Executive summary (1 page)

RISK-IR was a four-year (extended to 4.5 years) project that examined stem cell (SC) responses to ionising radiation. Stem and primitive progenitor cells are considered the cells of origin for most cancers. Understanding the responses of SCs to radiation over a range of doses aimed to improve the knowledge of the processes that contribute to radiation carcinogenesis, this knowledge is important for judgements on cancer risk extrapolation. Radiation cancer risk estimates in humans are largely derived from the studies of the survivors of the atomic bombings in Japan. Statistical power becomes limiting at doses of ≤ 100 mGy; by contrast, current occupational and public exposures to radiation are substantially less and delivered over long periods of time. Key responses were observed in the 10-100mGy range by RISK-IR studies in cellular model systems such as embryonic stem cells and induced pluripotent stem (iPS) cells, ex vivo models such as mesenchymal stem cells, and in vivo experiments observing responses of intact tissues, including skin, esophagus, brain and bone marrow that link SC exposures in the low dose and dose rate range to cancer risk. Findings include:

1. Demonstration of SC sensitivity to doses in the range 10-100 mGy (similar to single CT scan doses), that may be attributed to reactive oxygen species (ROS) generation and subsequent 'metabolic shift'. ROS were also found to contribute to genetic damage (micronuclei) in embryonic stem cells (ESC) at low doses and to longer term effects in skin and haematopoietic SC (HSC) populations. In the most primitive HSCs there appears to be a cell-intrinsic hyper-radiosensitivity driven by ROS from internal sources associated with metabolic re-programming and altered cell adhesion properties.
2. Activation of cell cycle arrest and its consequences were often different at low compared to high doses. While high-dose SC killing would likely reduce cancer risk (eliminating damaged cells), mutation risk could remain and be elevated at low dose because of the absence of cycle arrest and/or DNA repair. Overall, for some endpoints the data suggest that low dose effects on SCs do not necessarily back-extrapolate linearly from higher doses, and different SC populations can respond differently. In contrast, after 100mGy and 1Gy doses, ESC phospho-proteomic responses were linearly dose-related, although the impact of these findings for cancer risk are not entirely clear as they relate to early post-irradiation response
3. Some skin SC populations display specific sensitivity to cancer induction by 50mGy doses, but this was not the case for other skin SC populations. Thus, lineage is a critical factor determining cancer risk to low dose exposures. Lineage-dependent variation was also observed in HSC low dose hypersensitivity responses, in vivo protection by the SC niche may account for the differential responses. The relevance of these findings to in vivo cancer induction requires further investigation.
4. Radiogenic leukaemia risk was increased at 2.5mGy/h in a model system driven by specific chromosome deletions. At this dose rate, single track events in single cells are expected to predominate; lower dose rates simply reduce the number of cells with track traversals. At low dose rate there is an approximately two fold lesser effect on lifespan shortening than at acute exposure.
5. A better understanding of the age/developmental stage dependence of cancer risk was obtained from a study of brain/neural SC sensitivity to apoptosis that potentially also impacts other radiation-induced neurological conditions. Age dependant responses have also been observed by mesenchymal SCs for a range of endpoints, with doses of around 200-500 mGy affecting long term clonogenicity
6. Unique models of iPS have been developed to explore radiation reprogramming. Results to date suggest that secretion of IL6 induces senescence and promotes reprogramming. Radiation itself does not substantially affect the expression levels of the key 'Yamanaka' SC transcription factors, but doses of 90 – 500 mGy and above (6 Gy) seem to reduce reprogramming. Further investigation is needed at low doses, but this model provides useful tools to examine the implications of radiation-induced reprogramming on cancer risk, and whether this is direct or through secretion of factors. Interestingly exposure of human MSCs to 40 mGy x-ray increased their senescence.
7. In vivo epithelial cell fate tracking models showed that 50mGy doses trigger cell loss through differentiation, and identify the pathways that stimulate compensatory repopulation by proliferation that could be relevant to risk. Low doses lead to the preferential growth of clones carrying p53 mutations, suggesting that low doses could act as promotors of carcinogenesis.

Taken together these results uniquely and clearly indicate that SCs respond to radiation delivered at low doses and dose rates, with some of the responses likely to increase cancer risk.

Summary description of project context and objectives (4 pages)

The RISK-IR was a 4 year (latterly extended to 4.5 year) project that aimed to improve the knowledge of the response of stem cells to low doses of ionising radiation. Ionising radiation is well established to have detrimental effects on human health, with induced cancers being the main health effect that the internationally agreed system of radiological protection aims to limit to very low levels. Stem cells are considered to be the cells of origin for most cancers, therefore understanding the responses to radiation in these 'target' cell populations will improve knowledge of the processes that contribute to radiation carcinogenesis.

