcinogenesis may be influenced by the irradiated microenvironment. In skin (Annex F) there is clear evidence of a short hierarchical lineage, and a model for human skin cancer proposed that stem cells were likely target cells for basal cell carcinoma (BCC), early progenitor cells for squamous cell carcinoma (SCC), and late progenitor cells for papillomas. Concerning bone (Annex G), mesenchymal (stromal)/stem cells (MSCs) for the osteoblast lineage reside in the bone marrow. CD34-negative stem cells and mesenchymal precursors are possible target cells for radiation-induced bone cancers.

(l) Target cell location is an important consideration in the calculation of doses received from short-range particulate emissions from radionuclides retained in body tissues, including α particles and low energy electrons. The extent to which the immediate progeny of stem cells may also be targets for the development of particular cancer types varies between tissues and is not well established in many cases. For some tissues, this may not have implications for the definition of targets for dosimetric purposes because stem cells and their immediate progeny occupy the same microenvironment. Possible target cell locations are summarised in Table 3.2.

(m) In summary, answers to the questions posed in Chapter 1, Section 1.2, are as follows:

- *What are the target cells for carcinogenesis, and where are they located?* In most cases the target cells are the stem cells residing in the stem cell niche. In some cases also early progenitor cells (daughter cells of the stem cells) may be target cells.
- *Does the LNT model fit with considerations of stem-cell-based radiation carcinogenesis, and if so, how?* A single stem-cell origin of radiation-induced cancer and mutational

theory are consistent with an LNT approach for some tissues and organs, albeit with the caveat of the uncertain influence of radiation-induced epigenetic effects.

- *Is the current DDREF value supported by information concerning stem-cell-based radiation carcinogenesis?* Suggested mechanisms, and evidence for their support (i.e. stem cell competition and dose-rate dependent decrease in the slope of the linear term of the LQ dose response curve) give less risk from chronic exposures than expected from consideration of solely the linear component. These mechanisms support a DREF value greater than unity.
- *What are the mechanisms related to the stem cell response, and do those mechanisms help to explain the tissue differences in sensitivity to radiation carcinogenesis?* Carcinogenesis depends primarily on three mechanistic factors: (1) the number and sensitivity of stem cells to radiation-induced mutation; (2) the retention of mutated stem cells in a tissue; and (3) the population size of stem cells with a sufficient number of predisposing mutations. At present, there is a lack of definitive evidence for the various contributions of these factors to radiation carcinogenesis and hence to radiation risk in different tissues.
- *What could be an underlying mechanism related to stem cells for the age-dependent sensitivity to radiation carcinogenesis, and hence to risk?* Stem cells exposed at the fetal stage of development are less likely to be retained during neonatal growth, where radiation-injured stem cells are under strong competition with unirradiated stem cells to settle in the limited number of newly-established stem-cell niches. In contrast, high sensitivity in childhood can be understood if stem cell competition is less stringent, because the stem-cell/niches increase in number to cope with the increase in the tissue volume during childhood growth.

GLOSSARY

α/β value or ratio
: A measure of the curvature of the cell survival curve. The α/β is also the dose at which the linear and quadratic components of cell killing are equal. For tissues, the α/β value is a measure of their sensitivity to changes in dose fractionation. *In vivo*, the α component describes the dose-response slope at low doses, which is often considered independent of dose-rate but likely it can be modified in chronic radiation scenarios by cell renewal and cell competition processes. The β component describes the increase in slope at higher doses due to cumulative damage, which is repairable during fractionated or low-dose-rate exposures.

Absolute risk (AR)
: The risk of an adverse health effect – the probability or rate of the occurrence of a particular health event (e.g. disease incidence) over a specific period.

Absorbed dose, D
: The energy imparted per unit mass by ionising radiation to matter at a specific point. The SI unit for absorbed dose is joule per kilogram (J/kg) and its special name is gray (Gy).

Active (red) bone marrow
: The organ system bone marrow contains the cell systems for the formation of blood cells starting from the pluripotent haematopoietic stem cells (HSCs) to the mature blood cells.

Adaptive response
: Increased resistance of cells or tissues to radiation following a priming dose, or adjustment to radiation exposure which enables an organism to retain viability, maintain fertility and normal functional stability of all tissues, organs and systems under the conditions of chronic exposure. The principal criterion of radiation adaptation is an increased radioresistance (tolerance) of the organism and the cells of its critical organs.

Adenoma
: A benign tumour of glandular origin. Adenomas can grow from many organs including the colon, adrenal glands, pituitary gland, thyroid, prostate, etc. Although these growths are benign, over time they may progress to become malignant, at which point they are called adenocarcinomas (ADCs).

Apoptosis
: A mode of cell death in which the cell nucleus displays characteristic densely staining globules, and at least some of the DNA is subsequently broken down into internucleosomal units. Sometimes postulated to be a 'programmed' and therefore a potentially controllable process.

Asymmetric division
: Cell division producing two different types of daughter cells, e.g. a tissue stem cell producing both a stem cell and a progenitor cell (see also Immortal strand hypothesis).

Baseline disease rates

The annual disease incidence observed in a population in the absence of exposure to the agent under study.

Blastocyst

Structure formed in the early gestation of vertebrates. It is preceded by the morula. It possesses an inner cell mass, or embryoblast, which subsequently forms the embryo, and an outer layer of cells, or trophoblast, surrounding the inner cell mass and a fluid-filled cavity known as the blastocoele. The human blastocyst comprises 70-100 cells.

Caretaker genes

Caretaker genes encode gene products that stabilise the genome.

Cell death

In the context of radiobiology, cell death is generally equated with any process that leads to the permanent loss of clonogenic capacity, often termed loss of reproductive integrity. Cell death can also refer to physical death through a variety of processes such as apoptosis, necrosis and autophagy, and also sometimes, premature senescence and premature differentiation.

Checkpoint

A point in the cell cycle at which injured cells are arrested and then released after recovery to progress to the next phase of the cell cycle.

Chromothripsis

Multiple genomic rearrangements with sharply circumscribed regions of one or a few chromosomes, crisscrossing back and forth across involved regions.

Clonogenic cells

Cells that have the capacity to produce an expanding family of descendants (usually at least 50). Also called ‘colony-forming cells’ or ‘clonogens’.

Clonogenic surviving fraction

The fraction of clonogenic cells that survive exposure to, or treatment with, an agent that causes cell death. Only cells that are able to form colonies (clonogenic cells) are considered to have survived the treatment (*see* Cell death).

CO-FISH

Chromosome orientation fluorescence *in situ* hybridisation technique. Can be used to check if the segregation of sister chromatids is random or not.

Colony

The family of cells derived from a single clonogenic cell.

Complex tissues (also called flexible tissues or conditional renewing tissues)

Cell populations in which both function and proliferation can take place alternatively in the same cells.

Confidence limits or intervals

An interval giving the lowest and highest estimate of a parameter that is statistically compatible with the data. For a 95% confidence interval (CI), there is a 95% chance that the interval contains the parameter.

Cytokines

Organic molecules with biological function, originally defined as being polypeptides released from lymphocytes and involved in maintenance of the immune system. These factors have pleiotropic effects on not only haematopoietic cells but many other cell types as well. Often synonymously termed growth factors.

Cumulative dose

The total absorbed dose resulting from repeated exposures to ionising radiation over a period of time.

D_0

A parameter in the multitarget equation for cell survival: the radiation dose that reduces survival to e^{-1} (i.e. 0.37) of its previous value on the exponential portion of the survival curve.

Dose and dose-rate effectiveness factor (DDREF)

A judged factor that generalises the usually lower biological effectiveness (per unit of dose) of radiation exposures at low doses and low dose rates as compared with exposures at high doses and high dose rates; includes dose effectiveness factor (DEF) and dose-rate effectiveness factor (DREF).

Dose rate

The absorbed radiation dose delivered per unit time and measured, for example, in gray per hour.

Dose-rate effect

Decreasing radiation response with decreasing radiation dose rate.

Elemental dose

The lowest dose given by a single track of radiation to a nucleus of a cell.

Embryonic Stem (ES) cells

Cells in the inner cell mass of the blastocysts, responsible for further development of the entire embryo proper.

Epigenetic effects

Epigenetic changes consist of changes in the properties of a cell that are inherited but that do not represent a change in genetic information, e.g. methylation effects. They influence the phenotype without alteration in the genotype.

Epithelium

Membranous tissue composed of one or more layers of cells, forming the covering of most external and internal surfaces of the body and its organs.

Erythropoietin

Cytokine that regulates erythrocyte levels and stimulates late erythroid progenitor cells to form small colonies of erythrocytes.

Excess absolute risk (EAR)

The additional risk (or rate) from radiation exposure above the underlying (baseline) risk (or rate) of the disease. This is often expressed as the EAR per Gy or per Sv.

Excess relative risk (ERR)

The excess proportion (or percentage) of the rate of radiation-induced disease in an exposed population divided by the rate of disease in an unexposed population which has the same background risk factors (age, sex, race, etc.). This is often expressed as the ERR per Gy or per Sv.

Exponential survival curve

A survival curve without a threshold or shoulder region, which is a straight line on a semi-logarithmic plot.

FACS

Fluorescence-activated cell sorting, which can be used to identify stem cells using particular cell surface markers.

Fractionation and dose delivery patterns

The dose per fraction of radiation is the total dose divided into a particular number of fractions. A very large number of extremely small dose fractions becomes equivalent to low-dose-rate exposure. Very low dose-rates protracted over long durations are called chronic exposures.

Fractionation sensitivity

The dependence of the isoeffective absorbed radiation dose on the dose per fraction. Usually quantified by the α/β value – a high fractionation sensitivity is characterised by a low α/β value (see α/β value).

γH2AX foci

Identification of the broken ends of DNA caused by ionising radiation. H2AX is one of several genes coding for histone H2A. H2AX becomes phosphorylated on serine 139, then called γH2AX, as a reaction on DNA double-strand breaks (DSBs). γH2AX is a sensitive target for looking at DSBs in cells.

Gatekeeper genes

Gatekeeper genes encode gene products that act to prevent growth of potential cancer cells and prevent accumulation of mutations that directly lead to increased cellular proliferation.

Genomic integrity

Preservation of the structural and functional content of the genome of cells.

Granulocyte colony-stimulating factor (G-CSF)

Cytokine that stimulates proliferation and differentiation of progenitor cells into granulocytes.

Granulocyte-macrophage colony-stimulating factor (GM-CSF)
: Cytokine that stimulates proliferation and differentiation of progenitor cells into granulocytes, macrophages, and eosinophils.

Gray (Gy)
: The special name for the SI unit of absorbed dose: 1 Gy = 1 J/kg.

Growth factor
: An organic molecule which stimulates cell proliferation when it binds to its cell surface receptor. Often synonymously termed cytokine.

Growth fraction
: Proportion of viable cells in active cell proliferation.

Hierarchical tissues
: Tissues comprising a lineage of stem cells, transit (amplifying) cells, and postmitotic (differentiated or mature) cells.

High linear energy transfer radiation
: Radiation having a high linear energy transfer (LET), for example, α particles, heavy ions and interaction productions of fast neutrons. The ionisation density along the radiation track is high.

Homologous recombination (HR)
: HR takes place in S and G_2 phase cells to repair a damaged region of DNA by copying the intact counterpart of the sister DNA strand. HR is potentially error-free.

Hypoplasia
: Reduction in cell numbers in a tissue, e.g. owing to radiation-induced impairment of proliferation in early-responding tissues.

Hypoxanthine-guanine phosphoribosyltransferase (Hprt) mutations
: The Hprt assay is an *in vitro* mammalian cell gene mutation test. The estimation of mutant frequency in the reporter gene, called Hprt located on the X chromosome, can provide information on the biological effect of an absorbed dose in the cell type studied, and hence is a useful biodosimetry tool.

Immortal strand hypothesis
: Asymmetric segregation of DNA strands to minimise the replication error in the stem cells. The stem cell retains the template DNA strand after a round of DNA synthesis, while the progenitor cells inherit the daughter strand.

Incidence (incidence rate)
: The rate of occurrence of a disease in a population within a specified period of time, often expressed as the number of cases of a disease arising per 100,000 individuals per year, or per 100,000 person-years (PY).

Initial slope

The steepness of the initial part of the cell survival curve, usually indicated by the value of α in the linear-quadratic (LQ) model.

Interphase death
The death of irradiated cells before they reach mitosis. Sometimes used as a synonym for apoptosis.

Knockout mice
Mice in which one (or more) gene has been inactivated.

Label-retaining cells (LRCs)
Cells which retain a DNA label through multiple rounds of cell division.

Lifetime risk
The cumulated risk of morbidity or dying of some particular cause up to a given age.

Linear energy transfer (LET)
The rate of energy loss along the track of an ionising particle, usually expressed in keV/µm.

Linear-no-threshold (LNT) dose-response model
A dose-response model which is based on the assumption that, in the low dose range, any radiation doses greater than zero will increase the risk of excess cancer and/or heritable disease in a simple proportionate manner.

Linear-quadratic (LQ) dose-response model
A statistical model that expresses the risk of an effect E (e.g. disease, death, or abnormality) as the sum of two components, one proportional to dose (linear term) and the other one proportional to the square of dose (quadratic term). $E = \alpha D + \beta D^2$, where D is dose. For cell survival: $S = \exp - (\alpha D + \beta D^2)$.

Low-LET radiation
Radiation having a low LET, for example electrons, x-rays, and γ-rays.

Lymphatic system
A network of lymphatic vessels of varying calibre that collects tissue fluids from all over the body and returns these fluids to the blood. Accumulations of lymphocytes, called lymph nodes, are situated along the course of lymphatic vessels.

Macrophage colony-stimulating factor (M-CSF)
Cytokine that stimulates formation of macrophages from pluripotent haematopoietic cells.

Mammosphere
A spheroid of cells derived from single mammary gland cells. A single cell from a mammosphere can regenerate an entire mammary gland when transplanted into a mammary fat pad.

Multiple intestinal neoplasia (Min) mouse

Min mice are genetically heterozygous for a germ-line truncating mutation of the adenomatous polyposis coli (Apc) gene (i.e. $Apc^{Min/+}$), and develop multiple intestinal tumours and sporadic colon tumours in their intestinal tracts within several weeks of birth. The Min mouse provides a sensitive model for the study of tumourigenesis in irradiated mice.

Multistage carcinogenesis model
: Carcinogenesis model associated with a stepwise acquisition of mutations of oncogenes and tumour suppressor genes, associated with a progressive loss of external proliferative factors.

Necrosis
: Cell death associated with loss of cellular membrane integrity. Occurs for example in anoxic areas of tumours and is also a cause of cell death after irradiation.

Never-smokers
: People who have never smoked.

Niche (or stem cell niche)
: Specific microenvironment in a tissue where stem cells reside and are maintaind by various signals controlling proliferation and differentiation.

Non-homologous end joining (NHEJ)
: NHEJ repair takes place in non-cycling cells and variously in all cycle phases, and is dependent on the repair proteins Ku70, Ku80 and DNA-dependent protein kinase catalytic subunit (DNA-PKcs).

Non-smokers
: People who do not smoke.

Non-targeted effects
: Indirect effects of radiation exposure, including bystander effects and the induction of genomic instability.

Oncogene
: A gene that when mutated or overexpressed, contributes to converting a normal cell into a cancer cell.

Platelet-derived growth factor (PDGF)
: A cytokine which induces growth of fibroblasts and is involved in wound healing. Also acts on some epithelial and endothelial cells, and on mesenchymal cells.

Poisson distribution
: Distribution applicable when the probability of an event happening is small but the number of observations is large. The distribution of probabilities runs from zero to infinity, and an important characteristic of the distribution is that the mean equals the variance.

Potentially-lethal damage repair (PLDR)

DNA repair occurring in a delay period after irradiation, before cell division occurs.

Programmed cell death

Cell death that occurs as the result of an active process carried out by molecules in the cell. Examples include apoptosis, autophagy, terminal differentiation, senescence, and in some cases even necrosis.

Protection quantities

Dose quantities that the Commission has developed for radiological protection, that allow quantification of the extent of exposure of the human body to ionising radiation from both whole and partial body external irradiation and from intakes of radionuclides.

Radioresponsiveness

Temporal rate of response of a tissue to irradiation (in contrast to the magnitude of response, i.e. radiation tolerance). This depends on multiple factors, one of them hypothesised to be cellular radiosensitivity.

Radiosensitiser

In general, any agent that increases the sensitivity of cells and tissues to radiation. Commonly applied to electron-affinic chemicals that mimic oxygen in fixing free-radical damage, although these should more correctly be referred to as hypoxic cell sensitisers.

Radiosensitivity, cellular

The sensitivity of cells to surviving exposure to ionising radiation. Usually indicated by the surviving fraction at 2 Gy (i.e. SF_2) or by the parameters of the LQ or multitarget equations.

Reactive oxygen species (ROS)

Molecular species such as superoxide, hydrogen peroxide, and hydroxyl radicals. These species may function in cell signalling processes. At higher levels, these species may damage cellular macromolecules (such as DNA and RNA) and participate in cell death processes.

Recovery

At the cellular level: an increase in cell survival as a function of time between dose fractions or during irradiation at low dose rates. At the tissue level: an increase in tissue equi-effective total dose with an increase in time interval between fractions and a decrease in dose per fraction, or with irradiation at low dose rates.

Relative biological effectiveness (RBE)

The ratio of a dose of a low-LET reference radiation (usually of ^{60}Co γ–rays or kilovoltage x-ray quality) to a dose of the test radiation considered that gives an identical biological effect. RBE values vary with the dose, dose fractionation, dose rate, and a biological endpoint considered.

Relative risk (RR)

An expression of overall risk (i.e. including the radiation-induced risk) relative to the underlying baseline risk. If the total risk is twice the underlying baseline risk then the RR is 2.

Reproductive cellular integrity
: Ability of cells to divide many times (usually >5) and thus be 'clonogenic'.

Senescence
: A permanent arrest of cell proliferation associated with differentiation, ageing, or cellular damage.

Side population (SP)
: In flow cytometry, a subpopulation of cells that is distinct from the main population on the basis of the markers employed - often cells that show higher efflux of DNA-binding dye Hoechst 33342. By definition, cells in a side population have distinguishing biological characteristics (for example, they may exhibit stem cell-like characteristics), but the exact nature of this distinction depends on the markers used in identifying the side population.

Sievert (Sv)
: The special name for the SI unit of equivalent dose, effective dose, and operational dose quantities in radiation protection. The unit is joule per kilogram (J/kg).

SKY analysis
: Spectral karyotyping (SKY) of the chromosomal content of cells using metaphase cells pretreated and hybridised with a SKY probe mixture containing uniquely labelled chromosome-specific probes.

Slow repair
: Long-term restoration of radiation tolerance that takes place on a time scale of weeks to years, often associated with long-term intracellular repair.

Spheroids
: Aggregates of cells produced in culture by multiple divisions of a single cell. Spheroids can be produced both from some normal and malignant cells, the latter being often used as a model of tumour metastases.

Stem cells
: Cells with an unlimited proliferative capacity, capable of self-renewal and of differentiation to produce all the various types of cells in a lineage system. Stem cells are described as being totipotent (producing all lineages), whereas their daughter progenitor cells can be multipotent (producing several lineages) or unipotent (one lineage).

Stemness
: Stem cell characteristics that underlie self-renewal and the ability to generate differentiated progeny. There are differing degrees of stemness among more primitive and less primitive stem cells in some hierarchical lineage systems.

Stochastic effects of radiation
: Malignant disease or heritable effects; the probability of an effect occurring, but not its severity, is regarded as a function of dose without threshold.

Sublethal damage repair (SLDR)
: DNA repair occurring during low-dose-rate exposure or between dose fractions, resulting in less cell kill or less tissue reaction than if the total dose was delivered acutely.

Targeted effects
: Effects occurring in irradiated cells.

Telomeres
: The ends of chromosomes. One of the determinants of cellular senescence is the loss of telomeres through DNA replication. Rapidly replicating cells usually have telomerase activity to avoid telomere shortening. Shortening of telomeres is also associated with genomic instability and carcinogenesis.

Teratocarcinoma
: A malignant neoplasm consisting of elements of teratoma with those of embryonal carcinoma or choriocarcinoma, or both; occurring most often in the testis.

Tissue weighting factor, w_T
: The factor by which the equivalent dose in a tissue or organ T is weighted to represent the relative contribution of that tissue or organ to the total health detriment resulting from uniform irradiation of the body.

Transforming growth factor beta (TGFβ)
: A cytokine that regulates many of the biological processes essential for embryo development and tissue homeostasis, and which therefore plays a role in the healing of some tissues. The effects of TGFβ may differ depending on the tissue involved, e.g. TGFβ inhibits the proliferation of epithelial cells but stimulates proliferation, differentiation and collagen synthesis in fibroblasts.

Transit cells
: Differentiating proliferative cells that amplify cell production in a hierarchical tissue.

Translocations
: Chromosomal abnormalities which occur when chromosomes break and the fragments rejoin to other chromosomes. There are many structurally different types of translocations.

Trophectoderm
: The outer layer of the mammalian blastocyst after differentiation of the ectoderm, mesoderm, and endoderm, when the outer layer is continuous with the ectoderm of the embryo.

Tumour suppressor gene
: A tumour suppressor gene, or anti-oncogene, is a gene that protects a cell from one step on the path to cancer. When this gene is mutated to cause a loss or reduction in its function, the cell can progress to cancer, usually in combination with other genetic changes.

Working level month (WLM)

The cumulative exposure from breathing an atmosphere at a concentration of 1 Working Level (WL) for a working month of 170 hours. In the case of radon, 1WL = any combination of the short-lived progeny of radon in 1 litre of air that will result in the emission of 1.3×10^5 MeV of potential alpha energy. 1 WL = 2.08×10^5 J/m^3.

Main reference source for the Glossary

ICRP, 2012. ICRP Statement on Tissue Reactions / Early and Late Effects of Radiation in Normal Tissues and Organs – Threshold Doses for Tissue Reactions in a Radiation Protection Context. ICRP Publication 118. Ann. ICRP 41(1/2).

ABBREVIATIONS

53BP1	p53-binding protein 1
ADC	adenocarcinoma
ALL	acute lymphoblastic leukaemia
AML	acute myeloid leukaemia
Ang-1	Tie2/angiopoietin 1
AR	additive risk
ATM	ataxia telangiectasia mutated
BASC	bronchioalveolar stem cell
BCC	basal cell carcinoma
Bcrp1	breakpoint cluster region pseudogene 1
bFGF	basic fibroblast growth factor
BrdU	bromodeoxyuridine
CBCC	crypt base columnar cells
CFU-F	fibroblastoid colony-forming unit
CML	chronic myeloid leukaemia
CMP	common myeloid progenitor
CNS	central nervous system
DDREF	dose and dose-rate effectiveness factor
DEF	dose effectiveness factor
DREF	dose-rate effectiveness factor
DSB	double strand break
EAR	excess absolute risk
EGF	epidermal growth factor
EpiSC	epidermal stem cell
ERR	excess relative risk
ES	embryonic stem
FACS	fluorescence-activated cell sorting
FAP	familial adenomatous polyposis
FISH	fluorescent in situ hybridisation
G-CSF	granulocyte colony-stimulating factor
GFP	green fluorescent protein
GM-CSF	granulocyte-macrophage colony-stimulating factor
GMP	granulocyte/macrophage progenitor
Hprt	hypoxanthine-guanine phosphoribosyltransferase
HR	homologous recombination
HSC	haematopoietic stem cell
HSPC	haematopoietic stem and progenitor cell
IGF1	insulin-like growth factor 1
Klf4	Kruppel-like factor 4
Lgr5	leucine-rich repeat-containing G protein-coupled receptor 5
LNT	linear no threshold
LQ	linear quadratic
LRC	label-retaining cell
MAPK	mitogen-activated protein kinase
MaSC	mammary gland stem cells
M-CSF	macrophage colony-stimulating factor

Min	multiple intestinal neoplasia
miRNA	microRNA (microribonucleic acid)
Mre11	meiotic recombination 11
MSC	mesenchymal (stromal)/stem cell
mTert	mouse telomerase reverse transcriptase
NHEJ	non-homologous end joining
NSC	neural stem cell
Oct3/4	octamer-binding transcription factors 3/4
OSCC	Oxford Survey of Childhood Cancers
PDGF	platelet-derived growth factor
PLDR	potentially-lethal damage repair
RBE	relative biological effectiveness
ROS	reactive oxygen species
RR	relative risk
SCA-1	stem cell antigen-1
Sca-1	type II cell marker surfactant protein C
SCC	squamous cell carcinoma
SCF	c-kit tyrosine kinase receptor
SCGBa1a	secretoglobin a1a
SCLC	small-cell lung carcinoma
SCN	solid cell nest
SI	small intestine
SLDR	sublethal damage repair
Sox2	sex determining region Y-box 2
SP	side population
TA	transit amplifying
TGFβ	transforming growth factor beta
WLM	working level month

1. INTRODUCTION

1.1. Purpose of the report

(1) The risks of radiation-induced cancer have continued to dominate the reasons behind recommendations on restricting radiation exposures of workers and the public for many decades. Carcinogenesis from radiation is considered to be a stochastic event, originating in a single transformed target cell. Generally, the target cells are considered to be the stem cells and possibly also some of their daughter progenitor cells in each tissue. These target cells have tissue-specific characteristics, and they reside in a microenvironmental "niche" that regulates their proliferation and differentiation (see Fig. 2.1.). Knowledge about the stem cells and their regulation may help underpin extrapolated risk estimates for different tissue and organ systems, and may also help understand risk projections in different exposure scenarios.

(2) The location of target cells in different tissues is vitally important regarding the risks of carcinogenesis from short-range radionuclides and lightly-penetrating radiation beams. In its Publications, the Commission has made some judgments and assumptions about the location of these cells. In the skin, the target cells are considered to be in the hair follicles as well as in the basal interfollicular epidermis (ICRP, 1991). In the respiratory tract, the target cells are considered to be in the basal layer of the mucosa and in the alveoli of the lung (ICRP, 1995). In the intestine, the target cells are considered to be near the bottom of the intestinal crypts, but there are uncertainties about the carcinogenic potential of cells further up the crypts (ICRP, 2007). In the skeleton, the target cells responsible for radiation-induced osteosarcomas are considered to be the osteoblasts in bone cavities, as well as mesenchymal stem cells (MSCs) in the bone marrow (ICRP, 1996). The information in the present report is likely to contribute further to clarification of the target cell locations in the tissue of interest.

(3) The report comprises a review of advances in knowledge of the biology and radiation response of stem cells and progenitor cells in the context of the tissue microenvironment, in relation to mechanisms of radiation carcinogenesis. Recent progress in stem cell biology and radiobiology is described, including tissue architecture, the dynamic nature of tissue maintenance, stem cell radiosensitivity and renewal, dose-rate effects and age dependence. This information is then evaluated for deducing the implications of the role of stem cells in the mechanisms of carcinogenic risk as a function of acute and chronic radiation dose, including projections of risk for short-range radiations.

(4) In order to form a basis of scientific knowledge regarding stochastic radiation effects in different tissues and organs, a series of organ systems was selected as examples with known radiation-induced risks. As such reviews are not available currently, a detailed series of Annexes was produced for these individual examples. Not all organ systems were covered because of the enormity of such a task. Selection was made on the basis of importance for radiation protection purposes and of the extent of available radiobiological knowledge and interest. Firstly, those with the highest ICRP tissue weighting factor of 0.12 were selected, which include bone marrow, breast, digestive tract and lung. Among these, haematopoietic tissue is best studied for the stem cell aspect (Annex A), and there is good knowledge of stem cell locations and other hierarchical aspects of tissue turnover in the digestive tract (Annex D). The presence of stem cells is well proven for the mammary gland (Annex B), but those of the lung have yet to be fully characterised (Annex E). Thyroid was selected for its strong age dependence of susceptibility to radiation carcinogenesis (Annex C). Bone was selected because it is the best model system for cancer induction by internal emitters (Annex G). Skin

was selected for its simple hierarchical tissue structure which facilitates the understanding regarding tissue cell types, cancer sub-types and their susceptibility to radiation (Annex F), and the large, but rather uncertain, skin cancer incidence rate (ICRP, 1991, 1992, 2007; Annex F). The format of the Annexes was designed so as to present similar information for each tissue, in order to facilitate comparisons.

1.2. Radiation carcinogenesis models and stem cell biology

(5) Current understanding of mechanisms for radiation-induced cancer relies on the multistep model of Armitage and Doll (1954), and its extension to the molecular changes postulated by Vogelstein et al. (1988). However, these mechanisms have limited impacts since they were not thoroughly evaluated in the context of radiation carcinogenesis. Improvements in this situation can be attained by positioning the LNT and RR models in the context of the multistep model. Improvements in this situation may arise from the identification of target cells within tissues, understanding of radiation responses of these cells, and the kinetics of turnover and cell-cell interactions that control the potential of these target cells to progress in the carcinogenic process. These issues are important for external exposures particularly for low chronic exposures but they take on added importance when considering risks from internal emitters, particularly those with short ranges in tissues. The newly emerging stem cell biology provides a real opportunity to clarify these issues and provide a foundation to better understand the dose and dose-rate effects and better define the target-cell locations for dose effects from internal emitters. It may also contribute to reasons supporting the way to transport risks across different populations.

(6) The number, sensitivity, location and renewal characteristics of target cells are important biological parameters with respect to carcinogenesis from radiation. Many people consider that the target cells for carcinogenesis are the tissue stem cells and some of their daughter cells. The number of stem cells is often ill-defined and estimated using various assays in different tissues in experimental animal systems. Stem cell radiosensitivity varies both within and among tissues. For example, there are both resistant slow-cycling and sensitive fast-cycling stem cells in spermatogenic epithelium, sensitive apoptosis-susceptible cells and more resistant clonogenic cells in intestinal mucosa, and more sensitive stem cells in haematopoietic than in epithelial tissues. The microenvironment within tissues differs in a variety of parameters including cell-to-cell and cytokine-mediated signalling.

(7) The location of target cells in different tissues is vitally important regarding the risks of carcinogenesis following radiation doses from short-range radionuclides and lightly-penetrating radiation beams. Stem cells are often found residing in a specific microenvironment known as a stem cell “niche”. The target cells for leukaemia are considered to be HSCs and possibly some of their daughter cells, and their niches are situated within small bone cavities as well as near sinusoids. The density of some stem cell types near the central blood vessel is lower than closer to the bone surface, although there is some evidence that the reverse is true regarding their renewal ability. However, the general conclusion is that the primary stem cells are protected in hypoxic niches near the bone surface (Parmar et al., 2007). These uncertainties in target cell type and position have hampered attempts to provide accurate projections of radiation risks pertaining to short-range radiations, based on risks for homogeneous external irradiations. Moreover, the stem cell niche and its morphological features are still to be better defined for many tissue types such as mammary gland and thyroid.

(8) For purposes of radiological protection programmes, the ICRP applied the LNT model to the risk assessment of chronic exposures, with the use of a DDREF. The numerical value

of DDREF remains controversial, and various proposals have been made (BEIR VII, 2006). Evaluation of stem cells and tissue turnover is expected to help clarify the appropriateness of using LNT for low dose extrapolation and a DDREF value for adjustment of risk for chronic exposures. The latter is dependent to some extent on the uncertain repair capacity and the renewal/retention rate of the target cells in tissues. Although DNA repair has been discussed regarding its role in the DDREF, the cell renewal rate is considered to be more important for cancer types where the target cells are not only the stem cells, but also their daughter progenitor cells, which are subject to more vigorous renewal. In addition, recent studies have shown that even the damaged tissue stem cells in some cases are subject to constant removal competition from undamaged stem cells. This would be expected to result in a lower risk from chronic exposures than that predicted simply by the cumulative dose to the initial complement of target cells, which are being slowly replaced by new cells.

(9) The following chapters discuss the following questions and topics in the light of recent progress in stem cell biology: What are the target cells for carcinogenesis, and where are they located? Does the LNT model fit with considerations of stem-cell-based radiation carcinogenesis, and if so, how? Is the current DDREF value supported by information concerning stem-cell-based radiation carcinogenesis? What are the mechanisms related to stem cell response, and do those mechanisms help to explain the tissue differences in sensitivity to radiation carcinogenesis? What could be an underlying mechanism related to stem cells for the age-dependent sensitivity to radiation carcinogenesis, and hence risk?

2. GENERAL FEATURES OF TISSUE STEM CELLS

2.1. Cell division and differentiation in adult tissues

(10) Adult tissues fulfill various bodily needs with specialised sets of functional cells. Functional cells are terminally differentiated cells with a limited, often no, capacity to proliferate and they have to be replaced when needed. Adult tissues can be divided into two types, complex (flexible) and hierarchical types. In the flexible tissue such as liver, the functional cells have a potential to divide and can increase their number in special occasions especially when injured, but they are thought to be supplied by stem cells under normal conditions. In contrast, the turnover rate of the hierarchical tissues is high and functional cells in this tissue type are lost rapidly from the body. For the supply of a large number of a variety of functional cell types, the hierarchical tissues have a discrete lineage consisting of stem cells, progenitor cells and differentiated cells. The hierarchical tissues are the main target for radiation carcinogenesis and they are the focus of the present report.

(11) Stem cells differ between early embryogenesis, fetal development and after establishment of adult tissues. The stem cells of the embryonic stage are ES cells which are totipotent and have the capacity to differentiate into all tissue types of later organogenesis. In the fetal stage, stem cells are lineage-committed to a certain extent in order to contribute to specific tissues of the adult stages. In these two stages of early life, stem cells mostly undergo symmetric division to produce two equal daughter stem cells, associated with an increase in the size of the embryo and the fetus (see Section 2.3.5 and Fig. 2.5.). By contrast, adult tissue stem cells are mainly fully committed with restricted differentiation capabilities, and they divide in an asymmetric fashion, although they undergo symmetric division to various extents especially when repairing tissue injuries.

(12) The study of adult tissue stem cells started as early as 1960s. Haematopoietic progenitor cells (originally considered as stem cells) were first identified in bone marrow as those cells capable of forming splenic colonies after intravenous injection into lethally-irradiated mice (Till and McCulloch, 1961). Those early studies focussed on the regenerative capacity of tissue progenitor cells, but later studies *in vivo* revealed many important characteristics of tissue stem and progenitor cells including asymmetrical division (Potten and Loeffler, 1990).

(13) Asymmetric division of adult tissue stem-cells produces both a stem cell and a progenitor cell. Progenitor cells are also called transit amplifying (TA) cells, especially for epithelial tissues. Although the terms of progenitor cells and TA cells are used somewhat differently in the context of the haematopoietic system, these two terms are used interchangeably in the present report. The progenitor cells divide further to increase in number and they then differentiate into functional cells which are eventually lost by senescence from the tissue after serving their required functions. In this general scheme of tissue turnover, the stem cells are frequently quiescent while progenitors divide more rapidly with a limited proliferative capacity. This differential role sharing between tissue stem cells and progenitor cells is the strategy for life-long preservation of stem cells by minimising replication-mediated mutations while supplying a large number of cells to the functional compartment of a tissue by vigorous division of progenitor cells. The progression from stem cells to differentiated cells is usually unidirectional, but can be reversible under certain conditions such as when stem cells are lost for some reason where the vacant stem cell niche becomes occupied by a neighbouring stem cell or by a dedifferentiated progenitor cell. The latter scenario was demonstrated for germ cells of fruit flies and mice (Cheng et al., 2008;

Barrocal et al., 2009). The balance between the production and loss of cells maintains the bodily tissue mass.

(14) Hierarchical tissues contain three cellular compartments: the stem cell compartment; the progenitor cell compartment; and the functional cell compartment. The cells in the former two compartments have a capacity to divide while those in the last compartment generally do not. The steps from stem cells to the differentiated cells vary from tissue to tissue. This somewhat oversimplified scheme is depicted in Fig. 2.1. Importantly, while each cell resides in a defined compartment, the population as a whole consists of a generally unidirectional gradient of cells between compartments.

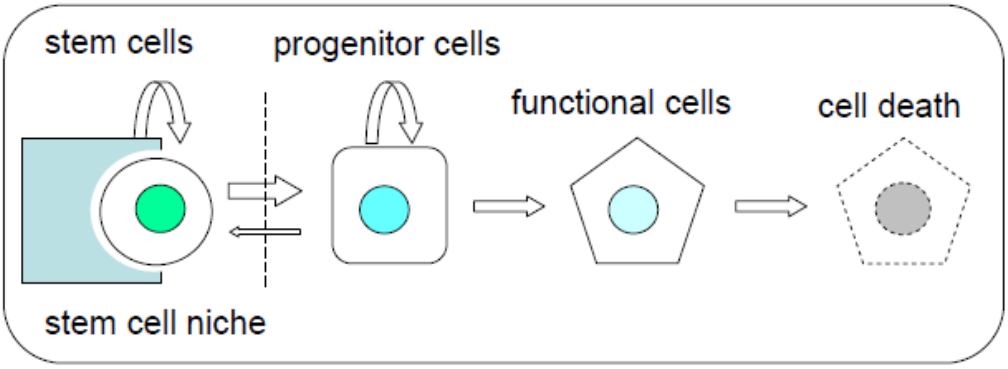

Fig. 2.1. Stem cell division and the maintenance of tissue dynamics. A niche is the location for stem cells, which divide to replace themselves or to produce progenitor cells. The latter divide further, producing functional mature cells. The functional cells have a limited lifespan before they die, requiring replacement by more divisions in the cell lineage.

(15) The number of cell stages in a lineage in a tissue varies greatly. In some tissues, stem cells supply the relatively limited number of lineages as in the case of the epidermis, while those in other tissues supply a variety of lineages as exemplified by HSCs in bone marrow. In addition, the number of divisions from the stem cells to functional cells is variable among tissues as schematised in Fig. 2.2. (Potten and Wilson, 2007). As mentioned earlier, differentiated cells are capable of cell division in flexible tissues such as liver, thyroid and lung when injured. (permission needed)

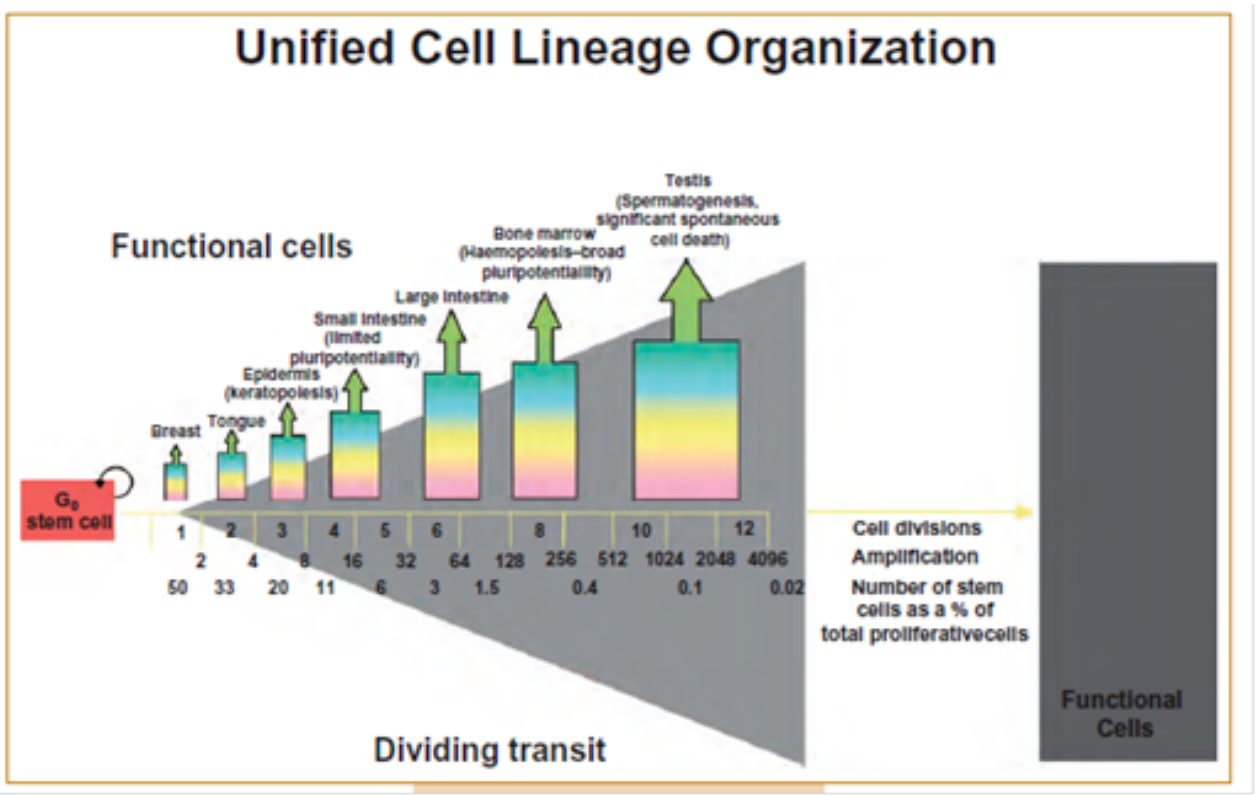

Fig. 2.2. A diagrammatic representation of a stem-cell-derived cell lineage. The number of cell generations in the lineage varies considerably between tissues, with stratified and glandular epithelia having the fewest amplifying divisions of dividing transit cells. For example, in the large-intestinal crypt, there are 5-9 amplifying cell divisions. Seven divisions would produce 128 post-mitotic maturing cells from 64 cells that had multiplied (proliferative cells) from one stem cell. Hence the percentage of stem cells in the proliferative cells in this example would be 1/65 x 100 = 1.5 %. In breast or tongue epithelium the lineage is short with likely only 1-2 amplifying cell divisions. Two divisions would produce 4 maturing cells from 2 proliferative cells arising from 1 stem cell. Hence the stem cells form 1/3 x 100 = 33 % of the proliferative cells (Diagram reproduced from Potten and Wilson, 2007). (Permission needed)

(16) The rate of turnover varies markedly between tissues. The ^{14}C dating technnique was used to estimate directly the rate of cell turnover in various tissues and demonstrated that the majority of cells in the brain stays for the entire life span of individuals without being replaced, while those in the blood and intestine turn over rapidly (Spalding et al., 2005). Since the technnique measures the level of ^{14}C isotope of all the cells in a tissue, it is not sensitive enough to exclude the possible existence of small number of cells which turn over. In fact, stem cells are known to be present in adult brain (Quinones-Hinojosa et al., 2007), but their contribution to the total turnover of brain tissue is too small to be detected by this technique.

(17) The same technology demonstrated the turnover rate of the fat cells in human to be 10% annually (Spalding et al., 2008). The turnover rate varied with age, and in cardiomyocytes it decreased from 1% annually at the age of 25 to 0.45% at the age of 75 years (Bergmann et al., 2009). The turnover rate and the number and the location of various cell types in a tissue are believed to be important determinants of tissue-specific risk of radiation carcinogenesis by external exposures as well as by internal exposures. In adult tissues, hierarchical tissues with higher renewal rates are generally more sensitive to radiation

carcinogenesis (small intestine is an important exception) than flexible/complex tissues with lower renewal rates. Also, the turnover rate varies by the developmental stage and age of individuals, and is one of the major determinants of the age dependence of radiation carcinogenesis (see Sections 2.5.4 and 3.6).

2.2. Functional identification and isolation of tissue stem cells

(18) Tissue stem cells are defined by their ability to self-renew and to produce progenitor cells of particular lineages, which in turn give rise to all the cells of the tissue. The serial transplantation of marrow into irradiated mice demonstrates that the cells in the colonies have a capacity to self-renew. Also, histological examination demonstrates the presence of several cell types in a colony, indicating that the transplanted colony-forming cells have the capacity to differentiate into various haematopoietic cell lineages. In addition, colony survival analyses enable the estimation of radiosensitivity of the bone marrow stem/progenitor cells. Studies of total bone marrow transplantation in combination with limited dilution of the cell preparation estimated the fraction of the stem cells to be 1/10,000 to 1/100,000 (Harrison et al., 1993; Szilvassy et al., 1990).

(19) The functional identification of stem/progenitor cells by *in vivo* colony formation was successfully applied for other tissues including the skin epidermis and intestinal epithelium (Withers, 1967; Withers and Elkind, 1969). Transplantation of mono-dispersed cells and tissue fragments was also used to demonstrate the presence of stem cells in mouse and rat mammary glands, and in rat thyroid (Daniel et al., 1971; Clifton et al., 1986). The colony assay was used also for quantifying the radiation induction of carcinogenic events in rat mammary and thyroid clonogenic cells (Kamiya et al., 1995; Watanabe et al., 1988). Although these *in vivo* studies identified stem cells by their functions, lack of isolated stem cells hampered further studies on the nature of the cells.

2.2.1. Isolation of embryonic and adult tissue stem cells

(20) ES cells were established by explant culture of mouse blastocysts *in vitro* (Evans and Kaufman, 1981; Martin, 1981). There are 20-40 ES cells in the inner cell mass of the blastocysts and they are responsible for further development of the entire embryo proper. ES cells can be identified by their characteristic colony morphology *in vitro* of small and tightly-packed cells. These cells are immortal and express telomerase to protect their chromosome ends (Carpenter et al., 2003). Also they are totipotent since ES cells contribute to almost all tissues, except for the trophectoderm. This totipotency was shared by embryonic carcinoma cells and early stage embryos, and ES cells readily form teratocarcinomas when transplanted into allogenic sites (Rossant and Papaioannou, 1984).

(21) ES cells have been isolated from a variety of mammalian species including humans (Thomson et al., 1998). ES cells serve as the target of gene ablation techniques and are playing a pivotal role in creating ‘knockout’ mice, thus contributing to the study of gene functions in the tissue and whole-body contexts. A variety of repair-gene knockout mice has been created which offers great opportunities for the functional analyses of these genes in terms of radiosensitivity, DNA repair, mutagenesis and carcinogenesis in mice (Griffin et al., 2005; Zha et al., 2007). In subsequent sections of this report, however, ES cells are discussed only when necessary, because they have no direct relevance to radiation carcinogenesis.

(22) Isolation and *in vitro* cultivation of cells facilitate qualitative and quantitative analyses of tissue stem cells. Long term *in vitro* cultivation of tissue stem cells was accomplished for mouse haematopoietic cells in 1976, but only to produce granulocytic cells

(Allen and Dexter, 1976). Numerous attempts have been made since then which resulted in identification of various cytokines for the growth and differentiation of HSCs. HSCs and their progenitors now can be maintained in a defined culture medium in the presence of cytokines (Miller and Eaves, 1997). However, the degree of the expansion of HSCs under *in vitro* cultivation is still modest, while such expansion through serial transplantation in irradiated mice was shown to be more than 8000-fold (Iscove and Nawa, 1997; Sauvageau et al., 2004).

(23) Isolation and *in vitro* cultivation of tissue stem cells are now greatly facilitated by identification of various stem cell-specific marker proteins (http://stemcells.nih.gov/info/scireport/appendixe). Among these, cell-surface marker proteins are particularly useful to isolate tissue stem cells by fluorescence-activated cell sorting (FACS) (Gundry et al., 2008). FACS sorting of HSCs relies on specific cell surface markers, such as c-kit tyrosine kinase receptor (SCF), stem cell antigen-1 (SCA-1) and CD34 (Shizuru et al., 2005). Cell surface markers (Table 3.2.) are described in the Annexes for tissue specific stem cells.

(24) In addition to the cell surface markers, a unique cellular property of the side population (SP) phenotype is shared by many tissue stem cells. This was exploited to enrich and isolate stem cells using flow cytometry. When bone marrow cells are stained with fluorescence dyes of Rhodamine 123 and Hoechst 33342, the most-weakly-stained fraction is found to contain long-term HSCs. This weak staining is associated with the low metabolic and mitotic activities of quiescent HSCs (Bertoncello and Williams, 2004). A high expression of the ABC transporter, breakpoint cluster region psueudogene 1 (Bcrp1), and the resulting efficient efflux of the dye are responsible for the SP phenotype of the quiescent HSCs (Zhou et al., 2001).

(25) Isolation and cultivation of stem cells were also accomplished by exploiting another unique feature. Neural stem cells (NSCs) and mammary gland stem cells (MaSCs) exhibit the SP phenotype as in HSCs, but in addition, they form spheroids when cultured *in vitro* (Annex B). A majority of cells died when a single cell suspension of the periventricular region of the adult mouse brain was cultured in a medium supplemented with epidermal growth factor (EGF). However, a small population of cells (about 1%) grew and formed spheroids (Reynolds and Weiss, 1992). Neurospheres, as they were called, were enriched with NSCs and their progenitor cells. Neurosphere formation is a valuable tool for quantitative assessment of radiation effects on the neural cells and such assay has been conducted on rat spinal cord stem cells (Lu and Wong, 2005). Spheroid formation was also noted for human MaSCs cultivated in the presence of EGF and/or basic fibroblast growth factor (bFGF) (Dontu et al., 2003). As is the case for the neurospheres, mammospheres also can be serially passaged. In addition, a single cell from a mammosphere can regenerate an entire mammary gland when transplanted into a mammary fat pad (Shackleton et al., 2006). *In vitro* cultivation and mammosphere formation offer a great opportunity in analyzing radioresponse and radiosensitivity of MaSCs, considering that mammary gland is one of the highly susceptible tissues for radiation carcinogenesis.

(26) HSCs originate from endodermal tissues, and NSCs and MaSCs from ectodermal tissues (Annex A). In addition, MSCs can be propagated successfully *in vitro* (Chamberlain et al., 2007). Thus, stem cells are expected to be isolatable from almost all of the tissue types. One problem of the current systems of FACS-mediated isolation and *in vitro* cultivation of stem cells is that the propagated population, whilst enriched for stem cells, still contains their descendants. Isolation of a pure stem cell population is yet to be accomplished.

(27) Recent advances of stem cell research have demonstrated a hierarchy of stem cells, especially for tissues of rapid turnover rate. For example, stem cells of the haematopoietic system can be classified into at least long-term HSCs and short-term HSCs, the former being

more primitive than the latter (Annex A). As for the small intestine, three types of stem cells have been identified within the various cell populations present in murine crypts (Annex D). There are two distinct stem cell populations located at the 4th position from the crypt base, which are highly apoptosis-sensitive P4 stem cells and highly-radioresistant stem cells with mouse telomerase reverse transcriptase (mTert) expression. The third type of stem cells are rapidly-cycling columnar cells at the crypt base, which are positive with leucine-rich repeat-containing G protein-coupled receptor 5 (Lgr5). In the lung, there are region-specific stem cells (Annex E); bronchioalveolar stem cells (BASCs), Clara cells and Clara variant cells whose hierarchical interplay needs to be further clarified. Human skin seems to have a more clear structure of epidermal stem cells (EpiSCs), early progenitors and late progenitors, each giving rise respectively to basal cell carcinoma (BCC), squamous cell carcinoma (SCC) and papilloma (Annex F).

2.3. Radiosensitivity and DNA damage response of tissue stem cells

2.3.1. Basic strategies of tissue stem cells for the maintenance of genomic integrity

(28) Current knowledge on DNA damage response pathways was summarised by ICRP in Publication 99 (2005). DNA damage response is particularly important for maintaining the genomic integrity of stem cells, since they have to supplement all the functional cells in a tissue throughout life. This can be achieved by minimising DNA damage, cell divisions and DNA replication, maximising DNA damage repair, and eliminating damaged/mutated cells. Indeed, failures of DNA damage response and DNA repair have been implicated repeatedly in human premature ageing syndromes, many of which can be viewed as the consequence of a premature exhaustion of tissue stem cells, and human cancer-prone syndromes (Friedberg et al., 2006). Abundance of antioxidants in stem cells and the provision of a hypoxic microenvironment by the stem cell niche contribute to minimising DNA damage for some tissue stem cells. Quiescence is a feature shared by stem cells of a variety of tissues which facilitates DNA repair and minimises accumulation of replication-mediated mutation. Alternatively, damaged stem cells can be eliminated by apoptosis and progression to more differentiated compartments of a tissue such as progenitor and functional compartments. Competition of stem cells for occupancy of the tissue stem cell niche is likely to help in eliminating damaged stem cells.

2.3.2. Radiosensitivity of tissue stem cells

(29) The DNA repair capacity of tissue stem cells is reflected in their radiosensitivity. Radiosensitivity of stem cells can be assessed by clonogenic assays *in vitro* or *in vivo* using transplantation or *in situ* techniques. The results of such analyses are shown for epithelial colony-forming cells (Fig. 2.3.). However, these assays generally cannot distinguish radiosensitivity of stem cells and progenitor cells of epithelial tissues since both types can be clonogenic cells whenever necessary.

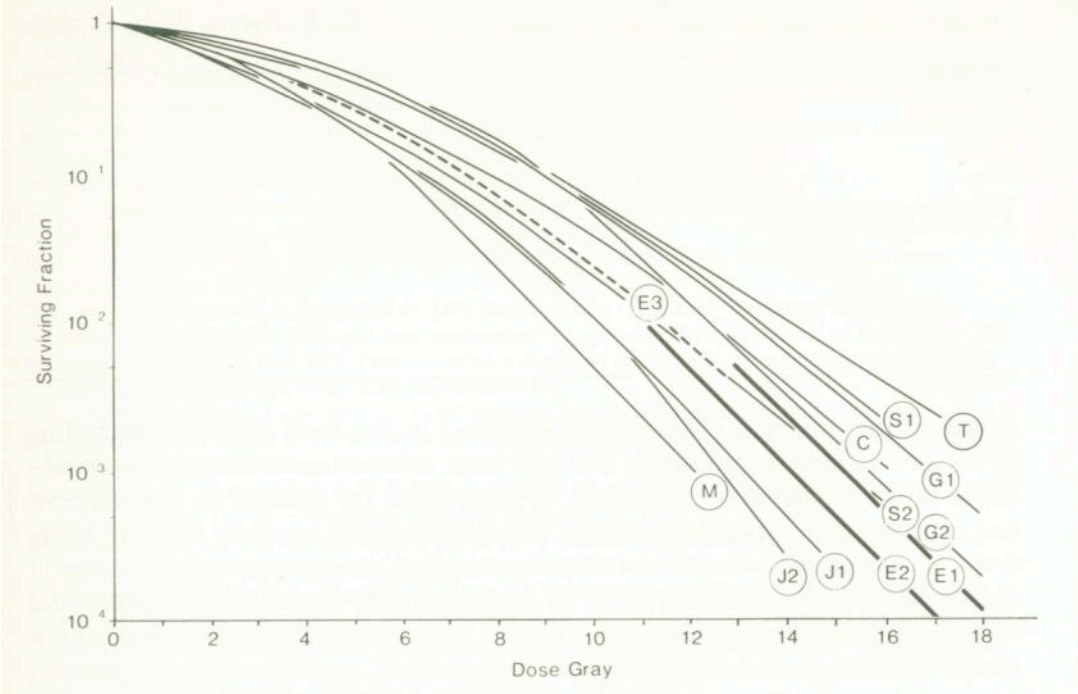

Fig. 2.3. Deduced survival curves for epithelial clonogenic cells in various tissues (Potten and Hendry, 1983). (Permission needed)
J1, J2 – cells regenerating jejunal crypts.
G1, G2 – cells regenerating gastric crypts.
C – cells regenerating colonic crypts.
E1, E2, E3 – cells generating macroscopic epidermal clones.
S1 – cells regenerating spermatogenic tubules.
M – mammary tissue-forming units. Cells transplanted at 24 hours after irradiation *in situ*.
T – thyroid follicle-forming units. Cells transplanted at 24 hours after irradiation *in situ*.

(30) A moderate PLDR capacity was demonstrated for clonogenic cells of rat mammary gland, thyroid, liver and bone marrow (Gould et al., 1984; Mulcahy et al., 1980; Jirtle et al., 1985; Kamiya et al., 1991). A general feature in the epithelial systems was the increase in survival when the cells were left *in situ* for 24 hours before transplantation *in vivo* to measure colony formation. This had the effect of shifting the survival curve to higher doses (Fig. 2.4.). The doses used in those PLDR experiments were mostly >6 Gy, and hence any changes in the 'α' component at lower doses were not directly measured. But the survival curve, assessed after a 24-hour recovery period, did have more curvature, i.e. the α/β ratio was lower, which indicates that the decrease in α was more than any decrease in β. Examination of the effect of further delay times before assay was performed using quiescent liver *in vivo*, with hepatocytes transplanted into fat pads for colony growth (Fisher et al., 1988). For these particular cells, the change at 24 hours was a decrease in survival-curve slope, which continued decreasing to the maximum 11 months delay examined. Cell proliferation was insufficient to explain the long-term reduction in radiosensitivity in terms of a dose-dependent replacement of damaged cells. Although there was a reduction in the frequency of cells with micronuclei postirradiation, the magnitude of this decrease was relatively small. Thus the long-term increase in clonogenicity could be explained only partially in terms of long-term repair of chromosome injury, assessed by the production of micronuclei. In addition, dose fractionation experiments were conducted, where the hepatocytes were assayed for survival either early or late after irradiation *in situ* (Fisher and Hendry, 1988). When the

assay was delayed 10 months, the value of α showed a tendency to decrease only slightly. The β component showed the greatest decrease with time, and the α/β ratio (1-1.6 Gy at 24 hours) remained low but slightly increased to 1.9-2.1 Gy at 10 months. It should be noted that these tissue systems (thyroid, mammary gland, liver), all have different kinetic, lineage, and structural properties, and hence postirradiation temporal changes may be tissue specific. These clonogenic assays also showed that bone-marrow-derived progenitor cells were more radiosensitive than epithelial clonogenic cells (Fig. 2.4.) (Hendry, 1985). The exact molecular mechanism of PLDR has not been elucidated, but ATM, the master gene of radiation damage response, has been suggested to be involved in the process although a number of uncertainties are yet to be resolved (Lobrich and Jeggo, 2005).

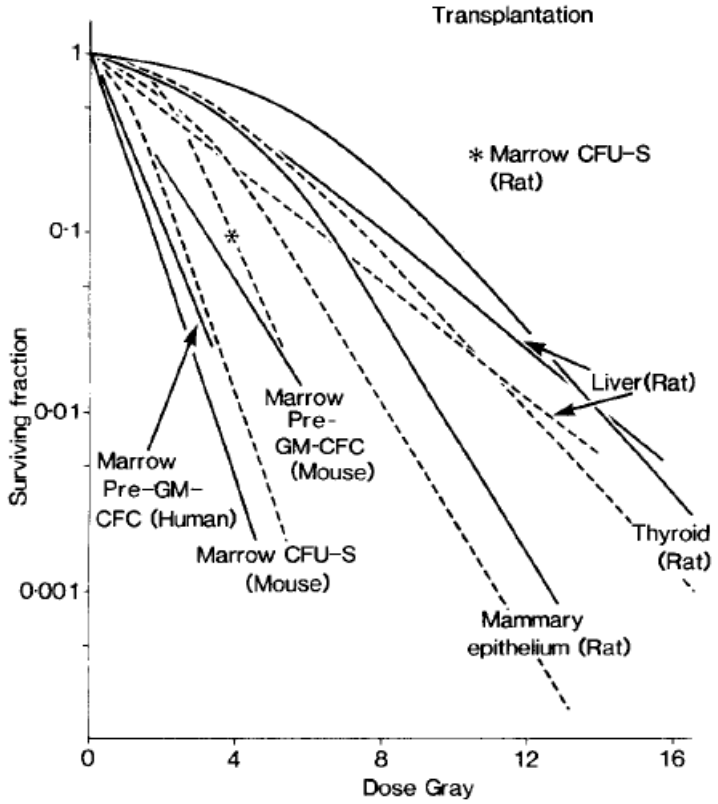

Fig. 2.4. Survival curves assessed *in vivo* by transplantation. Dashed lines: cells transplanted immediately after irradiation. Solid lines: cells transplanted at 24 hours after irradiation (or 3-4 hours in the case of marrow Pre-GM-CFS). The separation between the dashed and solid lines for each tissue type indicates PLDR; note the positive effect in the case of mammary and thyroid epithelial cells, and liver (hepatocytes), and in contrast the negative effect for marrow CFU-S considered due to a radiation-induced differentiation effect (Hendry, 1985). (Permission needed)

(31) Recent technological advances enabled *in vitro* propagation of relatively pure population of tissue stem cells which permits direct analyses of tissue stem cells. Human bone-marrow-derived clonally-expanded MSCs were directly analysed for their radiosensitivity. It was found that they were more radioresistant than human lung and breast cancer cell lines and this was found to be due to a better antioxidant capacity of the cells (Chen et al., 2006). As discussed in Annex F, skin stem cells when tested *in vitro* were more radioresistant than progenitor cells (Harfouche et al., 2010).

(32) A summary of radiosensitivities in relation to cellular stage in various hierarchical lineages is given in Table 2.1. Of note is the high radiosensitivity observed for cells that

undergo predominantly an apoptotic form of cell killing, e.g. some intestinal stem cells (ISCs), and types A, intermediate, and B spermatogonia.

Table 2.1. Radiosensitivity in relation to hierarchical status (updated from Potten and Hendry, 1983).

Tissue hierarchy	*Radiosensitivity*	*D_0 (Gy)*	Annex
Bone marrow, stem/progenitor (clonogenic)	High	0.8-1.2	A
Transit granulocytic	Medium	1.2-1.8	
Transit erythroid	Very high	0.5-0.7	
Mature cells	Low	-	
Fibroblastoid CFU-F	Medium	2.2	
Mammary stem cells	Medium	~2	B
Thyroid stem cells	Medium	2.0-3.5	C
Intestinal epithelium			
some P4 stem cells (SI)	Very high	0.1-0.2	D
CBCC	Medium	-	
m-Tert	Resistant	-	
Clonogenic cells (SI and colon)	Medium	1.0	
Transit cells	Low	-	
Functional cells	Very low	-	
Epidermis: stem (clonogenic) cells	Medium	1.4	F
Transit cells	Low	-	
Functional cells	Very low	-	
Testis Spermatogonia stem (clonogenic) cells	Medium	1.7-2.4	-
Type A	High	0.3-0.8	
Intermediate	Very high	0.3	
Type B	Very high	0.2	
Functional (spermatocytes)	Low	-	
Sertoli cells	Very low	-	

CFU-F, fibroblastoid colony-forming units; SI, small intestine; CBCC, crypt base columnar cells.

2.3.3. Quiescence/dormancy of stem cells and DNA damage response

(33) Mouse embryos before the compaction stage were shown to possess a unique p53-dependent S-phase checkpoint with no activation of p21 (Shimura et al., 2002; Adiga et al., 2007). ES cells derived from the blastocyst stage were shown to lack p53-dependent p21-activation after X-irradiation (Aladjem et al., 1998; Malashicheva et al., 2000). The cells of these stages are rapidly dividing, yet their DNA damage response differs considerably from that studied in cells *in vitro*.

(34) The DNA damage responses of adult tissue stem cells also vary, especially when analysed in the context of tissue microenvironments. For example, the P4 stem cells of mouse small intestine are known for their high sensitivity to radiation induced apoptosis (Potten, 1977; Potten et al., 2002; Potten, 2004b) (Annex D). This altruistic cell death was thought to

be a mechanism to eliminate damaged cells and therefore to maintain genomic integrity. Interestingly, some of the P4 cells were the first to undergo DNA replication after irradiation and pass through the p53/p21 repair pathway (Potten et al., 2009). Apoptosis of P4 stem cells occurs in two phases. Early p53-dependent apoptosis at 4.5 hours after irradiation was induced at doses below 1 Gy and delayed p53-independent apoptosis at 24 hours after higher doses such as 8 Gy (Dove et al., 1998). Absence of the early p53-dependent apoptosis of crypt stem cells in p53-null mice was restored when combined with the homozygous loss of the DNA-PKcs gene (Gurley et al., 2009).

(35) In addition to P4 stem cells, $Lgr5^{+}$ crypt base columnar cells (CBCCs) were claimed to be the primary stem cells. These cells divide rapidly at a cycling time of 24 hours and are less sensitive to apoptosis (Barker et al., 2007). Extensive evaluation of the past and more recent publications, however, led to a conclusion that the P4 cells are the likely stem cells of small intestine, and that the CBCCs are the intermediate progenitors possibly differentiating into Paneth cells (Potten et al., 2009). Furthermore, a very small subpopulation of cells was identified among the P4 stem cells of mTert-expressing mouse intestinal crypt. These $mTert^{+}$ cells were quiescent and did not exhibit apoptotic cell death even after 10 Gy (Montgomery et al., 2011). They gave rise to all the cell types in the small intestine, including $Lgr5^{+}$ cells. Although further experiments need to be conducted, $mTert^{+}$ P4 cells are probably the most primitive stem cells of the small intestinal mucosa. They are rare and quiescent, and they are insensitive to radiation-induced apoptosis. This suggests that sensitivity to altruistic cell death may not be a universal radiation response of tissue stem cells.

(36) Dormancy or quiescence is a general feature of many tissue stem cells. For the quiescence of stem cells, the damage sensor ataxia telangiectasia mutated (ATM) serves an essential role for HSCs (Ito, 2004). $ATM^{-/-}$ mice displayed premature depletion of HSCs in the bone marrow. The ROS level was high in $ATM^{-/-}$ mice and the high ROS level activated p38 mitogen-activated protein kinase (MAPK) to force the quiescent HSC into cell cycling, which then resulted in the exhaustion of HSCs (Ito et al., 2006; Liu and Finkel, 2006). Thus, cellular senescence eliminates overly-replicated stem cells, and quiescence is a mechanism to maintain stem cell potential. Further studies demonstrated that p53 and p21 are also involved in the quiescent state of HSCs (Liu et al., 2010; Cheng et al., 2000).

(37) The DNA damage response of stem cells includes loss of stemness (characteristics which underlie self-renewal and the ability to generate differentiated progeny) which results in differentiation. A recent study has demonstrated that melanocyte stem cells undergo terminal differentiation in the niche when exposed to radiation. ATM was found to be involved in this terminal differentiation of melanocyte stem cells since the lack of its function sensitises mouse skin to radiation induction of hair greying (Inomata et al., 2009). As in the case of quiescence, p53 is also involved in regulating cellular senescence, in addition to ATM kinase (Vigneron and Vousden, 2010).

2.3.4. DNA repair in stem cells

(38) DNA repair in tissue stem cells might well differ from cells of other types since it is known that the DNA repair pathway is dependent on their stage of differentiation. For example, the nucleotide excision repair pathway was reported to be attenuated in terminally differentiated cells (Rasko et al., 1993; Nouspikel and Hanawalt, 2002; Hsu et al., 2007). Mouse models defective in excision repair pathways exhibited tissue specific differences in mutagenesis and carcinogenesis, demonstrating that the repair system may differ between stem cells of different origins (Wijnhoven et al., 2007). It is tempting to speculate that the DNA repair pathways are responding to the demands of specific cell types. Mice have been

created with specific defects in damage response and repair, and they provide excellent models to study the role of tissue stem cells. As for the ageing of mice, the effect of repair defects usually manifests more severely in tissues of rapid turnover such as haematopoietic tissue (Park et al., 2005). HSCs from ageing mice were shown to have upregulated expression of a series of stress-responsive genes, suggesting a strong correlation of ageing, DNA damage and stress responses (Chambers et al., 2007). These studies demonstrate that DNA repair and damage response play important roles for tissue stem cells, to stay quiescent and to preserve genomic stability.

(39) Ionising radiations induce DSBs which are repaired either by HR or NHEJ. HR is potentially error-free since it takes place in S and G2 phase cells to repair the damaged region of DNA by copying the intact counterpart of the sister DNA strand. SLDR was shown to be dependent on Rad54, and therefore, represents the repair activity of HR (Rao et al., 2007). NHEJ takes place in non-cycling cells and in all cycle phases to a varying degree, and is dependent on the repair proteins Ku70, Ku80 and DNA-PKcs. PLDR is the repair of non-cycling cells and thus represents the repair activity of NHEJ.

(40) ES cells with either $Rad54^{-/-}$ or $Ku70^{-/-}$ were found to be equally sensitive to ionising radiation, showing the importance of both repair pathways (Gu et al., 1997). In contrast, although adult $Ku80^{-/-}$ mice and $DNA\text{-}PKcs^{-/-}$ mice are sensitive to radiation, adult $Rad54^{-/-}$ mice exhibit hypersensitivity only when combined with the DNA-PK deficiency (Essers et al., 2000). A series of mice defective in DSB repair has been generated and characterised (Brugmans et al., 2007). Among these mice, DNA ligase IV defective mice exhibit premature ageing of HSCs (Nijnik et al., 2007). These indicate that NHEJ is likely to be the major pathway of radiation damage repair in adult tissue stem cells. The dormancy/quiescence of tissue stem cells is essential especially for the tissues producing vast numbers of cells such as haematopoietic and gastrointestinal (GI) tissues. In the non-cycling quiescent tissue stem cells, the NHEJ pathway is the only way to repair DNA damage. Thus, the NHEJ pathway is associated with PLDR, which is defined operationally as the repair occurring in the stationary phase non-cycling cells. Hence it is reasonable that tissue stem cells exhibit a large capacity of PLDR as shown by *in vivo* and *in situ* clonogenic assays (Fig. 2.4.) (Hendry, 1985).

(41) Quiescence of stem cells poses two problems to the strategy of tissue stem cells in maintaining the integrity of the genome. Firstly, quiescent stem cells rely on NHEJ, but this repair pathway is considered to be error-prone. In addition, DNA damage accumulates in the quiescent stem cells, as demonstrated in HSCs by the occurrence of spontaneous γH2AX foci in ageing mice (Rossi et al., 2007). Radiosensitivity was tested for haematopoietic stem and progenitor cells (HSPCs: $Sca\text{-}1^{+}$, $CD34^{-}$), common myeloid progenitors (CMPs: $Sca\text{-}1^{-}$, $CD34^{+}$) and granulocyte/macrophage progenitors (GMPs: $Sca\text{-}1^{-}$, $CD34^{+}$). Their radiosensitivity as tested directly by the *in vitro* clonogenic assay demonstrated that quiescent HSPCs were more radioresistant than CMPs and GMPs, as expected (Mohrin et al., 2010). However, the frequency of chromosome aberrations after 2 Gy irradiation, as measured by SKY analyses, is higher by more than two-fold for the quiescent $CD34^{-}$ HSPCs than the $CD34^{+}$ two cell types. A stem-cell-enriched haematopoietic cell population was reported to exhibit similar chromosome sensitivity to that of peripheral lymphocytes, but the cell population in that study was $CD34^{+}$. Therefore, conclusive judgment cannot be made at present from the study on the chromosome sensitivity of $CD34^{+}$ HSPCs (Becker et al., 2009).

(42) These results may suggest that PLDR executed by the NHEJ pathway confers better survival of quiescent tissue stem cells, but it may also cause more chromosome mutations. Consistent with this notion, the classic study of PLDR using V79 cells also indicated that the irradiated cells kept in the stationary phase exhibited better survival, but the HPRT mutation

frequency stayed the same irrespective of the holding time (Thacker and Stretch, 1983). Yet, a recent study indicated that irradiated human diploid fibroblasts have less chromosome aberrations when they are in the non-cycling G_0 phase than in cycling G_1 phase (Liu et al., 2010). Further analyses need to be made to clarify the role of DNA repair in relation to colony survival and mutagenesis of quiescent tissue stem cells.

2.3.5. Cairns' hypothesis: suppression of replication-mediated mutation in tissue stem cells

(43) Since high dose radiation is not common in natural conditions, coping with radiation damage is not of importance to the normal maintenance and function of tissue stem cells. Rather, avoiding naturally occurring mutagenic events is more important. Among such events, DNA replication is an unavoidable source of mutation. An interesting hypothesis was proposed by Cairns (1975). The 'immortal strand' hypothesis proposed asymmetric segregation of DNA strands to minimise the replication error in the tissue stem cells. The stem cell retains the template DNA strand after a round of DNA synthesis, while the progenitor cells inherit the daughter strand. Since the template strand is never replaced, the replication error is minimised for tissue stem cells, while the strands with possible errors are passed to the progenitors which are eventually lost by differentiation/maturation into functional cells. The evidence supporting the hypothesis was presented in mice injected with ^{3}H-thymidine at infancy which carried the long-term LRCs in the stem cell region of rapidly proliferating intestinal crypts (Potten et al., 2002). In order for the template strand to stay on one side of the sister chromatids, there should be no recombination in the tissue stem cells. This requirement is likely to make stem cells to be 'recombination minus' which then leaves NHEJ as the only legitimate repair system in stem cells. Also, the template-strand hypothesis requires that all sister chromatids with the template strands have to segregate to the stem-cell side of the spindle poles at mitosis, with the one-to-one connection of old centrosomes and old centromeres. However, such requirements still have to be shown on a molecular basis.

(44) Proponents of the immortal strand hypothesis rely mostly on histological data indicating the presence of LRCs, and P4 stem cells in mouse intestinal crypts were shown to retain ^{3}H-thymidine or bromodeoxyuridine (BrdU) for a long time (Potten et al., 2009). Asymmetric chromosome segregation is currently not generalised for all tissue stem cells, and the immortal strand hypothesis remains under critical debate (Rando, 2007; Lansdorp, 2007). It was demonstrated that asymmetric segregation of DNA strands does not take place at least in HSCs and in hair follicle stem cells (Kiel et al., 2007; Waghmare et al., 2008). However, strand-specific segregation was reported on chromosome 7 in mouse neuronal cells (Armakolas and Klar, 2006). Furthermore, CO-FISH technique was used to address the problem. The results indicated that whereas the segregation of sister chromatids was random in mouse fibroblasts and ES cells, the segregation in mouse colon cells was not random (Falconer et al., 2010). The asymmetric segregation of the immortal sister chromatid requires suppression of the HR pathway, and at least, this is consistent with the reliance of tissue stem cells on the NHEJ repair pathway, rather than the HR pathway. The immortal strand hypothesis is likely to remain controversial until a more definitive analysis becomes possible.

2.4. Ageing and exhaustion of tissue stem cells

2.4.1. Mortal nature of tissue stem cells

(45) Tissue stem cells divide and replenish the tissue for the entire life of an individual. Tissue stem cells were once thought to be immortal and immune to cellular senescence. One of the determinants of cellular senescence is the loss of telomeres through DNA replication. Rapidly replicating cells therefore usually have telomerase activity to avoid telomere shortening (Blasco, 2007). Indeed, tissue stem cells were reported to possess telomerase activity, and this has been confirmed in a variety of tissue stem cells (Harrington, 2004). However, mouse and human HSCs nevertheless lose telomeric DNA with serial passages of cells *in vitro* and *in vivo* ageing of animals (Vaziri et al., 1994; Allsopp et al., 2001). Thus, adult tissue stem cells are unlikely to be immortal, although their division potential is enormous, e.g. as shown in bone marrow and intestine. Telomerase-deficient mice were reported to exhibit pronounced ageing with atrophy in rapidly proliferating tissues such as bone marrow, intestine and testis (Lee et al., 1998; Rudolph et al., 1999). There is now ample evidence to demonstrate that telomere shortening takes place in stem cells of an ageing body (Flores et al., 2006).

2.4.2. Telomere length of stem cells

(46) As discussed, tissue stem cells have limited telomerase activity so that erosion of telomere ends through rounds of DNA replication is inevitable. Therefore tissue stem cells have to possess mechanisms to prevent the loss of telomeres. One way is to have efficient repair of DNA damage, and failure to do this results in the loss of cells which necessitates compensatory replication of stem cells in a tissue. Therefore, damage checkpoints and DNA repair are important for tissue stem cells. Also, quiescence in the well-protected microenvironment of the tissue stem cell niche acts to promote the genomic integrity of tissue stem cells. Indeed, tissue stem cells in their niche seem to have the longest telomeres, as quantitative FISH has revealed for mouse hair follicles, small intestine, testis, cornea and brain (Flores et al., 2008). It is interesting to note that in this particular study, fluorescent signals for telomeres of the $Lgr5^{+}$ CBCCs were less than those of the P4 cells, implying the former being higher in the hierarchy of ISCs. Thus, even though tissue stem cells have telomerase activity and the longest telomeres, they nevertheless shorten their length in ageing mice. Shortening of telomeres can also be accelerated when HSCs are forced to replicate by serial bone marrow transplantation (Allsopp et al., 2001).

2.4.3. Telomere shortening and carcinogenesis

(47) When telomeres become critically short, the chromosome ends lose protection. The exposed chromosome ends are recognised as DSBs. DSBs are frequently rejoined incorrectly to create dicentric chromosomes which either block cell division or break apart to create new double-strand ends at mitosis. This breakage-fusion-bridge cycle can induce genomic instability in the cells, as human embryonic kidney cells undergoing replicative shortening of telomeres were shown to exhibit chromosome instability (Counter et al., 1992). Telomere shortening and chromosome instability lead to other consequences by activating DNA damage responses including focus formation of γH2AX, p53-binding protein 1 (53BP1), meiotic recombination 11 (Mre11) complexes and phosphorylated ATM (Takai et al., 2003). Resulting DNA damage responses culminate in activation of a series of p53-mediated responses including apoptotic cell death and/or cellular senescence (Karlseder et al., 1999; d'Adda di Fagagna et al., 2003). Senescence acts as a powerful block for carcinogenesis, as telomerase-deficient mice were found to resist chemical induction of skin cancer (Gonzalez-Suarez et al., 2000). Cellular senescence and apoptosis led to loss of stem cells, forcing them

to undergo further replication in order to maintain tissue homeostasis. This creates a vicious cycle of the additional loss of stem cells in tissues. Erosion of telomeres due to natural or forced replications thus leads to stem cell exhaustion, which is a hallmark of ageing tissues.

(48) Cellular senescence brought about by short telomeres may act as a block to carcinogenesis. However, short telomeres and the resulting genomic instability are associated with cancer induction in mice and humans (Murnane, 2012). Although mice lacking telomerase exhibit premature ageing phenotypes, the same mice on the p53 null genetic background are highly prone to developing epithelial cancers (Artandi and DePinho, 2010). Interestingly, the p53-null allele does not have to be homozygous, and heterozygosity was enough for high rates of cancers in these mice. Also, a $p53^{+/-}$ genetic background restituted the premature ageing and stem-cell exhaustion phenotypes of telomerase^{-} mice (Flores et al., 2009). Thus, the status of p53 is the major determinant of the two opposing outcomes of telomere erosion in tissue stem cells: namely, loss of cellularity in ageing tissue, and unregulated cell proliferation in carcinogenesis.

2.5. Tissue stem cell niche

2.5.1. Stem cell niche

(49) In homeostasis conditions, a tissue stem cell in adults is considered to divide asymmetrically to produce a stem cell and a progenitor cell. This asymmetry of cell division requires spatial asymmetry of the stem cell microenvironment, and the stem cell niche provides such a cue for the asymmetry (Watt et al., 2000). In the stem cell niche, stem cells attach proximal to specific stromas while the daughter cell positions are distal to them. Asymmetric expression of adhesion molecules on one side of a cell assures the specific interaction, which in turn results in the signalling by short acting factors mainly tissue specific cytokines to maintain the stemness of the cells. In addition, a study on the germline stem cells in the Drosophila testis demonstrated that an alignment of centrosomes plays a critical role in the division of stem cells perpendicular to the stroma (Yamashita et al., 2007). In the Drosophila testis, the stem cells are positioned proximal to the stromal cells in the germline niche and green fluorescent protein (GFP)-tagged centrosomes align with the proximal to distal direction. This structural feature of stem cells and their centrosomes are shared by mammalian tissue (Fuchs, 2004).

(50) Positional information of the stem cell niche is therefore pivotal to the hierarchial structuring of cells in a tissue by activating expression of a series of genes. This means that the gene expression patterns and the fates of stem cells, progenitors and terminally differentiated cells could be reversibly modified by changing the positional information, or by directly modifying the expression of fate-determining genes. In the Drosophila testis, progenitor cells were demonstrated to become stem cells by taking over the vacant stem cell niche (Cheng, 2008; Cheng et al., 2008). This dedifferentiation is associated with a specific pattern of gene expression. Direct manipulation of genes led to dedifferentiation and trans-differentiation of cells as demonstrated by a case of Pax5-deficient mature B cells which dedifferentiate and then trans-differentiate into T cells (Cobaleda et al., 2007). An extreme case of dedifferentiation and trans-differentiation is the conversion of mouse fibroblasts to induced pluripotent stem (iPS) cells by ectopic expression of 4 stem-specific genes: octamer-binding transcription factors 3/4 (Oct3/4), sex determining region Y-box 2 (Sox2), c-Myc and Kruppel-like factor 4 (Klf4) (Takahashi and Yamanaka, 2006). All of the recent studies thus indicate that the fate of cells is re-programmable.

2.5.2. Stem cell niche as a shelter

(51) The stem cell niche was shown to promote rapid establishment of the stem cell pool after genotoxic insults such as radiation exposure, and transplantation of MSCc rescues lethally-irradiated mice through their HSC-niche modulating activity (Lange et al., 2011). In the steady state, the stem cell niche was shown to provide a shelter from various genotoxic stresses. Tissue stem cells have to sustain themselves for the entire life span of an individual, and they have a number of strategies to circumvent genotoxic stresses. One of the strategies to escape from ROS is to rely on the intracellular antioxidants, as in the case of MSCs (Chen et al., 2006). Another strategy is to stay quiescent in a low oxygen environment, and the stem cell niche of HSC is such an example (Suda et al., 2007).

(52) There are three niches of HSCs in the bone marrow, an osteoblastic niche, a vascular niche and a medullary niche (Annex A) (Shiozawa and Taichman, 2012). HSCs in the former niche are in close association with osteoblastic cells, thus two most important targets of radiation carcinogenesis are sharing the same microenvironment in a tissue (Zhang et al., 2003; Calvi et al., 2003). HSCs are quiescent and this state of cells is dependent on their residence in the niche. The Tie2/angiopoietin 1 (Ang-1) signalling between the two cell types regulate quiescence of HSCs in the osteoblastic niche (Arai et al., 2004). Quiescence is a property shared by stem cells in other tissues, including EpiSCs (Nishikawa and Ozawa, 2007). Also, many types of tissue stem cells, such as those of neural, mammary, mesenchymal and adipose tissues, as well as ES, favour a hypoxic condition for their stable persistence *in vitro* and *in vivo* (Ezashi et al., 2005; Danet et al., 2003; Ivanovic et al., 2000; Grayson et al., 2007; Zhu et al., 2005; Lin et al., 2006). The osteoblastic niche of HSCs is poorly vascularised, and some HSCs were shown to be stained strongly by hypoxia-sensitive pimonidazole, suggesting that the oxygen concentration of the niche is less than 2% (Parmar et al., 2007). HSCs residing in the osteoblastic niche are those of primitive and less committed types, and hypoxia is favoured to protect cells from endogenous ROS. Hence the role of protection given by the stem cell niche has to be considered when assessing the effect of radiation on tissue stem cells.

(53) Although hypoxia in the niche microenvironment is important, it is interesting to note that an appropriate level of ROS is also required for the maintenance of genomic stability in ES cells in culture (Li and Marban, 2010). The frequency of chromosome aberrations analysed in ES cells decreased with decreasing levels of oxygen, but increased when the cells were treated with antioxidants. This increase was associated with the depletion of expression of repair-related genes when ROS were completely absent.

2.5.3. Stem cell competition for residence of the niche

(54) In contrast to the classic concept of asymmetric cell division of stem cells, a number of studies revealed that stem cells often divide symmetrically to produce two stem cells, or to produce two committed stem cells/progenitor cells (Fig. 2.5.). In the former case, the surplus of stem cells can compete for residence in the niche, and inferior stem cells are eliminated. In the latter case, a vacant stem cell niche is created with two committed cells leaving the niche. These processes lead to turnover of stem cells, which once were thought to reside in a tissue for a lifelong period.

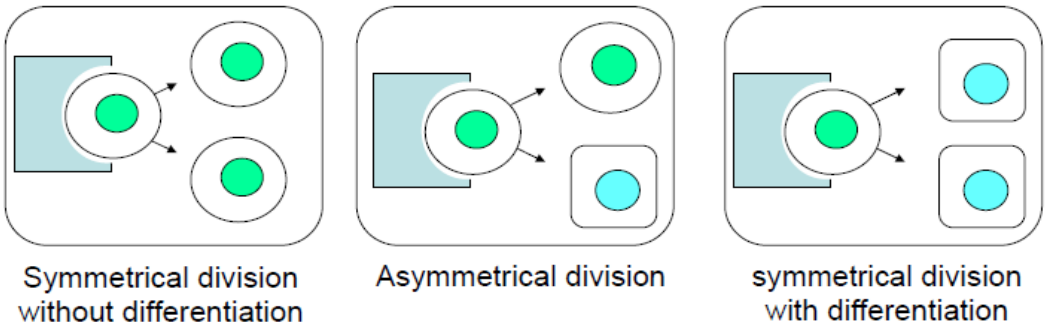

Fig. 2.5. Three division patterns of tissue stem cells.

(55) The first evidence of such competition and turnover comes from the analyses of mutant stem cells of intestinal crypts (Potten et al., 2009). For example, a mutant stem cell takes over the entire crypt in 5 to 7 weeks in colon and 12 weeks in small intestine after mutagen treatment of mice (Loeffler et al., 1993). Population expansion from a single stem cell and the resulting monoclonality of the crypt have also been shown by the lineage tagging of $Lgr5^{+}$ stem cells in intestinal crypts. From the pattern of the clone size distribution, it was concluded that ISCs are equipotential in replacing the neighbour, or being replaced by the neighbour, in a neutral drift fashion without directional pressures. Thus, ISCs are sometime lost, or take over the entire crypt (Lopez-Garcia et al., 2010). The lineage tagging method was applied to a variety of tissues and a similar competition and resulting turnover of stem cells were demonstrated in testicular germline stem cells (Lopez-Garcia et al., 2010; Klein et al., 2010) and in skin stem cells (Clayton et al., 2007).

2.5.4. Competition of stem cells during establishment of the adult stem cell niche

(56) Although dependent on the tissue type, the adult stem cell niche is usually established around the postnatal period after birth. For example, the intestinal tract in the mouse is formed as a simple tube of epithelial cells with high proliferative activity. The first differentiation of these equipotential epithelial cells, or fetal stem cells, is the formation of the villi at embryonic day 15, which are necessary for absorption of nutrition after birth. The formation of crypts which provide an adult-type stem-cell niche for the maintenance of tissue proliferation appears only at postnatal day 7 (Crosnier et al., 2006). It is important to note that while the stem cells left aside in the niche of newly-formed adult-type intestine are few, the number of fetal stem cells in the fetal intestine is substantial. This suggests a strong competition of stem cells for the occupancy of the niche. It is also interesting to note that the neonatal crypt is occupied by a polyclonal population of stem cells, and that the monoclonality is established at about 2 weeks after birth (Schmidt et al., 1988). This monoclonal conversion supports competition of fetal stem cells to result in the occupancy of a niche by a dominant clone.

(57) Haematopoietic development is first detected in the yolk sac region of embryos, which then shifts to an aorta/gonad/mesonephros site. A major site of haematopoiesis at the fetal stage is the liver, while the site in the adult is the red bone marrow. HSCs in the fetal liver migrate and settle in the bone marrow niche (Orkin and Zon, 2008). Although the migration of HSCs from liver to bone marrow is generally preserved among mammalian species, there appears to be distinct differences between the temporal patterns and major sites of haematopoiesis in the developing fetus of mice and humans: e.g. in the developing human

fetus, marrow steadily increases haematopoietic activity in the second half of gestation. In contrast, in the mouse, the liver is the dominant haematopoietic site from mid-gestation to birth, and only very late in gestation and into early postnatal period is there active migration of HSCs from the liver to skeletal sites. The process of 'adult niche development' appears to occur at different times postnatally in the two species.

(58) The fetal liver HSCs differ in their characteristics from adult bone marrow HSCs, in that the former are rapidly cycling while the latter are in a state of quiescence. The gradual shift in characteristics of HSCs takes place in the mouse 3 weeks after birth. Redistribution to bone marrow is selective rather than random at least in the mouse, so that HSCs are deficient in engraftment when they are transiting $S/G_2/M$ while those in G_1 successfully settle in the bone marrow niche (Bowie et al., 2006). Thus, the homing into the adult stem-cell niche during the neonatal stage development functions as a selective process in the case of mouse HSCs, where stem cells compete for residence the niche with retention of favourable cells and elimination of unwanted cells (Fig. 2.6.).

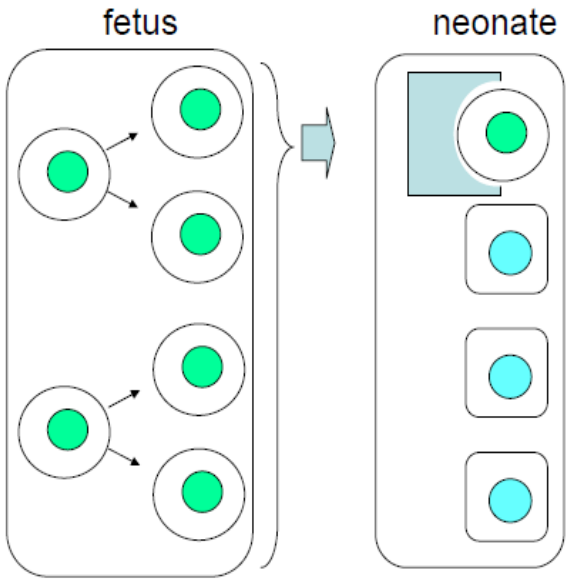

Fig. 2.6. Stem cell competition in the neonate. The competition is particularly strong when the adult tissue stem cell niche is established during the neonatal stage. (permission needed)

2.5.5. Effects of radiation on stem cell competition

(59) Irradiation affects the competition of HSCs for their residence in the bone marrow niche. When two marked populations were mixed and transplanted to lethally-irradiated mice, both populations contributed to the reconstituted HSCs equally. However, when one of the two populations was first exposed to 1 Gy and subsequently mixed with the second non-irradiated population prior to transplantation, the non-irradiated population predominated in the reconstituted marrow HSCs (Bondar and Medzhitov, 2010). In this competition, p53 played a crucial role in sensing the stress of irradiation, since the HSCs of a $p53^{+/-}$ or $p53^{-/-}$ genotype exposed to radiation were not outcompeted by the unirradiated wild-type population. The competitiveness was solely dependent on the p53 level, since bone marrow cells of $Mdm2^{+/-}$ mice with higher levels of p53 protein were outcompeted by those from the wild-type mice even without irradiation. Somewhat similar observations were made using bone marrow cells with the retrovirally-transduced p53 with functionally blocked dimerisation

domain, in which the cells were dominant over the wild-type cells after 2.5 Gy X irradiation (Marusyk et al., 2010). Altogether, stem cell competition for the residence of the tissue stem cell niche is sensitive to radiation stress which is sensed by p53.

3. THE ROLE OF TISSUE STEM CELLS IN RADIATION CARCINOGENESIS

3.1. The role of stem cells in radiation carcinogenesis

(60) This chapter aims to depict subjects of importance for basic processes of radiological health risk assessments and the development of guidelines deemed essential to the ICRP system of radiological protection. The first two sections deal with the mechanisms of carcinogenesis in tissues with a special emphasis on the role of tissue stem cells and other possible target cells with regard to the LNT model and the RR model. The discussion then moves into the radiation biology of stem cells, and the dynamic nature of stem cell competition in the tissue stem cell niche. This tissue level dynamism is likely to play a role when considering the dose rate effect, which in the past has been considered only from the cellular repair point of view. Stem cell competition is a new concept but it can explain some features of radiation risk, and more importantly, the age dependence of radiation carcinogenesis. Altogether, this chapter is devoted to bridge the advancement of knowledge of stem cell biology and issues of importance to radiation risk assessment.

3.1.1. Multistage carcinogenesis

(61) Cancer in adulthood is envisaged to arise as a result of accumulation of oncogenic mutations occurring mainly after birth, whereas some childhood cancers are characterised by mutations acquired during fetal development or inherited from the parents. The incidence of cancer in adults, especially solid cancer, exhibits a steady increase by age. Armitage and Doll (1954) noted that this increase follows roughly the fifth power of age and proposed the multistage carcinogenesis model. This was later supported by the molecular analysis of human colon cancer, in which the conversion of normal epithelial cells to adenoma and its progression to carcinoma were shown to be associated with a stepwise acquisition of mutations of oncogenes and tumour suppressor genes (Fig. D.8. of Annex D; Vogelstein et al., 1988). Acquisition of multiple mutations by spontaneous processes takes a long time, which explains why adulthood cancers arise late in life.

(62) Cancers in childhood form a unique group of neoplasias, which occur before puberty, between birth and 15 years of age. In contrast to adulthood cancer, childhood cancer such as retinoblastoma was found to require only two steps (Knudson, 1971). This two-step carcinogenesis process can explain why childhood cancer occurs with relatively short latency in early life before puberty. The reason for the difference in the number of steps (or mutations) for cancer of adulthood and childhood onsets is not fully understood. However, it is likely that these two differ in the target cell types, with some of the former being fetal-stage primitive cells and the resulting cancer often carries the suffix of “blastoma”, while the latter being adult tissue stem cells and progenitor cells.

(63) Animal experiments have shown that the process of stepwise carcinogenesis can be categorised into four steps: the initiation step with the irreversible change of a normal cell into a preneoplastic state, the promotion step with the proliferation and the clonal expansion of initiated cells, the malignant conversion step with an acquisition of neoplastic characteristics of cells, and the progression step with further accumulation of changes in cells with invasion to normal tissue territories. Each step is associated with functional changes of genes regulating cell proliferation, quiescence, differentiation, senescence and apoptosis (Perez-Losada and Balmain, 2003). These changes in gene functions are often brought about

by the genetic mechanism of mutation induction and by the epigenetic mechanism of transcription factors, chromatin modifications, DNA methylation and regulatory microRNA (miRNA) (Sharma et al., 2007). However, the present report attempts to restrict the discussion so as to focus on the multistage carcinogenesis model where mutations are the determinant. This restriction is made in order to simplify the discussion on the numerical aspects of radiation carcinogenesis. For the same reason, mechanistic models involving epigenetics and promotion/progression aspects are not considered.

3.1.2. Target cells for carcinogenesis

(64) The target cells for carcinogenesis are considered to be the tissue stem cells and their proximal progenitors (Reya et al., 2001). This assumption is considered reasonable because many characteristics of tissue stem cells resemble those of cancer cells, and the resemblance became particularly strong after the discovery of cancer stem cells in human leukaemia (Lapidot et al., 1994). Cancer stem cells are also found in a variety of solid tumour types arising in breast (Dick, 2003), brain (Singh et al., 2003; Hemmati et al., 2003), prostate (Lawson and Witte, 2007), liver (Roskams, 2006), and many other tissues. Cancer stem cells resemble normal tissue stem cells in many respects. They have capacities to self-renew and can initiate tumour formation when transplanted into appropriate hosts. Cancer stem cells exhibit the SP phenotype and therefore are resistant to chemotherapeutic agents as are normal tissue stem cells. Also, cancer stem cells *in vivo* are often quiescent, in contrast to rapidly proliferating non-stem cancer cells because of constitutive upregulation of damage checkpoints (Bao et al., 2006).

(65) These similarities suggest that cancer stem cells may arise from normal tissue stem cells and those of their proximal progenitors, which are able to regain stemness. A stem cell origin of cancer is suggested because the pool size of tissue stem cells in some cases correlates with the risk of carcinogenesis. For example, the number of mammary stem cells (MaSCs) is determined by the level of hormones *in utero*, and this suggests influences on subsequent breast cancer risk after birth (Trichopoulos, 1990). Similarly, body size of newborn babies is known to correlate with the leukaemia risk (Caughey and Michels, 2009). Involvement of insulin-like growth factor 1 (IGF1) was recently implicated in this correlation suggesting that either a larger target cell number or a higher proliferation rate of HSCs is related to leukaemogenesis (Chokkalingam et al., 2012).

(66) A stem cell origin of cancer is also reasonable, because this is the only cell type which has a long enough time of residence in the body to accumulate multiple mutations to gain malignant phenotypes, while progenitor cells do not have sufficient time (ICRP, 2005). It is interesting to note that the small intestine with high proliferation is refractory to carcinogenesis in humans, while the adjacent large intestine is prone to radiation carcinogenesis, demonstrating that not all stem cells give rise to cancer. A high sensitivity to apoptosis of small intestinal P4 stem cells was proposed as a reason for an extremely low cancer incidence in the small intestine (Li et al., 1992; Potten et al., 1992). Therefore, in addition to the number of target cells, their behaviour also plays a decisive role in carcinogenesis.

(67) There are examples, which suggest that lower-ranking stem cells and progenitor cells can be the target for carcinogenesis. The Cre recombinase-mediated loss of the APC gene in $Lgr5^{+}$ CBCCs, lower ranking to the P4 stem cells, was found to result in the formation of full adenomas (Barker et al., 2009). Also, in the human brain, precancerous lesions are known to arise first in the differentiated compartment, and poorly differentiated glioblastoma then evolves from a well-differentiated astrocytoma with a latency of 5-10 years

(Klihues and Cavenee, 2000). At the molecular level, inactivation of Ink4a/Arf was shown to trigger dedifferentiation of astrocytes, and further introduction of constitutionally active EGF receptor (EGFR) conferred the malignant glioma phenotype to the cells (Bachoo et al., 2002).

(68) In haematopoietic malignancy, progenitor cells retain a large proliferative capacity and are a common target of carcinogenesis, as many leukaemias and lymphomas carry committed phenotypes of particular lineages. In addition, progenitor cells were shown to become leukaemia stem cells by direct introduction of a fusion oncogene, MLL-AF9 (Krivtsov et al., 2006). The classic mouse model of radiation-induced thymic lymphomas was shown to arise from the $CD4^-/CD8^-$ progenitors in the thymic environment (Kominami and Niwa, 2006). Proliferation of leukaemia cells requires a bone marrow microenvironment, and that of lymphoma cells needs a lymph node environment. These two haematopoietic malignancies carry phenotypes of stem cells and committed progenitor cells, respectively.

(69) Skin is another tissue where progenitor cells in addition to the stem cells can form cancer. As discussed in Annex F, three cancer types are known in the skin; BCC, SCC and papilloma (section F.5, Fig. F.5.). EpiSCs were proposed to give rise to BCC commonly found in people of the European ancestry, early progenitor cells to give rise to more malignant SCC, and late progenitor cells to form benign papillomas. Although this is an interesting and informative model, how the early progenitor cells can resist the polarised flow of cells without being discarded is elusive.

3.1.3. Role of radiation in carcinogenesis

(70) The current model of radiation carcinogenesis assumes that radiation acts as a mutagen, and gives possibly one or two carcinogenic mutations to a target cell (ICRP, 2007). Radiation is known to induce DSBs especially those with clustered DNA damage, which are prone to inducing large mutations such as deletions and translocations. Induction of translocations follows an LQ dose response, while induction of small deletions is likely to follow a linear dose response. Deletion mutations inactivate tumour suppressor genes, while translocations activate proto-oncogenes by juxtaposing them to strong transcription promoter-elements or making fusion genes with oncogenic functions. These targeted actions of radiation are the theoretical foundation for the LNT model, which is used for projection of health risk at low dose and low dose rate.

(71) The direct involvement of radiation in inducing the oncogenic mutation has been tested experimentally by examining the presence of the transcripts of the rearranged genes, characteristic of leukaemias and thyroid cancer, in cells in culture. However, the dose required for such rearrangements was found to be 50 to 100 Gy, which is extremely high compared with the real situation where a few Gy induced those cancers (Ito et al., 1993a; Ito et al., 1993b). In addition, the characteristic rearrangement of RET/PTC elements in childhood thyroid cancer showed a strong age dependence of occurrence, casting doubt on whether such translocation is really due to radiation exposures (Annex C). These indicate that a signature of radiation in radiation-induced cancer has yet to be identified.

(72) Recently, an interesting mechanism was discovered which suggests a direct involvement of radiation in induction of multiple carcinogenic mutations. In contrast to the stepwise acquisition of mutations, genomic analyses of human cancer have demonstrated that 2-3% of all cancers and about 25% of bone cancers acquire multiple mutations by a single event (Stephens et al., 2011). Chromothripsis, as it is called, is multiple genomic rearrangements with sharply circumscribed regions of one or a few chromosomes, crisscrossing back and forth across involved regions. Involvement of a micronucleus in the generation of chromothripsis was demonstrated recently (Crasta et al., 2012). Micronuclei are

easily induced by radiation through a single hit process, yet its role has been implicated in cell death, and not in carcinogenesis. Thus, the role of chromothripsis in radiation carcinogenesis needs to be further investigated.

(73) In addition to these targeted actions, radiation is also known to act in a non-targeted fashion, which includes bystander effects and the induction of genomic instability especially in the low dose range (ICRP, 2007). Radiation has long been known to induce transient changes in gene expression, but some of these changes persist for a long time after irradiation. Indeed, the epigenetic mechanism of DNA methylation was recently shown to underlie the non-targeted effect of radiation (Goetz et al., 2011). Nevertheless, involvement of non-targeted and epigenetic effects have not yet been fully shown, since the deviation of dose responses for incidence and mortality from linearity is still weak in the low dose range for cancer among atomic bomb (A-bomb) survivors (ICRP, 2005, 2007). Furthermore, radiation is also known to change cell-to-cell and cell-to-tissue interactions in a tissue's microenvironment. In the case of the mammary gland, radiation has been shown to induce TGFβ from the matrix, which plays a significant role in mammary carcinogenesis (Nguyen et al., 2011). Irradiation modifies stem-cell-to-niche interaction by giving selective advantage to the Notch1 and p53-null stem cells for residence in the bone marrow niche, contributing to the further development of leukaemia (Marusyk, 2009; Marusyk et al., 2010). These studies suggest a variety of roles played by radiation in the induction of cancer. Nevertheless, the LNT model based on the targeted mechanism of radiation is still used widely to assess the risk at low dose and low dose-rate exposures since it fits fairly well with the epidemiological data of induction of cancer in radiation exposed human populations.

3.1.4. Models and the risk of radiation carcinogenesis

(74) The radiation dose-incidence relationship regarding solid cancers is fairly linear for A-bomb survivors, although there is recent evidence for curvilinearity when the dose range is limited to <2 Gy (Ozasa et al., 2012). In addition, a majority of solid tumours among the survivors occurred at a so-called 'cancer-prone' age, with a similar age-incidence trend as in the non-exposed population, but with the incidence being higher among the former in a dose-dependent manner. These effects are consistent with the assumption that radiation contributes one mutation out of multiple mutations necessary for full malignancy (ICRP, 2007). Assuming five mutations for cancer development, four of them are contributed by mutagenic factors other than radiation, including errors in DNA replication and chromosome segregation, and mutations induced by internal/external mutagens. Under this scenario, the RR model, also called the multiplicative model, is adequate for the risk assessment of cancer of adulthood onset since radiation is expected to increase, linearly with the dose, the proportion of cells with five mutations in populations, which already gained four mutations or to gain four mutations in the future. Thus, the net effect is that radiation increases the risk of cancer linearly to the dose and proportionally above the background incidence. However, the above considerations of mutations indicate that the role of radiation is relatively small in comparison to that of other mutagenic factors. This raises a question of whether or not it is reasonable to call a cancer radiation-induced, since radiation is not a major contributor to carcinogenesis. One may call such cancer radiation-associated, or radiation-related. However, the term radiation-induced has been kept for historical reasons and for simplicity of the discussion. In contrast to the RR model, the AR model (sometimes called the additive model) assumes that the radiation risk is independent of the background risk and depends linearly on the dose. Today, both RR and AR models appear to fit well the risk of solid cancers in the A-

bomb survivors' cohort, with coherent results about the modifying effects of age at exposure and attained age (Ozasa et al., 2012).

(75) Apart from its role as a descriptive model, the RR model implies an interesting practical application in the prospective management of carcinogenic risk of people after exposure to radiation, which is assumed to be impossible because of the nature of radiation as an absolute mutagen. As mentioned above, with the RR model, the extent of the risk from a dose of radiation is proportional to the background incidence. Hence any action taken which reduces the background incidence may also result in a reduction in the radiation-related increase of EAR. An example of this can be seen in the case of lung cancer from residential radon among smokers and non-smokers. The ERR for radon is similar at about 0.16 per 100 Bq/m^3 for *both* smokers and non-smokers, yet the background incidence at age 75 years differed about 25-fold between smokers and non-smokers (Darby et al., 2006). In experimental systems, caloric restriction was shown to reduce the spontaneous occurrence of myelocytic leukaemia in mice. In addition, the reduction was also noted in irradiated mice when caloric restriction was applied even after the radiation exposure (Yoshida et al., 1997). Application of caloric restriction and other measures were reviewed recently for their various effects on the incidence of radiation-induced cancer in experimental animals (Oliai and Young, 2013). These experimental findings imply that human health promoting actions like stopping smoking and improving dietary habits might lower not only the background incidence but also the radiation-related increase of some types of cancer. The benefit of such measures may be expected in theory for the cancer types where the RR model applies. Epidemiological studies could be designed to test whether such measures effectively reduce the future occurrence of cancer.

(76) In contrast to solid tumours, the dose response for leukaemia among A-bomb survivors, especially acute myeloid leukaemia (AML), has a strong quadratic as well as a linear component (Hsu et al., 2013). In addition, AML has a relatively short latency time after radiation exposure. These suggest the involvement of relatively fewer mutations such as two or so for this neoplasm. If the number of mutations is small, radiation can act as absolute carcinogen to supply all such mutations. Similarly, childhood cancer is known to be induced by a relatively smaller number of mutations such as two with shorter latency than adult-onset cancers, as discussed in section 3.1.1. The AR model is likely to fit with these cancer types, leukaemia and childhood cancer, since radiation is sufficient to induce cancer regardless of the background incidence in a particular target population.

(77) From the above observations based on current results, the following paradigm can be derived concerning the role of radiation, shape of dose response, latency and the radiation carcinogenesis model. Radiation induces mutations with increasing dose either in a linear or in an LQ fashion. The role of radiation is to contribute only a few mutations such as one or two to the carcinogenic process. Adult-onset solid tumours require a relatively large number of mutations, and one mutation by radiation has to be supplemented with additional mutations from other processes before acquisition of full malignancy. This requires a long latency after the radiation exposure. The dose response for such cancers follows a linear dose response, fitting well with the LNT model, and the risk is predicted by the RR model. In contrast, leukaemia and childhood cancer require much fewer mutations such as two, which can be supplied by radiation alone. For such cancers, the dose-response is LQ with relatively short latency, and the associated risk can best be assessed by the AR model. Such characterisation for the dose response, latency and radiation carcinogenesis model as summarised above is oversimplified, but it offers a foundation for future studies and better understanding of the mechanism.

(78) An exception can be found for some cancer types such as radiation-induced childhood thyroid cancer. This appeared with a short minimum latency of four years after the Chernobyl accident in the presence of screening programmes. Yet, the dose responses of childhood thyroid cancer were reported to be linear (Ron et al., 2012). With the above rule in mind, it is tempting to speculate that childhood thyroid cancer requires two mutations, and the linear dose response with the short latency occurred for those carrying the pre-existing *RET/PTC* rearrangement, with radiation responsible for inducing the second hit necessary for conversion of the cells to full malignancy. Indeed, the rearrangement does exist frequently in benign nodules in the thyroid, suggesting the possibility of such a mechanism (Marotta et al., 2011).

(79) The choice of risk models for radiation protection has been made empirically when transferring the risk between two populations differing in the background risk (ICRP, 2007). A comprehensive review is available on this important subject (Wakeford, 2012). Currently, the ICRP uses a number of risk transfer models: 1) a mixture of 50% RR model and 50% AR model for all types of cancer, except for thyroid and skin with 100% RR model, breast and leukaemia with 100% AR model, and lung cancer with a mixture of 30% RR model and 70% AR model. Among these cancer types, breast cancer with the AR model is clearly an outlier of the above simplified description. Its dose response for women exposed under age 40 is linear with a slight upward curvature instead of expected LQ, the latency is relatively long instead of just a few years, and the differences in background rates between Japan and Europe or US are so large that an AR model was considered more prudent and a better fit in age-specific comparisons (Preston et al., 2007). Thus, the working hypothesis as projected here is too simple to predict the trend of cancer occurrence after radiation exposures. Yet, the present consideration is useful to gain a possible mechanistic insight of radiation carcinogenesis and its implications for the choice of a risk model for the risk transfer.

(80) The risk and the sensitivity of radiation carcinogenesis are often evaluated by the functions EAR and ERR. The EAR quantifies the increment of the cancer incidence rate due to radiation, while the ERR describes the relative increase over the background (control) incidence rate due to radiation exposure. Both ERR and EAR not only differ among different age groups at the time of exposure but also change with time since exposure. ERR is usually higher following exposure at young ages, but the estimates decline with increasing years since exposure or with increasing attained age. In contrast, EAR estimates at early years since exposure (i.e. when ERR is highest) are usually smaller and increase along with the increase in years since exposure, because the background rate sharply increases at older ages. When attained age is the same, a younger age at exposure in A-bomb survivors may give rise to a higher EAR for some solid cancer types but not for others.

(81) If it is assumed that cancer occurs as a result of accumulation of certain number of somatic mutations, the occurrence of excess cancers as a result of inducing one cancer-related mutation may be expressed as a function of three factors: (1) the sensitivity of stem cells (and of proximal progenitor or even differentiated cells for certain cancer types) to radiation induced mutation; (2) the retention of stem cells which had undergone any mutation in any gene related to cancer development; and (3) the population size of stem cells, at the time of exposure, which may in the future accumulate a critical number of mutations (say 4 mutations) so that one mutation added by irradiation may result in the elevated rate of cancer. The number of stem cells with sufficient predisposing mutations is expected to be proportional to the number with full (say 5) mutations, which results in the background cancer incidence rate. The EAR is obtained by subtracting the background absolute incidence rate (BAR) from the total absolute incidence rate of cancer (TAR) in an exposed population (EAR = TAR – BAR). In contrast, the excess relative increase in radiosensitivity is quantified

by the ERR which is obtained by dividing the EAR by BAR as in the equation: ERR = EAR/BAR. The EAR depends on the BAR especially for the cancer types where the RR model provides a better fit when making risk transfer between different populations. A good example of where the EAR is strongly affected by the BAR can be found in radon-induced lung cancer in the smoking and non-smoking populations (Darby et al., 2005, 2006). The EAR was much larger for the smoking population but the ERR was insensitive to the smoking characteristic. A larger EAR for smokers may reflect a larger BAR of that population which has an increased number of predisposed target stem cells. The ERR was the same for both populations. It is tempting to speculate that the smoking-induced conditions did not markedly affect the sensitivity of target stem cells to radiation induction of carcinogenic mutations from radon. Altogether, the EAR and ERR functions represent different mechanistic properties of stem cell behaviour during radiation carcinogenesis (see section 3.6.2).

(82) Representative values of the EAR and ERR functions for some of the tissues considered in this report are taken from ICRP (2007) and quoted in Table 3.1. These values are standardised to the risk at attained age 70 following exposure to 1 Gy at age 30. The uncertainties in the values quoted are highest for breast, lowest for thyroid, with stomach, colon and lung in the middle. The decline in EAR per decade after exposure at age 30 is greatest for breast, lower and similar for thyroid, stomach and colon, with no change for lung. The values for ERR are of the same order for all tissues quoted, with declines per decade after exposure at age 30 being highest for thyroid, lower for stomach and colon, zero for breast and even a positive increase for lung. The values for bone marrow, skin and bone surface are not quoted in Table 3.1. because the values are not directly comparable in the same way. For bone marrow, the preferred dose-response model is LQ (Hsu et al., 2013), and hence the coefficients cannot be compared directly with those in Table 3.1., which are based solely on a linear model. The EAR dose coefficients for leukaemia at 1 Gy (at age 70 after exposure at age 30) were 0.70 (linear term) and 0.71 (quadratic term) for women, and 1.06 and 1.09 per 10^4 PY respectively for men. The corresponding ERR values for men and women were 0.79 (linear term) and 0.95 (quadratic term). Of note, compared to decreases for the other tissues in Table 3.1., the 51% increase in EAR per decade increase in age at exposure. Skin is a special case, as explained in detail in Annex F, section F.2.1. Risk coefficients were calculated in a different way from those in Table 3.1. and the values are very uncertain. For bone cancer incidence in the Life Span Study (LSS) of A-bomb survivors and assuming a linear dose response, an EAR value of 0.39 (0.08 to 1.04, 90% CI) per 10^4 PY per Gy for individuals at age 70 exposed at age 30, and an ERR value of 0.48 (0.07 to 1.4, 90% CI), were calculated (Preston et al., 2007). A quadratic model has been suggested (UNSCEAR, 2006), and in addition a more recent analysis indicated that a linear ERR model with a threshold at about 0.85 Gy appeared to be the most plausible model from statistical and biological points of view (Samartzis et al., 2013). A further EAR and LNT model for bone cancer induction has been developed based on ^{224}Ra data (EPA, 2011), although the LNT model is inconsistent with the ^{226}Ra data where a sigmoid response provides the best fit (Rowland et al., 1978). In view of the above differences and the complexities in making direct comparisons, in particular for skin and bone versus the other tissues in Table 3.1., a more detailed discussion of EAR and ERR risk values with reference to stem cell-based mechanisms would be very speculative (see section 3.6.2.).

(83) Values of a tissue weighting factor w_T are also quoted in Table 3.1. for completeness, because these formed part of the rationale for the initial choice of tissues for this report (see Introduction). The w_T is one of the basic elements of the ICRP risk model. It represents the relative contribution of a tissue or organ to the total health risk mainly due to cancer and used

to weight the equivalent dose of such organs (ICRP, 1991). The weighted dose thus derived is the effective dose from which the nominal risk for a dose can be calculated. The w_T values were revised in 2007 (ICRP, 2007), and again confirmed that the ERR of cancer at age 70 for people acutely exposed to a unit dose at age 30 years, varies between tissues. The ICRP recommended a w_T value of 0.12 for bone marrow, colon, lung, stomach, breast, 0.04 for bladder, oesophagus, liver and thyroid, and 0.01 for bone surface, brain, salivary glands and skin. The weighting factors refer to total human health risk, i.e. mortality, which is a combination of incidence and the probability that a particular cancer type will be lethal, adjusted for quality of life and years of life lost (see Box A.1, ICRP, 2007). The detriment-adjusted nominal risk coefficient for cancer is based upon lethality/life-impairment-weighted data on cancer incidence with adjustment for relative life lost.

	EAR		ERR		w_T
Tissue	Excess cases per 10^4 persons per year per Gy at age 70 after exposure at age 30	Age at exposure: % change in EAR per decade increase	ERR per Gy at age 70 after exposure at age 30	Age at exposure: % change in ERR per decade increase	Tissue weighting factor w_T
Breast	F 10.9	-39%	F 0.87	0	0.12
Thyroid	M 0.69	-24%	M 0.53	-56%	0.04
	F 2.33		F 1.05		

Table 3.1. Coefficients in the cancer-incidence based EAR and ERR models, and tissue weighting factors, for some of the different tissues considered in this report. Taken from Tables A.4.7., A.4.6. and A.4.3. in ICRP *Publication 103* (ICRP, 2007).

M = Male; F = Female

3.2. Stem cells and stem cell niche in radiation carcinogenesis

3.2.1. Stem cell radiation biology and radiation carcinogenesis

Stomach	M 6.63 F 9.18	-24%	M 0.23 F 0.38	-17%	0.12
Colon	M 5.76 F 2.40	-24%	M 0.68 F 0.33	-17%	0.12
Lung	M 6.47 F 8.97	+1%	M 0.29 F 1.36	+17%	0.12

(84) Two factors are essential in carcinogenesis, especially for cancers occurring in adults. One is the acquisition of oncogenic mutations by the target cells, and the other is the retention of such predisposed cells in the body to allow further accumulation of mutations to gain full malignancy. Cellular radiosensitivity and mutagenesis determine the former process, and the dynamics of stem cells in the tissue microenvironment determine the latter.

(85) Radiobiological analyses of pure tissue stem cell populations are still limited. Nevertheless, past studies and emerging evidence suggest that the radiosensitivity of tissue stem cells varies considerably between tissues and within a tissue. How these differences relate to radiosensitivity for carcinogenesis is not clear at present. In the case of the small intestine, P4 stem cells are highly sensitive to apoptosis by radiation dose as low as 100 mGy while the telomerase-positive P4 stem cells, putatively the most primitive stem cells of the intestine, survive even 10 Gy of radiation (see paragraph 37 in Chapter 2). This high radioresistance is difficult to understand because after such a dose the cells are unlikely to remain error-free even with extremely efficient DNA repair. This raises a possibility that the telomerase-positive P4 cells serve as the reserve for emergency purposes, rather than serving as the house-keeping supplier of lower ranking stem cells. HSCs are one of the most radiosensitive of all tissues, but the long-term HSCs are more radioresistant than short-term HSCs (Annex A, paragraph A75). In the skin, stem cells are more radioresistant than progenitor cells (Annex F, section F4.1). In general, primitive tissue stem cells are more radioresistant than their committed counterparts, but the relevance of this to their sensitivity to radiation carcinogenesis is not known.

(86) Quiescence plays an important role for the radioresistance of stem cells, as discussed in the previous chapter. In addition, staying quiescent is the best way for tissue stem cells to avoid replication-mediated mutations and exhaustion of tissue stem cells. At the same time, quiescence results in accumulation of spontaneous DNA damage as shown in mouse HSCs (Rossi et al., 2007). In addition, quiescent cells have to be dependent on the error-prone NHEJ repair for coping with DNA damage. Thus, the benefit of quiescence is dependent on the trade-off of avoiding replication-mediated mutation versus taking a chance of damage accumulation and resulting mutations. Another way to avoid replication-mediated mutation is the unique mechanism of immortal DNA strand retention and stem cell-specific chromosome segregation, which are likely to operate in stem cells of the intestine and mammary gland. It seems that stem cells have evolved multiple strategies to avoid replication-mediated mutations. Radiobiological characteristics of quiescent tissue stem cells *in vivo* need to be further analysed in order to understand the cellular processes of radiation carcinogenesis.

3.2.2. Tissue radiation biology and radiation carcinogenesis

(87) The steady-state maintenance of a tissue involves three tissue compartments: the stem cell compartment, the progenitor compartment and the functional cell compartment. Once cells move out of the first compartment, they will be discarded eventually from the body, except for some progenitors of long residential time in a tissue. Thus, stem cells are the

major target with long enough time of residence in the body to accumulate mutations and thus acquire a malignant phenotype. However, as discussed in section 3.1.2, even stem cells are not immune to being replaced by other stem cells since they are under constant competition for residence in the stem cell niche. This competition is due to occasional symmetric division of tissue stem cells in which extra stem cells compete for the residence in the niche.

(88) Analyses of Drosophilla germ cells revealed E-cadherin as the major gene involved in the competitiveness of the germline stem cells in the niche (Zhao and Xi, 2010). In this Drosophilla system, rapid turnover of stem cells functions as an efficient mechanism for the removal of aberrant stem cells in the gonad. This is likely to be so for mammalian stem cell systems where stem cells are in contact with various niche cells. HSCs interact with osteoblasts through N-cadherin and integrin, and receive the Tie2/Ang-1 signalling to regulate the quiescence (Suda, 2007). As for $Lgr5^+$ stem cells in the small intestine, adhesion to Paneth cells was found to be essential for keeping the stemness of the cells through essential signals received for their maintenance (Sato et al., 2011). Change in the expression of these genes, brought about by any stress including radiation and/or by mutations in relevant genes, is likely to affect the competitiveness of stem cells and their subsequent occupancy in the niche. The effect of low dose radiation on the niche interaction of stem cells has not been studied. Expression of E-cadherin was studied at >2 Gy with contradicting results; downregulation in mammary epithelial cells and upregulation in the rat liver (Andarawewa et al., 2007; Moriconi et al., 2009). The consequence of such changes is expected to result in the less-fit stem cells being competed out of the niche. Indeed, it was reported that irradiation of bone marrow with >0.5 Gy decreased the competitiveness of HSCs in the recipient hosts (Marusyk et al., 2010). This tissue microenvironment-based selection process is likely to function as a tissue-based quality control, independently from the molecular and cellular-based quality controls such as DNA repair and apoptosis.

3.3. Dose rate effect for radiation carcinogenesis

3.3.1. Cell-based considerations

(89) For radiological protection, the risk of radiation has to be estimated especially for low dose and low dose rate exposures, where the risk to be taken into consideration is that for stochastic effects (ICRP, 2007). The ICRP estimates such risk by the use of the LNT model and DDREF to adjust for the conditions of low dose and low dose rate exposures. The ICRP made a choice of 2.0 for the value of the DDREF and is continuing to use it (ICRP, 1991; ICRP, 2007). The dose response of cancer for acute exposures generally follows an upward concave curve which can be adequately described by a number of models. Two of the cell-based biophysical models have been well received: the sublesion model of Kellerer and Rossi (1972) and the repair saturation model of Goodhead (1985). In particular, the sublesion model is widely used with an LQ equation as below (UNSCEAR, 2006):

$$E(D) = \alpha D + \beta D^2 \qquad (1)$$

(90) The linear term of the equation (1) represents single-track events in cells which are supposed to be dose-rate independent. The quadratic term represents two-track events which are subject to cellular repair, and therefore this term becomes negligible at low dose and at low dose rate. As for the definition of low dose, any doses below 200 mSv were proposed by UNSCEAR since in this dose range, the linear term dominates the magnitude of the dose

response (UNSCEAR, 1993). In addition, under the assumption of equation (1), the risk of radiation carcinogenesis becomes identical for low dose and low dose rate. This is the reason why the low dose and low dose rate can be handled by one factor of DDREF. Then, DDREF can be described by the following equation:

$$\mathrm{DDREF} = (\alpha D + \beta D^2) / \alpha D = 1 + (\beta/\alpha)\, D \qquad (2)$$

(91) The US Biological Effects of Ionizing Radiation (BEIR) VII Committee applied equation (2) to the epidemiological data of the A-bomb survivors and animal data using a Bayesian approach, and obtained a DDREF value of 1.5 (BEIR VII, 2006). Furthermore, studies of radiation workers exposed at low dose rates reported similar risk coefficients for solid cancer as those observed for acute exposures in the LSS, suggesting a DDREF value of 1.0 (Jacob et al., 2009). UNSCEAR abandoned the use of DDREF and applied the LQ model to the LSS data to directly obtain the low-dose/low-dose-rate risk coefficient (UNSCEAR, 2006). The risk coefficient thus obtained was consistent with the ICRP risk values based on the LSS data adjusted by the DDREF value of 2.0.

(92) The calculation-based derivation of the DDREF by BEIR VII and UNSCEAR (UNSCEAR, 2006) relies on the validity of the LQ equation, and especially on the dose-rate independence of the linear term. At the cellular level, there is ample evidence that the linear term of the mutation induction rate by radiation is independent of dose rate. However, at the tissue level, there are cases in which the slope of the linear dose response of mutation and cancer decreases by lowering the dose rate. Such a decrease can be expected when the tissue level elimination of aberrant cells by stem cell competition is in operation.

3.3.2. Tissue-based considerations

(93) As discussed in the Chapter 2, HSCs of mice irradiated with ≥1 Gy are effectively competed out by those of unirradiated mice as demonstrated by co-transplantation into lethally-irradiated mice (Bondar and Medzhitov, 2010). This suggests that any deviation from total stemness by irradiation leads to elimination of such cells from the tissue. Stem cell competition is regarded as a quality control at the tissue level to eliminate phenotypically-unfit cells. Thus, there are three levels of quality control systems in a body: the molecular level quality control of DNA repair; the cellular level quality control of apoptosis; and the tissue level quality control of stem cell competition.

(94) Stem cell elimination is expected to affect the linear term of the LQ equation (1). One example of such case is seen in the radiation induction of germline mutations in mice. In the case of the male mouse, the induction is a linear function of radiation dose, yet the induction rate was lowered when the dose rate was reduced (Russell and Kelly, 1982). In the case of the female mouse, the dose-rate effect is extreme in that the linear dose response became completely flat, when the dose rate was decreased (Searle, 1974). Molecular and cellular quality controls cannot explain such data. As discussed in the previous section, the low dose-rate sparing of mutation by DNA repair should affect only the quadratic term of the LQ equation, and the linear term should not be affected by the dose rate. In addition, cellular quality control of apoptosis functions is less efficient when the dose rate becomes low. Thus, loss of the irradiated cells by whatever mechanism is likely to contribute not to the DEF, but to the DREF.

(95) Key issues in this topic are whether low dose exposure influences the stemness and if it does, how low can be the dose to affect the stemness. If the dose is as low as an elemental dose of radiation, that is, the lowest dose given by a single track of radiation to a

nucleus of a cell, then an interesting possibility emerges. An elemental dose of ^{60}Co γ rays is around 1 mGy for the typical mammalian cellular nucleus of 8 μm in diameter (Feinendegen, 1985). Chronic exposures at a dose rate of a few mGy per year mean that every cell in the body is hit by a track of radiation every few months. This then makes a hit stem cell, at any time, compete against surrounding non-hit stem cells within a niche. Thus, if the elemental dose affects the stemness, the hit cell will be preferentially lost by competition from the tissue stem cell niche. This elimination theory lowers the linear term. Hence, stem cell competition at the tissue level leaves an ample possibility for a DREF value larger than unity, as in the case of the current DDREF value used by the ICRP.

3.4. Experimental animal studies to supplement human data

(96) There have been many studies of radiation induced tumours in animal systems which have contributed knowledge of dose-incidence relationships and dose-rate/fractionation effects. These have focussed mainly on AML and solid tumours of the Harderian gland, pituitary, ovary, lung, breast, skin, and bone (ICRP, 2006; NCRP, 2005). Overall, it was considered by the Commission that the animal tumour data tended to support the hypothesis of a linear relationship of incidence versus dose at low doses and at low dose rates, with no threshold dose (ICRP, 2005). However, there are various caveats to this conclusion, notably that some of the tumour types studied in experimental animals are not the most relevant types in humans, and there are many strain and species differences in sensitivity. In addition, over a broad range of doses, all neoplasms are not increased in frequency within a given species or strains and certain neoplasms are decreased in frequency by irradiation. It has been argued that "From the diversity of observed dose-incidence relationships, it is clear that no one mathematical model for relating incidence to dose is universally applicable" (Upton, 1985). Nonetheless, the study of radiation induced tumours in one or more defined animal strains can help elucidate the molecular, cellular and tissue mechanisms which are fundamental to any animal species including humans.

(97) Concerning radiation-induced stochastic events in the haematopoietic system (Annex A), AML is the type of leukaemia in mice which has been studied in most detail. AML comprises less than 5% of all childhood leukaemias, but is one of three radiation-inducible leukaemia subtypes: AML; chronic myeloid leukaemia (CML); and acute lymphoblastic leukaemia (ALL). Regarding the target cells for AML, recent evidence in mice suggests that the initial radiation-induced AML stem cell may originate not only from irradiated HSCs but also from multipotent progenitor cells and common myeloid progenitors (Hirouchi et al., 2011; and Section A.5). Also, hemizygous deletion of *Dusp2* in chromosome 2 may contribute to the self-renewal potential of radiation-induced AML stem cells. A detailed study of the "immortal strand hypothesis" in highly purified HSCs revealed that BrdU had poor specificity and poor sensitivity as an HSC marker (Kiel et al., 2007). All HSC segregated their chromosomes randomly, and division of individual HSC in culture revealed no asymmetric segregation of the label. Hence, HSCs did not retain older DNA strands during division. The multistage theory of carcinogenesis and the importance of the microenvironment in promotional events continue to suggest that the target cells are likely to be the more slowly renewing cells in the lineage, and those cells are generally the more primitive stem cells in the population. Indeed, the correlation between chronic radionuclide doses/location and AML incidence was closest for target cells in the central marrow sinusoidal region (Lord et al., 2001), which is a principal site of such primitive cells. These are identified in marker studies as $CD34^{-}CD48^{-}CD150^{hi}LSK$.

(98) As noted in Section A.1.1, the incidence of AML at very low levels of exposure (from background levels to approximately 1 Gy) is fairly linear with increasing dose. At doses from 1 to 2-3 Gy, the dose-incidence curve tends to exhibit upward curvature, and at >3 Gy, the curve tends to bend over downwards. Also, DDREF values estimated from such data vary quite a lot, but generally fall in the range of 2 to 5 over that dose range (UNSCEAR, 1993). Values at smaller doses are expected to be lower, reflecting mainly the single-hit (α component) of the dose-response curve. In often-quoted mouse experiments delivering continuous irradiation for 28 days at 0.04-0.11 mGy/min to a total dose of 1.5 Gy, the AML incidence of 5% (Mole et al., 1983) was also produced by an acute dose of about 0.5 Gy (Mole and Major, 1983), which gives a DREF value of ≤3 at ≤1.5 Gy. In addition, after higher doses of 3.0 and 4.5 Gy delivered at the same low dose rate, the AML incidence was inexplicably also 5%. This led to the postulate of some "biological factors" related to stem/clonogenic cells, influencing the response to dose protraction (Mole and Major, 1983), as discussed further in section A.2.2.

(99) A number of studies have been published on the effects of dose and dose rate on mammary tumour induction in rodents (Annex B; UNSCEAR, 1993). Studies with Sprague-Dawley rats gave an approximately-linear incidence with increasing dose. The DDREF values ranged from less than 2 to about 4, for dose rates varying by a factor of ≥150 and for doses (at high dose rate) between about 2 to 3 Gy (Shellabarger et al., 1966; Gragtmans et al., 1984). With BALB/c mice, the dose-incidence curve at high dose rate was LQ up to about 0.25 Gy, and the linear term was similar to that obtained after low dose rate (0.07 mGy/min) exposures (Ullrich, 1983). Dose-fractionation studies showed a significant contribution from the quadratic component at doses as low as 0.1 Gy/fraction, and acute daily fractions of 0.01 Gy gave a tumour incidence similar to that observed after the low-dose-rate exposure to a total dose of 0.25 Gy in both cases (Ullrich et al., 1987). The DDREF decreased with decreasing dose from 11.7 at 0.25 Gy, and would be predicted to be near unity at a dose of between 0.1 and 0.01 Gy. The mouse studies showed that dose fractionation/protraction to 25 days (0.25/0.01) produced the incidence of mammary cancer predicted from the LQ analysis of the acute exposure response.

(100) Regarding age-at-exposure effects, the relatively restricted window of carcinogen susceptibility that is evident during or around puberty in both rodents and humans has been postulated to either contain the greatest number of target cells or be a critical period of stem cell regulation. There is a clear hierarchical lineage in mammary epithelium, and the many factors that control it. For example, the $CD24^{+}$ $CD29^{high}$ population in the mouse mammary epithelium is highly enriched for cells with multilineage and self-renewal potentials, the two properties that define a MaSC. Also, there is evidence that epithelial LRCs in mouse mammary gland divide asymmetrically and retain their template DNA strands (Smith, 2005). However, the target cell origin of radiation-induced breast cancers in terms of stem and progenitor cells is not yet elucidated.

(101) Concerning the thyroid (Annex C), radiation induces both papillary and follicular carcinomas, but in humans the former type predominates whereas in the common rat model the latter type predominates. Dose-incidence relationships for carcinomas (mostly follicular) in 3000 female Long-Evans rats showed a rising incidence with increasing x-ray dose from 0.8 Gy, flattening off at the higher doses to 10.6 Gy (Lee et al., 1982). However, adenomas were in the majority, and in contrast these showed a continuously rising dose-incidence curve. Hence the curves for the two tumour types appeared to be significantly different in shape. In addition, concurrent studies with ^{131}I and detailed dosimetry (Lee et al., 1979), showed a similar response to the high-dose-rate x-ray results for the carcinomas, but there was a tendency towards a lower incidence of adenomas at the higher doses of ^{131}I compared to x-

rays. If the adenoma yields are interpreted on an LQ basis, it can be estimated that the solely-linear component (not modified by dose rate) may be at doses up to about the first dose point of 0.8 Gy, and a DDREF of around 2 may apply at about 2 Gy. However, for the aggregated yields of both tumour types, the solely-linear component could be higher and the DDREF lower, albeit with large uncertainties. The similarity of tumour yields in the rats at low doses of acute x-rays and low dose rate ^{131}I is compatible with the human data, i.e. the ERR for external radiation exposure was compatible with the ERR estimates for internal radiation exposure following the Chernobyl accident (section C.2). Although there is evidence for the presence of a stem cell-type lineage in the thyroid epithelium (section C.3), there is no knowledge of whether the different tumour types originate from the same or different target cells in the lineage.

(102) Cancers of the stomach and colon (Annex D) in rodents are induced only by high radiation doses, e.g. ≥8 Gy (Boice and Fry, 1995), and are only very rarely found in the small intestine. A possible reason invoked for the latter is the radiation-induced apoptosis of mutated stem cells in the small intestine, which is prevented in the large bowel by the expression of the survival (anti-apoptotic) gene *bcl-2* (Merritt et al., 1995). Nonetheless, the stomach and colon are fairly resistant to cancer induction. The Min mouse provides a sensitive model for the study of tumourigenesis in irradiated mice. Min mice are genetically heterozygous for a germline truncating mutation of the Apc gene (i.e. $Apc^{Min/+}$) and develop multiple intestinal tumours and sporadic colon tumours in their intestinal tracts within several weeks of birth. The following yields of tumours were observed in CHB6 $Apc^{Min/+}$ mice exposed to 2 Gy x-rays *in utero* on day 7 (30 tumours/mouse, not significantly raised above numbers in unirradiated controls) or day 14 post-conception (44 tumours/mouse), as 2-day neonates (85/mouse) or as 10-day neonates (130/mouse), and 35-day young adults (70/mouse) (Ellender et al., 2006). Hence, neonates were more sensitive to tumour induction than young adults. The x-ray dose-incidence curve for adenomas was LQ over the range 0-5 Gy, and strikingly there were more tumours in the small intestine than in the caecum and colorectum (Ellender et al., 2011). In the caecum, the tumour incidence was elevated after ≥2 Gy, and in the colorectum after ≥1 Gy. In general, adenomas in the small intestine were sessile while the smaller numbers of adenomas in the large intestine were pedunculated. There was also an incidence of microadenomas in the small intestine, which was greater after the higher doses in the range used, but none was found in the large intestine.

(103) The target cell for colonic tumours is still considered to be the crypt stem cells, and the potential inclusion of progenitor cells as target cells is not yet resolved. An interesting development is the finding of rare (1 per 150 crypts), slowly-cycling, long-lived and radioresistant $mTert^{+}$ stem cells in both small and large intestine, giving rise to all differentiated intestinal cell types. These are probably the best candidate target cells in the colon in terms of the multistage model for carcinogenesis. Germline mutation of the APC gene predisposes both humans and mice to intestinal carcinogenesis. In humans, inheritance of mutant APC is associated with the cancer predisposing disorder, familial adenomatous polyposis (FAP), and mutation of APC is an early somatic event in sporadic colon cancer. Individuals carrying germline mutations in the APC gene develop hundreds to thousands of colorectal adenomatous polyps, some of which will progress to carcinomas if left untreated.

(104) From studies of A-bomb survivors and uranium miners, radiation-induced lung cancers (Annex E) appeared to be more likely small-cell lung carcinomas (SCLCs), and less likely to be ADCs (Land et al., 1993). In mice, SCLC does not occur, and ADCs are the most common type of lung cancer. The induction of ADCs in female BALB/c mice following acute irradiation was shown to be consistent with an LQ model in which the linear term was independent of dose rate, by comparing responses using 0.4 Gy/min and 0.06 mGy/min

(Ullrich, 1983). Also, the DDREF was about 4.2 at 3 Gy, and about 3.2 at 2 Gy, and from dose fractionation studies, the DDREF was predicted to be about 1.1 at 0.1 Gy (Ullrich et al., 1987). Like for mammary tumours induced in this same strain of mouse, the induced frequencies of lung tumours from acute, and protracted fractionated or low-dose-rate exposures, were consistent with each other in an LQ analysis.

(105) In the respiratory tract, the target cells for radiation-associated carcinogenesis are considered to be in the basal cells in the trachea and larger bronchi of the central lung, and in the Clara variant and type II alveolar cells of the peripheral lung (Annex E). An epithelial stem cell niche has been identified in the zone where airways terminate and form alveoli. The putative mouse BASCs in the bronchiolar alveolar junction, coexpress secretoglobin a1a (SCGBa1a), the type II cell marker surfactant protein C, Sca-1, and are negative for CD45 and CD31. Molecular analysis showed that, despite their distinct histopathological phenotypes, in human ADC and SCC, genomic profiles showed a nearly complete overlap, with only one clear SCC-specific amplicon (Tonon et al., 2005). Hence the common or different cellular origin of lung cancer types may become better understood. In addition, there may be influences from the irradiated microenvironment. For example, migration of MSCs into irradiated and stressed regions has been invoked as a potential alternative or contributory mechanism in carcinogenesis.

(106) Radiation induced skin cancers (Annex F) in humans are predominantly BCC. The traditional view was that a threshold dose exists for radiation-induced skin cancer, in the range of 8 to 10 Gy, but the A-bomb survivor data indicated that BCC can be induced by acute exposure at moderate doses, in the range of 1-4 Gy. In mice, radiation readily produces SCC but no BCC, whereas in rats about 20% of induced skin tumours are BCC. The dose-response curve for total tumours in rats was compatible with an LQ model (and linear for high LET radiation), albeit with a tendency for adnexal (hair follicle and sebaceous) tumours to be more common and later after low doses compared to epidermoid tumours being more common and earlier after high doses. With repeated weekly doses of 0.75 or 1.5 Gy over a lifetime, more tumours were produced than expected from single-dose responses, suggesting either the number of events increased per unit dose (i.e. induced sensitisation) or that clonal growth expanded the number of early transformed cells (Burns and Albert, 1986a and 1986b). The radiation had to penetrate at least about 180 μm to induce tumours, and 300 μm was about optimum irrespective of follicle growth phase and size, so demonstrating that the main target cells were in the stem cell zone of the hair follicle. Also, there was a marked effect of age on radiation-induced cancer incidence. In the rat, the dose to induce 2 tumours per rat (the mid-range induced) by 70 weeks postirradiation increased from 10 Gy (1 day old) to 15 Gy (28 days), 17.5 Gy (58 days) and 30 Gy (99 days). If this increasing resistance is expressed as an iso-effect per unit area of skin irradiated, the factor of 3 in iso-effective dose per rat between 1 and 99 days of age would be greater by likely 2 orders of magnitude (i.e. 3 x ~100), when allowing for the smaller skin area of the irradiated newborn compared to the much larger adult rat.

(107) A model for human skin cancer proposed that stem cells were likely target cells for BCC, early progenitor cells for SCC, and late progenitor cells for papillomas (Sell, 2004). Molecular characterisation of these different cell populations is continuing, and the most potent quiescent stem cells appear to be marked as $\alpha6^{bri}CD71^{dim}$. There are quantitative data of mouse follicle-bulge cell divisions marked with BrdU, which support the long-standing infrequent SC-division model (Waghmare et al., 2008). However, it was shown that hair follicle stem cells do not retain the older DNA strands or sort their chromosomes. To date, there are no distinct markers for the target cells of the different types of skin cancer.

(108) Radiation-induced bone sarcoma has been associated with high doses of ionising radiation from therapeutic or occupation-related exposures (Annex G). However, the development of bone sarcoma following lower doses remains speculative. Analysis of 80,000 individuals in the LSS cohort to assess the development of bone sarcoma (most commonly osteosarcoma) in A-bomb survivors, showed a preferred fit with a dose threshold at ~0.85 Gy (95% CI, 0.12 to 1.85 Gy) and a linear dose-response association above this threshold (Samartzis et al., 2011). Chadwick et al. (1995) fitted the radium dial painter data using a two-mutation carcinogenic model with clonal expansion (Chadwick et al., 1995). The analysis showed that an LQ dose-effect relationship can be applied and, because of the very low natural incidence of bone sarcoma, is consistent with very low AR at low doses and dose rates. Much of the experimental work on radiation-induced bone cancers has been performed using dogs. For the (low-LET) β emitter, ^{90}Sr, the dose response was non-linear with no tumours occurring at doses below 18 Gy cumulative average bone dose (Annex G). This much higher threshold dose may reflect differences in dosimetry, the protraction of the radionuclide dose, and the much shorter lifespan of dogs versus humans. Mesenchymal (osteogenic) stem cells (MSCs) for the osteoblast lineage reside in the bone marrow. CD34-negative stem cells as well as mesenchymal precursors are possible target cells for radiation-induced bone cancer. In mice, these target cells for bone-associated α-emitters lie within about 40 µm from the endosteal surface.

3.5. Location of target cells in a tissue

(109) The location of target cells in different tissues is an important consideration in the calculation of doses received from short-range particulate emissions from radionuclides retained in body tissues, including α particles and low energy electrons. Thus, the Commission has made some judgements and assumptions about the location of target cells in the skin, the respiratory and alimentary tracts, and the skeleton (ICRP, 1991, 1995, 1996, 2007). Based on the data in the present report and recent publications, the location and characteristics of target cells are assembled in Table 3.2.

(110) For tissues where the stem cell location is understood and well-defined, it may be reasonable to estimate doses specifically to this location since stem cells are the primary target for accumulation of mutations in the initiation and development of carcinogenesis. However, the extent to which the immediate progeny of stem cells may also be targets for the development of particular cancer types varies between tissues and is not well established in many cases. For some tissues, this possibility may not have implications for the definition of targets for dosimetric purposes because stem cells and their immediate progeny occupy the same microenvironment. For example, in the epidermis, the stem cells and immediate daughter cells are found in the basal layer. The nominal depth of target cells in human epidermis is taken to be 70 µm for dosimetric protection calculations, although there is significant variation in depth of the skin undulations. For the example of the colon, the distance from the mucosal surface is taken to be the crypt base at 280–300 µm depth, and widening this band to include progeny cells makes little difference to dosimetric calculations when considering irradiation predominantly from radionuclides in the intestinal lumen. For the respiratory tract, however, assumptions regarding the type and location of target cells within the airways can be a major determinant of estimated doses for some radionuclides, including the α particle emitting progeny of radon-222. For bone cancer, until recently the target has been taken to be a 10 µm layer adjacent to bone surfaces, but now it is recognised that a 40-50 µm layer would be more appropriate. For leukaemia, while it is known that stem cells are located in endosteal and vascular niches, refinement from the calculation of average

red bone marrow dose has not proved feasible, although it is recognised that risks of leukaemia from bone-seeking α particle emitters (plutonium-239, radium-226) are substantially overestimated by such calculations. For other tissues such as mammary gland and thyroid, regional distributions of potential stem cells are not considered, and radiation doses are calculated as tissue averages.

Table 3.2. Locations and characteristics of target cells for radiation-induced cancers in different tissues.

Annex, Cancer	Human target cells	Markers	Location
A, Leukaemia	HSCs (and potentially some progenitor cells)	$CD34^{+}$, $CD59^{+}$, $Thy1^{+}$, $CD38^{low/-}$, c-$Kit^{-/low}$, Lin^{-}	Endosteal and vascular niches
B, Breast	Mammary stem cells	Possibly $CD24^{+}$ $CD29^{high}$, and possibly K6	Mammary MaSC niche (not well defined)
C, Thyroid	Follicular stem cells	Possibly $Oct4^{+}Pax8^{+}Tg^{-}$	Solid cell nests (SCNs)
D, Stomach	Mucosal stem cells	Possible LRC (defensin5^{-}, Muc2^{-}, chromograninA^{-})	Gastric pits, 60–100 µm depth
D, Colon	Mucosal stem cells (possibly also some daughter cells)	$Lgr5^{+}$; $mTert^{+}$; possibly also DCAMKL-1	Crypt base, 280–300 µm depth
E, Lung	Possibly Clara, Clara variant, or BASC cells	SCGBa1a, surfactant protein C, Sca-1, $CD45^{-}$, $CD31^{-}$; also possibly (c-KIT^{+}, Nanog, Oct ¾, KLF4, Sox2) cells	Bronchiolar–alveolar duct junction zone, also possible distal lung niche
F, Skin	EpiSCs – BCC (also early progenitors – SCC, late progenitors – papillomas)	$α6^{bri}CD71^{dim}$; also $β6^{bri}/CD71^{dim}$	Interfollicular basal layer, nominal 70 µm depth
G, Bone	MSCs	CD90, CD73, CD105, and possibly Stro-1, CD106, VCAM-1	MSC niches and perivascular
	Some HSCs	$CD34^{-}$	Bone marrow

3.6. Cell-based and tissue-based considerations

(111) Regarding the relationship between the cancer incidence parameters (Table 3.1.) and the three considered mechanistic factors of (1) the number and sensitivity of stem cells to radiation induced mutation, (2) the retention of mutated stem cells in a tissue, and (3) the population size of stem cells with a sufficient number of predisposing mutations, there is a

lack of definitive evidence for the contribution of one or more factors to the values of radiation risk for the different tissues. One example is that on current estimates, differences in the stem cell number among tissues are unlikely to be an important factor, albeit with the caveat of large uncertainties in the numbers. Some estimates of total stem cells per human in bone marrow are ~10^8 (Annex A), colon ~4 x10^7 (Annex D), and ~2 x10^7 for skin (Annex F; 1 functional stem cell per 35,000 epidermal cells, and 8 x10^{10} total epidermal cells per human). These numbers span more than 3 orders of magnitude, and their ranking clearly does not show a similar ranking of sensitivities regarding cancer incidence expressed by either EAR or ERR functions. An extreme example is the small intestine with estimated more stem cells (~2x10^8) than in the colon (~4x10^7), and yet the risk of radiation-induced cancer in the small intestine is virtually zero compared to positive values in the colon. Biological reasons for this have been proposed based on more p53-mediated apoptosis in the stem cell zone in the small intestine, compared to its prevention by bcl-2 expression in the colon. Also there may be differences in the true target cell population in the two sites, depending on whether it includes extra 'potential' stem cells and hence is larger, or whether it consists of a minority of radioresistant mTert$^+$ stem cells which differs between sites and hence forms a smaller population.

(112) Mechanisms behind any tissue differences in the sensitivity to radiation carcinogenesis are likely to be multiple, ranging from tissue specific mechanisms of stem-cell number and turnover, and cell-type specific mechanisms of DNA replication, DNA repair, cell cycle control and apoptosis. Knowledge in these areas is still lacking, especially for the tissue stem cells. One general trend is that the risk of radiation carcinogenesis is higher for some tissues with higher rates of renewal such as skin, colon and stomach, while lower for those with lower rates of renewal such as oesophagus, liver, thyroid, and bone surfaces, although there are exceptions, e.g. bone marrow. It has been thought that a higher rate of stem cell proliferation could contribute to a more rapid rate of mutation accumulation, but also a higher turnover rate of progenitor cells may not allow time for the cells to accumulate enough mutations to acquire full malignancy. Adult tissues without proliferation are almost refractory to radiation carcinogenesis and brain can be such an example, although a small number of NSCs are slowly proliferating even in adult primate brain (Gould et al., 1999). In contrast, some highly sensitive tissues like breast are not particularly active in proliferation (Annex B). Similarly, bladder is known to be sensitive to radiation carcinogenesis, yet this tissue is rather quiescent. Thus, simple proliferative activity of tissues does not appear to predict tissue sensitivity to radiation carcinogenesis.

(113) Some tissue stem cells are characterised by specific mechanisms such as asymmetric segregation of template strands when replicating DNA, use of a specific DNA repair system such as NHEJ, altruistic apoptotic cell death and cellular differentiation. These cellular features are tightly regulated by the tissue microenvironment of the stem cell niche, which is essential in maintaining the stemness of the stem cells. Thus, any perturbation in these features is likely to lead to accumulation of mutations. In the case of mammary gland, the niche was shown to be the target of radiation carcinogenesis (Section B.4.2). For some tissues, stem cells are under constant competition for the occupancy of the niche, which leads to elimination of some of its stem cells. This elimination can be a contributing factor for the tissue-specific sensitivity to radiation. It is concluded that a number of mechanisms are known or speculated upon in this report which may contribute in some way to cancer susceptibility among tissues, but to date their applicability and importance are unknown.

3.7. Age dependence of radiation carcinogenesis

3.7.1. Age dependent occurrence of spontaneous cancers

(114) Childhood cancer is defined as those occurring after birth until puberty, and supposed to arise in children carrying predisposing mutations inherited from their parent. The mutation may also be acquired during fetal development or childhood growth. The incidence of childhood cancer is roughly 1 x 10^{-4} per live birth. The type of childhood cancer is restricted, and each cancer type has a specific age window of occurrence (Ries et al., 1999). For example, retinoblastoma of the hereditary form develops within a year after birth. In addition, these carriers develop bone sarcoma around the time of adolescence (Knudson, 1971; Abramson et al., 1984; Friend et al., 1986). This bimodal pattern of occurrence is shared by the non-hereditary form of retinoblastoma and bone sarcoma as well (Ries et al., 1999).

(115) Adult cancers are supposed to occur as the result of mutations acquired somatically, and exhibit a steady increase in the incidence with age (Armitage and Doll, 1954). This age-dependent increase makes cancer the leading cause of death in developed countries with a long life expectancy. The incidence of cancer differs by gender. It is slightly higher for males up to adolescence and almost twice as high for males than females after 70 years of age (Bleyer et al., 2006). The incidence of adulthood cancer is twice as high in females as in males in their 40s due to the female-specific cancers in this age group.

3.7.2. Risks from fetal-stage radiation exposures

(116) Russell and Russell (1954) were the first to show a strong developmental-stage dependence of radiation effects in mice. They exposed pregnant female mice to radiation, and found that the pre-implantation stage was highly sensitive to embryonic death, but no malformation was identified. The fetal stage was sensitive to radiation induction of malformations, but the occurrence of cancer was not reported. Another classic experiment performed by Bissell and her group noted that chick embryos lacked tumour formation even when inoculated with highly oncogenic Rous sarcoma virus, suggesting strong suppression of transformed phenotype in embryos (Howlett et al., 1988). Interestingly, such chicks after hatching developed tumours at the site where the injury occurred. Based on these classic studies, it is tempting to speculate that cancer is the disease taking place in the established adult-type tissue architecture including the tissue stem-cell niche.

(117) One of the largest human studies on the effect of fetal stage exposures was the Oxford Survey of Childhood Cancers (OSCC). OSCC, a case/control study of mortality from childhood cancer in Great Britain found an association with an intra-uterine x-ray examination, and indicated that fetal stages were highly sensitive to radiation with an ERR per Gy of 50 for childhood leukaemia and other childhood cancers alike (Wakeford and Little, 2003). The epidemiological study of the incidence of cancer among the *in utero* exposed A-bomb survivors suggested a high ERR for childhood cancers other than leukaemia of 22 per Gy (although based upon just two incident cases and was thus not statistically meaningful), but no raised risk of childhood leukaemia (Wakeford and Little, 2003). The study of Ohtaki et al. (2004) of chromosome translocations in peripheral blood lymphocytes sampled from A-bomb survivors exposed *in utero* found no dose response above a dose of around 100 mGy, in contrast to the dose response found in some of the mothers and other adults. The authors suggested the lack of chromosome aberrations being due to a high sensitivity of the fetal stage haematopoietic cells to killing by radiation at moderate doses, i.e. doses much lower

than those normally associated with cell killing affecting cancer risks. Competition-mediated elimination of stem cells from newly established bone marrow niches may also explain the lack of chromosome aberrations in lymphocytes of *in utero* exposed A-bomb survivors. A moderate ERR of 1.0 per Gy was found for mainly adult-onset solid tumours, with an overall risk lower than that for the childhood exposures with an ERR value of 1.7 (Preston et al., 2008). It is interesting to note that a combined analysis of the *in utero* exposed and the childhood-exposed indicated a dose-response of upward curvature, suggesting a quadratic component in the induction of cancer for these cohorts. The Commission made an extensive review, but at that time was unable to reach a clear-cut conclusion on the risks of fetal stage exposures (ICRP, 2003). Based on the data available, and uncertainty on the development of solid cancer for the *in utero* exposed, the ICRP made a judgment that the life-time cancer risk following *in utero* exposure is similar to that following irradiation in early childhood (ICRP, 2007). Based on more recent follow-up, this assumption appears to overestimate the lifetime risk of *in utero* exposure (Preston et al., 2008).

(118) Ideally, the unresolved issue of the epidemiological studies has to be augmented with the help of experimental studies. However, one problem of such an experimental approach is the lack of an appropriate animal model of human childhood cancer. For example, human childhood cancer is relatively rare with the cumulative incidence of roughly 10^{-4} from birth to 15 years of age. Experimental studies usually utilise a group of less than 100 animals which are too small to detect cancers arising at a frequency of 10^{-4}. Therefore, experimental studies are conducted to analyse the effects of *in utero* exposures on the lifetime occurrence of cancer, which mimics adult-onset cancer in humans. Such studies using laboratory mice and rats demonstrated that *in utero* exposures are less effective than neonatal exposures in inducing leukaemia and various solid tumours (Upton et al., 1960; Sasaki, 1991; Inano et al., 1996; Di Majo et al., 2003). Detailed studies on the age-dependent sensitivity of the $Apc^{Min/+}$ model mice to radiation, indicated that the sensitivity was highest for 10 day-old neonates and decreased in the order of 2 day old neonates, 35 day young adults, 14 day fetuses and 7 day embryos (Ellender et al., 2006). Mouse studies thus indicate that the fetal stage in general is less sensitive than neonates, and that the earlier embryonic stage is much less sensitive to radiation induction of leukaemia and solid tumours. The fetal stage has a shorter duration in mouse than in man and this could be one of the reasons for the different results between these two species. The OSCC reported that the first trimester was most sensitive to induction of childhood cancer (Bithell and Stiller, 1988), and this is in contradiction to the mouse data where the early embryogenesis stages are generally insensitive to radiation carcinogenesis. One possible reason is that the doses to the first trimester could have been greater than those received during the second and third trimesters (Mole, 1990), and the apparent greater sensitivity could just be related to the dose received during an examination (Doll and Wakeford, 1997). It is important to note that none of the case-control studies of prenatal exposure and childhood cancer risk performed individual dose reconstructions on the cases and the controls involved. Dose estimates were based on national surveys and there are uncertainties with regard to machine parameters, repeat examinations, and undocumented procedures.

(119) When considering cellular characteristics, it is difficult to discern the difference between stem cells of fetal stages and after birth. Therefore, the difference in the sensitivity to radiation carcinogenesis has to be sought at the tissue level. One distinction of fetal stage tissues is the lack of a clear niche-like microarchitecture while in the adult, tissue stem cells are thought to reside in a distinct microenvironment of a stem-cell niche. The adult-type niche is established after birth for many tissues. As discussed in Chapter 2 (section 2.5.4) for example, a major site of HSC proliferation is the liver during fetal development. HSCs then

migrate and colonise the bone marrow niche. Numerous HSCs migrate to the newly established bone marrow niche housing a limited number, and it was shown that the only HSCs which are able to settle in the niche are those in the G_0 phase (Bowie et al., 2006). This selective settlement functions as an efficient mechanism to remove aberrant HSCs.

(120) Nakamura and colleagues found that *in utero* exposed A-bomb survivors generally did not carry chromosome aberrations in their lymphocytes, although they did observe a small increase in the aberration yield at doses below 50 mGy (Ohtaki et al., 2004). This could be the underlying mechanism for the lack of leukaemia among *in utero* exposed A-bomb survivors. A similar lack of chromosome aberrations was observed for fetal irradiation in mice (Nakano et al., 2007). A recent study by the same group has indicated that the removal of *in utero* exposed HSCs is somewhat "leaky", and clonal expansion of surviving HSCs can be detected (Nakano et al., 2012). These studies can be explained by two assumptions. Firstly, fetal HSCs do not have to be more sensitive to cell killing by radiation. Secondly, fetal HSCs with chromosome mutations are preferentially removed during fetal to neonate stages, possibly by competition for residency in the bone marrow niche. Although further studies are definitely needed, the tissue level competition is likely to serve as an effective filter to remove aberrant HSCs. A similar explanation can be made for the low sensitivity of fetal exposures to radiation induction of intestinal tumours in the $Apc^{Min/+}$ model mice, as discussed in the previous section (Ellender et al., 2006). During fetal development, the intestine is formed as a simple tube with a layer of stem cells (Crosnier et al., 2006). In the case of mice, the first differentiation of the villus formation takes place in day-15 fetuses. However, crypt formation begins only on day 7 after birth. This suggests that the stem cells settling into the crypt stem cell niche are few compared with a large number of fetal-stage ISCs. This leads to strong competition among fetal stem cells which is likely to remove such aberrant cells for contributing to the maintenance of the adult intestinal tissue.

(121) The competition-mediated elimination of aberrant cells during neonatal stages is likely to operate not only for radiation-damaged stem cells but also for spontaneously aberrant cells (Nakamura, 2005). ALL is a major childhood cancer with characteristic translocations specific to certain types of leukaemia. With the examination of cord blood samples by the polymerase chain reaction (PCR), around 1% of newborns were found to carry the TEL/AML1 fusion gene, one of the major translocations specific to childhood ALL. This translocation was shown to be generated during normal fetal development (Mori et al., 2002). In fact, the number of translocation carriers is higher by two orders of magnitude than the incidence of childhood leukaemia of this type, suggesting a possible elimination of the cells with predisposing mutations during childhood growth. The elimination of predisposing cells after birth was suggested by the fact that the background incidence of ALL is highest at around 3 years of age, then declines rapidly with an increase in age to 20 years, and it increases again in the elderly (Smith, 2005). It is tempting to speculate that the stable lodging of HSCs into the bone marrow niche during postnatal stages of development, may serve as a barrier to select out unfavourable cells with any predisposing mutations.

(122) Such elimination of cells with cancer-predisposing mutations may operate for many other childhood cancers of sporadic types (Ries et al., 1999). Neuroblastoma, the tumour of neural crest origin, is one of the commonest malignancies among children and its incidence, before 15 years of age is roughly 8 in 10,000. However, necropsy samples of babies dying within 3 months after birth demonstrated that precancerous lesions are rather common, and found at a rate of roughly 1 in 200 (Beckwith and Perrin, 1963). Thus, the occurrence of the lesion in newborn babies and the neuroblastoma incidence differ considerably, suggesting that most of the precancerous lesions are eliminated by some mechanisms. In accordance to this, the incidence of tumours is highest during the first few months after birth, and declines

rapidly to almost zero at an age of 15 years (Goodman et al., 1999). Similar patterns of a rapid decline of the incidence after birth are noted for other childhood cancers such as retinoblastoma, Wilms' tumour and hepatoblastoma. For those tumours, terminal differentiation of precancerous cells may be involved as a mechanism of the decline, in addition to real elimination of aberrant/premalignant stem cells as in the case of ALL.

(123) Mouse data on fetal exposures tend to demonstrate that the risk is minimal at early stages of gestation. However, exposure at 17 days of gestation was shown to induce cancers of lung and pituitary gland (Sasaki, 1991). It is interesting to note that these two organs are well developed at day 17 of mouse gestation (Yu et al., 2004; Sheng and Westphal, 1999). Thus, even the fetal exposure is capable of inducing cancer at the perinatal stage, where certain tissues are well developed. In contrast to mice, humans have a much longer period of fetal development, and therefore it is likely that certain tissues at perinatal stages are as sensitive as after birth. Wakeford (2008) noted that 90% of the pelvimetric diagnoses in the OSCC were performed at the last month of gestation. So, such late stages of fetal development might exhibit a similar sensitivity to radiation carcinogenesis as that of the neonatal stage after birth. On the other hand, the remarkable similarity in the RRs of all childhood cancer types in the study raises a concern on the causal relationship to prenatal x-ray examinations (ICRP, 2003; Boice and Miller, 1999). It is also noteworthy that the lifetime RRs of childhood exposures are quite variable among cancer types (UNSCEAR, 2013).

3.7.3. High sensitivity of children to cancer induction from radiation

(124) It is well recognised that children are highly sensitive to radiation induction of leukaemia and some solid tumours (ICRP, 2003). The sensitivity of children to radiation-induced cancer has been reviewed extensively in a recent publication from UNSCEAR, and the high sensitivity is strongly dependent on the cancer type (UNSCEAR, 2013). Children are more sensitive for about 25% of 23 cancer types analysed when compared to adults. These types include leukaemia, thyroid, skin and brain cancer. Children have the same sensitivity as adults for about 15% of cancer types including bladder, and less sensitivity for about 10% of cancer types including lung. As for 20% of cancer types, the data are not sufficient for concluding a difference in the sensitivity, and for 30% of cancer types, no increase in the risk is observed after radiation exposure. The ERR of radiogenic cancer is inversely related to the age at exposure for many cancer types, i.e. high for the young with an age-dependent decline of the risk (Preston et al., 2007). The ERR per Gy for ALL is more than 15 for children less than 10 years old and the risk declines rapidly with increasing age (Hsu et al., 2013). It is noteworthy that even with the high ERR for early onset ALL, the EAR for such cancer is rather low because the background incidence of cancer is considerably lower in children than in adults. The ERR per Gy for adult solid cancer following exposure in childhood is as high as 3-5 which declines sharply as the age at exposure increases. In addition, the latency of cancer development is relatively short for some cancers after childhood exposures, e.g. the minimum latency interval for thyroid cancer occurred within 4 years in children after the Chernobyl accident (Section C.1.). As a consequence, the lifetime ERR per unit dose for solid cancer after childhood exposures is around 1.0 (Pierce et al., 1996).

(125) The higher sensitivities of children for radiation induction of leukaemia and some adult solid cancers are considered to be due to the high proliferation rate of the stem cells and progenitor cells in children. However, these cells are also proliferating rapidly in embryos and fetuses, and the sensitivity to radiation carcinogenesis for these stages does not appear as sensitive as in childhood as discussed at length in the previous sections. Therefore, a cellular feature such as proliferation alone is unlikely to explain the high sensitivity to radiation-

induced cancer after exposure in childhood. However, there is one feature of a tissue that differs considerably between children and adults, which could contribute to the high sensitivity of children to radiation carcinogenesis. As discussed, the adult stem cell niche is established around perinatal stages, although the time of establishment is likely to vary tissue to tissue, and species to species. During childhood growth, the stem cell niche together with its stem cells increases in number as a unit to match the demand of the body growth, as shown in Fig. 3.1.

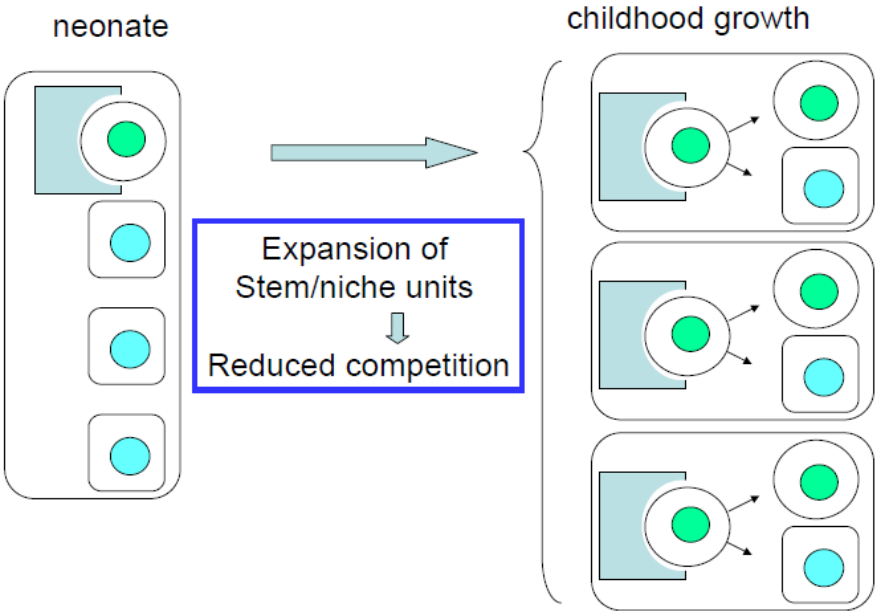

Fig. 3.1. Expansion of tissue stem-cell niche multiplicity during childhood growth. (permission needed)

(126) In the case of the intestine, this process of stem cell/niche expansion is accomplished by fission of crypts (Fujimitsu et al., 1996). The fission starts with an increase in crypt size which then splits from the bottom to form two crypts. The increased size of a crypt means an increased availability of crypt niches which eases the competition of stem cells within a niche. In addition, competition between niches is also eased. The end result of these is a better chance for an aberrant stem cell to remain to accumulate more mutations in the intestine. Thus, the process of expansion of the number of stem/niche units could potentially contribute to a higher sensitivity to children for the development of cancer after radiation exposures.

3.7.4. Risk of carcinogenesis from exposures in adulthood

(127) The risk of radiation carcinogenesis in general decreases inversely with age at exposure. The risk also decreases by attained age after radiation exposure. Adulthood exposures give a moderate risk as compared with childhood exposures, and this can partly be explained by a change in the cellular features of stem cells. For example, childhood stem cells divide frequently in a symmetrical fashion to cope with demands for the bodily growth, while adult stem cells do so less frequently. This makes the former more prone to mutate than the latter. Also, the stem cells during childhood growth are expected to experience less competition when the stem cell niche increases in number in response to the demand of

bodily growth as discussed above. Indeed, adult stem cells are under stronger competition than stem cells of childhood growth, which is likely to keep the risk low, as discussed in section 3.3.2.

(128) Elimination of aberrant stem cells has been demonstrated in animal models. In the case of mouse mammary carcinogenesis, radiation exposures induce many more initiated cells than those progressing to full malignancy (Adams et al., 1987; Kamiya et al., 1995). Indeed, it was shown in a rat mammary carcinogenesis model that the frequency was as high as 1 in 13 irradiated mammary clonogenic cells which could not be accounted for by a specific mutation induced by irradiation (see paragraph B55 of Annex B). Even with highly frequent initiated cells, neoplasias arise much less frequently, indicating either efficient elimination of such cells, or suppression of their aberrant phenotypes.

(129) As for radiation exposure in adulthood, the ERR at some specified time after exposure is generally smaller than that for childhood exposures. The risk rises within a few years for leukaemia and after 10 years or more for solid cancer, and the elevated ERR eventually starts to decline with an increasing attained age of those exposed. This pattern of elevation and decline of cancer risk has been observed repeatedly, but the most reliable data come from epidemiological studies (Boice et al., 1985) and notably of A-bomb survivors (Preston et al., 2007; Richardson, 2009; Hsu et al., 2013). Based on the multistage carcinogenesis model by Armitage and Doll (1954), the RR of a population given one hit by acute irradiation was predicted to decline over the attained age by a rate of 1/age (Pierce and Mendelsohn, 1999). The rates of decline of RR were estimated in the recent compilation of the A-bomb survivor data. Although the estimates varied among cancer types, the rates were in general in the range of around $1/age^2$ (Preston et al., 2007). In addition, a study of the radon exposed uranium miners indicated that the RR of lung cancer declined about 50% for each 10 years after they stopped working in mines, suggesting the decline to be proportional to around $1/age^3$ (Tomasek et al., 2008). These rates of the RR decline are higher than 1/age and may suggest the loss of initiated/precancerous cells in a tissue over time, as discussed earlier.

(130) Overall, the above-described age-dependent sensitivities to radiation carcinogenesis can be summarised as follows: embryo and fetal stages to be low to moderate, children to be high and adult to be low. However, mechanistic insight for this pattern of radiosensitivity is still lacking. In the past, it was naively assumed that the high sensitivity to radiation carcinogenesis for children could be attributed to high rates of proliferation of somatic cells at this stage of human life. However, this simple assumption makes it difficult to explain why the fetal stages with even higher rates of proliferation are not associated in general with extremely high sensitivity to radiation carcinogenesis, although the latter remains somewhat controversial.

3.8. Summary

- There is evidence for a hierarchical-type cell renewal lineage and stem cell niche in all the animal tissues considered in detail in this report. Most evidence pertains to haematopoiesis, GI tract and epidermis, which have greater renewal rates than in mammary tissue, lung, thyroid and bone.
- The target cells for carcinogenesis continue to be considered primarily the tissue stem cells. In haematopoietic tissue and colonic mucosa, there is evidence of an age structure within the stem cell population. In haematopoietic tissue, colonic mucosa and epidermis, there is some evidence for progenitor cells being target cells as well. The microenvironmental

niche is an important regulator of stem cell maintenance and a modifier of stem cell response.

- The immortal DNA-strand hypothesis was proposed which would protect stem cells from replication-mediated mutation. There is evidence in support of such a mechanism in small intestinal crypts, mammary epithelium, some muscle satellite cells and progenitor cells, and some CNS cells. It was also inferred from studies of tongue epithelium. However, it was found not to apply in HSCs. Since it is not a universal phenomenon, the relevance of this highly interesting hypothesis is yet to be proven.
- The radiation-induced incidence of cancer for different organs and its associated tissue weighting factors relate to carcinogenesis of target cells. Carcinogenesis depends primarily on three mechanistic factors: (1) the number and sensitivity of stem cells to radiation-induced mutation; (2) the retention of mutated stem cells in a tissue; and (3) the population size of stem cells with a sufficient number of predisposing mutations. It is postulated in this report that the ERR function reflects largely the cellular sensitivity to radiation-induced mutation and the retention of predisposed stem cells. The EAR function reflects in addition to those two factors, the population size of the predisposed stem cells, thus being more complex but comprehensive in relation to radiation carcinogenesis of stem cells and stem cell populations. At present, there is a lack of definitive evidence for one or more of these factors contributing to radiation risk for the tissues considered in this report.
- An LQ model describes fairly well the dose-response relationships for tumour induction in animal systems, and values of the DDREF are generally in the range 2 to 10 (UNSCEAR, 1993; NCRP, 2005), i.e. generally a higher range than that proposed for human application. With dose fractionation and protraction to 25 days, the results for mammary tissue and lung are consistent with LQ predictions, and for thyroid, the low-dose-rate ^{131}I and low-dose acute x-ray data are consistent. For AML, further protraction *reduces* tumour yield, whereas for skin cancer further protraction *increases* the yield. Hence, protraction effects are clearly tissue dependent. The stem cell competition model, where damaged stem cells are outcompeted by undamaged stem cells for residence in the stem cell niche, is theoretical but with some supporting evidence which might help explain the different risks among tissues from chronic irradiation scenarios.
- The LNT model continues to form the foundation of the ICRP risk assessment for low dose and low dose rate exposures. For tissues where transfer of risk is based mainly on ERR, the LNT model in combination with the RR model implies that any risk-reducing actions which decrease the underlying background risk of some cancer types also might be effective in reducing the risk of radiation-related cancer. It is, however, clear that the LNT model is a tool for radiation protection purposes to assess the risk of all tumour types combined and does not apply for some cancer types such as leukaemia and skin cancers. Furthermore, it is not applicable to those cancers not consistently increased following radiation exposures, such as the rectum, small intestine, pancreas, uterus, prostate and kidney parenchyma (Ozasa et al., 2012) as well as the testis, cervix and lymphomas.
- In addition to the quality control systems of DNA repair at the molecular level and apoptosis at the cellular level, stem cell competition is likely to function as a quality control at the tissue level which could adequately explain the age dependence of radiation carcinogenesis. In addition, stem cell competition offers a new mechanism for the sparing of risks of stochastic effects at extremely low dose rates such as a few mSv per year.
- Age-dependent sensitivities to radiation carcinogenesis can be summarised as follows: embryo and fetal stages to be low to moderate, children to be high and adult to be low,

with possibly higher sensitivity as people age further. However, mechanistic insight for this pattern of radiosensitivity is still lacking at present.

3.9. Recommendations for future research

1. Knowledge of the stem-cell/niche systems and their sensitivity to radiation injury resulting in carcinogenesis in bone marrow, large intestine and skin is much more developed than for other tissues at risk such as mammary gland, thyroid, lung and bone. Hence there is a need to further investigate the stem cell systems in these latter more-slowly renewing tissues, their controlling factors, and their mutational/mechanistic responses to acute and protracted irradiation.
2. The immortal-DNA-strand hypothesis, whereby the parental DNA template is retained during asymmetric stem-cell divisions so that the mutational burden in the stem-cell population is kept low, might act to protect against radiation carcinogenesis. There is evidence in support of this mechanism in some tissues considered in the present report (e.g. small-intestinal crypts, mammary epithelium, and epidermis), but the mechanism was found not to apply in HSCs. Hence it would be informative to study further tissue types, and to attempt to measure purportedly-relevant carcinogenic mutations in stem cells following acute or protracted irradiations in tissues which show this phenomenon.
3. The concept of competition between normal and radiation-injured stem cells for residence in the stem-cell niche, which might act to give less carcinogenic events than expected after irradiation, is supported by studies in haematopoietic tissue after acute doses of 1 Gy or more. It would be useful to study this effect after lower doses and in particular after low chronic-radiation exposures, as well as in other tissues where possible.
4. The numerical value of the DDREF appears to be lower from recent evidence in some human populations than in experimental animal systems, and the reasons for this are currently unclear. Also, regarding the effect of age-at-exposure, there are inconsistencies between the evidence in human populations and in experimental animal systems regarding carcinogenesis at the fetal and neonatal stages of development. Reconciling these differences at the biological level should help in underpinning the consistency and robustness of the protection system.