Understanding mechanistic aspects of radiation carcinogenesis is particularly important in the context of low dose (<100 mSv low LET radiation) risk estimation. The magnitude of risk at these levels is uncertain and based largely on extrapolation of risk assessed in populations exposed to higher levels of radiation, using a linear non-threshold model of dose versus effect. The main population study that provides quantitative information on radiation health risk is the Life Span Study of the survivors of atomic bombings in Japan. These and other population studies of groups exposed occupationally, medically or as a consequence of accidents and incidents have been successful in providing quantitative estimates of health risks, primarily cancer risks, down to doses of 100 – 200 mSv low LET radiation. The prediction of health risks at lower levels of exposure requires extrapolation of dose-effect, and the mathematical model utilised for extrapolation is informed and supported by mechanistic understanding of the processes that contribute to radiation carcinogenesis. A number of international bodies have contributed to the review of evidence of radiation risk from quantitative and mechanistic perspectives, and make recommendations on the protection of the population from radiation health risks. Notable amongst these bodies are the United Nations Scientific Committee on the Effects of Atomic Radiation (UNSCEAR) that carries out review and consolidation of scientific evidence on the levels and effects of ionising radiation, and the International Commission on Radiological Protection (ICRP) that develops and recommends an internationally recognised system of radiological protection. Both of these bodies recognise the importance of mechanistic understanding of radiation carcinogenesis to inform and support judgements on low dose risk. For example, UNSCEAR in 2012 published a brief 'white paper' on the 'Biological mechanisms of action of radiation at low doses', and ICRP has specifically recognised the importance of stem cells in radiation carcinogenesis in its 2015 report, ICRP Publication 131 'Stem cell biology with respect to carcinogenesis aspects of radiological protection'.

Furthermore, there is increasing recognition that the refinement of radiation risk estimates based solely on epidemiological evidence has statistical limitations. The size of population groups required to detect significantly increased risk at lower doses can present major challenges. This limitation has been recognised by, amongst others, the EU supported High Level and Expert Group (HLEG) on Low Dose Risk Research (www.hleg.de). The HLEG proposed the formation of an EU-wide platform – the Multidisciplinary European Low Dose Risk Research Initiative (MELODI) to help encourage, facilitate and co-ordinate European low dose risk research. MELODI has now operated for some years (see www.melodi-online.eu) aided by EC funded activities such as DoReMi (www.doremi-noe.net), OPERRA (http://www.melodi-online.eu/operra.htm) and CONCERT (http://www.concert-h2020.eu/en). The HLEG report identified three major scientific issues requiring investigation to improve low dose radiation risk assessment, and these can be summarised as (i) The shape of the dose-response curve for radiation cancer (ii) non-cancer disease and (iii) individual human

sensitivity. The RISK-IR project addressed issues that relate to the shape of the dose response for cancer and to some extent individual sensitivity, especially in relation to age. The 2016 MELODI SRA also specifically recognised amongst is priority research areas the important role of research on stem cells, 'To determine the nature of the target cells for radiation carcinogenesis. These are generally taken to be stem and progenitor cell populations, which may have specific responses to radiation.' Additionally, the 2016 MELODI statement included as a priority, 'To explore the role of specific target cells for radiation-induced late-developing health effects.'

Therefore, there has been a growing consensus internationally before and during the project life of RISK-IR that research on the effects of radiation at low doses and at low dose rates can be informative on issues relating to radiation risk assessment. In this sense the project has been both timely and relevant.

Thus, the RISK-IR project brought together four groups specialising in aspects of stem cell biology with partners having a track record in low dose research and a particular interest in effects on stem cells. Basic understanding of stem cell biology has improved rapidly in recent years but the application of this knowledge and associated technical developments has not yet been fully explored. This was noted in ICRP Publication 131.

The overarching concept of the RISK-IR project was to bring together expertise in low dose ionising radiation risk research with laboratories working primarily on fundamental aspects of stem cell biology and tissue kinetics research. The interaction between partners and resulting research activity will help address a number of issues of relevance to understanding the mechanisms of radiation carcinogenesis, to estimation of the magnitude of radiation cancer risk at low doses (<100 mSv) and to developing biologically realistic risk extrapolation models, including:

1. The identification and enumeration of stem/progenitor cells at risk of developing cancers.

2. Understanding the low dose radiosensitivity of stem cells and tissues, and dose-response relationships.

3. Improving the understanding of mechanisms of age-dependent cancer risk.

4. Improving the understanding of mechanisms contributing to tissue specific differences in cancer risk.

5. Identification of key events and individual susceptibility factors associated with cancer development.

The work undertaken utilised animal models, human cellular systems and a range of state-of-the-art methods in stem cell biology, tissue kinetics research and molecular analysis of response to radiation. The primary outputs are scientific publications to strengthen the evidence base for low dose radiation risk estimation. Efforts have also been made to place the primary scientific findings in context such that the relevance and value for health risk estimation can be more readily appreciated.

Description of main S&T results/foregrounds (25 pages)

1. Cellular models

Cancer is a multi-step process originating from single cells that have accumulated genetic alterations often as a result of inflicted DNA damage. A key question related to biological effects of low dose ionising radiation (LDIR) concerns a possible threshold for the induction of cancer and relevant genetic damage due to the action of efficient DNA damage responses (DDR) i.e. repair, cell cycle control, apoptosis. Stem cells are considered to be the cells of origin for most cancers, and examination of stem cell responses to radiation is important to understand radiation carcinogenesis. Embryonic stem cells (ES cells) that are derived from the inner cell mass of a mammalian blastocyst exhibit long-term proliferative potential in culture, have stable karyotypes and are distinguished by their ability to differentiate into any cell type. Mouse embryonic stem cells (mESC) can be differentiated into mouse neural stem cells (mNSC) to allow direct comparisons of cellular responses in isogenic pluripotent (mESC) versus multipotent (mNSC) stem cells following low and high radiation doses.

Checkpoint activation
mESC lack the G1 checkpoint (Hong and Stambrook, 2004, Proc Natl Acad Sci U S A. 2004;101(40):14443-8) while the existence of a G1 checkpoint in mNSC is unclear. To address this issue, we analysed the S phase entry of G1-phase-irradiated cells (Deckbar et al., 2010, Cancer Res. 2010;70(11):4412-21). High dose ionizing radiation (HDIR) exposed mNSC continued S phase entry with the same kinetics as unirradiated cells. Only 6 hr after irradiation a slight reduction in S phase entry was observed. Further analysis using flow cytometry revealed that S phase entry is slower in mNSC than mESC at times >4 hr post 2 Gy. Thus, mNSC express an intermediate G1 checkpoint behaviour.