ANNEX A: HAEMATOPOIETIC TISSUES: ROLE PLAYED BY STEM CELLS AND LINEAGE-COMMITTED PROGENITOR CELLS IN RADIATION-INDUCED LEUKAEMIA

A.1. Radiation-induced leukaemias and lymphomas

(A1) Leukaemias and related neoplasias of the blood-forming system of body are prominent, late-arising pathologies of prior, sufficiently damaging radiation exposures. As a general class of radiation-induced "cancer", leukaemia is the earliest arising type that manifests following exposure, and is arguably the best defined, most radiogenic form of cancer. Induction of leukaemia by ionising radiation is well documented in both humans and in animals (ICRP, 2005, 2012; UNSCEAR, 2000, 2006; 1990; 2006).

A.1.1. Epidemiological studies

(A2) As a single disease entity, radiogenic leukaemia has an appreciably higher ERR than any of the other late-arising pathologies that have manifest within the A-bomb survivor cohort. As shown in Table A.1., the estimated ERR per Gy of exposure for leukaemia (in aggregate for both sexes, using a linear dose model) within the A-bomb (LSS) survivor cohort is approximately 8-10 fold higher than it is for the solid cancers, and about 30-35 fold higher than for the non-cancer diseases. The latter category selectively excludes a group of non-neoplastic blood disorders, e.g. the myelodysplastic syndromes (MDS), which have pathological relationships with evolving leukaemia and in turn have comparably elevated ERRs to that noted for the radiogenic leukaemias (Iwanaga et al., 2011), although the time pattern is peculiar in that increased risk are high some 40 years after exposures and does not seem to decline as is the case for radiogenic leukaemia over time. MDS was not a disease classification until the 1980s, so that early occurrence could not be effectively evalued. Gender differences have been observed, albeit relatively small: for males and females, the estimated ERRs for leukaemia mortality have been estimated to be respectively 4.6 (95% CI: 3.0, 6.9) and 3.9 (2.5, 6.1) per Gy, based on a linear dose-effect model (Ozasa et al., 2012). Estimates of absolute rates (i.e. EARs), by contrast, tend to indicate greater differences between the sexes: e.g. BEIR VII (2006) reported an EAR for leukaemic deaths in males to be 1.62 per 10^4 PY-Sv, whereas for females the estimate was considerably lower, 0.93 per 10^4 PY-Sv. Additional estimates of leukaemic risk based on alternative radiation-exposed cohorts have been developed and reported, and many indicate generally comparable levels of elevated risk, but with a much greater level of uncertainty; e.g. an ERR/Gy estimate of 1.93, with 95% CI (<0, 8.47), was reported for a massive cohort of occupationally exposed nuclear workers (~400,000) from 15 countries (Cardis et al., 2007). The lack of statistical significance in such large studies indicates that chance cannot be discounted as one possible explanation for the observed association, related in part to the relatively low dose and narrow dose range of the exposed population.

(A3) The risk of developing radiogenic leukaemia is related to the extent of radiation exposure. Within the LSS cohort of A-bomb survivors, at very low doses ranging from 0.005 to 0.1 Gy, an estimated 5% of the leukaemias likely developed as a result of the irradiation. At higher exposure levels of 0.1 to 0.5 Gy, an estimated 36% of the cases were radiation associated. At a still higher range of doses of 0.5-1.0 Gy, 66% of the cases were estimated to be radiation related, and at or above 1.0 Gy, it was 86% (Table A.2.).

Table A.1. Estimated ERR per Gy for major causes of death within the A-bomb (LSS) surviving cohort 1950 to 2003[1]

Cause of death	Gender	Number[2]	Model	P-value	Est ERR/Gy[3,4]	95% CI
All causes	M&F	50,620	L	<0.001	0.22	0.18,0.26
Leukaemia	M&F	318	L/Q at 1Gy	<0.001	3.1	1.8,4.3
Leukaemia	M&F	318	L/Q at 0.1Gy	<0.001	0.15	-0.01,0.31
"	M	163	L	<0.001	4.6*	3.0.6.9
"	F	155	L	<0.001	3.9*	2.5,6.1
Leukaemia[5]	*m/f*	*310*	*L*		*4.7*	*3.5,6.4*
AML Leukaemia[5]	*M&F*	*124*	*L*		*4.3*	*2.7,6.6*
CML leukaemia[5]	*M&F*	*58*	*L*		*6.4*	*3.0,13.7*
ALL leukaemia[5]	*M&F*	*19*	*L*		*3.7*	*0.8,13.0*
MDS[6]	*m/f*	*47*	*L*	*<0.001*	*4.3*	*1.6,9.5*
Lymphoma	M&F	284	L	>0.05	0.16	-0.13, 0.59
Multiple myeloma	m/f	93	L	>0.05	0.54	-0.04, 1.58
Solid cancers	m/f	10929	L	< 0.001	0.47	0.38,0.56
Non-cancer diseases[7,8]	m/f	25618	L	< 0.001	0.13	0.08,0.18
Blood diseases	*m/f*	*238*	*L*	*<0.001*	*1.7*	*0.96,2.7*

[1] Data obtained from Ozasa et al. 2012 (except where otherwise indicated).
[2] Numbers listed from the LSS cohort registry (~120,000 individuals).
[3] Estimates for ERR values are sex-averaged (* indicates ERR estimate is for a single sex) and based on a linear dose model for all causes of death (all categories), except for the 'leukaemia' value which is based on a LQ model and for an exposure of 1 Gy (at a lower 0.1 Gy exposure, the ERR estimate is 0.15 with 95% CI of -0.01 and 0.31).
[4] ERR estimates for leukaemia, based on a linear dose model, yields higher ERRs for both males (4.6/Gy with 95% CI: 3.0, 6.9) and females (3.9/Gy with 95% CI: 2.5, 6.1).
[5] ERR estimates (without effect modification), along with 90% CI, are listed for both the aggregate (sex-combined) leukaemia-related mortality response, as well as for the leukaemia mortality responses of major subtypes of the radiogenic leukaemias, as listed in Richardson et al. (2009).
[6] MDS, Myelodysplastic syndrome: a preclinical haematological disease that often precedes the onset of myeloid leukaemia. Data presented are from Iwanaga et al. (2011): 47 MDS patients within the LSS cohort of 22,245 Nagasaki subjects.
[7] ERR estimate (excluding non-neoplastic blood diseases) made using a linear dose-effect model, and based on the aggregate of non-cancer deaths recorded from 1966-2003.
[8] Non-neoplastic blood diseases that were excluded from the non-cancer disease category.

(A4) The above updated estimates closely correspond to those reported earlier (Pierce et al., 1996; Preston et al., 1994), when approximately 44% of the total leukaemic deaths (78 of 176) observed in the LSS cohort for doses above 0.005 Sv were attributable to the A-bomb irradiation.

(A5) Points to note are: (1) estimates clearly indicate a rising proportion of exposure-related leukaemias related to dose; (2) the dose-dependent rise is not simply linear, but has upward curvature (Fig. A.1.) which can be fitted using an LQ function; (3) estimates and models pertain to exposure levels ranging from ~0.005 Gy to 3.0 Gy; and (4) specific types of leukaemia (e.g. acute, chronic, myeloid, lymphocytic, etc) give rise to different risk estimates. This is illustrated in Table A.3 by the marked variance in the excess number of leukaemic deaths resulting from spinal irradiation for ankylosing spondylitis (Darby et al., 1987; 1990). Note that in the Darby et al. study, the "observed to expected ratio" of leukaemic cases was

calculated to be ~3.17, based on the 39 leukaemic deaths observed, and 12.29 which had been expected within a cohort of 14,106 patients and a follow up period of ~48 years. An earlier study of the same cohort by Smith and Doll (1982) reported that these patients had average bone marrow doses ~3.21 Gy and a calculated ERR of 0.98 per Gy. Also, note that a more recent follow-up study on this same cohort was report by Weiss et al. 1995: in contrast to earlier reports, the ratio of 'observed-to-expected' cases of leukaemia, excluding CLL, was ~11 during a period 1-5 years following initial radiation treatment; this ratio declined to ~1.9 for the 25-year period following initial exposure; the ERR for ALL, a leukaemia subtype, was estimated to be ~12/Gy using an LQ dose model and an average spinal marrow radiation dose of ~4 Gy.

Table A.2. Leukaemic mortality 1950-2000 within the A-bomb LSS cohort of survivors[1]

Marrow dose (Gy)	Subjects	Person-years	Cases	Estimated excess	Estimated attributable risk (%) [2]
<0.005	37,407	1,376,521	92	0.1	<0.2
0.005-0.1	30,387	1,125,891	69	4.0	5.6
0.1-0.2	5,841	208,445	14	4.7	33.6
0.2-0.5	6,304	231,149	27	10.4	38.5
0.5-1.0	3,963	144,276	30	18.9	63.0
1.0-2.0	1,972	71,485	39	27.7	71.0
2.0+	737	26,589	25	28.2	>100
Total	86,611	3,184,356	296	93	31.4

[1] From Preston et al. Radiat Res 162: 377-389, 2004, with added 'attributable risks'.
[2] Estimates of 'attributable risk' for leukaemia added to the Preston et al. table by the present authors.

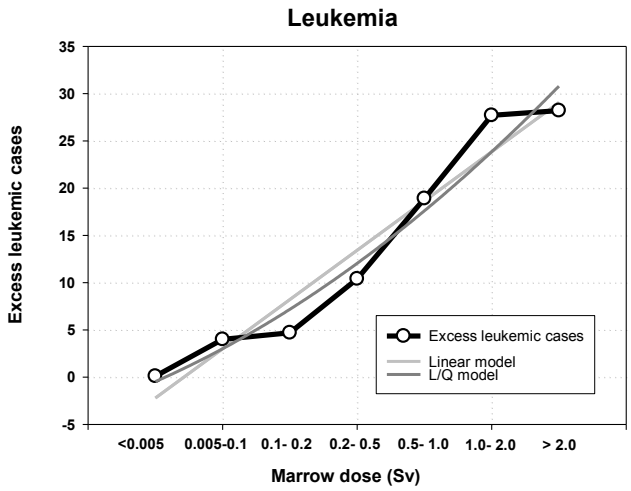

Fig. A.1. Linear-quadratic (LQ)-fitted radiation exposure versus leukaemic incidence relationship. Excess number of total leukaemic cases recorded within the LSS cohort of the A-bomb survivors. Note the marked and significant (P=0.002) upward curvature of the response at the higher doses (graph drawn from tabulated data of Preston et al. 2004). (Permission needed)

Table A.3. Excess number of leukaemic deaths resulting from spinal irradiation for ankylosing spondylitis[1, 2]

Leukaemic subtype	Number of deaths		
	Observed number	Expected number	Ratio of observed/expected
Myeloid leukaemia:			
Acute	17	4.34	3.92
Chronic	3	2.05	1.46
Unspecified	4	0.71	5.63
all subtypes	24	7.10	3.38
Lymphocytic leukaemia:			
Acute*	2	0.93	2.15
Chronic	2	2.38	0.84
Unspecified	3	0.38	7.89
all types	7	3.69	1.89
Unspecified leukaemia:	3	0.28	10.71
all types	36	11.29	3.198*

[1] Darby et al.1987, reported in BEIR V 1990.

[2] Patients received fractionated regimens of partial-body, x irradiation of the spine; average, cumulative exposures to the bone marrow were estimated to be 3.21 Gy (Smith and Doll 1982).

* Follow-up study by Weiss et al. (1995) reported on 60 cases of leukaemia among 13,914 patients with ankylosing spondylitis and treated by fractionated radiotherapy: (a) average radiation dose to bone marrow was estimated at 4.38 Gy; (b) observed-to-expected ratios for periods 1-5 years and 1-25 years post initial treatments were ~11 and 1.9 respectively; and (c) using an LQ dose model, the ERR at 1 Gy exposure for ALL was estimated at ~7/Gy.

(A6) Estimates of leukaemic risk vary depending on both (a) the subject's 'age' at the time of radiation exposure and (b) the subject's 'attained age' following exposure (Preston et al., 2004). For the very young (0-9 years), the EAR estimate is 0.66 per 10,000 PY Sv (90% CI: 0.13, 1.3) and with a negative, 'time-since-exposure' power function of -1.1 (-1.6, -0.07); for younger adults, 20-39 years of years, the EAR estimate is elevated at 1.3 (0.3, 2.5) and has a small, but positive, 'time-since exposure' power function of 0.03 (-0.6, 0.7); older adults (≥40 years), the EAR estimate is further elevated at 1.9 (0.4, 3.9), with a relatively strong, positive 'time-since-exposure' power function of 0.5 (-0.3, 1.3). Estimates of ERRs vary markedly according to 'age' as well: a temporal 'wave' of markedly elevated risk estimates (e.g. ERR ~10-100/Gy) has been noted in very young exposed cohorts over the initial decade or two post-exposure (Little, 2009; Richardson D, 2009; Wakeford, 2008, 2012; Weiss et al., 1995). The most recent analysis of leukaemia among A-bomb survivors, however, has not shown as large a risk as seen in these previously analyses (Hsu et al., 2013). As the age of the exposed cohort increases (i.e. both the age-at-exposure, as well as the attained age at the time of analyses), the magnitude of the RR declines appreciably, but still remains significant (Kodama et al., 2012).

(A7) Risk estimates for leukaemia associated with *in utero* radiations have varied widely: for example, markedly elevated and statistically significant estimates (ERR ~50/Gy) of childhood leukaemia associated with antenatal diagnostic radiography continue to be reported from updated case-control studies of the OSCC (Wakeford, 2008); whereas, by contrast, significantly increased leukaemic risks have not been demonstrated in any cohort study of *in utero* irradiated individuals (NCRP Report 174, 2014), e.g. offspring of Mayak female nuclear workers (Schonfeld et al., 2012); children of Japanese A-bomb survivors (Jablon and

Kato, 1970), or 40,000 prenatally exposed children in the UK followed prospectively (Court Brown et al., 1960).

A.1.2. Experimental animal studies

(A8) The results from experimental animal studies are generally consistent with the human epidemiological observations and associated risk estimates. Although the dose-dependent nature of induced leukaemia from both acute and fractionated/chronic whole-body ionising radiation has been demonstrated in a wide variety of experimental animal species under different experimental conditions, the relationship between exposure dose and leukaemic incidence is: (a) quite variable, depending on the nature of the induced leukaemic subtype; (b) dependent on substantial levels of bone marrow exposure and damage; and (c) tends not to conform to a simple, LNT function (1990). Experimental studies have not found consistent evidence that *in utero* exposure to radiation increases the risk of leukaemia in offspring (NCRP Report 174, 2014; Upton 1960).

(A9) Of the experimental radiation-induced lymphohaematopoietic malignancies, the thymus-associated T-cell lymphomas have been most studied: for instance, in select strains of mice, these T-cell lymphomas can be readily and reproducibility induced at extremely high frequency by minimal, fractionated dose regimens (e.g. 4 x 2 Gy fractions delivered at weekly intervals for a month) (Humblet et al., 1997; Utsuyama and Hirokawa, 2003). With use of the latter model, exposure thresholds can be readily demonstrated, as well as the protective actions of bone marrow shielding or infusions of normal, unirradiated marrow (Humblet et al., 1989; Kaplan, 1974; Sado et al., 1991). Age of the animal and of the thymus itself plays a critical role following such lymphoma/leukaemic-inducing regimens of ionising radiation; i.e. malignant process(es) are strongly influenced by age-related factors of the thymic microenvironment, the bone marrow and the host environment (Utsuyama and Hirokawa, 2003). Early work suggested that these thymus-associated T-cell lymphomas (as well as radiation-induced myeloid leukaemias) in experimental mice were largely the result of radiation-induced activation of latent leukaemia retrovirus (Gross, 1959; Janowski M. et al., 1987). However, it is now recognised that in a number of strains of mice (e.g. NFS, C57BL/6 mice) with well characterised susceptibilities to radiation-induced thymic lymphomas, exposure-related malignancies are manifested that are largely free of transforming endogenous ecotropic retrovirus (Ihle et al., 1976; Kominami and Niwa, 2006; Okumoto et al., 1990).

(A10) Molecular/cytogenetic analyses of radiation-induced leukaemias in a number of mouse strains have revealed a number of predisposing genetic lesions, e.g. an allelic loss on chromosome 4 of the LYrs/TLSR5 locus in >95% of pre-B lympho-myeloid leukaemias of irradiated CBA/H mice (Cleary et al., 2001).

(A11) Experimental myeloid leukaemia has been somewhat less studied than the lymphomas or lymphocytic leukaemias. The nature and patterns of induction of myeloid leukaemia seem to align more closely to the noted disease induction patterns in humans. For example, in select strains of myeloid leukaemia-susceptible mice (CBA, RFM, C3H), the radiation exposure versus myeloid leukaemia incidence curve rises proportionately with increasing doses of acute, whole-body γ or x irradiation (Major and Mole, 1978; Mole et al., 1983; Otsu et al., 1995; Upton, 1985). The response at very low levels of exposure (from background levels to approximately 1 Gy) is fairly linear. At doses from 1 to 2-3 Gy, the incidence curve tends to exhibit upward curvature; and at doses greater than 3 Gy, the curve tends to bend over downwards.

A.2. Relevant data for various radiations and exposure types

A.2.1. Epidemiological data

(A12) Although radiobiological relationships concerning protracted, low-dose/dose-rate exposures are clearly less well-defined in humans compared to experimental animals, it seems highly likely that basic relationships are comparable and that further, detailed epidemiological work will show this to be the case. In support of this contention, BEIR-VII (BEIR VII, 2006) ERR estimates for leukaemia (excluding CLL) within select, chronically exposed 'worker populations' were significantly lower than those reported for A-bomb survivors (i.e. an ERR of 2.2/Gy for 'all ages' of 'workers' chronically exposed to low doses of radiation is significantly lower than the earlier reported ERR value of 4.2/Gy for A-bomb survivors who were acutely exposed to relatively high doses of low-LET radiation).

(A13) Epidemiological studies of leukaemia incidence within the chronically exposed Techa river cohort, however, have indicated a strong, dose-dependent response with ERR values of 4.2 and 4.9 for all subclasses of leukaemias, except CLL (Krestinina et al., 2010 ; Krestinina et al., 2005). These elevated ERR values might be due to the nature of the chronic exposures: namely either mixed external/internal exposures of both high and low-LET radiation, or perhaps due to variable rates of exposure over time. If the latter is correct, then the elevated ERR values might be more consistent with prevailing radiobiology theory (i.e. little to no sparing of cancer/leukaemia incidence with protraction of low dose/dose-rate, high-LET type exposures).

(A14) The overall leukaemia-inducing potential of internally-deposited radionuclides, especially bone-seeking, α-emitting nuclides (e.g. in ^{226}Ra/^{228}Rn exposed dial painters (Spiers et al., 1983) and in ^{239}Pu exposed nuclear workers at the Russian Mayak nuclear complex (WHO, 2001), is somewhat less than what one might expect by comparison to the leukaemogenic efficiencies of external, whole-body exposures with low-LET x- or γ-rays. This is despite accounting for estimated dose-equivalents to tissue targets such as the endosteal layer at the bone/marrow interface, and the substantial differences in LET of various types of radiations. There is no question that internally-deposited radionuclides have the potential to cause leukaemia in humans: this has been amply documented under a variety of exposure conditions and with various radionuclides (Andersson et al., 1993; Wick et al., 2008). The difference seems to lie in terms of the leukaemogenic efficiency of the different radiation qualities.

A.2.2. Experimentally-based animal data

(A15) The effect of both radiation quality and radiation dose rate can been seen readily by the change in slope of the myeloid leukaemia incidence curve between total doses ranging from background levels to doses of 1-3 Gy. Experimentally (in various strains of mice; e.g. RFM and CBA), myeloid leukaemia incidence per Gy markedly declines with increasing dose fractionation (or continuous exposures at very low dose rates) (Mole and Major, 1983; Otsu et al., 1995; Upton et al., 1970). From such studies, DDREF values have been estimated and range from 3-9 (Otsu et al., 1995). These estimates are comparable to those DDREF estimates (i.e. 2-10) made for humans exposed chronically to low doses/dose-rates of low-LET radiation (NCRP-64, 1980).

(A16) The dose-fractionation effect on myeloid leukaemia incidence is considerably more pronounced with low-LET x- or γ–ray exposures than with high-LET neutrons (Upton, 1985; Upton et al., 1970). The latter LET/dose-rate effect on myeloid leukaemia incidence has a

very significant effect on the estimated RBE values for neutrons, and probably for other high LET irradiations, For example, the estimated RBE for neutrons at high dose-rate is in the range of 2-3 for myeloid leukaemia incidence, while at very low dose rates (~ 0.01 Gy min^{-1}) the RBE dramatically increases to 16 or more (Ullrich and Preston, 1987; Upton et al., 1970). Nevertheless, the latter values are much less relative to the RBE estimates of 20-50 as reported for the induction of solid tumours of neutron-exposed rodents. Regardless, these markedly and significantly elevated values of RBE at low doses/dose-rates of high LET fission-neutron exposures clearly have the potential to impact leukaemia/cancer risk assessment process(es) (BEIR VII, 2006).

(A17) The leukaemogenic potential of internally deposited radionuclides has been verified using various animal models under a variety of treatment protocols (Boecker, 1995; Humphreys et al., 1987).

(A18) Short-range α-particle emitters have been used to help identify the location of the target cells for radiation-induced leukaemia and osteosarcoma. After radionuclide injection in mice, cumulative radiation dose to different regions of the bone and marrow was assessed in detail from sequential microradiographs taken over a period of 15 months (Lord et al., 2001). The relative incidence of osteosarcoma after uptake and redistribution of ^{239}Pu, ^{241}Am, or ^{233}U correlated preferentially with the dose to target cells averaged over just 0-40 μm from the bone surface, and for AML risk the correlation was closest for target cells in the central sinusoidal region. Although there are caveats to this, such as cell migration during the dosimetry period, the results support the general contention of the importance of the endosteal and sinusoidal regions in radiation carcinogenesis.

A.3. General features of haematopoietic tissues

(A19) The haematopoietic system is an essential organ system whose primary activity is manufacture of various species of mature, functional cells which circulate in the blood. These blood cells include: erythrocytes for oxygen transport; leukocytes for immune defence; and platelets for haemostasis. The cell producing capacity and fidelity are great: e.g. approximately 2.5 x 10^9 erythrocytes, ~2.5 x 10^9 platelets, and ~10 x 10^9 granulocytes per kg body weight are produced on a daily basis in humans (Williams et al., 1990).

(A20) Haematopoietic tissue is a hierarchical, self-renewing, and cell amplifying tissue, maintained by small numbers of stem cells and early progenitor cells, whose primary functions respectively are to asymmetrically self-renew following infrequent divisional cycles and to commit to differentiate into specific blood cell lineages. The most primitive HSCs (marrow repopulating HSCs) of humans, in a steady state, are thought to undergo 50 or more divisional cycles during an average lifespan, producing upward of ~10^{15} blood cells (Kay, 1965). Note that reported estimates for replicative capacity can vary widely, ranging from ~5 to ~1000 divisions per lifetime, depending on, but not limited to, such factors as the nature of HSC population/subpopulation and physiological state of the marrow at the time of HSC isolation and, of course, the species from which the marrow was derived. To illustrate the later, small subpopulations of largely-quiescent HSCs in steady-state marrow of humans and mice replicate at significantly different rates, namely ~280 days and 145 days, respectively; therefore, suggesting markedly different numbers of HSC replications per lifespan for individuals within these species (Catlin et al., 2011; Kay, 1965; Harrison, 1979a, b; Pietras et al., 2011; Shepherd et al., 2004; Shepherd et al., 2007). These stem cells do not function independently, but rather in a well-orchestrated manner dictated largely by stromal cell niches and associated endogenous signalling networks (Fig. A.2.) (NCRP-150, 2005).

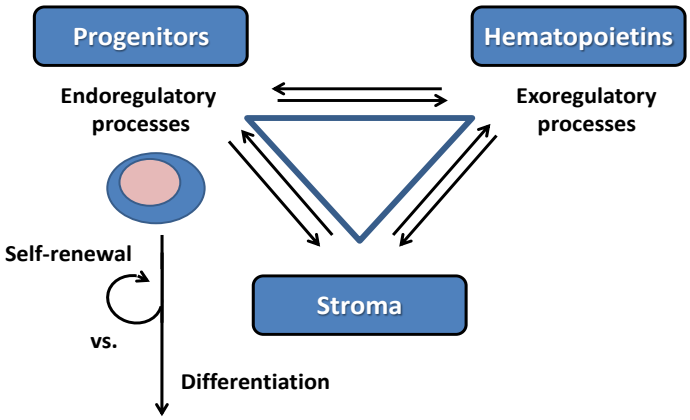

Fig. A.2. Interrelationships of bone marrow stroma, haematopoietins, and haematopoietic progenitor cells. (Figure reproduced courtesy of the NCRP) (permission needed)

(A21) The most primitive of the HSCs is exceedingly rare cell types, occurring at estimated frequencies from 10^{-4} to 10^{-5}. The current consensus is that there are two major HSC compartments within the marrow. The first, often labelled the Lt-HSC compartment, is more primitive, quiescent by nature. They are responsible for the long-term renewal and maintenance of haematopoiesis. The second, labelled the St-HSC compartment, somewhat quiescent, with higher self-renewal, and shorter term responsibilities for repopulating precursor marrow niches (Yin and Li, 2006). Under steady-state conditions, these cells are largely quiescent (>95% in a non-cycling G_0 state), but when forced into a divisional cycle they preferentially self-renew. These HSCs reside within three domains: (i) endosteal; (ii) parenchyma cord; and (iii) vascular sinus regions (Shiozawa and Taichman, 2012; Fig. A.3). Through an ill-defined process, selected daughters of the stem cells take alternative steps and begin to commit to one specific lineage, thereby losing some self-renewing capacity but gaining proliferative activity. Through successive divisional cycles, sufficient numbers of lineage-committed progenitor cells are produced in order to satisfy the production requirements for specific cell lineages, and at this stage, proliferation stops and terminal maturation processes begin. Count estimates for a number of these marrow compartments are listed in Fig. A.4.

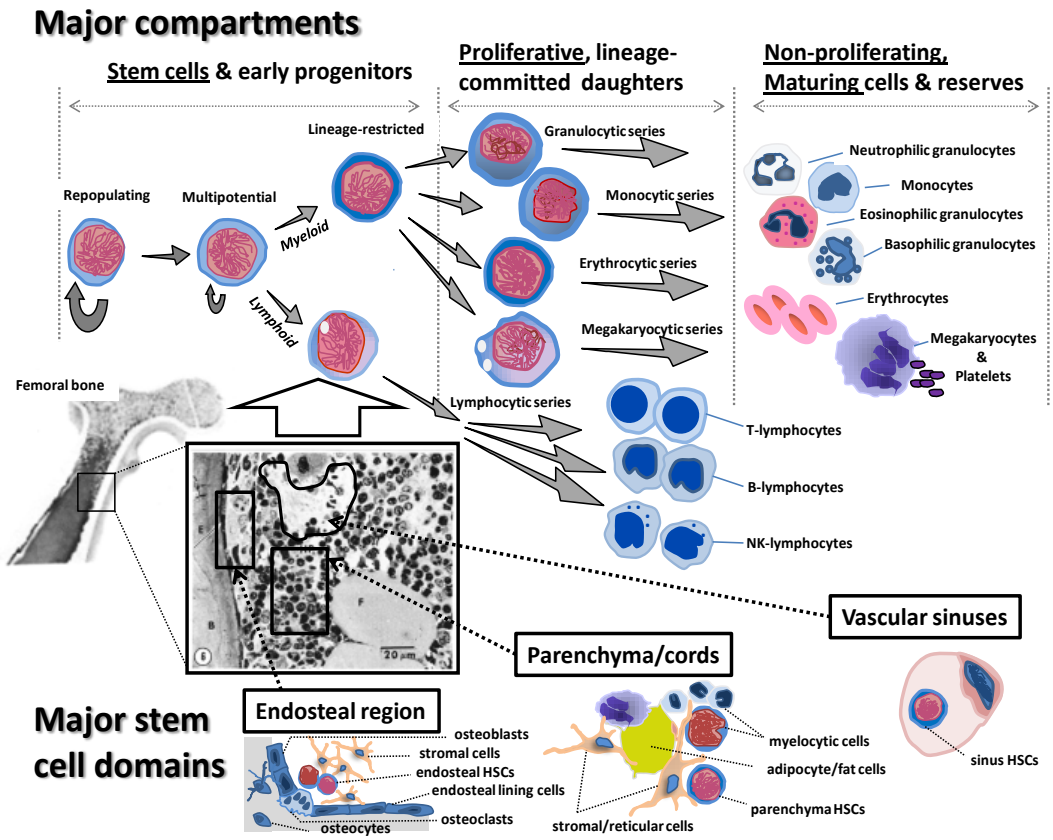

Fig. A.3. General features of haematopoietic tissues, modified from an NIH online report (NIH Report, 2008). (Bone cross-section drawing reproduced with the courtesy and permission of Terese Winslow LLC, Alexandria, VA). (permission needed)

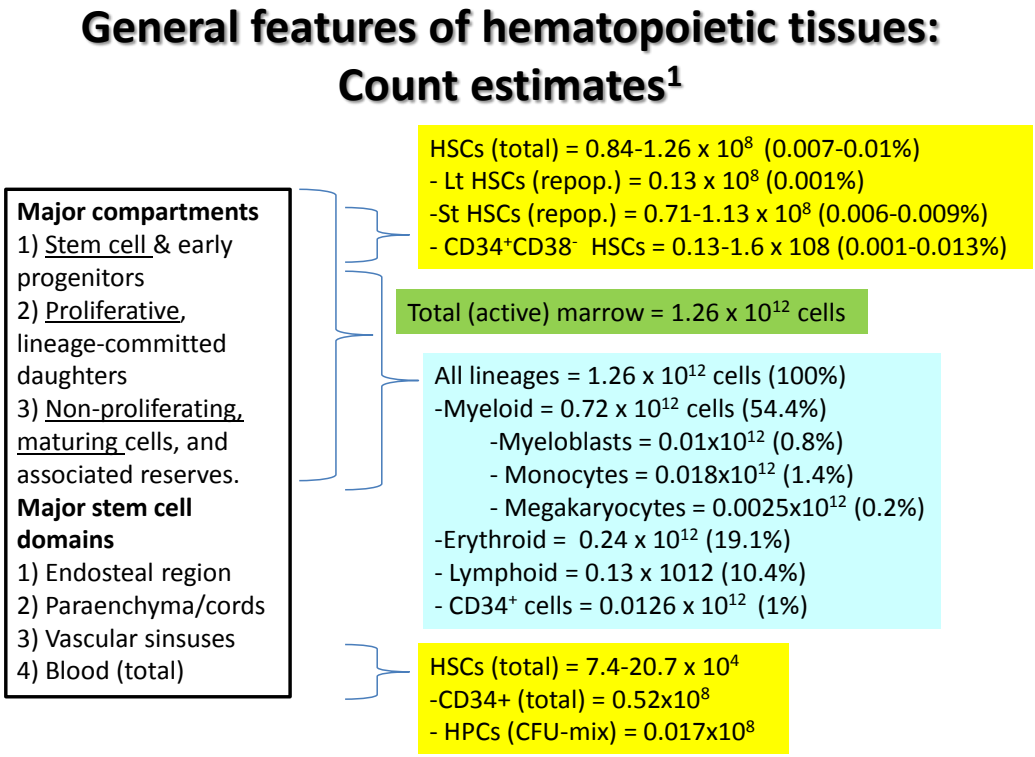

Fig. A.4. Estimates of cell numbers for various major haematopoietic tissue compartments. (permission needed)

(A22) The effect of collateral GI injury and selected types of microbial flora (e.g. *Helicobacter sp.*) on leukaemic progression is fairly well recognised. In contrast to conventionally-reared laboratory mice, gnotobiotic (germ-free) RFM mice appear highly resistant to radiation-induced leukaemia (Walburg et al., 1965). In contrast, humans with various types of chronic infections that generate genome-modifying free radicals, appear susceptible to leukaemia and related lympho-haematopoietic disorders (Karaoglu et al., 2004; Maeda, 1998; zur Hausen, 2009). The precise mechanism(s) by which chronic infection and ionising radiation might synergise relative to stem cell transformation remains speculative.

A.3.1. Turnover rate

(A23) There are at least 8 blood cell types that circulate within the blood, each having defined turnover rates, distinct cell origins and lineages, with separate reproductive and maturational histories. As such, "turnover" (as defined by "cell replacement") of haematopoietic tissue needs to be considered in the light of its multilineage nature. As indicated earlier, the origin of these multiple, blood-forming cell lineages lies within the progenitor cell compartments of the bone marrow. These small but vital cell compartments contain a continuum of very primitive and less-primitive progenitor cells having a full range of self-renewal potentials and reproductive capacities, as well as varying cell cycling activities and pre-programmed commitments for lineage differentiation. It remains uncertain, however, whether or not the most primitive of these progenitor marrow compartments ever renew completely during the lifespan of the individual. Nevertheless it is the lifespan and

change in number of circulating, functionally mature cells in the blood that ultimately dictate the turnover of haematopoietic tissue.

(A24) Under steady-state conditions, turnover rates of the different types of blood cells within the circulation can differ by orders of magnitude: for example, the circulating pool of erythrocytes in man turns over completely every 120 days or so, while circulating pools of blood platelets and granulocytes are replaced approximately every 5-9 days and 12-24 hours, respectively. Under a variety of abnormal haematological conditions, these turnover rates can be altered appreciably. As such, the trilineal haematopoietic tissues of the marrow (i.e., myelopoietic, erythropoietic, and megakaryocytopoietic elements) need to be both flexible and robust, not only on a daily basis, but also throughout life. In terms of erythropoietic capacity, approximately 1.75×10^{11} fully functional, fully mature red cells (per average ~70 kg person) are produced in the marrow and released into the blood on a daily basis in order to replenish the daily loss of approximately 0.8% of the circulating red cell pool. Megakaryocytic elements of the marrow need to produce and release a comparable number of 1.75×10^{11} platelets in order to make up for the daily loss of ~16% of the circulating platelet pool. Also, the granulocytopoietic elements of the marrow need to produce daily $\sim 7 \times 10^{11}$ blood granulocytes in order to completely replenish the circulating pool about every 12 hours.

(A25) Blood cell losses can be accentuated appreciably under a variety of disease states (including ionising radiation induced cytopenias), and the durability and capacity of the marrow's blood-cell production are quite impressive. It is estimated that there are ~2600 grams of active bone marrow distributed somewhat unevenly within some 206 bones of the body (not all bones contain active bone marrow, but under select physiological/pathophysiological conditions, all can be induced to produce active marrow). This marrow contains approximately 1.26×10^{12} bone marrow cells (Fig. A.4.) and has a turnover rate of 3.16×10^{11} cells per 70 kg body weight per day (Fliedner, 1998). From these numbers it would appear that in aggregate, approximately a quarter of the marrow renews itself daily. However, the latter estimate is an average over all the marrow compartments, and turnover within specific compartments (e.g. progenitor versus post-proliferative, maturing compartments) can vary by orders of magnitude.

(A26) When the haematopoietic system comes under stress and circulating blood cell levels fall (or rise) abnormally, the marrow responds in a strictly controlled and graded fashion. First, the tissue releases (or retains, in cases of excess cell numbers) stored reserves of fully mature functional cells. Second, the tissue alters (shortens in cases of deficit, and lengthens in cases of excess) the transit times of maturing, non-dividing cells. Third, there can be additional divisional cycles within the proliferative cell compartments. Fourth, there can be selective enhancement of differentiation of progenitor cells at the expense of self-renewal. Experimental data support the contention that all of the latter processes are operative during or following periods of severe cytopenia, especially those that arise as a consequence of high-dose radiation exposure (Carsten, 1984; Fliedner et al., 2002).

(A27) Turnover rates of marrow HSCs have been estimated for a number of species, including humans. The estimates have been made using indirect approaches, i.e. measures of downstream responses of HSC daughter cells as reflections of the true kinetics of HSCs themselves (Shepherd et al., 2004; Shepherd et al., 2007). Using a combination of techniques, i.e. telomere length measurements using fully mature blood granulocytes and selective genetic lineage tracing of marrow progenitors, turnover rates of marrow HSCs in different species have been estimated more accurately. Average HSC turnover rates for the following species were reported: ~2.5 weeks in mouse (via telomere shortening assays); ~8.3 weeks in cat; ~23 to 36 weeks in non-human primate (baboon) (depending on the use of telomere shortening or gene tracking); and ~45 weeks in humans (Shepherd et al., 2004; Shepherd et

al., 2007). From these data, Shepherd et al. suggested that there is a highly conserved, species-independent constancy for HSC replication over the lifespan of the individual. This estimate ranged from 80 to 200 HSC replications per individual per lifespan.

A.3.2. Age dependence

(A28) Age has a profound effect on both structure and function (and radiosensitivity) of the body's haematopoietic tissues. The earliest, 'primitive' form of haematopoiesis is within the yolk sack (3-4 weeks post-conception), subsequently it moves to the fetal liver (4-8 weeks) and only after 8-9 weeks does it start to move to its final destination in fetal bone (Kelemen and Calvo, 1982). In addition to this initial ephemeral site of haematopoiesis within the yolk sack, a second, more stable and 'definitive' site of haematopoiesis containing self-renewing, multipotent adult-type HSCs emerges autonomously within the aorta/gonad/mesonephros (AGM) region of the embryo (Tavian et al. 2010). Haematopoiesis in fetal bone results from the seeding of liver-derived, circulating HSCs into a bony-encapsulated receptive matrix comprised of innervative vascular and mesenchymal elements (Fliedner et al., 2002). The cellular processes and sequence of steps in developing this receptive matrix within the primitive bone cavity appear to be similar in most mammals, e.g. cartilage within fetal bone cavities becomes necrobiotic, leaving centralised cavities into which primitive mesenchymal elements of the perichondrium penetrate, followed by infiltrating nerves and vasculature (Fliedner, 1998). The change from skeletal cartilage to pre-haematopoietic stroma to haematopoiesis seems to be the same among mammalian species, although the temporal patterns expressed by different bones appear to differ. The latter sequence of matrix development to stem cell seeding to haematopoiesis, starts at about 6 to 8 weeks of gestation, but appears not to be completed (all bone marrow sites initiated) until ~15 weeks or later. Only during these later gestational periods (≥16 weeks post-conception) does the bone marrow become the dominant site of haematopoiesis in the developing human fetus (Kardel et al. 2012). High concentrations of circulating stem cells and progenitor cells, along with an abundance of receptive stromal niches within bones, seem to drive the seeding process and the establishment of new sites of haematopoiesis within fetal bones. By 21-22 weeks post-conception, long bones, ribs and sternum, and vertebra exhibit robust haematopoiesis (red marrow dominates and yellow marrow is absent). This accounts for ~70% of the active marrow and its estimated 1.5-2.5 x 10^{10} nucleated marrow cells at birth (Kelemen and Calvo, 1982). During prenatal periods (>12 weeks post-conception), the marrow is rich in myeloid-committed progenitors (e.g. GM-CFU), and estimates are in the range of ~2-10 x10^6. Similarly, fetal blood concentrations of stem cells and progenitor cells appear enriched, an order of magnitude larger than those concentrations found in adults under normal steady-state conditions.

(A29) In contrast to the quiescent nature of primitive HSCs from bone marrow of adults, prenatal progenitor cell populations are non-quiescent and actively cycling; it is only during the early postnatal period (i.e. ~3-4 weeks in mice and probably ~2-4 years in humans) that the "quiescent state" becomes a characteristic feature of marrow HSCs (Bowie et al., 2006). Further, judging from the report by Bowie et al. (2006), full 'engrafting' potential of HSCs is reached only when this active cell-cycling ends, along with the transient overexpression of an essential, but countervailing HSC-homing chemokine (CXCL12).

(A30) The risk of radiation-induced leukaemia appears to decline with age at the time of exposure (Table A.4.), but increases with the elapsed time following exposure (BEIR VII, 2006). The relative leukaemic risk within the A-bomb survivor cohort declined appreciably with increasing age at the time of exposure (Pierce et al., 1996; Preston et al., 2004). In

contrast, the estimated risk increased with time following exposure. In very young survivors (0-9 years of age), the risk steeply increased during the early years following exposure, whereas in the older survivors, the rise in risk was significantly delayed and more gradual.

(A31) Estimates of leukaemic risk for the irradiated, developing fetus are less clear than for the young or for the adult. Earlier work stemming from a Study of Childhood Cancers in the United Kingdom reported RRs in the range of 1.4-1.5 for pregnant women who underwent obstetric x-ray examinations, and had received very low doses estimated to be in the range of 10-20 mGy (Doll and Wakeford, 1997). Follow-up analyses of these studies have questioned these elevated ERRs on the grounds of bias and confounding factors (Boice and Miller, 1999; ICRP Publication 90, 2003; NCRP Publication 174, 2014). Similarly, Delongchamp et al. (1997) reported elevated leukaemia mortality among *in utero* irradiated A-bomb survivors. However, this apparent rise in "radiation-associated leukaemia" was based on an extremely small, statistically-challenging number of observed cases, so that chance could not be discounted as an explanation. More recent analyses of leukaemia within this cohort have failed to detect a significant increase in leukaemia, especially when compared to the elevated number seen in the postnatally-exposed A-bomb survivors (DL Preston, personal communication; Hsu et al., 2013). Further, in a report of cancer risks (solid cancers and leukaemias) within 8,000 children of female Mayak nuclear workers who were exposed *in utero* to low doses (mean doses of ~55 mGy) of ionising radiation, any significant elevated cancer risks were not shown (Schonfeld et al., 2012). Similar differences in the apparent estimated risk of fetal exposures have also been noted experimentally. For example, a low incidence or even an absence of induced leukaemia or cancer was found in fetal irradiated mice (Upton, 1960; Ellender et al., 2006; Di Majo et al., 1990) and irradiated canines (Seed et al., 1987), yet increased leukaemia or cancer rates were observed following postnatal irradiation.

Table A.4 a & b. Age-dependent responses relative to lifetime attributable risks of leukaemia and other cancers: incidence and mortality[1,2]

a. Incidence

Cancer type/ gender	Age at exposure (years)									
	0	5	10	20	30	40	50	60	70	80
Leukaemia/ male	237	149	120	96	84	84	84	82	73	48
Leukaemia/ female	185	112	86	71	63	62	62	57	51	37
Solid cancers/ male	2326	1667	1325	881	602	564	507	407	270	126
Solid cancers/ female	4592	3265	2525	1575	1002	824	678	529	358	177

b. Mortality

Cancer type/ gender	Age at exposure (years)									
	0	5	10	20	30	40	50	60	70	80
Leukaemia/ male	71	71	71	67	64	67	71	73	69	51
Leukaemia/ female	53	52	53	51	51	52	54	55	52	38
Solid cancers/ male	1028	781	641	444	317	310	259	246	181	102
Solid cancers/ female	1717	1295	1051	711	491	455	415	354	265	152

[1] Number of cases per 100,000 persons exposed to 0.1 Gy

[2] Data from BEIR VII, phase 2 (2006). Tables 12D-1 & 12D-2 in Annex 12D, pp 311

A.3.3. Cellular features of HSCs

(A32) HSCs and their early progeny, i.e., haematopoietic progenitor cells (HPCs), are generally characterised either by (a) their functional attributes, (b) their cell surface properties,

or (c) a combination of these. This is in contrast to the conventional morphological definitions commonly applied to the fully mature and functional blood cells within the circulation. The most primitive of the HSCs (independent of species of origin) is not clearly identifiable morphologically. They have rather primitive, monocytic-like features, not dissimilar to that of small lymphocytes. These HSCs are small, roundish cells, ~7-10 µm in diameter, with large, oval to round nuclei, small cytoplasmic rims, and generally relatively smooth cell surfaces (van Bekkum, 1976). HPCs tend to be a little larger and somewhat more pleomorphic in size and shape, often reflecting cellular features characteristic of lineage commitment.

(A33) *Functional descriptors:* HSCs can be routinely identified, enumerated, and characterised using functional assays that utilise basic characteristics that serve to define the HSC's self-renewing and cell-amplifying properties and its potential for differentiation. Invariably, these assays are indirect by nature, employing the serial dilution and transfer of test marrow cells or marrow cell fractions into recipient animals or cultures, with subsequent time-based monitoring of either (a) tissue repopulation, or (b) tissue or animal survival as endpoints.

(A34) Lineage-committed HPCs, by contrast, can be quantified and functional properties examined somewhat more directly through the use of colony assays performed either *in vitro* (CFU-C) or *in vivo* (CFU-S) (Metcalf, 1971, 1977). Serial dilution and transfer plating (or infusion) of HPC-enriched cell fractions, with subsequent monitoring of clonogenic responses are exceedingly useful procedures for determining the numbers, proliferative and differentiative potentials of marrow-derived HPCs, regardless of their multi-, bi-, or uni-lineage potentials. These assays have provided, and continue to provide, an opportunity to examine and assess in detail the function of both individual and populations of HPCs.

(A35) Assays of myeloid-committed HPCs have long been used as clinically relevant, "surrogate" markers of marrow HSCs within both donor and recipient marrow prior to, or following, autologous or allogeneic bone marrow transplantation (Mangalik et al., 1979).

(A36) *Cell surface characteristics*: With the advent of multicolour FACS protocols, and arrays of cell-type specific monoclonal antibodies, phenotying via cell surface markers of rare lympho-haematopoietic progenitors was made possible (Spangrude et al., 1988). Currently, HSCs and HPCs can readily be distinguished using flow cytometry. Specific subsets of HSCs and lineage-specific HPCs can be identified, counted, and analysed in both steady-state conditions and after exposure to physiochemical toxicants (e.g. benzene and ionising radiation), as well as in evolving pathological conditions.

(A37) The major distinguishing cell surface features of these main classes of lympho-haematopoietic progenitor cells are as follows. First, progenitor cells of the marrow, i.e. HSCs plus HPCs, are characterised by a select few surface markers. For example in mice, high levels of Sca-1^{+} and stem cell factor receptor (cKit^{+}) along with low levels of thymocyte surface antigen-1 (Thy-1^{lo}) and low levels of CD34$^{+/lo}$, define the progenitorial class, whereas in primates, high levels of the CD34$^{+/hi}$ marker serves to define the progenitor cell group (NIH Report, 2008). Other defining surface markers for select HSC subsets in primates have been reported as well. These markers include, but certainly are not limited to, KDR^{+} which is a VEGRF2 surface receptor (Ziegler et al., 1999), the SLAM family receptors (Kiel et al., 2005), and myeloid leukaemia 1 (MCL-1) which is an apoptosis response modulator that belongs to the BCL-2 gene family (Opferman et al., 2005). Further, an HSC surface adhesion molecule, CD49f, has been recently described as a specific HSC marker in humans; HSCs bearing CD49f were characterised as being highly efficient in generating long-term, multilineage grafts (using an optimised HSC xenograft assay of intrafemorally injected, highly-enriched HSCs into female NOD-scid-IL2Rgc$^{-/-}$ mice) while the loss of the surface

marker identified a transiently-engrafting multipotential progenitor (MPP)-cell subpopulation (Notta et al., 2011). Second, HSCs are defined as being "lineage negative (Lin^-), whereas HPCs are defined by lineage-positivity (Lin^+) for either all, or some of specific lineages identified using a battery of monoclonal antibodies directed to specific blood cell types. Other sorting strategies using different arrays of cell surface markers have also been successfully employed. One sorting strategy used the differential expression of the surface marker "Rho" on HPCs and HSCs (Eckfeldt et al., 2005). Low Rho (Rho^{lo}) expression on $CD34^+CD33^-CD38^-$ cells served to define the HSC population, whereas the high Rho expression (Rho^{hi}) on $CD34^+CD33^-CD38^-$ cells identified them as HPCs. In turn, these sorted HSCs and HPCs were used in a unique functional screen (a morpholino antisense oligonucleotide-based screen in zebrafish) to identify and assess essential function of HSC- and HPC-specific genes during very early stages of organogenesis.

(A38) *Nuclear proteins and genes:* As HSCs reproduce and also produce progenitor daughter cells committed to either myeloid or lymphoid lineage development (HPCs), silent or low-functioning lineage-specific genes become activated (Krause, 2002). HSC-to-HPC transition and the associated shift from a non-lineage-committed state to one that is committed, occur when lineage-associated genes are activated by selective demethylation of specific lineage-repressive amino acids within bounding histones. At the macromolecular level, regions of condensed chromatin become more open and permissive of active transcription of associated genes. Good examples of the latter are data indicating that: (a) the activation of normally-silent B- and T-lymphoid related genes within human HSCs ($CD34^+CD39^{lo}$) is through the selective loss of repressive methylation marks on lysine 9; and (b) selective demethylation of H3 lysine 4 within B-cell specific gene loci (Maes et al., 2008).

(A39) There is general support for the concept that lymphoid-specific genes are already "primed" for expression prior to lineage commitment. A continuum of molecular events or stages exists within HSCs during the priming and full commitment to transition to HPCs. In contrast, HSC/HPC transition-related commitment either to erythroid, granulocyte and T-cell lineages appears more global and less specific, with loss of histone acetylation at non-lineage-associated genes. Nevertheless, the essential nature of activating select types or classes of genes in lineage commitment and subsequent lineage development has been well documented: e.g. c-Myb for T-cell development (Allen et al., 1999), and calmodulin-dependent protein kinase (CaM kinase) IV in regulating erythroid lineage commitment and survival (Wayman et al., 2000).

(A40) *Cytokines, cytokine receptors and cell signalling:* The precise nature of HSC self-renewal and lineage commitment remains ill defined. Regulation of HSC involves a combination of extrinsic and intrinsic signalling pathways that need to converge in order to properly regulate HSC's decisions on whether to self-renew or to initiate differentiation (Fig. A.2.) (Eckfeldt et al., 2005). Although a large number of cytokines, chemokines, and respective surface receptors have been identified, cloned and analysed for targeting activity and specificity, none has been demonstrated to have exclusivity relative to these vital "fate decision responses" by the HSC in a steady state as well as following irradiation. Nontheless, a number of novel regulators of HSC fate decisions have been identified. Overexpression of homeobox b4 (Hoxb4) has been shown to enhance replication of both murine and human HSCs, and in turn, to increase their repopulation potential (Antonchuk et al., 2001; Buske et al., 2002; Sauvageau et al., 1995). In contrast, maintenance of HSC quiescence appears to be intrinsically controlled, at least in part, by p21, a G_1-checkpoint, cyclin-dependent kinase inhibitor (Cheng et al., 2000). Extrinsic regulators of HSC self-renewal have been identified, including Notch (Varnum-Finney et al., 2000), mammalian homologues of Drosophila

wingless (Wnt) (Murdoch et al., 2003; Reya et al., 2003), sonic hedgehog (SHH) and bone morphogenetic protein 4 (BMP4) (Bhardwaj et al., 2001).

(A41) *In situ*, HSCs reside within growth-regulating stromal-cell niches of the marrow. "Fate decisions" made by resident HSCs represent in part the net result of signalling pathways emanating from the niche. A host of haematopoietic stimulators, e.g. cytokines, growth factors, soluble cytokine/growth factor receptors, such as SCF, fms-related tyrosine kinase 3 (flt3), erythropoietin, thrombopoietin, interleukin (IL) 6, soluble IL6 receptors, have been shown to enhance HSC and early HPC proliferation (Henschler et al., 1994; Zandstra et al., 1997).

A.4. Radiosensitivity

(A42) HSCs and lineage-committed progenitor cells (HPCs), as well as more-differentiated, proliferative daughter cells, all exhibit a high degree of heterogeneity regarding radiosensitivity (Wagemaker, 1995). The origin of this heterogeneity is not entirely clear, owing to the considerable differences in composition and function of largely-undefined subsets contained within these larger, progenitor haematopoietic compartments. Radiosensitivities are dependent on the species (e.g. rodents, canines, primates), the subclass of cells being tested, cell-cycle status, growth factor and microenvironmental influences (Hendry and Yang, 1995). In mice, these relationships have been well studied and documented, and hence are fairly well accepted as being accurate and reflective of the situation in other mammals, including primates. As one moves from the most primitive of the HSCs in the marrow (cells with marrow repopulating ability, MRA cells) to slightly less primitive, multipotential, lineage-committed cells such as CFU-S-day-12, to more mature, lineage-restricted HPCs (G-CFU or GM-CFU), the radiosensitivity of these cells shifts, and dramatically so, but not in a unidirectional manner (Gidali, 2002; Imai and Nakao, 1987; Ploemacher et al., 1992; Scheding et al., 1996). Further, minor subpopulations of radioresistant cells within larger, more dominant radiosensitive HSC and HPC marrow compartments have been clearly identified and documented within marrow of a variety of species, including mice, dogs, and primates (Inoue et al., 1995; Seed et al., 1982; van Bekkum, 1991). Based on a continuous monitoring of such shifts in radiosensitivity of myeloid-committed progenitor cells in marrow of chronically irradiated dogs, it has been suggested that these small radioresistant subpopulations become dominant following extended periods of radiation exposure, often associated with evolving myelodysplasia and/or frank myeloid leukaemia (Seed et al., 1985).

(A43) Shifts in radiosensitivity during very early phases of lineage committment (e.g. from quiescent, long-term marrow repopulating HSCs to short-term, non-quiescent, repopulating and cycling multipotential/multilineage progenitors) are associated with equally dramatic shifts in terms of type of basic DNA repair pathways employed to repair potentially-lethal DSBs following acute radiation exposure. Specifically, the most primitive and most quiescent of the marrow HSCs (in adult mice) appear to employ mainly error-prone NHEJ, whereas MPPs employ a high fidelity HR during cell reproduction in order to correct major lesions (Mohrin et al., 2010).

(A44) Radiosensitivity tends to rise during early HSC-to-HPC transition, and associated early phases of lineage commitment, and sequentially tends to fall selectively with further lineage-restriction and progenitor cell maturation (Fig. A.5.). Meijne et al. (1991) showed that within mouse marrow progenitor cell populations, (1) MRA cells are moderately radioresistant with measured D_0 values in the range of 1.13-1.18 Gy; (2) less mature CFU-

S_{day12} and more mature CFU-S_{day7}, both with multilineage potentials, have D_0 values of 0.94 Gy and 0.71 Gy respectively; and (3) more mature lineage-restricted bi- or uni-potential myeloid progenitor cells have significantly higher D_0 values (~1.2-1.6 Gy). In contrast, murine erythroid lineage-restricted progenitors are considerably more radiosensitive with lower D_0 values: 0.68 Gy for burst-forming units erythroid (BFUe) and 0.53 Gy for colony-forming units erythroid (CFUe) (Imai and Nakao, 1987).

(A45) Shapes of 'radiation dose-cell survival' curves are fairly consistent for the above mentioned progenitor subtypes within various mammalian species irradiated under steady-state haematopoiesis. In general, the decrease in survival of either HSCs or HPCs tends to be exponential with increasing radiation dose, without a substantial threshold at the lowest doses. This common pattern is illustrated in Fig. A.6a. by the single-dose/response relationship for CFU-S_{day9} (Bond, 1995). Further, relationships between the extent of radiation-induced loss of these vital progenitor cell types, the overall severity of marrow injury, and 30-day mortality of the irradiated animals are illustrated in the complementary Fig. A.6b. These figures highlight the fact that only a very small fraction of surviving progenitors is required to maintain organ function. For survival of the larger species (man, dog, Rhesus monkey), a significantly greater surviving fraction of these vital marrow HSCs/HPCs after irradiation is required, than for the smaller rodent species (mouse, rat) (Vriesendorp and van Bekkum, 1984).

(A46) Despite the very common features of the dose/response patterns of the various progenitor cell subtypes, specific differences do exist and largely relate to dose-dependent rates of cell lethality (as reflected by differences in the slopes of the survival responses). Fig. A.7a-d shows representative "radiation dose-survival response" patterns, along with estimated "D_0 values" of several major progenitor subtypes from $B6D2F_1$ male mice 24 hours following acute, whole-body, γ ray (^{137}Cs) exposures.

(A47) Haematopoietic disequilibria, regardless of aetiology, will result in marked shifts in assayable, progenitor cell survival as well. For example, growth factor-mediated myelopoietic stimulation of HSCs/HPCs will elicit marked changes in both sublethal and potentially lethal damage capacities (Fitzgerald et al., 1989; Goff et al., 1997). Similarly, the disequilibria associated with chronic, low daily dose γ irradiation (7.5 cGy day^{-1}) of experimental canines fosters outgrowth and repopulation of normally minor, radioresistant subsets of myeloid progenitors (Seed et al., 1982).

(A48) Similar shifts in radiosensitivity of human HSCs/HPCs have been noted as well. However, the results are not quite as clear due to a variety of technical limitations in testing (e.g. relatively low purity of test cells, coupled with low plating efficiencies *in vitro*). Nevertheless, workers such as Kreja et al. (1993) reported on the *in vitro* growth characteristics and the radiosensitivity of $CD34^+$ enriched cells from human umbilical cord blood. Following immunomagnetic bead selection, $CD34^+$ cells were irradiated over a full range of doses with x-rays, plated under clonogenically-permissive growth conditions (complete tissue culture media, supplemented with a cytokine cocktail containing recombinant human SCF or bFGF, erythropoietin, and placenta conditioned medium, and assessed for the types and numbers of specific colonies formed. Not surprisingly and consistent with work done in rodents, the less mature, multipotential HPCs were considerably more radiosensitive than the more mature, lineage-restricted HPCs (e.g. mixed colony-forming cells had D_0 values ~95 cGy, whereas the uni- or bipotential BFUe and GM-CFU had D_0 values of ~136 cGy).

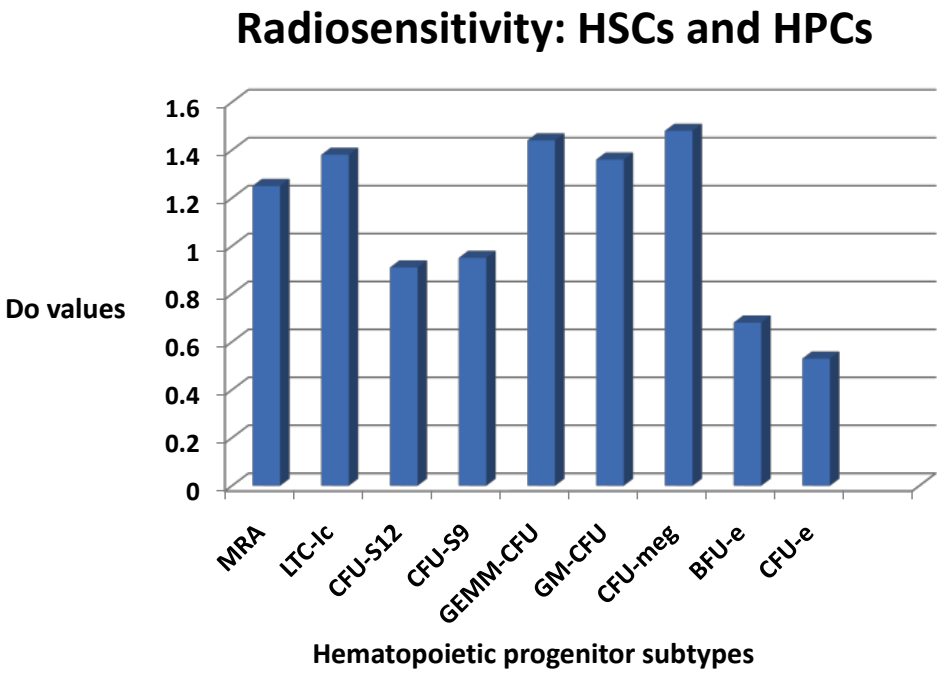

Fig. A.5. Radiation sensitivities (measured in terms of D_0 values) of various murine HSC and HPC subsets are shown. HSCs and HPCs, as well as more differentiated proliferative daughter cells all exhibit a high degree of heterogeneity regarding radiosensitivity. D_0 values are variable: primitive repopulating HSCs (MRA and longterm cultured cells (LTC-Ic)) are relatively radioresistant; multipotential, lineage-restricted spleen colony-forming cells (CFU-S assayed at day 12 or 9) are less resistant; whereas multi- or bipotential, granulocyte/erythroid/monocyte/megakaryocyte lineage-restricted *in vitro* colony-forming cells (GEMM-CFU and CFU-meg) are more resistant compared to erythroid-restricted progenitors (BFUe or CFUe). Data were taken from the following references (Gidali, 2002; Imai and Nakao, 1987; Ploemacher et al., 1992; Scheding et al., 1996; Testa and Lajtha, 1973; Till and McCulloch, 1961). (permission needed)

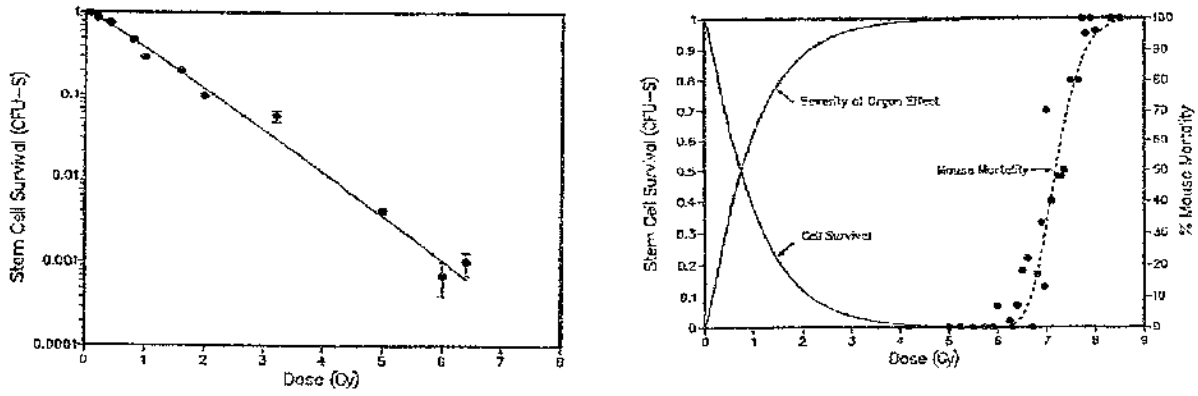

Fig. A.6. Left panel: A representative radiation dose/ haematopoietic progenitor cell survival response is shown (with survival plotted on a log-linear scale). Right panel: An inverse relationship of the declining survival of marrow CFU-S and rising severity of haematopoietic tissue damage is shown relative to a lower range of radiation doses, whereas a rapidly rising incidence of mortality is shown

to occur at a significantly higher range of radiation doses (Bond, 1995). (Figures reproduced with courtesy and permission from John Wiley & Sons Inc.) (permission needed)

(A49) When the radiosensitivities of three major HSC/HPC classes, namely MRA, CFU-S_{day7}, and CFU-C, were assayed both *in vivo* and *in vivo* and under different levels of oxygenation, following either 1 MeV fission neutrons or 300 kVp x-rays, the differences in radiosensitivity expressed by these maturationally-distinct progenitor cells were found to be due largely to intrinsic factors and not extrinsic factors such as niche sites of progenitor localisation or oxygenation (Meijne et al., 1996). However, later work showed that the most primitive stem cells were protected from low-LET radiation by the hypoxic nature of the niche (Kubota, 2008; Parmar et al., 2007).

(A50) The multipotential cells (CFU-S) show little dose-rate effect, and this is consistent with their fairly high sensitivity to low acute doses which can be characterised by a large α-kill component in the LQ formalism. This lack of dose-rate effect was observed even down to 2 decades of depopulation caused by irradiation at 0.45-0.9 Gy/day continuous irradiation (Chu-Tse and Lajtha, 1975), but after an accumulated dose of 2.5 Gy, there was a plateau in numbers followed by a dose-rate-dependent repopulation rate. Similarly, effects of dose fractionation are small, until concomitant protraction of exposure results in marked repopulation effects (Hendry and Lajtha, 1975). Animal studies have shown that the haematopoietic system is capable of maintaining an adequate number of cells during chronic low-dose and low-dose rate radiation exposure. This is due to increased rates of cell production resulting from shortening of the cell cycle and maturation time, increased proliferative activity of stem cells and precursor cells, and overall stimulation of haemopoiesis (Fliedner et al., 2002; Gidali, 2002; Lord, 1965).

(A51) The haematopoietic microenvironment, which normally maintains homeostasis of the stem cell pool by interaction with stem cells and multipotent progenitor cells, plays an important role in recovery after damage (Molineux et al., 1987). Extramedullary haematopoiesis and migration of HSCs from bone marrow to the spleen, liver and lymph nodes can also occur. Recovery of haematopoiesis is more complete after exposure at low dose rate than at high dose rate. For example in mice, recovery of haematopoietic and stromal progenitor cells was almost complete by one year after 12.5 Gy delivered at 0.0005 Gy/minute compared with incomplete recovery after only 6.5 Gy given at 0.7 Gy/minute (Gallini, 1988). Nonetheless, in other studies after low-dose rate exposure, CFU-S was not restored to baseline levels during the lifetime of the animals, demonstrating some very long-term residual injury (as reported in ICRP, 2012).

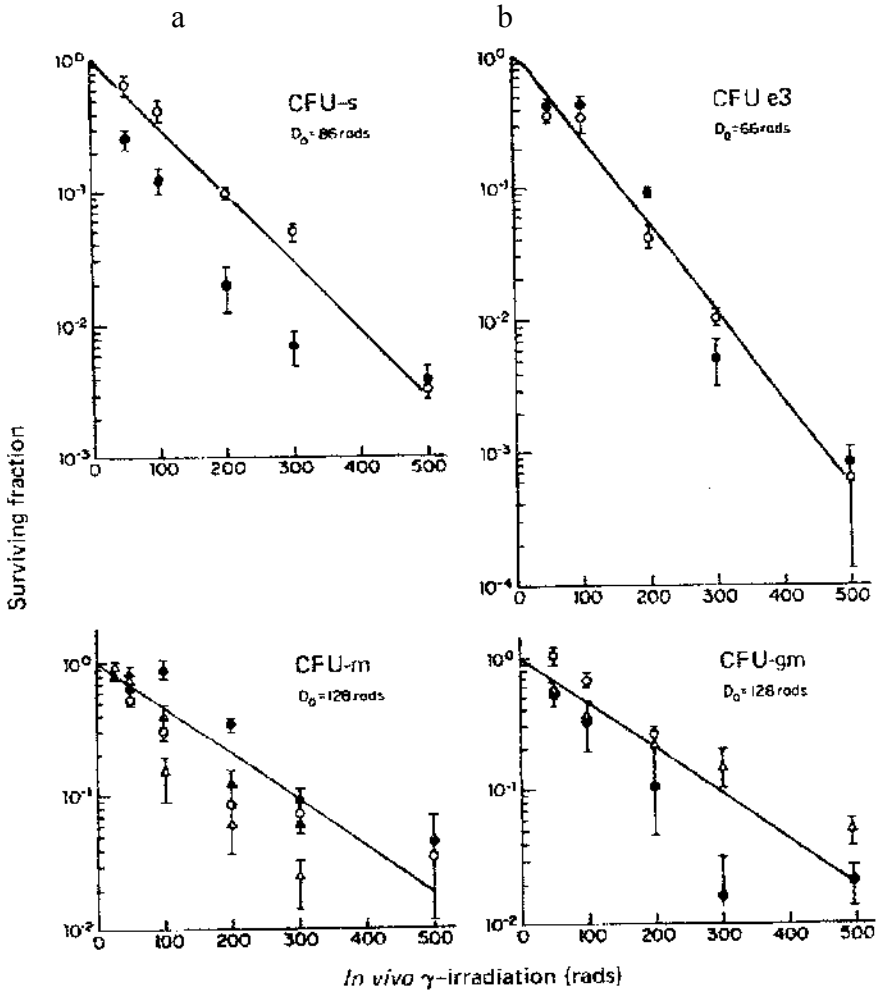

Fig. A.7. Representative radiation dose-survival response patterns for various murine haematopoietic progenitor subtypes: (a, upper left panel) colony-forming units assayed in spleen at day 9 (CFU-s_{day9}); (b, upper right panel) erythroid colony-forming units assayed at day 3 (CFUe3); (c, lower left panel) megakaryocyte colony-forming units (CFU-m); (d, lower right panel) granulocyte/macrophage colony-forming units (CFU-gm) (Nakeff, 1979). Note the common features of dose-response linearity, lack of a substantial response threshold, and the differences in the slopes of the dose-survival responses. (Figure reproduced with courtesy of Springer Inc.) (permission needed)

A.5. Characteristics of single-cell responses

(A52) Strategies that employ FACS isolation of potential HSC/HPC marrow cell targets based on cell surface determinants, followed by *in vitro* plating of single cell targets bearing defined surface markers, and subsequent time-dependent, single cell growth, clonal, biochemical and/or molecular analyses have been reported. $CD34^+$ Lin^- bone marrow cells were FACS isolated and analysed for molecular signatures of myeloid and erythroid cell lineages. Approximately 50% of $CD34^+$ Lin^- marrow cells expressed mRNA for both β-globin and myeloperoxidase, suggesting that a large fraction of these marrow HSCs were simultaneously primed at the gene level for both erythroid and myeloid lineage commitment

(Hu et al., 1997). It has been suggested that such low-level priming of lineage-specific genes across multiple lineages is a characteristic of HSCs, (Krause, 2002). Also that full lineage commitment is more a function of suppressing low-level gene activity within those lineage-specific gene sets not selected for, rather than specifically activating and enhancing gene activities within the lineage that is ultimately selected for commitment. The influence of radiation exposure on these pre-commitment processes is unknown.

(A53) A fundamental regulatory control of HSCs' cycling status and lineage-commitment process(es) is exerted directly by local environmental signals generated by the bounding stromal niche (Trentin, 1989). Further, the niche appears to contribute not only to the HSC's repopulating capacity, but also to the maintenance of quiescence, thus allowing these cells to stay in a radioprotective, noncycling state (Greenberger and Epperly, 2009). The more importance of these HSC-regulatory signals certainly includes the Tie2/Ang-1 signalling pathway. In this pathway, the stromal cell niche supplies the functional ligand, Ang-1, that interacts with specific tyrosine kinase surface receptors, Tie2, on HSCs in order to maintain not only the stem cell-defining features of quiescence and self-renewal, but also adhesiveness to key stromal elements (e.g. osteoblasts) within the niche itself (Arai et al., 2004). Other niche-associated factors, e.g. oxygenation, also play key roles in preservation of "stemness" (Parmar et al., 2007; Suda T, 2011).

A.6. Mutagenesis

(A54) Mutagenesis of the blood-forming system of mammals, including man, is well founded and documented (Bartel et al., 2000; Caddle et al., 2008; Germeshausen et al., 2008; Mattison et al., 2007). Although most mutations can be found within fully mature, circulating blood cells, these mutated cells originate largely within the progenitor cell compartments of the marrow. Mutations within HSCs and HPCs appear to arise either spontaneously, or following exposure to a wide variety of physiochemical toxicants and/or biological agents. Also, all progenitor cell classes have the potential to serve as targets for ionising radiation-associated mutagenesis. Mutant subpopulations of fully mature, non-proliferating blood cells (e.g. glycophorin A (GPA)-mutated erythrocytes, PIG-A-mutated leukocytes) have been detected within the circulation of individuals long after A-bomb exposure (Dong et al., 2001; Kusunoki et al., 1995; Kyoizumi et al., 1996; Kyoizumi et al., 2005). This implies (a) a lineage-specific nature and progenitor origin of these mutations; and (b) lineage-committed progenitors are the targets for the mutational process. The doubling dose for GPA mutations has been estimated to be ~1.2 Sv, with a threshold dose of ~0.24 Sv (Kyoizumi et al., 1996). A sizeable threshold dose for these GPA-related mutations is most likely responsible for largely negative findings of a number of investigators who have used the GPA assay to monitor mutations within erythrocytes of individuals with a prior history of very low radiation exposures (Tawn et al., 2003).

(A55) The timing and expression of radiation induced mutations within haematopoietic progenitor cells have been investigated (Kadhim et al., 1994; Kadhim et al., 1992; Kadhim and Wright, 1998; MacDonald et al., 2001; McIlrath et al., 2003; Wang et al., 1996; Watson et al., 1996; Watson et al., 2001). A delayed expression was reported of nonclonal chromosomal aberrations within colonies of non-irradiated haematopoietic cells that had been derived from irradiated haematopoietic progenitor cells many cell generations earlier (Kadhim et al., 1994; Kadhim et al., 1992). Initially, these delayed mutations were thought to be produced only by high LET α particle irradiation, but later were shown to occur with other radiation qualities (x and γ rays) (Watson et al., 2001).

(A56) These delayed genomic lesions are now considered part of a larger class of radiation-associated genomic responses, commonly referred to as "non-targeted" effects. These include both manifestations of 'genomic instability' and 'bystander responses' within downstream non-irradiated haematopoietic daughters of the original irradiated parental cell or of haematopoietic progenitor cells residing in close proximity to irradiated neighbouring cells (Wright, 2010). Wright and colleagues have shown that at least within the lymphohaematopoietic system, these delayed responses are highly 'genotype-dependent' and tied to aberrant molecular signalling related to inflammatory processes. Specifically, these signals (i.e. partially identified as tumour necrosis factor α, nitric oxide, and superoxide) have been shown to emanate from heavily, but not lightly irradiated (bystander) macrophages (Lorimore et al., 2008; Zyuzikov et al., 2011).

(A57) The role of radiation-induced genomic instability in humans is uncertain. Kodama et al. (2005) evaluated in detail the cytogenetics of blood T-cells of A-bomb long-term survivors and found no evidence to support the concept of exposure-induced chromosomal instabilities. Nakamura (2005) speculated that the radiation-induced leukaemias within the A-bomb surviving cohort might be associated with a very small subset of individuals, predisposed to the radiation effects by virtue of pre-existing, naturally-acquired chromosomal lesion(s). Delayed-type Hprt mutation frequencies were consistently and significantly elevated by prior irradiation of stem cells (more likely a mix of multipotential and more lineage-restricted HPCs) (Harper et al., 1997). Elevated mutation frequencies were noted not only for α particle irradiation, but also for 250 kVp x-rays, and even more markedly for fast neutrons. Further, mutation frequencies of the delayed type of mutations were consistently elevated, albeit marginally for the neutron and x-ray induced mutations, relative to the more immediate-type of mutations. The estimated mutation frequencies per Gy for both these classes of mutations are listed in Table A.5. (Harper et al., 1997).

(A58) Radiation mutagenesis within more primitive haematopoietic progenitors (e.g. marrow repopulating stem cells) is less well defined. Long-persisting chromosomal instability within fully reconstituted marrow of recipient mice was detected following an earlier (1 year) total-body irradiation conditioning and subsequent syngeneic transplantation of *in vitro,* α-particle-irradiated marrow repopulating stem cells (Watson et al., 1996). Long-term radiation-induced chromosomal instability was also reported within Lt-HSCs of repopulated marrow of adult Swiss mice given 0.5-1.5 Gy of γ rays while *in utero* (Devi and Satyamitra, 2005). The dose range used in this study and the long-term mutational effects noted provide a rough estimate of the radiosensitivity (relative to mutagenesis) of marrow repopulating Lt-HSCs in mice.

Table A.5. Estimated Hprt mutation frequencies for irradiated marrow HPCs [1,2]

Radiation type/ quality	Hprt mutation frequency per Gy	
	Immediate	Delayed
α particles	9.6×10^{-6}	18.0×10^{-6}
Neutrons	21.3×10^{-6}	26.3×10^{-6}
x-rays	4.6×10^{-6}	6.9×10^{-6}

[1] Estimates based on raw mutation frequencies (Harper et al., 1997) and with assumed linearity relative to radiation dose delivered.

[2] Background mutation frequencies of unirradiated mouse marrow HPCs were 2.6×10^{-6} and 4.5×10^{-6} for immediate- and delayed-type Hprt mutations, respectively.

(A59) In contrast to the little information concerning radiation mutagenesis of primitive marrow HSCs, information on radiation mutagenesis within the lymphocytic cell lineage,

especially circulating blood lymphocytes, is relatively plentiful, and clearly instructive in terms of the nature of radiation mutagenesis within the lympho-haematopoietic system. For the induction of Hprt mutations in splenic T lymphocytes of ^{137}Cs γ irradiated mice, the mutation frequency depended markedly on dose, dose rate and time after exposure (Lorenz et al., 1994). Dose-response relationships for Hprt mutations were best fitted at high dose rate by an LQ function (i.e., MFs $_{\text{(high dose rates)}} = 6.9 \times 10^{-6}$ Gy + 1.2×10^{-6} Gy^2), whereas at low dose rates, a simple linear function seemed best suited (i.e. MFs$_{\text{(low dose rates)}} = 3 \times 10^{-6}$). The DDREF was 1.5 for doses of <2 Gy and dose rates of 1 Gy/day or less, and 3-5 for high doses and dose rates (Lorenz et al., 1994).

(A60) Kataoka et al. (1993) reported the frequencies of Hprt mutations induced by both single and fractionated doses of fission-spectrum neutrons and compared those estimates with ^{60}Co γ ray induced mutations. The estimated mutation frequencies for single, acutely delivered doses (1.5 Gy) of fission neutrons were not significantly different from the estimated mutation frequencies for single doses (7.5 Gy) of ^{60}Co γ rays (i.e. (5.98 ± 1.51 SE) x 10^{-5} versus (5.56 ± 3.09 SE) x 10^{-5}). By contrast, the fractionated regimen of fission neutrons (0.25 Gy x 6, to total doses of 1.5 Gy) elicited marginally, but not significantly higher mutant frequencies compared to comparably fractionated doses (1.5 Gy x 6) of γ rays delivered to total doses of 9.0 Gy: estimated mutation frequencies for fractionated neutron exposures = (8.71 ± 5.39 SE) x 10^{-5}; MFs for γ-rays = (2.30×10^{-5}) ± (9.07×10^{-6}) SE. Not surprisingly, these results support the concept that the genotoxic potency of high LET fission neutrons, relative to low-LET γ-rays, appears to increase, albeit marginally, with fractionated exposure regimens.

(A61) Consistent with the above mentioned reports, Griffiths et al. (1994) compared the estimated Hprt mutation frequency of x irradiated B-cell precursors with other haematopoietic cell types and found them to be quite comparable. Interestingly, in a latter study (Griffiths et al., 1997), these workers found that the apparent high levels of Hprt mutations within x irradiated p53 null cells were the result of a preferential survival of the mutant, p53 null cells, rather than from a p53-dependent increase in mutation rate. This observation clearly highlights the need for due caution when interpreting these estimated mutation frequencies.

(A62) Over a decade ago, Kadhim and Wright (1998) noted that "...delayed chromosomal abnormalities, delayed cell death by apoptosis and late-arising specific gene mutations may reflect diverse consequences of radiation-induced genomic instability". They also pointed that the relationship(s) between these radiation-associated changes was not well established, and that "…the expression of these delayed heritable effects is determined by the type of radiation exposure, type of cell and a variety of genetic factors…". In terms of radiation-induced leukaemia, it is clear that this type of non-specific genetic damage abounds within radiation-induced leukaemic cells, and is attributable to the accumulation over time of genetic lesions within irradiated HSC (and/or early HPC) targets. This is, of course, compatible with the general concept that radiation leukaemogenesis is a multistage process, requiring extended processing/development times, involving genetic instability, with subsequent stem cell crisis and premalignant/malignant transformation(s) (MacDonald et al., 2001; Seed et al., 1989; Seed, 1991).

(A63) How a specifically identified genetic lesion, or a collection of genetic lesions, within marrow cell targets at defined post-exposure/preclinical periods, relates to the penultimate leukaemic cell transformation, remains ill defined. Some light has been shed into this "black box", however, by virtue of the progress made in understanding the nature and aetiology of CML, and the role played by a key gene alteration, namely the bcr/abl gene and

its p210 product. This gene is formed by an aberrant reciprocal translocation of chromosomes 9 and 22 to form the pathonomic "Philadelphia chromosome" and its unique fusion gene (bcr/abl) and gene product (p210). By limiting the formation or controlling function of this gene, one can control the disease, regardless of its aetiology (spontaneous, radiation-, or chemical-induced). The chimeric gene, bcr/abl, can be directly induced within targeted haematopoietic cells *in vitro* by very high (i.e., 50-100 Gy) doses of low-LET irradiation (Ito et al., 1993b; Spencer and Granter, 1999), thus providing direct evidence of its induction by x irradiation *in vivo*. However, the relevance of the latter studies has been seriously questioned, and rightfully so, as the "inductive" doses used in these experiments are at least an order of magnitude greater than what one would normally perceive as being "biologically relevant". Clearly, these observations, however interesting, need to be confirmed at a much lower range of radiation doses.

(A64) It is interesting to note that Ito and his colleagues (1993) reported that these artificially-created fusion genes contained not only CML-specific bcr/abl rearrangements, but also other forms of bcr/abl fusions as well: e.g. fusion genes with junctions of bcr exon 4/abl exon 2 intervened by a segment of DNA of unknown origin, bcr exon 5/abl exon 2, and bcr exon 4/abl exon 2. These authors speculated that only those progenitor cells that bear certain CML-related bcr/abl fusion genes are positively selected by virtue of a growth advantage *in vivo*. Further, the results of the Spencer and Granter study (1999) showing significantly elevated levels of bcr/abl transcripts within *in vitro* irradiated leukaemic cells, when compared to the lower frequencies found in comparably-irradiated non-leukaemic cells, prompted the suggestion that the radiation-induced illegitimate recombination may be due to aberrant DSB repair mechanisms within individuals predisposed to CML.

(A65) Other leukaemia-type specific chimeric genes (e.g. the AML t(8,21)-associated chimeric gene, aml1/eto) have been shown to be directly radiation-inducible as well (Deininger et al., 1998). Further, these hybrid mutant genes appear to originate within multipotential, trilineal-committed marrow progenitors (Miyamoto et al., 1996).

(A66) Despite the intriguing and insightful nature of the bcr/abl story, the consensus is that leukaemic cell transformation by ionising radiation, or any other known leukaemogen, is not merely a function of an aberrant, single gene- chimeric or otherwise; but rather that the transforming process is both multigenic and multi-staged by nature (Fig. A.8.). Although it is still relevant to think of radiation leukaemogenesis in terms of cooperative networks of aberrant proto-oncogenes (e.g. Ras, Myc, Myb) and tumour suppressor genes (p53, Rb, etc), the role of chimeric genes, global genomic instabilities, bystander effects, altered or loss of exogenous, stromal niche-mediated growth controls, etc. need to be considered as well.

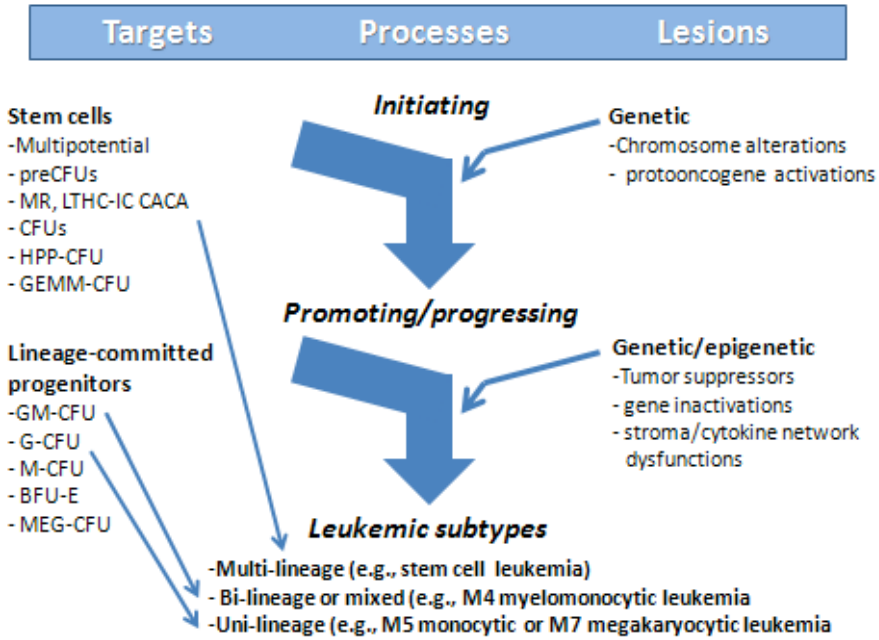

Fig. A.8. Potential cell targets, processes and lesions associated with radiation leukaemogenesis (NCRP-150, 2005). (Figure reproduced with courtesy and permission from NCRP.) (permission needed)

(A67) Although it is beyond the scope of this report to detail various genes and their interplay during leukaemia development, a select few examples are given below in order to illustrate the latter points. First, the cooperative action of c-Myb and bcr/abl fusion gene on the clonal capacity of two subclasses of marrow progenitors, i.e. Lin^{-} $sca\text{-}1^{+}$ and Lin^{-} $sca\text{-}1^{+}$ Kit^{+} progenitor cells, has been recently documented (Lidonnici et al., 2008). With allelic loss of c-Myb, normal colony-forming capacity was markedly suppressed in progenitor cells expressing p210(bcr/abl), while the clonal response was only modestly affected in the non-p210(bcr/abl)-expressing progenitor cells. The results support the contention that c-Myb is required for p210(BCR/ABL)-dependent leukaemogenesis. Second, $CD34^{+}$ progenitor cells bearing the bcr/abl chimeric gene exhibit a wide array of gene-based functional alterations linked either directly or indirectly to the CML progenitor phenotype (Kronenwett et al., 2005), e.g. increased cell-cycling and proteasome activity, downregulation of DNA repair proteins and detoxification enzymes, decreased expression of CXC chemokine receptors, and up regulation of GATA-2, etc. The upregulation of GATA-2 is particularly interesting, as it might provide the basis of enhanced self-renewal of targeted pre-leukaemic HSCs. Third, a recent study of gene expression analyses of AML blood cells by Casas et al. (2003) provides additional support for the above mentioned concept of a cooperative imbalance between a select set of activated proto-oncogenes (e.g. Jun, growh factor receptor-bound ptotein 10: GRB10) that are upregulated and enhance cell cycling and cell proliferation, and another set of tumour suppressor genes (e.g. p53, p16). The latter normally act in a countervailing manner, but during patent AML, they appear to be downregulated, promoting genomic instability and deregulated apoptotic pathways. Comparable changes are readily envisioned during radiation-induced AML as well.