After IR, cells induce the G2 checkpoint to prevent cell division in the presence of unrepaired DSBs. An unregulated G2 checkpoint can be detrimental as DSBs may result in broken chromosomes after chromosome condensation, which increases risk of losing genetic information during mitosis. Determination of the fraction of mitotic cells at 90 min. post-irradiation showed that while LDIR up to 100 mGy did not affect the mitotic index of mNSC significantly, a clear reduction could be observed in mESC. Moreover, to halve the mitotic index, 200-300 mGy were sufficient for mESC while mNSC needed approximately 500 mGy to achieve the same effect. These results indicate that mESC display a more sensitive G2 checkpoint than mNSC. To assess whether checkpoint abrogation also displays different sensitivity levels in mESC and mNSC, cells were irradiated with different doses and the mitotic index was monitored over several hours. In both cell types, an increase in radiation dose prolonged the G2 arrest, demonstrating permanent signalling from unrepaired DSBs to the checkpoint machinery. At all doses, even though mNSC started to re-enter mitosis earlier than mESC, the rate of mitotic entry increased faster in mESC than in mNSC, leading to higher mitotic indices at later time points. Non-homologous-end-joining (NHEJ) deficient Ku80 and Lig4 mESC displayed a more drastic reduction in the fraction of mitotic cells after 100 mGy, yet at 4 hours post-IR the proportion of cells proceeding through mitosis was similar to the control level although at this time point repair of DSB was not complete. These results demonstrate differences between mESC and mNSC in the duration of the radiation-induced G2 arrest, indicating that not only the checkpoint induction but also the checkpoint maintenance is less sensitive to DSB numbers in mNSC than in mESC. Importantly, stem cells with low levels of unrepaired DNA damage can enter and pass through mitosis

potentially causing the formation of genetic damage (e.g. micronuclei (MN)) in the next cell cycle.

These findings were further corroborated by examining cell growth after a more chronic high dose ionising radiation (HDIR) or LDIR exposure regime. For this purpose, cells were irradiated every 48 hours (at 3 doses: 0, 0.1 and 1 Gy) and passaged 24 hours after each irradiation, in a total of 11 times over 22 days. mESC irradiated with HDIR (1 Gy) displayed an approximately 1.5 fold extended doubling time compared to control and LDIR (0.1 Gy) irradiated cells. No significant difference in growth rate was observed between LDIR exposed cells and unirradiated control mESC. As expected, NHEJ deficient mESC (Lig4 and Ku80) displayed a much more pronounced increment of doubling time after HDIR and lost the ability to divide after 5-6 irradiations. As defective NHEJ did not lead to an increased doubling time after LDIR, it was concluded that low levels of DSB are insufficient to significantly reduce cell proliferation. As a consequence, stem cells with low levels of unrepaired DNA damage can enter and pass through mitosis potentially causing the formation of genetic damage (e.g. micronuclei (MN)) in the next cell cycle.

DNA repair pathways

Another aim of these studies was to investigate the DSB repair mechanisms in a cell cycle-specific manner and to uncover whether there are differences between mESC and mNSC. In differentiated cells, the majority of IR-induced breaks are repaired quickly via NHEJ with DNA-PK being one of the core enzymes. In contrast, a sub-fraction of breaks is repaired with slow kinetics in an ATM-dependent manner which represents homologous recombination (HR) in G2 and a resection-dependent end-joining pathway in G1 (Beucher et al., 2009, EMBO J. 28:3413-27; Biehs et al., 2017, Mol Cell 65:671-684.e5). Since the molecular mechanisms of DSB repair are only poorly defined in stem cells, the contribution of NHEJ and ATM-dependent repair in G1 and G2 phase in embryonic and neuronal stem cells was evaluated. Using DSB markers (γH2AX or 53BP1) in combination with cell-cycle markers, DSB induction and the repair behaviour of WT mESC and mNSC were compared. Both cell types exhibited an induction of ~20 DSBs (at 15 min post-IR) in G1 and ~40 DSBs in G2 after 2 Gy irradiation. Nearly all breaks were repaired within 8 hr. Interestingly, and in contrast to the aforementioned observations with differentiated cells, the repair in mESC and mNSC did not display biphasic kinetics suggesting that these various DSB repair pathways act more or less simultaneously and/or that more than two repair pathways can work hand in hand to remove DNA breaks.

The role of ATM in the repair of IR-induced DSBs in G1 and G2 phase mESC and mNSC was investigated by treatment with a specific small molecule inhibitor. Inhibition of ATM revealed for both cell types a repair defect that became apparent at later time points in G1 and G2 phase, resulting in 40 to 50 % of unrepaired breaks. In differentiated cells, ATM-dependent repair represents HR in G2 and a resection-dependent pathway in G1 (Beucher et al., 2009, EMBO J. 28:3413-27; Biehs et al., 2017, Mol Cell 65:671-684.e5). To verify whether ATM-dependent repair in G2 phase stem cells is the same as in differentiated cells, the HR-protein Rad51 was inhibited and DSB repair was monitored. After Rad51 inhibition (Rad51i), neither mESC nor mNSC displayed a repair defect at 2 hr post damage induction, consistent with NHEJ representing the main repair pathway during the fast repair component. At 6 hr after irradiation, a repair defect became apparent in mNSC that was around the same magnitude (~50%) as the defect that was previously observed in ATM-deficient stem cells. In contrast, mESC did not exhibit any impairment in DSB repair after Rad51 inhibition. This was

surprising; especially in the light of reports demonstrating that HR plays an even more prominent role in stem cells than it does in differentiated cells.