(A68) A number of candidate gene targets have been identified as well, and these appear to be associated with radiation leukaemogenesis (in mice), e.g. the HLX1 homeobox gene which is essential for haematopoietic development, and RAD51, a DNA repair gene, etc. A

combined analysis was performed on the latter genes, i.e. RAD51 and HLX1 variant alleles, and a synergistic 9.5-fold increase (95% CI: 2.22, 40.64) in the risk of radiation-induced AML was observed (Jawad et al., 2006).

(A69) Radiation-induced genetic instability has been documented both at the molecular level and at the chromosomal level. For example, Plumb et al. (1997) reported significantly elevated instability of the Y-chromosome within haematopoietic cells of male mice with acute, radiation-induced leukaemia. Similarly, elevated frequencies of chromosome-2 aberrations, along with associated interstitial deletions, are common cytogenetic findings in acutely-irradiated, leukaemia-prone CBA mice (Bouffler et al., 1996). However, the formation of these aberrations does not seem to determine the individual mouse's leukaemogenic sensitivity, since only 20-25% of these irradiated animals eventually progressed to patent AML. This would imply that these radiation-induced chromosome-2 lesions are indeed associated with early pre-leukaemic events (perhaps acting as "initiating events"), but clearly they are not self-sufficient for leukaemogenesis (in the 3 Gy irradiated CBA/H male mouse). Further, the radiation-induced chromosome-2 lesion appears not to be rate limiting in terms of leukaemogenesis. In this regard, the work by Rigat et al. (2001) is instructive: using a PCR-based molecular approach, these workers showed that the frequency of the radiation-induced "loss of heterozygosity" (LOH) of chromosome 2 within stem cells of CBA/H x C57BL/6 F_1 mice was no greater than the induced LOH in other control chromosomal regions evaluated.

(A70) So what are the rate limiting steps? It has been suggested that the absolute number of marrow stem cells defines the number of potentially radiation transformable genomes, and in turn, overall leukaemogenic risk (Jawad et al., 2007). If this is the case, then it is the number of available targets within the defined radiation volume that defines the leukaemic rate, and in turn the efficiency of the irradiation. Further, if the available "targets" include not only the very rare, primitive, endosteal layer-associated stem cells (Lt-HSCs), but also the more plentiful, centrally-located progenitor/daughter cells (e.g., St-HSCs, early HPCs), this should substantially favour any/all of the radiation-associated stochastic events of the early leukaemogenic process.

(A71) In the light of this possibility, recent studies have been performed to help identify the target-cell type for AML. Hirouchi et al. (2011) gave 3 Gy to induce AML in mice. They extracted marrow, sorted it using a range of different cell-type-specific markers, then transplanted populations of HSC, MPP, CMP (common myeloid progenitors), CLP (common lymphoid progenitors), B-cell/natural killer-cell, and T-cell types into normal mice. Bone marrow was analysed in those grafted mice which developed AML. They concluded that (a) there was a possibility that hemizygous deletion of Dusp2 in chromosome 2 contributed to the self-renewal potential of radiation-induced AML stem cells, and that (b) the initial radiation-induced AML stem-cell may originate not only from irradiated HSC but also from MPP and CMP.

(A72) The plausibility of possible multiple target-cell types is further strengthened by the recent work on induced genetic reprogramming of differentiated somatic cells into embryonic-like stem cells (referred to as iPS cells). The latter appears to share many growth and maturation potentials seen in immortalised, pre-transformed (pre-cancerous) cells (Meissner et al., 2007). Also, supporting this suggestion are the older studies on retroviral targeting of lineage-committed progenitors and the comparable "reprogramming" of virus-targeted, lineage-committed haematopoietic progenitor cells into more primitive, stem-like precursor cells with enhanced, but often aberrant proliferation and differentiation potentials (Minucci et al., 2002).

(A73) Lastly, as a contribution to the debate on leukaemogenesis mechanisms, a detailed study was carried out regarding the "immortal strand hypothesis" (see section 2.3.5.) in highly purified HSC, to detect if the parental DNA strand was retained during division cycles to preserve its integrity (Kiel et al., 2007). They administered BrdU to newborn mice, mice treated with cyclophosphamide and granulocyte colony-stimulating factor, and normal adult mice for 4 to 10 days, followed by 70 days without BrdU. In each case, less than 6% of HSCs retained BrdU and less than 0.5% of all BrdU-retaining haematopoietic cells were HSCs, revealing that BrdU has poor specificity and poor sensitivity as an HSC marker. Sequential administration of 5-chloro-2-deoxyuridine and 5-iodo-2-deoxyuridine indicated that all HSCs segregate their chromosomes randomly. Division of individual HSCs in culture revealed no asymmetric segregation of the label. It was concluded that HSCs cannot be identified on the basis of BrdU-label retention and do not retain older DNA strands during division, indicating that these are not general properties of stem cells.

A.7. Summary and conclusions

(A74) *Radiation-induced leukaemias:* Leukaemia is a rare, but very prominent disease of the blood forming system. Although the a,y of most cases of leukaemia is unknown, a relatively small number of the diseases have been causally associated with prior exposures to a variety of physicochemical agents, most specifically ionising radiation and various classes of myelotoxic chemicals. Following extensive and detailed epidemiological evaluations of leukaemia incidence within a Japanese cohort of A-bomb survivors, the AR of developing the disease (in aggregate) is estimated at 2.7×10^{-4} per Sv per year, while risk estimates for the major leukaemic subtypes- ALL, AML, or CML- are 0.6, 1.1, and 0.9×10^{-4} per Sv respectively (Preston et al., 1994). CLL has not been consistently or convincingly increased following exposure to ionising radiation.

(A75) Leukaemia, as a complex disease, is characterised by a highly aberrant, dysfunctional blood-cell forming system of the body, a system that is normally highly efficient and very reliable. This "cell producing factory" resides in the bone marrow and relies heavily on the reproductive capacities of both primitive, and the not-so-primitive progenitor cells. These cells appear to exist in a continuum of dual, reproductive capacities that allows for balanced cycles of self-renewal and selective, lineage-specific commitment to differentiate. It is a combination of the latter two cellular functions that become abnormal, which is responsible for the disease.

(A76) *Relevant data of various radiations and exposure types:* In principal, ionising radiation of all qualities, dose levels and conditions has leukaemogenic potential; however, disease risk can vary by orders of magnitude. Acute doses of whole-body exposures with deeply-penetrating low-LET x- or γ–rays seemingly carry relatively high risks when compared to exposures from select types of internalised, bone-seeking, high LET α-particle-emitting radionuclides (e.g. ^{226}Ra), in all likeliehood related to differences in the anatomical distribution of dose in bone marrow.

(A77) *General features of haematopoietic tissues:* The marrow's blood-forming capacity is totally reliant on the well-orchestrated and coordinated activities of the major marrow compartments: (1) stem cells and early haematopoietic progenitor cells; (2) proliferative, lineage-committed daughter cells; and (3) non-proliferating maturing cells and associated reserves. Tissues are bounded by encapsulating bone (spongy or compact), supported both structurally and functionally by intra- and inter-stromal matrix, and both are vascularised and innervated. The tissue construct is analogous to a tree in full foliage: the root system, represented by the nutrient vasculature, with the endosteal matrix between enveloping bone

and marrow and its mesenchymal osteo-haematopoietic precursor cells; the tree stem, representing the supporting stem cell and early progenitor compartments; the rising major branches, representing major cell lineages, in turn to a multitude of terminal branches, representing amplified, maturing and finally, functional cells. Damage to the "stem", i.e., the stem cell/progenitor compartment(s), will most certainly limit subsequent compartmental cell amplifications, cell transitions, and maturation processes, and ultimately limit the capacity to produce and to maintain circulating blood cell pools.

(A78) Being rather indiscriminate by nature (in terms of energy deposition), ionising radiation will (if given the opportunity) produce damage along the entire haematopoietic chain from primitive stem cell to the fully functional and mature blood cells. However, in general, it is the stem cell/progenitor compartment(s) that is at pathological risk following exposure.

(A79) *Turnover rates:* The estimated turnover rate for the bone marrow with the average adult human is ~4.5 x 10^{-9} cells per day per kg of body weight (Fliedner, 1998). In human marrow, approximately two thirds of this activity is devoted to myelopoiesis, while the remaining third is dedicated to erythropoiesis. In total, this marrow activity serves to supply and to renew on a daily basis circulating blood pools of ~2.5 x 10^{9} erythrocytes, ~2.5 x 10^{9} platelets, and ~10 x 10^{9} granulocytes per kg body weight (Williams et al., 1990). The stem cell compartment of the marrow comprises approximately 0.1 to 0.05% of the total tissue. Precise turnover rates of stem cells and early progenitor cells within the marrow are uncertain and remain to be determined. Nevertheless, rough estimates for marrow HSCs of several species have been garnered via indirect methods: in humans, HSCs within marrow were estimated to turn over once every ~45 weeks (Shepherd et al., 2004). Further, it is generally accepted that the more primitive the species of stem cell/progenitor cell, the lower is its turnover rate. Taking this to the extreme, it is quite possible that small numbers of marrow stem cells remain quiescent for the entire lifespan of the individual. This is another area of debate that will require further work.

(A80) In terms of leukaemogenesis, a low turnover within virtually-quiescent marrow stem cells might well serve to reduce the number of potential, transformable cell targets for the leukaemogenic action of ionising irradiation, regardless of LET or exposure intensity, by allowing time for repair of genomic damage prior to replicative transmission of that damage to their daughter cells. This effect may be counteracted to an uncertain extent if some daughter cells are also target cells.

(A81) *Age dependence:* Chronological age plays a significant role in radiation leukaemogenesis. Preston et al. (1994) reported on the relative leukaemic risk within the A-bomb survivor cohort, and found that with increasing age at the time of exposure, the risk declined appreciably. In contrast, the estimated RR increased with time following exposure: in the very young survivor (0-9 years of age) sub-cohort, the risk steeply increased during the early years following exposure; whereas in the older sub-cohorts, the rise in risk was significantly delayed and more gradual in slope. In general, animal-based studies support these epidemiological findings.

(A82) *Cellular features:* A unique array of cell surface markers serves to distinguish HSCs from other nucleated cells within bone marrow tissues. In the bone marrow of humans, HSCs can be distinguished by the phenotype of $CD34^{+}$, $CD59^{+}$, $Thy1^{+}$, $CD38^{low/-}$, $c\text{-}Kit^{-/low}$, and Lin^{-}. The latter Lin^{-} "marker" represents that absence of some 13 to 14 different surface markers which characterise mature blood cells of the various blood cell lineages. By contrast, HSCs of rodent (mouse) marrow, are distinguished by $CD34^{low/-}$, $SCA\text{-}1^{+}$, $Thy1^{+/low}$, $CD38^{+}$, $c\text{-}Kit^{+}$, and Lin^{-} (NIH Report, 2008).

(A83) The major subpopulations of HSCs, namely Lt-HSCs and St-HSCs, can be distinguished via functional, time-based transplantation assays. In turn, the HSCs of various marrow domains (e.g. endosteal, vascular) can be identified, as well as being distinguished from multipotential HPCs by differential FACS analyses using SLAM, KDR, and other related surface markers (Kiel et al., 2005; Ziegler et al., 1999).

(A84) *Radiosensitivity:* The radiosensitivity of HSCs and HPCs varies, and significantly so, in ways which might seem counter institutive. The response (i.e. change in radiosensitivity with HSC/HPC maturation) appears "U" shaped, and not at all linear. The most primitive of the HSCs (long-term marrow repopulating) are quite radioresistant, endowed with substantial SLDR and PLDR capacities, when compared to the more radiosensitive CFU-S_{day7} with selective lineage restriction, but still clearly multipotential by nature. Like the MRAs, the more primitive CFU-S_{day12} appears more resistant than the CFU-S_{day7}. A similar shift in radioresistance is noted as daughters of multipotential HPCs become lineage-restricted and committed to specific differentiation lineages, e.g. granulopoiesis and associated HPC transitions from GEMM-CFUs to GM-CFUs to M-CFUs.

(A85) *Characteristics of single-cell responses:* While it is true that the biotechnology of monitoring haematopoietic progenitor cells has significantly improved, still our inability to isolate from marrow, especially human marrow, the primitive HSCs to homogeneity in a consistent and routine fashion, severely limits the application and utility of the more standard biochemical and molecular approaches that are currently available. New cloning procedures both *in vitro* and *in vivo* have been forthcoming, but still the results obtained from these techniques assume that the initial "seeding" cell is indeed the stem cell of interest and not a more mature progenitor cell which contaminates the seeding population.

(A86) Nevertheless, despite these technical limitations, progress has been made toward a better understanding of the molecular and biochemical make-up of the more primitive blood cell precursor cells found in the marrow. A good example of the latter progress is the application of differential genomic arrays in evaluating RNA transcript profiles of primitive, pre-committed HSCs and finding low levels of transcription specific for multiple lineages. This work has given rise to a new paradigm suggesting perhaps that lineage-commitment is a balanced, competitive process that involves not only the activation of lineage-specific genes, or sets of genes, but also, more importantly, shutting off activity of genes/gene sets, associated with the unselected lineages. Another excellent example has been the use of single-cell cloning of HSCs to identify and to characterise radiation-induced genetic alterations that are passed down to daughter cells during early reproductive cycles.

(A87) *Mutagenesis:* Mutagenesis within the most primitive and rare HSCs remains ill-defined, regardless of aetiology, due to the reasons mentioned above. The information that we do have comes indirectly from the analyses of cloned daughter progenitor cells that are many generations from the primary HSCs of interest. If it is assumed that the patterns and frequencies of radiation-induced mutations are relatively constant for the different classes of marrow HSCs and HPCs, then the Hprt mutation analyses of *in vivo* (CFU-S_{day11}) and *in vitro* (CFUc) cloned marrow progenitors might be reflective of radiation mutagenesis within more primitive, long-term marrow-repopulating precursors. That is, the mutability of HSCs and HPCs alike would be comparable to the mutability of progenitor cells from other body tissues, e.g. for Hprt mutations induced by low-LET x- or γ-rays, the mutation frequencies would be in the range of ~2 x 10^{-5} per Gy, while the spontaneous rates would be at least a factor of 10 lower.

ANNEX B: MAMMARY GLAND STEM CELLS

B.1. Radiation-induced breast cancer

B.1.1. Radiation exposure and breast cancer risk: Epidemiology and physiology

(B1) Breast cancer is the most commonly diagnosed cancer in women. Rates vary between countries but in general the rate increases to age 50, approximately the age of menopause, after which the rate of increase is less. Together with the recognised contribution of life history, which includes age of menarche, age at first pregnancy, number and timing of pregnancies, ovarian integrity, these data are indicative of the importance of ovarian hormonal factors in breast cancer incidence.

(B2) Radiation exposure is a well-documented risk factor for breast cancer. The UNSCEAR 2006 report (UNSCEAR, 2008) concluded that female breast tissue is moderately radiosensitive, and that there is compelling evidence of excess cancer which increases linearly with dose. There are complex modifying effects of age at exposure and attained age, as well as interactions with physiological processes, including pregnancy, and other risk factors. Fourteen cohort studies provided quantitative information on the level of risk following a wide range of doses in different populations around the world, as reviewed by Boice (2001). Three key features were discussed: (1) linearity of dose response; (2) the modification of risk by age at exposure, where the risk is inversely related to exposure age and exposures past the menopausal ages appear to carry a reduced excess risk; and (3) the effect of fractionating the dose on the subsequent risk is minimal. Although observed in individual studies, a combined analysis of almost 78,000 women and 1,500 breast cancer cases from eight cohorts confirmed linearity of dose response and little effect of dose fractionation on AR and a decreasing risk with age at exposure (Preston et al., 2002). Interestingly, a 6-fold decrease in risk was associated with protracted (as opposed to fractionated) exposures compared with acute exposures in childhood.

(B3) Medical treatments are one source of radiation exposures linked to breast cancer. Increased risk of breast cancer was noted in populations of girls with scoliosis, who were usually monitored using x-rays from the onset of the growth spurt through puberty and adolescence (Boice Jr. et al., 1991). Tuberculosis patients who spanned a wide range of ages also showed a greater breast cancer incidence later in life. The average dose to the breast was lower in the scoliosis cohort (0.11 Sv) compared to two fluoroscopy cohorts (0.79 and 0.89 Sv). x-rays were used for treatment of benign breast disease (BBD) and acute postpartum mastitis in women of child-bearing ages (Mattsson et al., 1993; Shore et al., 1986). Doses to the breast were considerably higher compared to the above mentioned studies. Women with BBD had a 3.58 fold increased risk for breast cancer when compared to a control group never treated with radiation (Mattsson et al., 1993). Women treated for postpartum mastitis who were exposed to >1.5 Sv were at 2- to 4-fold increased risk of breast cancer compared to a combined control group of siblings and unirradiated mastitis patients (Shore et al., 1986). Radiation doses of several Gy used to treat haemangiomas on the chest region of children under age 1 year were linked to elevated risks of breast cancer (Lundell et al., 1999).

(B4) Radiotherapy for breast cancer can result in a dose of several Gy in the contralateral breast; radiation treatment for breast cancer was found to be related to development of secondary breast cancers among women younger than 45 years of age (Boice et al., 1992; Stovall et al., 2008). Land et al. (1994) conducted a nested case-control study of 196 breast cancer patients and 566 matched controls among Japanese A-bomb survivors. Statistical

modelling indicated that early first birth, multiple births and long cumulative lactation history protected not only against breast cancer *per se*, but also against radiation-induced breast cancer in this population. The protective effect of age at first birth held for parous women at exposure and for women who completed a first pregnancy following radiation exposure. The protective nature of pregnancy may depend on additional factors. A pooled analysis of eight radiation-exposed cohorts showed that women with BBD or postpartum mastitis had the highest excess rates of breast cancer following radiation therapy for those disorders, an effect most pronounced among young women with BBD (Preston et al., 2002). The observation might be partly related to the underlying association of BBD and breast cancer. The long latency (approximately 40 years) between exposure and disease occurrence, the absence of excess when exposure occurs after age 45, and the effect of tissue and reproductive processes suggest that breast biology is a strong determinant of radiation risk.

(B5) The rate of breast cancer in Japan is among the lowest in the world, but breast cancer contributes a disproportionately large fraction of radiation-related cancer burden in A-bomb survivors (Preston et al., 2007). The data from the Hiroshima and Nagasaki survivors provide strong evidence for increased breast cancer following single acute doses of the order of 20 cGy and linearity of risk with increasing dose (Pawel et al., 2008; Pierce et al., 1996; Preston et al., 2003b).

(B6) These studies also support the strong effect of age on modifying breast cancer risk, in that there was no significant elevation among women exposed after the age of about 40 years in 1945. Girls (approximately aged 10-14 years) exposed to ionising radiation at Nagasaki-Hiroshima were much more likely to develop breast cancer than older girls or adult women exposed to comparable radiation doses (Tokunaga et al., 1993). The risk was inversely related to age at exposure, with the youngest children having the highest risk. The latency, that is the time from exposure to development of breast cancer, was inversely related to age at exposure, i.e. the young children had to live until the ages later in life when breast cancer rates were high for the excess breast cancers to be manifest. A similar age effect was found for high dose radiation exposures to the breast from fluoroscopy for tuberculosis and radiation therapy for Hodgkin's disease (Boice Jr. et al., 1991; Hancock et al., 1993; Howe and McLaughlin, 1996).

(B7) More than 50,000 women in the US have been treated with chest radiation (≥20 Gy) for paediatric or young adult cancers. According to the Childhood Cancer Survivor Study, breast cancer risk is greatest among women treated for Hodgkin's lymphoma (HD) with high-dose mantle radiation, but it is also elevated among women who received moderate-dose chest radiation (e.g. mediastinal, lung) for other paediatric and young adult cancers, such as non-Hodgkin's lymphoma, Wilms tumour, leukaemia, bone cancer, neuroblastoma and soft tissue sarcoma (Mertens et al., 2008). Children treated for cancer with radiotherapy have a 2.9 RR of subsequent malignancy compared to those without (Mertens et al., 2008). These women are at significantly increased risk of breast cancer and breast cancer mortality following cure of their primary malignancy. In a review of 11 retrospective studies and 3 case-control studies (Henderson et al., 2010), the authors concluded that risk of breast cancer increased as early as 8 years following chest radiation and did not plateau with increasing length of follow-up. The authors point out that the cumulative incidence of breast cancer by 40–45 years of age ranged from 13–20%, and by 25–30 years of follow-up ranged from 12–26%. This incidence is similar to that in women with a BRCA gene mutation, where by age 40 the cumulative incidence ranges from 10–19% and is substantially higher than in young women in the general population in whom the cumulative incidence of invasive breast cancer by age 45 is only 1% (Satagopan et al., 2001). However, estimates of ERR 0.06 (95% CI: 0.01, 0.13) Gy^{-1} for HD survivors as a function of dose provides a lower ERR than that

estimated for the LSS, which is attributed to cell killing at high doses (Travis et al., 2003). Moreover, those patients in which the ovaries receive ≥5 Gy, and are treated with certain chemotherapies, have decreased risk due to ovarian deficit and early menopause, respectively.

(B8) A study from Milan of the molecular and marker analysis of the breast cancers of women exposed to therapeutic radiation for childhood/young adult cancers, revealed a high risk of breast cancer diagnosed at an early age (39 compared to 57 in a sporadic consecutive series) and a higher frequency of developing estrogen receptor (ER)-negative tumours (Castiglioni et al., 2007). More than half (53%) of the breast carcinomas from irradiated women showed features of basal-like tumours compared to 11% in a consecutive series of breast cancers not preceded by radiation. When compared to age-matched controls, incidences of basal-like cancer were significantly higher (P = 0.0001) for women irradiated after breast maturation at the expense of human EGFR2 (HER2)-positive and luminal cancer subtypes. Interestingly, there is little evidence of a disproportionate frequency of contralateral ER-negative breast cancer in women treated with radiation for breast cancer, suggesting a physiological basis for the shift identified in the Milan study. Consistent with this idea, the breast cancer subtype in women treated with radiation after maturation was much more likely to be ER- and progesterone (PR)-negative, p53 and cytokeratin (K) 5/6-positive, and less likely to be $HER2^{+}$, compared to those whose radiation exposure occurred before maturation (Castiglioni et al., 2007). Additionally, Broeks and colleagues used expression profiling to compare sporadic and radiation-preceded breast cancer. Unsupervised hierarchical clustering of the profile data resulted in a clustering of 22 tumours obtained from patients who developed breast cancer after HD compared to 20 control tumours. The radiation–preceded tumours were characterised by genes associated with a more aggressive tumour type (Broeks et al., 2010).

(B9) Both human and experimental animal data suggest that there are additional poorly-understood factors associated with ageing or other life events that are permissive of, or actively promote, carcinogenesis. The preneoplastic phenotype *in vivo* is usually initially recognised as a focus of autonomously proliferating cells that often exhibit surface antigen or cytosolic enzymatic alterations. The subsequent appearance of neoplasia at the sites of such lesions has been well-documented in experimental models in a variety of tissues (Farber, 1987), but it is not clear what factors dictate the eventual development of clinical cancer. The relatively restricted window of carcinogen susceptibility that is evident during or around puberty in both rodents and humans has been postulated to either contain the greatest number of target cells or be a critical period of stem cell regulation (Boice Jr., 2001). Based on the idea that stem cell transformation can lead to unlimited progeny, tissue-specific stem cells or early progenitor cells are considered to be the critical cellular target in carcinogenesis as has been discussed from many perspectives (Clifton et al., 1986; Potten and Loeffler, 1990; Reya et al., 2001; Sell, 2004; Welm et al., 2002; Zeps et al., 1996).

B.1.2. Dose rate and radiation quality data

(B10) As noted above, total dose is associated with excess cancer, regardless of fractionation, e.g. low radiation doses as occurred with repeated diagnostic fluoroscopy (Boice Jr. et al., 1991; Hoffman et al., 1989). In contrast to other organs like thyroid in which risk is greater for high dose rate than low dose rate, cancer risk for breast is different. Either EAR or ERR for breast cancer risk in three high-dose-rate studies was not consistently different of those from two dose-rate studies (UNSCEAR, 2008). Fluoroscopy studies indicate breast sensitivity with no effect on lung cancer (Howe et al., 1995).

(B11) In regards to radiation quality, there is little evidence that internal low-LET or high-LET emitters are associated with excess breast cancer (UNSCEAR, 2006). Medical exposures are photons in the range of 250 kVp, while the relatively high-energy γ-rays produced by the A-bombs used in Hiroshima and Nagasaki would be less biologically effective by a factor of 3. No elevation of breast cancer risk has been observed among several studies of airline crews, who receive an average of 6 mSv/year, with 20-50% of the absorbed dose attributed to the neutron component.

B.2. General features of the mammary gland and carcinogenesis

B.2.1. Tissue architecture

(B12) The tissue architecture consists of the parenchyma that resides in a dense fibroblastic stroma embedded in an adipocyte stroma. As in other tissues, the stroma and epithelial components can be separated from each other using mechanical and enzymatic dissociation. But unlike most other tissues, the epithelium can be destroyed and the stromal fat pad maintained separately *in vivo*. The gland-free fat pad can become at any point the recipient of transplanted epithelial cells that will repopulate the gland (DeOme et al., 1959). This feature provided both unequivocal evidence of the MaSC, which has been a focus of investigation for more than 60 years, and a functional assay for putative MaSC isolated from disaggregated tissue.

(B13) The stroma plays an important role in determining tissue function. The embryonic mammary epithelial interaction with the dense fibroblast stroma determines the ability of the epithelia to interact with the fatty stroma (Sakakura et al., 1982). Rudimentary mammary epithelium undergoes normal mammary morphogenesis in the mouse mammary fat pad of females in the absence of reproductive hormones (Sakakura et al., 1979), but if the epithelium does not come into contact with mesenchymal cells in the postnatal period, ductal morphogenesis fails to occur (Kratochwil, 1969). Fetal mammary epithelial rudiments are supported by adult stroma to fully differentiate (Sakakura et al., 1976). The tissue-specific pattern of ductal branching is dictated by stromal signals (Daniel et al., 1984; Sakakura et al., 1976). Sakakura and colleagues showed that heterotypic recombinants of fetal salivary mesenchyme induce mammary epithelium to undergo ductal branching patterns typical of salivary gland, but at lactation, the isografts synthesised specific milk proteins (Sakakura et al., 1976). It was observed that the ductal-alveolar nodules formed by mammary epithelium-salivary mesenchyme chimeras gave rise to a greater incidence of tumours (Sakakura et al., 1981). The mechanism by which perturbed tissue interactions contribute to the process of neoplasia is not understood.

(B14) The processes of mammary gland development, differentiation and involution are regulated by the ovarian hormones, estrogen and progesterone (Shyamala, 1997). Although the mouse mammary gland is organised differently from the human breast, these general features are similar and both are ovarian hormone dependent (Shyamala, 1997). Studies in mice have shown that estrogen provides the primary stimulus for ductal morphogenesis during puberty while concomitantly priming the gland for lobular-alveolar differentiation via its induction of PR. Progesterone is subsequently necessary for elaboration of the ductal tree via side-branching in the virgin animal and alveolar expansion during pregnancy (Atwood et al., 2000). While estrogen and progesterone are critical for proliferation, it is clear that mammary epithelial cells differ in their ability to respond to these signals. During both ductal and lobular-alveolar mammary growth, the distribution of proliferating cells is heterogeneous,

suggesting the involvement of local factors in dictating the specific response to systemic hormones (Bresciani, 1968; Christov et al., 1993; Daniel et al., 1987).

(B15) Estrogen signalling is mediated initially by ERα or β, members of the nuclear receptor superfamily of transcription factors. The precise roles of these receptors in regulating mammary development are complex (Shyamala et al., 2002). Both receptors impact on mammary carcinogenesis and are expressed in a subset of tumours and hence, also impact on their therapeutics. However, ERβ is dispensable for mammary development in mice, while ERα is required. This will be discussed in detail because the ER status of breast cancer is an important prognostic feature, a critical therapeutic target, and the ER status of breast cancer has been shown to be affected by radiation in both humans and mice (Castiglioni et al., 2007; Nguyen et al., 2011).

(B16) Studies in adult human breast indicate that ER expressing luminal epithelial cells also express PR, and constitute 20-30% of the epithelium (Clarke et al., 1997b). The frequency of ERα cells increases with age in human breast paralleling the rise in breast cancer risk (Shoker et al., 1999). Lawson et al. (1999) proposed that an increased frequency of cells increases breast cancer risk. Women at higher risk of breast cancer have more ERα cells compared with those women in a low risk population. Japanese women living in Hawaii had more ERα than those in Japan, which parallels cancer risk (Lawson et al., 2002). ERα cells are increased in normal tissue of tumour-bearing breasts (Khan et al., 1994), in postmenopausal women (Shoker et al., 1999), and in postmenopausal women using hormone replacement therapy (Lawson et al., 2001).

(B17) Anderson and colleagues showed that in contrast to the uterus, ERα does not co-localise with DNA synthesis or with broad markers of proliferation in human breast (Clarke et al., 1997b). Additional studies have confirmed this in human and in normal mouse and rat mammary glands (Ewan et al., 2005; Russo and Russo, 1998; Saji et al., 2000; Turgeon et al., 2001; Zeps et al., 1998). Nonetheless, proliferation of ER^{+} cells does occur, albeit at a low frequency that is highly restricted. The overall frequency of ERα cells co-localised with markers of proliferation was 0.2% in the study from Anderson and colleagues (Clarke et al., 1997b). Russo et al. (1999) showed that the distribution of proliferating ERα cells depended on the type of lobule in human breast, and demonstrated an inverse relationship between the degree of human breast lobular differentiation and co-localisation. The least-differentiated type 1 lobule averaged 0.48% co-localisation of Ki67 and ERα, while the more-differentiated type 3 lobule exhibited only 0.01 % dual labelling. Proliferating ERα cells are thought to represent a distinct lineage compared to non-proliferative cells. Dual-labelled cells are significantly more numerous in all proliferative lesions compared to normal pre- or post-menopausal lobules and correlate positively with increasing level of breast cancer risk (Shoker et al., 1999), but curiously do not occur frequently in breast cancers themselves (Jensen et al., 2001).

(B18) Mouse models have been used to determine whether ERα cells cannot or do not proliferate, i.e. are they incapable or restrained from responding to signals? Mammary epithelial proliferation is increased approximately 4-fold in adult $TGF\beta1^{+/-}$ mice, at oestrus (Ewan et al., 2002). The majority of the cells were ER-negative, indicating that decreased exposure to TGFβ permits cells to more readily respond to hormone-mediated signal. Notably, $TGF\beta1^{+/-}$ mice also exhibit a significant increase in cells dual-labelled for ER and Ki67 or BrdU (1 hour labelling) compared to wild-type mice at oestrus (Ewan et al., 2005). Thus, at least some ER cells are capable of proliferating, supporting the idea that they represent a progenitor population.

(B19) Studies in rats also showed that radiation exposure followed by pregnancy reduced tumourigenesis (Clifton et al., 1975). It was speculated that differentiation of a given

mammary cell for milk secretion markedly reduced the proliferative potentiality of that cell. In other words, in the presence of hormones that stimulate end-differentiation of the mammary cells, irradiation-altered mammary cells are lost from the high proliferative-potential population. Along these lines, Sivaraman et al. (2001) proposed a cell-fate hypothesis to explain the protective effect of pregnancy on breast cancer risk in general. They suggested that the hormonal milieu of pregnancy affects the developmental fate of a subset of mammary epithelial cells, perhaps by altering signal transduction and/or gene expression, which thereby reduces the risk of breast tumour development. This hypothesis would support the data from A-bomb survivors showing that early pregnancy reduces the risk of radiation-induced breast cancer.

B.2.2. Features of mammary stem cells *in situ*

(B20) It is now generally accepted that "stemness" is not a single property, but a number of properties that can be manifested under different conditions (Booth et al., 2000). As stated by Potten, a stem cell must be undifferentiated (relative to other epithelial cell types, but not necessarily relative to embryonic cells) and capable of proliferation, self-maintenance, and regeneration of the tissue after injury (Potten and Loeffler, 1990). It must be capable of producing many differentiated progeny, and retaining the ability to switch between these options when appropriate. These properties also make stem cells an important target for neoplastic transformation.

(B21) Great advances have been made in the first decade of this century in isolating putative MaSCs from rodent and human tissues in laboratories around the world. As mentioned above, the demonstration that mouse mammary epithelial cells could serially repopulate the mammary gland provided the first evidence that the mammary gland contains a stem cell (Daniel and Deome, 1965). It is widely believed that understanding the biology of MaSC in human breast and rodent mammary gland is fundamental to understanding the development of cancer. However it is now well appreciated that the properties, and probably the number, of stem cells may change in response to circumstances, including experimental manipulations.

(B22) Experiments by Smith and colleagues first demonstrated the clonal origin of murine mammary outgrowths (Kordon and Smith, 1998). Mammary epithelial cells were marked by random insertion of mouse mammary tumour virus and serially transplanted to show that outgrowths arose from a single cell based on the pattern of viral insertion sites. Large cell patches with the same blueprint X-chromosome inactivation in the human breast support the existence of a stem cell (Tsai et al., 1996). Terminal ductal-lobular units, the structures that cap the ducts in the human gland, are mono-phenotypic regarding X-chromosome inactivation (Diallo et al., 2001), as also occurs at the smaller ducts (Preston et al., 2003c). Interestingly, both luminal cells lining the ducts and surrounding myoepithelial cells present matching patterns of inactivation, suggesting that they are derived from a common precursor.

(B23) The stem cell distribution is restricted within the architecture of many adult tissues, like the crypt of the small intestine (Potten and Loeffler, 1990) or the hair follicle in epidermis (Jensen et al., 1999). But samples taken from any portion of the mammary gland at any age and at any developmental stage, including full functional differentiation, give rise to mammary epithelial outgrowths with complete developmental capacity. Smith and Medina (1988) proposed morphological features which can be used to distinguish MaSC populations in mouse, rat and human mammary glands. Cytological examination of mouse mammary gland explants revealed the presence of morphologically distinct cells distributed sporadically among the mammary epithelium, whose behaviour *in vivo* and *in vitro* suggested that they

might represent a latent epithelial stem cell population. The authors of these studies suggested that rare small cells that possess relatively few cytoplasmic organelles and dispersed chromatin are undifferentiated MaSCs. Using osmium tetroxide staining and electron microscopy, Chepko and colleagues identified small light cells (SLCs), undifferentiated large light cells (ULLCs), and an intermediate population between SLCs and ULLCs, which they suggested, might indicate that SLCs differentiate into ULLCs (Smith and Chepko, 2001). Both SLCs and ULLCs are division-competent, as evidenced by the presence of mitotic chromosomes (Chepko and Smith, 1997). Interestingly, both cell types can appear as single cells, but are also found as homogeneous pairs (two SLCs or two ULLCs), suggestive of symmetric division, or in mixed pairs formed by one SLC and one ULLC, suggestive of asymmetric cell division. SLCs are suprabasal and do not have contact with the lumen, while ULLCs can appear in contact or not with luminal areas.

(B24) The observation that SLCs constitute a constant fraction of the mammary epithelium through pregnancy, lactation and involution, suggests that this population increases and decreases as necessary, indicative of a stem-like behaviour for these cells (Chepko and Smith, 1997). However, the frequency of these cells (about 3% of the epithelium) is too large for a MaSC population, thus suggesting that a committed progenitor cell also might be included in the SLC pool. Division competence, staining properties and the presence of mixed pairs of SLCs and ULLCs make these latter cells a candidate progenitor cell population. These ULLCs were postulated then to give rise to both secretory and myoepithelial cells. Notably, both SLCs and ULLCs are depleted in growth-senescent tissue (Smith et al., 2002), i.e. mammary outgrowths from a third-generation transplant or beyond that are unable to give rise to further mammary tissue upon transplantation into a cleared fat pad. In contrast, SLCs and ULLCs are readily detectable in hyperplastic alveolar outgrowths, which can be propagated indefinitely by serial transplantation, which was suggested to be consistent with a MaSC-like population. Moreover, rat SLCs seem to be surrounded by a niche, formed by differentiated luminal cells and myoepithelial cells, as well as stretches of direct contact with the basement membrane (Chepko and Dickson, 2003). These niches contain 1 to 4 SLCs, with one of them always close to the basement membrane. While conceptually attractive, these studies lack functional tests that SLCs or ULLCs are stem cells since preparation of the tissue for electron microscopy eliminates the possibility of either isolating or staining for other markers (e.g. hormone receptors).

(B25) A functional assay of long-lived populations, so-called LRCs, has been used in mammary glands (Kenney et al., 2001; Welm et al., 2002; Zeps et al., 1996). Depending on physiological status, stem cells are generally thought to be quiescent during normal tissue homeostasis (Cheng et al., 2000; Lowry et al., 2005; McCroskery et al., 2003). ^{3}H-thymidine, BrdU or a fluorescent cell tracker can be used to follow proliferation and persistence of cells in which label was incorporated. The label is provided for a short period (e.g. 2 weeks) during which the majority of cells undergo DNA synthesis. A wash-out period of several weeks is then observed during which the label is either diluted beyond detection, which is approximately 5 divisions, or the cell is lost. Cells that retain label many weeks (e.g. 8-9) after the initial labelling period are considered to be early progenitors based on the model in which stem cells and progenitor cells divide infrequently (Potten and Loeffler, 1990).

(B26) LRCs have been previously studied in the mouse mammary gland, although the protocols used to identify these cells have been different every time. When mature mice were labelled by injecting them with ^{3}H-thymidine, different distributions in the number of cells that incorporated and retained the label over a two-week chase were observed depending of the stage of the oestrus cycle at the time of label injection (Zeps et al., 1996). The distribution of label intensity was also variable. This is due to the fact that different hormonal levels

during the oestrus cycle regulate the size of the proliferating compartment in the mammary gland. However, in all cases, the most heavily labelled cells were at some distance from the closest cells with similar degrees of label retention. This would point towards the presence of discrete units randomly distributed along the mammary tree containing one stem cell and its closest progeny. Most LRCs thus identified had a luminal location and expressed the ER (Zeps et al., 1998), although a subpopulation of heavily labelled, ER^- basal cells was also described. Interestingly, the frequency of these cells is more consistent with that of a stem cell population, and their basal location agrees with some other reports which indicate that the MaSCs have a basal (Deugnier et al., 2002) or suprabasal (Chepko and Smith, 1997) location. The fact that the initial labelling was done on adult, non-stimulated mice, indicates that most mammary MaSC, quiescent during tissue homeostasis, should not have incorporated the label and therefore, would not be included in the LRC population identified in these studies.

(B27) LRCs seemed to be located into discrete, periodical units along the mammary ducts in mammary tissue transplants following BrdU administration in drinking water and with an 8-week 'chase' (Kenney et al., 2001). These units likely contain both stem cells and their earliest progeny. LRCs did not express markers of milk production, like whey acidic protein (WAP) or β-casein, and a subpopulation of them did not express adhesion molecules, like α-catenin and zonula occludens 1 (ZO-1). This might be related to the maintenance of these cells in a niche. Inexplicably, some ducts seemed not to possess any LRCs.

(B28) BrdU can also be delivered using micro-osmotic pumps that release their content at a constant rate (Theeuwes and Yum, 1976). Using this approach, it was possible to label 3-week-old mice (entering puberty, where the mammary MaSC pool is supposed to divide symmetrically) for two weeks (Welm et al., 2002). This was then followed by a 9-week 'chase'. This approach presents several advantages. First, BrdU release is continuous rather than in pulses. Second, the stage in the oestrus cycle of the mice did not influence the results, since both labelling and chase periods are relatively long and include several complete cycles. After the chase, very few LRC were found that expressed the progesterone receptor. A subpopulation not expressing luminal or myoepithelial markers was also described within the LRC compartment. Finally, when the SP of these mice was analysed, LRCs were shown to be four times more abundant in the SP than in the remaining cells.

(B29) An alternative interpretation of LRCs proposed by Cairns is that stem cells maintain differential strand segregation that protects the stem cell DNA from replication errors (Potten et al., 2002). Double-labelling experiments using ^{3}H-thymidine and BrdU provide support for this mechanism in mouse mammary gland (Smith, 2005). The tissue was first labelled with radioactive thymidine after transplantation into cleared fat pads, which mimics mammary development, when MaSCs are supposed to divide symmetrically. After a chase period, BrdU coupled with a proliferative stimulus (hormonal treatment, which should induce asymmetric MaSC division) was used to further label the dividing cells. Most epithelial LRCs incorporated the second label, thus showing that, although quiescent, they have a high proliferative potential. Stromal LRCs did not seem to be able to incorporate the second label, but this might have been due to an inadequate proliferative stimulus. When a second chase was carried out, most LRCs lost the second label, but not the first label. This indicates a selective segregation of DNA in these cells in agreement with the Cairns hypothesis, and therefore validates the use of LRCs in the mouse mammary gland, as long as the initial labelling is done during tissue development (normal or post-transplantation).

(B30) Transplants of human breast epithelium into athymic mice have recently been used to study human LRCs (Clarke et al., 2005). Most LRCs were shown to be out of cycle (they expressed p27, a cyclin-dependent kinase inhibitor) and enriched for expression of p21 and

Musashi 1 (Msi-1), both putative stem cell markers. However, these two markers are expressed in distinct subfractions of the LRC pool, with $p21^+$ LRCs in suprabasal locations and Msi-1^+ LRCs in luminal or suprabasal positions. This might indicate the presence of two different stem cell compartments with different potentials, or a stem cell hierarchy where a multipotent stem cell gives rise to a unipotent stem cell. Interestingly, most of the cells expressing p21 or Msi-1 in the outgrowths were hormone receptor positive.

(B31) An advantage of LRC techniques is information *in situ* unavailable with disaggregation approaches like cell sorting profiles or mammosphere-forming cells. A systematic characterisation using image analysis techniques, together with the classic immunofluorescence and microscopy approaches, has catalogued the properties, frequency and distribution of LRCs (Fernandez-Gonzalez et al., 2010; Fernandez-Gonzalez et al., 2009). Immunofluorescence was used to identify LRCs that incorporated BrdU while undergoing DNA synthesis during puberty (3-5 weeks of age) and retained this label in the adult gland (14-18 weeks), as well as epithelial cells expressing PR and a transcription factor p63, indicative of specific functions. Approximately 4% of luminal cells were LRCs, the majority of which did not express PR, a luminal epithelial differentiation marker, indicative of undifferentiated luminal cells. Multi-scale analysis *in situ*, which is high-content image analysis measurements of multiple features (e.g. population size and distribution, local organisation or cellular properties) linked at multiple scales (e.g. organ, tissue, single cell), revealed that luminal LRCs have a distinct nuclear morphology, are enriched 3.4-fold in large ducts, and are distributed asymmetrically across the tissue. A population of suprabasal cells, located between the luminal and the myoepithelial layers, was highly enriched for LRCs. Myoepithelial cells express p63 and represent a differentiated population. Many suprabasal LRCs exhibited both the luminal LRC nuclear morphology and the myoepithelial marker p63, which suggests that they have bi-lineage differentiation potential. Together, these data suggested that the ventral-most, large ducts contain a reservoir of MaSCs. In support of this, CD24 and CD49f epithelial cells were enriched in preparations from the ventral versus the dorsal gland.

(B32) Lineage tracing is used to experimentally test whether a marker is truly expressed in stem or progenitor cells. An example is that of an orphan 7-transmembrane receptor, Lgr5, which was identified using lineage tracing as a marker of cycling stem cells in the gut (Barker et al., 2007). This transgenic technology creates a mouse in which the expression of a reporter protein, often fluorescent, is transiently driven by the promoter of the protein in question to mark all the progeny of the cell expressing the designated marker at the time of induction. For example, Lgr5, which is a Wnt target gene, also marks stem cell compartments of the skin and stomach. However, in the mammary gland, the progeny of $Lgr5^+$ cells does not mark stem cells *per se* but marks lineage specific cells (de Visser et al., 2012). Interestingly, Lgr5 was shown to mark luminal cells immediately after birth but switches to myoepithelial cells at 12 days postnatal. Adding to this complexity is cytokeratin lineage tracing experiments from Blanplain that suggest there are restricted basal and luminal stem cell compartments in the mouse mammary gland (Van Keymeulen et al., 2011).

B.2.3. Turnover rate and age dependence

(B33) Cell turnover in the mammary gland is a response to a complex life cycle dictated by systemic factors. The mammary gland is established via mesenchymal induction of ectoderm specialisation. At birth, the gland consists of an epithelial anlagen that is mainly quiescent until proliferation and morphogenesis are stimulated by the hormones of puberty. Proliferation within the epithelium varies from highly proliferative endbuds to relative

quiescence among the epithelium of the nipple. Short periods of proliferation occur with oestrus in mammals, followed by apoptosis which maintains the ductal tree throughout life. Ovariectomy leads to regression and an essential static state. Pregnancy induces considerable expansion (i.e. from ~10% epithelium to 90%) accompanied by secretory differentiation. The period of lactation is sustained by suckling and can persist for years in humans. Cessation of suckling leads to rapid apoptotic involution and tissue remodelling to a quasi-nulliparous state.

(B34) Whether the unusual age dependence of radiation breast cancer risk is linked to physiological processes unique to mammary development remains widely debated. An estimate of the number of stem cells (clonogens) in the mammary gland was first reported by Clifton and Gould. They reported on the successful takes and extent of growth of serially-diluted mammary epithelial cells into the interscapular fat pad of rats, and estimated that mammary epithelial clonogens were present at a frequency of approximately 0.05% in the cell population of the virgin rat mammary gland (Gould et al., 1977). Experiments performed in mice have demonstrated that any portion of the mammary tree (i.e. primary duct, tertiary duct), any developmental stage (i.e. virgin, lactating), or any age (i.e. 3 weeks, 80 weeks) contains cells capable of repopulating the mammary stroma and undergoing the complete developmental cycle of the parenchyma (Neville and Daniel, 1987). These experiments convincingly demonstrated that totipotent stem cells exist throughout the mammary parenchyma tree and are not localised to just the terminal portions of the mammary tree.

(B35) The number of the clonogenic cell subpopulation increases during ductal morphogenesis during puberty, and is apparently stable subsequently despite cycles of differentiation and involution. Rapid and extensive proliferation during pregnancy is primarily that of TA cells that will undergo secretory differentiation, followed by apoptosis during involution upon cessation of suckling. There are some experimental data suggesting that stem cells expand with hormonal stimulation (Joshi et al., 2010), but this remains controversial due to interpretation of functional assays. Involution returns the gland to a state similar to that of the nulliparous animal, but with distinct changes that are transiently associated with increased risk of cancer but confer long-term protection, at least if occurring in young women (Schedin, 2006). Notably, the repopulation potential of the epithelium, as evidenced by serial transplantation of the mouse mammary gland, is maintained into old age, but the capacity of the host to support mammary gland outgrowth decreases with age (Daniel et al., 1968). Thus, the increase of stem cells in adults compared to puberty appears to be roughly inversely proportional to susceptibility to carcinogenesis.

B.2.4. Cellular features: stem cell markers

(B36) Many markers have been used to enrich or identify stem cells but none are definitive in the mammary gland or breast epithelium at the time of this publication. Cytokeratins, lineage commitment antigens and stem cells markers from other tissues have been used to characterise MaSCs. Thus, for example, keratin 6 (K6), which is very rarely expressed in mouse luminal epithelial cells, has been postulated as a MaSC marker (Smith and Chepko, 2001). This is based on two features: (1) in both mouse and human cultures, only colonies formed from luminal cells display multiple phenotypes (Pechoux et al., 1999; Smalley et al., 1999); and (2) K6 is expressed in the proliferative area of primary cultures of mammary epithelium (Smith and Chepko, 2001). Thus, it is likely that the MaSC population resides in the luminal epithelium, and that since K6 seems to mark cells with high proliferative potential, the rare luminal epithelial $K6^{+}$ cells might represent a stem cell

population. However, no functional characterisation of this population has been successful at proving enrichment in stemness potential.

(B37) A population of suprabasal, luminal cells was isolated from human mammary tissue based on the expression of epithelial cell surface antigen (ESA, a luminal marker expressed at the basolateral surface of the cells) and the absence of muchin 1 (MUC1) expression (an apically-expressed luminal marker) (Gudjonsson et al., 2002). These cells were then immortalised to generate a suprabasal cell line, which was able to generate both luminal (expressing K18) and myoepithelial (expressing K14) cells in clonal assays. The suprabasal cell line was formed by cells that also expressed K19, known to be restricted to a luminal subpopulation *in situ*. Interestingly, in clonal cultures, this cell line also showed multipotency regarding K19 expression in its progeny. However, no hormone-receptor positive cells were obtained *in vitro*. These data seem to indicate that the suprabasal cell line contains not a stem, but a progenitor, cell population. However, functional characterisation of these cells by transplantation into cleared mouse fat pads might shed light on the hormone sensitivity of the resulting outgrowths. Noted above is that basal and luminal cytokeratins appear to mark distinct lineages in the mouse (Van Keymeulen et al., 2011), which may point to a species difference.

(B38) Luminal and myoepithelial lineage markers, epithelial membrane antigen (EMA) and common acute lymphoblastic leukaemia antigen (CALLA), respectively, seemingly expressed later in the commitment process than cytokeratins (see above), have been used to identify candidate stem cell populations in the human breast (Clayton et al., 2004). Interestingly, populations that are either double positive (0.47% of the epithelium) or double negative (30%) for both of these markers were able to generate mixed (luminal and myoepithelial) colonies, but double negative cells generated more colonies with a luminal phenotype. Most double negative cells expressed K18, a luminal marker. This might indicate that double negative cells contain a luminal progenitor population. In that case, the myoepithelial colonies generated by these double negative cells would confirm the hypothesis of a myoepithelial progenitor within the luminal compartment [see above, (Gudjonsson et al., 2002)]. Only double positive cells showed proliferation markers (very few cells) or expressed ER (at low levels). When these cells were grown in the presence of estrogen, they generated fewer luminal colonies, thus suggesting a role for estrogen in cell fate decision. However, the loss of ER expression after two days in culture (Kothari et al., 2003), together with other potential changes in gene expression that the cells undergo *in vitro*, prevent drawing any definitive conclusions from these results regarding the role of ER in regulation of cell commitment. In any case, both the double positive and double negative populations seem to contain progenitors with some degree of commitment, as shown by expression of ER (double positive cells) or K18 (double negative cells).

B.2.5. Functional analysis of mammary stem cells

(B39) Functional analysis of stem cells has been the gold standard for HSCs using bone marrow repopulation following lethal irradiation. Likewise, MaSCs can be demonstrated by their high repopulation potential. A series of break-through experiments was carried out in the Cancer Research Lab at Berkeley in the 1950s, where mouse mammary epithelial cells were transplanted into inguinal mammary fat pads from which the epithelial compartment had been excised (so-called cleared fat pads) (DeOme et al., 1959). Transplanted tissue or dissociated cells proliferate, undergo ductal morphogenesis to fill the fat pad, and pregnancy initiates differentiation to produce milk. The age of the donor does not have an effect on the outgrowth potential of the transplant (Young et al., 1971). These experiments clearly showed

that there must be a pool of cells within the mammary epithelium with extensive regenerative potential. Furthermore, the fact that serial transplantation experiments can be made up to four to six serial iterations with similar efficiency (Daniel et al., 1968) indicates that these cells not only can give rise to a fully functional mammary gland, but also are able to self-renew many times. Similar properties have been shown for rats (Gould et al., 1977; Kamiya et al., 1998; Kim et al., 2000) and for human mammary epithelial cells transplanted into mouse fat pads (Clarke et al., 1997a; Laidlaw et al., 1995).

(B40) The rational for the SP sorting scheme for stem cells is based on the hypothesis that stem cells are resistant to chemical damage to their DNA because they express ATP-binding cassette (ABC) transporter family proteins which are able to "pump out" certain chemical products (Jonker et al., 2005; Zhou et al., 2002). One of these products is Hoechst 33342. Using flow cytometry on cells counterstained with this dye, a subpopulation of cells that have excluded it from their nuclei, the SP can be identified. In the bone marrow, this (SP) subpopulation has been shown to be highly enriched for HSCs (Goodell et al., 1996; Goodell et al., 1997).

(B41) A mouse mammary SP also has been identified (Welm et al., 2002). This population, which constitutes about 2-3% of the epithelium, is formed by relatively small cells, and is enriched for expression of Sca-1, a stem cell marker in the haematopoietic system (although it is also broadly expressed in the mammary epithelium). The mammary SP is also enriched for LRCs (see below), which might be indicative of a quiescent phenotype *in situ*. Transplantation experiments revealed that approximately 1/4000 SP cells can give rise to a functional outgrowth in a cleared mammary fat pad. However, the cells had to be cultured for 3-5 days before sorting and transplantation. This step, which might have induced cell transformation (e.g. by enhancing the ability of cells to efflux the dye (Alvi et al., 2003)), was necessary to increase the cell viability through Hoechst staining and sorting. Interestingly, in a different study (Alvi et al., 2003), a mouse mammary SP was isolated without intervening cell culture, and a much smaller fraction of cells (0.45% of the epithelium) was obtained.

(B42) A human SP, constituting 0.18% of the epithelium, was likewise isolated in that latter study (Alvi et al., 2003). These cells have comparable expression profiles to non-SP cells regarding luminal (K18) and myoepithelial (K14) markers. However, when different luminal (EMA) and myoepithelial (CALLA) markers were assayed (Clarke et al., 2005; Clayton et al., 2004), the human SP cells were shown to be mostly negative for both of them. This might indicate that the different lineage markers used are expressed at different stages of the differentiation process, with keratins being "turned on" before EMA or CALLA. When assayed for colony formation, single human mammary SP cells were able to form colonies expressing K18, K14 and both markers, thus suggesting a multilineage potential for these cells. Self-renewal of the SP has also been shown by the fact that virtually all the mammosphere-forming cells (see below) are included in the SP in human tissue (Dontu et al., 2003).

(B43) Contradictory results regarding ER expression in these cells have been reported (Alvi et al., 2003; Clarke et al., 2005; Clayton et al., 2004), and it is therefore difficult to conclude whether they are sensitive or not to the mitogenic stimulus provided by estrogen. SP cells seem to be enriched for cells expressing telomerase (Alvi et al., 2003), as well as other potential stem cell markers, like p21 or Msi-1 (Clarke et al., 2005). Also, activation of the Wnt pathway (involved in stem cell self-renewal (Reya and Clevers, 2005)) increases the size of the mouse mammary SP both in primary cultures and in transgenic mice (Liu et al., 2004). All this evidence, although sometimes contradictory, points towards the existence of a stem (or early progenitor) subpopulation within the SP compartment. However, SP identification

requires destruction of the tissue microenvironment, thus rendering impossible the study of these cells within their tissue context.

(B44) Sca-1, a MaSC marker in the haematopoietic system, has also been used to isolate a population of luminal, mouse mammary epithelial cells with enriched regenerative potential upon transplantation into cleared fat pads (Welm et al., 2002). Sca-1^{+} cells are very frequent in the SP (75% positive cells versus 20% in the entire epithelium), and they contain twice as many LRCs as the Sca-1^{-} fraction. When labelled *in situ*, Sca-1^{+} cells do not express PR, another steroid hormone-related protein, or differentiation markers. However, they constitute about 20-30% of the epithelium, which makes unlikely that Sca-1 labels a stem-cell-only population, but a progenitor or TA cell population.

(B45) Along these lines, it has been shown that a population of mouse mammary epithelial cells, where 1 in 20 cells is able to repopulate a fat pad, expresses low levels of Sca-1 (Stingl et al., 2006). These cells were sorted based on their high expression of CD24, a neural MaSC marker, and CD49f (α6-integrin), which labels epidermal MaSCs. Gene expression analysis showed that these cells have very similar profiles to myoepithelial cells, with no significant differences: most of them were positive for smooth muscle actin or K14, a few were positive for K18, and none expressed both markers. Also, very few of these cells expressed K6, previously postulated as a MaSC marker (Smith and Chepko, 2001). The SP contained less than 10% of these cells (Stingl et al., 2006), thus indicating that the SP may represent more-differentiated progenitor cells, because its repopulation efficiency was not as high as it was for the $CD24^{+}$ $CD49f^{high}$ population. CD49f is expressed as a heterodimer with CD29 (β1-integrin). In a very systematic study using CD24 and CD29 expression (Shackleton et al., 2006), a similar enrichment for regenerative potential in the $CD24^{+}$ $CD29^{high}$ population (1 in 64) was shown, and this confirmed the keratin expression patterns observed previously. The $CD24^{+}$ $CD29^{high}$ epithelial cells had increased colony-forming potential in two-dimensional (2D) cell cultures, while in three-dimentional (3D) culture they gave rise to both ductal and alveolar structures with luminal and myoepithelial populations. This study demonstrated for the first time, regeneration of a fully functional outgrowth after transplantation of a single $CD24^{+}$ $CD29^{high}$ cell into a cleared fat pad (with and without support cells). The outgrowths obtained recapitulated the CD24/CD29 expression profile of intact glands. All these pieces of data demonstrate the multilineage potential of the $CD24^{+}$ $CD29^{high}$ cells. When cells from one of these outgrowths (generated from a single $CD24^{+}$ $CD29^{high}$ cell) were transplanted into multiple fat pads, a functional outgrowth was obtained in each of the recipient fat pads. This shows that the $CD24^{+}$ $CD29^{high}$ cell that gave rise to the outgrowth must have self-renewed. Therefore, this study demonstrates that the $CD24^{+}$ $CD29^{high}$ population in the mouse mammary epithelium is highly enriched for cells with multilineage and self-renewal potentials, the two properties that define a MaSC. Interestingly, these cells are enriched for LRC content, but not for Sca-1 expression or Hoechst exclusion (SP), and they do not form mammospheres.

(B46) Transcriptional profiling of FACS-sorted $CD44^{high}$ $CD24^{low}$ breast epithelial stem cells demonstrated an enrichment in transcripts determining cell motility, cell adhesion, cell proliferation, chemotaxis and angiogenesis. A striking observation was the enrichment in transcripts for TGFβ and Wnt signalling components in these stem cells (Shipitsin et al., 2007). Indeed, the stem cell compartment was responsive to TGFβ, and targeted by TGFβ inhibition, whereas $CD44^{low}$ $CD24^{high}$ progenitor cells had lost responsiveness due to downregulation of the TGFBR2 gene. Clinical evidence demonstrated that expression of a "TGFβ cassette" of genes (expressed in $CD44^{high}$ $CD24^{low}$ > $CD44^{low}$ $CD24^{high}$) in breast tumours is associated with a shorter metastasis-free survival of breast cancer patients with ERα-negative tumours (Shipitsin et al., 2007). The transcriptional similarity between normal

and neoplastic stem cells was greater than that between stem cells and their $CD44^{low}$ $CD24^{high}$ progeny within the same tissue. It would appear that, as in embryonic stem cells, TGFβ signalling plays a role in cancer stem cell maintenance.

(B47) Taking this observation one step further, it was shown that inducing 'Snail'-overexpression leads to epithelial-mesenchymal transition or transformation (EMT) in human mammary epithelial cells that then exhibit stem-cell-like properties in terms of expression of stem cell markers, increased mammosphere seeding activity *in vitro* and tumourigenicity *in vivo* (Mani et al., 2008). TGFβ frequently mediates the transcriptional EMT programme (Zavadil and Bottinger, 2005; Zavadil et al., 2004). It is noteworthy that radiation exposure induces TGFβ activation in mouse mammary gland (Barcellos-Hoff, 1993, 1994), and that irradiated human mammary epithelial cells activate TGFβ and are predisposed to undergo TGFβ mediated EMT (Andarawewa et al., 2007). Excessive TGFβ levels in the irradiated microenvironment may therefore not only maintain stem cells, but also contribute to their formation via EMT of more differentiated progenitors. This possibility remains to be tested.

(B48) An important caveat to these experimental studies is that most of studies make use of cell sorting techniques that require disruption of the tissue microenvironment before obtaining a stem cell-enriched population. Most of the antibodies used in flow cytometry analysis have ligands on the cell membrane or in the cytosol. When tissue sections were stained for $CD24^{+}$ $CD29^{high}$, it was hard to identify cells that met the criteria due to the broad expression of these markers *in situ* coupled with the compactness of epithelial tissues (Shackleton et al., 2006). In ducts, $CD24^{+}$ $CD29^{high}$ cells seemed to be basolateral, while in end buds (the growing portion of the ducts during gland development) expression was higher in cap cells, the invading cell layer, supposed to be a stem cell reservoir (Williams and Daniel, 1983). Thus, although $CD24^{+}$ $CD29^{high}$ can be immunostained *in vitro* (where cells are nicely isolated or form relatively small clumps), when these markers are stained *in situ*, large populations of positive cells are identified. Furthermore, it is often difficult to tell what cell is positive and which one is not, especially when dealing with cell surface labels. This prevents the use of these markers for studies of the *in situ* organisation of stem-like populations and their microenvironment.

(B49) The frequency of mammary repopulating cells isolated from dissociated mammary gland is generally assumed to accurately reflect the frequency and behaviour of MaSC *in situ*, i.e. the presumption is that stemness is a property inherent to certain cells rather than being conferred by the microenvironment or context. The recent identification of heterogeneous populations with mammary repopulating properties, yet lacking multipotent lineage commitment evidence *in situ,* together with the struggle to obtain pure MaSC populations, suggests that both cell intrinsic and extrinsic properties are important in stem cell behaviour. This underscores the need to identify the niche that harbours stem cells in order to understand and control stem cells themselves. As of now, identification of the indisputable mammary MaSC niche has been elusive.

B.3. Radiosensitivity

B.3.1. Stem cell and progenitor cell radiosensitivity

(B50) Few studies have attempted to assess the radiation sensitivity of the MaSCs or progenitor cells. A comprehensive analysis of the radiosensitivity of rat mammary clonogens has been conducted by Gould and colleagues (Kamiya et al., 1990; Kamiya et al., 1998; Kamiya et al., 1991). In this model, monodispersed rat mammary epithelial cells are injected into the intrascarpal fat pad where they form clonogenic organoids. The surviving fraction of

clonogenic mammary cells was measured in groups of virgin rats from 1 to 12 weeks after birth after single exposures to ^{137}Cs γ rays (Shimada et al., 1994). The radiosensitivity of clonogens from prepubertal rats was high and changed with the onset of puberty at between 4 and 6 weeks of age. Additional studies suggest that the ability of immature tissue to process chemical carcinogen damage is different form that of the mature tissue (Ariazi et al., 2005).

(B51) Studies to test the hypothesis that mammary progenitor cells are resistant to radiation suggest that the relative resistance of a progenitor population is mediated at least in part by Wnt signalling, which is implicated in stem cell survival (Woodward et al., 2007). Freshly isolated mammary epithelial cells following *in vivo* radiation (4 Gy) and sorted by FACS for cells similar to that published (Shackleton et al., 2006), did not enrich for this stem cell population and in contrast, decreased this population by approximately one-third. However, radiation selectively increased the Sca-1$^+$ population *in vivo*, the SP of primary mammary epithelial cells after 3 days of culture, and the Sca-1$^+$ fraction of MCF7 breast cancer cells and COMMA-D murine epithelial cells. The authors concluded that mammary progenitor cells are relatively resistant to radiation, which they attribute to increased survivin signalling (Chen et al., 2007; Woodward et al., 2007).

B.3.2. Single cell responses

(B52) When irradiated clonogenic mammary epithelial cells are transplanted and hormonally stimulated, they give rise to clonal glandular structures within which carcinomas may arise (Gould et al., 1991). Using this experimental strategy in breast and thyroid, Clifton and colleagues found frequencies as high as one cancer per 10-300 clonogens (Kamiya et al., 1995). Similarly, Kennedy and Little found similarly high initiation of transformation in irradiated C3H10T1/2 mouse fibroblasts (Kennedy et al., 1980; Kennedy and Little, 1978). Both epithelial and fibroblast events were suppressed by high density or addition of unirradiated cells. These data and others led to the conclusion that initiation can be a high frequency epigenetic event rather than a low frequency mutational process (Kamiya et al., 1995).

(B53) Growth in suspension culture systems has been known to be a property of cancer cells (Freedman and Shin, 1974) but is also attributed to stem cells. Dissociated human mammary epithelial cells cultured on a non-adherent substratum undergo anoikis, i.e. death due to the lack of anchorage. A few cells survive, however (Soule and McGrath, 1986), and under specific culture conditions they give rise to multicellular spheroids, which in the case of breast are called mammospheres (Dontu et al., 2003). To demonstrate stem cell activity, dissociated mammosphere cells were shown to give rise to luminal, myoepithelial and alveolar cells in monolayer culture (seeded on tissue culture plastic at clonogenic densities), and to complex, branching, ductal-alveolar systems in 3D culture conditions (Dontu et al., 2003). Furthermore, when mammospheres are dissociated into single cells and grown again in suspension, they can form new mammospheres with multilineage potential, which can be repeated indefinitely. The more passages, the more bipotent progenitors (measured by the frequency of mixed luminal and epithelial colonies obtained in monolayer) are present in the mammospheres. Successive passaging does not alter the frequency of mammosphere formation or their size, indicating that all the spheroids arise from cells with self-renewal competence. This shows that mammospheres contain cells with multilineage and self-renewal potentials, the two properties expected of a stem cell population.

(B54) This approach allowed protein expression and transcriptional profiling studies in order to identify human MaSC markers (Dontu et al., 2003). For example, CD10, α6-integrin or K5 were all shown to be expressed by cells in the centre (and not in the periphery) of

mammospheres. Transcriptional profiling showed that mammosphere cells shared upregulation of many genes with ES, NSC and HSC. For example, the Notch pathway has been involved in cell fate decision (Artavanis-Tsakonas et al., 1999). Using the mammosphere system, it was shown that treatment with an inhibitor of Notch signalling reduced the size of primary mammospheres, and inhibited formation of secondaries (Dontu et al., 2004). On the other hand, addition of an activator of that same pathway induced an increase in the frequency of mammospheres with each passage, and also with respect to untreated controls, suggesting an increased number of mammosphere-forming cells in each generation due to activation of self-renewal. These Notch-activated mammospheres also contain an enlarged fraction of myoepithelial progenitors as shown by the increased frequency of mixed and myoepithelial colonies obtained in monolayer cultures of mammosphere-dissociated cells. This indicates a role for Notch in inducing proliferation of bi-potent and myoepithelial progenitor cells. However, this mammosphere-forming potential cannot be identified *in situ*, and therefore, as with the SP, it is not possible to study the location and arrangement of these cells within the mammary gland or the potential presence of a niche around them.

B.4. Experimental models of carcinogenesis

B.4.1. Initiation

(B55) Evidence from rodent models indicates that neoplastic initiation by radiation or chemical carcinogen is relatively frequent (Clifton et al., 1986), while progression to neoplasia is relatively less frequent (Medina et al., 1986). Studies from Gould and Clifton showed that one in approximately 13 irradiated (7 Gy) rat mammary clonogens gave rise to cancer, which indicated that at least 1 of approximately 95 clonogens was radiogenically initiated (Kamiya et al., 1995). A similar high initiation frequency was seen in grafts of methylnitrosourea (MNU)-treated clonogens. Such initiation is thus far more frequent than specific locus mutations. In sites grafted with larger cell inocula, cancer incidence per clonogen was suppressed inversely as the number of irradiated or MNU-treated clonogens per graft increased. Addition of unirradiated cells to small irradiated graft inoculates also suppressed progression. In a variety of systems, the expression of dysplasia *in vivo* and neoplastic transformation in culture was inversely correlated with the number of cells seeded (Clifton et al., 1986; DeOme et al., 1978; Ethier and Ullrich, 1982; Terzaghi-Howe, 1990; Terzaghi and Nettesheim, 1979). The nature of the cell dose dependence is unclear but appears to be due to suppression of the initiated cells' expression of its altered phenotype by the normal cells. These epithelial-epithelial interactions are clearly involved in a variety of normal and neoplastic processes.

B.4.2. Progression

(B56) In mouse mammary gland, hyperplastic nodule formation is one distinct morphological marker of preneoplasia (Banerjee et al., 1987). Depending on the carcinogen, parous status and hormonal environment of the gland, such hyperplasias will appear to be ductal, alveolar or ductal-alveolar. Some carcinogens, such as 7,12-dimethylbenz(a)anthracene (DMBA) and radiation, produce ductal hyperplasia and dysplasia (Ethier and Ullrich, 1982), which are similar in their range of severity and histology to those abnormalities observed in the pathogenesis of human cancer (Ethier et al., 1984). As with mouse mammary tumour virus (MMTV) lesions (DeOme et al., 1978), dissociation and

transplantation of exposed cells into host glands increase the expression of lesions over those observed in intact mammary glands, indicating that the potential for expressing preneoplastic lesions is present long before they are expressed in the intact gland (Ethier and Ullrich, 1982). The size of the inocula and the age of the animal at analysis also affect the frequency and severity of the dysplasia (Ethier et al., 1984). Interestingly, a 10-fold smaller inoculum is most effective. This is thought to reflect the increased number of cell divisions required to fill the mammary gland, which allows for a greater opportunity to express the altered phenotype, but also could be due to a suppressive action of more non-transformed cells (Kamiya et al., 1995).

(B57) Analysis of the mammary outgrowths from irradiated cells showed that analysis of an actively growing (immature) or in a resting state (mature) gland allows a distinction to be made between the expression of the preneoplastic phenotype, which is greatest in the growing gland, and whether the altered phenotype persists in the resting gland (Ethier and Cundiff, 1987). Some lesions regress or remodel following the cessation of growth. However, the altered phenotype could be re-expressed upon retransplantation even in an apparently normal recombinant gland. Persistence of a lesion following cessation of gland growth is considered to reflect the acquisition of the ductal dysplasia for some autonomy from tissue regulatory mechanisms. Increasing the time between carcinogen exposure and transplantation increased the frequency of persistent preneoplastic lesions, which also indicates progression of cells that continue to acquire new characteristics with time. If a year is allowed to elapse between exposure and transplantation then a third characteristic is observed: the ability to elicit a host response (Adams et al., 1987). The dynamics of neoplastic progression were found to be similar for exposure to DMBA or radiation at these low doses, and this reveals commonalities between two qualitatively different carcinogens. However, DMBA did induce more persistent lesions than radiation, although when 1 year elapsed between exposure and transplantation, this difference was eliminated.

(B58) A qualitative relationship between preneoplastic lesions and tumourigenesis is generally accepted, because such lesions have been noted to precede neoplasia and progressively acquire phenotypes consistent with increasingly autonomous behaviour. However the quantitative relationship is less well understood. The efficiency of tumour induction is usually related to dose of a given agent, but other factors such as the toxicity of the agent or the hormonal status of the tissue can influence tumour incidence. For example, at DMBA exposures below 0.125 mg, tumour incidence is highly dose-dependent, at doses above 0.25 mg, the toxic and carcinogenic effects in other tissues become important, and at higher doses, the histological tumour type changes from ADC to adenocanthomas (Ethier and Ullrich, 1982). The preneoplastic lesions preceding such dose effects have not been studied. Experimental models that maximise the progression to frank tumours for radiation have been developed by altering the hormonal environment of the gland (Clifton et al., 1986) but have not yet been studied in terms of their specific effects on preneoplastic lesions.

(B59) As discussed above, Ullrich developed a novel assay to show that radiation influences progression using mammary cells dissociated from mice 24 hours to 16 weeks after being irradiated and subsequently injected into gland-free fat pads to assess the frequency and characteristics of ductal dysplasias in outgrowths (Ethier and Cundiff, 1987; Ullrich, 1986). Notably, this assay suggests that the acquisition of the altered growth potential which resulted in ductal dysplasias and the ability of these lesions to gain some autonomy from growth-regulatory mechanisms were separate events that occurred at different times after carcinogen treatment. Radiation quality and dose rate affect both characteristics. For example, mice irradiated with fission-spectrum neutrons at dose rates of 1 cGy/min or 1 cGy/day were assessed (Ullrich, 1986). Dysplasias from cells irradiated at high or low dose

rates were similar, but a large fraction of the dysplasias in outgrowths derived from mice irradiated at low dose rate persisted, whether collected at 24 hours or 16 weeks, while those from mice irradiated with a high dose rate did not persist. These data suggest that low-dose-rate neutron exposures enhance the probability of progression of carcinogen-altered cells rather than increase the number of initiated cells.

B.4.3. Tumourigenesis

(B60) The efficiency of radiation-induced tumourigenesis depends on dose and the quality of radiation (Fry et al., 1983). Rats stimulated with high prolactin levels and glucocorticoid deficiency (Prl^{+}/Glc^{-}) for 48 days increased ^{3}H-thymidine uptake by nearly 4-fold, and total mammary clonogens by about 5-fold (Kamiya et al., 1999). Irradiation with 4, 40, and 80 cGy x-rays after hormone stimulation increased total mammary carcinomas per rat-day-at-risk linearly with dose, while a dose of 40 cGy x-rays before hormone treatment yielded tumour frequencies insignificantly different from unirradiated controls. In contrast to results with x-rays, 10 cGy neutrons prior to hormone treatment yielded tumour frequencies and latencies insignificantly different from those for 10 cGy neutrons after hormones. The authors concluded that the carcinogenic action of x-rays, but not of neutrons, was thus influenced by total clonogen numbers and/or their proliferation rates.

(B61) A series of studies in rodents demonstrates the age, dose and radiation quality dependence of mammary carcinogenesis. When 7–8-week-old ACI, F344, Wistar, and Sprague-Dawley rats were evaluated until 1 year of age after irradiation (0.05–2 Gy) with either 290 MeV/u carbon ions with a spread-out Bragg peak (LET 40-90 keV/μm) generated from the Heavy-Ion Medical Accelerator in Chiba compared to ^{137}Cs γ rays. Carbon ions significantly induced mammary carcinomas in Sprague-Dawley rats but less so in other strains. The dose-effect relationship for carcinoma incidence in the Sprague-Dawley rats provided an RBE of 2 at high dose per fraction but was estimated to be 10 at low doses (Imaoka et al., 2007). Metastasis was noted only in animals exposed to high LET radiation.

(B62) Recent studies from Shimada and colleagues support the concept that physiological status is critical factor in determining the subsequent cancer risk (Imaoka et al., 2011). Female Sprague-Dawley rats irradiated at a dose of 0.2 or 1 Gy with either ^{137}Cs γ rays or a 290-MeV/u monoenergetic carbon ions (LET 13 keV/μm) at embryonic days 3, 13, and 17 or 15 weeks after birth did not develop more tumours over a lifetime (90 weeks) compared with the non-irradiated group. However, among the groups of rats irradiated 1, 3 and 7 weeks after birth, similar dose responses (0.2-2.0 Gy) to γ rays were evident. Moreover, the effect of carbon ions increased along with the age at the time of irradiation, indicating RBE values of 0.2 (-0.3, 0.7), 1.3 (1.0, 1.6) and 2.8 (1.8, 3.9) (mean and 95% CI) for animals that were 1, 3, and 7 weeks of age, respectively.

B.4.4. Microenvironmental influences

(B63) The stroma is also a possible target of radiation effects. Epithelial-stromal interactions, mediated by the extracellular matrix, play a pivotal role in normal mammary gland biology (Bissell and Aggeler, 1987). Normal stroma can suppress neoplastic progression (Cooper and Pinkus, 1977; Decosse et al., 1973; Fujii et al., 1982; Kamiya et al., 1995). Specific stromas are induced by breast cancer (Finak et al., 2008). Indeed it has been postulated that cancer can be promoted by an abnormal stroma (reviewed in (Barcellos-Hoff, 1998; Bissell, 2001; Farber, 1984; Tlsty, 1998)).

(B64) Barcellos-Hoff developed a mammary chimera model to test whether radiation effects on the stromal microenvironment contribute to its carcinogenic potential (Barcellos-Hoff and Ravani, 2000; Nguyen et al., 2011). This model consists of surgically removing the endogenous epithelium of the mammary gland at puberty, the mouse irradiated and the gland subsequently transplanted orthotopically with unirradiated, non-malignant epithelial cells. In one set of experiments, host irradiation with a high dose (4 Gy) up to two weeks before transplantation with unirradiated, immortalised mammary epithelial cells, led to rapid formation of large, aggressive tumours even though normal outgrowths formed in non-irradiated hosts (Barcellos-Hoff and Ravani, 2000).

(B65) There are several other examples of such 'non-targeted' radiation effect. A series of experiments from the laboratory of Kaplan in the late 1950's showed that mice irradiated (~7 Gy) and then transplanted with an unirradiated thymus develop thymic lymphoma at the same incidence and latency as intact irradiated mice (Kaplan et al., 1956). An immortal myogenic cell line forms tumours rapidly in irradiated mice but gives rise to normal tissue in unirradiated host muscle (Morgan et al., 2002). Likewise, brain tumours develop in Ptch mutant mice following partial body irradiation (3 Gy) even when the brain is shielded (Mancuso et al., 2008). Together, these studies are evidence for mechanisms of radiation acting via persistent changes in the host or microenvironment.

(B66) A recent study used the radiation chimera model consisting of donor epithelium primed to undergo neoplastic transformation by genetic loss of p53 transplanted to mice irradiated with a low dose (<0.1 Gy) (Nguyen et al., 2011). Syngeneic p53 null mammary gland transplanted to wild-type hosts develops normal ductal outgrowths but undergoes a high frequency of spontaneous transformation. This is evident histologically around 8 months post transplantation as ductal carcinoma *in situ* and genomic instability. By 12 months, most (~60-70%) transplanted glands exhibit palpable tumours that are classified as carcinomas (Medina et al., 2002). In radiation-chimera experiments in which mice were exposed to doses of 0.1-1 Gy, the first tumours were detected at about 170 days post transplantation in both irradiated and non-irradiated hosts. By 300 days, 100% of transplants in hosts irradiated with either 0.1 or 1 Gy had developed tumours compared to 54% of transplants in unirradiated hosts. Tumour development was significantly accelerated by more than two months in hosts exposed to low dose irradiation. Surprisingly, host irradiation significantly increased development of ER-negative tumours. The effect of host irradiation on ER-negative tumour frequency was not associated with the effect of radiation on latency *per se*.

(B67) ER is perhaps the most important clinical marker in breast cancer and is associated with distinct risk factors, pathological features and clinical behaviour (Jensen et al., 2003), but what determines the prevalence of ER negative cancer is not well-understood (Allred and Medina, 2008). ER negative breast cancer is most frequent in young women and certain racial groups, particularly African-American women (Parise et al., 2010). A single study by Castiglioni et al. (2007) revealed that tumours from women exposed to therapeutic radiation for childhood/young adult cancers were more likely to be ER-negative, specifically triple-negative breast cancer, compared to age-matched controls. Broeks et al. (2010) reported that gene expression profiles of radiation-preceded breast cancer were consistent with basal-like intrinsic subtypes, more aggressive, and could be clustered compared to those occurring in women diagnosed at the same age without radiation treatment. Shimada and colleagues reported that irradiation of Sprague-Dawley rats at 3 weeks of age produced carcinomas that were negative for either ER or PR, whereas the majority of carcinomas arising in rats irradiated at 7 weeks (post puberty) were positive for both ER and PR (Imaoka et al., 2011). The observation from Nguyen et al. that irradiated hosts were significantly more likely to give rise to ER- and PR-negative tumours implicates radiation-induced heterotypic signalling

in determining critical clinical features of breast cancer. These data in both experimental models and humans suggest that radiation can not only increase the risk of cancer, but also ultimately affect the type of cancer.

(B68) It has been proposed that breast cancer heterogeneity is determined in part by the cell of origin and its position within the epithelial lineage hierarchy of normal organs (Sell, 2004). A corollary is that the tumours retain fundamental programming that remains evident in the biology, behaviour, and signature of the cancer subtype. Indeed, the expression profiles of isolated MaSCs, which are thought to give rise to luminal progenitor cells that in turn generate mature luminal cells, segregate breast cancers with specific markers and prognoses (Lim et al., 2010). Mouse p53 null tumours are similar to claudin-low breast cancer (Prat et al., 2010) and both are enriched in the MaSC signature (Lim et al., 2010). Moreover, neoplastic transformation in p53 null mammary gland is thought to be enhanced by a propensity for stem cell self-renewal (Cicalese et al., 2009), which is mediated by Notch signalling (Li and Marban, 2010). Notch is preferentially activated in the normal ductal luminal epithelium and promotes commitment of MaSCs *in vivo* (Bouras et al., 2008). Notch nuclear co-localisation was significantly increased by radiation (Nguyen et al., 2011). Thus, radiation could affect stem cell activity by inducing key regulators of mammary self-renewal and lineage commitment. Gene expression profiling was carried out to evaluate the signalling pathways that mediate the communication between cell types after radiation exposure. The tumours arising in the irradiated host have a strong MaSC profile, as does the irradiated mammary gland (Nguyen et al., 2011).

(B69) Together, these data suggested the hypothesis that low dose host irradiation might affect the mammary lineage hierarchy by altering self-renewal in MaSCs. To test this idea, mice were irradiated with graded low doses at 3 weeks of age, and cells isolated from fully mature glands were analysed by FACS using $CD24^{medium}$ $CD49^{high}$ mammary repopulation markers (Shackleton et al., 2006). The proportion of Lin^{-} $CD24^{medium}$ $CD49^{high}$ cells in irradiated mice was significantly increased compared to sham-irradiated mice. The absence of dose dependence indicates that this effect is not mediated by cell kill *per se*. Functional analysis of repopulating potential is the gold standard to assess MaSCs (Purton and Scadden, 2007). Mammary repopulating activity increased nearly 2-fold in mice irradiated at 3 weeks of age compared to sham-irradiated mice, again without evidence of dose dependence between 0.1 and 1 Gy. Thus, low doses of ionising radiation induce a tumour promoting microenvironment by induction of Notch pathway that in turn increases MaSC activity, and correlates with the increased frequency of ER-negative tumours (Arvold et al., 2011).

B.5. Summary and conclusions

(B70) The most striking feature of breast cancer risk following radiation, as compared to other organs, is the strong and consistent age-at-exposure effect (Boice Jr., 2001). Why is there a window of susceptibility during adolescence? Six fundamental alterations in cell physiology underlie cancer progression: self-support in growth signals; insensitivity to growth-inhibitory signals; escape from apoptosis; infinite replication; sustained angiogenesis; and tissue invasion and metastasis (Hanahan and Weinberg, 2000). These same underlying mechanisms that contribute to cancer progression affect cell processes needed for normal mammary development. The biology of breast differs in that the parenchyma is generated post-natal, primarily due to action of ovarian hormones whose action is initiated with puberty. The nascent epithelial bud expands to generate an adult ductal tree that is capable of preparatory cycles of proliferation as a function of the menstrual cycle, and explosive proliferation and differentiation under the hormones of pregnancy. The capacity of the organ

to undergo repeated expansion, differentiation and involution throughout adult life speaks to a huge reservoir of regenerative capacity. This capacity is thought to reside in MaSCs whose self-renewal during puberty leads to their distribution throughout the gland.

(B71) Weissman and colleagues have argued that understanding stem cell biology can provide insight into the origins of cancer (Reya et al., 2001). They propose that the similarities between self-renewal in stem cells and cancer cells could be exploited therapeutically if more was known about the MaSC populations. Low doses of radiation significantly increased the mammary repopulating activity, and could thereby increase the number of target cells that could initiate cancer (Nguyen et al., 2011). Understanding the effect of radiation on the MaSC is likely to provide key insights into physiological and genetic determinants of cancer risk. The data in the radiation-chimera mammary model (Nguyen et al., 2011) suggest that radiation exposure early in life can alter heterotypic interactions, set the stage for stem cell expansion and increase the risk of developing ER-negative breast cancer observed in women treated with radiation for childhood cancers (Castiglioni et al., 2007). A plausible scenario is that radiation elicits a transient change in signalling or a persistent change in the inflammatory, macrophage, or vasculature compartment of the gland. This altered microenvironment in turn permanently alters the pool of mammary epithelial stem/progenitor cells.

(B72) Moreover, gene expression profiling of radiation-preceded tumours is a new avenue for exploring the effects of radiation both on the source cells and host biology. Mao et al. (2005) suggested the hypothesis that specific early events after radiation exposure induce changes in caretaker genes, and the nature of the early events may determine the overall genomic signature observed in the resulting tumour. Consistent with this, analysis of the expression profiles of radiation-preceded breast cancer implied that a large number of these genes are indeed caretakers and gatekeepers (Broeks et al., 2010). The radiation-chimera mouse model suggested that host irradiation alone can ‘imprint’ the tumour with the MaSC signature and shift the biology toward a more aggressive phenotype (Nguyen et al., 2011). This ‘imprint’ or metaprofile can actually cluster radiation-preceded thyroid cancers from spontaneous cancers (Nguyen et al., 2013).

(B73) Stem cell biology has thus provided important insights both in the biology of breast cancer and a growing body of evidence that radiation may affect breast cancer risk by altering tissue composition and stem cell regulation. The rapidly advancing knowledge of critical pathways, as well as definitive stem cell markers in breast in humans and mammary gland in experimental models, will likely provide greater insight into the age dependence of breast cancer risk in humans.

ANNEX C: THYROID STEM CELLS

C.1. Radiation-induced thyroid carcinogenesis

(C1) Ionising radiation is a well-established causative agent for thyroid cancer, especially papillary thyroid carcinoma (PTC), in humans. The relationship between external irradiation and late occurrence of thyroid cancer was reported originally by Duffy and Fitzgerald (1950). Thyroid cancer was the first solid tumour that showed significant increases among A-bomb survivors (Thompson et al., 1994; Wood JW, 1969). Pooled analytical studies of paediatric patients exposed to external radiotherapy demonstrated a strong positive association between radiation exposure and thyroid carcinoma (Ron et al., 2012). Based on the ERR model, a linear dose-response relationship fitted the data well (Ron et al., 2012; Shore, 1992). The elevated risk among aromic bomb survivors has persisted for over several decades after the initial exposure. It began to decline after 30 years, but was still elevated at >50 years (Furukawa et al., 2013; Ron et al., 2012).

(C2) An age-dependent susceptibility to external radiation of the thyroid gland exists. The increased incidence of thyroid cancer was observed among the A-bomb survivors under 20 years of age at exposure (Furukawa et al., 2013; Thompson et al., 1994), whereas the elevated risk was no longer observed among persons exposed after the age of 30 years. Recent analysis using data on cancer incidence during 1958-2005 demonstrated that persons exposed early in life had a very high risk of thyroid cancer (Furukawa et al., 2013; Preston et al., 2007). In the latest A-bomb data (Furukawa et al., 2012), the ERR was estimated to decrease 4.5-fold between ages 10 and 30 at exposure and yet further at older ages. Although one analyst reported that thyroid cancer incidence among females exposed at over the age of 20 was dose-related in the A-bomb study (Richardson, 2009), the most recent data indicate little or no effect from adult exposures. Similarly, a pooled analysis of studies of external radiation exposure and subsequent thyroid cancer reported that there was “little risk apparent after 20 years” (Ron et al., 2012). The RR tended to be greater in women, but the difference between men and women was not statistically significant.

(C3) On 26 April 1986, the nuclear reactor No. 4 at the Chernobyl nuclear power plant released approximately 1,760 PBq of ^{131}I into the environment. More than 90% of the thyroid dose was attributed to ingested ^{131}I, while less than 10% was from short-lived isotopes, such as ^{132}I, ^{133}I and ^{135}I. Individual thyroid doses ranged from a few milligrays to several grays (Ron 2007). A few years after the accident, an increase of thyroid cancer among children in the contaminated area was reported (Prisyazhiuk A, 1991). Subsequently, several studies revealed a substantial increase in thyroid cancer incidence among children exposed in the three affected countries (Abelin et al., 1994; Baverstock et al., 1992; Likhtarev IA, 1995; Stsjazhko et al., 1995; Tsyb et al., 1994). A recent report on a large population-based case-control study included 276 children with thyroid cancer diagnosed between 1992 and 1998 versus 1,300 matched controls (Cardis, 2005). A linear dose-response relationship was observed up to 2 Gy, and an estimated ERR was approximately 4.5 per Gy. The study also demonstrated that the risks were higher in children in iodine-deficient areas. The first cohort study of thyroid cancer following the Chernobyl accident screened 13,127 people in Ukraine who were younger than 18 years of age at the time of the accident. A strong and approximately linear dose-response relationship was demonstrated, which yielded an estimated ERR of 5.25 per Gy (Tronko et al., 2006) for thyroid cancer prevalence. Subsequent rounds of screening found an ERR for thyroid cancer incidence of 1.91 per Gy

(Brenner et al., 2011). A similar dose response was reported from a screening of 11,970 people in Belarus with an ERR per Gy of 2.15 (Zablotska et al., 2011).

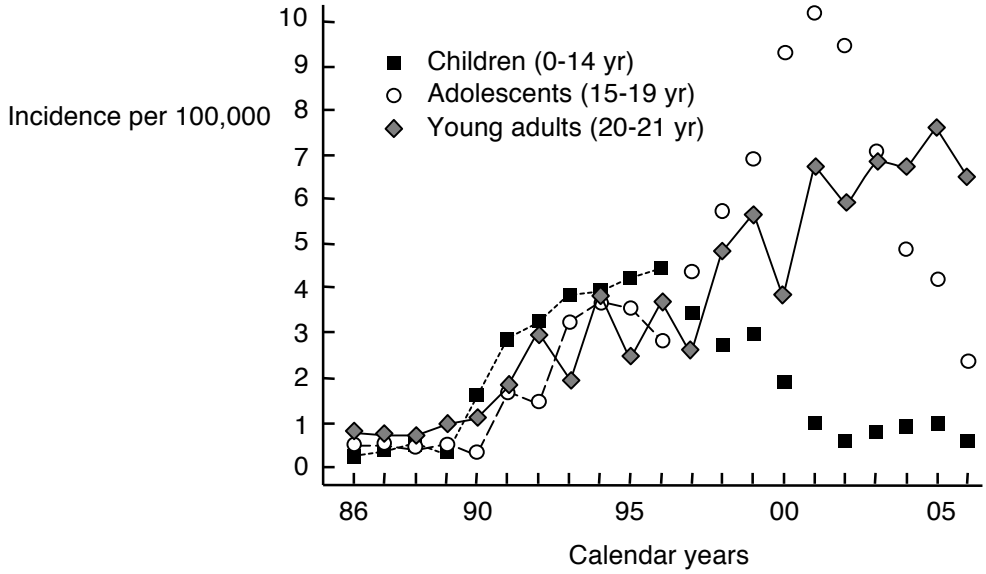

Fig. C.1. Incidence of thyroid cancer in different age groups in Belarus between 1986 and 2006 (Demidchik, 2007). (Permission needed)

(C4) The annual incidence of thyroid cancer, examined in patients of different age groups in Belarus, documented a striking increase in the incidence of thyroid cancers. In children (aged 0-14 years), the incidence of thyroid cancers started increasing 3-4 years after ^{131}I exposure in the presence of population screening (Demidchik, 2007). The cohorts exposed to ^{131}I in childhood or adolescence continued to show excess risk of thyroid cancer as they progressed through adolescence and their 20's and early 30's, whereas those born after 1986 showed no excess thyroid cancer risk.

(C5) Development of thyroid carcinomas after an injection of ^{131}I as well as external exposure to x-rays, was also demonstrated in experimental animals (Goldberg and Chaikoff, 1951; Doniach, 1956; Lee et al., 1982). Both internal irradiation with ^{131}I (0.925-1.48 MBq) and external x rradiation (5-10 Gy) resulted in destructive lesions in the thyroid gland, which in turn induced sequential changes from focal regenerative hyperplasia to benign nodule formation (Lindsay and Chaikoff 1964). Higher doses of ^{131}I such as 7.4 or 14.8 MBq, or 20 Gy of x-rays, almost completely destroyed the thyroid gland, preventing epithelial regeneration (Frantz, 1957; Field, 1959). As the focal hyperplasia and benign nodule formation were dependent on thyroid stimulating hormone (TSH), TSH has been considered to be a promoting factor in development of thyroid carcinoma (Hall, 1948). Cardis et al. (2005) reported that thyroid cancer risk was 3 times higher in areas with deficient dietary iodine. While α-particle radiation was reported to cause degenerative lesions in the thyroid gland (Hamilton, 1950), its carcinogenic effect is unclear.

C.2. Human data on radiation qualities and type of exposure

(C6) Internal radiation exposure from ^{131}I used for diagnostic testing and therapeutic treatments in adults showed no increase in the risk of thyroid cancer, while therapeutic use of ^{131}I in adults with hyperthyroidism suggested a small effect regarding thyroid carcinogenesis

(Hall and Holm 1998; Schneider and Sarne 2005). Prior to the Chernobyl accident, an excess risk of thyroid cancer was observed among children living in the Marshall Islands, where only a small contribution was from ^{131}I (Schneider and Ron, 2000). A study of the children exposed to nuclear fallout from nuclear weapons at the Nevada Test Site demonstrated a small albeit statistically insiginificant excess of thyroid cancer (Kerber, 1993; Lyon et al., 2006). Data from the study of childhood exposure to radioactive iodine released from the Hanford Nuclear site showed no evidence for an increased risk of thyroid neoplasms (Davis, 2004), while a recent report claimed that the results could be due to inadequate statistical power and proposed to interpret the results as inconclusive (Hoffman, 2007).

(C7) Although a pooled analysis has shown that external irradiation with thyroid doses of 0.5 Gy or more increase the risk of human thyroid carcinoma (Ron et al., 1995), there were some studies examining the doses of the order of 0.1 Gy (Ron 1989, Pottern 1990, Shore 1992). The effects of fractionated external radiation were also examined in nuclear workers, radiologists and radiologic technologists (Polednak 1986, Wang 1990, Boice, 1992, Zabel, 2006). Some associations between occupational exposure and thyroid cancer risk were observed; however, the results need confirmation with more accurate historical dose estimation (Zabel, 2006).

(C8) For childhood exposure to external radiation, the pooled ERR per Gy was 7.7 (95% CI: 2.1, 28.7) (Ron et al., 1995). After the Chernobyl accident, most of the thyroid dose was caused by internal exposure. In fact, reconstitution of radiation doses in a case-control study revealed that 92.3% and 84.1% of thyroid exposures were internal radiation from ^{131}I in Belarus and Russia, respectively (Drozdovitch et al., 2010). In the Ukrainian arm of the first cohort study, the ERR per Gy was 5.25 (95% CI: 1.7, 27.5) (Tronko, 2006). The ERR estimate per Gy in the case-control study conducted in Belarus and Russia was 4.5 (95% CI: 1.2, 7.8) (Cardis et al., 2005). Thus, the ERR for external radiation exposure was compatible with the ERR estimates for internal radiation exposure following the Chernobyl accident.

(C9) The thyroid is one of the sensitive organs to radiation regarding carcinogenesis. Studies of children treated with external radiation demonstrated that doses as low as 0.1 Gy could induce thyroid cancer (Shore 1992). The ERR per Gy was highest among children exposed at the age of 0 to 4 years, and it decreased with increasing age (Ron, 1995). Among A-bomb survivors, the risk also decreased at increasing age at exposure (Thompson, 1994). The incidence of thyroid cancer among the population exposed after the Chernobyl accident was inversely correlated with age at exposure in many studies (Saad, 2006). It is generally believed that a higher risk of thyroid cancer from childhood exposure is likely due to a higher proliferative activity of the thyroid in children. Since the proliferation rate of thyroid cells is significantly higher before birth (Table C.1.) (Saad, 2006), it is expected that *in utero* exposure may increase the incidence of thyroid cancer. However, no evidence so far has shown a statistically significant increase, while *in utero* exposure exhibited a statistically significant increase in the incidence rates of solid cancer (Preston, 2008). For example, A-bomb survivors exposed *in utero* showed a similar risk for solid thyroid nodules to that observed in those exposed in childhood (Imaizumi, 2008). Although the information on thyroid cancer risks associated with *in utero* exposure to the Chernobyl fallout is very limited, no significant increase in thyroid cancer was observed 15 years after the accident (Shibata, 2001). A more-recent screening study suggested that *in utero* exposure may have increased the risk of thyroid carcinoma approximately 20 years later (Hatch, 2009); however, the estimate was not statistically significant. Apparently, additional studies are required to clarify the issue of *in utero* exposure (Cardis and Hatch, 2011). So far, possible explanations for the discrepancy is that thyroid stem cell (TSC) proliferation in early fetal life may eliminate damaged cells more efficiently, or repair DNA damage more efficiently, or enhance the ROS-

scavenging system, and the small size of the Chernobyl *in utero* cohorts compared to the postnatal Chernobyl cohorts.

Table C.1. Proliferation rate of thyroid cells (Saad 2006)

Age	Labelling index (%)
Fetal (weeks gestation)	
11~15	16.3
16~20	11.7
21~25	8.1
26~30	7.6
31~35	1.9
36~40	0.4
Paediatric (years)	
0~12	0.07~0.38
13~19	0.11~0.18
Adult (years)	
20~39	0.08~0.11
40~60	0.08~0.09

C.3. General features of the thyroid

C.3.1. Human thyroid development and stem cells

(C10) Mice and humans possess the same set of endocrine organs, as well as the common orthologous factors that have been implicated in the development and maintenance of the thyroid during the embryonal period and after birth (Trueba et al., 2005). In humans, the thyroid is the largest classical endocrine gland weighing about 20~40 g in adults. The thyroid gland is butterfly-shaped and composed of two lobes with an isthmus connecting them. The thyroid is comprised of spherical follicles which contain colloid (Fig. C.2.). The follicles are surrounded by a single layer of thyroid epithelial cells called follicular cells. Colloid is produced by the follicular cells and is rich in thyroglobulin (Tg) protein. Between the spherical follicles is another type of thyroid cells, parafollicular or C cells, which secrete calcitonin. Outside the follicles, there are two other types of cells, which are endothelial cells and fibroblasts.

(C11) Thyroxine (T4) is the hormone synthesised from Tg by thyroid follicular cells. After the sodium/iodide symporter (NIS)-dependent accumulation of iodide into the follicular lumen, iodide is rapidly oxidised by thyroperoxidase (TPO) in the presence of hydrogen peroxide, generated by dual oxidases (Ris-Stalpers, 2006; Sumimoto, 2008), and is attached to tyrosyl residues in Tg, followed by coupling of iodotyrosines to form T4.

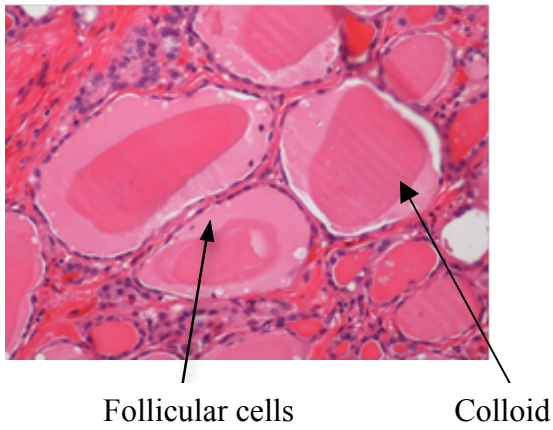

Fig. C.2. Histological structure of thyroid follicles

(C12) Human ES-cell-derived, endoderm-positive cells have been shown to express several early thyroid markers, including thyroid stimulating hormone receptor (TSHR) and paired box gene 8 (Pax8), but not Tg. Therefore, with the restrictions of human ES studies, the main efforts have been directed to the identification of adult TSCs. It has been speculated for a long time that a subpopulation of pluripotent stem cells, present in the inner cell mass of the pre-implantation embryo, may never differentiate; instead, these cells might persist and seed adult tissues (Weissman, 2000). Such cells can generate diverse cell types during tissue renewal in response to environmental signals. Recently, the cells expressing markers of pluripotency have been identified in the thyroid. The steady synthesis of octamer-binding transcription factor 4 (OCT4) mRNA can be detected in a culture of adult primary thyrocytes indicative of the possible presence of cells with an embryonic-like phenotype (Thomas et al., 2006). Oct4 belongs to the family of POU-domain transcription factors abundantly expressed in embryonal tissues, whereas it is downregulated in most adult somatic cells (Pesce and Scholer, 2001; Pesce, 1999). In cultures of adult thyrocytes, the expression of OCT4 mRNA remains stable over several passages without signs of reduction, suggesting the ability of $Oct4^+$ cells for self-renewal and reconstitution of the population after subculturing. Immunohistochemical and FACS analysis also confirmed the existence of $Oct4^+$ cells, which account for 0.2% of the total thyroid cell population. Sorted $Oct4^+$ cells express PAX8, but not TG mRNA, indicating that despite the synthesis of the embryonic marker of pluripotency, the cells have already acquired some degree of differentiation (Thomas et al., 2006).

(C13) In human fetal thyroid, Pax8 is expressed from Carnegie stage (CS) 14, while Tg appears only at CS19 (Trueba et al., 2005). It is therefore possible to speculate that $Oct4^+Pax8^+Tg^-$ cells in adult thyroid may represent the group of embryonic cells, whose maturation is stalled at an embryonic age of CS14-19. These cells may be the candidates for adult TSCs. The $Oct4^+$ cells are not always equal and specific to stem cells because of a mixture of stem cell-derived progenitor cells (Lan, 2007).

(C14) The presence of cells with an embryonic phenotype in the thyroid might represent a situation in which immature cells and adult thyrocytes with “facultative stem-cell properties” coexist in the gland as indicated in the pancreas (Dor, 2004), and both may contribute to tissue homeostasis. In the thyroid, the solid cell nests (SCNs) have been speculated for a long time to be the “niche” and main repository of embryonic cells. However, only by pathological examination, SCNs are considered to represent the embryonic remnants of ultimobranchial bodies (UBBs) and are composed of thyroid transcription factor 1 (TITF-1) positive C-cells

(Cameselle-Teijeiro et al., 1994; Damante et al., 2001; Harach, 1988; Reis-Filho et al., 2003). TITF-1 is a transcription factor, which protects the fetal primordium and UBBs against apoptosis in the embryonal period (Damante et al., 2001; De Felice and Di Lauro, 2004; De Felice et al., 2004; Kusakabe et al., 2006). Once activated at CS14, TITF-1 expression remains for a lifespan in the follicular and C-cells (Ordonez, 2000; Reis-Filho et al., 2003). Among the cells of SCNs, there is a distinct group of so-called "main cells", which show no TITF-1 staining. It has been suggested that the main cells of SCNs could transdifferentiate to follicular cells under certain circumstances (Burstein et al., 2004). The presence of colloid-containing follicles in SCNs of some patients (Harach, 1991) and in mice (Manley and Capecchi, 1995), as well as the high expression of p63 (Preto et al., 2002; Reis-Filho et al., 2003) and telomerase (Burstein et al., 2004; Preto et al., 2004) have been demonstrated in the main cells, highlighting their unique properties. p63 is usually detected in the basal and progenitor layers of many epithelial tissues, such as breast and prostate, where it is responsible for survival of basal layers of epithelium and stem-like properties (Di Como et al., 2002; Preto et al., 2002; Signoretti et al., 2000; Yang et al., 1999). Also, the telomerase elongates the protective caps at the ends of chromosomes (Hiyama et al., 1995a) and can confer increased lifespan without eliciting a cancer-associated phenotype (Jiang et al., 1999; Vaziri and Benchimol, 1998). It may allow the stem cells to overcome replicative senescence (Mathon and Lloyd, 2001; Wynford-Thomas, 2000) and maintain the original DNA matrix in an intact state. Therefore, the co-expression of p63 and telomerase in the main cells of SCNs may reflect a unique role of these cells in the thyroid and point to their stem-like properties. This combination of features gives SCNs strong evidence for being considered as a "residence of stem cells" (Preto et al., 2004; Preto et al., 2002).

C.3.2. Tissue turnover rate

(C15) The turnover rate of normal human thyroid tissue was estimated using *in vitro* labelling experiments. Normal human thyroid tissues were obtained from adult patients in the age 22-56 years with solitary benign nodules or thyroid cysts. The estimated minimal labelling index (LI) was 1.34×10^{-4}. Assuming an S phase of 10 hours, it corresponds to a turnover rate of 8.5 years (Coclet et al., 1989). Estimated turnover rate was between 8.5 and 14.4 years, which implies that the thyroid follicular cells divide approximately 4 or 5 times during adulthood.

(C16) Also, other studies have shown that if the culture medium is supplemented with certain growth factors, the thyrocytes can divide up to 40 times (Curcio et al., 1994), and that entire follicles can be regenerated through the division of a single thyrocyte and assembling of daughter cells (Toda et al., 2003, 2001a, b). The neofolliculogenesis also occurs in inflamed thyroid: in subacute thyroiditis, the progressive inflammation and gradual destruction of parenchyma paralleled partial restoration through the division of differentiated thyrocytes (Toda et al., 2000). Thus, it confirms that the proliferative capacities of thyrocytes themselves might be enough for the turnover in normal and damaged tissue. The thyrocytes acting as facultative stem cells are an attractive hypothesis. In this model, long-lived thyrocytes can maintain the follicles' structure without intermediate progeny, and they also can accumulate multiple genetic alterations until they acquire cancer hallmarks for clonal expansion.

(C17) The number of regenerative cells, which corresponds to either progenitors or stem cells, was assessed by an experiment in which monodispersed rat thyroid follicular cells were transplanted into the fat pads of thyroidectomised rats. The number of clonogens (colony-forming cells) per follicle was calculated to be approximately 3, which represents about 3%

of all follicular cells (Clifton et al., 1978; Watanabe and Hendry, 1991). It has also recently been shown that human thyroid follicles are able to be transplanted into severe-combined immunodeficiency (SCID) mice (Nomura T, 2008). However, the number of clonogens in human thyroid has not yet been determined.

C.3.3. Age and gender specificity of tissue turnover

(C18) From a temporal point of view, the human thyroid primordium starts to bud at CS12, analogous to embryonic day 8 (E8) in the mouse, that is paralleled by the early expression of TITF2 and PAX8 genes during the migration and differentiation of thyroid cells. Once thyroid organogenesis is finished by 12-13 weeks gestation, the thyroid becomes functionally active (De Felice and Di Lauro, 2004). Follicle precursors are detectable, and Tg is secreted into follicular spaces at this stage. Thyroid weight between 95 and 100 days gestation is 80 mg and it becomes 1,430 mg by the time of birth (Table C.2.).

Table C.2. Weight of the thyroid at fetal ages (Fisher, 1974)

Fetal age (days)	Fetal weight (g)	Thyroid weight (mg)
95~100	75	80
105~120	146	131
122~138	203	139
145~165	465	261
166~230	1080	723
Term	3270	1,430

(C19) The highest cell proliferation rate, as judged by Ki-67 nuclear staining, was observed in early fetal life (Saad, 2006). At 11 to 15 weeks gestation, Ki-67 staining was positive in about 16% of cells. Then, it continuously decreased to 0.4% at 36 to 40 weeks gestation. After birth, the proliferation rate was between 0.2 to 0.3% in the paediatric group and close to 0.1% in the adult group, which are comparable to the results obtained by others (Katoh, 1995; Shimizu, 1993).

(C20) At the time of birth in the full-term newborn, an abrupt rise in the serum TSH level occurs within 30 minutes of delivery. This results in a dramatic stimulation of thyroid function. Following the neonatal period, there is a gradual decrease in the T4 production rate. In infants, it is about 5 to 6 mg/kg per day, and over the first few years, it decreases to 2 to 3 mg/kg per day at ages 3 to 9 years. This is in contrast to the rate in adults, which is about 1.5 mg/kg per day. The weight of the thyroid of the newborn is approximately 1 g and it increases about 1 g per year until age 15 years, when it reaches the adult size of about 15 to 20 g. Average weights of thyroid glands in males in the age ranges 20-29 years and 30-69 years were 16.4 g and 18.5 g, respectively. The average weight of the female thyroid gland in the age range 20-69 years was 14.4 g (Pankow, 1985).

(C21) As discussed in the tissue turnover rate section, *in vitro* labelling of human thyroid slices demonstrated the LI of 1.34 x 10^{-4} for normal human follicular cells obtained from adults between 22 to 56 years old, which corresponds to a turnover rate of the order of 8.5 years (Coclet et al., 1989). A different LI was obtained in one 13-year-old subject, 2.49 x 10^{-3}, which corresponds to a turnover rate of the order of 167 days. Higher LIs have also been observed for young rats versus old rats (Sheline, 1969).

C.3.4. Mouse thyroid development and embryonal stem cells

(C22) The mouse is the best-studied animal model for embryonic development of the thyroid gland. During gastrulation, the epiblast, consisting of multipotential cells, generates three embryonal layers: endoderm, mesoderm and ectoderm. Cells within the specified areas of the endoderm respond to inductive signals and differentiate toward a specific lineage, such as pancreatic or hepatic (Fukuda and Kikuchi, 2005; Tam et al., 2003). The formation of the thyroid begins at embryonic day 8, when the thyroid primordium from a ventral outpocketing of the pharynx starts to migrate caudally to join with lateral outpocketings from the fourth pharyngeal pouch, called UBBs. In the mouse, these series of events take place before E14 (De Felice and Di Lauro, 2004; De Felice et al., 1998; De Felice et al., 2004). The functional differentiation of the thyroid gland starts at E14, thus completing organogenesis. From this moment, thyroid-specific genes such as TPO, Tg and TSHR become transcriptionally active, followed by NIS expression and follicle formation at E16 (Postiglione et al., 2002).

(C23) Considering that thyroid parenchyma is composed of the follicular and C-cells, differentiation of thyrocytes induced in the culture may trigger the differentiation of C-cells as well. NeuroD1 is the neuroectodermal transcriptional factor, expressed in C-cells from E15 and known to coordinate the terminal differentiation of neuroendocrine cells by controlling the cell cycle and activating the transcription of hormonal genes (Kameda et al., 2007; Mutoh et al., 1997). In the culture of ES cells, the thyroid differentiation is not associated with the increase of NeuroD1, showing that thyrocytes and C-cells are developmentally dissociated and require distinct factors to enter the differentiation programme, at least under these experimental conditions (Lin et al., 2003).

C.3.5. Cellular features

(C24) Thyroid specific gene expression has been well-studied in the mouse. *In vivo*, Titf2 and Pax8 are involved in the early stages of thyroid formation. During mouse development, Titf2 is expressed within E8-13 and is responsible for functional repression of thyroid-specific genes until migration of the thyrocytes through the mesoderm has been fully completed. The Titf2 knockout mouse develops normal follicular and C-cells, whereas thyroid primordium fails to migrate and remains ectopically attached to the pharynx (De Felice et al., 1998; Zannini et al., 1997). PAX8 is crucial for fetal thyrocytes from E10. In the $PAX8^{-/-}$ mouse, the thyroid is the only organ which cannot develop, while other tissues easily overcome the effects of the gene knockout, indicating its importance for survival of follicular cells (Mansouri et al., 1998; Plachov et al., 1990). In the murine fetal thyroid, both PAX8 and Titf2 are simultaneously expressed only for a short time during E10-13, before the transcriptional activation of NIS, TPO and TSHR (De Felice and Di Lauro, 2004; Japon et al., 1994). In culture, the concerted expression of PAX8, Titf2 and TSHR in embryoid bodies clearly indicates cell differentiation into the thyroid lineage; however, the temporal gene expression in this system is different from the temporal pattern *in vivo*.

(C25) The TSH/TSHR axis regulates the expression of thyroid-specific genes, such as TPO and NIS (Kogai et al., 1997; Levy et al., 1997; Zarrilli et al., 1990), whereas it is not required for the onset of TG expression in mouse fetal thyroid (Postiglione et al., 2002). From this viewpoint, the acquisition of thyro-phenotype by cells of embryoid bodies would probably occur under the control of TSH. The addition of TSH to the growing ES cells not only augments NIS and TPO expression, but also induces the expression of TSHR and PAX8, thus confirming the leading role of TSH in maintaining the thyroid-like phenotype. In this model, and likewise *in vivo*, TSH shows no effect on induction of TG mRNA synthesis in embryoid bodies (Lin et al., 2003). Therefore, despite differences in the temporal pattern of gene

expression in an *in vitro* model, the main mechanisms involved in thyroid differentiation remain highly similar to those occurring *in vivo*.

(C26) The directed differentiation of ES cells toward the thyroid lineage enables us to look at early thyroid progeny and to trace the fate of fetal follicular cells under different conditions. Current approaches in manipulation with mouse embryos allow isolation of ES cells from blastocysts and maintenance of them in an undifferentiated state. When plated on feeder layers of mitotically inactivated fibroblasts, the cells keep their embryonic morphology and abundantly express the stem cell marker Oct4 (Pan et al., 2002; Sun et al., 2006; Tai et al., 2005). However, when appropriately prompted to differentiate, ES cells undergo dramatic changes in gene expression profile and can give rise to the thyrocyte-like cells (Lin et al., 2003). This is seen as a formation of embryoid bodies in the culture plate and the expression of PAX8, NIS and TSHR mRNA at day 6 of differentiation, and the appearance of Titf2, Pax8 and Tshr proteins at day 8. The thyrocyte-like cells protrude from the main cell mass of the embryoid body and tend to form a monolayer of differentiated cells (Lin et al., 2003). In a study to facilitate the tracing of early thyroid cells, ES cells have been genetically modified and the GFP-NEO construct has been introduced to ES cells under the control of a TSHR promoter, therefore allowing visualization and isolation of the early thyroid progeny by cell sorting (Arufe et al., 2006). In genetically modified ES cells, the acquisition of thyroid phenotype by ES cells leads to appearance of GFP-positive cells at day 2 of differentiation. When sorted, GFP fluorescent cells show characteristic features of the thyroid phenotype: they express NIS, TPO and assemble into follicle-like structures. While TSH-directed thyroid cell differentiation from ES cells has been well documented, ES cells exposed to activin A also underwent endoderm differentiation, which is a prerequisite for thyroid differentiation with the aid of TSH and IGF1 (Ma, 2009).

(C27) More recently, recombinant ES cell lines, in which TITF-1 and Pax8 expressions are independently regulated by the addition of doxycyclin, were established (Antonica, 2012). Transient concurrent expression of TITF-1 and Pax-8 efficiently promoted the expression of TSHR, TPO, NIS and TG mRNA. In combination with TSH, ES cell committed thyroid follicular cells gave rise to 3D structures reminiscent of thyroid follicles, which are functional *in vivo* as demonstrated by transplantation into hypothyroid mice.

(C28) The ability to generate and isolate embryonal thyrocytes is a strong impulse for further study of the signals and mechanisms involved in maturation of TSCs. The molecular mechanism of mouse thyroid carcinogenesis should be examined using more sophisticated approaches, especially focussing on age-dependent cell characterisation.

C.4. Radiosensitivity

(C29) The thyroid is very radioresistant regarding dysfunction. Several studies demonstrated that thyroid dysfunction, such as hypothyroidism and thyroiditis, were caused only by high-dose radiation exposure from radioactive iodine therapy and external radiotherapy. For example, acute radiation thyroiditis occurs within 2 weeks after exposure of the thyroid to radioiodines. The symptoms include inflammation and necrosis of some or all cells in the thyroid gland. From several clinical observations, clinically significant acute radiation thyroiditis may not be seen at thyroid doses below approximately 200 Gy from ^{131}I (Maxon and Saenger, 2000). Above this threshold dose, each 100 Gy increment is estimated to develop acute radiation thyroiditis in an additional 5% of persons. Hypothyroidism is a condition caused by insufficient thyroid hormone from the thyroid gland. A linear dose-response relationship was observed between radiation doses to the thyroid from ^{131}I and the

probability of development of hypothyroidism (Maxon, 1977). The lowest dose for developing hypothyroidism was approximately 270 Gy from ^{131}I (Maxon and Saenger, 2000).

(C30) Dormant thyroid cells are very radioresistant, but their radiosensitivity to a proliferative stimulus *in vivo* was determined by transplantation of thyroid cells into the fat pads of thyroidectomised rats. The dose-response curve for the clonogens gave a mean D_0 value of 197 cGy (Mulcahy et al., 1980). This is lower but of the same order as the value of 350 cGy measured by assessing the survival of whole transplanted thyroid follicles (Watanabe and Hendry, 1991). Although the number of clonogens in human thyroid is still to be determined, the thyroid epithelial cells derived from surgically removed thyroid tissues have been cultured *in vitro*, and radiation sensitivity determined by colony forming assay. The mean D_0 dose was estimated to be 0.93-0.94 Gy (Hiraoka et al., 1985; Miller et al., 1987).

C.4.1. Characteristics of damage response at the cell level

(C31) DNA damage checkpoint activation was examined in primary thyroid cells obtained from thyroid tissues, which were taken by thyroidectomy from patients with hyperthyroid diseases. A p53-p21-dependent G_1 checkpoint activation was demonstrated in response to radiation exposure *in vitro* (Namba, 1995). The ATM-dependent DNA-damage checkpoint pathway was active, because phosphorylation of histone H2AX, which is one of the downstream effectors of the ATM pathway, was demonstrated in primary human thyroid cells (Galleani et al., 2009). The γH2AX assay revealed that DNA repair kinetics in primary thyroid cells was comparable to those reported in other normal human cells.

(C32) There is, however, little information available for human TSCs. Recently, it was demonstrated that human thyroid tissues could be successfully maintained in the improved SCID mice up to 2 years (Nomura, 2008). Histological features of human thyroids in improved SCID mice were well preserved. After irradiation, follicles disappeared and thyroid hormone secretion was dramatically decreased in a dose-dependent manner. 3D-reconstituted follicles were demonstrated from thyroid cells embedded in collagen (Toda et al., 2001a). These approaches could be applicable for mimicking thyroid folliculogenesis *in vivo*.

C.5. Mutagenesis

(C33) Oncogenic mutations found in thyroid cancer are different depending upon the patients' age at exposure and diagnosis, clinicopathological manifestations, and the individual's genetic characteristics. According to the current histological classification, papillary, follicular, medullary, and undifferentiated carcinomas are the major diagnostic types of malignant thyroid tumours. Among them, PTC is the most prevailing type of thyroid cancer in both children and adults (Table C.3.) (Ciampi and Nikiforov, 2007; Fagin and Mitsiades, 2008; Yamashita, 2007).

(C34) Abnormal thyroid follicles with reversed polarity have been induced by radiation (Watanabe and Hendry, 1994). These were produced by irradiating rat thyroids *in vivo* to 5.5 Gy, disaggregating the follicles, injecting the cells into fat pads of thyroidectomised recipients, and scoring the frequency of abnormal follicular structures at 6 weeks later. These dysplastic follicular structures imply the induction of stable mutants, reminiscent of the radiation-induced ductal dysplasias produced in a similar mammary tissue model (Annex B) and dysplastic crypts induced in the colon (Annex D).

Table C.3. Histological types of human thyroid cancer (Yamashita and Saenko, 2007).

Tumour types	Prevalence (%)

	Children and adolescents	Adults
Papillary carcinoma	67~98	85~90
Follicular carcinoma	4~23	<10
Medullary carcinoma	2~8; 17	3
Poorly differentiated and undifferentiated carcinoma	<0.1	2-7

Table C.4. Prevalence of genetic alterations in human thyroid cancer (Yamashita, 2007).

Molecular alterations	Prevalence (%)	
	Children and adolescents	Adults
Gene rearrangements:		
RET/PTC	38~87	0~35
NTRK1	5~11	5~13
AKAP9-BRAF	11	1
PAX8-PPARG	Unknown	0~50
Point mutations:		
BRAF	0~16	25~69
RAS family	0~6	0~43
GNAS	0	~11
p53	0~23	0~20

(C35) Gene rearrangements are the most prevailing genetic alterations in childhood PTC (Fagin, 2004; Fenton et al., 2000; Nikiforov, 1997) (Table C.4.). Fusions of the RET proto-oncogene with several partner genes, which collectively been designated RET/PTC (Fusco et al., 1987; Grieco et al., 1990), are the most frequent gene rearrangement in PTC in children. The RET gene encodes protein for a membrane receptor tyrosine kinase (RTK). It consists of three functional domains: an extracellular ligand-binding domain, a transmembrane domain, and a tyrosine kinase domain. Binding of the ligands, growth factors belonging to the glial cell line-derived neutrophic factor family, causes receptor dimerisation, autophosphorylation of the intracellular domain, and activation of tyrosine kinase activity. The partner gene products are highly expressed in thyroid follicular cells, and they possess coiled-coil domains that enable homodimerisation of RET/PTC proteins. As a result, RET/PTC proteins are activated independently of the ligand. Other types of rearrangements include juxtaposition of the A kinase anchor protein 9 gene (AKAP9) and v-raf murine sarcoma viral oncogene homologue B1 (BRAF), designated AKAP9-BRAF, and rearrangement of the neurotrophic tyrosine kinase receptor type 1 (NTRK1) gene (Table C.4.).

(C36) An age-dependent disproportion in the prevalence of gene rearrangements and point mutations is demonstrated in childhood and adult PTC (Yamashita, 2007). In contrast to RET/PTC rearrangement, point mutations are rather infrequent events in childhood thyroid carcinoma irrespective of radiation exposure (Table C.5.). For example, a point mutation in codon 600 of the BRAF gene is the most prevailing mutation in adult PTC (Xing, 2005). However, it is quite uncommon in paediatric patients (Kumagai et al., 2004; Lima, 2004; Nikiforova, 2004).

Table C.5. Prevalence of BRAF mutation and RET/PTC rearrangements in PTC (Xing, 2005).

Number of cases/Total (%)			
BRAF mutation		RET/PTC rearrangements	
Radiation-exposed	Non-exposed	Radiation-exposed	Non-exposed
7/109 (6)	2/55 (4)	92/175 (53)	29/56 (52)

(C37) After the Chernobyl accident, the highest risk for radiation-induced thyroid cancer was observed among children exposed at the age of 0-4 years. The childhood PTC showed a significantly higher prevalence of gene rearrangement than later-onset tumours. In particular, RET/PTC3 and less frequently RET/PTC1 rearrangements were predominant in PTC in paediatric patients (Fugazzola et al., 1995; Klugbauer, 1995; Nikiforov, 1997; Williams, 2009). Direct connection between radiation exposure and induction of thyroid tumours suggests that radiation-induced mutagenesis is likely involved in tumour development. In A-bomb survivors, RET/PTC rearrangements have been correlated with the radiation dose (Hamatani et al., 2008); however, a similar correlation was not found in Russia after the Chernobyl accident (Tuttle, 2008). *In vitro* experiments using primary thyroid tissue transplanted into SCID mice demonstrated radiation-induced RET/PTC rearrangements (Ito and Cotsarelis, 2008; Mizuno, 2000). Chromosomal loci participating in RET/PTC1 rearrangement have been shown to be in spatial proximity in human interphase thyrocytes, which possibly facilitates the rearrangements by radiation exposure (Nikiforova, 2000). Thus, while radiation exposure is a potent inducer for gene rearrangements, the RET/PTC rearrangements observed in childhood thyroid cancers after the Chernobyl accident showed less evidence of a common radiation signature. Therefore, they may not be a direct consequence of radiation exposure, rather it augments the frequency of the same events occurring in sporadic cases.

(C38) In relation to mutagenesis, another unique characteristic of the thyroid gland is the evidence that thyrocytes continuously generate ROS, particularly hydrogen peroxide (H_2O_2), in addition to those ROS produced by mitochondrial aerobic respiration (Krohn et al., 2007). Hydrogen peroxide is a ROS that is a key component of hormonogenesis, and it is generated by dual oxidases (DUOX1/2), also known as thyroid oxidase (ThOX1/2) (Sumimoto, 2008). High concentrations of H_2O_2 together with oxidised iodine, which is converted from iodide via a reaction catalysed by TPO, are required for iodination of tyrosine residues on Tg. A variety of ROS defense systems are upregulated in thyrocytes, although there are studies showing that excess H_2O_2 levels could be hazardous for the genome of thyroid cells (Poncin, 2009). For example, an antibody against 8-oxo-2'-deoxyguanosine (8-OHdG), one of the well-known DNA adducts, showed the most intensive staining in the follicular cells around the lumen, where H_2O_2 is generated (Maier, 2006). Such oxidative stress is likely to be involved in spontaneous DNA damage induction, which possibly results in the elevation of the spontaneous mutation rate demonstrated in the thyroid gland from LacZ transgenic mice (Maier, 2006). Thus, although there is no study showing a direct connection between elevated ROS level and BRAF mutations so far, oxidative stress during hormone production could be a cause of mutagenesis in thyroid cells (Krohn et al., 2007).

(C39) While several studies have described RET/PTC rearrangements in childhood thyroid cancer after the Chernobyl accident, the principal difference in terms of molecular events between sporadic and radiation-induced thyroid cancers needs to be determined in future studies. The molecular analysis of the genomic sequence of RET/PTC fusion point found evidence for the use of NHEJ to repair radiation-induced DSBs (Gandhi et al., 2010). However, this would not be sufficient enough to identify the role of radiation in RET/PTC

rearrangements. Furthermore, the most common point mutation in the *BRAF* gene, although it is not the major event in childhood thyroid cancer after the Chernobyl accident, involves T:A to A:T transversion, which could not be the transversion induced by oxidative DNA damage, such as 8-oxo-guanine (Loon et al., 2010; Nakabeppu, 2006). Thus, at the present time, it is improbable to identify radiation signatures in RET/PTC rearrangements and in BRAF mutation. Since the mutation type corresponds more to age-of-onset of thyroid cancer rather than radiation, the role of radiation for the induction of oncogenic mutation remains to be elucidated.

C.6. Summary

(C40) *Fundamental knowledge of thyroid stem cells:* The term "stem cell" is widely used to describe a cell capable of both prolonged self-renewal and differentiating into one or more functional cell types. Their property of symmetrical cell division is a critical phenomenon. So far, there is no definite marker to identify thyroid-specific stem cells from embryonic as well as adult thyroid glands. The thyroid gland, despite being an epithelial organ, is believed to show an extremely low rate of self-renewal *in vivo*, and primary thyrocytes barely proliferate *in vitro*. The neonates and children, however, have a growth capacity for every organ in the body, including the thyroid gland. Preliminary data also suggest that primary cultured human thyroid cells contain "proliferative follicular cells" in certain culture conditions (Suzuki, 2011). Furthermore, goitorous thyroid glands, either simple or multinodular, or benign or malignant, may have relatively a large number of progenitor or stem-cell-like thyroid cells. This evidence suggests a different capacity and reaction between neonate/childhood and adult thyroid cells.

(C41) Recent review articles provide some hints and insights into the pure isolation of TSCs (Lin, 2007; Thomas, 2008). However, the niche or microenvironment of TSCs, including the time and spatial effects of paracrine factors, are unknown or ignored in TSC research *in vitro*. Specific culture conditions with EGF and bFGF are suitable for obtaining thyroid spheroids (Lan et al., 2007), which are similar in morphological appearance and long-term culture characteristics to neurospheres (Fierabracci et al., 2008). These thyrosphere cell lines may be a good tool for further TSC research. Their purification and analysis of interactions between signalling pathways probably would be the main future direction in TSC research.

(C42) *Questions from human thyroid carcinogenesis by radiation*: In view of a rapid increase of childhood thyroid cancers after the Chernobyl accident (Demidchik et al., 2007), identification of the effect of radiation on TSCs is being sought (Yamashita, 2007). Several reports implied that accidental or medical radiation exposures are likely cause the RET gene rearrangements (Bounacer et al., 1997; Fugazzola et al., 1995; Klugbauer, 1995). *In vitro* studies have confirmed that radiation is able to induce RET/PTC rearrangements (Ito et al., 1993b; Mizuno, 2000). However, such RET/PTC rearrangements are observed not only in thyroid cancer from radiation exposed patients, but also those occurring in unexposed thyroids (Yamashita et al., 2007). The principal difference in terms of molecular events between sporadic and radiation-induced thyroid cancers is not yet clear. Therefore, it is still questionable whether exposures to relevant doses of ionising radiation are the primary cause for the RET/PTC rearrangements. Since the mutation type corresponds more to age-of-onset of thyroid cancer rather than radiation, the role of radiation for the induction of oncogenic mutation remains to be elucidated. In this regard, a critical question is whether or not the late-occurring thyroid cancers of the childhood-exposed population in the Chernobyl area carry BRAF mutations.

(C43) The characteristics of the thyroid gland and its radiation response described in this Annex are summarised in Table C.6.

Table C.6. Characteristics of the thyroid gland

Stem cells and other cells	The human thyroid primordium starts to bud at Carnegie stage (CS) 12, and Pax8, a marker for early thyroid, is expressed from CS14, indicating that thyroid-committed stem cells already exists by CS12. In adults, *in vitro* labelling of human thyroid slices demonstrated the LI of 1.34×10^{-4}, which corresponds to 0.2% of Oct-4 positive cells in thyroid tissue. No such information is available for newborn, infant, adolescent, and other aged periods.
Turnover rate	From *in vitro* labelling experiments, and assuming an S phase of 10 hours, a tissue turnover rate is estimated to be between 8.5 and 14.4 years. The same labelling experiments demonstrated that a subject from a 13 year-old patient showed a turnover rate of the order of 167 days.
Tissue architecture	Thyroid organogenesis is finished by 12-13 weeks gestation, and the thyroid becomes functionally active. Follicle precursors are detectable, and Tg is secreted into follicular spaces at this stage. The solid cell nest could be a niche for TSCs.
Cellular radiosensitivity	D_0 values of 0.9-3.5 Gy have been reported for thyroid clonogenic cells/regenerative cells. No reports have compared the radiosensitivity of stem and progenitor cells.
Susceptibility to radiation carcinogenesis	The ERR of thyroid cancer is 1.28 per Gy at age 60 years after exposure at age 10 years. An age-dependent decrease in the ERR is clear. There is little cancer risk for those exposed after the age of 20.

ANNEX D: DIGESTIVE TRACT STEM CELLS

D.1. Radiation induced cancer in the digestive tract

(D1) Evidence that ionising radiation causes cancer of the GI tract in humans has been reviewed (Boice and Fry, 1995; UNSCEAR, 2000, 2008). The evidence comes from epidemiological studies of the Japanese A-bomb survivors (Ron et al., 1994; Thompson et al., 1994), patients given radiotherapy for cervical cancer (Boice et al., 1987; Boice et al., 1985; Boice et al., 1988), and patients treated with radiation for benign conditions such as ankylosing spondylitis (Darby et al., 1987), gynaecological disorders (Darby et al., 1994; Inskip et al., 1990), and peptic ulcer (Griem et al., 1994). The studies of A-bomb survivors have followed subjects prospectively for over 40 years at the level of mortality (Davis et al., 1989; Shimizu et al., 1990) as well as cancer incidence (Thompson et al., 1994; Ozasa et al., 2012). The average whole-body dose was relatively low, approximately 0.23 Gy, but the dose distribution ranged up to 4 Gy, permitting dose–response evaluations. This is the single most informative study on radiation risks to date, including exposures of both men and women, and children and adults. Its major limitation is that risks apply to acute brief radiation exposures, and is not directly applicable to the chronic or periodic exposures experienced in daily life from environmental, occupational, or medical sources.

(D2) The risk of radiation-induced cancer in the mouth and small intestine is vastly smaller than in the other regions of the alimentary tract, and they are not given specific values of tissue weighting factor but are included in remainder tissues (ICRP, 2007a). This section of the report will focus on oesophagus, stomach and colon/rectum.

(D3) The study of patients irradiated for ankylosing spondylitis was the only study of medically exposed populations to report a significant risk of radiation-associated oesophageal cancer. These patients received the highest estimated dose of 4 Gy, and were at a significantly increased risk of mortality in comparison with the general population. The RR at 1 Gy was estimated as 1.3 (Darby et al., 1987) and AR was estimated as 0.21×10^{-4} PY/Gy. There was some association of radiation exposure and oesophageal cancer in the LSS. Since oesophageal cancer is relatively infrequent, there was insufficient statistical sensitivity to detect an excess in the several low-dose occupational studies. There are insufficient data to characterise the shape of the dose-response curve (UNSCEAR, 2008). Information on the distribution of cancers arising in the oesophagus suggests that the greatest proportions are seen in the distal region.

(D4) Stomach cancer accounted for 16% of the total excess cancer attributable to radiation in A-bomb survivors (Thompson et al., 1994). The ERR of fatal stomach cancer at 1 Gy was estimated from the A-bomb studies as 0.65 in females and 0.2 in males, with EAR of 3.3 in females and 2.1×10^{-4} PY/Gy (Preston et al., 2003b). The ERR per Gy declined substantially with increasing age at exposure, but declined very little with increasing attained age. Significant excess occurred in two other series: cervical cancer patients (Boice et al., 1988) and peptic ulcer patients (Carr et al., 2002). In the US peptic ulcer study, the ERR per Gy was 0.20 (95% CI: 0, 0.73) based on 11 stomach cancer deaths in 309 patients irradiated with doses of $\leq$10 Gy.

(D5) In general, with whole body exposures, large regional differences in dose between different parts of the stomach would not be expected, and in that scenario, it is reasonable to calculate an average dose. The target was taken to be the stem cell zone, treated as a continuous uniform layer in the stomach wall (ICRP, 2007).

(D6) The small intestine is very resistant to cancer induction by radiation. There are a few individual medical studies but there are no reliable estimates of ERR (UNSCEAR, 2008).

(D7) Colon cancer has occurred in excess in most irradiated populations, with the notable exception of cervical cancer patients (Thompson et al., 1994). Conceivably, at the cytotoxic doses used to treat cancer of the uterine cervix, cell killing prevented significant cell transformation (Boice et al., 1987). Estimates of RR at 1 Gy ranged from 1.13 to 1.67, and the AR ranged from 0.45 x 10^{-4} PY/Gy to 2.18 x 10^{-4} PY/Gy.

(D8) Colon cancer incidence was calculated for 1950–1980 in the A-bomb LSS tumour cancer registry. Very similar linear dose-response coefficients were obtained for cancers located in the caecum and ascending colon (ERR per Sv = 0.80, 90% CI: 0.07, 1.96), transverse and descending colon (ERR per Sv = 1.09, 90% CI: 0.17, 2.59), and sigmoid colon (ERR per Sv = 0.96, 90% CI: 0.33, 1.87) (Nakatsuka et al., 1992). There was a significant decrease in ERR per Sv by age at exposure. In the latest follow-up of the LSS cohort for colon cancer mortality during the period 1950–1997, the estimate for ERR per Gy based on a linear model, for exposure at age 30 with no assumed variation by attained age, was 0.54 (90% CI: 0.13, 1.2) for males and 0.49 (90% CI: 0.11, 1.1) for females, with a 25% decrease per decade of age at exposure (Preston et al., 2003c). UNSCEAR (2000) concluded that there was strong evidence of an ionising radiation effect on colon cancer risks that was consistent with a linear dose response, and this was reconfirmed more recently (UNSCEAR, 2008).

(D9) In the analysis of cancer mortality among A-bomb survivors (Preston et al., 2003b), rectal cancer mortality was not associated with radiation dose among men. However it was positively and significantly associated with dose among women (ERR per Gy = 0.75, 90% CI: 0.16, 1.6), for exposure at age 30 years. Increased rectal cancer incidence has also been observed among cervix (Kleinerman et al., 1995) and prostate cancer patients (Baxter et al., 2005) given radiotherapy. UNSCEAR (2008) concluded that it was difficult to characterise radiation-related risk of rectal cancer at doses under about 1 Gy, but it seemed reasonably clear that there was a radiation-related excess risk for rectal doses of tens of Gy.

D.1.1. Data for internal exposure

(D10) Very limited information is available on cancer induction in the alimentary tract by ingested radionuclides. Tumours induced by internal irradiation have been observed in the intestinal tract of rodents after administration of ^{137}Cs, ^{95}Nb, ^{144}Ce, ^{90}Y, and ^{106}Ru (Casarett, 1973). Intestinal polyps in dogs and rats fed ^{210}Po or ^{144}Ce were also reported, mostly occurring in the large intestine, with a tendency to malignant change and showing different latencies in the two species (Lebedeva, 1973). UNSCEAR (2000) reviewed the sparse evidence from human follow-up studies.

(D11) For the purposes of the ICRP report on the human alimentary tract (ICRP, 2007), ingested radionuclides were assumed to deliver dose uniformly during their transit through the oesophagus. Dose was averaged for the oesophagus assuming a uniform distribution of target cells throughout the basal layer of the stratified squamous epithelium.

(D12) UNSCEAR (2000) reviewed data for stomach cancer in patients treated with ^{131}I for hyperthyroidism. A significant excess in terms of incidence and mortality was reported in a Swedish study, with risks consistent with the estimate from the A-bomb study. However, UNSCEAR (2000) cautioned that because of the small numbers of stomach cancers and uncertainties in the risk estimate for ^{131}I exposure, it was not possible to draw conclusions about the relative effect of acute external and protracted internal radiation exposures.

(D13) Stomach cancer was shown to be significantly increased in radon-exposed cohorts of underground miners (Lo et al., 1995). However, there was no trend in stomach cancer

mortality with the low levels of cumulative radon exposure to the stomach, and excesses of stomach cancer have been reported for other groups of miners. This suggests that factors other than radon exposure were responsible in each case. Female radium dial painters starting work after 1930 showed an increase in stomach cancer mortality (Stebbings et al., 1984), but those starting work before 1930, with generally higher radium exposure, did not show an increased risk. These data do not provide convincing evidence of stomach cancer induction by α-emitting radionuclides.

(D14) Concerning internal exposures to low-LET (^{131}I) and high-LET radiation (radon and radium), UNSCEAR (2000) noted that the low doses to the colon did not allow conclusions to be drawn

D.2. General features of the digestive tract

(D15) The length of the oesophagus, from the pharynx to the stomach, is typically in the range of 23–30 cm in adult males and 20–26 cm in adult females (ICRP, 2002). Autopsy measurements on infants and children indicate an oesophageal length of approximately 8–10 cm at birth, 12 cm at 1 year, 18 cm at 10 years, and 19 cm at 15 years (ICRP, 1975). The similarity in the values for 10- and 15-year-old children is inconsistent with the rate of growth of the upper body during that period, and suggests that the subjects may not have been representative.

(D16) The length of the oesophagus has been determined in a number of modern studies by external imaging techniques. Reported values vary considerably, apparently due mainly to differences in the definition of 'oesophageal length' in medical studies and, to a lesser extent, to intersubject variability. In a study of 51 normal adults (27 males and 24 females) from the US, oesophageal length, defined as the average distance from the proximal end of the upper oesophageal sphincter and the distal end of the lower oesophageal sphincter, was given as 28.3 ± 2.4 cm (Awad et al., 1999). On the basis of these data, values for the length of the oesophagus were given in *Publication 89* (ICRP, 2002) as shown in Table D.1.

Table D.1. Reference values for length of the oesophagus (cm)

Newborn	1 year	5 years	10 years	15 years		adult	
				male	female	male	female
10	13	18	23	27	26	28	26

ICRP *Publication 89*, 2002.

(D17) The oesophageal wall of the adult is approximately 3.5–5.6 mm thick (ICRP, 1975). The stratified squamous epithelium lining the oesophagus, illustrated in Fig. D.1, is taken to be 200 µm thick in the adult. The target layer is assumed to be at a depth of 190–200 µm. The oesophageal wall of the newborn is thinner than that of the adult, but the epithelium thickens rapidly after birth, and the depth of the target layer is assumed to be independent of age for the purpose of this report. The presence of a layer of mucus on the lumenal surface of the oesophagus has been ignored. The thickness of the mucus layer, of perhaps 10–30 µm, is within the range of uncertainties in the overall average depth of the basal cell target layer. The epithelial lining of the oesophagus is a thick layer of protective tissue, many cells deep, classed as non-keratinised stratified squamous epithelium (Fig. D.1.). The basal layer contains stem cells and proliferative cells. The diameters of the oesophagus were given in *Publication 100* (ICRP, 2007) as shown in Table D.2.

Table D.2. Assumed values for the internal diameter of the oesophagus (cm) (ICRP, 2007).

Newborn	1 year	5 years	10 years	15 years	Adult
0.5	0.6	0.7	0.8	1	1

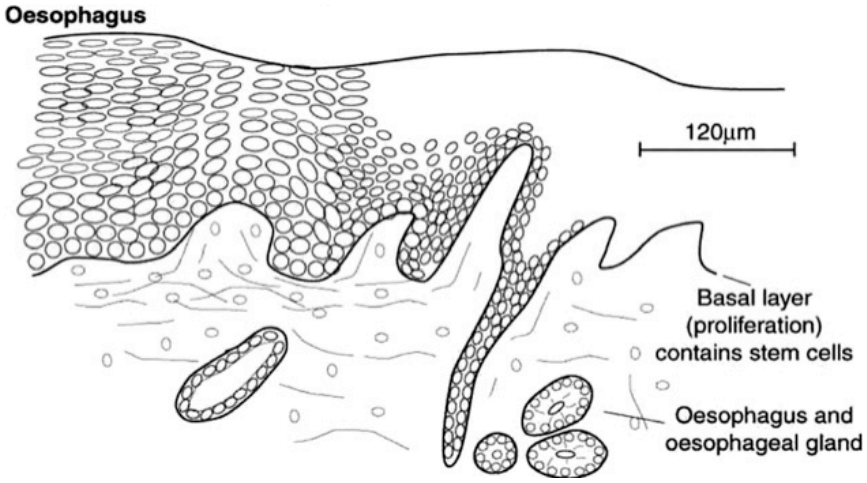

Fig. D.1. Illustration of the cross-sectional structure and dimensions of the human oesophageal stratified squamous epithelium (ICRP, 2007). Courtesy of Chris Potten, Epistem Ltd, UK.

(D18) The stomach is highly variable in volume, being capable of distension to accommodate a large amount of ingested material. It is connected to the oesophagus at the cardiac sphincter and to the small intestine at the pyloric sphincter. The stomach wall exhibits numerous longitudinal folds, or rugae, when the organ is not distended with food. For the purpose of this report, it was assumed that the stomach is a simple sphere of volume 175 cm^3 in adults. Volumes at different ages (Table D.3.) were based on values of mucosal surface area given in *Publication 23* (ICRP, 1975). To calculate age-dependent volumes, a constant relationship was assumed between measured mucosal areas and the surface area of the reference spherical stomach (volume 175 cm^3).

(D19) The gastric epithelium is a single layer of cells, continuous with the basal layer of the stratified epithelium of the oesophagus. The lining of the stomach is indented by numerous pits that supply several million tubular glands. The glands are divided into three categories: the cardiac glands occur in the first 5–40 mm from the cardiac orifice; the pyloric glands occur near the intestine; and the gastric glands lie between these two regions. The cells of the cardiac and pyloric glands all appear to be of the mucous type. The epithelium of the gastric glands is more diversified, containing enzyme- and acid-secreting cells as well as mucous cells.

(D20) The gastric epithelium is a single layer of cells, a simple or unilaminar epithelium, which lines numerous glandular indentations into the stomach wall (the gastric pits) (Fig. D.2.). Differentiated epithelial cells within the gastric pits secrete hydrochloric acid (oxyntic cells), digestive enzymes (zymogen cells), and mucous. The stem cells are assumed to be towards the neck of the gastric pit at approximately one-third of the total pit depth (Fig. D.2).

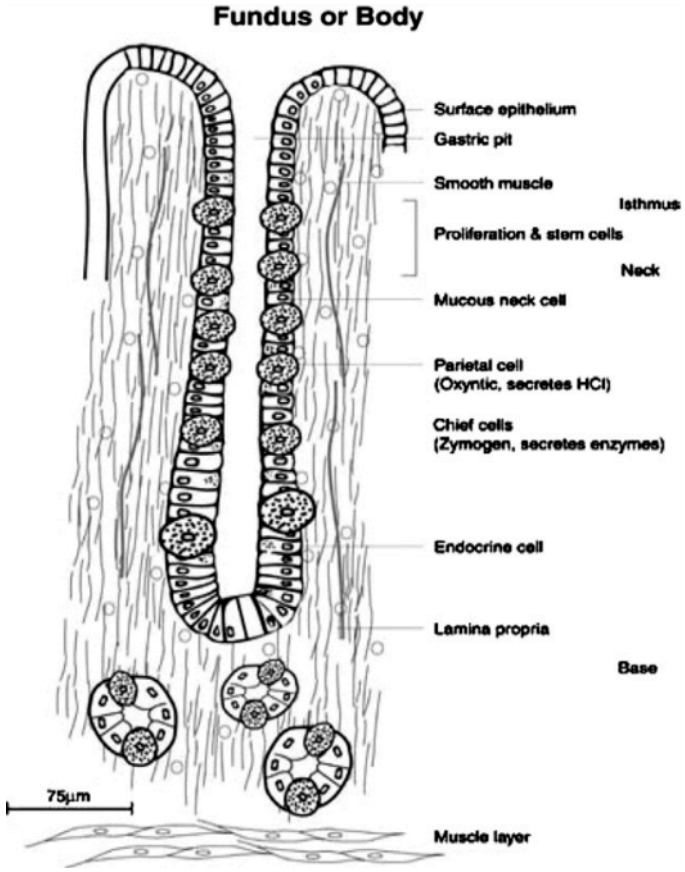

Fig. D.2. Illustration of the cross-sectional structure and dimensions of a typical gastric gland in the human stomach, lined with a single layer of columnar epithelial cells (ICRP, 2007). Courtesy of Chris Potten, Epistem Ltd, UK. (permission needed)

Table D.3. Values for the volume of the stomach (cm^3)[a]. Taken from ICRP *Publication 100* (2006).

Newborn	1 year	5 years	10 years	15 years	Adult
30	40	60	80	120	175

[a] Calculated using rounded values for mucosal surface area based on data given in ICRP *Publication 23* (ICRP, 1975): 150 cm^2 at 3 months (applied here to newborn), 200 cm^2 at 1 year, 250 cm^2 at 5 years, 300 cm^2 at 10 years, 400 cm^2 at 15 years, and 525 cm^2 in adults.

(D21) The mucosal lining of the stomach is of variable thickness. However, for dosimetric purposes, in situations of retention of radionuclides in the stomach wall, the source region is taken to extend to a depth of 300 µm into the mucosa. Fig. D.2. illustrates the cross-section of a typical gastric gland, showing that the proliferative cells, and target stem cells, are thought to be towards the upper lumenal ends of the glands. A target depth of 60–100 µm is assumed to apply at all ages as a uniform target at this depth over the entire inner surface of the stomach.

(D22) Data on the large intestine, particularly data related to the motility of the lumenal contents, are often reported in terms of the right colon, left colon, and rectosigmoid. The right colon is defined as the ascending colon, including the caecum, plus the proximal half of the transverse colon. The left colon is defined as the distal half of the transverse colon plus the descending colon. The rectosigmoid is defined as the sigmoid colon plus the rectum.

(D23) Central estimates for the physiological length of the large intestine of newborns and adults are approximately 45 cm (range 20–70 cm) and 110 cm (range 91–125 cm), respectively (ICRP, 1975). These central estimates are used in ICRP *Publication 89* (2002) as reference values for newborn infants and adults. Values for children aged 1–15 years are based on the assumption that the length of the large intestine is linearly related to body height (see Table D.4.).

(D24) The diameter of the large intestine varies along its length, reducing from caecum to rectosigmoid. The values used in this report are given in Table D.5., based on data reviewed in ICRP *Publication 23* (1975). As for the small intestine, it is assumed that the internal diameters of the regions in infants are one-half of the values used for adults, and intermediate values are used for 1–10-year-old children.

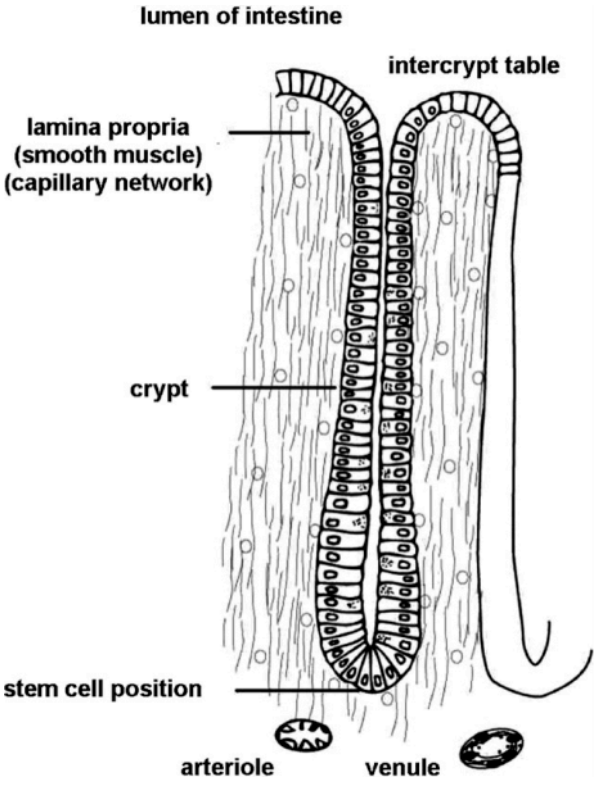

Fig. D.3. Illustration of the cross-sectional structure of the epithelial lining of the human large intestine, showing crypt and stem cell position (ICRP, 2007). Courtesy of Chris Potten, Epistem Ltd, UK.)

(D25) In the large intestine, deep, straight crypts penetrate into the lamina propria from a lumenal intercryptal plate; there are no villi. The stem cells are in the base of the crypts; there are no Paneth cells as found in the small intestine. Cell division leads to a constant flow of cells up the crypts, with loss of cells in the large intestine from the intercryptal plate. It has been reported that there are about 82 cells per crypt column, about 46 cells per crypt circumference, about 2,250 cells per crypt, with >5-9 transit cell generations (Potten, 1995).

(D26) Potten (see ICRP 100, 2006) measured crypt depths of 311 ± 9 µm for the ascending colon (48 crypts from one adult), 358 ± 16 µm for the sigmoid colon (28 crypts from three adults), and 245 ± 10 µm for the rectum. It was assumed here that the target layer is at a depth of 280–300 µm at all ages. As for the small intestine, the target cells were taken to

form a continuous layer at this depth in a cylindrical tube (see Fig. D.3.). In situations where retention of radionuclides in the wall of the large intestine is considered, distribution is assumed to be uniform within the mucosa to a depth of 300 μm from the lumenal surface.

(D27) The rectum is divided into an upper region and the anal canal. In the anal canal, the epithelium changes abruptly from simple columnar to stratified. The rectum is treated here as part of the rectosigmoid for dosimetric purposes.

Table D.4. Reference values for the physiological length of the large intestine (cm) (ICRP, 2007).

Segment	Newborn	1 year	5 years	10 years	15 years		adult	
					Male	female	male	female
Right colon	14	18	23	28	30	30	34	30
Left colon	16	21	26	31	35	35	38	35
Rectosigmoid	15	21	26	31	35	35	38	35
Total length	45	60	75	90	100	100	110	100

Table D.5. Assumed values for the internal diameter of the large intestine (cm) (ICRP, 2007).

Segment	Newborn	1 year	5 years	10 years	15 years	Adult
Right colon	3	4	4.5	5	6	6
Left colon	2.5	3	3.5	4	5	5
Rectosigmoid	1.5	2	2.3	2.5	3	3

D.2.1. Cell kinetics

(D28) Stem cells in stratified squamous epithelium, as in the lining of the mouth, the tongue, and the oesophagus, are taken to be located in the basal cell layer, adjacent to the basement membrane, as assumed for skin (ICRP, 1992). In the stomach, stem cells are thought to be located towards the upper regions of the gastric pits, renewing the epithelial layer by both upward flow of daughter cells towards the luminal surface of the stomach and downward flow into the gastric glands (Karam et al., 2003; Modlin et al., 2003).

(D29) In the colon, the site where most intestinal cancers arise, the stem cells are situated at the very base of the crypts. This has been deduced from a variety of cell kinetic, mutational, and regeneration studies in mouse models (Potten, 1995). Their position in man is likely to be qualitatively similar. The number of stem cells per colonic crypt in mice has been estimated to be in the range of one to eight. Colonic crypts in man are around six times as large in all dimensions as in mice. It is considered that the number of stem cells per crypt may be similar in man and mouse, but with more transit cell divisions in man.

(D30) The lower third of the crypt constitutes the replicative zone where newly generated cells undergo two to three more divisions as intermediate cells while they begin their migration up the crypt to the luminal surface, where they are shed at the midpoint between two adjacent crypts. Estimates of the average cell cycle time of proliferative cells have been reported in the range 34-69 hours, with S phase duration of 9 hours (Potten, 1995). A peak in the LI was reported in both the human colon and rectum over the range of 10-25 cell positions from the crypt base (Potten et al., 1992). The maximum LI was about 29% in the colon and about 22% in the rectum. These figures suggest that in the region of maximum LI, the cell cycle time may be of the order of 30 hours for the colon and 39 hours for the rectum. The stem cell cycle time in human colonic crypts was stated as ≥36 hours (Potten, 1995). The turnover times of the epithelial cells of the colon are similar to those of the small intestine, i.e.

about 6 days for the absorptive cells and goblet cells, and up to 4 weeks for the entero-endocrine cells. Senescent epithelial cells are shed into the lumen.

(D31) The division potential of ISCs is enormous. There may be up to 1,000 stem-cell divisions over the 3-year lifetime of a mouse, and up to 5,000 in man over a lifetime (Marshman, 2002). Several mechanisms have been considered to ensure stem-cell integrity over this extremely large number of divisions: (a) selective retention of the parental template DNA strand during cell division (the "immortal strand" hypothesis), demonstrated in the intestine (Potten, 1978); (b) cell cycle checkpoint genes such as p53 and p21 to arrest cell cycle progression and prompt repair when DNA damage is detected; and (c) removal of damaged stem cells by apoptosis after low radiation doses.

(D32) In the past, the number of stem cells per crypt in mice was often inferred from calculations using dose-survival relationships for whole crypts after high acute doses of radiation. It was assumed that a crypt would repopulate from one or more surviving stem cells (called variously colony-forming cells or clonogens). One estimate of the number of repopulating cells in a mouse colonic crypt was 88 (Tucker et al., 1983) or 105-116 (Tucker et al., 1991), and even higher in the small intestine. Although it was recognised that some post-stem cells could also possibly regenerate crypts, the calculated numbers were unrealistic on the basis of the normal cell renewal processes in the crypt. Various technical reasons were considered to be associated with the high calculated numbers, and lower numbers were later calculated (Cai et al., 1997). At low doses, values of 5-10 clonogenic cells were calculated, and these were similar to the lower limit to the number of stem cells of about 6 per colonic crypt estimated from the maximum number of apoptotic cells in the crypt at short times after irradiation (Potten and Grant, 1998). This was based on the presumption that altruistic cell suicide would occur in damaged stem cells, and that the number of apoptotic cells would increase as the dose increased. Then, as the dose increased further, the number of apoptotic cells would reach some lower limit to the number of stem cells, governed by the increasing resistance of post-stem cells to apoptosis. Similarities between number of apoptotic cells and clonogens have not been examined in stomach crypts.

(D33) Genetic modification of crypt cell radiosensitivity provides a further test of the correlation between numbers of clonogenic/stem cells and apoptotic cells. This has been addressed only in the small intestine. In that case (Table D.6.), mice were used which were separately null for ATM, p53, or Bcl-2. Low dose-rate radiation was used in order to back-extrapolate the dose response curve semi-logarithmically to zero dose, and estimate the clonogen number more accurately. Null-ATM decreased slightly the apoptotic yield, clonogen number was unchanged, but clonogen radiosensitivity increased 3-4 fold. With null-p53, apoptotic yield decreased, and clonogen number and radiosensitivity tended to decrease. With null-Bcl-2, apoptotic yield was unaffected, whereas clonogen number and radiosensitivity tended to increase. It was concluded that there were some relative shifts in apoptotic and clonogenic population sizes caused by these mutations, but it was not possible to relate these to detailed cell positional data because of the uncertainties involved in the values (Hendry, 2002). Hence, the respective roles of apoptosis and mitotic death in the radiation-induced sterilisation of stem cells in the small intestine remain unclear. In addition, recent data showed that deletion of p21 resulted in protection of crypt stem/progenitor cells from radiation-induced cell death (George et al., 2009). In the colon, Bcl-2 prevents stem-cell apoptosis after irradiation, and apoptosis is random throughout colonic crypts. Hence, in this site, apoptosis does not play a significant role in stem cell killing after radiation, which is probably mediated by mitotic death following attempted repair involving p53 and p21 checkpoint controls.

Table D.6. Apoptosis levels and clonogen numbers in small-intestinal crypts in mutant mice compared to those in the wild-type strains FVB and C57 (Hendry, 2002). (Permission needed)

		Apoptoses/crypt		Clonogens/crypt	
		@1 Gy	@8 Gy	n	α (Gy^{-1})
atm	−/−	2.1	5.6	12 ± 6	0.60 ± 0.10
FVB	+/+	3.5	6.3	13 ± 6	0.17 ± 0.02
p53	−/−	0.6	–	20 ± 33	0.12 ± 0.05
FVB	+/+	5.0	7.2	65 ± 44	0.23 ± 0.03
bcl2	−/−	5.2	–	13 ± 5	0.19 ± 0.02
C57	+/+	5.2	–	8 ± 3	0.14 ± 0.01

(D34) It has been estimated that there are about 5 x 10^7 crypts in the small intestine in man (Potten, 1995). The length of the human small intestine is about 550 cm, the diameter is about 5 cm, the surface area is about 8,250 cm^2, and hence the crypt density is about 6,060 per cm^2. Crypts in the colon are larger than in the small intestine, so the density may be around 4,000 per cm^2. The human colon is about 110 cm long, the diameter is about 5 cm, and the surface area is about 1,650 cm^2 which is 1/5 that of the small intestine. Hence, there should be about 6.6 x 10^6 crypts in the large intestine in man. In a mouse colonic crypt, it is estimated that there are about 6 (to within a factor of 2-3) stem cells, and probably the same in man. Hence there may be a total colonic stem cell population in man of about 4 x 10^7. This is a very rough estimate with large uncertainties.

(D35) There is also the possibility that some daughter cells on the first step towards commitment and differentiation also might be capable of regenerating all crypt cell populations, so-called "potential stem cells", if they can relocate to the stem cell zone after radiation-induced cell depletion and function as stem cells. This possibility was raised because of the general discrepancy between many early estimates of high numbers of clonogens and the low numbers of stem cells expected from cell lineage and kinetic considerations. The idea is also consistent with the scenario in haematopoietic tissue of its repopulation by "cord blood stem cells" - cells with lower self-renewal capacity than the native niche stem cells; nonetheless, they can regenerate depleted marrow quite extensively. However, in the crypt, later work showed that the higher clonogen estimates could be accounted for instead by a second mode of clonogen kill at higher doses caused for example by some radiation damage to the niche (Roberts et al., 2003). Hence, the "potential stem cell" scenario in the crypt remains uncertain, but if true, it could provide more target cells for carcinogenesis.

(D36) In the stomach, there are less extensive data. The mucosal surface is 525 cm^2. If it assumed that the parameter values for stomach and colon are similar except for mucosal area, which in stomach is about 1/3 of that in the colon, the total stomach crypt stem cell population would be about 10^7.

(D37) The cell population structure and lineage characteristics for oesophageal epithelium are under current investigation (Barbera et al., 2014).

D.2.2. Age dependence

(D38) Table D.7. lists typical masses for segments of the alimentary tract as a function of age and gender. Values for adult males and females are rounded central estimates based on the data of Tipton and Cook (1969). Age-specific estimates for the stomach are based on data

from Scammon (1919), who collected measurements of stomach mass for 543 subjects in the first two decades of life. These data indicate that the growth rate of the stomach in postnatal life is equal to or slightly greater than that of the body as a whole. Age-specific estimates for the oesophagus and divisions of the intestines are based on the assumption that the rate of growth from birth to maturity parallels that of the stomach. Tissue masses are assumed to be independent of gender to 10 years of age.

Table D.7. Summary of typical values for masses (g) of walls in the GI tract. (Table A.2. in ICRP *Publication 100*, 2006).

Component	Newborn	1 year	5 years	10 years	15 years		adult	
					male	female	male	female
Oesophagus	2	5	10	18	30	30	40	35
Stomach	7	20	50	85	120	120	150	140
Small intestine	30	85	220	370	520	520	650	600
Large intestine:								
right colon	7	20	49	85	122	122	150	145
left colon	7	20	49	85	122	122	150	145
rectosigmoid	3	10	22	40	56	56	70	70
Total mass	56	160	400	683	970	970	1210	1135

(D39) Around birth, epithelial proliferation is confined to shallow pockets residing between the villi of the small intestine of mice. Mature small intestinal crypts appear in the first weeks after birth, by a process in which the intervillus pockets invade the wall of the small intestine. Similarly, colonic crypts become progressively deeper in early postnatal life. Intervillus pockets are initially polyclonal, but rapidly become monoclonal through a poorly understood process of refinement (Schmidt et al., 1988). In order to accommodate the growth of the organ into adulthood, the number of crypt units steadily increases by crypt fission, a process in which new crypts form by branching off existing crypts (Totafurno et al., 1987).

(D40) The response of the developing mouse intestine to x radiation using neonates (1 day postpartum), infants (2 weeks postpartum) and adults (7 weeks postpartum) has been examined (Miyoshi-Imamura et al., 2010). Irradiated adult small intestinal crypts displayed two waves of apoptosis. The first wave peaked at 3 hours, followed by a broad wave with a peak persisting from 24 to 48 hours. p53 was expressed during the first wave but not the second wave. For the infant small intestine, the intensity of the first wave was approximately half that of the adult wave, and for the colon, the intensity was even smaller. In neonates, apoptosis was delayed, peaking at 6 hours for small intestinal crypts, and at 24 hours for colonic crypts. Although no apoptosis occurred at 3 hours postirradiation in neonates, p53 was present in both the small intestine and colon, owing at least in part to the inability of p53 to increase the level of Noxa, a p53-dependent pro-apoptosis protein, suggesting a discontinuity in the p53-Noxa-caspase pathway in neonates. By contrast, the induction of p21, a pro-survival protein, was greater in neonatal cells than in adult cells. Thus, it appears that the developing and adult intestine mount different apoptotic responses to radiation.

(D41) As the mammalian GI tract develops from the embryonic gut, it is made up of an endodermally-derived epithelium surrounded by cells of mesodermal origin. Cell signalling between these two tissue layers plays a critical role in coordinating patterning and organogenesis of the gut and its derivatives. Many lines of evidence have revealed that 'Wnt' signalling is the most dominant force in controlling cell proliferation, differentiation, and apoptosis along the crypt-villus axis. Wnt mRNA expression in intestinal subepithelial

myofibroblasts and Fzd mRNA expression has been found in both myofibroblasts and crypt epithelium, as part of the stem cell "niche" (Yen and Wright, 2006). Moreover, there are many other factors, for example, BMP, 'homeobox', 'forkhead', 'hedgehog', 'homeodomain', and PDGF that are also important to stem cell signalling in the GI tract.

(D42) The effect of ageing has been studied, but only in the small intestine. After high doses of irradiation, the surviving crypts in old mice (28-30 months of age) were both smaller and fewer in number than in young mice (6-7 months of age). There was also a growth delay of 1-1.5 days in the older mice. Surprisingly, the number of clonogenic cells per crypt was estimated to be greater in the older mice. These studies indicated important age-related alterations in the capacity to regenerate the crypts after radiation damage (Martin et al., 1998a). Also, a twofold increase in the level of apoptosis was seen following 1 Gy γ irradiation in 29-month-old animals, compared to the young and middle-age groups. After 8 Gy irradiation, the level of apoptosis in all age groups was high and the age effect was less pronounced. The data suggest that stem cells do undergo some functional alteration with age (Martin et al., 1998b). There was also alteration in the level of p53 and p21 expression, suggesting an age-related defect in the capacity to recognise damage and initiate apoptosis or repair (Potten et al., 2001). It is not known if these effects are also present in the large intestine.

D.2.3. Cellular features

(D43) In human oesophagus and stomach, LRCs have many features of stem cells (long-lived, slow cycling, uncommitted, and multipotent), and these have been detected recently in a recognised stem cell niche (Pan et al., 2012). Further analyses of these cells, in healthy and metaplastic epithelia, are required.

(D44) One of the first suggested specific markers for stem cells was an antibody to Msi-1, an RNA-binding protein identified as playing a role in asymmetric division control in NSCs, which appeared to be expressed in very early lineage cells in the small intestine (Kayahara et al., 2003; Potten et al., 2003).

(D45) Also, a large series of studies indicate that the Wnt signalling pathway has a unique and central role in the (patho-) physiology of the intestine (Barker et al., 2008). Multiple secreted Wnt factors are produced by the epithelial cells at the crypt bottom (Gregorieff et al., 2005), potentially generating a morphogen-like gradient of Wnt signals along the crypt–villus axis. Barker and Clevers (2007) detected several Wnt target genes with a very restricted expression within crypts. One of these, the Lgr5/Gpr49 gene, was expressed in a particularly unique fashion. The Lgr5 gene encodes an orphan G protein-coupled receptor, characterised by a large leucine-rich extracellular domain. It is closely related to receptors with glycoprotein hormone ligands, such as the TSH, FSH, and LH receptors. *In situ* hybridisation on small intestinal tissue revealed highly restricted expression at the crypt bottom, also observed in colon. This expression pattern clearly differed from that obtained with any of the other 80 genes in the Wnt signature. Thus, the $Lgr5^{+}$ cells in small intestine and colon cells fulfilled the definition of stemness in displaying longevity and multipotency. The observations confirmed earlier estimates that each crypt contains approximately six independent, long-lived stem cells. Counter-intuitively, although these cells are putative stem cells, they appeared never to be quiescent. Rather, they completed a cell cycle every day (in mice). Tian et al. (2011) employed lineage tracing and cell ablation studies, and reported that elimination of the rapidly-cycling stem-cell population had no effect on intestinal homeostasis, leading them to conclude that Lgr5-expressing cells are dispensable. This would be consistent with the presence of a second lineage pathway.

(D46) Another study found that the stem-cell marker Bmi1 was expressed in discrete cells predominantly at cell position 4 above the base of the small-intestinal crypt in mice (Sangiorgi and Capecchi, 2008). Over time, these cells proliferated, expanded, self-renewed and gave rise to all the differentiated cell lineages. Ablation of the $Bmi1^+$ cells led to crypt loss. Bmi1-expressing cells were dramatically increased in number following Lgr5-cell ablation and they appeared to function as a reserve stem cell pool contributing to intestinal lineage development via the Lgr5 cell-independent pathway. When the ablation signal was removed, the Bmi1-expressing cells rapidly gave rise to Lgr5-expressing cells, thereby restoring the Lgr5-dependent lineage pathway. Also, the induction of a stable form of β-catenin in the $Bmi1^+$ cells was sufficient to rapidly generate adenomas.

(D47) A further study identified a rare population (one cell per 150 small-intestinal crypts) of slowly-cycling stem cells (90-95% in G_0, located mostly between cell positions 5-8 from the crypt base), marked by mTert expression (Montgomery et al., 2011). The $mTert^+$ cells were distinct from $Lgr5^+$ cells, but they included a subpopulation of Bmi-1+ cells. The $mTert^+$ cells were distributed in a pattern along the crypt–villus axis similar to that of long-term LRCs, and they gave rise to $Lgr5^+$ cells. Lineage-tracing studies demonstrated that $mTert^+$ cells gave rise to all differentiated intestinal cell types, persisted in the long term, and contributed to the regenerative response following injury. In addition, Takeda et al. (2011) identified Hopx gene expression as another marker of slowly-cycling stem cells at cell position 4 in the crypt. Hopx-expressing cells appeared to be present in virtually every crypt, and could give rise to $Lgr5^+$ cells, and vice versa, indicating the plasticity of the lineages.

(D48) A picture is emerging of a stem cell lineage in the small-intestinal crypt comprising both slowly-cycling and rapidly cycling stem cells at about cell position 4, with the latter producing several progenitor cell lineages. Much less knowledge is available for colonic crypts, but $mTert^+$ cells and $Lgr5^+$ cells have been detected there.

(D49) Other genetic clonal marking strategies exploit mutation of the X-linked glucose-6-phosphate dehydrogenase gene in male mice (Griffiths et al., 1988; Park et al., 1995), of a hypothetical enzyme involved in O-acetylation (Campbell et al., 1996), or of mitochondrial 'cytochrome c' oxidase (Greaves et al., 2006; Taylor et al., 2003) in human colon. Another elegant tracing strategy follows epigenetic changes in gene methylation patterns as a lineage marker (Shibata, 2008; Yatabe et al., 2001). Other data support the use of doublecortin and CaM kinase-like-1 (DCAMKL-1), a microtubule-associated kinase, as an intestinal and possibly colonic stem cell marker (Giannakis et al., 2006; May et al., 2008). On the other hand, the CD133 marker, which is expressed by normal primitive cells of the neural, haematopoietic, epithelial and endothelial lineages, was reported to be extremely infrequent in normal colon tissues (Ricci-Vitiani et al., 2007).

D.3. Radiosensitivity

(D50) The radiosensitivity of crypt stem cells is usually measured using clonogenic radiosensitivity, assuming that stem cells will be capable of regenerating all crypt cell populations. Clonogenic radiosensitivity measures the most resistant population of clonogens, because as the dose increases crypt numbers start to decline only when the last clonogen has been killed in a small proportion of the crypts. Hence if there are some more-sensitive clonogens these will be killed by lower doses and will not contribute to the terminal shape of the dose-survival curve, but they will contribute to the threshold dose for crypt killing.

(D51) Crypt clonogens are slightly more resistant to acute doses in the stomach (Fig. D.4.) and colon (Fig. D.5.) than in the small intestine. In the colon, this is due to the presence of p53, because in the p53-null mouse, the sensitivities of colonic and jejunal clonogens are the

same (Hendry et al., 1997). A possible explanation is the greater involvement of p53 in the large than in the small intestine, regarding repair and a G_2-phase checkpoint delay.

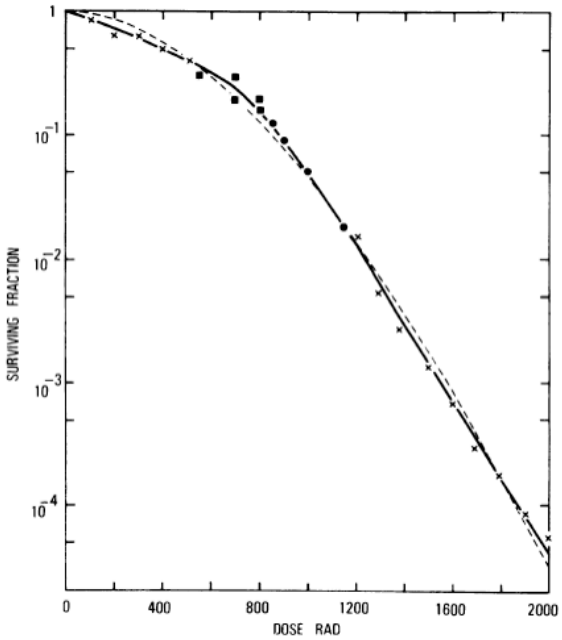

Fig. D.4. Survival of gastric clonogenic cells exposed to γ rays. Circled points, extrapolated data from a figure published in Chen and Withers (1972). Squared points, deduced using extrapolated values. 1 rad = 1 cGy. Reproduced from Hendry (1979). (Permission needed)

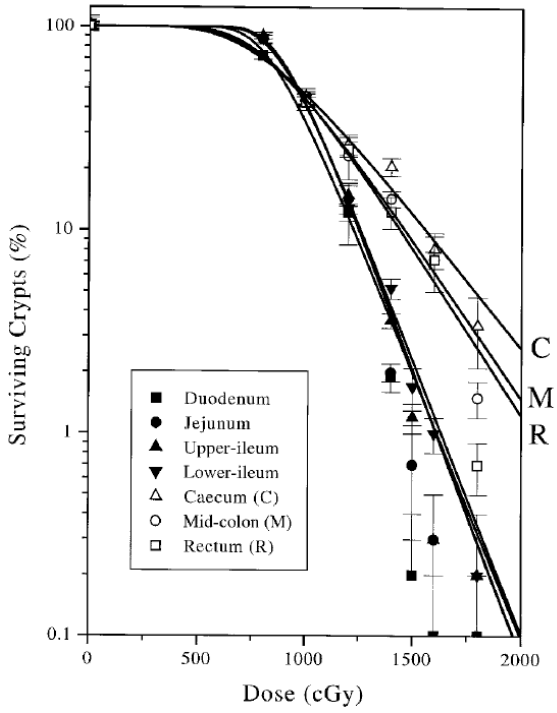

Fig. D.5. Crypt survival curves obtained for the four regions of the small intestine (closed symbols) and three regions of the large intestine (open symbols). The small intestinal data were obtained 3 days after irradiation using haematoxylin- and eosin (HE)-stained sections with autoradiography and a threshold of >10 labelled cells and the large intestinal data 5 days after irradiation using vincristine-

treated animals and a threshold of >3 mitotic cells to define a viable crypt (Reproduced from Cai et al. 1997b). [Note that these are survival curves for whole crypts containing many clonogens, not for individual clonogenic cells, and hence the presence of the initial plateau portion of the curves. A more detailed curve in the lower dose region for single clonogenic cells can be found in (Tucker et al., 1983)]. (permission required)

(D52) Clonogenic radiosensitivity is lowest at low doses or when the dose rate is low. The lowest reported value of the α (initial slope) sensitivity parameter for colonic crypt clonogens is 0.15 Gy^{-1} (or initial D_0 = 5.4 Gy) derived from fractionated dose studies (Tucker et al., 1983). For stomach clonogens, the initial D_0 was similar at about 5.5 Gy (Hendry, 1979). GI clonogenic cells show conventional dose-rate, dose fractionation, and dose protraction effects, characteristic of cells proficient in repair and repopulation (Figs. D.4. and D.5.; Potten, 1995).