As a measure for HR involvement in DSB repair Rad51 foci formation was monitored in G2 phase mESC and mNSC. Interestingly, especially at short times the absolute numbers of Rad51 foci were much higher in mESC than in mNSC, arguing that usage of HR for DSB repair is more prevalent in mESC than in mNSC. This result appears to be in contradiction with the observed lack of a repair defect in Rad51-inhibited mESC. One explanation for these results might be that while HR is more prominent in mESC than it is in mNSC, a lack of HR in mESC can be readily compensated through the usage of alternative pathways. To confirm that HR is Rad51-dependent in mESC, mESC were used expressing Rad54 S572A, a non-phosphorylatable form of Rad54, which impedes the removal of Rad51 from DNA. Following Rad51-dependent strand invasion, these mutant cells are unable to switch back to alternative pathways like single-strand annealing, leading to a DSB repair defect. While at 6 hours after irradiation almost all DSBs were repaired in WT cells, Rad51 foci persisted in Rad54 S572A cells. Thus, even though Rad51i did not have any effect on DSB repair in mESC, inhibiting the late steps of HR prevented the use of alternative and more deleterious pathways indicating that only a defect in early steps of HR can be compensated in mESC. Finally, it was aimed to establish whether the efficient repair in the absence of HR, which was observed in Rad51-inhibited mESC, is conducted in a PARP-dependent manner. Indeed, inhibition of PARP resulted in a substantial repair defect in Rad51-inhibited mESC but not in an additional repair defect in mNSC. Thus, mNSC cannot switch to an alt-NHEJ pathway when breaks have been committed to repair via HR e.g. through extended resection. In contrast, mESC seem to be more flexible in their pathway choice and can compensate for an HR-deficiency by using a PARP-dependent repair pathway. These experiments demonstrate that PARP-dependent alternative EJ takes place in different cell types to a different extent. It was concluded that (i) the repair defect which can be observed in G2 phase mESC and mNSC after ATM inhibition is due to an inhibition of ATM-dependent resection and thus an abolition of HR, and that (ii) a defect on the early steps of HR in mESC might be compensated by the efficient usage of alternative repair pathways.

Since different studies using human fibroblasts indicate that efficient DSB repair is only activated at doses above 20 mGy (Rothkamm and Löbrich, 2003, Proc Natl Acad Sci U S A. 100:5057-62; Grudzenski et al., 2010, Proc Natl Acad Sci U S A. 107:14205-10), it was investigated whether this is also the case in stem cells. After 100 mGy, both mNSC and mESC displayed efficient repair similar to what was observed after 2 Gy irradiation. In contrast, repair was massively impaired after 10 mGy in mNSC. While G2 cells still repaired ~50% of breaks induced, nearly no repair could be detected in G1 phase cells. The latter finding is consistent with the repair in G2 consisting of NHEJ and HR. It was previously shown that NHEJ is compromised after low dose irradiation (Rothkamm and Löbrich, 2003, Proc Natl Acad Sci U S A. 100:5057-62; Grudzenski et al., 2010, Proc Natl Acad Sci U S A. 107:14205-10), therefore the observed repair in G2 phase after 100 mGy probably represents repair via HR. The inability of mNSC to repair DSBs induced by LDIR is consistent with previously published data for differentiated cells (Grudzenski et al., 2010, Proc Natl Acad Sci U S A. 107:14205-10) and a possible consequence of low levels of oxidative stress. Indeed, artificially induced oxidative stress by H_2O_2 treatment was able to restore repair nearly to background levels after 10 mGy in mNSC. Using small molecule inhibitors for ATM and DNA-PK, it was found that after HDIR (1Gy) the activity of either kinase was sufficient to stimulate

the formation of repair foci. In contrast, only inhibition of ATM resulted in reduced foci levels after LDIR (10mGy). The inability of H_2O_2 treatment to stimulate DSB repair after LDIR in ATM-inhibited cells suggests that the observed reconstitution of DSB repair in WT cells by artificially induced oxidative stress is not a consequence of an oxidative stress-dependent activation of DNA-PK. These data indicate that ATM constitutes the main kinase that phosphorylates H2AX after LDIR. Interestingly, and in contrast to high doses, DNA-PK is not able to compensate for the loss of ATM after low doses.

Induction of genetic damage

Faithfull repair of radiation-induced DNA damage is essential to maintain genome integrity and prevent the formation of genetic damage. To identify dose response relationships for genetic effects in mESC after LDIR was investigated by the formation of micronuclei (MN) in a high throughput fashion. A role for MN in cancer development has recently evolved as they may be responsible for the occurrence of massive DNA rearrangements in isolated chromosomes, a phenomenon being referred to as chromothripsis, that is observed in various types of tumours (Hintzsche et al, 2017, Mutat Res. 771:85-98; Crasta et al, 2012, Nature. 482:53-8). MN frequencies were observed that best fit a linear induction with dose over a range of 10 mGy up to 200 mGy with no evidence for a threshold. These results are consistent with recently published data on LDIR induced MN in cultured human fibroblasts (Boei et al, 2011, Radiat Res. 177:602-13.) and mouse reticulocytes (Manning et al, 2014, Mutat Res. 770:29-36). Inactivation of NHEJ resulted in up to 3-fold increase in IR-induced MN formation compared to the wild type cells after LDIR (200 mGy). Interestingly, deletion of PolQ recently shown to be involved in alternative end-joining did not enhance the formation of MN. It is concluded that while efficient DSB repair is required to minimalize the extent of induced genetic damage (MN), there is no evidence that repair after LDIR is more efficient than after HDIR.

Genome wide analysis of cellular responses

Identification of the various cellular processes that are affected by LDIR and HDIR requires a genome wide and integrated analysis of the induced cellular responses (Puigvert et al, 2013, Sci Signal. 22:59). Using a quantitative Stable Isotope Labelling in Cell Culture (SILAC) based proteomics strategy, the temporal dynamics were investigated of global-, phospho-, and redox- proteomes of mESC in response to LDIR (100 mGy) and HDIR (1 Gy). Remarkably, numbers of differentially regulated proteins, phosphosites and oxidation sites did not differ between LDIR and HDIR and did not alter dramatically with time with one exception: after HDIR the number of phosphosites clearly increased after 4 hours with a relative reduction of sites that are common to LDIR suggesting the induction of HDIR-specific phosphosites. The data also suggest that for HDIR the phosphosites are more persistent over time which is most likely related to a dose dependent induction of DDR. As expected the Atm kinase was activated in a dose dependent way by IR; in contrast, the Atr kinase was not activated at any time point or dose. Pathway analysis revealed a high similarity in affected pathways after LDIR and HDIR that clustered with time. Among these pathways were many radiation relevant pathways such as DNA damage response related pathways (DNA damage signalling, cell cycle and mitotic checkpoints, apoptosis), stress signalling, regulation of transcription, regulation of (PI3K) kinase signalling, nuclear receptor activation, proteases, redox metabolism. It is concluded that although the magnitude of response differs between LDIR and HDIR, the repertoire of affected pathways is highly similar for the two treatments and the data cluster with time. Although the impact is not understood of the phosphorylation events

and dynamics on the function of the target protein (except for some well-studied kinases such as Atm), the data clearly reveal the complexity of the response to IR and the dose dependent level of phosphorylation of Atm targets. Moreover, the high similarity of phosphorylation dynamics after LDIR and HDIR is evident.

To understand the impact of LDIR and HDIR on the level of transcription regulation (initiation, elongation, termination) nascent RNA transcripts were identified after irradiation by selective labelling and isolation of newly synthesized RNA followed by its sequencing and data analysis. This approach allows very sensitive analysis of the RNA response at the level of individual genes since it excludes the bulk of existing RNAs from the analysis. The transcriptional response after HDIR was much stronger than after LDIR but a biphasic response was observed for both types of exposures. In the early response, genes/pathways are linked to expression of ribosomal genes both for LDIR and HDIR. Interestingly, ATM dependent trans inhibition of ribosomal RNA synthesis has been reported after HDIR (Kruhlak et al, 2007, Nature. 447:730-4). Indeed, the present ATM data reveal significant phosphorylation even after LDIR that might lead to repression of RNA Polymerase 1 transcription after LDIR and HDIR. At the 1 hr time point a strong increase was observed after HDIR indicating transcription regulation of genes involved in cell division and signal transduction (kinases) as well as transcription and translation. This response very likely represents the p53 mediated regulation of p53 responsive genes as recently demonstrated (Narayanan et al, 2017, Sci Rep. 7: 43598). This response is present but weak after LDIR probably related to the modest p53 phosphorylation after LDIR in comparison to HDIR. It is concluded that although the transcription response after HDIR is much stronger then after LDIR, the repertoire of affected genes/pathways is quite similar for the two treatments. Notably, the magnitude of responses might at least partly represent the differences of p53 activation (phosphorylation) after LDIR and HDIR.

To summarise, LDIR (100 mGy) exposure of mESC and mNSC results in a transient induction of the G2 checkpoint. The magnitude of checkpoint induction after LDIR is enhanced in mutants with NHEJ deficiency, but not the recovery time. As a consequence the population doubling time of NHEJ mutant cells is unaffected following eleven repeated exposures of 100 mGy 48 hours apart, whereas their cell proliferation is completely halted after only 5 repeated exposures of 1 Gy. For the repair of IR-induced DSB in the G2 phase of the cell cycle, mESC more frequently than mNSC use error-free ATM-dependent homologous recombination. Furthermore, mESC are more proficient than mNSC in switching to the usage of alternative pathways when HR is inhibited. However, despite the efficient repair of DSB in mESC and probably as a consequence of the transient nature of the G2 arrest, the induction of genetic damage (MN) increased linearly with dose from 10 mGy upwards suggesting that there is no threshold for the induction of genetic damage at low IR doses. Moreover, measurement of DSB repair kinetics at very low doses (10 mGy) indicated that this is very inefficient in mNSC although it can be stimulated by the induction of oxidative stress. Genome-wide analyses of post-translational protein modifications and transcriptional changes indicated (in)activation of the same cellular pathways after LDIR and HDIR, as for instance the activation of cellular checkpoints, with similar time kinetics but varying in the extent of induction. Finally, the observation that mouse stem cells after LDIR can proliferate in the presence of unrepaired DSB and thereby generate cancer relevant damage (MN) points to potential risks for carcinogenesis after LDIR.

2. iPS cell models

The impact of radiation exposure on direct or indirect, secreted factor (or 'secretome')-mediated re-programming of differentiated cells into cells with stem cell characteristics may well be of relevance to carcinogenesis because of the potential to modulate the frequency of long-lived target cells with stem cell-like properties.