(D53) Generally, the sensitivity of cells that undergo apoptosis is much greater than that of clonogenic cells (n.b. an exception to this is the rare population of $mTert^+$ stem cells, not showing apoptosis at the conventional scoring time of 3 hours after 1 Gy or 10 Gy (Montgomery et al., 2011)). Survival curves for apoptically-susceptible cells were generated for the small intestine by generating a dose-incidence curve over the dose range 0.01-1 Gy, noting the maximum incidence as the dose was increased, and expressing "the maximum number minus the number observed at any lower dose" as a surviving proportion of that maximum. The resultant D_0 was 0.1-0.2 Gy (Hendry and Potten, 1982; Potten, 1977), very much smaller than the value for clonogens. It was recognised that this high sensitivity was very dependent on the maximum value chosen, with the use of higher maximum values leading to lower sensitivities, but still the big difference remained (Hendry and Potten, 1982). The sensitivity to apoptosis was independent of dose rate between 0.0027 and 0.45 Gy per minute (Hendry et al., 1982). In addition, it was shown that when apoptosis in the stem cell zone was induced by an acute dose of 0.5 Gy, the apoptosis-susceptible cells were restored within 2 days (Ijiri, 1984). This suggests that during chronic irradiation at a very low dose rate, continuous deletion of damaged cells and their replacement might occur, but this has not yet been studied. The apoptosis-susceptible cells are within the stem cell zone in the small intestine.

(D54) Radiosensitivity of the apoptosis-susceptible cells was greater in the case of high-LET neutron irradiation (Hendry et al., 1982). It was calculated that a single radiation track from neutrons would initiate the apoptotic process. For both high and low dose-rate irradiations using low-LET or neutron sources, the distribution of apoptotic cells in the crypt was similar, indicating that the differential sensitivity of the affected cell populations remained the same. Further, the 'clustering' of apoptotic cells was examined, and found not to be significantly different from a random distribution. This indicated that there was no evidence for a local 'bystander' effect in neighbouring cells, and in the case of the 600 MeV (maximum energy) neutron beam, no evidence for multiple cells hit by nuclear fragmentation 'stars'.

(D55) Cycling CBCCs, another population of stem cells at crypt positions +1-+3 in mouse small intestine, were shown to be relatively radioresistant, repairing DNA by HR significantly more efficiently than TA progenitor cells or villus cells. CBCCs underwent apoptosis less than 24 hours after irradiation (32% ± 2% of total lethality) or mitotic death at 24-48 hours (Hua et al., 2012).

(D56) In the mouse colon, the frequency of radiation-induced apoptosis was highest at the crypt base, and it declined gradually to more than halfway up the crypt (Pritchard, 2000; Fig. D.6.). This is consistent with radiation killing of the stem cells at the crypt base as well as some transit daughter cells as they differentiate up the lineage. Also, the frequency of

apoptosis in the colon continued to rise as doses increased from 1 to 8 Gy (Fig. D.6.), rather than almost saturating at around 1 Gy as in the small intestine. This suggests that the transit cells have increasing resistance to apoptosis. Bcl-w-null animals exhibited more apoptosis than their wild-type counterparts (Fig. D.6.), indicating the importance of this bcl-2 family member in causing resistance to radiation-induced apoptosis in these colonic progenitor cells.

(D57) In the mouse stomach, maximum numbers of apoptotic cells were observed in both antrum and corpus at 48 hours after radiation doses greater than 12 Gy (Przemeck, 2007; Fig. D.7.). The incidence of apoptosis was much lower than observed in the small intestine or colon after similar doses of radiation. The highest numbers of apoptotic cells were observed at cell positions 5–6 in the antrum and cell positions 15–18 in the corpus. These distributions coincided with the distributions of proliferating cell nuclear antigen (PCNA)-labelled proliferating cells. In the gastric corpus, the putative stem cell zone and the proliferative zone are located at the isthmus of the gland. Decreased numbers of apoptotic gastric epithelial cells were observed in p53-null, bak-null, and bax-null mice, and increased numbers in bcl-2-null mice, compared with wild-type counterparts 6 and 48 hours after 12 Gy γ-radiation. Detailed lineage characteristics have not yet been established for stomach crypts.

(D58) Protection of clonogens in the small intestine against killing by radiation has been achieved by using high levels of certain growth factors and cytokines, such as TGFβ3, IL-11, and keratinocyte growth factor (KGF) (Booth and Potten, 2001), as well as a variety of other agents including antioxidants and prostaglandins (reviewed in ICRP, 2012). Most of these studies have been directed towards pre-irradiation protective measures for the small intestine subject to high doses in a therapeutic or accident context. Few studies have focussed on other regions of the GI tract relevant for carcinogenesis such as stomach and colon. Examples are a moderate radioprotective effect on murine colonic crypts by pre-irradiation treatment using the aminothiol compound WR-2721 (Ito et al., 1986), and retinoic acid and interferon (Mason, 1994).

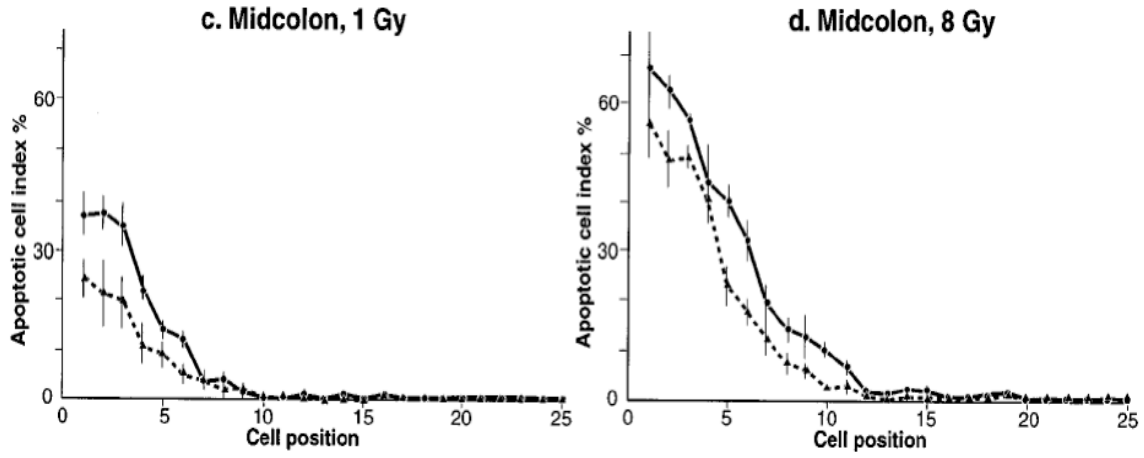

Fig. D.6. Cell positional distribution of apoptosis induced by γ-irradiation in colonic intestinal crypts. The bcl-w wild-type (dashed curves) and homozygously-null (solid curves) mice (four animals in each experimental group) had received ^{60}Co γ-irradiation 4.5 hours earlier. (c) Midcolon, after 1 Gy. (d) Midcolon, after 8 Gy. The standard error of the mean is shown for each point (Pritchard et al., 2000). (Permission needed)

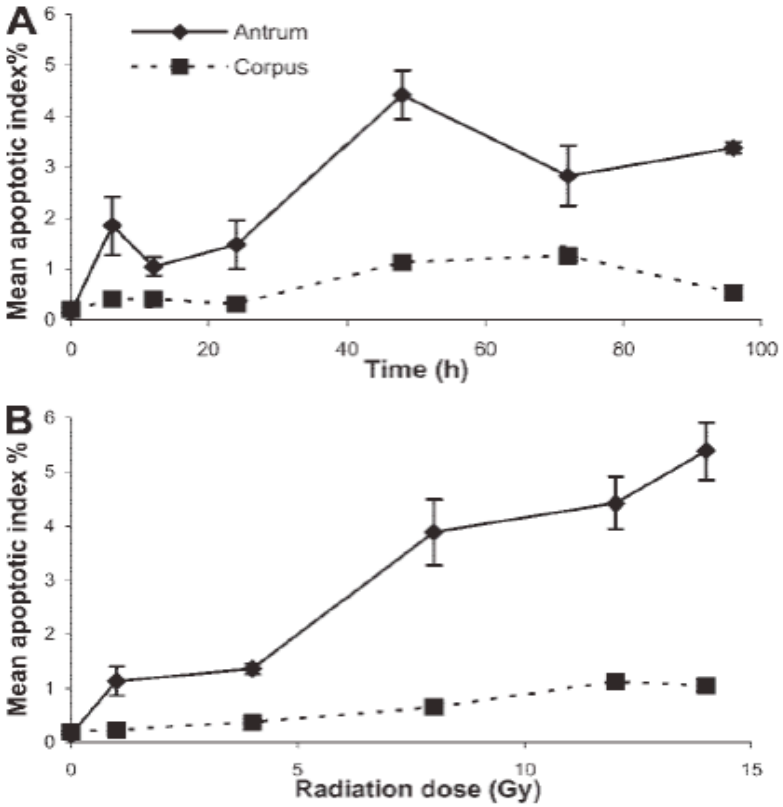

Fig. D.7. A: Time course showing mean (± SE) apoptotic index assessed by HE staining in antrum and corpus of male CD1 mouse stomach up to 96 hours following 12 Gy γ-radiation (n = 9 at 48 hours, n = 4–6 at other time points). B: dose response showing mean ± SE apoptotic index induced in antrum and corpus of male CD1 mouse stomach 48 hours following 1–14 Gy γ-radiation (n = 9 at 12 Gy, n = 5 at other doses) (Przemeck et al., 2007). (Permission needed)

D.3.1. Characteristics of single-cell responses

(D59) The radiation survival curve for a normal rat intestinal epithelial cell line, IEC-17, which has features of undifferentiated small intestinal crypt cells, exhibited no initial shoulder and was bimodal (Sierra et al., 1985). The D_0 for the first component was about 0.2 Gy and for the second component, representative of about 10% of the cell population, was 5.0 Gy. These values are strikingly similar to the D_0 of 0.1-0.2 Gy for the apoptotically-sensitive cells *in vivo* in the mouse (paragraph D48), and the initial slope of the survival curves for the clonogenic cells with D_0 5.4 Gy (paragraph D47). These results indicate that the bimodal radiation response observed *in vivo* can be recapitulated *in vitro* using a crypt-derived cell line. Extended age of IEC-17 cells in culture (greater than 100 passages) resulted in altered morphology, decreased doubling time, increased chromosome number, and loss of anchorage dependence, all features characterising spontaneously-transformed high-passage IEC-17 cells (DeRose and Claycamp, 1989). These high-passage cells also exhibited a bimodal response to x-rays, suggesting that the different radiosensitivity of the subpopulations remained throughout the spontaneous transformation of high-passage IEC-17 cells.

(D60) Single cells have been cloned from disaggregated human and murine normal colonic crypts (Whitehead RH, 1999), and an epithelial cell line has been established from the colonic mucosa of protein tyrosine kinase 6 (Ptk6) null mice (Whitehead RH, 2008). PTK6 is an intracellular src-related tyrosine kinase that regulates differentiation in the intestine, and

these cells have the characteristics of a stable progenitor cell. When cultured in collagen gel, the Ptk6 null cells form complex organoids, some of which resemble cups of cells, containing cells with differentiated phenotypes. Also, differential cell surface abundance of ephrin type-B receptor 2 (EPHB2) allows the purification of different cell types from human colon mucosa biopsies. The highest EPHB2 surface levels correspond to epithelial colonic cells with the longest telomeres and elevated expression of ISC marker genes. Using culturing conditions that recreate the ISC niche, a substantial proportion of EPHB2-high cells can be expanded *in vitro* as an undifferentiated and multipotent population (Jung et al., 2011). Such cell lines and organoids may be useful for studying aspects of radiation responses at the cellular level.

(D61) Information on the requirements of the crypt niche for stem-cell maintenance and growth was reviewed by Shaker and Rubin (2010). It has been shown that mesenchymal-derived cells are required for long-term culture of stem cells (Ootani et al., 2009). Using a microenvironment consisting of an air-liquid interface 3D collagen gel to improve oxygenation, as well as myofibroblasts and stem-cell niche signalling molecules, long-term culture of intestine and colon was established. Also, $Lgr5^{+}$ stem cells can be maintained in long-term culture and differentiate into crypt–villus-like units in the presence of a limited set of appropriate extracellular growth signals, which normally are derived from the underlying mesenchyme *in vivo* (Sato et al., 2009).

D.4. Mutagenesis

(D62) A concept of early tumour development has evolved, as described in the UNSCEAR (2000) report and reproduced here. The concept requires a relatively tissue-specific "gatekeeper" gene to be mutated in order for stem-like cells to enter a phase of inappropriate clonal expansion (Kinzler and Vogelstein, 1996; Sidransky, 1996); this expansion then allows for the accumulation of further mutations. According to the concept, the accumulation of other mutations in the neoplastic pathway in the absence of gatekeeper defects will result only infrequently in the clonal development of recognisable tissue lesions. In essence, the temporal order of mutational events is likely to be important for productive neoplastic growth with loss of specific gatekeeper genes as critical early events.

(D63) A key element in this hypothesis, as it relates to colorectal cancer, is that the first consistent mutation in tissue lesions should be monoclonal mutation of the APC gatekeeper gene, which acts as a transcriptional regulator (Nakamura, 1997). In the main, the data (Kinzler and Vogelstein, 1996) support this, but investigations of the temporal sequence of gene mutations add considerable weight to the argument.

(D64) Using tumour microdissection and allelotyping methods, the sequence and tempo of allelic losses in a series of colorectal cancers at different stages of development were followed (Boland et al., 1995). The principal losses that were tracked were those associated with deletion of APC (5q21), p53 (17p13), and deleted in colorectal cancer (DCC) (18q21). In brief, LOH via allelic loss was not recorded in normal tissue surrounding colorectal tumours. However, 5q but not 17p losses arose abruptly and consistently at the transition from normal tissue to benign adenoma; a proportion of adenomas also showed 18q losses. Losses to 17p occurred equally abruptly and consistently at the adenoma to carcinoma transition border, and in highly advanced and invasive carcinomas, there was a high level of allelic variation indicative of clonal heterogeneity due to genomic instability.

(D65) Thus, commencing with APC loss from cells in normal tissue, the development of colonic tumours is characterised by abrupt waves of clonal expansion, with p53 loss and chaotic allelic variation being critical watersheds in the evolution of the fully malignant

phenotype. Considering these and other molecular genetic observations with colorectal cancer, a temporal model of neoplastic initiation and malignant development has been proposed (Boland et al., 1995). This is illustrated in Fig. D.8.

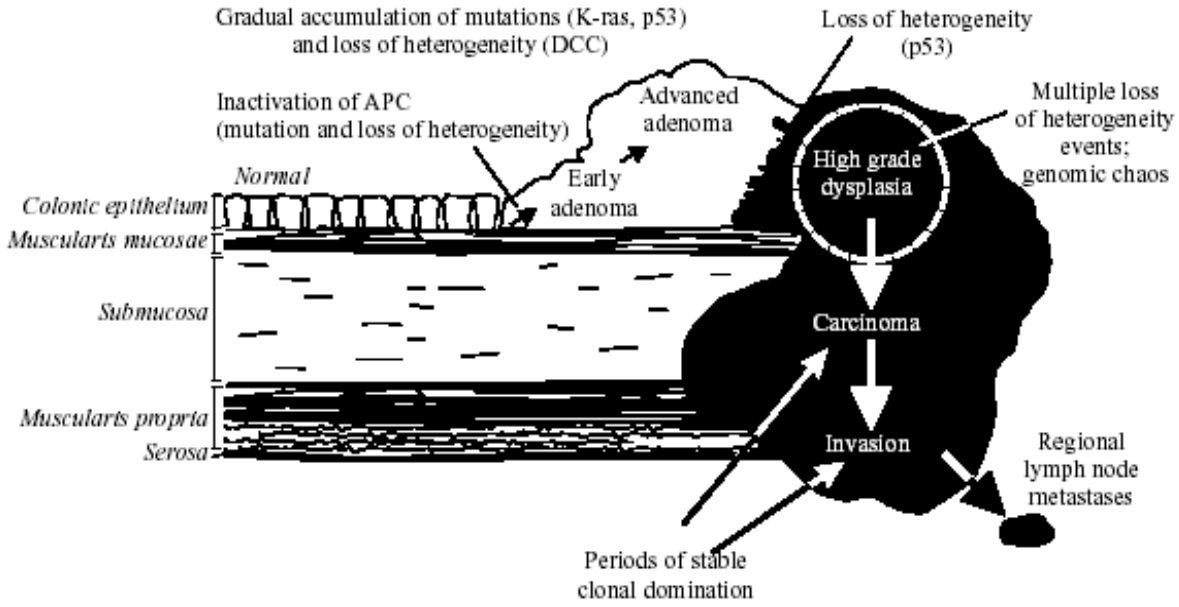

Fig. D.8. A model of the sequence of genetic events in neoplastic development in the human colon (Boland et al., 1995; UNSCEAR, 2000). The colon is one of the clearest examples of elucidation of the mutational steps involved in carcinogenesis. Initial mutations of LOH at the APC locus of a colonic epithelial cell produce dysplastic crypts followed by adenoma development involving k-ras mutation and DCC loss. Loss of p53 from advanced adenoma marks the transition between benign and malignant disease characterised in turn by the development of genomic instability, multiple gene losses and invasive behaviour/metastasis to regional lymph nodes. (permission needed)

(D66) Mutation/loss of the tumour-suppressor gene APC has for some time been believed to be a critical early event in the development of human colon cancer. Up to about 70% of early colonic adenomas show apparently monoclonal structural/functional loss of this gene (Powell et al., 1992), and a critical role in tumour initiation seems likely (Boland et al., 1995). With use of a mouse (Min) model of intestinal carcinogenesis, this view of monoclonal tumour initiation has been strengthened. In essence, aberrant crypts, the earliest intestinal lesions detectable microscopically, have been microdissected from Min mice and shown to be monoclonal with respect to Apc loss (Levy et al., 1994; Luongo et al., 1994). On the other hand, it has been found that a large proportion of intestinal adenomas in the GI tracts of human FAP patients, who were also XO/XY in genotype and therefore mosaic for the Y-chromosome, was apparently polyclonal. In this study, polyclonality was judged by the presence within single adenomas of a mixed population of cells with respect to the Y-chromosome sequence. By this measure, up to 76% of adenomas were polyclonal. However, early Y-chromosome loss and field effects creating tumour clustering and collision (UNSCEAR, 2000) might contribute to this finding.

(D67) Tumour clustering may also explain data on the apparent polyclonality of a proportion of spontaneously arising intestinal adenomas in Min mice as assessed by genetic features other than Apc loss (Dove et al., 1998). Thus, polyclonality may be acquired during adenoma development rather than arising *de novo* at the time of initiation. For example,

fusion of independent Apc-deficient microclones may allow for cooperative growth. These data illustrate some of the problems that remain in resolving the early molecular events and complex cellular interactions of tumour development. In spite of these uncertainties, there remain experimental data on intestinal tumourigenesis that forcibly support monoclonality for induced neoplasms (Griffiths et al., 1989).

(D68) Based on investigation of the earliest colonic tissue alteration in FAP patients, Boman et al. (2008) presented the hypothesis that initiation of colorectal cancer by APC mutation is mediated by dysregulation of two cellular mechanisms. One involves differentiation, which normally decreases the proportion (proliferative fraction) of colonic crypt cells that can proliferate; the other is a cell cycle mechanism that simultaneously increases the probability that proliferative cells are in S phase. In normal crypts, stem cells at the crypt bottom generate rapidly proliferating cells, which undergo differentiation while migrating up the crypt. Modelling studies of normal crypts suggest that these transitions are mediated by mechanisms that regulate the proliferative fraction and the S-phase probability. In FAP crypts, the population of rapidly proliferating cells is shifted upwards, as indicated by the LI. Analysis of FAP indicates that these transitions are delayed because the proliferative fraction and S-phase probability change more slowly as a function of crypt level. This leads to expansion of the proliferative cell population, including a subpopulation that has a low frequency of S-phase cells.. Boman et al. (2008) reported that stem cells (or cells having high stemness) are proliferative cells with a low probability of being in S phase. Thus, it was concluded that dysregulation of mechanisms that control proliferative fraction and S-phase probability explains how APC mutations induce system cell overpopulation at the crypt bottom, shift the rapidly proliferating cell population upwards, and initiate colon tumourigenesis. It has also been shown that $Lgr5^{+}$ stem cells, which represent about 5 to 10% of the cells in mouse intestinal adenomas, generate additional $Lgr5^{+}$ cells which fuel adenoma growth, as well as all other adenoma cell types (Schepers et al., 2012). The $Lgr5^{+}$ cells were intermingled with Paneth cells near the adenoma base, a pattern reminiscent of the architecture of the normal crypt niche.

(D69) A model for colonic crypt organisation, patterns of stem cell divisions, niche succession and clonal conversion (Fig. D.9.), was described by Humphries and Wright (2007).

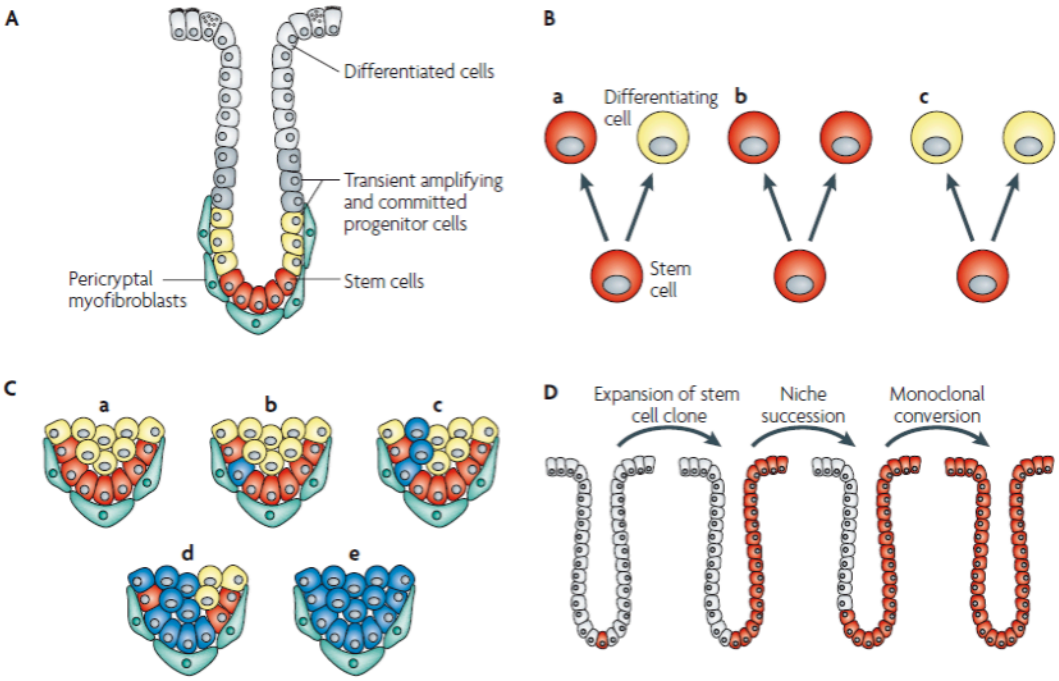

Fig. D.9. Colonic crypt organisation, patterns of stem cell divisions, niche succession and clonal conversion. Panel A. A diagrammatic representation of the colonic crypt. Stem cells are housed in the base of the crypts where they communicate with the niche cells — the pericryptal myofibroblasts, which are outside the crypt but communicate by cell signalling. Stem cells feed the TA compartment, where most cell production occurs. This portion of the crypt is also thought to house the committed progenitor cells: cells committed to one or more cell lineages. Panel B. Patterns of stem-cell divisions (a) shows an asymmetric stem-cell division, in which a stem cell gives two daughters, one remaining in the niche as a stem cell and the other committed for differentiation, sometimes called q divisions. If all stem cells underwent such asymmetric divisions, stem cells would remain immortal: their progeny remain within the niche. However, several strands of evidence indicate that other stem-cell divisions occur: symmetric divisions, producing either two stem cells — so-called p divisions (b) — or two cells destined to differentiate — r divisions (c). If the incidence of r divisions or apoptosis is >0, then a stem cell will eventually come to dominate the niche. Thus, in a stem-cell niche, all three types of stem cell divisions are found. Panel C. Niche succession. (a) shows the colonic crypt niche, housing the stem cells. In (b), a stem cell has developed a mutation (blue) — say in the cytochrome c oxidase gene. By asymmetric division, a clone of mutated cells in the above dividing transit compartment develops from this mutant stem cell, shown in (c). At this stage, the clone could be lost if the mutant stem cell should undergo apoptosis or a symmetric r division. However, if adjacent stem cells undergo apoptosis or an r division, the mutant stem cell may expand through p divisions to colonise the niche, as shown in (d). Finally, the mutant stem cell comes to dominate the niche (e). Of course, if the mutation occurs in a tumour suppressor gene or an oncogene rather than a housekeeping gene, which confers a selective advantage, then niche succession would occur more rapidly. Panel D. Clonal conversion: the progeny of a mutant stem cell replaces all other cells in the crypt (Humphries and Wright, 2008). (Permission needed)

(D70) Cancers are generally considered to originate from stem cells, i.e. cells that possess unlimited reproductive capacity. These are transformed by carcinogenic agents so that their differentiation patterns are altered in such a way that cell renewal predominates over differentiation, leading to growth of an abnormal cell population. It has also been considered that the carcinogen target cells are the actual stem cells in small intestine which are protected

from tumour induction in the small bowel by an altruistic suicide of cells bearing carcinogenic lesions, while those in the large bowel are not protected by this altruistic cell suicide and hence can initiate cancers (Potten et al., 1992). The altruistic apoptosis in the large bowel is prevented by the expression of the survival (anti-apoptotic) gene bcl-2, which is not expressed significantly in the small intestine (Merritt et al., 1995).

(D71) In contrast, it was suggested that tumours in the human colon may originate in cells on the intercryptal plate rather than, or in addition to, stem cells at the base of the crypt (Shih et al., 2001). That study indicated that most early neoplastic lesions of the colon contain dysplastic cells only at the orifices of crypts and on the luminal surface between crypts. Analysis showed loss of the APC gene and high expression of β-catenin in such dysplastic cells but not in cells with normal appearance within the crypts. Mutations in the APC gene are the earliest genetic alterations in the genesis of colorectal tumours, and appear to be required to initiate clonal evolution, involving overexpression of β-catenin (Fodde et al., 2001).

(D72) This suggestion of target cells on the lumenal surface is contentious. In normal tissue, differentiated epithelial cells on the intercryptal surface would have a very limited lifespan of a few days, destined to be lost in the intestinal lumen in the normal process of cell renewal. To develop into a tumour, these dysplastic cells would need to escape this process to allow time for progression to malignancy, involving a sequential number of mutational events (Goyette et al., 1992; Vogelstein et al., 1988). Preston et al. (2003b) reported a detailed examination of early lesions from patients who had undergone colectomies for FAP and also of sporadic colorectal adenomas, from which they concluded that adenomas originate in single crypts and grow initially by crypt fission. Only in established sporadic adenomas was there evidence of growth downwards into adjacent crypts. There has been considerable debate about what happens next in the progression to an established adenoma: whether dysplastic cells spill over to invade and colonise the territory of adjacent crypts — a top-down process; or whether the monocryptal adenoma spreads by fission, in a manner described to account for field cancerisation in the colon — the bottom-up proposal. Although it is difficult to obtain definitive dynamic proof in humans, clonal expansion by crypt fission appears to be the prominent mode of spread of an adenomatous crypt, and is now generally accepted as the most important mechanism for clonal expansion in early adenomas (Humphries and Wright, 2008; Renehan, 2002; Zeki et al., 2011).

D.5. Target cells and radiation protection

(D73) In the ICRP (2006) report on the human alimentary tract, doses were calculated to the estimated position of the stem cells. However, in considering uncertainties in dose estimates, the possibility that cells higher in the crypts may also be targets was addressed, including the extreme possibility of target cells on the lumenal surface. Doses were calculated separately for the mucosal layer of each region of the human alimentary tract (ICRP, 2006). For penetrating radiations, it was considered reasonable to use the average dose to the walls of each region as a measure of the dose to the mucosal layer. For non-penetrating α and β particle emissions, the dose is dependent on the assumptions made regarding the location of target cells for cancer induction. For each region of the alimentary tract, the target was taken to be the stem cells that are located in the basal layer of the stratified squamous epithelia of the oesophagus and within the crypts that penetrate the mucosal layer in the stomach and large intestine.

(D74) Uncertainties were illustrated for the specific case of doses to the colon, because colon doses are generally the major contributors to relevant alimentary tract doses concerning

carcinogenesis. Although it is generally accepted that the stem cells in the base of the crypts are the targets for colon cancer induction, some uncertainty was raised by observations of dysplastic cells on the lumenal surface of the colon between apparently normal crypts (Shih et al., 2001), although this finding was challenged by other authors (Preston et al., 2003c). Thus, as well as uncertainties in the depth of the crypts and hence the depth of the stem cells, there is also uncertainty as to whether it is only the stem cells that should be regarded as targets.

(D75) Table D.8. compares colon doses for different assumptions of target cell location, normalised to the default assumption that they form a continuous layer at a depth of 280–300 μm from the lumenal surface of the colon. Thus, uncertainties in the depth of the crypts and hence the depth of the stem cells, represented by Columns 2 and 3, result in differences of about ±10% for ^{115}Cd and smaller differences for the other examples considered. For ^{234}U and ^{239}Pu, there is no dose to the colon wall from activity in the lumen, and thus no change with differing assumptions regarding stem cell depth. Similarly, widening the target to include cells at higher positions up the crypts (200–300 μm), and thus increasing the mass of target tissue, results in a maximum change in colon dose of about 10% for ^{115}Cd. The extreme assumption that the target may include all epithelial cells from the base of the crypts to the luminal surface (0–300 μm) results in a larger increase in dose. The increase by factors of about 1.5 for ^{234}U and 3 for ^{239}Pu is relative to the dose to the colon resulting from activity absorbed to blood. However, these increases in colon doses from ^{234}U and ^{239}Pu will make negligible differences to committed effective doses, which are dominated by contributions from doses to tissues and organs from activity absorbed to blood.

Table D.8. Differences (%) in dose coefficients (h) for the colon, compared with the default case[a], resulting from considerations of target depth in the mucosa, considering ingestion by adult males. Table reproduced from Table 8.5. in ICRP (2006).

Nuclide	Assumed location of the target region depth from lumen (μm)			
	220-240	340-360	200-300	0-300
^{55}Fe	0%	0%	0%	0%
^{59}Fe	1%	1%	1%	6%
^{90}Sr	7%	6%	5%	21%
^{106}Ru	3%	2%	2%	8%
^{115}Cd	13%	9%	9%	38%
^{234}U	0%	0%	0%	148%
^{239}Pu	0%	0%	0%	317%

[a]Default case assumes a target depth of 280–300 μm.

(D76) Regarding potential committed lineage cell targets for carcinogenesis, there also may be evidence of long-lived committed progenitor cells in the human colon: crypts can be identified where the cyclooxygenase (COX)-mutated clone is present well above the stem-cell zone (Humphries and Wright, 2008). This might represent a clone which originated in the stem-cell region but now is in transit to be lost eventually at the top of the crypt by extrusion; but it may also represent a mutation occurring in such a committed progenitor cell. The dynamics of this process are not yet known, but interpolation from the rarity of such events suggests that they last for a long time. This raises the possibility that the cell which undergoes the first mutation and selection event is not the stem cell, but a committed progenitor cell. Although there is no direct evidence for this, particularly in humans, this occurrence would explain several common observations: tumours arise from the colon that are composed

predominantly, or exclusively, of mucin-secreting cells, endocrine cells or even Paneth cells (Humphries and Wright, 2008).

D.6. Summary

(D77) ICRP radiation risk weighting factors in the alimentary canal are highest in stomach and colon (0.12 each), lower in oesophagus (0.04), and extremely low in small intestine (near zero, classified in remainder tissues). The target cells for carcinogenesis are considered to be predominantly the mucosal stem cells, situated in the basal layer of the oesophageal epithelium or in crypts (gastric pits) of the stomach (at 1/3 of the crypt depth from the top), small intestine (at cell position 4 from the base), and colon (at the crypt base). The location influences the risk of short-range radiations from radionuclides by varying extents. The possibility that stem-cell daughter cells are also target cells for carcinogenesis remains unclear, and this could also influence risk variously.

(D78) The cytotoxic response of most of the normal stem cells to increasing doses of radiation is bimodal. Low doses of a few cGy induce apoptosis in the stem cell zone in the small intestine, compared to throughout the crypt in the colon. In the small intestine, this effect is considered protective by deleting injured stem cells. In the colon, anti-apoptotic bcl-2 is expressed which prevents this. After higher doses, some stem and other progenitor cells are reproductively sterilised, and after doses above 8 Gy, whole crypts are sterilised. The increasing proportion of sterilised crypts observed as dose is increased, enables deduction of the radiosensitivity of clonogenic cells. The radiosensitivity is slightly higher in the small intestine than in the colon, but this difference is due to the differential effect of p53 in the two sites.

(D79) When high cytotoxic doses of radiation are used, crypt clonogenic cells show marked sparing effects when the radiation dose rate is reduced, the dose is fractionated, or the dose is protracted so as to allow repopulation from surviving stem cells. When low doses are used, most damaged stem cells are removed by apoptosis and replaced, except the rare mTert-expressing cells and those stem/clonogenic cells in the colon expressing bcl-2. The recent findings of some stem cells that are either slowly cycling or in G_0 provides a mechanism for the multiple mutation and multistage development of malignancy. The most plausible pathological mechanism is the fission and expansion of dysplastic/aberrant crypts leading to adenomas.

ANNEX E. LUNG STEM CELLS

E.1. Lung cancer

E.1.1. Occurrence of lung cancer

(E1) Lung cancer is the most common cancer world-wide accounting for 1.6 million new cases annually (resulting in about 19% of all cancer deaths) (Ferlay et al., 2010; ICRP, 2007). While smoking is the major cause for about 80% of lung cancer cases, there are a significant number of lung cancers that occur in never smokers. Specific mutations in the tyrosine kinase domain of EGFR are implicated in lung cancers arising in non-smokers indicating that there are significant non-smoker related exposures leading to lung cancer (Lynch et al., 2004; Paez et al., 2004). Indeed, non-smoker lung cancers are now the seventh leading world-wide cause of cancer related deaths, and more common than cancers of cervix, pancreatic or prostate (Parkin, 2002).

(E2) The most common form of lung cancer in never-smokers is ADC (Brownson et al., 1998; Dibble et al., 2005; Kabat and Wynder, 1984; Ko et al., 1997; Muscat and Wynder, 1995; Radzikowska et al., 2002; Stockwell et al., 1992; Toh et al., 2006; Yu et al., 2006; Zhong et al., 1999), and the reason for this type of lung cancer is not known. Lung cancer in never-smokers occurs more frequently in women and varies considerably by geographical region (Brennan et al., 2006; Brownson et al., 1998; Cerfolio et al., 2006; Dibble et al., 2005; (Lynch et al., 2004; Paez et al., 2004; Parkin et al., 2002). Du et al., 1996; Gorgoulis et al., 2005; Kabat and Wynder, 1984; Koyi et al., 2002; Kreuzer et al., 1999; Muscat and Wynder, 1995; Radzikowska et al., 2002; Shimizu et al., 1984; Toh et al., 2006; Wakai et al., 2006). Paradoxically, the rate of lung cancer in male never-smokers is less variable, suggesting a greater contribution of risk factors other than smoking in females, including hormonal regulation. Irrespective of gender, there are many environmental risk factors that are associated with lung cancer in never-smokers including asbestos, chromium, arsenic, nickel, indoor radiation (radon) and outdoor air pollution exposures (Alberg et al., 2005; Brennan et al., 2006; Subramanian and Govindan, 2007).

E.1.2. Multistep process of lung cancer pathogenesis

(E3) Lung cancer develops in a series of multiple steps extending over years. Conceptually, this is divided into 3 phases: initiation (accumulation of genetic and potentially epigenetic changes); promotion (selective growth of cells with these changes over normal cells); and progression (development of invasive cancer and the metastatic phenotype). It is important to consider the concept of “field carcinogenesis” where clones of multiple independent preneoplastic lesions occur within the lung stochastically or after exposure to carcinogens including radiation.

(E4) The above steps are associated with numerous genetic and epigenetic lesions caused by exposure to cigarette smoke, carcinogens including radiation, and inter-individual variation in risk of developing lung cancer. It is considered that multiple genetic and epigenetic abnormalities must occur before a lung tumour becomes clinically evident (Minna et al., 2002; Sekido et al., 2003; Zochbauer-Muller et al., 2002). Alterations can occur at the genetic level through chromosomal changes such as large or small gains and deletions, and at the nucleotide level, or through epigenetic changes such as DNA methylation. There have been many detailed studies characterising the number and type of both genetic and epigenetic

changes, as well as the relationship to smoking and geographical and gender influences which help to provide quantitative estimates of the number of steps involved (Girard et al., 2000; Toyooka et al., 2004; Toyooka et al., 2001; Toyooka et al., 2003; Zochbauer-Muller et al., 2001). The genetic changes in lung cancer include activation of oncogenes and other growth promoting genes, and inactivation of tumour suppressor genes (Hanahan and Weinberg, 2000). Also, it will be important to test for the other potential changes, as well as for variables such as gender, race, and environmental exposure differences.

E.2. Radiation induction of lung cancer in humans

(E5) Compared with smoking, radiation is much less potent, but it does contribute to the development of lung cancer. The lung is one of the most sensitive tissues to induction of cancer and ICRP gave a tissue weighting factor of 0.12 to the lung (ICRP, 2007). Two sources of information for radiation related lung cancer cases come from A-bomb survivors with external radiation, and miners and the public with internal exposures to radon.

(E6) The A-bomb survivor data include a large cohort, long follow-up and relatively good dosimetry. The dose response for lung cancer incidence among A-bomb survivors is linear for lung doses up to 1.5 Gy with a gender-averaged ERR of 0.81 per Gy (Preston et al., 2003a). The ERR for females is larger than for males by a factor of 4-5.

(E7) Radon is an important environmental risk factor known to be associated with lung cancer, and UNSCEAR assembled publications to assess the effects of radon in animal model systems and in humans (UNSCEAR, 2006). The assembled studies implicated radon in cancer of the lung but not that of other tissues. A pooled analysis of 13 European case-control studies demonstrated a linear dose-response for lung cancer incidence against residential radon concentration up to 1,200 Bq/m^3 with an ERR of 0.16 at 100 Bq/m^3 (Preston et al., 2003a). When assessed separately for cancer types, ERRs per 100 Bq/m^3 were 0.31, 0.06 and 0.04, for small-cell lung cancer (SCLC), ADC and squamous cancer, respectively.

(E8) ICRP *Publication 115* (2010) on radon and progeny examined the risk for lung and other cancers after either residential exposure or exposures of underground miners. A compelling case was made for the induction of lung cancer and leukaemia as a result of residential and underground mining exposures, but not for other solid tumours. For underground miners, based upon recent analyses of Czech and French mining cohorts (where exposures were considered low, and where exposure estimates were of good quality), a significant association between lung cancer and low-level radon exposures was described with ERRs of 2.0 and 3.4 per 100 working level months (WLM) (Tomasek et al., 2008; Vacquier et al., 2009). Most studies of underground miners do not take into account smoking status. However, *Publication 115* noted that the estimated ERR for smokers is generally larger than for non-smokers. One study suggested that there was a relationship between the increasing ERR per 100 WLM and the increasing number of cigarettes smoked daily (Villeneuve et al., 2007). *Publication 115* summarised the estimates of ERR per 100 WLM. Although there are variations in study populations, data in three studies used for the analysis (Lubin et al., 1995; UNSCEAR, 1999) were highly concordant, with ERRs per WLM of 0.49, 0.59 and 0.59, respectively. These studies used the largest numbers of cohorts and the largest numbers of miners. At sufficiently high exposures, an inverse effect of exposure rate was also observed (Lubin et al., 1995) (see below).

E.2.1. Effects of age at exposure and age since exposure

(E9) Analyses of the lung cancer incidence among A-bomb survivors demonstrated that lung is one of the few sites for which the ERR increased with increasing age at exposure (Preston et al., 2003b). Thus, the survivors exposed at the age of 40 years showed a higher lung cancer risk than those exposed at the age of 10 years. This is interesting since other solid cancers exhibit the opposite age-at-exposure effect where a younger age-at-exposure gives a higher ERR. Part of this lung-specific age-at-exposure effect could possibly be biased by a change in the smoking habit. Smoking was considerably less prevalent among those with older ages at the time of bombing than among the younger people. Indeed, an analysis for a sub-cohort of A-bomb survivors with information on smoking did not reproduce an increase of the ERR with age at exposure (Preston et al., 2003b). A high ERR of lung cancer after radiation exposure decreased with attained age (Preston et al., 2003c). In contrast, EAR increased drastically with attained age, and decreased with age at exposure.

(E10) The BEIR VI (1999) analysis found a decrease of the ERR with attained age for lung cancer risk after exposure to radon. In the age group 75 years or older, the ERR was lower than in the age group younger than 55 years by a factor of about four (Preston et al., 2003b). The pooled study of Czech and French miner cohorts also demonstrated the dependence of the ERR on time after exposure with a decrease in the ERR by 55% per decade (Preston et al., 2003b). Thus, the trend of decreasing ERR by attained age since exposure is shared in lung cancers associated with both low-LET A-bomb radiation and high-LET radon-related α-particle radiation.

E.2.2. Effect of smoking

(E11) Smoking interacts multiplicatively with radiation in the case of radon. Thus, the RR of radon for non-smokers is 0.16 per 100 Bq/m^3. While smokers exhibit roughly a 20 times higher incidence of lung cancer than non-smokers, their RR for radon is the same as that of the non-smokers (Preston et al., 2003b). As for low-LET A-bomb radiation, the effect of smoking was once thought to be additive (Preston et al., 2003b). However, recent re-analyses of the LSS cohort has indicated the generalised interaction model in which the joint effect of radiation and smoking is super-multiplicative for light to moderate smokers with less than a pack of cigarettes a day, while the effect was additive or even sub-additive for heavy smokers (Furukawa et al., 2010).

E.2.3. Data on different external/internal/high-LET/dose-rate exposures

(E12) Radiation carcinogenesis is assumed to operate as though there is no threshold dose. However, quantification of that risk for any cancer is problematic, and there are very large uncertainties. Mullenders et al. (2009) described current biophysical modelling and some of the challenges for researchers examining low dose or protracted radiation exposures (Mullenders et al., 2009). Up to a decade ago, the two main sources of information for lung cancer risk from exposure to ionising radiation for humans were A-bomb survivors, who were exposed to external radiation, and uranium miners, whose main exposure pathway was inhalation of radon. In the past years, our knowledge on lung cancer risk after exposure to ionising radiation was improved considerably by studies of lung cancer related to residential radon, and by studies of the Mayak workers, who were exposed to plutonium and external radiation.

(E13) *A-bomb survivors.* The latest analysis of site-specific cancer incidence among A-bomb survivors from Hiroshima and Nagasaki includes 112,952 cohort members at the start of the reporting period on 1 January 1950 (Preston et al., 2007). At the end of the reporting

period (31 December 1998), 52% of them were still alive. In total, 1,759 cases of lung cancer were registered, 43% of them among females. Histological confirmation was obtained for 73%. Similarly to other cancer registers in Japan, the lung cancer incidence rate among A-bomb survivors increased from 25 (7.5) per 105 males (females) in the period 1975-1979 to 37 (11) per 105 males (females) in 1995-1999. About 117 of the lung cancer cases were estimated to be related to radiation exposure from the A-bomb detonation. This amounts to 14% of all cancer cases that were attributed to radiation exposure. The role of smoking in this cohort is limited to one analysis to date (Pierce et al., 2003). Lung cancer among A-bomb survivors is significantly associated with lung dose with no evidence of non-linearity over the 0- to 2-Sv dose range (Preston et al., 2007). Lung cancer incidence has a similar ERR per dose as mortality (Preston et al., 2003b). The ERR for females is larger than for males by a factor of 2 for mortality and a factor of 5 for incidence. The latter value is higher than for all other site-specific cancer incidence data among A-bomb survivors.

(E14) Animal studies of lung cancer from external radiation sources have compared γ-irradiation with fast or fission neutron exposures at varying dose rates (Ullrich, 1983; Ullrich et al., 1979; Ullrich et al., 1977; Ullrich and Storer, 1979; Upton et al., 1970). While these studies examined the neoplastic process overall, data specific to lung cancers were generated across different strains of mice. RBE values of 15-20 were determined for acute neutron exposures while chronic neutron exposures were less effective at low total doses but more effective at high total doses. Subsequently, Storer et al. (1988) determined that the RR for lung cancer was not significantly different between humans and mice.

(E15) A comparison of neutrons and γ-rays in a rat model determined an RBE of 30-40 at a neutron dose of 0.1 Gy and more than 50 if the neutron dose was reduced to 0.016 Gy (Lafuma et al., 1989). Subsequently, both a two-step and a two-mutation model were proposed as models for lung tumours in rats exposed to radon (Heidenreich et al., 1999; Moolgavkar et al., 1990). In both models, it was assumed that radiation is associated with the first step and less so with the second step. In addition, an inverse dose rate effect was described for neutrons (Heidenreich et al., 1999). Other animal studies that focus on the spectrum of mutations or other molecular events that may drive lung cancer induction after the inhalation or radon or other isotopes are described below.

(E16) *Miners.* In 1999, the BEIR VI Committee gave a comprehensive overview and analysis of data on lung cancer related to exposure to radon (1999). BEIR VI based its risk models on analyses of combined data from 11 miner studies. Data were available for 60,606 miners including 2,674 lung cancer cases. BEIR VI developed sophisticated models to describe effect modification.

(E17) ICRP *Publication 115* (2010) tabulated the lifetime excess absolute risk (LEAR) generated from models that included multiple cohorts of miners. Tomasek et al. (2008) derived similar LEAR values using the risk model from *Publication 65* while using background reference rates from *Publication 60* and *Publication 103*. However, if the BEIR VI model including time since exposure (TSE), age and concentration was used, the LEAR nearly doubled from 2.8×10^{-4} to 5.3×10^{-4} per WLM. By using the Czech-French risk model (Tomasek et al., 2008) that considered to have the best quality of exposure assessment, the LEAR dropped to an intermediate value of 4.4×10^{-4} per WLM. *Publication 115* argued that the increase in LEAR is the result of the consideration of low dose rate exposures and the increased ERR per WLM of recent studies as mentioned earlier. As described in *Publication 115* (2010), other independent calculations confirmed the BEIR VI and Czech-French modelling, while inclusion of analyses of more recent single groups generates a range of LEAR of $3\text{-}7 \times 10^{-4}$ per WLM. *Publication 115* notes the sensitivity of such models regarding sex and ethnic background, for which background lung cancer risks can vary. The

Commission now recommends a LEAR of 5 x 10^{-4} per WLM for radon and progeny-induced lung cancers, instead of the *Publication 65* value of 2.8 x 10^{-4} per WLM.

(E18) BEIR VI used models in which the ERR depends linearly on exposure. For miners, exposure was specified by a quantity related to the cumulated concentration of short-lived progeny of radon at the workplace using the unit 'WLM'. Assuming some typical conditions, an exposure rate of miners of 1.6 WLM $year^{-1}$ corresponds to the exposure by residential radon with a concentration of 400 Bq m^{-3}. Thus a 30-year exposure at this level corresponds to about 50 WLM. For exposures up to 50 WLM, no dependence of the risk on exposure rate was detected. At higher exposures, however, BEIR VI found an inverse dose-rate effect, i.e., for the same exposure level, a lower exposure rate or a longer lasting exposure causes a higher lung cancer risk.

(E19) The analysis of the German Wismut miner data was also based on a linear dependence of the lung cancer risk on exposure (Grosche et al., 2006). The resulting ERR-per-exposure value of 0.21 (95% CI: 0.18, 0.24) per 100 WLM was lower than the BEIR VI result by a factor of more than three. The presence of an inverse exposure-rate effect at high exposures was confirmed. After adjustment for smoking and asbestos exposure, the result for the ERR per exposure in the Wismut case-control study was even lower than in the cohort study by a factor of two (Brüske-Hohlfeld et al., 2006). An exposure-rate effect was not detected in that study.

(E20) UNSCEAR (2009) calculated a weighted mean average ERR per exposure from the results of 9 large miners studies (excluding the Wismut cohort). The studies included a total of 3,380 lung cancer cases. An estimate of 0.59 (95% CI: 0.35, 1.0) per 100 WLM was obtained.

(E21) *Residential radon*. Darby performed a collaborative analysis of individual data from 13 case-control studies in Europe (Darby et al., 2005). Before starting most of the single studies, considerable efforts were made to have comparable study designs. The analysis included 7,148 persons with lung cancer and 14,208 without lung cancer. The mean concentrations of residential radon were assessed for the period of 5 to 34 years before the diagnosis of, or death due to, lung cancer, and for the corresponding time period for the control persons. The strengths of the study include the large number of lung cancer cases, the homogeneity of the study designs, and the detailed information on smoking behaviour including analyses performed using stratification by smoking history. Relative to a radon concentration of 0 Bq m^{-3}, the ERR was significant in the radon concentration category 100-199 Bq m^{-3}, and in the two categories with radon concentrations exceeding 400 Bq m^{-3}. The estimate of the ERR per radon concentration was 0.08 (95% CI: 0.03, 0.16) per 100 Bq m^{-3}. The relation remained significant when the analysis was limited to measured radon concentrations smaller than 200 Bq m^{-3}. Correcting for a bias due to uncertainties in the measured radon concentrations doubled the ERR-per-radon-concentration estimate.

(E22) There was no evidence for a threshold or a non-linear response to the radon concentration (Darby et al., 2005). The upper 95% CI for a threshold was 150 Bq m^{-3}. For males, the best estimate of the ERR per radon concentration was higher than that for females by a factor of four, although it was not significant. The results of a North American pooling study (Krewski et al., 2005) on the ERR per radon concentration are consistent with the European pooling study, when uncertainties in measured radon concentration were taken into account. *Publication 115* (2010) described the pooled analysis of three residential case control studies including Europe, North America and China, and estimated that the risk of lung cancer increased by at least 8% for radon concentrations of 100 Bq/m^3, with an ERR of 0.16 per 100 Bq/m^3 increase. These values take into account an exposure period of at least 25 years. Furthermore, these values are considered to be robust enough on their own to provide

protection of the public without the use of dose conversions based on underground miner exposures as was suggested in ICRP (1993).

(E23) *Mayak workers*. The Mayak Production Association in Southern Urals was established to produce plutonium for the nuclear weapons of the former Soviet Union. Sokolnikov et al. (2008) evaluated lung cancer in a cohort of 17,740 Mayak workers, who were initially hired during the period 1948-1972. Among them, 3,924 were exposed to external radiation only, for 5,572 the lung dose from α-radiation was assessed based on measurements of plutonium in the urine, and 8,244 were potentially exposed to plutonium but not monitored. By the end of 2003, 681 lung cancer deaths had occurred. External radiation was related to 8.6% of cases, while internal radiation from incorporated plutonium was related to 29% of cases. For external exposures, excess risks depended linearly on lung dose without any evidence for a threshold (Jacob et al., 2009; Sokolnikov et al., 2008). The ERR per dose was compatible with the corresponding result for A-bomb survivors.

(E24) For plutonium exposures, Sokolnikov et al. (2008) found, in a non-parametric analysis of lung cancer mortality among Mayak workers, no excess risks for lung doses smaller than 100 mGy, a non-significant excess risk in the dose range of 100-200 mGy, and significantly increased risks for larger doses. In a recent analysis of a sub-cohort of male workers with information on smoking and alcohol consumption, the description of the data by a linear ERR model was significantly improved by introducing a dose threshold in the range of 100-200 mGy (Jacob et al., 2009). An even better description was obtained by a model of carcinogenesis, in which the dose rate dependences of the initiation rate and of the hyperplastic growth rate had a threshold in the order of 10 mGy year^{-1}.

(E25) *Summary*. For external exposures, there is no evidence for a deviation from an LNT description of the excess lung cancer risk versus lung dose, and this is also the case with most studies on internal exposures to α-radiation. However, a recent analysis of lung cancer mortality among Mayak workers showed evidence for a threshold in the dose response at about 100 mGy, or in the dose-rate response at about 10 mGy year^{-1}. A weighting factor of 20 was proposed for radon by the ICRP (2007). However, the direct measurement of radiation dose given by inhaled radon to the target cells in the respiratory tract was not considered possible. *Publication 115* (2010) reviews effective dose estimates on radon but does not address the potential for threshold effects. Published values are listed for effective dose from inhalation of radon and progeny using various dosimetric models for indoor, outdoor, home, workplace and mines. The effective dose is dependent upon a number of factors but appears to be age insensitive. Using the human respiratory tract model, values of effective dose per WLM ranged from 10–20 mSv. For homes and mines, the effective dose was 13 mSv per WLM, while it was 20 mSv per WLM for indoor workplaces, when the breathing rate was adjusted to that of a standard worker. The Commission concluded that doses from radon and progeny should be calculated using ICRP biokinetic and dosimetric models, which will allow doses to be calculated to other organs besides the lung. Also, dose coefficients, equilibrium factors and aerosol characteristics for domestic and occupational exposure conditions would be provided subsequently.

E.3. General architectural features of lung tissue

(E26) Airflow passes through the upper respiratory tract (nasal passages, pharynx and larynx) and then enters the lower respiratory tract. The airflow then enters a series of between 221 and 223 branches of the adult human airway (Fig. E.1.) covered by a pseudostratified columnar epithelium in the larger branches (trachea and larger bronchi) of the conducting system of the lung (Mercer et al., 1994; Weibel, 1963, 1997). As these branches in the human

lung become smaller, the epithelium becomes more cuboidal. Eventually, the smallest branches merge with the alveolar epithelium where air exchange occurs (Fig. E.2.).

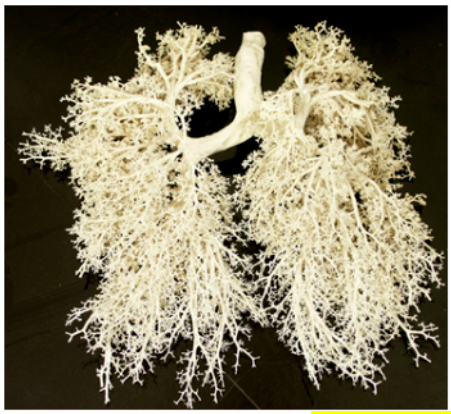

Fig. E.1. The adult human airway. (permission needed)

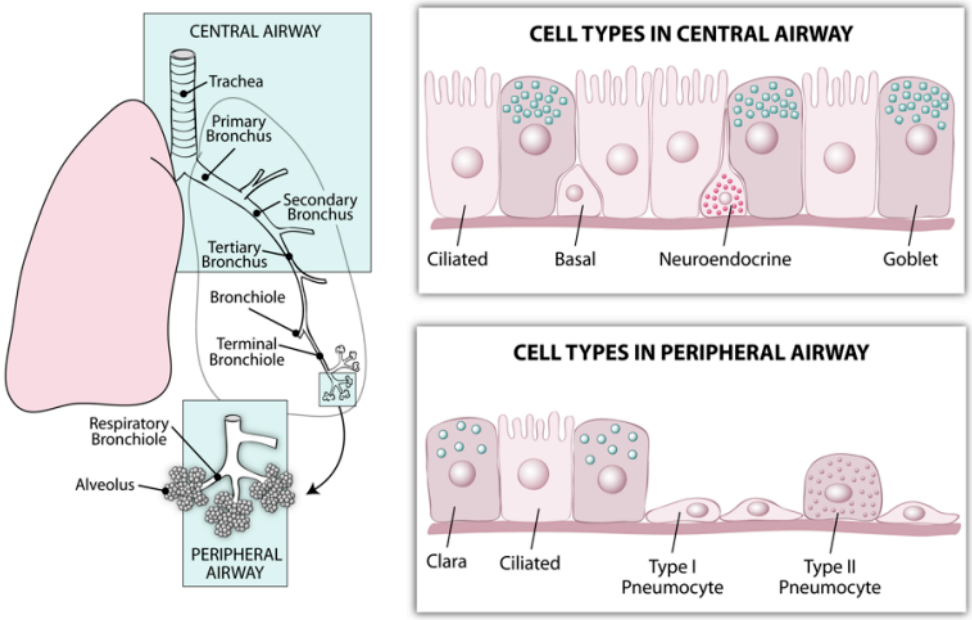

Fig. E.2. Illustrations depicting the major transitions in the human lung from large bronchi (central airway) down to respiratory bronchioles connecting to the air exchange cells of the alveloli (peripheral airway). The cells lining the airways include ciliated and goblet cells in the central airway. The basal cell is believed to have stem cell characteristics. The neuroendocrine cells may be involved in small cell carcinoma. In the peripheral airways, there is a variety of types of Clara cells in addition to cuboidal ciliated cells. In the alveoli, there are type I and type II pneumocytes. The stem cell(s) of the peripheral airway are still somewhat controversial. (permission needed)

(E27) The continuous layer of epithelial cells lining the conducting exchange portions of the lung (termed the respiratory epithelium) plays a critical role in maintaining efficient clearance of mucous, in defense processes, as well as for the passage of air (Knight and Holgate, 2003). There are about 30,000 terminal bronchioles (Lane et al., 2007) consisting of Clara domed-shaped cells, which have a protective and detoxifying function, and simple low columnar ciliated cells. As the terminal bronchioles branch into respiratory bronchioles which are contiguous with the septated saccular alveolar structures, the conducting portion of the

airways becomes the respiratory airways (where gas exchange occurs). In summary, at each terminal bronchiole, several respiratory bronchioles emerge, and up to 11 alveolar ducts arise from each respiratory bronchiole. These alveolar ducts each finally branch into about 5-6 alveolar sacs. To provide maximal surface areas for gas exchange to occur, there are about 300 million alveoli in the human lung giving a surface area of about 80 m^2. The lung is also unique among all internal organs by the way it is exposed directly and continuously to the surrounding atmosphere in which hostile agents are often abundant. This could influence the susceptibility of the lung parenchyma to inflammatory responses. Finally, the weight of the lungs is about 1.4 kg, and only the skin (2.7 kg), the liver (1.8 kg), and the blood (~7% of total body weight) are heavier organs.

(E28) There are three cell types that are found within the alveolar spaces (Type I and Type II pneumocytes, and alveolar macrophages). Type I pneumocytes are squamous cells that cover over 95% of the alveolar wall, and lie in close apposition to the endothelial cells of small capillaries where gas exchange occurs (Fig. E.2.). More cuboidal Type II pneumocytes are interspersed among the Type I pneumocytes, and they secrete several surfactant proteins that reduce the surface tension within the alveolar sacs. In addition, the type II cells may have stem cell characteristics and help replenish damaged type I cells. The alveolar macrophages do not attach to the alveolar wall and serve to engulf particles and infectious agents that have penetrated the alveoli. They may also be important in mediating inflammatory responses.

(E29) While most of our knowledge of adult stem cells is based on experimental evidence on the cells dedicated to the renewal of tissues such as the skin, gut and bone marrow, there is mounting evidence that organs such as the lung, liver, and pancreas, which turn over more slowly, may use alternative strategies, including the self-renewal of more differentiated cells (Neuringer and Randell, 2004; Rawlins and Hogan, 2006). The response of these organs to radiation may also reveal the potential of differentiated cells to act as stem cells. The lung shows both slow turnover and rapid repair. New experimental approaches are needed to identify putative lung stem cells and strategies of lung homeostasis and repair. The terms "stem cell" and "progenitor cell" are well defined in tissues with rapid turnover. Another term that may be more appropriate for tissues with minimal turnover, except in cases of damage, may be "reparative cells".

(E30) Several putative adult lung epithelial stem or reparative cells have been identified in mouse model systems. However, the *in vivo* capabilities of these putative stem cells to contribute to different lineages, and their control mechanisms, remain unclear.

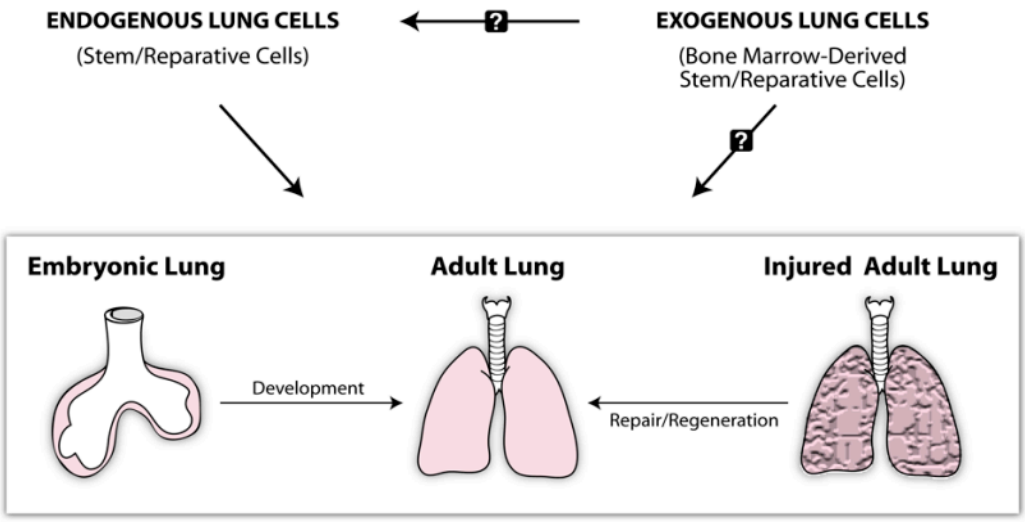

Fig. E.3. One model of lung homeostasis (which is still controversial), includes endogenous cells for normal lung turnover which is very slow, and both endogenous and exogenous cells that may be required in the cases of acute injury to the lung. (permission needed)

(E31) Many seminal studies with new isolation and characterisation techniques suggest that stem cells exist in the adult lung. The picture that is emerging from mouse and rat models is that different regions of the respiratory system, the trachea/large airways, and the distal bronchioles and alveoli, use different populations of stem cells and strategies for maintenance and repair (Fig. E.2.). Moreover, there is evidence that differentiated epithelial cell types are able to proliferate and trans-differentiate in response to some conditions. However, the precise mechanisms involved in any of these processes are only beginning to be understood (Fig. E.3.).

E.3.1. Tissue turnover rate

(E32) The steady-state turnover of epithelial cells in the lung and trachea is highly relevant to investigations of endogenous adult stem cells, and the potential target cells of radiation-associated carcinogenesis. Early work indicated that the airway epithelium is a dynamic tissue normally undergoing slow, but constant renewal. On the basis of studies in experimental animals and limited studies in humans, the airway epithelium was believed initially to renew every 30 to 50 days (Bowden, 1983). Upon injury, the airway epithelium responds rapidly to reestablish an epithelial sheet with normal structure and function, with resident cells thought to be the source of the new cell population (Erjefalt 1995; Erjefalt and Persson, 1997). Although there may be some contribution from circulating stem/progenitor cells, most evidence supports the concept that stem/progenitor cells distributed throughout the airway epithelium are the source of the new epithelial cells, and that these stem/progenitor reparative cells have the potential to differentiate into all of the cell types of the normal epithelium (Rawlins and Hogan, 2006; Kim 2005). At present, there is no definitive understanding of progenitor cell–progeny relationships in the airway epithelium. In addition, there is not much evidence if “rare” lung epithelial stem/progenitor cells residing in specific niches (e.g. basal cells in the larger central airways and Clara or rarer c-kit^{+} cells in the peripheral airways) proliferate, migrate, and differentiate in a highly orchestrated process similar to classical models of high turnover tissues.

(E33) Recent studies have reexamined the average lifetime of different cell types in the airways. Using a Cre/loxP genetic technology to label a random fraction of ciliated cells throughout the airways of a cohort of mice, investigators followed the fate of these cells for 18 months (Rawlins and Hogan, 2008). The results demonstrated that ciliated airway epithelial cells are a terminally differentiated population with a significant number of LRCs still being present at the end of the experiment. The results demonstrated that the average half-life of ciliated cells in the trachea is 6 months and approximately 17 months in the remaining compartments of the lung. Thus, in the absence of acute or chronic damage, the residence time in the central and peripheral airway cells is much longer than previously estimated (Rawlins and Hogan, 2008).

E.3.2. Age dependence: Lung development

(E34) Based on morphology at different stages during embryonic and fetal development, the following major stages of lung development have been described (Fig. E.4.). The stages include the embryonic, pseudoglandular, canalicular, saccular and alveolar periods. The embryonic stage (Fig. E.4., top) is when the lung bud emerges from the primitive foregut to produce the trachea and major bronchial branches. The early growth of the emerging lung buds is regulated by a series of growth factors and transcription factors including FGF10, forkhead box A2 (FOXA2), TITF1, Sprouty, SHHH and TGFβ signalling. A pseudoglandular stage between the embryonic and canalicular periods is when the lung resembles an endocrine gland and branching continues to form bronchioles. At this stage, there is initiation of formation of ciliated and goblet cells. In the canalicular stage (Fig. E.4.), alveolar ducts containing type I pneumocytes first appear. The saccular stage initiates the time when alveoli ducts become sac-like structures which contain both type I and II pneumocytes. Finally, around birth and continuing after birth, the alveolar stage (Fig. E.4., bottom) involves the final maturation of the alveolar sacs, including septation of the alveoli by the surrounding mesenchyme and maturation of the vasculature surrounding the alveoli. Surfactant protein, which reduces surface tension, is secreted by the type II cells of the alveloli and Clara cells of the smaller bronchioles.

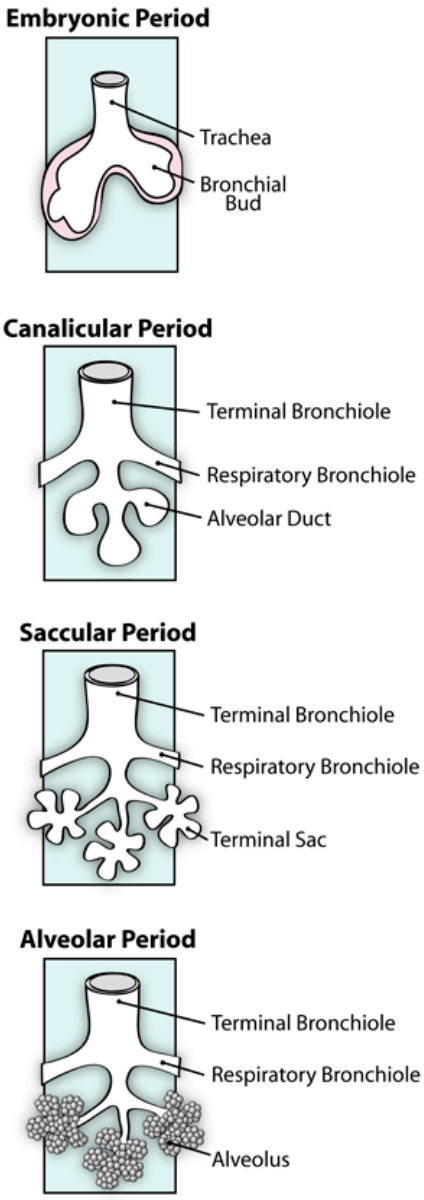

Fig. E.4. Development of the mammalian lung goes through specific stages. (permission needed)

E.3.3. Cellular features

(E35) *Basal cells in the lung central airways*. Until recently, somatic stem cells in the pseudostratified mucociliary epithelium of the large airways remain largely unknown because of its cellular complexity, low baseline proliferation, and potential cellular plasticity of different cell types participating after injury. However, identification and isolation of specific cell populations from the trachea using advanced techniques are leading to the consensus that at least one major class of lung stem cells, the basal cell, may represent at least one type of lung stem cells (Fig. E.5.).

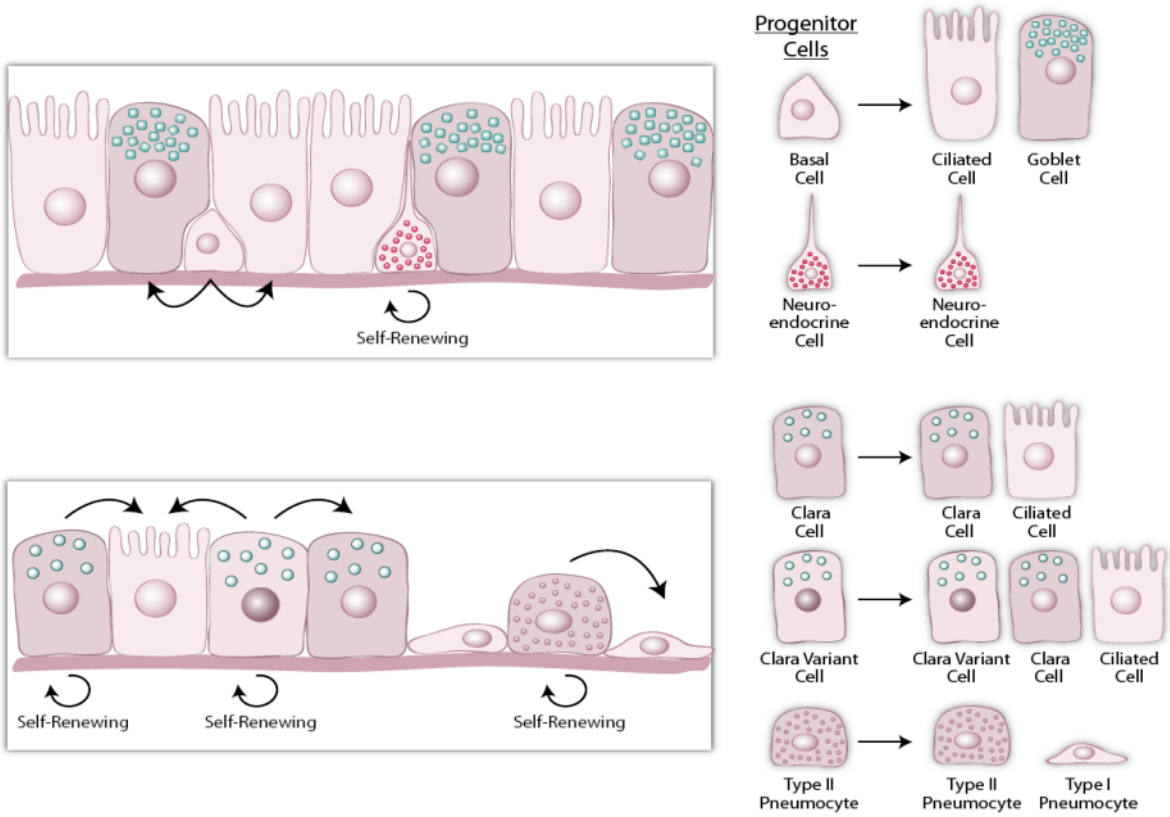

Fig. E.5. There is solid evidence that basal type cells in the central airways can differentiate into both ciliated and goblet cells. Much less is known about the neuroendocrine cells, but they are believed to be capable of self-renewal and only into the additional neuroendocrine cell. It is more complex and controversial in the peripheral airways as to which cells have stem cell characteristics. There is evidence for a Clara or Clara-variant cell being important as well as type II cells dedifferentiating upon acute injury. More recently, there is evidence that a rare c-KIT$^+$ cell may exisit in niches in the distal airway and be responsible for replacing most, if not all the cells of the peripheral airways. (permission needed)

(E36) Basal cells make up approximately 30% of the pseudostratified mucociliary epithelium of the large airways of the lung (Rock et al., 2009). They are considered to be undifferentiated, express p63, and express K5/K14 (Daniely et al., 2004; Evans et al., 2001; Schoch et al., 2004). In the rodent, basal cells are confined to the trachea, where they are interspersed among the ciliated, secretory (goblet), and neuroendocrine cells (Fig. E.5.). In contrast, in the human lung, basal cells may be present throughout the airways, including small bronchioles (Boers et al., 1999; Evans et al., 2001; Nakajima et al., 1998).

(E37) While cell turnover in the adult trachea is normally very low (Kauffman, 1980), epithelial injury elicits rapid proliferation of surviving cells, except ciliated cells (Rawlins et al., 2007), and the tissue is soon repaired. Several lines of evidence suggest that tracheal basal cells function as stem cells for this repair. First, lineage tracing of K14-CreER–expressing cells after naphthalene injury, which depletes secretory goblet cells, demonstrates that some basal cells can both self-renew and give rise to ciliated and goblet cells (Hong et al., 2004). Second, a subset of basal cells retain BrdU label in the long-term after epithelial damage by SO_2 inhalation (Borthwick et al., 2001). Third, basal cells isolated on the basis of high expression of a K5-GFP transgene have a greater capacity to proliferate and give rise to larger colonies *in vitro* compared to K5-GFP$^-$ cells (Schoch et al., 2004). Fourth, fractionated rat tracheal basal cells can restore the entire epithelium of a denuded trachea in a xenograft

model (Randell et al., 1991). Finally, isolated basal cells of the mouse trachea function as progenitor cells both during postnatal growth, in the adult at steady state, and in a repair model. Using a clonal assay, basal cells of both mouse and human airways can self-renew and differentiate in the absence of stroma or columnar epithelial cells (Rock et al., 2009). Basal cells do not produce neuroendocrine cells, and these appear to be another type of stem cell in the lung, but neuroendocrine cells appear to give rise only to other neuroendocrine cells (Fig. E.5.).

(E38) *BASCs, Clara, Clara variant, and facultative stem cells of the peripheral airways*. The existence of distinct proximal (tracheobronchial, large bronchiolar) and distal (smaller bronchiolar and terminal bronchiolar) stem cells hierarchies suggests that discrete regenerative units maintain the conducting airway epithelium. Characteristics of the proximal and distal epithelial compartments are potentially a reflection of distinctions in the properties of region-specific stem cells, as well as the influence of the niche/microenvironment on the activity of these cells. While in the trachea and large bronchi, the basal cell is considered to be the main stem cell type, in the more distal airways (small bronchi and bronchioles), the epithelium changes from columnar to cuboidal and Clara cells predominate over ciliated cells (Fig. E.5.). Importantly, there are no basal cells in mouse or rat peripheral lung, so that they are unlikely to be involved in turnover and repair (Pack et al., 1981; Randell et al., 1991) of the small bronchioles or alveoli. However, in the human lung, some type of basal cells may persist in the distal airways, although this remains uncertain.

(E39) The most distal region of the lung is organised into a complex system of alveoli, where there are two types of epithelial cells. Type I cells (also called type I pneumocytes), provide the thin-walled gas exchange surface. Type II pneumocytes contain numerous secretory vesicles (lamellar bodies) filled with surfactant material, including surfactant protein C. There is evidence that type II pneumocytes are another peripheral stem cell of the lung, but that type II cells give rise only to other type II cells. Also, when there is acute damage, type II cells are capable of replicating and differentiating into type I cells (Fig. E.5.).

(E40) The transitional region between the terminal bronchioles and the alveoli is known as the bronchioalveolar duct junction (BADJ). There is a variety of cell types at this junction: one such cell type expresses SCGB1a1, also known as Clara cell 10-kDa protein (CC10) or Clara cell secretor protein (CCSP), and is often used as a marker for Clara and Clara variant cells. There have been many studies of SCGB1a1 expression in relation to cell lineages in mice, but in some instances, the definition of the Clara cell has become confusing, and there may be more than one type of Clara cell (Fig. E.5.). If Clara cells are morphologically defined as domed cuboidal, nonciliated cells of the simple bronchiolar epithelium as originally described, then SCGB1a1-expressing secretory cells in the pseudostratified portions of the airway are distinct from bronchiolar Clara cells. However, Clara cells are commonly defined based on SCGB1a1 expression. This suggests that Clara cells likely represent a spectrum of cells, present in different airway levels, with different stem cell potentials. A generic term for this spectrum of cells is a facultative stem cell. This term refers to a differentiated cell that is normally quiescent but responds to injury by dividing and self-renewing, and giving rise to progeny that differentiate into one or more cell types.

(E41) After injury by oxidant gases that damage ciliated cells, surviving bronchiolar Clara cells proliferate to restore the bronchiolar epithelium (Evans et al., 1986); but this observation, in itself, does not provide evidence for a stem cell hierarchy within the Clara cell population. When naphthalene is administered to mice, almost all Clara cells are killed due to selective metabolic activation of the toxin. Ciliated cells shed their cilia and cover the denuded bronchiolar basement membrane, and the few surviving Clara cells (some term these Clara variant cells) then proliferate (Van Winkle et al., 1995). The naphthalene-resistant progenitor

cells represent a subset of Clara cells that are label retaining thus suggesting that this may constitute the actual distal lung stem cell (Hong et al., 2001). After naphthalene injury, pulmonary neuroendocrine cells also proliferate (Stripp et al., 1995), but they are a distinct lineage system not requiring nor generating Clara cells (Reynolds et al., 2000). These are similar to the neuroendocrine cells found in the larger airways. An epithelial stem cell niche has been identified in the zone where airways terminate and form alveoli (Giangreco et al., 2002; Hong et al., 2001). Specific cells in the bronchiolar alveolar junction, co-express SCGBa1a, the type II cell marker surfactant protein C, Sca-1 (Kim et al., 2005), and are negative for CD45 and CD31.

(E42) The putative mouse BASCc proliferate in response to naphthalene or bleomycin injury, and demonstrate a high clonal growth capacity and differentiation potential to form both Clara cells and distal lung epithelium composed of cells expressing type I or type II cell markers (Kim et al., 2005). There are only limited data suggesting that BASCs can form type I or II cells, and these are based on cell markers. There are no direct *in vivo* data demonstrating that isolated BASCs can form alveolar cell types, and it is entirely possible that type II cells may dedifferentiate upon damage and resemble BASCs. In mouse models of lung cancer, BASCs expand when an active K-ras oncogene is induced to generate ADCs (Kim et al., 2005). Thus, Clara, Clara variant, BASCs, or facultative stem cells exhibit transit stem cell characteristics after injury, and may contribute to lung cancer. This is supported by Xu et al. (2012), where, using a Cre-knock-in approach to express a mutant K-Ras in $CC10^{+}$ epithelial cells and surfactant protein C-positive type II alveolar cells in an adult mouse, type II cells, Clara cells of the terminal bronchiole and BASCs were identified as the cells of origin for K-Ras-induced lung hyperplasia. Interestingly, only type II cells progressed to ADC.

(E43) In summary, an area of much uncertainty is if Clara, Clara-variant, or BASC cells can actually give rise to type I or II cells. However, the regeneration of type I and II cells after injury using a transgenic mouse model where Scgb1a1-expressing cells and their progeny can be labelled with the enhanced GFP (EGFP) was described (Zheng et al., 2012). They argued that these EGFP-expressing progenitor cells are most likely Clara cells and it is these cells that respond to injury of the alveolar epithelia after bleomycin exposure. Given the similarity of the lung response between bleomycin and radiation injury, it is conceivable that the same model would be appropriate to test the radiation response.

(E44) It is also important to determine if similar multipotent cells exist in the human bronchiolar–alveolar duct junction zone, as it does in the mouse. More detailed *in vivo* molecular and cellular characterisation of BASCs, other putative lung stem and progenitor cells, and differentiated cells, is needed to determine the lineage relationships in the adult human lung. The studies conducted so far highlight the existence of region-specific stem cells in the conducting airways and the complexity of analysing the differentiation potential of stem cells at different sites. While most knowledge about peripheral lung stem cells has been obtained using mice, a major deficiency is our understanding of these processes in the human lung. Recently, but controversially, there is evidence for the existence of a human lung stem cell expressing c-KIT that may be able to replace all the cells in the distal lung (Kajstura et al., 2011). This cell type is proposed to reside in rare niches in the distal lung and also to be capable of regenerating vascular endothelial cells after lung injury. These $c\text{-}KIT^{+}$ cells also express the same transcription factors that are present in ES cells (Nanog, Oct 3/4, KLF4 and Sox2), suggesting that they may actually be exogenous cells from the bone marrow.

(E45) Human lung diseases such as cystic fibrosis or chronic obstructive pulmonary disease, idiopathic pulmonary fibrosis, as well as the most common forms of lung cancer in the US, may involve bronchiolar and alveolar cell defects. It is likely that the delicate balance

of stem, progenitor, and differentiated cell functions in the lung is critically affected in patients with these devastating diseases. Thus, the discovery and a complete understanding of putative lung stem cells will lay the foundation for new inroads to understanding lung biology related to lung disease.

(E46) *Lung stem cell summary*. Adult stem cells are still ill defined in the human lung, and the mechanisms that control their proliferation and differentiation are almost completely unknown. Nevertheless, the possibility that lung disorders may one day be treated by manipulating endogenous lung stem cells, or with exogenously applied stem cells, is the focus of much current research effort. However, if this approach is to be successful, it is necessary to understand the normal behaviour of endogenous lung stem cells, and how they are controlled by their environment.

(E47) *Adult marrow-derived cells in lung biology and disease*. There is mounting interest in the role of bone marrow-derived progenitor cells in the repair of massive and chronic lung damage. While there are a number of current controversies regarding assessments of lung chimerism by adult bone marrow-derived cells, at the present time, a consensus on the functional role of adult marrow-derived cells in lung repair and remodelling after injury has not been clarified (Fig. E.3.). It is well established that adult bone marrow cells serve as precursors for HSCs. However, a substantial number of studies report that cell populations derived from bone marrow of adults including HSCs, stromal-derived MSCs and circulating fibrocytes, can localise to and acquire phenotypic and functional markers of mature tissues including lung (Pereira et al., 1995; Suratt et al., 2003) (Fig. E.2.). Whether the bone marrow–derived cells are truly "stem" cells has not been rigorously demonstrated, and most of these studies are controversial. In some cases, the marrow-derived cells may fuse with existing organ-specific cells rather than trans-differentiating into mature organ-specific cells. For example, in humans, lung specimens from some clinical bone marrow transplant recipients demonstrate chimerism for both epithelial and endothelial cells (Mattsson et al., 2004). Similarly, lung specimens from some lung transplant patients demonstrate chimerism of lung epithelium (Kleeberger et al., 2003; Spencer et al., 2005). However, chimerism or lung engraftment with adult marrow–derived cells has not been found in all studies. Irrespectively, even fusion of normal adult marrow–derived cells with irradiated differentiated adult lung tissue may influence our current models of lung cancer risk assessment after ionising radiation.

(E48) In summary, using more recent sophisticated techniques, the current consensus is that local cells within the lung are primarily responsible for maintaining or reconstituting the lung epithelium, and that bone-marrow derived cells contribute few, if any, cells directly to the structure of the airway or alveolar epithelium under normal homeostatic conditions.

(E49) *Molecular characterisation of lung cancer*. As briefly described above, lung cancer develops in a series of steps extending over years. Conceptually, this is divided into 3 phases: initiation, which is the accumulation of genetic and epigenetic changes; promotion, where there is a selective growth of cells with these changes over normal cells; and progression, which leads to the development of invasive cancer and the metastatic phenotype. Lung cancers are of three main types: SCLC; non-small cell lung cancer (NSCLC); and ADC. NSCLC accounts for 85% of all lung cancers. Lung cancer is principally caused by tobacco smoke: 98% of all SCLC patients and 85% of all NSCLC patients smoke. And, while the numbers of lung cancers is decreasing in the US and the UK, in China 2/3 of all adult males smoke and they represent 1/3 of all smokers worldwide. Interestingly, some 20% of ADCs occur in non- or never-smokers, arise predominantly in the peripheral lung, and the development of these tumours is unique from those lung cancers seen in smokers or former smokers (Sun et al., 2007). Epidemiological studies have also identified a major lung cancer

susceptibility locus at 6q23-25 that is associated with a 2.5-fold increased risk (Amos et al., 1999; Bailey-Wilson et al., 2004).

(E50) NSCLC, SCLC and ADCs arise from different compartments of the lung, and are thought to arise from one of two putative stem cells that are found in different locations of the lung and which are responsible for specific differentiated cell types. The basal bronchial cell found in the central region of the bronchial airways can differentiate into ciliated or mucosal cells and can give rise to ADC, NSCLC and SCLC. The BASC, or Clara cell, gives rise to the terminal respiratory unit of bronchioles and alveoli which can give rise to peripheral ADCs.

(E51) There have been extensive molecular and genetic studies of lung cancer (Ding et al., 2008; Sekido et al., 2003; Thomas et al., 2007; Weir et al., 2007) as recent examples which describe genetic and epigenetic changes associated with the development of lung cancer. Indeed, detailed microdissection and molecular analyses of the bronchial epithelium from people with and without lung cancer have described histologically normal bronchial epithelium that contains clones of cells with genetic changes seen along the pathway to lung cancer, while bronchial brushes and sputum of people exposed to cigarette smoke without lung cancer contain DNA methylation (epigenetic) changes (Wistuba et al., 2000, 1997; Zochbauer-Muller et al., 2003). The genetic alterations that occur in the development of lung cancer are substantial. Sekido et al. (2003) describe more than 20 alterations, genetic and epigenetic, in overt lung cancers. These alterations can and should be categorised by the biological consequence of the signalling pathways altered.

(E52) *Tumour suppressor genes*. p53, the most frequently mutated gene in lung cancers impinges upon several signalling pathways (Olivier et al., 2009). Mutations within the DNA-binding region of p53 can result in inactivation, or gain of function mutations can result in mdm2 binding and subsequent p53-mediated proteolysis. There is a distinct mutational spectrum described for smoking. Upstream regulators of p53 include mdm2 and p14. Both loss and gain of function alterations in either protein result in the disregulation of p53. Loss of p14 is a frequent event in SCLC, large cell neuroendocrine cancer (LCNEC) but less so in ADC (Brambilla et al., 1998). Altered p53 signalling has adverse consequences for cell cycle checkpoint control, regulation of either pro- or anti-apoptotic proteins via mitochondrial apoptosis, or death receptor signalling via the FAS/tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) pathway, and DNA repair.

(E53) A lack of retinobalstoma protein Rb is also common to some lung cancers. Rb regulates G_0/G_1 transition via cyclin D, and is the most frequent factor in loss of cell cycle control in SCLC. Interestingly, hyperphosphorylation of Rb is more common in NSCLC. The mutation status of Rb in ADC also reflects a loss of RB function through either mutation, LOH, or hyperphosphorylation (Ding et al., 2008; Weir et al., 2007). Hyperphosphorylation in NSCLC is via loss of p16 or overexpression of Cyclin D1 (40-60% of NSCLC), and p16 activity can be suppressed via methylation, deletion or mutation (Brambilla et al., 1999; Weir et al., 2007). These are early events associated with dysplasia. Cyclins D and E are frequently found amplified in ADC (Weir et al., 2007), and overexpression is an early event in bronchial pre-invasive lesions (Jeanmart et al., 2003).

(E54) *Oncogene addiction*. Oncogene addiction refers to the addiction of a cell to the abnormal function of oncogenic proteins. These oncogenic proteins generally drive cells toward proliferation and subsequent malignancy. In lung cancer, it is members of the RTK family that likely drive oncogenic processes because of their position at the pinnacle of a number of signalling pathways that control cell cycle and proliferation, angiogenesis, evasion of apoptosis, and DNA repair. In particular, the EGFR, one of 4 RTKs that homo- or heterodimerise, allow for a diverse set of cytoplasmic signalling events. These signalling events include activation of the phosphotidylitositol 3-kinase (PI3K) pathway, signal

transducers and activators of transcription (STAT) signalling and the RAS pathway. EGFR is often found amplified in cancer; however in NSCLC, EGFR is often mutated as well as amplified (Eberhard et al., 2005; Hirsch et al., 2003; Shigematsu and Gazdar, 2006). For ADCs, geographical location, never-smoking status and female sex are independently associated with EGFR mutation, and EGFR overexpression is associated with poor prognosis (Nicholson et al., 2001). Mutations in EGFR target the tyrosine kinase domain, and include mutations, deletions, insertions and activating point mutations. A point mutation resulting in an arginine for leucine substution at amino acid 858 (L858R) and a deletion in exon 19 account for approximately 85% of all mutations. EGFR has become a significant biological target for targeted therapy.

(E55) The PI3/v-akt murine thymoma viral oncogene homolog (AKT) pathways, which are regulated by ERBB members, lead to enhanced cell survival and proliferation (Sharma et al., 2007). PI3K catalytic subunit p110α (PI3KCA), along with KRAS is a gene found mutated in many cancers; however, amplification of the 3q25-27 region, which contains PI3KCA, often occurs in NSCLC and especially in SCC (Garnis et al., 2006). PI3KCA is one of the few known genes that is found more frequently disregulated in SCC than ADC.

(E56) Similar to EGFR, the extent of mutations in KRAS (10-20% in NSCLC) is associated with geographical location. However, KRAS mutation is rare in Asians. KRAS mutations are strongly associated with ADC and appear more in males and smokers. KRAS mutations appear to target smokers (Gazdar et al., 2004). Inactivating mutations within the liver kinase B1 (LKB1) gene are often associated with KRAS tumours. These inactivating mutations are associated with the loss of barriers to initiation and altered cell polarity and metastasis (Ji et al., 2007). LKB1 mutations are frequently seen in ADC, although they are uncommon in most tumours (Herbst et al., 2008; Matsumoto et al., 2007).

(E57) Other oncogenic addictions may include TITF1, which codes for the TTF1 protein, and which is a lineage-specific marker for peripheral ADCs. These tumours of the terminal respiratory unit usually express TTF1, and when deprived, will undergo growth arrest or die by apoptosis (Tanaka et al., 2007). Lastly, in regard to oncogenic addiction, there is amplification of the MYC family. The MYC genes control cell growth and apoptosis, and they are often amplified in lung cancers. c-MYC is more often associated with NSCLC while L-MYC is predominantly associated with SCLC. In either cancer, enhanced MYC expression is also seen as a result of transcriptional upregulation without gene amplification (Gazzeri et al., 1994; Gazzeri et al., 1990).

(E58) *Evasion of cell death*. Mitochondrial apoptosis is regulated by the interaction of the BAX and BCL-2 proteins. The anti-apoptotic protein BCL-2 blocks the activity of the pro-apoptotic protein BAX through dimerisation (Danial, 2007). BCL-2 is overexpressed in most SCLC, and to a limited extent, in NSCLC (Brambilla et al., 1996; Gazzeri et al., 1998). BAX on the other hand is downregulated in SCLC, and upregulated in NSCLC. The result is dramatically altered BAX/BCL2 ratios that favour cell survival in NSCLCs, and this is seen early in the generation of dysplasia (Brambilla et al., 1998). Impinging upon the apoptotic pathway via caspase-8 activation is the Fas signalling pathway, which is activated when Fas ligand (FasL) binds the death receptor Fas. Both Fas and FasL are downregulated in the majority of NSCLCs. This is in contrast to SCLC where the Fas receptor is downregulated or not expressed at all, yet FasL is highly expressed. This disrupts the apoptotic pathway ordinarily regulated by the Fas receptor.

(E59) Normally, the shortening of telomeres, the repetitive sequences at the end of chromosomes that stabilise chromosome integrity and chromosome length, shortens after each cell division. This limits the lifetime of cells through the activation of apoptosis or cellular senescence. Because cells see short telomeres as DNA damage, the inactivation of

p53 signalling limits the activation of DNA repair and cell cycle checkpoint pathways at the early stages of the carcinogenic process (Bartkova et al., 2005; Gorgoulis et al., 2005; Nuciforo et al., 2007). Without p53 or Rb checkpoint controls, telomerase activity is upregulated which, through selection, results in production of immortalised cells that are genomically unstable. Human telomerase reverse transcriptase (hTERT), which maintains telomere length, is overexpressed in alveolar hyperplasia (75%) and in some broncho-alveolar carcinomas (Lantuejoul et al., 2004), and is seen in 80% of NSCLC and nearly all SCLCs (Hiyama et al., 1995b; Lantuejoul et al., 2004).

(E60) *Molecular alterations*. Molecular epidemiological studies have now described different molecular alterations in never-smokers when compared to lung cancer in smokers, suggesting that these cancers arise by different mechanisms. For example, mutations in both KRAS and EGFR are associated with ADCs (Chiosea et al., 2007; Johnson et al., 2001; Politi et al., 2006). However, mutated KRAS is found almost exclusively in lung cancers from smokers, while EGFR mutations are more frequent in never-smokers. In fact, EGFR mutations were the first specific genetic mutation associated with never-smokers (Kosaka et al., 2004; Pham et al., 2006). p53 mutations, while occurring in both smokers and never-smokers, occur less frequently in tumours from non-smokers. Furthermore, the mutational spectrum for p53 is unique to smoking status (Denissenko et al., 1997; Le Calvez et al., 2005; Vahakangas et al., 2001). More broadly, a number of studies examining gene expression have shown the expression profiles to be very different from one another (Gorgoulis et al., 2005; Lam et al., 2006; Takeuchi et al., 2006), including high levels of expression of AKT and p27 (Dutu et al., 2005). Methylation patterns in lung cancers identified in never-smokers versus smokers may also be distinct (Marchetti et al., 1998).

(E61) Given that over 50% of lung cancer in females are seen in never-smokers, while the same can be said for only 15% of male never smokers, the likelihood of sex-specific hormones as a risk factor for lung cancer is possible. Estrogen metabolism, for example, may result in mutagenic DNA adducts (Cavalieri and Rogan, 2006; Yager and Liehr, 1996). Other studies have looked at the role of estrogen as a proliferative agent in NSCLCs, as well as the role of ERα and ERβ. ER, for instance, is seen more often in tumours from never-smokers and more frequently in tumours from females rather than male never-smokers (Wu et al., 2005). Expression of ER is also considered to be a negative therapeutic prognosis factor in NSCLCs (Kawai et al., 2005). Mechanistically, however, it is the interaction of the EGFR and ER pathways that is most compelling. When estrogen is stimulated in lung cancer cell lines, EGFR ligand is rapidly released, which then activates the EGFR/MAPK1 growth pathways (Stabile et al., 2005). Indeed, EGFR protein expression is downregulated in the presence of estrogen and upregulated in the presence of anti-estrogens. Furthermore, in a mouse model of lung cancer that employed a conditional knockout of K-ras and deletion of p53, female mice had twice the number of tumours, larger tumours, and tumours of a higher grade, compared to male counterparts, a finding that was eliminated in ovariectomised females (Hammoud et al., 2008).

(E62) Radon was the first environmental cause of lung cancer identified. As early as the 1920's, lung cancer was attributed to radon exposures in underground miners. The risk for lung cancer in underground miners classified as never-smokers as a result of radon exposure has been shown to be elevated in several studies (Archer et al., 1973; Gilliland et al., 2000; Roscoe et al., 1995; Roscoe et al., 1989; Samet et al., 1984). The determination of cellular and molecular features of radiation-induced cancers developed in parallel to the determination of the molecular phenotype of lung cancer in general. Indeed, risk models for radiation-induced lung cancers are now beginning to incorporate such biological markers of lung cancer.

(E63) One of the first studies to examine the molecular phenotype of radiation exposed lung cells was that of Vahakangas et al. (1992), who described the mutational spectrum of the p53 and Kras genes in underground uranium miners. Interestingly, they observed a different mutational spectrum for lung cancer for radon exposure compared to that of tobacco smokers. They identified no kras mutations in exons 12-13. Although 9 mutations were seen within exons 5-9 of the p53 gene, none of these mutations were in the hotspot codons described for lung cancer. This study was followed up by several other studies in different populations of miners. In 1994, Taylor et al. (1994) described a single mutation at codon 249 of the p53 gene as a mutation hotspot in radon-associated lung cancer, including non-smokers. This hotspot was not found in either of three comparable datasets (Bartsch et al., 1995; Tierney et al., 1996; Yang et al., 2000) nor in a study of lung tumours associated with domestic radon exposure (Lo et al., 1995). In a recent analysis of p53 mutational status, Ruano-Ravina et al. (2009) examined the data from 8 studies that included 578 individuals, and concluded that the results do not support a radon-associated mutational hotspot for the p53 gene.

(E64) A number of animal systems including mouse, rat, dog, and baboon have been used to determine biological data for lung cancer induction via inhalation of radioactive elements such as radon or plutonium (Bair WJ, 1989; Bruenger et al., 1991; Guilmette et al., 1987; Lundgren et al., 1995; Morgan, 1989; Oghiso et al., 1994; Sanders et al., 1993; Simmons and Richards, 1984; Yuile et al., 1970) or irradiation via external sources. The role of KRAS or p53 mutations in radiation-induced lung carcinogenesis was examined in γ-irradiated F344/N rats (Belinsky et al., 1996) and in plutonium exposed rats (Stegelmeier et al., 1991). Unique to these studies was the identification of K-ras mutations in 33% of tumours after $^{239}PuO_2$ exposure, yet only a single K-ras mutation was identified in 35 x-ray induced tumours (18 squamous and 17 adeno). This suggests that there is not a specific signature nor a major role for p53 or K-ras mutations in lung carcinogenesis after low-LET radiation, but that there may be for high LET radiation. As suggested by Tierney et al. (1996), high-LET radiation-induced cancers may be through species-specific molecular mechanisms, as their studies of p53 and K-ras mutations in canines differed from those of Stegelmeier et al. (1991), who employed rats as their model system, but concurred with those of Vahakangas (Vahakangas et al., 1992) for K-ras mutations. p53 mutations, on the other hand, were one-third of those seen in lung cancers in uranium miners (Taylor et al., 1994; Vahakangas et al., 1992), and in line with the frequency seen in rat lung tumours (Kelly et al., 1995; Leach et al., 1993), again suggesting that the p53 abnormalities associated with radiation-induced lung cancers in animals and humans may be different.

(E65) *Epigenetic events*. Regulation of gene expression without changing DNA sequence is referred to as epigenetic regulation. This can occur through methylation of CpG islands within genes and their promoter regions, which silences the gene, or through gene regulation by miRNA. In addition, loss of methylation in non-coding regions is common in tumours, and is considered to lead to genomic instability. There are many genes that have been silenced in lung cancers (Hanahan and Weinberg, 2000; Shames et al., 2006), including retinoic acid receptor β (RARB), p16, O^6-methylguanine-DNA methyltransferase (MGMT), tissue inhibitor of metalloproteinase 3 (TIMP3) and Ras association domain family member 1A (RASSF1A) as examples (Zochbauer-Muller et al., 2001), and this process of gene silencing begins early in the pathogenesis of lung cancer (Zochbauer-Muller et al., 2003). Epigenomic analysis has shown that alterations of histone proteins are linked with DNA methylation patterns and is likely causal for lung cancer (Esteller, 2007).

(E66) miRNA, non-coding small RNAs, regulate gene expression by controlling the activity of specific mRNA via precise base pairing interactions (Boyd, 2008; Cowland et al., 2007), and miRNA expression is now known to be disregulated in lung and other cancers

(Nana-Sinkam and Geraci, 2006; Ramdas et al., 2009; Takamizawa et al., 2004; Yanaihara et al., 2006). The targets for most miRNAs are as yet unknown; however, they can clearly act in a manner analogous to tumour suppressors or oncogenes through their targeted regulation of mRNA levels, or through translational silencing. Interestingly, they are often found at DNA fragile sites and in regions of amplification or deletion. Six miRNAs are unique to ADC. Let-7 is decreased in lung cancer and is known to regulate KRAS expression (Eder and Scherr, 2005), which is also mutated in many ADCs. Let-7 also regulates expression of cell cycle-related genes, cellular division and genes in the DNA repair pathways. miRNA 34 (Mir-34) is now linked to the p53 pathway including cell cycle arrest and apoptosis (He et al., 2007), and the deletion of miR-34 is seen often in lung cancer (Bommer et al., 2007; Calin et al., 2004). Other miRNAs impact cell cycle through myc activity (Hayashita et al., 2005), and the DICER protein, which is a miRNA-processing enzyme, is decreased in pre-invasive lesions in ADC hyperplasia (Chiosea et al., 2007).

(E67) *Epigenetic events associated with radiation exposure*. As described above, altered DNA methylation patterns play a significant role in the lung carcinogenic process. Lyon et al. (2007) have described the methylation of the promoters of specific genes in ADCs of Mayak workers. Comparisons for a 4-gene panel in workers versus non-workers identified significant increases in methylation frequency for p16 and GATA-binding protein 5 (GATA5), as well as a dose response for plutonium exposure and the extent of gene methylation (Belinsky et al., 2004; Lyon et al., 2007). Furthermore, in addition to the increase in methylation, the multiplicity of gene inactivation was enhanced with increasing dose. Comparing the ADCs of γ-ray exposed versus plutonium-exposed individuals did not reveal a difference in the extent of multiplicity, suggesting that LET played no role.

(E68) Leng et al. (2008) argued that DSB repair, as measured by chromosome/chromatid rejoining capacity, influenced the extent to which genes were methylated. Using the Lovelace Smokers Cohort, single nucleotide polymorphisms in the DSB repair pathway [MRE11, checkpoint kinase 2 (CHEK2), x-ray repair cross-complementing group 3 (XRCC3), DNA-PKcs, and nibrin (NBN)] were scored. As the number of single nucleotide polymorphisms (SNPs) within these genes increased, the risk for gene methyation also increased. When the number of SNPs rose to seven, there was a 14.5-fold risk for a high methylation index. Also, although this cohort has no association with radiation exposure, these data support the notion that there are individuals who will carry a greater risk for promoter methylation, and perhaps carcinogenic risk, after radiation exposure based upon their intrinsic radiosensitivity via their capacity to repair DNA damage.

(E69) *Genetic factors*. Even though lung cancer is considered a disease related to environmental factors, some 80-90% of smokers will not get lung cancer. This suggests that for the 10-20% that develop lung cancer, genetic factors may play an important role in the susceptibility to the environmental factors that cause lung cancer in never-smokers. Epidemiological studies identifying familial clustering have shown an increased risk for lung cancer in never-smokers (Matakidou et al., 2005), racial differences (Cote et al., 2005), and a major susceptibility locus for lung cancer at 6q23-25 (Bailey-Wilson et al., 2004). This suggests that genetic factors play an important role in lung cancer incidence.

E.4. Radiosensitivity: Cells involved in lung repair after damage, and radiation as a lung carcinogen

(E70) After injury by bleomycin, naphthalene, sulphur dioxide and irradiation, epithelial repair is rapid with both dedifferentiation and migration of cells to cover defects, as determined by metabolic pulse-labelling studies. This not only indicates a substantial

plasticity of cells in the airways, but also that there is a high priority to repair such epithelial injuries. Even though there have been impressive recent research advances, a major area of uncertainty is the number, sensitivity, location and renewal characteristics of putative lung endogenous stem cells. In the respiratory tract, the target cells for radiation-associated carcinogenesis are considered to be in the basal cells in the trachea and larger bronchi of the central lung (Hong et al., 2004; Rawlins et al., 2007; Rock et al., 2009; Schoch et al., 2004), and in the Clara variant and type II alveolar cells of the peripheral lung (Giangreco et al., 2002; Kim et al., 2005). While it is commonly believed that the target cell of radiation-associated carcinogenesis is the stem or progenitor cell of various tissues, very little is known about radiosensitivity of specific stem cell populations in the lung. In addition, while in many tissues, the stem cell niche may be protected from atmospheric oxygen and potential ROS, the lung is chronically exposed to oxygen.

(E71) The question arises as to the target cell for carcinogenesis. As described by Greenberger and Epperley (2009), there are two distinct possibilities. The first possibility is that radiation-induced cancers are the product of malignant transformation of stem cells found within the irradiated field at the time of irradiation. The second possibility is that MSCs have migrated into the irradiated site and that it is the proliferation of these cells, in a microenvironment of increased free radical production in association with the onset of fibrosis, that is responsible for carcinogenesis. The appearance of donor origin cell markers in a number of studies of relapsed leukaemia in marrow transplant recipients (Katz et al., 1993; Thomas et al., 1972), and the donor bone marrow origin of gastric cancer in chronically inflamed *Helicobactor pylori*-infected mice (Houghton et al., 2004) suggest at least the potential for the second mechanism. While there is evidence that bone marrow-derived cells contribute to both tissue repair and late fibrosis of irradiated epithelial tissues (Epperly et al., 1999; Greenberger and Epperly, 2009), the evidence for mesenchymal stem cell differentiation into lung epithelial cells is controversial. As described earlier, marrow-derived cells may fuse with existing organ-specific cells rather than trans-differentiating into mature organ-specific cells. While this would limit the likelihood of the second mechanism in radiation-induced carcinogenesis, fusion of normal adult marrow–derived cells with irradiated differentiated adult lung tissue may influence our current models of lung cancer risk assessment after ionising radiation exposure.

(E72) In summary, there is a large knowledge gap regarding the radioresponse of endogenous lung stem cells: the extent to which stem cells are radiosensitive is unclear (Bao et al., 2006; McCord et al., 2009; Woodward et al., 2007); and any role for MSCs in lung carcinogenesis is controversial. Other questions concern the role of the microenvironment. If the MSC hypothesis is true, then the microenvironment is critical. Is the target cell dependent upon radiation delivery, e.g. inhalation vs external irradiation? After the initiating event(s), what molecular alterations are associated with progression?

(E73) In addition, it is increasingly clear that the molecular phenotype of radiation-induced lung cancer may be species specific, given the differences in radiation-induced molecular phenotypes between human, dog, and rodent. In humans, K-RAS mutations, commonly associated with lung cancer in smokers, appear not to be linked to radiation-induced lung cancers when examined in radiation transformed lung epithelial cell lines or in humans after domestic radon exposure, which is similar to K-RAS status in lung cancers in never-smokers. Epigenetic changes, however, are seen in both smoking-induced cancers and radiation-induced cancers. Indeed, there may be a radiation dose response for gene promoter methylation, although LET appears not to play a role (Goetz et al., 2011). Almost completely ignored is the possibility that there is an interplay between hormonal regulation (estrogen) and radiation exposure in the increased risk for lung cancer seen in women, particularly non-

smoking women. Is estrogen exposure a promotional event for initiated stem/progenitor cells? Finally, while we may be gaining insight into radiation-induced lung cancer by characterising the molecular phenotype, the link between stem cell/progenitor cells and overt lung cancer after radiation exposure has yet to be bridged.

E.4.1. Single cell responses: Endogenous lung tissue stem cells

(E74) While multipotent long-lived cells have been identified in distinct regions of the lung architecture, the identification of undifferentiated human lung stem cells has remained elusive. Only recently, such a cell type within the lung has been identified (Kajstura et al., 2011), based upon the expression of c-kit and negative for a host of markers of cell type. The consequences of radiation exposure in these cells have not yet been studied.

(E75) However, non-oncogenically immortalised and oncogenically immortalised bronchial epithelial cells have been used to elucidate molecular and cytological responses to radiation associated with the oncogenic process. Based upon the incidence of breast cancer in A-bomb survivors, the carcinogenic risk for epithelial cells has been estimated at 10^{-12}/Gy per cell (Hei et al., 1996; Hei et al., 1998; Zhou et al., 2004). Using an oncogenically immortalised [human papilloma virus (HPV)-immortalised] normal human bronchial epithelial cell system referred to as BEP2D cells, Hei et al. (1996, 2001) described malignant transformation of BEP2D cells after ^{4}He- and ^{56}Fe particle irradiation as measured by cellular transformation, e.g., growth in soft agar, to tumour production in immune-compromised mice after injection of transformed cells. Interestingly, no KRAS mutations were identified in the ^{4}He-particle or ^{56}Fe-irradiated cells and subsequent tumours. However, Betaig-h3 was identified as causally linked to tumour induction in BEP2D cells after ^{56}Fe irradiation (Zhou et al., 2004). Betaig-h3 expression is regulated by TGFβ1 signalling and when reintroduced into 3 tumourigenic cell lines, tumourigenicity was markedly reduced. In addition, mutated p53 was seen in BEP2D tumours where no p53 was seen in the unirradiated cell line. Because BEP2D is HPV-immortalised, the lack of p53 staining is consistent with the expression of the viral protein E6. However, the extent of expression of p53 in a single tumour was modest. Whether mutated p53 inhibits the expression of mutant p53 is unknown. In addition, Cyclin D1 was also upregulated in tumour cells leading to the suggestion that this may circumvent the inhibitory effects of Rb signalling (Hei et al., 1996).

(E76) Non-oncogenically-immortalised human bronchial epithelial-cell systems, which can be grown in traditional 2D culture as well as in 3D organotypic culture, are now surplanting oncogenically-immortalised systems (Ramirez et al., 2004). These differentiated 3D cultures were shown to have strikingly different DNA repair kinetics, particularly to high-charge, high energy (HZE) particle exposure (Asaithamby et al., 2011). Furthermore, gene expression analysis revealed the downregulation of multiple DNA repair pathways which would render cells susceptible to DNA damage and the promotion of genomic instability. Such organotypic cultures should shed new light on the molecular alterations after radiation exposure that lead to lung cancer, including the advantage of 3D growth and manipulation of genes associated with “stemness”.

E.4.2. Mutagenesis

(E77) As described above, in view of the only recent isolation of undifferentiated lung stem cells, the consequences of radiation-induced mutations in this cell population or in their pluripotent progenitors have yet to be analysed.

E.5. Models of carcinogenesis bridging the stem cell concept and epidemiology

(E78) Shown below is a list of important areas for future research:

1. Better functional analyses of putative lung stem cells (e.g. labelling, marker studies, stem-cell pool size) especially in human tissue.
2. Determine the target cells for radiation-associated carcinogenesis because this is crucial for understanding the temporal patterns of radiogenic cancer.
3. Isolate and propagate lung tissue stem cells to investigate the differential radioresponses between different types of stem cells.
4. Determine the location of stem cells in mouse studies: the microenvironmental influences, positional information, dormancy, and oxygen conditions in the niche.
5. Know more about changes in stem cells related to differences and risk as a function of age. As part of this, we need to know the increased risk for premalignant lesions as a process of increased age, and to determine if this increase in age-associated preneoplastic lesions counterbalances the decline in turnover rate through ageing processes which may decrease risk.

ANNEX F: SKIN STEM CELLS AND RADIATION CARCINOGENESIS

F.1. Skin carcinogenesis

(F1) Skin cancer is separated into two categories, melanoma and non-melanoma skin cancers. As there is little evidence concerning a possible link between radiation exposure and malignant melanoma incidence, this type of cancer has not been included in this annex. Emphasis is placed on carcinomas, because the great majority of skin cancers are of epithelial origin.

F.1.1. Basal cell carcinoma

(F2) BCC is the most frequent cancer in both men and women among populations of European ancestry, also called Caucasian populations, thus accounting for 29% of all cancers (Epstein, 2008). Its incidence appears to be increasing worldwide. In Caucasian populations, BCC accounts for 80% and SCC for 16% of human sporadic skin cancers. Despite this high frequency, the death rate from BCC is very low, because these tumours rarely metastasise. BCC, which arise from epidermal keratinocytes, are mostly localised tumours. They invade locally, where they can cause disfiguring and destruction of skin. The fact that they are slow growing with relatively stable genomes presumably limits the accumulation of additional DNA abnormalities that might confer a metastatic potential. Infrequently (<0.1% of cases), metastases can develop regionally in lymph nodes or at sites distant from the primary tumour, in the lung, bone and internal organs.

(F3) The principal aetiologic factors include ultraviolet (UV) radiation and ionising radiation, chemical carcinogens (arsenic, polycyclic aromatic hydrocarbons, psoralens) and possible infection with papilloma viruses. Sunlight is a major environmental factor for BCC development, as demonstrated by the fact that mutations are frequently UV specific. However, the exact relationship is complex. The use of sunscreens has been found not to correlate with reduced BCC risk (Green et al., 1999), even in the long term (van der Pols et al., 2006). Also, the incidence of BCC peaks at about twofold at 10,000 to 35,000 hours total exposure, and surprisingly, does not increase with further exposure. Several authors have postulated that BCC development may correlate better with intermittent sunlight exposure than with total exposure (Zanetti et al., 2006).

(F4) Although the majority of BCCs arise sporadically, genetic factors may also have a substantial role. Several genetic diseases are associated with the development of skin cancer, including xeroderma pigmentosum (XP) and Gorlin's syndrome. Apart from genetic diseases, very little is known about population genetic susceptibility to BCC. In contrast to Caucasian populations, persons of the South Asian and African ancestry with dark skin colour are resistant to BCC development. SNP technology was used to characterise DNA sequence variants that confer susceptibility to cancer. Some of these variants seem to operate through their association with fair pigmentation traits, resulting in reduced protection from the damaging effects of UV radiation (Han et al., 2006). Other sequence variants, such as K5, have no obvious role in pigmentation or UV susceptibility, but instead seem to operate in the context of growth and differentiation of the basal layer cells of the epidermis (Stacey et al., 2009).

(F5) Concerning the molecular mechanisms of development of this cancer type, the only known molecular abnormality for a long time was p53 mutations, which are found in 30% of sporadic BCC. This lack of knowledge was due to the difficulty of growing human BCC in

tissue culture or as xenografts. It was also due to the lack of an appropriate animal model, because mice do not develop BCC, either sporadically or after exposure to UV, ionising radiation or chemicals. Identification of a genetic event in a rare subset of patients who have a great propensity to develop BCC pointed to aberrant SHH signalling as one of the pivotal defects leading to formation of these tumours (Fig. F.1.). This rare heritable disorder is Gorlin's syndrome [also known as basal cell nevus syndrome (BCNS)] which is caused by mutations in the patched 1 (PTCH1) gene, encoding the receptor to which the HH ligand binds (Epstein, 2008; Hahn et al., 1996). The incidence of this syndrome ranges from 1/56,000 in UK to 1/256,000 in Italy (Lo Muzio, 2008). Aberrant activation of the SHH pathway has been observed in sporadic and hereditary BCC. Mutations in at least one allele of PTCH1 have been identified in 90% of sporadic BCC.

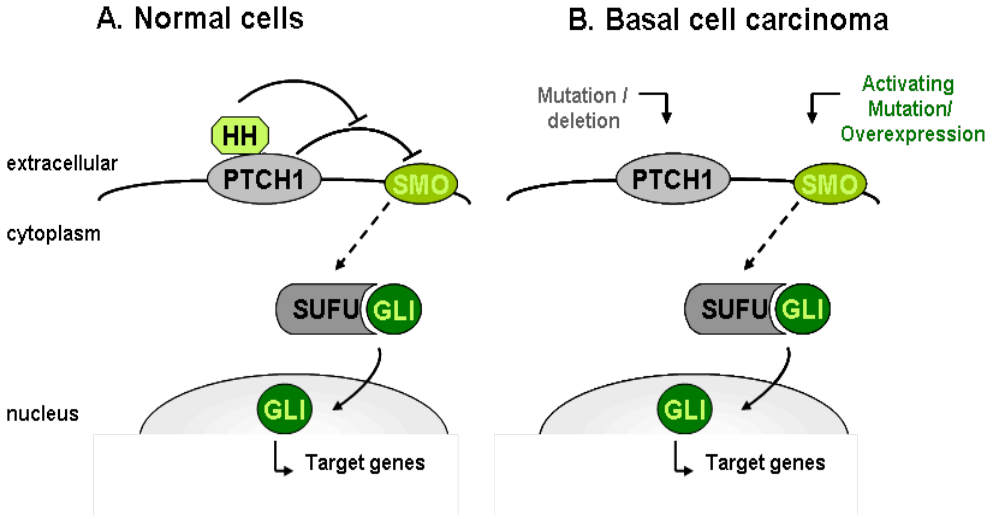

Fig. F.1. The SHH signalling pathway is frequently deregulated in BCC. Panel A. In the absence of extracellular hedgehog (HH) ligands (SHH, Indian HH or Desert HH), PTCH1 maintains the HH pathway in an inactive state by inhibiting the signalling effector smoothened (SMO). In the presence of HH ligand, the positive regulators of the HH pathway are shown in green and the inhibitors are shown in grey. During physiological HH signalling, binding of SHH to PTCH1 receptor relieves the normal repression of SMO. Then, SMO transduces the signal which leads to the activation of glioma-associated gene homologue (GLI), transcription factors allowing transcription of various target genes. Panel B. The HH pathway is always active in BCC in the absence of ligand, due to mutations in PTCH1 or SMO. Evidence for a role of a deregulated control of HH signalling in the BCC pathogenesis comes from studies of patients with Gorlin's syndrome and mouse models in which HH signalling is constitutively expressed. Germline mutations in the PTCH1 gene confer a strong susceptibility to develop multiple BCC in patients with Gorlin's syndrome, both spontaneously and after ionising irradiation (reproduced from Lo Muzio, 2008). (Permission needed)

(F6) Activation of the SHH pathway can be recapitulated by expressing the SHH protein or proteins of its signalling pathway ectopically in regenerated human skin, thereby inducing cancer in human tissue by using only one genetic element (Fan et al., 1997). High-SHH-expressing tissue showed cardinal BCC features. These data represented the first experimental induction of human neoplasia by a defined genetic alteration, as well as the first experimentally induced malignant conversion of human tissue (Khavari, 2006). Additionally, the surprising finding was the induction of features of the most common cancer by a single genetic alteration that activates a dominant developmental pathway, an observation that might help to explain how common is this cancer. Khavari's group used a specific model, which is grafting of *in vitro* reconstituted human skin in immunodeficient mice, which requires high

cell proliferation at several steps. This might explain why this model allows obtaining within months what requires years in human skin. *In vivo*, in undisturbed skin with a slower turnover, and after more moderate activation of the SHH pathway, a much longer latency period is required for BCC induction.

(F7) Another gene has been implicated in BCC development, the phosphatase and tensin homolog (PTEN) gene. PTEN is a tumour suppressor for skin cancer and loss of PTEN activity appears critical for UV-induced skin tumourigenesis (Suzuki, 2003).

F.1.2. Squamous cell carcinoma

(F8) Similar to BCC, SCC also arises from epidermal keratinocytes, but SCC is a more aggressive tumour that can form lethal metastases. Sunlight is clearly the major environmental factor for SCC development. The incidence rises with the total number of hours of sun exposure (Zanetti et al., 2006) and the use of sunscreens correlates with reduced SCC risk (Green et al., 1999), even in the long term (van der Pols et al., 2006). Spontaneous SCCs are associated with mutations in Ras, p53 and p16. The Ras GTPase is mutated to an activated form in 20% of sporadic SCC. Ras is also strongly activated biochemically in its GTP-bound form in most human SCCs through additional mechanisms besides direct Ras mutation. Such mechanisms might include Ras gene amplification, overexpression of upstream RTKs, or loss of inhibitory mRNAs. In addition, c-Myc overexpression and activation of Stat signalling occur frequently in skin SCC development, notably playing a role in the progression to the tumoural state. SCC arises most commonly as a sporadic tumour, but it is also characteristic of several inherited disorders, including those that impair DNA repair and genomic stability, such as XP and dyskeratosis congenita (DC), in which SCC might arise as a secondary result of increased genomic mutagenesis. SCC also affects approximately 75% of patients who are afflicted with recessive dystrophic epidermolysis bullosa (EB). In summary, SCC is a skin tumour that differs from BCC in many respects, including the aetiology, genetics, incidence rate and pathology.

F.2. Radiation carcinogenesis in human skin

F.2.1. Cancer types

(F9) The first type of cancer documented as being associated with exposure to ionising radiation was skin cancer, which was reported only 7 years after the discovery of x-rays (Frieben, 1902). Ionising radiation is a general risk factor for workers, as recently described by the European HELIOS study performed for 10 major occupational groups (Suarez et al., 2007). Radiation-induced tumours in skin are mainly carcinomas, developing from keratinocytes of the basal layer of epidermis, which is the deepest layer of this multilayered epithelium. An association between low-LET external exposures and the risk for these non-melanoma skin cancers has been demonstrated in a series of studies, based on accidental and medical treatment data (UNSCEAR, 2008). Some rare tumour types have also been described, such as trichoblastoma and trichoblastic carcinomas, which are tumours with differentiation towards hair structures. No significant relationship of melanoma with ionising radiation exposure has been demonstrated, including in A-bomb survivors (UNSCEAR, 2000, 2008).

(F10) BCCs are the more frequent type and continue to show an approximately steady ERR for 40 or more years after radiation exposure (Dutreix, 1986; Preston et al., 2007). Induction has been well documented, and it has been reported after occupational, therapeutic and accidental exposures (ICRP, 1992, 2000, 2007; Shore et al., 2002). In the survey of A-bomb

survivors for the time period 1958-1987, a strong positive dose-response trend for BCC was reported. The best fit dose response for BCC was non-linear, with an ERR Sv^{-1} of 0.7 (90% CI: 0.1, 2.8) below 1 Sv and 4.0 (1.9-9.0) above 1 Sv (Ron et al., 1998; Charles, 2007). This LSS cohort is very informative, because skin cancers are relatively rare in Japan, with estimated background incidence rates being 3 per 100,000 each for BCC and SCC. These rates are much lower than in the US Caucasian population (200 for BCC and 40 for SCC). The *tinea capitis* cohort studies in New York and Israel demonstrated significant BCC development after medical irradiation of the scalp (Ron et al., 1991; Shore et al., 2002), with further confirmatory evidence from a large population-based case-control study (Karagas, 2007). Histopathologic review of a case series suggested that up to 23% of nominal radiation-associated BCCs might be trichoblastomas or trichoblastic carcinomas, which are tumours with differentiation towards hair structures (Fazaa et al., 2007), although the representativeness of the series is unknown.

(F11) The evidence for induction of SCC by ionising radiation is much less clear than that for BCC. The proposed ratio of radiation-induced BCC:SCC incidence is generally 10:1, but it varies according to the particular study. For early radiation workers, who frequently had high and chronic exposures to the hands, there were numerous reports of SCC (Shore, 1990). A population-based study among men in Alberta, Canada, reported a 5- to 6-fold increase in incidence of BCC and SCC associated with nondiagnostic x-ray exposure (Gallagher et al., 1996). A more recent study performed in New Hampshire found an increased risk of both BCC and SCC in relation to therapeutic ionising radiation (Karagas et al., 2007; Lichter et al., 2000). Elevated risks were confined to the site of radiation exposure (BCC odds ratio, 3.30; SCC odds ratio, 2.94). One possibility is that SCC can be induced significantly only by high radiation doses (Charles, 2007). A mortality rate of 1% was evaluated for SCC (ICRP, 1992).

(F12) Radiation-induced skin tumours are very similar to sporadic skin tumours. Ionising radiation appears essentially to increase the natural rate. Although the nominal risk coefficient for skin cancer appears to be the highest among all tissues (Table 1.1.), the health detriment is much smaller for skin cancer than other radiation-induced cancers, and hence the ICRP has assigned a small value of tissue weighting factor (w_T) for skin of 0.01. Further details of this are as follows. As described in the ICRP *Publication 103*, the nominal risk estimates (which are excess rates, and not ERRs) for most of the tissue sites considered, were determined by the application of LSS-based radiation risk (ERR and EAR) estimates to rates in hypothetical "composite" Asian and Euro-American populations. However, the nominal coefficient for skin cancer was not determined in this way. Instead (as noted in *Publication 103*, paragraph A118), skin cancer was handled differently. In particular, the nominal skin cancer incidence risk coefficient was chosen to be consistent with the skin cancer mortality risk coefficient in *Publication 60*, which uses a nominal skin cancer mortality risk coefficient of 2 deaths per 10,000 people at 1 Sv and also indicates (Table B-19 in *Publication 60*) that the lethality fraction is 0.002. This translates into the nominal skin cancer incidence coefficient of 1,000 cases/10^4/Sv given in *Publication 103* for a whole population (the value used for a working age population is 670, Table 1.1). This nominal risk coefficient was not derived explicitly based on LSS results. Despite the large nominal incidence risk for skin cancer, the tissue-weighting factor for skin cancer is the same (0.01) in *Publications 103* and *60*. The reason for this is that radiation-associated skin cancer cases are given a very low weight in the computation of detriment. Regarding the present report on stem cells as target cells for induced cancers, the implication of the above discussion is that skin stem cells have a high sensitivity to carcinogenesis, but this has a large uncertainty as deduced from currently available publications.

F.2.1. Threshold dose

(F13) The traditional view was that a threshold dose exists for radiation-induced skin cancer, in the range of 8 to 10 Gy. In a cohort of radiotherapy patients (Lichter et al., 2000), the RR of developing skin carcinoma after receiving therapeutic ionising radiation was increased for total radiation doses higher than 30 Gy, which was consistent with other previous estimates for the radiation dose necessary to induce skin carcinoma after therapy protocols (using multiple doses of 2 Gy per fraction). The risks of BCC and SCC were increased specifically among those treated with 10 Gy per week or less, and less than or equal to 2 Gy per treatment. Surprisingly, more-highly-fractionated doses of ionising radiation, involving lower doses per fraction, appeared to be more carcinogenic than the classical protocols. In a study of skin cancers induced by radiotherapy in childhood, BCCs were strongly induced within the radiation field by (total) therapeutic doses ranging from 7 to 27 Gy (Levi et al., 2006a). No case of cutaneous SCC, or of malignant melanoma, was observed. In 6 different studies of the effects of medical exposures, where mean doses ranged from 2.25 to 6.8 Gy, the mean RR per Gy was 1.6, ranging from 1.1 to 2.1 (Shore, 2001). Other available data suggest that relatively small doses do have an effect. In the A-bomb cohort where the mean dose was <0.3 Gy, analyses of total non-melanoma skin cancer which allowed for a spline (i.e. a change in slope) at 1 Gy fitted the data better (P = 0.005) than a pure linear model (Preston et al., 2007). Using the spline model, the estimated ERR per Gy was 0.17 (non-significant) for doses below 1 Gy and 1.2 (90% CI 0.57; 2.3) for doses above 1 Gy. An analysis of skin BCC found an ERR Gy^{-1} of 2.0 (95% CI 0.7, 4.3) above about 0.6 Gy, but no dose trend below that (slope = -0.05) (Sugiyama, 2012). Thus, the A-bomb survivor data indicate that skin cancer, BCC in particular, can be induced by acute exposure at moderate doses above about 0.5-1 Gy, with little skin cancer risk at lower doses, so that there could be a threshold dose of ≤0.5 Gy.

F.2.2. Variation risk by age at exposure

(F14) Several studies have examined how risk varies by age at radiation exposure. In the A-bomb study, there was a 30-fold variation in ERR for BCC with age of exposure (Ron et al., 1998). The ERR was 21 for those aged 0-9 years at exposure and 0.7 when exposed at ages higher than 40. In a later analysis, the ERR also decreased rapidly with increasing age at exposure (P <0.001), but there was little change with attained age (P >0.5) (Preston et al., 2007). In the case of *tinea capitis*, both an Israeli study (Ron et al., 1991) and a New York study (Shore et al., 2002) showed clear age effects. The declines in risk amounted to 11% per year of age at exposure in the A-bomb study, 13% in the Israeli study and 13% in the New York study. A similar strong relation between BCC induction and radiation exposure in childhood was also shown in a study of secondary skin neoplasms in long-term survivors from cancer (Levi et al., 2006b; Schwartz et al., 2009; Watt et al., 2012). Thus, the available studies show that skin cancer risk is greater from radiation exposure at younger ages than at older ages (Shore, 2001).

F.2.3. Interaction with UV

(F15) An interesting biological question is whether UV and ionising radiation have an interactive effect on skin tumour induction. The initial study of A-bomb survivors concluded that the ERR per Sv was higher on UV-shielded skin than on the face and the hands (Ron et al., 1998). A more recent study of the same cohort showed that the EAR of BCC per unit skin surface area, related to radiation exposure, did not differ between UV-exposed and shielded

parts of the body, suggesting a simple additivity of the radiation-related and background BCC risks (Kishikawa et al., 2005). Mizuno et al. (2006) assessed the association with UV in BCC from the Japanese cohort, by characterising mutations in the p53 gene. They showed that 70% of BCC had p53 mutations independent of ionising radiation or UV. However, the distribution of mutation types depends on the UV or ionising radiation exposure, suggesting different modes of action of UV and ionising radiations in inactivating the gene.

(F16) After medical exposure, studies of patients irradiated for *tinea capitis* reported contradictory results, with RR greater on the relatively UV-shielded scalp than on the face and neck (Ron et al., 1991), or greater for the UV-exposed margin of the scalp (21/100 cm^2 per Gy) than for the (relatively) UV-shielded scalp (4.7/100 cm^2 per Gy) (Shore et al., 2002). In a study of second skin neoplasms in patients surviving from childhood cancer, all the BCCs were located within the radiation field, thus indicating that ionising radiation is the key aetiological factor, even in the absence of any meaningful interaction with UV (Levi et al., 2006b).

(F17) Thus, no clear evidence appears after accidental or medical exposure for an interaction between ionising radiation and UV exposure. However, there is some evidence for an association of ionising radiation sensitivity and UV sensitivity. The New York study of ionising irradiation for scalp ringworm found a substantial excess of BCCs among white patients but no skin cancer among the irradiated black patients (Shore et al., 1990). The radiation risk for skin cancer was 10 times higher among the Caucasians. For SCC, an association with radiotherapy was principally observed among persons with a sun-sensitive phenotype (Lichter et al., 2000). Finding few excess skin cancers among irradiated African-Americans as compared to Caucasians with a comparable dose, may indicate that skin susceptibility to UV exposure increases the excess risk from ionising radiation.

(F18) UNSCEAR (2000) considered that the question of a possible interaction between ionising and UV radiations remained unresolved, and that more data were needed to fully understand this complicated relationship.

F.2.4. Gender role

(F19) Male gender is a known risk factor for sporadic BCC in humans and is associated with more BCC than in females (rate ratio: 1.2). Although the role of estrogens in the development of skin cancer is controversial, the results point to an effect of hormonal status on BCC development. However, for radiation-induced skin cancer, none of the major studies has found a difference in cancer risk according to gender (Shore, 2001). In the A-bomb survivor cohort, no gender role was found in the initial description of skin cancer (Sadamori et al., 1989). This was confirmed in more recent studies on BCC. The incidence of BCC in sun-exposed sites was significantly higher in men than in women, but no gender effect was observed in the incidence of radiation-induced BCC at non-exposed sites among the survivors (Kishikawa et al., 2005; Naruke et al., 2009).

F.2.5. Genetic susceptibility to radiation-induced skin cancers

(F20) The relationship between ionising radiation exposure and cancer risk varies greatly among the different human cancer-prone genetic diseases. For ataxia telangiectasia (AT) patients, where cells exhibit a higher sensitivity to ionising radiation, no excess of radiation-induced skin cancer has been described. For XP, a genetic disease exhibiting hypersensitivity to UV radiation and a high incidence of skin cancers, no excess for radiation-induced skin cancer has been described. The DC syndrome is a very rare telomere biology disorder, with a

high risk of SCC induction. Both hypersensitivity (Cengiz et al., 2004; M'Kacher et al., 2003) and cancer induction by ionising radiation (Hyodo et al., 1999) have been described for this syndrome.

(F21) Evidence for a correlation between radiation exposure and increased risk in skin cancer due to genetic susceptibility comes mainly from studies of patients affected by Gorlin's syndrome (or NBCC) (Lo Muzio, 2008). This rare autosomal dominant disease is characterised by a wide range of developmental abnormalities and a predisposition to developing tumours, particularly BCC. In Gorlin's patients, BCC usually starts at puberty, and up to 90% of patients will have developed a BCC at the age of 40 years. For black Gorlin's patients, the incidence of BCC is lower (40%) and occurs at later ages, probably linked to protection due to skin pigmentation. After radiotherapy for medullablastoma in children with Gorlin's syndrome, multiple BCCs were found in the radiation field at 3 to 6 years after irradiation (Kleinerman, 2009; Mitchell et al., 2005; O'Malley et al., 1997). Fibroblasts isolated from the skin of three Gorlin's patients were described as sensitive to ionising radiation (Chan and Little, 1983). Data from Gorlin's syndrome probably point to differences between UV and ionising radiation, as Gorlin's skin keratinocytes are not hypersensitive to UV-induced cell death (Brellier et al., 2008).

F.3. Radiation carcinogenesis in rodent skin

(F22) *Limitations of mouse models*. Human and mouse differ in their skin organisation and homeostasis (Khavari, 2006). One example is hair follicle biology. Architecturally, the epithelium in adult, fur-covered murine skin is disproportionately comprised of densely-distributed hair follicles. In contrast, epithelium in human skin is proportionately much more inter-follicular (mean of 20 follicles per cm^2). Moreover, murine hair follicles undergo synchronous cycles that span the first 2 months of life, a time period when many neoplasia phenotypes are either initiated or studied, and the oscillation of follicular cycles can have dramatic effects on the proliferation of the cutaneous epithelial pool (Stenn and Paus, 2001). This situation differs from human skin, in which sparse hair follicles generally cycle asynchronously throughout life. Another example is epidermis thickness, around 25 µm with 2-3 cell layers in mice and 100 µm with 6-10 cell layers in humans, which results in greater percutaneous absorption and decreased barrier function in mice.

(F23) As a consequence of these differences, mice differ from humans in a number of general ways that are relevant to cancer. *First*, the spectrum of malignancies differs dramatically (Anisimov et al., 2005; Hahn and Weinberg, 2002; Khavari, 2006; Rangarajan et al., 2004; Rangarajan and Weinberg, 2003). Whereas most human tumours arise in epithelia, most murine tumours occur in the form of non-epithelial sarcomas and lymphomas. Epidermal skin cancers are very rare in these animals when they have not been exposed to UV or ionising radiation, which is different from the high basal rates seen in humans, notably from European ancestry. Moreover, all classical mouse skin carcinogenesis models readily produce tumours of the squamous type, but none of the basal type. *Second*, the origin of epidermal tumours is the interfolllicular epithelium for most human skin cancers, whereas most carcinomas are from hair follicle origin in mice. *Third*, there are species-specific differences in the mechanisms of cell transformation. Telomeres are approximately five times as long as human telomeres, a difference that might explain the ease of immortalising mouse cells compared with human cells (Rangarajan et al., 2004). Differences in oncogene signalling are illustrated by the action of oncogenic Ras through the Raf downstream effector cascade in murine fibroblasts, whereas the oncogenic function of Ras takes place through Ral pathway induction in human cells (Hamad et al., 2002).

(F24) These substantial cross-species differences argue for caution in applying data from studies of murine neoplasia directly to humans. Murine models suffer from various limitations, paramount among these being the uncertain applicability to cancers that arise in humans. This is also true when ionising radiation is the carcinogen. For example, BCC is the major skin tumour induced in Caucasian human populations, whereas in mice, ionising radiation readily produces SCC but no BCC. Rats may represent a better model than mice, at least concerning the type of tumours. In rats, epithelial tumours develop more frequently than mesodermal tumours (Anisimov et al., 2005). After exposure to either single or multiple doses of ionising radiation, the most frequent type of skin tumour in rats is BCC, followed by SCC (ICRP, 1992). However, the radiation had to penetrate at least about 180 μm to induce tumours, demonstrating that the main target was also the hair follicle (Albert et al., 1967).

(F25) *Cancer genes*. The mechanisms of development of skin tumours after radiation exposure are still largely unknown. It is clear that ionising radiation can act as an initiator, a promoter and a complete carcinogen. In rats, c-myc appears to play an important role, as activation of c-K-ras and c-myc oncogenes was found in SCC (Sawey et al., 1987), as well as amplification of c-myc during BCC and SCC progression (Garte et al., 1990).

(F26) Cancer gene manipulation is a field where mouse studies may be important, due to the power of gene-knockout technology in that species. Recently, the discovery of the role of the patched gene in the patients with Gorlin's syndrome stimulated the engineering of several mouse models, in which HH signalling could be manipulated and BCCs could be produced. These models have allowed more basic investigations into BCC tumourigenesis, including after ionising radiation. Ptch1 codes for a transmembrane protein that works as an antioncogene by negatively regulating the SHH pathway and cell proliferation. The group of Anna Saran observed BCC induction in irradiated $Ptch1^{+/-}$ mice. Unirradiated mice develop precursor lesions, that progress to BCCs only in irradiated mice. $Ptch1^{neo67/+}$ mice and wild-type littermates of both sexes were whole-body irradiated with 3 Gy of x-rays as adults (age 3 months). In these mice, BCC incidence was 12%, whereas no BCCs were observed in unirradiated mice. In addition, 2-month-old mice were subjected to local irradiation of the dorsal skin with a single x-ray dose of 4 Gy. These mice showed a 4.9% BCC incidence (Mancuso et al., 2004; Pazzaglia et al., 2004).

(F27) To test for interactions between SHH signalling and poly (ADP-ribose) polymerase 1 (PARP-1), a major protein of the repair of DNA strand breaks, PARP-1-null mice were crossed to Ptch1 heterozygous mice. Double-mutant mice were strikingly more susceptible to BCC, with over 50% of animals developing multiple, large, infiltrative tumours within 30 weeks of age. The results provide genetic evidence that, in mice, DNA-strand-break repair controlled by PARP-1 is required and cooperates with the SHH pathway to prevent BCC formation in response to DNA damage (Tanori et al., 2008).

(F28) Also, using cell-fate tracking of x-ray induced BCCs in $Ptch1^{+/-}$ mice, their essentially exclusive origin was found to be K15-expressing stem cells of the follicular bulge (Wang et al., 2011). However, conditional loss of p53 not only enhanced BCC carcinogenesis from the bulge, but also produced BCCs from the interfollicular epidermis, at least in part, by enhancing SMO expression. This latter finding is consistent with the lack of visible tumours on ears and tail, sites lacking SMO expression, in $Ptch1^{+/-}$ mice.

(F29) *Gender role*. The gender role has been assessed in the $Ptch1^{neo67/+}$ mouse model (Mancuso et al., 2004). Although microscopic basal cell lesions were observed in males and females, infiltrative BCCs only developed in males, showing a dramatic gender role in that model. The role of endogenous estrogen in that difference was addressed in $Ptch1^{+/-}$ mice, useful for BCC studies, and skin carcinogen-susceptible (Car-S) mice, elective for studies of papilloma and SCC induction. Susceptibility to radiation-induced BCC or chemically-

induced SCC was dramatically increased in ovariectomised Ptch1$^{+/-}$ and Car-S females and restored to levels observed in males. These findings underscore a highly protective role of endogenous estrogen against skin tumourigenesis in two independent mouse models of skin cancer (Mancuso et al., 2009).

(F30) *Type of exposure and radiation quality*. Experimental results suggest that reduction in dose rate does reduce the carcinogenic effect to some degree. The data of Hulse et al. (1983) and Papworth and Hulse (1983) suggested an acute threshold of about 16 Gy for induction of epidermal tumours in rats. The resistance to the induction of SCCs by protracted irradiation was well illustrated by the finding that 0.75 Gy given three times a week over their lifetime induced only a 3% incidence of SCC, whereas higher doses per fraction resulted in a 100% incidence. The LQ dose-response relationship for the induction of skin tumours in rats suggests that fractionating the exposures or lowering dose rate should reduce the effectiveness.

(F31) Burns et al. (1975) reported that the tumourigenic effect was decreased by splitting a dose and the reduction in effect increased with increasing time between the exposures. Split doses of 0.7 MeV electrons were used by Burns and Vanderlaan (1977) to study the repair and recovery of the carcinogenic events and found a repair half time of about 3 hours. The ability to repair the initial events was maintained for many exposures. The data for multiple daily exposures when plotted as tumours per rat at 48 weeks as a function of dose on a log-log scale gave a slope of 2.4 which is in approximate agreement with the D^2 term in the LQ model. The increased exponent for multiple doses delivered over a lifetime was much greater than expected if the effects of each individual dose were simply additive in each time increment. In the multistage theory of carcinogenesis, the increased exponent is interpretable either as an increased number of events occurring stochastically in time or as clonal growth of one or more of the intermediate stages (i.e. of mutated stem or progenitor cells in the lineage) (Burns and Albert, 1986b). There was also a direct effect of age, with older rats being less susceptible to tumour induction, but in contrast, the half time for persistence of carcinogenic damage became shorter, being 3.6 hours for 28-day-old rats, 1.2 hours for 112-day-old rats and 0.08 hours for 182-day-old rats (FJ Burns, personal communication). The use of different electron energies and penetrations identified the target cells as being at 0.3-0.4 mm depth, irrespective of the follicle size changes throughout the hair growth cycle (Burns et al., 1976).

(F32) The addition of solar spectrum UV radiation, of which 80% was absorbed in the epidermis, yielded more tumours after lower doses of ionising radiation but fewer tumours after higher doses. The reduction seemed to be a sterilising effect on the development of small tumours, because when the UV exposures were stopped, tumours began to appear at about the same rate as observed earlier in the groups that received ionising radiation without subsequent UV. Interestingly, the UV prevented the onset of all types of tumours including SCC and hair follicle tumours. If the sterilising effect was a result of direct action of the UV, it would be necessary to conclude that the presumptive tumour cells were in or just below the surface epidermis at the time of UV exposure (Burns and Albert, 1986a). This would implicate both this region and the region at 0.3 mm together in the carcinogenic process. There are various questions that remain to be answered: e.g. (1) Do the stem cells become more quiescent with increasing age and hence may have more time for efficient repair?; (2) Was the time-power/slope increase in tumour incidence with multiple exposures over a lifetime a result of radiation dose to cells that had already embarked on neoplastic pathways as a result of prior exposures?; (3) Are the target cells for carcinogenesis restricted to early stem cells or including daughter progenitor cells?; and (4) How important in tumour progression are radiation effects on the stem cell niche?

(F33) In the mouse, it is necessary to protract or fractionate the dose to obtain a dose that is sufficiently high to produce SCCs without excessive damage to other tissues. However, in the experiments of Coggle and Williams (1990), no reduction in effectiveness was found over a 1000-fold range of β-radiation doses down to 0.1 Gy/min. In contrast, Hulse et al. (1983) did find a reduction from a total of 30 tumours to 5 in comparable numbers of mice when the dose rate was reduced from 1.1-2.0 Gy/min to 0.017-0.024 Gy/min. Neither the dose rate nor the total dose were low in terms of radiation protection or carcinogenesis in the experiments of Hulse et al. (1983) and Williams et al. (1986). Thus, it is possible, as the experiments of Hulse et al. suggested, with very low dose rates, say 0.01 Gy per day, the reduction in effectiveness might be considerable.

(F34) There is a split-dose sparing effect on the survival of irradiated macrocolony-forming cells in mouse epidermis, irradiated using high doses (see Fig. F.6.), and hence dose-rate effects on clonogenic survival are expected.

(F35) Regarding LET, studies reviewed by Burns and Albert (1986a, b) indicated that in rats exposed to single doses, epithelial tumours appeared in about 10 weeks post irradiation, and then at an increasing rate over a period of at least 80 weeks (Burns and Albert, 1986a, b). With exposures to monoenergetic electrons (0.34 keV/μm) that penetrate at least 1 mm the data for the dose response for skin tumours/rat at 80 weeks as a function of dose could be fitted using a quadratic model, whereas irradiation with argon ions (125 keV/μm) resulted in an approximately linear dose response up to about 9.0 Gy. The authors concluded that the results were consistent with an LQ dose-response model with the linear component dependent on the LET of the radiation. The types of skin cancer induced in rat skin were independent of LET, and RBE values of around 5 were reported at the lowest dose of 1 Gy low-LET radiation where tumours were induced. The question of the RBE for skin cancer induction by high-LET radiations was stated by ICRP (1992) to be essentially unanswered, and the topic was further reviewed by Masse (Masse, 1995).

F.4. General features of skin

(F36) The skin is the largest organ of the human body. Adult skin comprises between 15 and 20 % of the total body weight, with a surface area of 2 m^2. For the average adult human, the skin is between 2-3 mm thick. Skin performs numerous functions, including protection, sensation, heat regulation, excretion, and absorption. It consists of two broad tissue types: the epidermis – an external stratified, non-vascularised, epithelium; and an underlying connective tissue called the dermis – consisting of dense fibrous components produced by fibroblasts. The dermis is usually less than 2 mm thick and houses many of the skin's functional components, including its vascular, neural and lymphatic systems and its multiple epidermal appendages. The latter include excretory and secretary glands (sebaceous, eccrine and apocrine glands), keratinising structures (hair follicles and nails), and sensory nerve receptors. Finally, anatomists include a third skin layer, the subcutis or hypodermis, consisting of fatty connective tissue that connects the dermis to underlying skeletal components. As the majority of skin cancers have an epithelial origin, this section focusses on epidermis and keratinocytes.

F.4.1. The epidermis

(F37) The epidermis is the outermost layer of the skin. It forms the waterproof, protective wrap over the body surface and is made up of a stratified squamous epithelium with an underlying basal lamina. The total thickness is usually between 20 to 80 μm thick, depending on body localisation, going up to 1.4 mm on palms and soles (Potten, 1986). The epidermis of

a single human being comprises approximately 8 x 10^{10} basal keratinocytes (Larcher et al., 2007).

(F38) The epidermis consists of four main types of cells: keratinocytes, melanocytes, Langerhans cells and Merkel cells. Keratinocytes, the cells that make the keratin proteins, are the predominant type of cells in the epidermis (Fig. F.2.). At the lowermost portion of the epidermis, named the basal layer (*stratum germinativum*), are immature, rapidly dividing keratinocytes. As they mature, keratinocytes flatten out and move upward to the suprabasal layers. At the end of their life cycle, they reach the uppermost layer called the horny layer (*stratum corneum)*. This layer consists mainly of dead keratinocytes, filled with keratins and lipids forming a protective barrier. Dead cells from the *stratum corneum* continuously slough off through the desquamation process and are replaced by new cells coming from below. The human epidermis completely renews itself every 3 to 5 weeks. Two to 3 billion skin cells are shed daily by the desquamation process. The body expends this effort to replace epidermis every month, because the skin constitutes the first line of defense against dehydration, infection, injuries and temperature extremes.

(F39) The salient features of the structure of the epidermis can be summarised as follows (ICRP, 1992):

(1) The epidermis is composed of viable and non-viable layers. The outer layers of dead cells, the *stratum corneum*, constitute ~25% of the total epidermal thickness;

(2) In the viable epidermis, stem cells are restricted to the basal layer, predominantly towards the bases of rete pegs, although cell divisions do occur in suprabasal cells;

(3) More than 50% of basal cells are to be found at a depth of >200 μm, distributed in the shaft of hair follicles at varying depths within the dermis; and

(4) The depth of the basal layer in the interfollicular epidermis varies greatly but is between 20-100 μm in most body sites. On the hands, the epidermis of the fingertips is thicker and the depth of the basal layer is >160 μm.

(F40) As described in ICRP (1991), the *stratum spinosum* is made up of a variable number of cell layers. The cells of these layers are rich in cell-to-cell contacts, desmosomes, which bind the cells firmly together. It is the structural and proliferative organisation of cells in the basal layer and the *stratum spinosum* which largely determines the response of the epidermis to radiation-induced injury. The basal layer, which is separated from the dermis by a basement membrane, is considerably undulated, and in many regions, distinct rete ridges or pegs can be seen.

(F41) The results of radiolabelling experiments with ^{3}H-thymidine demonstrated the presence of labelled suprabasal cells in human epidermis (Epstein and Maibach, 1965; Weinstein and Van Scott, 1965). In a quantitative study (Penneys et al., 1970), it was found that 32% of labelled nuclei were in a suprabasal position. The precise kinetic relationships between the proliferating cells of the basal and suprabasal layers have not been established; however, a number of models have been proposed which have been linked with the presence of the rete pegs or ridges.

(F42) Hume and Potten (1979) considered three possible models for the cell kinetic organisation of a thick epidermis as is seen in man. In all three models, it was assumed that the rate of cell desquamation from the surface of the *stratum corneum* overlying the rete pegs was equal to that from the viable epidermis between the rete pegs. In the model favoured by Hume and Potten (1979), because it was in better accord with the available cell kinetic data, it was proposed that only a small proportion of the keratoblasts in the basal layers are stem cells, with the majority of basal cells being proliferative transit cells. The stem cells are confined to the base of the rete pegs: therefore, the transit cells increase in age with distance from the base of the rete peg. Consequently, the proliferation indices would be highest in the region of

the rete pegs. In man, the exact relationship between the 68% of labelled cells located in the basal layer and the majority of the remaining labelled cells found in the first suprabasal layer remains uncertain. The latter may represent transit cells that still retain division capabilities.

(F43) Because of the complexity of the thick epidermis found in man, the stacking of cells into columns (as found in rodents) may not be precise or may be totally absent in some regions (Bergstresser and Chapman, 1980; Blair, 1968; MacKenzie, 1975). Where cell stacking has been demonstrated in human epidermis, it was limited to the *stratum corneum* and could not be traced beneath the granular layer (MacKenzie et al., 1981). In this respect, the organisation of a thick human epidermis is very different from the highly organised structure in the epidermis of rodents. In rodents, it has been proposed that each clearly defined column of cells has its own slow cycling stem cell and a population of cells which provide amplification of cell division. Such a cluster of cells was termed an epidermal proliferative unit (EPU) (Potten and Allen, 1975).

(F44) If an EPU exists in human epidermis, possibly based on the rete peg, then it would differ in many respects from the EPU postulated for a thin epidermis, such as that of the dorsum of the mouse. The rete peg unit is much larger than an EPU. In addition, the vertical orientation of a high proportion of mitoses precludes the precise sequence of an age-related cell migration as predicted by the EPU model. Another prominent difference between the two proposed units is the presence of suprabasal proliferative cells in the rete peg unit. If a fixed number of cell divisions take place in the transit-cell compartment, as is the case in the EPU model, the rete pegs would be regularly spaced; this does not appear to be the case in histological sections of the epidermis.

(F45) The epidermis contains no blood vessels, and is nourished by diffusion from the dermis. The epidermal homeostasis and the differentiation programme are regulated by diffusible factors regulating proliferation and differentiation, provided by the mesenchyme, by other neighbouring tissues or by the circulation. Skin epithelium also includes appendices, hairs, sebaceous and sweat glands, which are mainly located in the dermis.

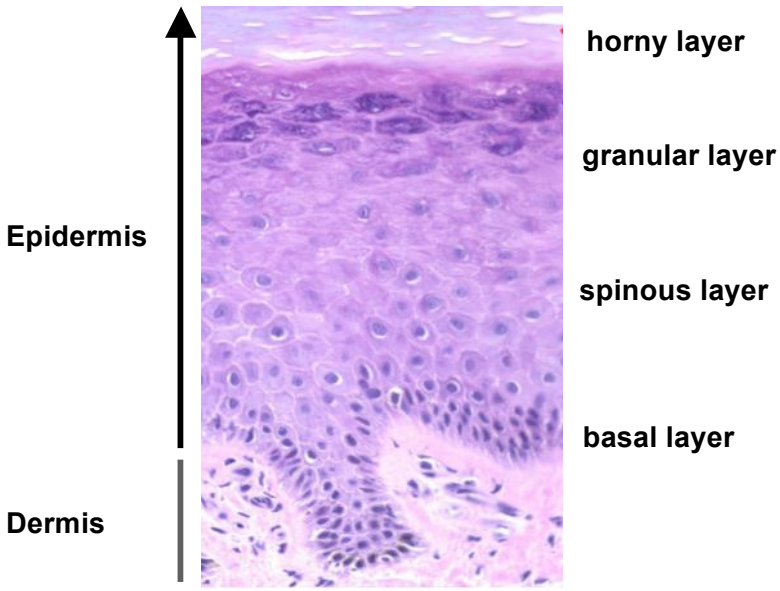

Fig. F.2. Organisation of human epidermis (courtesy of MT Martin, CEA, France). (permission needed)

(F46) The epidermis self-renews every 28 days through keratinopoiesis. It is organised in four main layers representative of the successive steps of keratinocyte differentiation. Proliferation of keratinocytes is mainly restricted to the basal layer. By moving to the upper layers, cells stop dividing and progressively develop terminal differentiation. Corneocytes are eliminated in the horny layer by desquamation.

(F47) *The hair follicle*. Hair is a keratinised protein filament that grows through the epidermis from follicles deep within the dermis. It has two distinct parts, the hair follicle and the hair shaft. The bulbar region of the hair follicle contains a pool of relatively undifferentiated epithelial cells, termed matrix cells. During the "growing phase" (anagen) of the hair cycle, these matrix cells proliferate extremely rapidly with a doubling time of 18-24 hours. This proliferation appears to be tightly controlled by the dermal papilla. After a period of active growth in anagen, matrix cells cease to divide, and the lower follicle regresses during catagen, which is a regressing phase. When regression is completed, the follicle enters telogen, a resting phase that can last for several months. The matrix cells then resume proliferation and produce a new hair bulb, thus reentering anagen and completing a hair cycle (Paus and Cotsarelis, 1999). The distribution of basal cells between the epidermis and the follicular epithelium has not been determined for human skin.

(F48) *Turnover rate*. Many of the reports of cell kinetic studies in human skin are, to a greater or lesser extent, less reliable than those obtained from studies in laboratory animals, because of the difficulties associated with the application of the experimental techniques as applied to man. These findings were extensively reviewed by Potten et al. (1987), where it was indicated that the majority of the reports dealt only with the basal layer of the epidermis. Mitotic index (MI) values vary greatly, both between individual subjects in a study and between studies. The few estimates of the time for mitosis suggest 1.0-1.5 hours. Studies involving *in vivo* labelling with ^{3}H-thymidine have produced values for the LI of 3.8-8.1% although many authors suggest values of between 5% and 6%. Estimates of the DNA synthesis time (T_S), using *in vivo* double labelling, indicate values of between 7.0 hours and 10.6 hours. In a major study in human skin using the fraction of labelled mitosis (FLM) technique, Weinstein and Frost (1969) estimated T_S to be 16 hours from an FLM curve that was fitted by eye. This was revised to 12.1 ± 6 hours following a retrospective computer model fitted to the same data (Potten et al., 1985). From the same analysis, the duration of $T_{G2} + T_M$ was estimated to be 8.7 ± 4.6 hours. Estimates of the cell production rate (k) for human epidermis, based on MI and LI data have varied from 5.1-8.8 cells/1000 basal cells/hour (Potten, 1975). Values for the turnover time of the basal cell population (TT) can also be calculated using estimates of T_S and LI.

(F49) Estimates of the cell cycle time (TC) for the basal cells of the normal epidermis have come from a number of sources. On the basis of the time elapsed between the first and second peak in an FLM curve, obtained from *in vitro* studies, a value of 59 hours was proposed (Chopra and Flaxman, 1974). Values of between 50 hours and 137 hours were reported using flow cytometry (Bauer et al., 1980; Bauer and Grood, 1975). The cell cycle of human EpiSCs is expected (by analogy with mouse data) to be longer than that for other basal cells, but no accurate values are known.

(F50) *Age and gender specificity of tissue turnover*. The above features of the tissue architecture and the turnover rate are dependent on age and gender, and this will be addressed in this paragraph. The age periods to be discussed include those of fetal development, childhood growth and adulthood. Thus, the tissue turnover rate and the relevant information in fetus, infant, young adult and adult are discussed. Of particular interest is the decline of the turnover rate through ageing process which contributes for the age-dependent decrease of the

sensitivity to radiation carcinogenesis. Aged epidermis is less proliferative than young epidermis, as exemplified by slower wound healing. However, it is not known whether quantitative and/or qualitative alterations in the stem and/or TA compartments are responsible for the decreased proliferation. Earlier studies found a normal or decreased frequency of putative EpiSCs with ageing. Using long-term repopulation *in vivo* and colony formation *in vitro*, it was shown that although no significant difference was detected in EpiSC frequency with ageing, TA cell frequency was increased (Charruyer et al., 2009). Moreover, aged TA cells persisted longer, whereas their younger counterparts had already differentiated. Underlying the alteration in TA cell kinetics in the aged was an increase in the proportion of cycling keratinocytes, as well as an increase in cell cycle duration. In summary, although no significant difference in EpiSC frequency was found, TA cell frequency was increased (as measured by *in vivo* repopulation, growth fraction, and colony formation). Furthermore, the proliferative capacity (cellular output) of individual aged EpiSCs and TA cells was decreased compared to that of young cells. Although longer cell cycle duration contributes to the decreased proliferative output from aged progenitors, the greater number of TA cells may be a compensatory mechanism tending to offset this deficit. Flow cytometric measurements of the DNA content were performed on a large number of skin biopsies by an automated technique. Expressed as a percentage of all viable cells in the epidermis, the figures for cells in S-phase averaged 1.8% and for G_2M 0.9%. No significant differences due to sex were found. Concomitantly with age, the ratio S/G_2M (representing the duration of S to the duration of G_2M) increased. Also, seasonal effects were clear, showing higher values for S and G_2M in June compared to November and December. Lastly, small differences were found dependent on body-site, the ratio S/G_2M being greater in legs than in arms.

F.4.2. Skin stem cells

(F51) *Two types of skin stem cells:* Specific properties have been proposed for the keratinocyte stem cells (KSCs) as compared to more differentiated keratinocytes. KSCs can be defined as:

- rare cells in the skin and the only permanent, possibly anchored, long-term residents;
- found in well-protected niches;
- cells on which the entire lineage and ultimately the tissue are dependent, through their self-renewal potential; they are responsible for the long-term maintenance of skin;
- undifferentiated cells both structurally and biochemically (cytoplasm filled with ribosomes and devoid of keratin filaments);
- slow cycling in homeostatic conditions;
- LRCs as a consequence of their slow cycling and their specialised DNA segregation process; and
- capable of very high proliferation in response to wounding and to certain growth stimuli.

(F52) *Follicular stem cells*. Hair follicles are self-renewing structures that cycle and reconstitute themselves throughout life, through the activity of follicular stem cells. In rodent hair follicles, it was demonstrated that LRCs, assumed to be slow cycling KSCs, localise to a region of the outer root sheath surrounding the rodent hair shaft termed the bulge (Cotsarelis et al., 1990). The bulge approximates the attachment site for the arrector pili muscle, and marks the bottom of the permanent portion of the follicle during cycling. Others argued for the localisation of stem cells in the upper half of the follicle: x-irradiation destroys the hair matrix, but cells in the outer root sheath can regenerate a complete hair bulb, lending strong support to the notion that the germinative source of each generation of hair follicles must reside in the outer root sheath and not in the bulb. Besides, the lower half of rat (vibrissa) hair

follicles can be surgically removed, and a new hair bulb can regenerate in response to the implantation of a new dermal papilla (Cotsarelis et al., 1990).

(F53) Using a specific model, the whisker follicle of the rat, the group of Barrandon demonstrated at the single-cell level that cultured stem cells retain their long-term potential to form hair follicles when grafted into athymic mice (Claudinot et al., 2005). They showed that (i) clonogenicity is an intrinsic property of the adult stem cells of the hair follicle; (ii) after cultivation for >140 poplulation doublings (PDs), these stem cells, transplanted to the dermo-epidermal junction of newborn mouse skin, form part or all of the developing follicles; (iii) the stem cells incorporated into follicles are multipotent, because they generate all of the lineages of the hair follicle and sebaceous gland; and (iv) thousands of hair follicles can be generated from the progeny of a single cultulred stem cell. Thus, accumulated evidence confirmed that in rodents, the bulge is the repository of multipotent stem cells that support hair follicle cycling and can repopulate interfollicular epidermis and sebaceous epithelium.

(F54) More recently, new data revealed a diversity amongst follicular stem cells that was previously unrecognised (Watt and Jensen, 2009). The rodent follicle now appears to be a continuum of at least five different populations, each with specific markers, including:

1. Cells of the junctional zone, which are positive with leucine-rich repeats and immunoglobulin domains 1 (Lrig1) and the cell-surface marker MTS24 (Watt and Jensen, 2009);
2. $MTS24^{+}/CD34^{-}$, integrin $\alpha6^{low}$ cells of the upper isthmus (Watt and Jensen, 2009);
3. $CD34^{+}/K15^{+}$ cells of the bulge, also LRCs (Blanpain et al., 2004; Lyle et al., 1998; Trempus et al., 2003);
4. $Lgr5^{+}$ cells, under the bulge (Jaks et al., 2008); and
5. $Lgr6^{+}$, above the bulge (Snippert et al., 2010).

Surprisingly, some of these populations (populations 2 and 4 in the above list) are actively cycling. The specific roles of all these populations remain to be determined. However, the bulge population still remains the major stem cell repository.

(F55) Regarding the stem cell niche, Wnt/β-catenin is required for follicle SC maintenance and niche biology, and β-catenin activation is essential for promoting quiescent follicle SCs to proliferate and terminally differentiate along the hair cell lineage (Yang and Peng, 2010). Further, β-catenin stabilisation promotes *de novo* HF morphogenesis, and constitutively active β-catenin expression results in pilomatricoma. Both BMP and TGFβ signals are required for quiescent niche maintenance: BMP deletion results in SC activation, whereas TGFβ may play a role in SC identity maintenance.

(F56) Concerning human skin, although several lines of evidence have suggested that the hair follicle also provides a niche for KSCs, anatomic boundaries, biochemical distinctiveness, and global gene expression pattern are ill defined. In contrast to the bulge of murine follicles, which can easily be outlined as a discrete projection, the human adult anagen bulge does not possess distinctive morphological features. Ohyama et al. (2006) first isolated and characterised stem cell–enriched human hair follicle cells. Some markers have been described for these cells, including CD200, K15, and K19. Interestingly, a role has been proposed for HH signalling in maintaining the human bulge cell phenotype in young and aged human skin (Rittie et al., 2009). Although it has been shown that under severe stress conditions, some bulge "stem" cells can undertake a variety of restorative-related functions, their role in the undisturbed state is unclear.

(F57) *Interfollicular stem cells in human skin*. The existence of interfollicular stem cells in human skin has been demonstrated, by discriminating three clonal types of keratinocyte with different capacities for multiplication: namely holoclones, meroclones and paraclones (Barrandon and Green, 1987). The entire epithelial compartment of the epidermis of an adult

human (8 x 10^{10} cells) can be generated from the progeny of a single stem cell or holoclone. In cell culture, holoclones are characterised by sustained proliferation, more than 100 PDs and organogenesis, epidermis reconstruction up to 50 PDs (Fortunel et al., 2010). The paraclones exclusively contain cells with a short replicative lifespan (<15 PDs), after which they uniformly abort and terminally differentiate. The meroclone contains a mixture of cells of different growth potential and is a transitional stage between the holoclone and the paraclone. The concept of the holoclone is still widely used as a criterion for human EpiSCs (Fig. F.3.).

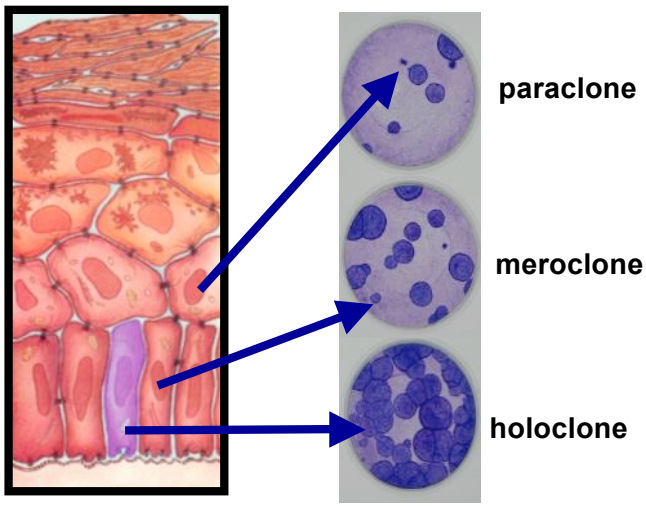

Fig. F.3. Keratinocyte stem cells are characterised as holoclones (courtesy of MT Martin, CEA, France). (permission needed)

(F58) The basal layer of human epidermis is composed of keratinocytes with different growth potential, as described by Barrandon and Green (1987). The long-term regeneration of the epidermis is due to the presence of rare cells, which can be isolated as holoclones in tissue culture.

(F59) The description of membrane markers in keratinocytes with characteristics of stem cells permitted another classification, according to which the basal layer of epidermis is composed of two major cell types, the rare stem cells and their progeny, the keratinocyte progenitors (Fig. F.4.). Keratinocyte progenitors, also termed TA keratinocytes, constitute the main population of the basal layer. As they divide frequently and give rise to the differentiated keratinocytes of the suprabasal layers of the epidermis, they are responsible for the short-term maintenance of the skin. This population is not homogeneous, ranging from early progenitors close to the stem cells up to late progenitors, prepared to migrate into the upper layers, and can be paralleled to the meroclone and paraclone classification.

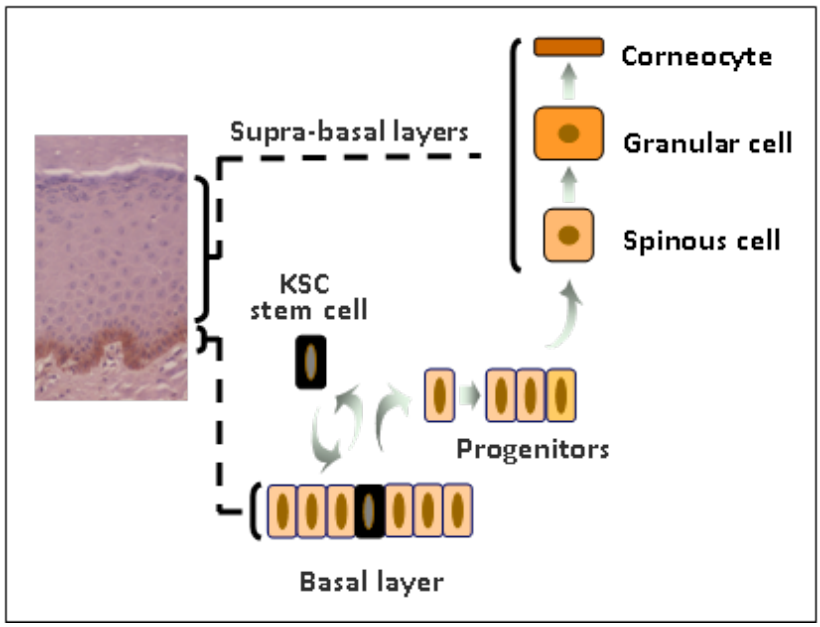

Fig. F.4. The classical model of the hierarchical organisation of human epidermis (modified from Harfouche and Martin, 2010b). (Permission needed)

(F60) In homeostatic conditions, KSCs are undifferentiated and slow cycling, which self-renew through asymmetric division. Their direct progeny, the keratinocyte progenitors, or TA cells, ensure short-term epidermis maintenance, as they proliferate in the basal compartment and then migrate to upper layers to give rise to the differentiated keratinocytes.

(F61) Although a variety of membrane markers have been described for KSCs, a unique marker is still unavailable and it remains impossible to identify precisely these cells *in situ* in human skin. However, several methods have been described that can select populations enriched in stem cells from a general population of keratinocytes through cell sorting, including selection on cell size, specific membrane markers, and drug exclusion (Fuchs, 2008; Kaur, 2006; Watt and Jensen, 2009). Small cell size and rapid adhesion to a matrix-coated culture dish have been demonstrated as a way to enrich a keratinocyte population for human stem cells from cells freshly isolated from a skin sample (Li et al., 2008). A population within rapidly adherent keratinocytes that ranged in size from 5–7 μm displayed the highest density of β1-integrin receptor, contained the highest percentage of cells in G_0/G_1 phase, showed the highest nucleus to cytoplasm ratio, and possessed the highest colony forming efficiency. When injected into murine blastocysts, these cells participated in multi-tissue formation.

(F62) Several membrane markers have been used to enrich cell populations for stem cells from skin. Combinations of markers based on two KSC properties, quiescence and strong adhesion to the basal layer of epidermis, are the most frequently used. For adhesion molecules, the expression of integrins β1 and β6 has been particularly studied. Thus, enrichment and isolation of a subpopulation of basal epidermal cells from human skin has been achieved (Li et al., 1998), based on high levels of the adhesion molecule integrin β6 ($\beta6^{bri}$), and low levels of the transferrin receptor CD71 ($CD71^{dim}$). It was shown that cells with the phenotype $\beta6^{bri}/CD71^{dim}$ represent the EpiSC population, based on the demonstration that these cells exhibit the greatest regenerative capacity of any basal cells, and they represent a minor subpopulation of basal cells (10%), which are quiescent at the time of isolation from the epidermis. Moreover, this strategy was the only one for which serial transplantation

potential could be demonstrated. The $\beta6^{bri}$/$CD71^{dim}$ keratinocytes were able to participate in three serial epidermis reconstructions in immuno-compromised mice (Terunuma et al., 2007). Another similar approach used the high expression of adhesion molecules, revealed by a high adhesion capacity, and the low expression of the EGFR ($Adh^{+++}EGFR^{low}$ phenotype) to isolate KSCs (Fortunel et al., 2003a). These cells exhibited high growth potential in tissue culture and the long-term capacity to form a pluristratified epidermis.

(F63) A hierarchical lineage structure was also shown by Schluter et al. (2011), using interfollicular neonatal foreskin epidermis. The tissue was disaggregated, fractionated into subsets based on markers of keratinocyte stem cells ($\alpha6^{bri}CD71^{dim}$), cycling progenitors ($\alpha6^{bri}CD71^{bri}$), and early differentiating cells ($\alpha6^{dim}$), followed by functional evaluation using a limiting dilution *in vivo* model for tissue reconstitution. The results showed the presence of a quiescent stem cell population with high long-term epidermal renewal, a cycling progenitor cell population with rapid but not sustained epidermal reconstitution, and a differentiated cell population with low regenerative capability.

(F64) One difficult aspect is SC isolation from cultured keratinocytes, because the specificity of most membrane markers is lost in tissue culture conditions. Cell sorting after Hoechst labelling has been demonstrated as an efficient method of SC isolation from primary keratinocyte cultures (Larderet et al., 2006). A family of drug-effluxing pumps, including the BCRP1/ATP-binding cassette subfamily G member 2 (ABCG2) transporter, allowing the exclusion of DNA dyes such as Hoechst 33342, is more active in stem cells. The small SP of cells that is able to rapidly exclude the dye, is enriched for stem cells (Redvers et al., 2006). SP keratinocytes represent 0.16% of the total population, exhibited increased colony-forming efficiency and long-term expansion potential as compared to other keratinocytes. Importantly, SP keratinocytes retained the potential to form a pluristratified epidermis even after long-term culturing.

(F65) *Interfollicular stem cells in mouse skin*. In mouse skin, the existence and role of interfollicular stem cells have long been debated, due to the importance of the follicular stem cell population. One of the most distinguishing features of stem cells is their slow-cycling nature. This means that a prolonged period of ^{3}H-thymidine or BrdU staining leads to the labelling of stem cells, and once labelled, these cells retain the isotope for an extended period of time during subsequent “chasing”; they can therefore be identified as LRCs. This technique has been largely used in mice and allowed demonstration that LRCs are found in mouse skin in the hair follicle and interspersed as single cells within the basal layer of interfollicular epidermis (Potten and Booth, 2002). These cells perform the long-term maintenance of epidermis, whereas follicular stem cells are involved in maintenance of the hair follicles.