At an early stage in RISK-IR, "reprogrammable" mice in which the transitory induction of the four factors Oct4, Sox2, Klf4 and c Myc results in full reprogramming in vivo of all tissues was reported (Abad et al.,2013). HDIR (5 - 9 Gy) has now been found to prevent reprogramming in vitro, exposure to lower doses (90 – 500 mGy) may have an inhibitory effect, but results are not yet clear and require replication. One assay of in vivo reprogramming in low-dose irradiated mice did not reveal any effect. This experiment needs to be repeated for robustness and consistency.

HDIR (6 Gy) induces cells to undergo senescence and to acquire the so-called senescence-associated secretory phenotype (SASP) secreting to the medium (conditioned medium) factors that potently promote reprogramming when added to normal healthy cells. It was known that interleukin-6 (IL6) is upregulated during in vitro reprogramming and IL6 has now been identified as a critical component of the radiation-induced secretome that promotes in vivo reprogramming. Furthermore, it has been established that p16 is essential for secretome-mediated reprogramming.

Studies of in vivo reprogramming indicate that radiation at high dose inhibits the process while locally administered bleomycin (a radiomimetic drug) enhances reprogramming. This difference may relate to a reduction in cytokine production, including the pro-reprogramming factor IL6, after high whole-body irradiation by comparison with local bleomycin treatment. Further investigation of the dose-dependence of senescence induction and the associated secretory phenotype will reveal if re-programming might be a contributor to high dose radiation effects alone or possibly over the lower dose range as well. The observation of radiation exposures at different dose levels having an impact on reprogramming in these model systems is of potential importance, hinting that the exposures may have an impact in the 'stem-ness' of cells and hence their longevity. If found to be robust and when quantified these phenomena will have a bearing on considerations of target cells at risk for radiation cancer in specific tissues.

3. Mesenchymal stem cells

Mesenchymal stem cells (MSC) are multipotent precursor cells in the adult organism of chordate animals. As such, they contribute to life-long regeneration of mesenchymal tissues (bone, cartilage, connective tissue, adipose tissue), support haematopoiesis by forming bone marrow stroma, and exert various immunomodulating functions. After completion of embryonal development, MSCs are not formed de-novo any more, but maintain there multipotency and long-term repopulation capacity by slow and asymmetric cell division. To ensure that MSCs can meet the demands of tissue repair after damage, can replace precursor cells for multiple differentiation lineages and exert their functions as supporting factors in heamatopoiesis and immunology, their genome has to be kept free of mutations, chromosomal alterations and epigenetic disturbances from birth until old age.

Ionising radiation represents a particular challenge for these cells, which could lead to an accumulation of DNA alterations and persistent cellular stress in cases of chronic low-dose rate or protracted exposure. Apart from our interest in the low-dose radiation response of MSCs under physiological conditions (i.e. IR exposure in-vivo), there is also a lot of interest in the problem of safety and therapeutic potential of those cells when they are removed from donors and expanded ex-vivo prior to an autologous or allogenic stem cell therapy. In this case, an accelerated cell-division under highly unphysiological conditions could change the radio-sensitivity, genetic stability and stem cell capacity of MSCs after implantation such that IR exposure to the recipient induces detrimental effects like tissue degeneration, increased risk for malignant diseases and others.

The experiments on MSCs within RISK-IR focused on a few defined problems: (1) How do human and murine MSCs respond to low-dose gamma irradiation (LD-IR) in terms of their stem-cell potency [senescence, spontaneous adipogenesis, telomere length], (2) How does LD-IR and cellular aging interact in the recognition and repair of DNA double-strand breaks and (3) are murine MSCs a valid model for the biology of human MSCs, in particular with regard to an impaired or mutated RB1 gene.

We have shown that gamma-doses as low as 40mGy (human) and 200mGy (murine) induces either senescence and/or spontaneous adipogenesis in-vitro, both processes leading to an irreversible loss of stem-cell capacity (Höfig et al 2016, Stem Cells Int. 2016:6429853; Alessio et al 2015 Oncotarget. 6:8155-66.). It is worth noting that we have not observed an increase in cellular apoptosis after LD irradiation, suggesting that MSCs do not succumb to cell death after genotoxic stress, but undergo changes in their cellular programme. One conclusion might be that in contrast to apoptosis-prone cells, MSCs survive IR by prematurely increasing their cellular program towards senescence or premature differentiation. It remains to be determined if those cells, after accumulating in tissue throughout life indeed harbour radiation-induced genetic alterations or, and if so whether they could later contribute to malignancies. In our studies on human MSCs undergoing LD-IR induced senescence we found that a specific pattern of pro-inflammatory cytokines (so called SASP for senescence-associated secretory program) is released into the medium (Capasso et al 2015 Oncotarget. 6:39457-68). Studies by other groups on this SASP have found evidence for its role in exocrine signaling promoting carcinogenesis.

The long-term repopulating potential of low-abundance stem cells is best studied by their clone-forming capacity in-vitro. We have compared the gamma-radiation survival curves of MSC, MEFs and osteoblasts (all of murine origin) and did not found any indication for major differences. We have also compared the gamma-radiation survival curves (in-vitro) of murine MSCs collected from mice irradiated with 0.2Gy in vivo early in life and within the sensitivity of our assay could not find an effect of this split dose experiment. In contrast to the absence of a detectable effect on cell survival, , cellular senescence went up following in-vivo LD irradiation (Tewary MSC thesis 2017, manuscript in prep).

The long term irreversible effects of MSCs (senescence, premature differentiation) after IR are classically interpreted as a result of residual, unrepaired DNA damage. It was therefore logical to compare the persisting cellular effects with the early and intermediate processes of post-radiation DNA repair. In both human and murine MSCs we saw the induction of gamma-H2AX/53BP1 foci after LD gamma-irradiation (significant increase starting from doses above 40mGy and 50mGy, in human and mouse MSCs, respectively). Here we found

an interaction with inherent biological features of the MSCs that point to a potentially important impact of cellular aging and cell fate. In human MSCs a sub-fraction of non-cycling (i.e. Ki67 negative) cells maintained unrepaired repair foci even after 2 days, and the fraction of those cells increased following RB1 knockdown (Alessio at al 2017 Cell Cycle. 16:251-258.). Since we could also show that in human MSCs knockdown of RB1 increases senescence one could argue that cells that lose their stem-cell potential also accumulate higher numbers of IR induced DNA breaks. In murine MSCs we observed a similar process, but here it was caused by aging and changes in stem cell potential following long-term in-vitro growth. The ability of MSCs to recognize and repair DNA double-strand breaks (as determined by gamma-H2AX/53BP1 immunofluorescence) gradually impaired during 1 – 12 weeks in-vitro growth. And this loss of DNA break repair was associated with an increase of LD gamma induced micronuclei (Hladik MSc thesis 2016, manuscript in prep.) starting at doses as low as 50mGy. Modelling the data of DNA DSB repair and of micro-nucleus (MN) induction suggests that in-vitro aging of murine MSCs reduces their capability to recognize and repair DNA damage (i.e. impaired DDR signaling) in a synergistic way, but that the increase of micronuclei with age is a mechanism separate from the radiation-induced increase of these chromosomal defects. A dispersion analysis of the distribution of MN in individual MSCs clearly demonstrated that the age-associated defects cluster in a few heavily affected cells, whereas the radiation-induced MNs in relatively young MSCs are evenly distributed over all cells. This suggests that radiation-induced DNA double-strand breaks and chromosomal instability accumulates in aging MSCs or senescent cells derived from them. We can therefore conclude that the increased fraction of non-cycling cells or aged MSCs (see findings above) indeed harbour a “reservoir” of cells with persisting genomic alterations that are refractory to cell death.

MSCs derived from older donors or after in-vitro aging exhibit some features that distinguish them clearly from pluripotent cells. In contrast to the later, MSCs show a slow but gradual loss of telomere length, and we have found that this telomere aging can influence the extent of IR induced losses of telomeric and chromosomal stability. As shown in an earlier study (Gonzalez-Vasconcellos et al 2013 Cancer Res. 2013 73:4247-55.), transiently-amplifying precursor cells of the osteoblast lineage depend on the integrity of the Rb1 pathway for maintenance of their telomeric stability, and a defect in this cellular pathway causes accelerated telomeric attrition and LD radiation induced anaphase bridges. The murine MSCs (progenitor cells of the osteoblast precursors) show a much slower loss of telomere length in vitro, and no detectable Rb1 dependence. To serve as target cells for a malignant transformation, however, MSCs have to undergo immortalisation. A specific process in tumors of mesenchymal origin is the mechanism of alternative telomeric lengthening (ALT), characterized by recombination between sister telomeres (T-SCE). We have shown before that this feature of ALT is prominent in unirradiated murine osteoblasts in-vitro, but rarely seen in unirradiated MSCs from the same mice. Following gamma-irradiation (0.5 – 2 Gy), however, this form of telomeric disturbances increase in MSCs but not in osteoblasts (Rosemann M, 2016 In: Genetics, Evolution and Radiation (ed. C.Mothersill, V.Korogodina) Springer Verlag 2016. 385-396). More detailed studies on the influence of in-vivo aging on this phenomenon have shown now that the frequency of radiation induced T-SCE in irradiated MSC strongly depends on the donor age. Whereas MSCs from > 9 month old mice show indeed the above mentioned susceptibility for telomeric recombination after 0.5 and 2 Gy, cells from younger mice (< 4 month) appeared largely refractory to this effect (manuscript in prep). This might explain why radiotherapy associated bone tumors in young patients

mainly show osteoblastic differentiation (i.e. derivatives of osteoblasts, rather than of MSCs), whereas in elder patients a larger fraction of bone tumors show features of undifferentiated or anaplastic sarcoma (also called malignant fibrohistiocytoma).

We also could show a donor-age effect of MSCs in vitro for the formation of abberant cell foci. The data suggest that in MSCs from 9 month old mice, but not from 4 month old mice, 150mGy gamma-irradiation can induce a 3-fold rise in the number of aberrant foci. We are still working on experiments to better characterize these aberrant foci and their potential involvement in the induction of malignancies.

The relative resistance of young donor derived MSCs for the formation of T-SCEs or ALT, however, was replaced by a clear increase of sister telomere fusion starting from 0.5 Gy and onwards. Whereas T-SCEs result from homologous recombination repair, simple telomere fusions are the outcome of non-homologous end joining. It is unclear however, how radiation doses as low as 0.5 Gy can have an effect on DNA loci as small as telomeres. It is unlikely that DNA double strand breaks are involved here, since their number is too small. It remains to be studied in future experiments as to what extent the effect of MSC aging on double-strand break repair after IR (see above) has anything to do with telomeric instability, or if the latter is caused by more abundant DNA damage such as single-strand breaks or ROS-induced base alterations.