(F66) Structural considerations of the epidermis on the back of the mouse suggested that the epidermis is subdivided into a series of functional groupings of cells, each comprising an individual cell lineage with its own stem cells (Potten, 1986; Potten and Hendry, 1973; Potten et al., 2002). The cornified layers consist of thin flat cellular elements (squames), which have a large hexagonal surface area, and these are arranged into columns like a stack of plates. The columns can be traced down to the basal layer within which are a group of about 10 cells with the responsibility for producing cells for that column, to compensate for the surface squames that are constantly being lost. As these structures have stability and a long life, they must be maintained by stem cells. This entire unit was referred to as EPU, and various studies suggested that each EPU contained a single stem cell situated towards the middle of the cluster of 10 basal cells (Potten, 1986; Potten and Hendry, 1973; Potten et al., 2002). A similar organisation may exist in human skin, but this has not been clearly demonstrated because of difficulties in tracing the columns.

(F67) Follicular stem cells can participate in the reconstitution of damaged epidermis, but this participation occurs only during wound healing and is transitent. The proof of the different roles of interfollicular and follicular SCs was provided using a model of ablation of hair follicles in mice. Grafted bulge cells responded to injury by migrating to the wound, participated in the early phases of epidermis regeneration, but were eliminated over several weeks. Follicular stem cells generated progenitor keratinocytes responsible only for acute wound repair (Ito and Cotsarelis, 2008; Ito et al., 2005). Similarly, mouse stem cells from the hair follicle bulge were found by another group to contribute transiently to interfollicular epidermis wound repair but not to normal homeostasis. In homeostatic conditions, follicular keratinocytes do not participate in interfollicular renewal (Claudinot et al., 2005).

(F68) The frequency of mouse interfollicular stem cells varies considerably according to different authors and is generally thought to be 1 to 10% of the basal cell (Bickenbach et al., 1986; Heenen and Galand, 1997; MacKenzie, 1985; Morris et al., 1985). A macroscopic, clonal regeneration assay for mouse epidermis was developed by Withers (Withers, 1967), which generates nodules very similar in appearance to haematopoietic colonies in the spleen of irradiated mice. Subsequently, Al-Barwari and Potten (1976) developed a microscopic clonal assay that required a shorter-time interval between irradiation and tissue sampling. Together, these clonal regeneration assays were interpreted to indicate that only about 10% of the basal cells have a regenerative capacity, i.e. are stem cells or at least cells capable of some fairly extensive renewal to form colonies. More recently, a functional assay of long-term repopulating cells found a very low frequency of 1 in 35,000 total epidermal cells, or in the order of 1 in 10^4 basal epidermal cells, similar to that of HSCs in the bone marrow (Schneider et al., 2003).

F.5. Cellular origin of skin tumours

(F69) Several models have been proposed concerning skin cell lineages and cancer type. Sell (2004) speculated that the phenotype of epidermal carcinoma is related to the stage of differentiation of the cell types in the skin where the malignant phenotype is expressed. Thus, in this model, mutated stem cells in the bulge of the hair follicle would give rise to trichoepithelioma, EpiSCs would give rise to BCC, early progenitors would form SCC, and late progenitor cells would be the origin of papillomas (Fig. F.5.).

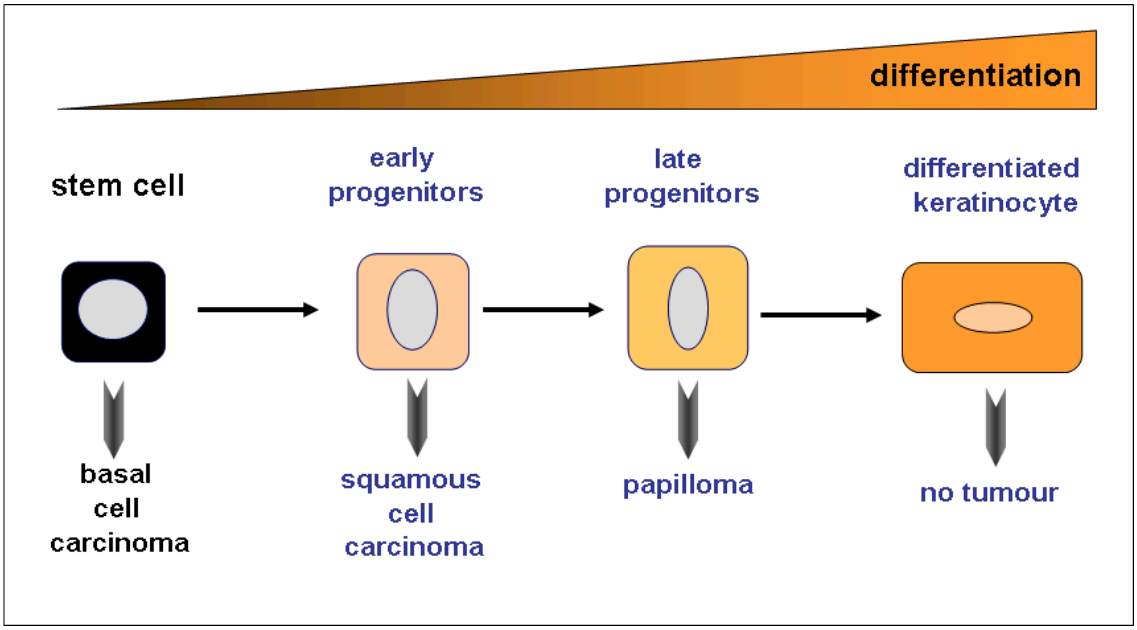

Fig. F.5. Modelling human skin cancer origin (modified from Sell, 2004). (Permission needed)

(F70) The principle of the involvement of stem cells and progenitors in human carcinogenesis is illustrated in the case of UV-induced p53 mutations in human interfollicular epidermis (Owens and Watt, 2003). Numerous cells with p53 mutations can be found in sun-exposed, clinically-normal human epidermis. Both scattered single cells and clonal patches of mutated cells are observed throughout exposed epidermis. The single mutated cells are predominantly suprabasal, terminally-differentiating cells. The size and distribution of p53-mutated patches can be compared with the distribution of stem and progenitors, identified given that stem cells have relatively higher β1-integrin expression and they tend not to be actively cycling. Some clones were small and restricted to the progenitor compartment, suggesting that they have arisen from a TA cell founder. Other clones were larger and their location was consistent with a stem-cell founder. Some of these large clones encompassed several clusters of stem cells. As the location of large patches of p53-mutated cells is selective for stem-cell-rich regions, the authors proposed that only EpiSCs have the capacity to substantially propagate UV-induced genetic alterations.

(F71) However, oncogenic events or tumour promoters might also reactivate a stem-cell programme with self-renewal ability in a target cell without these characteristics (Perez-Losada and Balmain, 2003). The initiated '*de novo* stem cell' might have an increased adhesion potential: so consequently, it would avoid being discarded by the normal desquamation process. In fact, skin stem cells express increased levels of adhesion molecules, such as β1 and β6-integrins, compared with their more differentiated counterparts. This newly acquired self-renewal capacity could allow these cells to remain within the epidermis long enough for other oncogenic events, which lead to stabilisation, to accumulate. '*De novo* stem cells' could therefore maintain the new tumoural tissue by establishing an altered hierarchical organisation within the previously normal tissue. The authors thus proposed that both stem and progenitor cells may be at the origin of cancer. Concerning the differentiated keratinocytes, as none of the benign skin lesions that arose from the suprabasal layers of epidermis progressed to malignancy, they also stated that reactivation probably does not occur in these differentiated keratinocytes.

(F72) Cancer stem cells have not yet been identified in hair follicle or sebaceous tumours. However, oral SCC initiating tumour cells have been identified and shown to express high levels of the hyaluronan receptor CD44 (Prince et al., 2007). However, CD44 is not expressed in human bulge stem cells. It is not known whether a small population of yet-to-be-identified epidermal cells express CD44, or whether *de novo* expression occurs in SCC tumour cells. K15 and K19 are highly enriched in hair follicle and sebaceous carcinomas, suggestive of a bulge origin (Bieniek et al., 2007; Kanitakis et al., 1999). However, as epithelial progenitors have been described in the sebaceous gland (Lo Celso et al., 2008), the origin of sebaceous carcinomas could be directly from these glandular cells. SCCs express high levels of proteins which are normally found in the basal layer of epidermis, including p63, which is required for proliferation and maintenance of basal keratinocytes (Blanpain and Fuchs, 2007; Rocco et al., 2006), and β6-integrin, whose expression is enriched in EpiSCs (Fortunel et al., 2003b). Although expression of stem-cell markers might reflect the tumour cell of origin, it is only a correlation, and it remains possible that these markers are induced by the oncogenic events that occur after the initiation of neoplastic growth. In summary, although most data point to a role of stem/progenitor cells, it is not yet demonstrated which cells are at the origin of human skin cancers.

F.5.1. Cell origin of SCC in mice

(F73) Evidence that tumours arise from stem and/or progenitor cells was provided by the two-step model of rodent skin carcinogenesis (Sell, 2004). The two steps are initiation and promotion. In the classic model, DMBA, the initiator, is painted on to the skin. This chemical binds to DNA in the skin cells, causing a permanent genetic alteration (initiation) at codon 61 in the Hras gene. However, cancers will not arise unless a proliferative stimulus is also given (promotion). This is provided by treating the skin with 12-*O*-tetradecanoylphorbor-13-acetate (TPA). Thus, the initiation event induces genetic damage, and the promoter then stimulates the damaged cells to proliferate, leading to cancer. Initiation must occur before promotion. If promotion is performed prior to initiation, cancers will not develop. The time between initiation and promotion is the critical factor in implicating the stem cell as the initiated cell. This interval can be days, or even months or years in length. In order for tumours to grow in this model, the initiated cells must survive from the time of initiation to the time of promotion. Given the well-established fact that all cells in the skin, except for (some of) the stem cells capable of self-renewal, turn over completely every 2–3 weeks in mice and about 1-2 months in humans, it is clear that the only way in which the initiated cells could still be present, if months or years have passed since initiation, would be for initiation to have occurred in a resting stem cell population. In the course of a year or more between initiation and promotion, all TA cells would have been replaced by newly generated cells from the basal stem cells. Thus, in the initiation-promotion model for skin carcinogenesis, the initiated cell must be a resting stem cell that is only called upon to proliferate under the stress of promotion (Sell, 2004).

(F74) In mouse skin, the hair follicle rather than the interfollicular epidermis is largely responsible for experimental tumour formation, as most of the mouse skin tumours are SCC follicle-related (Cotsarelis et al., 1990). As tumour initiation must involve primarily a population of long-lived cells, it has been proposed that the follicular stem cells produce mouse skin tumours. However, targets of tumour initiation can also be found in the mouse interfollicular epidermis. To determine the origin of skin tumours, the interfollicular epidermis of carcinogen-initiated mice was completely removed by an abrasion technique known to leave the hair follicles undisturbed (Morris et al., 2000). The interfollicular

epidermis of the abraded mice quickly regenerated from cells in the hair follicles, after which time tumour promotion by chemicals was begun. Mice in which the interfollicular epidermis had been removed developed papillomas and carcinomas; however, the number of papillomas was half that of the unabraded mice. These data were consistent with the hypothesis that the targets of tumour initiation are stem cells found in the hair follicles regarding most malignant carcinomas, whereas more differentiated cells give rise to papillomas with a low risk of malignant conversion.

(F75) Evidence that the consequences of an oncogenic injury depends on the cell that sustains it, comes from experiments in which the same oncogene is expressed in the hair follicle or in different layers of the epidermis of transgenic mice (Owens and Watt, 2003; Preto et al., 2004). When mice were produced with a Ras oncogene which is driven by promoters that are selectively expressed during terminal differentiation, the only tumours to form were benign papillomas that tended to regress (Bailleul et al., 1990). However, if Ras was driven by a truncated K5 promoter that was expressed exclusively in the proliferating cells of the hair follicle, the mice developed malignant carcinomas (Brown et al., 1998).

(F76) Stem cell markers can be studied to address the origin of SCC. In the murine epidermis, one such candidate marker is CD34, a cell-surface marker that has been used to enrich for multipotent stem cells from the hair follicle bulge (Trempus CS, 2003). Malanchi et al. (2008) characterised a subpopulation of $CD34^+$ keratinocytes in murine epidermal tumours induced by chemical (DMBA/TPA) carcinogenesis. The percentage of $CD34^+$ keratinocytes was increased in epidermal tumours (papillomas) and SCCs versus normal epidermis, although it was not clearly demonstrated whether these cells were progeny of preexisting $CD34^+$ cells or *de novo* CD34-expressing cells. However, the $CD34^+$ population exhibited phenotypic characteristics of bulge stem cells, including expression of bulge markers and an absence of the differentiation marker K10. Taken together, these results indicate that the $CD34^+$ population contains cancer stem cells.

(F77) In chemically induced skin tumours, nuclear β-catenin was enriched in $CD34^+$ versus $CD34^-$ tumour cells (Malanchi et al., 2008), suggesting a potential functional relevance for this pathway. More importantly, deletion of β-catenin in established tumours led to a reduction in the percentage of $CD34^+$ cells, and the tumours regressed, as marked by extensive terminal differentiation. The skin tumour regression phenotype was recapitulated following β-catenin deletion in another mouse skin tumour model (Tg.AC) that expresses activated H-Ras. Taken together, these data suggest that β-catenin is required for Ras-driven tumourigenesis in mouse skin, although the mechanism of cancer stem-cell loss following β-catenin deletion is not yet established. However, as noted by the authors, nuclear β-catenin expression is not strictly a marker of cancer stem cells, and CD34 is not expressed in human SCC, precluding a stem cell analysis of human tumours. In the human hair follicle, CD34 immunoreactivity is found; however, there is no evidence that this marker enriches for stem cells. In summary, Malanchi et al. elegantly demonstrated that murine SCCs contain a subpopulation of cancer stem cells that can be enriched by selection for the follicular bulge cell surface marker, CD34. They provided convincing evidence that β-catenin signalling is required for growth of murine SCC, most likely via the maintenance of stem cells, although the mechanism by which this occurs is still unclear. Finally, they demonstrated that the Wnt/β-catenin pathway is also activated in human malignant SCC. However, the challenge remains to identify cancer stem cells within human epidermal tumours.

(F78) Another group found similar results regarding CD34 and β-catenin. They identified a population of mouse cells in early epidermal tumours characterised by phenotypic and functional similarities to normal bulge skin stem cells. This population contains stem cells, which are the only cells with tumour initiation properties. Transplants derived from these

cells preserve the hierarchical organisation of the primary tumour. They described β-catenin signalling as being essential in sustaining the cancer stem cell phenotype. Ablation of the β-catenin gene resulted in the loss of stem cells and complete tumour regression. In addition, they provided evidence for the involvement of increased β-catenin signalling in malignant human SCCs. Because Wnt/β-catenin signalling is not essential for normal epidermal homeostasis, such a mechanistic difference may thus be targeted to eliminate cancer stem cells and consequently eradicate SCCs (Prince and Ailles, 2008). Although these data are interesting, the data based on markers are questionable, as it has been shown for BCCs that they cannot be used to unravel the cellular origin of skin tumours (Youssef et al., 2010).

(F79) In summary, concerning the cellular origin of mouse SCC, transforming events in stem cells and/or progenitors are the most frequent early events that lead to SCC formation, although the precise role of each basal keratinocyte population is still to be defined.

F.5.2. The cell origin of BCCs

(F80) As in Ptch1$^{neo67/+}$ mice, skin tumours can originate both from hair follicle and interfollicular epidermis, and these mice have been used to assess the origin of BCC skin tumours. BCC precursor lesions arose from both follicular and interfollicular epithelium in unirradiated mice, but these lesions progressed to nodular and infiltrative BCCs only in irradiated mice. Using K14 as a basal differentiation marker, the fact that BCCs originate from the basal layer of epidermis has been tested. In normal skin, the basal cell layer of the epidermis and the outer root sheath displayed marked immunoreactivity for anti-K14 antibody. No immunoreactivity was detected in the remaining follicular compartments such as the inner root sheath and the bulb. Basaloid hyperproliferation areas, as well as nodular and infiltrative BCCs, showed strong immunoreactivity for anti-K14 antibody, suggesting that they originate from a common progenitor localised in the basal layer of interfollicular epidermis (Mancuso et al., 2004). The origin of BCC was also addressed by the group of Blanpain in another model of mice, where they conditionally activate the SHH pathway in various types of skin cells. Activation was obtained by expressing an active form of the SMO mutant protein (SmoM2) (see Fig. F.1.). In this model, activation of SmoM2 in hair follicles did not result in BCC induction. BCC mainly arose without carcinogenic treatment from long-term resident progenitors of the interfollicular epidermis, and to a lesser extent from upper infundibulum (Youssef et al., 2010).

(F81) In summary, the data obtained from two models of mice engineered to modify the SHH pathway bring interesting findings regarding BCC. In SmoM2^{-} mice, which are models of sporadic tumours, stem/progenitors of the interfollicular epidermis were at the origin of BCCs. In Ptch1$^{+/-}$ mice, which are models for radiation-induced tumours (paragraph F28), BCCs also appeared to originate from the epidermis. Thus, both models, which may closely mimick the human BCC pathology, point to the stem/progenitor compartment, without going in more detail into cell origin.

F.6. Radiosensitivity of stem cells and progenitors

(F82) As skin stem cells self-renew during the whole life of an individual, representing the long-term reservoir for tissue regeneration, the maintenance of genomic stability should be particularly stringent in these cells (Harfouche et al., 2010). This maintenance can be realised through different mechanisms, some specific to stem cells and others which are common with the differentiated cells.

(F83) The first mechanism is quiescence, which is possible because a functional hierarchy exists in epidermis (Fig. F.4.). The most primitive cells, responsible for the long-term renewal of the tissue, divide at a low frequency. Thus, the risk of accumulation of replication errors as well as fixation of mutations induced by DNA-damaging agents, is reduced in the stem cell population. The high proliferation rate necessary for turnover of the epidermis is maintained by the direct progeny of the stem cells, or progenitors, which divide actively. These progenitors are regularly eliminated by the process of terminal differentiation.

(F84) Another protective mechanism specific to stem cells was proposed by Cairns (1975). This mechanism is the asymmetrical cell division, giving rise to a stem cell similar to the initial stem cell, and to another cell type, a progenitor. The group of Fuchs postulated that stratification occurs through asymmetrical cell divisions in which the mitotic spindle orients perpendicularly to the basement membrane. They showed in mice that basal epidermal cells use their polarity to divide asymmetrically, generating a committed suprabasal cell and a proliferative basal cell (Lechler and Fuchs, 2005). Cairns (1975) and then Potten (2004a) proposed an asymmetrical chromosome segregation resulting in an immortal template DNA strand remaining in the stem cell and a newly constituted strand being in the progenitor cell. Such a mechanism would also protect stem cells from replication errors in DNA. To address this question, the group of Tumbar developed a strategy to count bulge cell divisions in mouse hair follicle and marked them with BrdU. Their study provides quantitative data supporting the long-standing infrequent SC-division model. However, they showed that hair follicle stem cells do not retain the older DNA strands or sort their chromosomes (Waghmare et al., 2008).

(F85) To maintain their genomic stability, stem cells might have developed appropriate mechanisms of response to DNA-damaging agents. One possible mechanism is the elimination of damaged cells. Cell death, induction of differentiation or senescence of any damaged stem cells can be a definitive solution to avoid deleterious long-term effects. Keratinocytes usually die by necrotic processes and mitotic cell death, rather than by apoptosis, although it is often difficult to distinguish the mode of death in sheet preparations of epidermis.

(F86) In the mouse, survival data have been published after high doses, based on *in vivo* studies. The radiosensitivity of epidermal (macro) colony-forming cells, which possess some of the features of stem cells, was summarised by Potten et al. (1985). From several published data, an average D_0 of about 1.2 Gy was determined (Fig. F.6.).

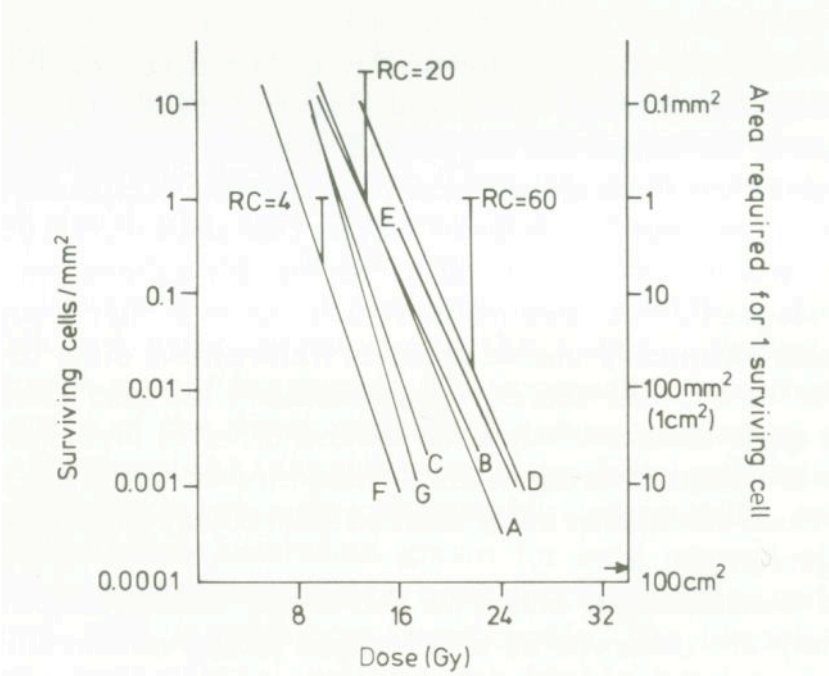

Fig. F.6. Survival curves for macroscopically visible colonies in mouse skin (Potten et al., 1985). The number of surviving cells per mm^2 of epidermal surface is plotted on a log scale against radiation dose. The scale on the right shows the surface area needed to provide one surviving cell. The arrow (bottom right) shows the approximate entire surface area of a mouse. For 3 of the studies, the size of the extrapolation number on the cell survival curve was estimated using split-dose techniques to provide a recovery factor, or repair capacity (RC). The letters A-G identify curves measured in different studies, as reviewed by Potten. (Permission needed)

(F87) Moreover, the curve for small microscopic colonies, evaluated 3 days after irradiation, crossed the curves for the larger colonies evaluated at later times. This suggested that most small colonies, produced by the 3 generations of progenitor cells in the epidermis, grew after irradiation and performed a normal small number of divisions before maturation. This would be consistent with a relative radioresistance of transit cells. That is in contrast to the high radiosensitivity of transit cells to UV. This difference is also presumed to explain why there is a longer latency for appearance of skin reactions in the case of x-irradiation compared to after UV-irradiation (e.g. acute sunburns) (Al-Barwari and Potten, 1979).

(F88) There have been a number of attempts over many years using standard rodent systems to gather quantitative information on the changes in the fraction of hairs remaining or surviving (i.e. those not epilated) with varying dose. This should generate a survival curve for follicular clonogenic cells (stem cells and probably some early progenitor cells). Although there is some scatter within the data from one set of experiments, each set of experiments tends to generate a reasonable survival curve. Unfortunately, when comparisons are made between sets, the range can be enormous (Hendry et al., 1980; Potten et al., 1985) (Fig. F.7.). Even with a single strain of mice, where there is considerable genetic uniformity, the results vary enormously with threshold doses ranging from 4 to 20 Gy and D_0 values ranging from 1.35 to 6 Gy or even higher. The lower D_0 estimates are consistent with the average value for interfollicular epidermal clonogenic cells. Some of the problems associated with the follicle survival studies are as follows (Potten et al., 1985):

1. Different endpoints have been used: some workers score variously the hairs shed, those that remain, the follicles that remain intact, or the follicles that regrow a new hair. Also,

many of these scores have been made at different times after irradiation. Clearly the most stringent test is whether or not a follicle can survive to reorganise a new cycle of hair growth after irradiation, but this does not necessarily provide the most sensitive survival curve;

2. The follicles (and the interfollicular epidermis) are subject to various degrees of mild hypoxia, sufficient to affect radiosensitivity, and this can be modified by environmental conditions or by the use of anaesthetics;
3. Hair follicles in mice may produce more than one hair, so that follicle and hair counts may differ;
4. There are different types of hair growing from different-sized follicles which may have different sensitivities. The proportions of the various follicle types may vary from mouse strain to strain and from species to species;
5. Follicles in different stages of the hair growth cycle contain different numbers of cells at the various stages of the cell cycle;
6. Damage to one follicle may result in the loss of the hair, but the follicle reorganises to regrow another, or alternatively, the follicle may be sterilised and be incapable of further hair growth. It is conceivable that it may continue to contain a hair for some time even though the follicle is sterilised; and
7. During the post-irradiation reorganisation of the epithelium, new follicles may be formed from surviving follicle cells, epidermal cells or sebaceous gland cells. This reorganisation may take a considerable time.

The range of dose-response curves for hair follicles and hair survival is illustrated in Fig. F.7. The enormous range in sensitivity is evident. In some cases, anagen hairs are reported to be more sensitive than telogen hairs, while in other cases, they are said to be more resistant.

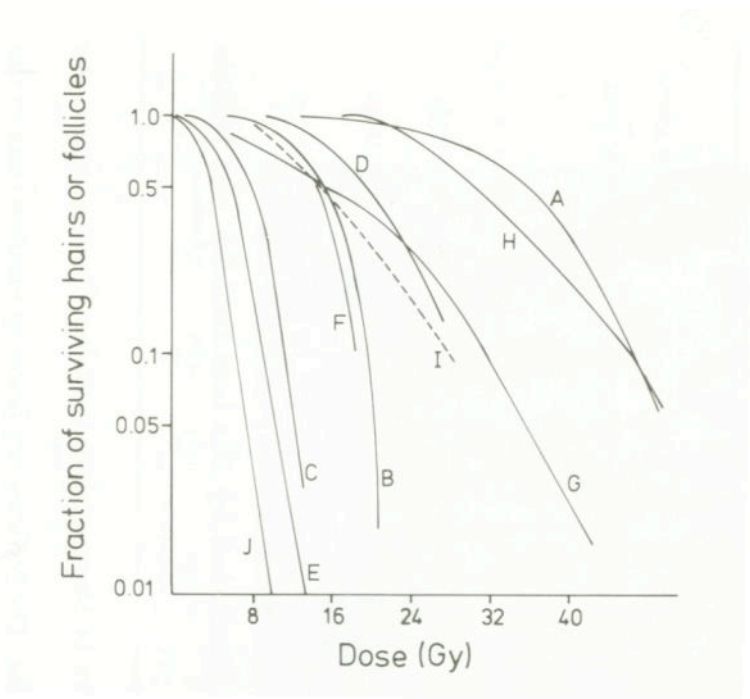

Fig. F.7. Survival curves for hairs or hair follicles where the surviving fraction has been plotted on a log scale against the dose on a linear scale (Potten et al., 1985). The labels A-J refer to individual studies, as reviewed by Potten. (Permission needed)

F.6.1. Radiation response *in vitro*

(F89) The radiosensitivity of colony-forming primary keratinocytes from childhood foreskin was measured *in vitro* (Dover and Potten, 1983). The survival curves showed that the cell population was more sensitive (D_0 of 0.7-0.9 Gy) than murine epidermal colony-forming cells *in vivo* (D_0 ~ 1.3 Gy). This difference is likely to reflect difference between *in vivo* versus *in vitro* conditions rather than intrinsic difference in radiosensitivity between species.

(F90) For human cells, protection from cell death was found in keratinocyte progenitors in tissue culture (Tiberio et al., 2002). After isolation from epidermis, cells rapidly adhering to collagen type IV, with a high level of expression of β1, α6 integrins and p63, were protected from cell death via an integrin signalling pathway in a Bcl-2 dependent manner. Another study, using the selection of keratinocytes based on the phenotype $\alpha6^{bri}CD71$ (Li et al., 1998), characterised the response of two cell populations isolated from the basal layer of human epidermis. The stem cell population ($\alpha6^{bri}CD71^{dim}$), was found radioresistant to γ-irradiation (2 Gy), whereas its direct progeny, the keratinocyte progenitors ($\alpha6^{bri}CD71^{bri}$), was much more sensitive to the same dose (Rachidi et al., 2007). A 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide salt (XTT) assay revealed 30% mortality in progenitors at 72 hours after exposure and none in the stem cells, and colony assays showed that 82 % of KSCs survived at 2 weeks, as compared to only 29% for the progenitors. A transcriptome analysis was performed to compare the genomic responses to radiation of the two populations. One of the most striking responses of stem cells was the repression of a network of genes involved in cell death processes. Such a repression can be related to an important function of stem cells, which is the maintenance of tissue homeostasis, which requires avoiding massive cell death that would lead to stem cell depletion.

(F91) A major mechanism of resistance to ionising radiation is activation of DNA repair. In keratinocytes selected with the α6/CD71 phenotype, human KSCs showed more rapid repair of global DNA damage than in progenitor cells. This result was obtained using the comet assay, which under alkaline conditions measures mainly single-strand breaks (SSBs). Moreover, DSBs, measured by the γH2AX assay, were also repaired more rapidly in KSCs than in progenitor cells. Taken together, these data show that the basal layer of human epidermis contains two cell populations with different DNA repair capacities, with the minor, quiescent stem cell population exhibiting a more rapid and efficient repair than the large progenitor cell population (Harfouche and Martin, 2010).

(F92) Similarly, stem cells from the mouse follicle bulge were found capable of activated DNA repair capacity. Keratinocytes isolated on the basis of an $\alpha6^{+}CD34^{+}$ phenotype (hair follicle bulge cells) were compared to cells with the $\alpha6^{+}CD34^{-}$ phenotype, which represent all the other keratinocytes, including those of the hair follicle and the basal layer of the epidermis. Although this comparison is poorly selective, it allowed demonstrating that the repair of both SSBs and DSBs was more rapid in the bulge cells. Moreover, the NHEJ pathway was found involved in this rapid repair, through DNA-PK activity (Sotiropoulou et al., 2010).

(F93) Finally, activated cell signalling was another possible mechanism of stem cell resistance. Using the phenotype α6/CD71, activation of the bFGF pathway by DNA damage was investigated in stem and progenitor cells. The results revealed that the bFGF signalling pathway was induced by DNA damage in stem cells, but not in progenitor cells. To examine the role of this endogenous bFGF in DNA repair, stem cells were exposed to bFGF pathway inhibitors. Blocking the bFGF receptor FGFR1 or the kinase MAPK1 resulted in inhibition of DNA SSB and DSB repair in the KSCs. Moreover, supplementing the progenitor cells with

exogenous bFGF activated their DNA repair. The authors proposed that bFGF helps to maintain genomic integrity in stem cells by activating stress-induced DNA repair (Harfouche and Martin, 2010).

(F94) The recent data obtained on human cells and in mice, highlight the importance of decrypting the DNA damage response in stem cells. If the increased repair rate observed is an intrinsic property of EpiSCs, the question of the repair fidelity is crucial concerning the possible late effects of radiation exposure, such as keratinocyte transformation. *In vitro* transformation of normal human keratinocytes is not a common process. Studies on whole populations of keratinocytes reported that exposing normal cells in tissue culture to various graded doses of irradiation failed to show any evidence of transformation (Thraves et al., 1990; Tuynder et al., 1991). When cells were irradiated with 8 fractions of 2 Gy over several months, and then selected in medium which caused cessation of growth (low EGF concentration and high Ca^{2+}), one clone escaped senescence but failed to form tumours in nude mice (Tuynder et al., 1991).

(F95) Recent studies (Harfouche et al., 2010; Sotiropoulou et al., 2010) point to NHEJ as a mechanism of repair for DNA damage in the stem cell population, which can be an error-prone mechanism of repair. On the other hand, keratinocyte progenitors exhibited a slow and incomplete repair, which appears potentially at high risk for cell transformation. In fact, a long-term study of genomic instability in cultured clones of progenitors irradiated with 2 Gy resulted in the occurrence of transformed clones with a surprising high incidence (M Martin, unpublished data). However, *in vivo*, the differentiation process should limit this risk, as keratinocyte progenitors regularly migrate to the upper layers of epidermis to progressively develop terminal differentiation. An important issue will be to develop cell transformation assays on purified populations to better characterise the risk for human stem cells and for the different types of keratinocyte progenitors. New models of 3D skin cultures or human grafts in immune-compromised mice could provide relevant conditions to address this issue.

F.7. Mutagenesis

(F96) Mutant $Ptch1^{neo67/+}$ mice have been used to assess the origin of BCC skin tumours, as described in section F.3. Also, the origin of BCC was also addressed by the group of Blanpain in another mouse model, where they conditionally activate the SHH pathway in various types of skin cells. Activation was obtained by expressing an active form of the SmoM2 (see section F.3).

F.8. Summary and conclusions

(F97) The role of KSCs in radiation carcinogenesis is still poorly defined. This is due to two main reasons. Firstly, knowledge about the biology of EpiSCs is still not sufficient, illustrated for example by the fact that specific markers of the different cell types found in the basal layer are lacking. We do not know precisely how to separate stem cells from progenitors directly from skin samples, and only enrichment is possible. We do not know how to characterise the different types of progenitors, from early progenitors close to the stem cells up to the late progenitors committed to migrate to the upper layers of the epidermis. This is a strong limitation to characterising their role in carcinogenesis.

(F98) Secondly, rodent skin is extremely different from human skin, thus limiting the comparisons. For example, most mouse tumours are SCCs arising from the stem cells of the bulge, a hair follicle structure. On the contrary, most human epithelial tumours are BCCs originating from the interfollicular epidermis.

(F99) Despite these strong limitations, one clear result is that most human epithelial skin tumours arise from the basal layer of the epidermis, whereas differentiated keratinocytes give rise to benign lesions such as papillomas. Multiple data argue that EpiSCs can be at the origin of BCCs, both with and without exposure to ionising radiation. However, some types of progenitor cells, still to be defined, also probably participate in carcinogenesis. Concerning the origin of SCC, there is very little evidence available. In conclusion, the model proposed by Sell (2004), in which the type of carcinoma depends on the differentiation of the initially-modified cells, is still a working hypothesis.

(F100) One important question is whether protein markers can be used to elucidate the target cells for radiation-induced cancer. Recent data on BCC mouse models demonstrate that this is probably not the case. Another important question is the management of the radiation stress by the cells. New data, obtained for human and mouse keratinocytes, demonstrate that more primitive cell populations are able to repair their DNA damage better than their progeny. Moreover, specific cell signalling upstream of DNA repair can be activated in the stem cells. It is important to define the type of DNA repair pathways used and the long-term consequences on genomic stability.

(F101) Trying to answer some of all the remaining questions will require fundamental studies to better characterise the different types of keratinocytes within the basal layer of human epidermis and the hair follicle. New skin cancer models, such as those derived by the group of Khavari, where tumour cells are cultured in 3D environments or after grafting in immune-compromised mice, will be necessary for improving radiobiological knowledge.

(F102) Finally, the development of cell reprogramming technology, which permits derivation of embryonic-like stem cells from the skin cells of patients, opens a totally new area of research. Deriving iPS cell lines from patients with hypersensitive syndromes such as Gorlin's syndrome or DC could help better understand the development of radiation-induced skin tumours.

ANNEX G: BONE STEM CELLS

G.1. Radiation carcinogenesis

(G1) Incorporation of radionuclides into the skeleton has been shown to cause bone cancer and leukaemia in humans and animals (ICRP, 1991; UNSCEAR, 2000; WHO, 2001). Radiation-induced tumours are characterised by a latent period, i.e. the time from radiation exposure to the clinical appearance of a tumour. The latent period consists of two periods: the true latent period, or interval from the initiation of cells to the beginning of unrestricted growth, and a second period of tumour growth, or the time to diagnosis or presentation. Although the minimum latent period is 3-4 years for bone cancer, the mean latent period for the induction of bone cancer following external irradiation is 10-15 years (Mettler and Upton, 1995).

G.1.1. Radiation-induced bone tumours in humans

(G2) An increased incidence of bone tumours has been observed in people exposed to long-lived α-emitting isotopes of radium, particularly in painters of luminous dials, but also in radium chemists and in people treated with radium salts in the belief that their effect was therapeutic (Rundo, 1986). Radium, as an alkaline earth element, behaves similarly to calcium and is retained predominantly in the skeleton. It is deposited on bone surface and incorporated into bone volume (Leggett et al., 1982). Because of the short range of α particles (40 – 50 μm in soft tissues and less in bone mineral), only atoms decaying on or near to bone surfaces will irradiate target cells for the induction of bone cancer. In the US, almost 5,000 workers, mainly female, were employed in the luminising industry, mainly between 1915 and 1930 and between 1940 and 1954. Fluorescence was achieved initially by addition of ^{226}Ra salts to paint; later a mixture of ^{226}Ra and ^{228}Ra was also used (Fry, 1998). ^{226}Ra (physical half-life, 1,600 years) emits α particles and decays to ^{222}Rn. ^{228}Ra (half-life 5.75 years) decays by β particle emission but radioactive progeny include the α particle emitters, ^{228}Th, ^{224}Ra, ^{220}Rn (called thoron), ^{216}Po, ^{212}Bi and ^{212}Po. By the end of 1983, 62 cases of bone sarcoma had occurred in a total of 2,352 people who had been measured to obtain an estimate of their body content and hence dose (Rundo, 1986). No bone sarcomas were observed at cumulative average bone doses of below 10 Gy (Rowland, 1997).

(G3) ^{224}Ra (half-life 3.6 days) was used in Germany as a treatment for arthritis, ankylosing spondylitis, and bone tuberculosis in the 1940s and 1950s. The most recent reports of cancer in patients exposed to high levels of ^{224}Ra (mean cumulative bone surface dose of around 30 Gy, high LET) have included 899 individuals exposed as adults or children (Nekolla et al., 1999; Nekolla et al., 2000; Nekolla et al., 2005; Spiess, 1995). A total of 56 malignant bone tumours have occurred (0.3 expected), with a peak incidence at 8 years after treatment; only 4 tumours were diagnosed after 1980. Younger ages at exposure, particularly at ages of active bone growth, appeared to be associated with a higher risk, depending on the dose estimate used (Henrichs et al., 1995; Nekolla et al., 2000; Nekolla et al., 2005). Lower levels of ^{224}Ra were given to adult ankylosing spondylitis patients (typical mean cumulative bone surface dose of about 5 Gy). Follow-up of about 1,500 patients (Wick, 2005; Wick et al., 1999) has noted small numbers of bone tumours (4 observed, 1.3 expected) and leukaemias (16 observed, 6.5 expected).

(G4) Mayak workers were exposed to very high levels of external and internal radiation, including plutonium exposures in the radiochemical plant and plutonium production plant.

Levels of irradiation were particularly high during the early years of plant operation (late 1940s to the mid-1950s). In the group of 11,000 workers who started work at Mayak during this time, 27 malignant bone tumours have been observed (19 bone and cartilage neoplasms, 4 myosarcomas, 3 synovial sarcomas and 1 fibrosarcoma) (Koshurnikova et al., 2000). Bone surface doses were considerably greater than 10 Gy. However, sufficiently reliable estimates of dose are not yet available, and risk estimates for ^{239}Pu-induced bone cancer have not yet been made (Harrison and Muirhead, 2003).

(G5) In the past, the terms bone sarcoma or bone cancer, have often been used synonymously with osteosarcoma. However, radiation-induced bone sarcomas have been shown to follow a number of different lines of differentiation. In addition to bone-producing osteosarcomas, a number of other tumour types have been observed, including fibrosarcomas, malignant fibrous histiocytoma (MFH) and chondrosarcoma. In two retrospective studies of bone sarcomas induced by ^{226,228}Ra (Schlenker, 1989) and ^{224}Ra (Gossner et al., 1995), the two most common histological types were found to be osteosarcoma (70% for ^{226,228}Ra, 53% for ^{224}Ra) and non-bone-producing sarcomas of the fibrosarcoma/MFH type (30% for ^{226,228}Ra, 33% for ^{224}Ra). The high incidence of fibrosarcoma/MFH type tumours, of about 30%, is significantly greater than the incidence of these tumours of about 10% in spontaneously-occurring bone sarcomas (Gossner, 1999, 2000). It is of interest to note that in the lower dose ^{224}Ra group referred to above (Wick, 2005; Wick et al., 1999), the small number of sarcomas observed were not osteosarcomas but included fibrosarcoma/MFH type tumours. The spectrum of radiation-induced bone tumours after exposure to radium isotopes is similar to that seen after external irradiation (Huvos, 1991; Unni, 1996) and in non-irradiated patients who developed tumours at the site of preexisting bone lesions, such as Paget's disease and bone infarct (Desai et al., 1996; Schajowicz, 1993).

(G6) It is possible that gross tissue damage plays an important role in the induction of bone sarcomas by radiation. Radiation is known to cause chronic disturbances of bone remodelling in conditions referred to as osteitis, osteodystrophy, and osteodysplasia. It appears that there is a low threshold for the induction of such lesions, of about 3 Gy cumulative α skeletal dose (Hahn et al., 1988). The damage caused is characterised by areas of bone infarction with bone necrosis, vascular damage and finally peritrabecular fibrosis. A proliferative fibro-osseous response is also frequently seen in the irradiated marrow. The histopathology of these responses is similar to the active phase of Paget's disease, osteitis deformans (Gossner, 1986; Luz, 1991). Such radiation-induced bone lesions have been described in individuals exposed to ^{226}Ra. For example, Lisco (1956) reported a fibrosarcoma associated with peritrabecular fibrosis in a radium dial painter. A detailed electron microscope examination of another case of fibrosarcoma in a radium dial painter by Lloyd and Henning (1983) showed a fibrotic layer with a thickness of up to 50 μm interposed between marrow cells and the bone surface in the region of the tumour.

G.1.2. Radiation-induced bone tumours in animals

(G7) The toxicity of radium isotopes and other bone-seeking radionuclides has been compared in beagle dogs in a number of large studies carried out in the US at the University of Utah, University of California, Inhalation Toxicology Research Institute and Pacific Northwest Laboratories (Boecker, 1995; Lloyd et al., 2001; WHO, 2001). The results have generally been expressed in terms of average bone dose. Comparing bone cancer rates from linear dose-response relationships for individual α-emitting nuclides injected systemically in soluble form, toxicity relative to ^{226}Ra was summarised by Boecker et al. (1995) as: 16 for ^{239}Pu, 9 for ^{228}Th, 6 for ^{241}Am, 4 for ^{224}Ra, and 2 for ^{228}Ra (Boecker, 1995). For the β emitter,

^{90}Sr, the dose response was non-linear with no tumours occurring at doses below 18 Gy cumulative average bone dose. Boecker et al. (1995) quoted values for ^{90}Sr toxicity relative to ^{226}Ra of 1 ± 0.5 at >40 Gy and 0.05 ± 0.03 at 5 – 40 Gy. The different toxicities of the α-emitting nuclides are attributable to differences in dose to the target region near to endosteal bone surfaces, which will depend on the affinity of nuclides for different bone surfaces, their incorporation or burial in bone, and their half-lives. The observed differences between ^{226}Ra and ^{90}Sr are largely attributable to RBE, with β emissions from ^{90}Sr being substantially less effective than α particles except at very high doses.

(G8) Muggenburg et al. (1995) compared the effect of single and multiple administration of ^{224}Ra at levels corresponding to cumulative average bone doses from 0.1–3 Gy (Muggenburg et al., 1995). Protraction of dose by administration of ^{224}Ra in 50 weekly injections increased the overall incidence of bone tumours per Gy by a factor of 4. Interpretation of the results is complicated by early deaths from marrow dyscrasia in the highest dose, single injection group. Muggenburg et al. (1995) concluded that the incidence per Gy following protracted administration was very similar to that after injection of ^{239}Pu or inhalation of ^{238}Pu oxide.

(G9) Raabe et al. (1995) analysed data for ^{226}Ra bone cancer induction in dogs in terms of average skeletal dose rate and time to death. No decrease in lifespan compared to controls was seen at dose rates of 1 mGy day^{-1} and less (cumulative doses of <3 Gy) and deaths from marrow dyscrasia occurred in the high dose region of >100 mGy day^{-1} (cumulative doses of >100 Gy). At dose rates of 1 – 100 mGy day^{-1}, there was a high incidence of tumours and a decreased latent period with increasing dose rate. Raabe et al. (1995) interpreted these data to imply that a practical threshold will exist at low dose rates because the time taken for tumour development will exceed the normal lifespan.

(G10) Osteosarcoma has been observed in mice exposed to ^{224}Ra, ^{239}Pu, ^{241}Am and ^{233}U (Humphreys et al., 1993; Humphreys et al., 1987; Muller et al., 1990). Ellender et al. (2001) compared the effect of ^{239}Pu, ^{241}Am and ^{233}U at three levels of activity giving cumulative average skeletal doses of 0.25–0.3 Gy, 0.5–1 Gy and 1–2 Gy. ^{233}U was considerably less effective than ^{239}Pu and ^{241}Am in causing osteosarcoma, consistent with its greater incorporation into bone mineral due to its chemical similarity to calcium. Osteosarcoma incidence increased with increasing dose of ^{239}Pu and ^{241}Am, but ^{239}Pu was 2–3 times more effective per unit average bone dose. Detailed analysis of dose distribution by image analysis of autoradiographs of bone sections showed that the difference between ^{239}Pu and ^{241}Am in osteosarcoma induction was consistent with their relative delivery of dose to the endosteal surface (Lord et al., 2001). The best fit between osteosarcoma induction and dose was obtained by considering dose to a 40-µm layer of marrow adjacent to endosteal surfaces, although dose to a 10-µm layer also provided a reasonable correlation.

G.1.3. Radiation quality and type of exposure

(G11) The ^{224}Ra data discussed above have been used by ICRP to derive a risk estimate for bone tumour mortality of 5 x 10^{-4} Sv^{-1}, assuming a radiation weighting factor of 20 for α particles (ICRP, 1991, 2007). This estimate applies to average skeletal dose and would be a factor of 9 lower if estimated on the basis of dose to the bone surface as currently calculated (Eckerman, 1995; Puskin et al., 1992; Spiess, 1995). The data from the follow-up of A-bomb survivors can be used to provide an estimate of the risk of bone tumours resulting from exposure to external, mainly low-LET radiation. Using these data, the risk of fatal bone cancer at low dose and dose rates, applicable to the population of England and Wales, was estimated as 1 x 10^{-4} Sv^{-1} (Muirhead et al., 1993). Grogan et al. (2001) used the analysis of the A-bomb data by Pierce et al. (1996) to provide an estimate of lifetime risk for the US

population of around 2–4 x 10^{-4} Sv^{-1}. The incidence of Paget's disease is low in Japan, relative to the US, which may result in underestimation of risk calculated on the basis of A-bomb data (Richardson and Cole, 2012). A recent report based on results from the LSS revealed osteosarcoma to be the most common bone sarcoma, with a dose threshold of 0.85 Gy (Samartzis et al., 2011). These estimates of risk may be regarded as reasonably consistent with those based on the radium studies, given the uncertainties associated with the A-bomb data and the dose estimates for the ^{224}Ra cases. However, consideration of the risk of bone cancer based on dose to the bone surface, rather than average bone dose, suggests that the α-particle RBE for bone cancer may be low, although there are substantial uncertainties associated with each estimate (Harrison and Muirhead, 2003).

(G12) Bone tumours may include types for which there is a threshold dose below which no tumours will be observed. It was reported that no sarcomas were observed in 1,339 cases with systemic intakes of ^{226}Ra/^{228}Ra estimated to give cumulative bone doses of less than 10 Gy; 46 sarcomas were observed in 191 cases with doses greater than 10 Gy (Rowland, 1997; Rowlands et al., 1995). Analysis of tumour types has shown an increase in radium cases in the numbers of fibrosarcoma and MFH, relative to osteosarcoma (Gossner, 1999). Tumours of this type are also known to occur at sites of prexisting non-tumourous bone lesions, including bone necrosis and fibrous dysplasia, and it may be that the high incidence of sarcomas of fibrohistiocytic and fibroblastic origin reflects the cell types involved in repair and remodelling processes. Deterministic tissue damage after high doses of radiation is well documented in radium-exposed individuals and may be the precursor of fibrosarcoma and MFH. Another implication of the prevalence of these tumour types is that the target cells may not be confined to the bone surface lining cells but include cells further into the marrow (Gossner, 2000).

(G13) However, Chadwick et al. (1995) fitted the radium dial painter data using a two mutation carcinogenic model with clonal expansion. The analysis showed that an LQ dose-effect relationship can be applied and, because of the very low natural incidence of bone sarcoma, is consistent with very low risk at low doses and dose rates (Chadwick et al., 1995).

G.2. General features of bone

G.2.1. Bone structure

(G14) The skeletal system consists of bone, bone marrow, periosteum, all cartilage of the body, teeth, and the blood vessels and nerves contained in these tissues. Bone consists largely of an organic matrix impregnated with inorganic salts and permeated by a complex cellular network (Fig. G.1.). The matrix of bone is composed of various proteins, carbohydrates, lipids, and other substances, but the bulk of the organic material is made up of a protein called collagen (Triffitt, 1980). The inorganic matter of bone consists mainly of submicroscopic deposits of forms of calcium phosphate (Fawcett, 1986; Neuman, 1980).

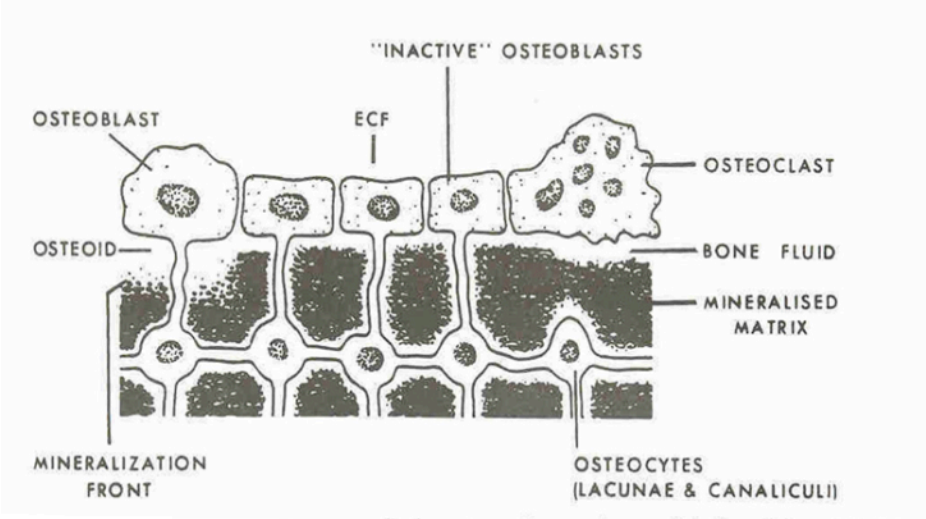

Fig. G.1. Schematic representation of the lacunar-canalicular system of bone as it opens into the cellular space on the bone surface. From Jaworski (1976). (Permission needed)

(G15) Bone develops by transformation of a preexisting connective tissue. Two different modes of osteogenesis are recognised in embryos. When bone formation occurs directly in primitive connective tissue, it is known as intramembranous ossification. When it takes place in preexisting cartilage, it is known as intracartilaginous or endochondral ossification (Fawcett, 1986). In endochondral ossification, the bulk of the cartilage must be removed before bone deposition begins, and neovascularisation of the cartilage anlage has an important role in the transition from cartilage to bone. The deposition of bone is essentially the same in both types of ossification. Bone is laid down first as spongy bone, but some of it is subsequently converted to compact bone by a filling in of the interstices between trabeculae.

(G16) In bone formation, bone-forming cells (osteoblasts) synthesise the organic matrix, and this pre-osseous tissue (osteoid) then undergoes mineralisation (Triffitt, 1980). This results in a hard, durable structure which is not permanent. Throughout life, there is a continual modification (remodelling) of bone by bone resorbing cells, called osteoclasts, and by osteoblasts to maintain the mechanical competence of the structure and to accommodate conditioning forces that are applied through locomotion, lifting, and the maintenance of posture (Frost, 1980). Bone remodelling also serves a role in calcium homeostasis (Frost, 1980).

(G17) The two main types of bone structure can be distinguished by differences in hardness, porosity, and soft tissue content: compact (cortical) bone and trabecular (cancellous, spongy) bone. Compact bone is the hard, dense bone that forms the outer wall of all bones, but the bulk of compact bone is found in the shafts of the long bones. Trabecular bone is a soft, spongy bone composed of a lattice-work of fragile appearance and located at the interior of flat bones and ends of long bones. Trabecular bone has a much higher porosity or soft tissue content (consisting mainly of bone marrow) and consequently a much lower fractional volume than compact bone. That is, a much lower portion of volume remains within external surfaces of bone after subtraction of volumes of all holes normally occupied by organic material (Frost, 1963). Not all bone tissue is easily classified as either compact or trabecular, since there is often a zone between the two bone types that is intermediate in porosity and surface-to-volume ratio (Parfitt, 1988).

(G18) Nearly-uniformly-spaced cavities, called lacunae, can be found throughout the interstitial substance of bone (Fig. G.2.). Each lacuna is filled by a bone cell or osteocyte, which is essentially an osteoblast that has become surrounded by bone matrix (Fawcett, 1986; Matthews, 1980). Radiating in all directions from each lacuna is slender, branching tubular passages, called canaliculi, which penetrate the interstitial substance and join with canaliculi of neighbouring lacunae. Thus, the lacunae form a continuous system of cavities connected by an extensive network of minute canals (Fawcett, 1986).

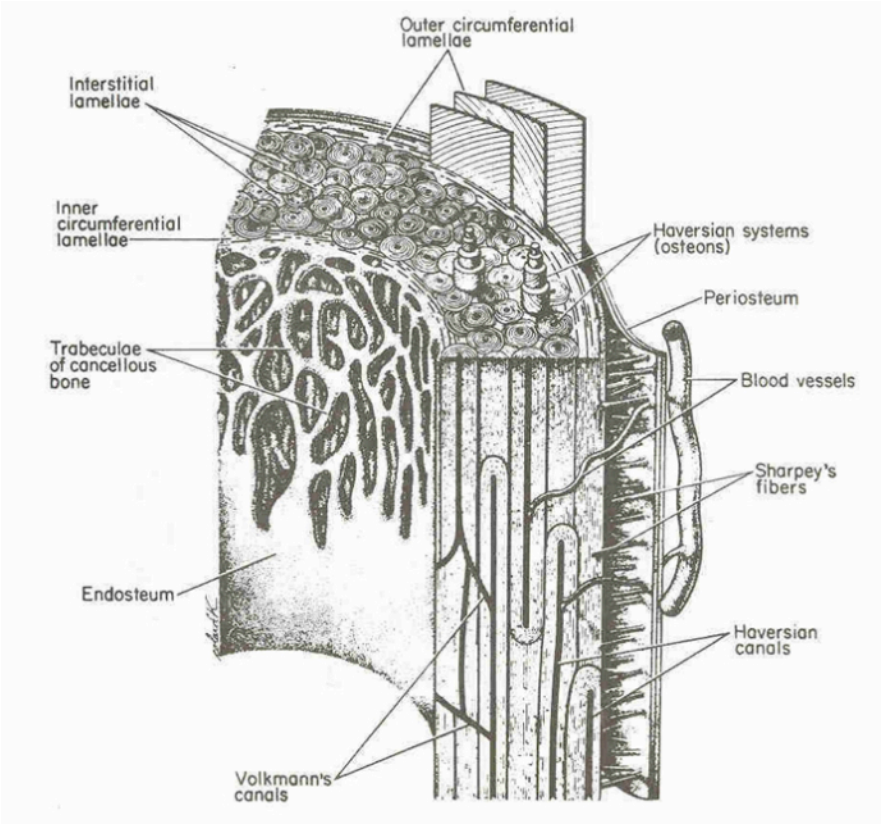

Fig. G.2. Diagram of a sector of the shaft of a long bone illustrating the Haversian systems, Volkmann's canals, interstitial lamellae, outer and inner circumferential lamellae, and attachment of periosteum of bone. From Fawcett (1986). (Permission needed)

(G19) The dominant microscopic structure of compact bone is the Haversian system or osteon (Fig. G.2.). The typical osteon is a cylinder running parallel to the long axis of bone and is about 200 µm in diameter, but there is considerable variation in the shape, direction, and size of these structures (Fawcett, 1986; Parfitt, 1983). Within the osteon is a central canal about 40 µm in diameter, containing blood vessels, lymphatics, nerves, and connective tissue. The walls of the osteon consist of concentric lamellae (layered bone) about 7 µm thick (Parfitt, 1983). Between the Haversian systems of compact bone are irregularly shaped systems of lamellar bone, called interstitial systems (Fig. G.2.), separated from the Haversian systems by thin lines of dense connective tissue called cement lines.

(G20) Haversian canals are connected with one another and communicate with the bone marrow and exterior surfaces of bone via supporting channels called Volkmann's canals (Fig. G.2.). Volkmann's canals are typically oblique or transverse, and are structurally distinct from Haversian canals in that they are not surrounded by concentrically arranged lamellae but traverse the lamellae around Haversian systems (Fawcett, 1986). The Haversian systems

together with Volkmann's canals, serve to supply nutrients to the canicular network, which in turn carries nutrients to the cells in the interior of compact bone. Cancellous bone has relatively few Haversian systems and usually consists primarily of angular pieces of lamellar bone (Fawcett, 1986). The bone cells are generally nourished by diffusion from the endosteal surface via minute canaliculi that interconnect the lacunae and extend to the surface (Fawcett, 1986).

(G21) All bones are covered in a fibrous sheath, called the periosteum, except at joint surfaces. The periosteum consists of a variably thick layer of fibrous connective tissue consisting of two layers, the outer fibrous layer, and an inner cambium layer (Ellender et al., 1988). The fibrous layer contains fibroblasts and the cambium layer contains progenitor cells with the potential to develop into bone-forming osteoblasts or cartilage-forming chondrocytes. In the adult, the progenitors revert to a resting form (Fawcett, 1986), unless activated by events such as physical damage (e.g. fracture) or novel mechanical stimulation. The periosteum is penetrated by blood vessels that communicate with Volkmann's canals, which in turn communicate with vessels of Haversian canals. The periosteum is abundantly supplied with nerves (Moss, 1966). Muscle tendons and ligaments may attach directly into the compact outer surface of a bone, or they may blend with outer layers of the periosteum (Moss, 1966). The numerous small blood vessels penetrating the periosteum may help keep the periosteum attached to the underlying bone (Fawcett, 1986). In addition, there are coarse bundles of collagenous fibres, called Sharpey's fibres or perforating fibres (Fig. G.2.), that turn inward from the outer layer of the periosteum and penetrate the outer circumferential lamellae and intersitital systems of the bone (Fawcett, 1986).

(G22) The endosteum is a layer of cells lining the walls of all cavities in bone that houses the bone marrow. The endosteum resembles the periosteum in its bone-forming potential but is much thinner, usually being composed of a single layer of cells without associated connective tissue fibres. All cavities of bone, including the Haversian canals and the marrow spaces within trabecular bone, are lined by endosteum (Fawcett, 1986).

(G23) In the adult human, the typical long bone is composed of a central cylindrical shaft called a diaphysis, two roughly spherical, terminal articular regions, called epiphyses and two intermediate cone-like regions, called metaphyses, that connect the shaft and articular ends. In growing children, the epiphysis is separated from the diaphysis by a cartilaginous epiphyseal plate, which is united to the diaphysis by columns of trabecular bone in the metaphysis.

(G24) The flat bones of the skull generally lack a central marrow region, but consist of two plates of compact bone with an intervening trabecular region, called the diploë (Fawcett, 1986; Moss, 1966). The outer surfaces of both plates are covered with a periosteum, and the diploic space is lined with an endosteum. In the case of the bones of the skull vault, the outer surface is lined by a connective tissue covering called the pericranium, and the inner surface is lined by the dura mater of the brain; these linings do not differ greatly in structure or function from the periosteum and endosteum of the long bones (Fawcett, 1986; Moss, 1966).

G.2.2. Cell types and location

(G25) Precursors of bone tumours must be cells with proliferative potential, and thus both stem cells and pluripotent progenitor cells are candidate precursors for radiation-induced bone tumours described above (Basu-Roy et al., 2012a). In the osteoblastic lineage, mature osteocytes and osteoblasts are non-proliferative cells. Preosteoblasts are dividing cells committed to osteoblast formation with a limited proliferation capacity (Dorfman and

Czerniak, 1998). Osteoprogenitor cells, precursor of the preosteoblast, have a higher proliferation capacity (Gossner, 2003).

(G26) Stem cells for the osteoblast lineage reside in the bone marrow and other soft tissues. Populations of marrow-derived stem cells that are capable of osteogenesis were first identified by Friedenstein et al. (1970) who showed that when plated at clonal density, a subpopulation of plastic-adherent, bone marrow-derived cells formed colonies (CFU-F) and could differentiate to form bone and cartilage tissue after heterotopic transplantation (Friedenstein et al., 1987). Over the years, pluripotent progenitors and stem cells with osteogenic potential were assigned various names and identified by diverse biological attributes in part due to population heterogeneity with respect to self-renewal. In 2005, new criteria and nomenclature for defining mesenchymal stromal/stem cells (MSCs) were proposed by the International Society for Cellular Therapy, and accepted widely in the field (Dominici et al., 2006; Horwitz et al., 2005; Keating, 2012). The minimum criteria for defining MSC (which refers to both mesenchymal stromal cells and mesenchymal stem cells) include adherence to plastic, pluripotent differentiation (adipogenic, chondrogenic and osteogenic cells) and expression of select cell-surface markers (see Section G.2.5); not all cells within a population of MSC retain the capacity to self-renew (Dominici et al., 2006; Horwitz et al., 2005; Keating, 2012). Although this is currently an active area of research, there remains uncertainty regarding self-renewal ('stemness') and pluripotency of MSC *in situ* (Bianco et al., 2008; Keating, 2012). Once transplanted heterotopically or grown under select culture conditions, both human and murine bone marrow-derived MSC, retain the potential to replicate and differentiate into different bone cell types, including chondrocytes, adipocytes, osteoblasts and fibroblasts (Pittenger et al., 1999). Circulating MSC also may contribute to tissue repair, including at sites of bone fracture and radiation damage (Khosla et al., 2010; Mouiseddine et al., 2007). Further, a subpopulation of circulatory stem cells with osteogenic potential for tissue repair may derive from a common HSC (Pignolo and Kassem, 2011), although further study is needed regarding lineage.

(G27) The bone marrow adjacent to bone surfaces is a complex tissue containing a connective tissue network, known as stroma. It consists of fibroblast-like stromal cells, MSCs, macrophages, and quiescent $CD34^-$ HSCs. Developments in stem cell biology indicate that there is a high degree of plasticity in the stem cell pool (Gossner, 2003; Huss, 2000). It has been shown that $CD34^-$ cells, as well as generating $CD34^+$ haematopoietic progenitors, can also regenerate committed mesenchymal precursors, such as osteoblasts, chondrocytes and myocytes. Thus, it appears that $CD34^-$ stem cells as well as mesenchymal precursors are possible target cells for radiation-induced bone cancer.

(G28) Bone surfaces include both quiescent areas covered by thin layers of inactive osteoblasts, referred to as lining cells (Eriksen, 2010) and active, formative surfaces covered by contiguous osteoblasts. These are recognised histologically by eccentric nuclei and basophilic cytoplasm, due to a high content of rough endoplasmic reticulum engaged in production of the extracellular matrix (osteoid). The ratio of these two surfaces will vary depending on the extent of ongoing bone remodelling required under normal steady-state conditions or as a consequence of radiation-associated pathology (Seed et al., 1982). Since preosteoblasts, their committed osteoprogenitor precursors, MSCs and $CD34^-$ stem cells are not normally located directly in contact with bone surfaces, the current assumption (ICRP, 1975) of a cell layer thickness as low as 10 μm being the target for radiation-induced bone cancer does not appear to be appropriate.

(G29) Gössner (2003) considered that in general terms, the targets for radiation tumourigenesis may not be confined to the stem cells and committed progenitor cells in which DNA mutation may result in transformation as a step towards malignancy. Such cells

exist in a microenvironment of surrounding normal cells, vasculature and extracellular matrix, each of which may become involved in the tumourigenic process. This view is in accord with *in vitro* observations of non-targeted (bystander) effects in which damage responses such as apoptosis and genomic instability are seen in cells that have not been directly irradiated, presumably by cell–cell signalling (Prise et al., 2002). This is an area of active experimental research, but current evidence for bystander effects occurring *in vivo* is limited and their role in tumourigenesis remains to be determined (Brooks, 2004; Kassis, 2004; Morgan and Bair, 2013).

G.2.3. Tissue turnover rate

(G30) As discussed above, bone has three distinct cell types: osteoblasts, or bone-forming cells, osteoclasts, or bone-resorbing cells and osteocytes, which are terminally differentiated osteoblasts entrapped within lacunae. Osteoblasts create and maintain skeletal architecture and are responsible for the deposition of bone matrix and for the regulation of osteoclasts. Osteoblasts are mononuclear, not terminally differentiated, specialised cells (Canhao et al., 2005) and form tight junctions with adjacent osteoblasts and regions of plasma membrane specialised in vesicular trafficking and secretion (Mackie, 2003). As they differentiate, they acquire the ability to secrete bone matrix (Gori et al., 2000). The vast majority of osteoblasts (60-90%) undergo apoptosis rather than become osteocytes or inactive lining cells (Jilka et al., 2007; Parfitt, 1990). Ultimately, some osteoblasts become trapped in their own bone matrix, giving rise to osteocytes, which gradually stop secreting osteoid (Mackie, 2003). Osteocytes are the most abundant cells in bone and communicate with each other and with the surrounding medium through extensions of their plasma membrane (Knothe Tate et al., 2004; Manolagas, 2000). Therefore, osteocytes are considered to act as mechanosensors, instructing osteoclasts where and when to resorb bone and osteoblasts, also where and when to form it (Manolagas, 2000; Nakashima et al., 2011; Seeman and Delmas, 2006).

(G31) Osteocytes can undergo apoptosis, and remain entrapped within the mineralised matrix. Apoptosis of osteocytes is thought to provide signals for resorption of bone in response to localised or humoral stimuli (Aguirre et al., 2006; Tomkinson et al., 1997). Although high dose radiation reduces osteocyte density and induces bone resorption (Sugimoto et al., 1993; Takahashi et al., 1994), animal studies suggest that osteocyte apoptosis does not account directly for acute, radiation-mediated bone resorption (Midgley et al., 1995).

(G32) In order to balance bone formation and resorption in healthy individuals, osteoblasts secrete factors that regulate the differentiation of osteoclasts, and osteocytes secrete factors regulating the activity of both osteoblasts (Hartmann, 2006) and osteoclasts (Seeman and Delmas, 2006). Bone is constantly remodelled in a dynamic system where osteoblasts are responsible for bone formation and osteoclasts for its resorption (Ducy et al., 2000). The net amount of bone formed is a function of the number and/or the activity of mature osteoblasts. Targeted ablation of 70-80% of osteocytes leads to cortical porosity and bone loss (Tatsumi et al., 2007). Thus, both proliferation of cells within the osteoblast lineage and death of osteocytes may each contribute to bone formation or the regulation of bone resorption, although neither is likely to be required for remodelling to proceed. Resorption is much faster than formation: an area of bone can be resorbed in 2-3 weeks but it takes at least 3 months to rebuild (Harada and Rodan, 2003). The typical remodelling cycle lasts 6-9 months in healthy adult humans and relies on the generation of new osteoblasts and osteoclasts. Therefore, remodelling is dependent throughout life on a continuous supply of stem cells and progenitors to replenish the pools of precursors depleted during remodelling.

G.2.4. Age and gender specificity of tissue turnover

(G33) The number of MSCs in the body decreases with age (Fibbe and Noort, 2003) and infirmity (Inoue et al., 1997). The greatest numbers of MSCs are found in neonates and numbers reduce throughout life to about half at the age of 80 (Fibbe and Noort, 2003). In the fetus, the highest number of circulating MSCs is detected during the first trimester and declines during the second trimester to about 0.0001 % and further to 0.00003 % of nucleated cells in cord blood (Campagnoli et al., 2001). It has been suggested that uncommitted MSCs circulate during gestation and travel from fetal sites into other tissues during early development (Makino et al., 1999).

(G34) As mentioned previously, bone sarcomas are most prevalent in the young relative to old adults, peaking in the 10-19 year age group with a second peak arising at a later age (>65 years) (Bleyer et al., 2006; Ottaviani and Jaffe, 2009). There is also a gender difference; young males are more frequently affected than young females, though mortality rates are comparable. One possible explanation for an increased tumour incidence in 10-19 years old is that puberty corresponds to a period of rapid skeletal growth and skeletal modelling, rendering conditions favourable to transformation and tumourigenesis, analogous to the sensitivity of sites associated with Paget's lesions.

G.2.5. Cellular features

(G35) MSCs isolated as plastic-adherent multipotent cells, capable of differentiating into bone, cartilage and fat cells, can be isolated from many tissues as well as from bone marrow (Davis and Zur Neiden, 2008; Kolf et al., 2007). They express a range of specific surface antigens, but a definitive marker for MSCs has yet to be identified, hampering studies of cell lineage and niche location. The most widely used MSC markers are CD90, CD73 and CD105 (Dominici et al., 2006). Stro-1 has been identified as an MSC marker and Stro-1^- cell populations are not capable of forming colonies (i.e. not containing CFU-F) (Simmons and Torok-Storb, 1991). Stro-1^+ cells can become HSC supporting fibroblasts, smooth muscle cells, adipocytes, osteoblasts and chondrocytes (Dennis and Charbord, 2002). However, Stro-1 is unlikely to be a general MSC marker since its expression is not exclusive to MSCs (Gronthos et al., 2003). Its mouse counterpart has not been known and its function remains to be elucidated. CD106 or vascular cell adhesion molecule 1 (VCAM-1) is expressed on blood vessel endothelial and adjacent cells, consistent with a perivascular location of MSCs. It is likely to be functional in MSCs, being involved in cell adhesion, chemotaxis and signal transduction (Carter and Wicks, 2001). CD106 singles out about 1% of Stro-1^+ cells, which are the only Stro-1^+ cells that form colonies and show stem cell characteristics such as multipotentiality, expression of telomerase, and high proliferation *in vitro* (Gronthos et al., 2003). Notably, primary human osteosarcomas and chondrosarcoma cells express key transcription factors characteristic of MSC and their differentiated progeny, including Sox9 (chondrocytes), runt-related transcription factor 2 (Runx2) (osteoblasts), Sox2 and MET (osteosarcoma) (Basu-Roy et al., 2012b; Dani et al., 2012; Tang et al., 2010; Wagner et al., 2011). Further, cloned cell lines from osteosarcomas or chondrosarcomas selected for CD133 continue to express stem cell makers, and retain both tumourigenicity and the ability to differentiate into osteoblasts and adipocytes (Grosse-Gehling et al., 2013; Martins-Neves et al., 2012; Tirino et al., 2011).

(G36) A number of factors have been implicated in the maintenance of MSCs as stem cells, including leukaemia inhibitory factor (LIF) (Jiang et al., 2002; Metcalf, 2003), FGFs

(Tsutsumi et al., 2001; Zaragosi et al., 2006), and Wnt (Boland et al., 2004; Kleber and Sommer, 2004). LIF, a pleiotropic cytokine, maintains the stem cell state of MSCs and other stem cells, and also regulates osteoblast/osteoclast activity. The MSC niche may be located perivascularly as indicated by the expression of α-smooth muscle actin in MSCs isolated from all tissue types tested (Meirelles, 2006) and the immunohistochemical localisation of $CD45^-/CD31^-/Sca\text{-}1^+/Thy\text{-}1^+$ cells to such sites (Blashki et al., 2006). MSCs identified by Stro-1 and CD146 markers have been located to blood vessel linings in bone marrow and dental pulp (Shi, 2003). Localisation of MSCs to perivascular niches is consistent with their having ready access to and a role in repair of damage in many different tissues. The capacity for MSCs to home to different tissues seems to be related in part to expression of Stro-1 (Bensidhoum et al., 2004). Whereas $Stro\text{-}1^-$ cells were better able to aid in the engraftment and survival of HSCs, $Stro\text{-}1^+$ cells were more capable of homing and engrafting to most tissues studied. *In vitro* studies show that MSC migration is regulated by stromal-derived factor-1/CXCR4 and hepatocyte growth factor/c-Met complexes, and involves matrix metalloproteinases (Son et al., 2006). *In vivo* studies have shown that injury alters the patterns of migration and differentiation of exogenously added sites but also promoted widespread engraftment to multiple organs (Francois et al., 2006).

(G37) The Wnt signalling pathway has been shown to play a vital role in the process of differentiation of osteoblasts, chondrocytes and adipocytes from stem and progenitor cells *in vivo* and *in vitro* (Davis and Zur Neiden, 2008). The Wnt family of ligands consists of a number of highly evolutionarily conserved secreted glycoproteins involved in many developmental processes, such as cell proliferation, differentiation, polarity, migration and regeneration (Huelsken J., 2002; Moon RT, 2002). Wnts are essential for normal embryogenesis and also control tissue-specific stem cell activity postnatally in the intestine, skin, and other tissues. The vertebrate Wnts have been divided into two functional groups: the canonical and non-canonical pathways. In the canonical pathway, Wnt molecules attach to the membrane-bound receptor Frizzled (Fzd) and to a coreceptor called low-density lipoprotein receptor related protein 5/6 (LRP5/6) (Cadigan and Liu, 2006; Krishnan et al., 2006). In the absence of a Wnt ligand, a multiprotein complex involving axin, casein kinase 1, glycogen synthase kinase 3β, APC, and Dishevelled (Dsh) mediate the degradation of β-catenin. Wnt signalling allows the accumulation of β-catenin which enters the nucleus and binds to the transcription factors lymphoid enhancore factor (LEF) and/or T-cell factor (TCF), triggering downstream gene transcription, including that of c-myc and Cyclin D1 (Logan and Nusse, 2004). The non-canonical Wnts are less well characterised, but do not signal through β-catenin, and in some cases, may inhibit nuclear β-catenin activity (Ishitani et al., 2003). The canonical and non-canonical pathways are not distinct, as some Wnts can signal through both pathways (Kuhl et al., 2000) and some downstream targets are involved in both, such as Dsh (Yang Y, 2003). The action of the pathway members appears to depend on the cellular contact. In stem cells, Wnt/β-catenin signalling regulates cellular function and maintains "stemness". However, both the canonical (nuclear β-catenin activity) and non-canonical cascade (blockage of nuclear β-catenin activity) also control differentiation.

(G38) As well as controlling self-renewal and early differentiation, the Wnt signalling pathway thereafter specifically regulates the lineage-specification of mesenchymal precursor cells into adipocytes, osteoblasts and chondrocytes (Ross et al., 2000; Sommer, 2002; Tosh and Slack, 2002). However, not only biochemical signals, but also physical conditions such as cell density and cell shape appear to play a role in lineage commitment. Mesenchymal condensations are characterised by increased cell density and cell-cell adhesion. Lower cell densities appear to support osteoblast differentiation whereas higher cell densities cause cells to condense, forcing differentiation into adipocytes (McBeath R, 2004). Cell shape may be

regulated by RhoA, a downstream target of the non-canonical Wnt pathway. Cell-cell adhesion involves N-cadherin which directly interacts with β-catenin at the cell membrane, specifically at the time of mesenchymal condensation prior to differentiation (Tuan, 2003). β-catenin activity is essential for the differentiation of mature osteoblasts and bone formation. Lack of β-catenin does not change the differentiation of osteoprogenitor cells into the early osteoblastic precursors, but blocks the expression of transcription factor osterix (Osx), and consequently, the cells acquire a chondrogenic phenotype (Hartmann, 2006).

(G39) During differentiation, osteoblasts express a characteristic pattern of genes that distinguish them from other cell types (Caetano-Lopes et al., 2007). Collagen type Iα1 (COL1) is expressed from the beginning of osteoblast differentiation and is the main structural component of bone matrix. Alkaline phosphatase (ALP) is upregulated early during osteoblast differentiation, and both osteopontin (OPN) and ALP are important in stabilising the matrix. Osteocalcin is another non-collagenous protein, which is almost exclusively expressed in bone and is upregulated in the late differentiation stage. This stage coincides with the onset of mineralisation, suggesting that osteocalcin may play a part in the regulation of bone matrix mineralisation (Thomas et al., 2001).

(G40) Osteoblasts have been shown to have a role in the regulation of bone resorption through receptor activator of nuclease factor-κB (RANK) ligand (RANKL) that links to its receptor, RANK, on the surface of preosteoclast cells, inducing their differentiation and fusion. Osteoblasts also secrete a soluble decoy receptor (osteoprotegerin, OPG) that blocks RANK/RANKL interaction by binding to RANKL, and thus prevents osteoclast differentiation and activation, reducing bone resorption (Krane, 2005). Therefore, the balance between RANKL and OPG determines the formation and activity of osteoclasts (Gori et al., 2000).

G.3. Radiosensitivity

(G41) MSCs are considered relatively radioresistant. Long-standing evidence for radioresistance of the stromal compartment is based on the findings that bone stromal cells and MSCs are typically of host origin following bone marrow ablation (Bartsch et al., 2009; Rieger et al., 2005). The relative radioresistance of MSCs is attributed to the fact that the cells are quiescent or divide very slowly *in situ* (Morikawa et al., 2009). Even when maintained *in vitro* in the presence of serum growth factors that stimulate proliferation, relatively high doses of radiation are required to trigger apoptosis and inhibit CFU in human MSCs (hMSCs) [D_0 = 2 Gy for hMSCs obtained from a commercial source (Chen et al., 2006)].

(G42) Cultures of cells obtained from bone marrow and capable of forming colonies when plated at clonal density include a subpopulation that is pluripotent and capable of osteogenesis, although there is not a one-to-one correlation between CFU-F and CFU-osteoblast (CFU-Ob; osteogenic cells) (Morikawa et al., 2009). Therefore, earlier lessons learned regarding radiosensitivity and genomic instability from experiments performed on stromal cells that were simply flushed from the marrow and seeded on to plastic, also are pertinent to what are now described as MSCs., e.g. (Epperly et al., 1999; Friedenstein et al., 1981; Greenberger et al., 1982). The D_0 for radiation-induced inhibition of colony formation (CFU-F) from guinea pig marrow is reported to be 2 Gy (Friedenstein et al., 1981) just as for hMSCs (Chen et al., 2006)). Substantial differences in cell culture conditions, purification methods and immunophenotyping and characterisation between experiments contribute substantial uncertainty to quantitative values reported in the literature, and this remains a significant challenge in this field.

(G43) With respect to MSCs, radiosensitivity can be considered in the context of differentiation, pluripotency and senescence, in addition to the more traditional survival assays for apoptosis and colony formation. Radiation-induced changes in differentiation potential are particularly relevant, considering the mounting evidence that stem cell niches and the marrow microenvironment produced by various differentiated progeny of MSCs, contribute to carcinogenesis. When interpreting results from cell culture experiments on MSC growth and differentiation, it is important to take into account the fact that the vast majority of studies do not employ clonal plating densities: therefore, observed changes in cell behaviour and molecular pathways typically reflect the behaviour of a heterogeneous population of cells that are not clonal in origin.

G.3.1. Characteristics of damage response at the cell level

(G44) Irradiation can adversely affect various key cell functions in MSCs, including proliferation, differentiation, apoptosis, senescence and perhaps pluripotency. Recent evidence demonstrates that mouse MSCs express high levels of various DNA damage response proteins (ATM, Chek2, and DNA Ligase IV), high levels of anti-apoptotic proteins and low levels of pro-apoptotic proteins, which together are likely to confer relative radioresistance of MSCs compared to other cell types (Sugrue et al., 2013a, b). Even after high dose irradiation, there is evidence that MSCs retain the capacity for osteogenic differentiation, albeit at reduced levels [e.g. 9 Gy *in vitro* irradiation of hMSC (Chen et al., 2006) and 7Gy of mouse MSC (Mehrara et al., 2010)]. Osteoblast differentiation in culture is highly density-dependent: therefore, in cases where irradiation inhibits differentiation, the responses actually may arise due to insufficient cell density. Nonetheless, a radiation-induced decrease in bone formation by osteoblasts has potential adverse consequences for both mineralised tissue and bone marrow.

(G45) The influence of radiation on MSC growth, differentiation and senescence in cell culture depends to a large extent on the stage of cell growth at the time of exposure, with confluent cells showing modest effects on osteoblast differentiation markers (Ikeda et al., 2000; Kurpinski et al., 2009; Wang and Jang, 2009). Irradiation of hMSCs with x-rays or ^{56}Fe (1 Gy) at 70% confluence, does not impair osteogenic differentiation, but does reduce growth and induce senescence (Kurpinski et al., 2008; Wang and Jang, 2009). In contrast, primary hMSCs irradiated in suspension, then plated to grow in osteogenic or adipogenic media, display reduced osteogenic differentiation and proliferation (Li et al., 2007). As in human cells, mouse bone marrow stromal cells immunophenotyped for MSC markers exposed to high doses (>7 Gy) during exponential growth showed reduced proliferation and osteogenic differentiation with increased markers for apoptosis (Mussano et al., 2010).

(G46) Animal studies support cell culture results showing that irradiation can impair the osteogenic differentiation in marrow progenitors and stem cells. In mice, total body irradiation (TBI) with 7 Gy of low-LET x-rays or 0.5 Gy or high-LET ^{56}Fe ions reduces subsequent *ex vivo* osteoblastogenesis from bone marrow cells (Mehrara et al., 2010; Yumoto et al., 2010). Irradiation of mice (4 Gy ^{137}Cs, TBI) reduces both the numbers and osteoblast differentiation of MSCs *ex vivo*, immunoselected using an endothelial progenitor cell marker, vascular endothelial growth factor receptor 2 (VEGFR2/Flk1) (Ma et al., 2007). A radiation-induced reduction in *ex vivo* osteogenesis is also associated with a reduction in mass (osteopenia) of rapid turnover, trabecular bone, although the acute loss of bone is most likely due to increased bone resorption by osteoclasts; radiation-induced deficits in bone formation by progeny of MSC are most likely to influence bone structure only at later times during subsequent bone turnover (Kondo et al., 2009; Willey et al., 2008; Yumoto et al., 2010). Thus,

results from animal studies support findings from the post-surgical clinical application of ionising radiation to prevent heterotopic ossification (Balboni et al., 2006), perhaps in part, by impairing MSC responses to local growth factors that are released as a consequence of tissue injury (Pohl et al., 2003).

(G47) Ionising radiation may also influence the differentiation fate of MSCs and pluripotent progenitors. In humans, radiogenic marrow ablation leads to increased marrow adiposity (Rosen et al., 2009). One hypothesis to explain these and related findings is that adipocytes differentiate at the expense of osteoblasts, due to a 'switch' in differentiation at the level of a shared progenitor cell (Calo et al., 2010). Differences in the dose dependence of radiation-induced changes in adipogenic versus osteogenic differentiation markers expressed by mesenchymal progenitors are reported in some but not all studies (Li et al., 2007; Schonmeyr et al., 2008).

(G48) In summary, the preponderance of evidence indicates that ionising radiation can influence MSC growth, senescence and differentiation, although further study is needed to determine if radiation can influence differentiation fate (Mehrara et al., 2010; Schonmeyr et al., 2008).

(G49) Irradiation of MSCs, progenitors, and pluripotent cell lines stimulates key radiosensitive signalling and transcriptional pathways including Rb, p53/p21, ATM and MAPK (Chen et al., 2006; Jin et al., 2008; Kurpinski et al., 2009; Liang et al., 2011; Wang and Jang, 2009). Radiation exposure can also influence cell behaviour by modulating cytokine signalling pathways. Irradiation of the pluripotent cell line C2C12 during exponential growth interferes with BMP2-receptor complex formation and inhibits osteogenic differentiation (Pohl et al., 2003), but has little effect on differentiation if cells are near confluence at the time of irradiation (Ikeda et al., 2000). In addition, treatment with the neuropeptide, Substance P, prolongs MAPK activation and mitigates radiation-induced apoptosis of hMSC *in vitro* and bone marrow-derived CFU-F *in vivo* (An et al., 2011).

(G50) As mentioned above, MSC and HSC reside *in situ* within a low oxygen-tension environment, which may protect cells from radiation damage. MSC appear to possess robust antioxidant ROS-scavenging capacity and DNA repair mechanisms, which may contribute to their relative radioresistance (Chen et al., 2006; Sugrue et al., 2013b).

G.4. Mutagenesis

(G51) Specific radiation-induced gene mutations leading to transformation in MSC have not been identified to date, although there is limited cell culture evidence that irradiation of hMSCs can lead to malignant transformation. hMSCs transduced to express elevated telomerase levels did not actively restore their chromosome-arm specific telomere-length pattern after exposure to ionising radiation (Graakjaer et al., 2007). Further, irradiation of a telomerase-transduced human mesenchymal stem cell line led to chromosomal instability and tumour formation in SCID mice (Christensen et al., 2008; Horwitz et al., 2005; Keating, 2012; Martins-Neves et al., 2012; Sugrue et al., 2013a; Sugrue et al., 2013b). These results argue that in principle, irradiation of bone marrow-derived MSCs can directly cause transformation.

G.5. Summary

- Incorporation of radionuclides into the skeleton causes bone cancer and leukaemia in humans and animals.

- Radiation-induced bone sarcomas include osteosarcomas, fibrosarcomas, MHF and chondrosarcoma.
- Tissue damage may play an important role in the induction of bone sarcomas by radiation, as radiation causes chronic disturbances of bone remodelling in conditions referred to as osteitis, osteodystrophy and osteodysplasia.
- Based on ^{224}Ra data, the risk estimate for bone tumour mortality is 5 x 10^{-4} Sv^{-1}, assuming a radiation weighting factor of 20 for α particles (applied to an average skeletal dose) (ICRP, 1991, 2007). For estimates of the risk for bone tumours resulting from exposure to external, mainly low-LET radiation, the A-bomb survivor data provide an estimate of 1 x 10^{-4} Sv^{-1} for the population of England and Wales and a lifetime risk around 2–4 x 10^{-4} Sv^{-1} for the US population.
- MSCs are identified as plastic-adherent pluripotent cells, capable of differentiating into bone, cartilage and fat cells, and can be isolated from many different tissues in addition to bone marrow. MSCs express high levels of DNA damage repair proteins, are relatively radioresistant (D_0 ~2 Gy), and possess robust antioxidant ROS-scavenging capacity.
- Exposure to ionising radiation can adversely affect various key cell functions of cultured MSCs (proliferation, differentiation, senescence) and also can cause loss of bone mass and skeletal fragility in both animals and humans.
- MSCs and quiescent $CD34^-$ HSCs are candidate precursors for radiation-induced bone tumours.
- MSCs and primitive HSCs reside *in situ* within a low oxygen-tension environment, which may help protect cells from radiation damage.

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