An important aim of this study was also to validate the use of murine MSCs as a model for radiation effects in human MSCs. This was necessary since some experiments (like effects of donor age, malignancy in vitro, studies on gene knockdown) still require murine studies. In conclusion we can say that for some, but not all mechanisms that we studied in parallel, the radiobiology of MSCs from murine or from human origin is comparable. This is true for the effect of LD radiation induced DNA double-strand breaks (gamma-H2AX/53BP1 foci) and effects of cellular age on break-repair. It is also true for the relative absence of radiation-induced apoptosis in both cell types. For the cellular pathway that governs loss of stem-ness, however, we saw marked differences between MSCs of both species. Whereas human MSCs after LD gamma irradiation mainly undergo cellular senescence, murine MSCs show premature differentiation. It is also likely that the absence of a RB1 effect in murine MSCs as compared to a promoting effect of RB1 deficiency onto cellular senescence in human MSCs and a partial compensation by overexpression of other retinoblastoma-family genes is related to this difference in senescence vs. differentiation.

4. Haematopoietic stem cells

The haematopoietic system is a classical hierarchical stem cell system, and a tissue at risk of radiation carcinogenesis with a fairly high nominal risk and lethality and thus substantial tissue weighting (0.12 of a total 1.0) in the ICRP 103 system. Identification of specific target cell populations for specific leukaemia types and understanding low dose responses are important issues that have been addressed by RISK-IR investigators using experimental animal models and human haematopoietic stem and progenitor cells (HSPCs).

Mouse hematopoietic stem compartment comprises three populations of cells which are functionally defined by their ability to serially reconstitute the entire blood system of lethally irradiated recipient mice: long-term engrafting HSCs (LT-HSC), short-term engrafting HSCs (ST-HSCs) and non-self-renewing multipotent progenitors (MPP). They can be isolated with high purity using combinations of cell surface markers (LT-HSC: lin-, Sca+, ckit+ (LSK)

FLK2-CD150+CD48-; ST-HSC: lin-, Sca+ckit+ (LSK) FLK2-CD150-CD48-; MPP: lin-, Sca+ckit+Flk2+ (LSKFlk2+); however, there is strain dependency of surface marker phenotypes and the expression of ckit and Sca are affected by radiation exposure.

In purified populations of LT-HSC, ST-HSC and MPP isolated from C57BL/6 mice irradiated in vitro and subsequently grown in vitro, differential sensitivity to low doses was observed with LT- and SC-HSC showing a marked hypersensitivity (HRS) that was not seen in MPP. Mathematical modelling of these results using Joiner's induced repair model has now been carried out; while there is marked spread in the data, the HRS models fit the data statistically better than non-HRS models. These survival results underline the need to consider well defined populations of cells and understand the relationship of each with specific cancers.

Transcriptome analysis followed by further more detailed functional characterisation has yielded results indicating that (i) HRS at low doses is independent of DNA double strand break DNA damage response and of transcriptional p53 activity, but is dependent on a mitochondrial response, (ii) HRS may be due to oxidative stress and the Nrf2/Keap1 pathway is important to limit the oxidative stress-dependent HRS; $Nrf2^{-/-}$ mice display an exaggerated HRS while $Keap1^{-/-}$ mice do not display the HRS phenotype, (iii) low dose exposures are associated with changes in the energy metabolism in LT-HSC; transiently decreasing ATP production along with loss of mitochondrial activity, and (iv) genes specifically activated at 20 mGy encode proteins involved in adhesion and this modification of expression could have an effect on the early steps of homing when the HSCs are transplanted into recipient mice. Taken together these results indicate that the low dose response to radiation in HSCs is dominated by reactive oxygen species (ROS) and metabolic alterations rather than DNA damage responses.

Human umbilical cord blood-derived hematopoietic stem/progenitor cells (HSPC) are defined as CD34+ CD38lo CD90+ cells by cell sorting. 20 mGy radiation exposure has been found to alter human HSPC self-renewal capacity in in vitro tests and in vivo transplantation studies, the latter demonstrating no effect on HSC differentiation. More differentiated multipotent progenitors (MPP) were even more sensitive to low dose irradiation, including 20 mGy (20 mGy/min) in a primary CFU assay with possible hypersensitivity to 20 mGy. The sensitivity to low doses was not dependent on ATM or p53 pathway activation, but was found to be affected by ROS. An increase in ROS and reduction in mitochondrial function is observed in human HSPC immediately after 20 mGy exposures and this is followed by induction of the p38MAPK pathway.

These data appear to indicate that doses in the region of 20 mGy may be particularly damaging to primitive hematopoietic cells of the mouse and human, but that there may be differences in sensitivity of specific populations of cells between the two species. Further work continues to be needed to consolidate these data.

In C57BL/6 mouse hematopoietic progenitor and stem cells, transcriptional analysis of acute cell response to 20 mGy pointed to involvement of cell adhesion proteins. To investigate whether this alteration could modify the early steps of homing, a confocal endoscopy analysis and in vivo tracking were performed. LT-HSC irradiated at low doses had an aberrant homing into the diaphysis of the femur compared to sham-irradiated or high dose-irradiated LT-HSC that home into the femoral head. Detailed analysis of results indicated that low doses of radiation are able to induce multiple long-term defects mediated by a persistent oxidative stress, namely:

1) a myeloid bias at the expense of lymphoid B-cells,

2) a decreased capacity of engraftment,

3) a self-renewal defect that leads at long term to exhaustion of LT-HSC.

These defects suggest a phenotype of aged HSC as already described in the literature. Some, but not all, phenotypic and molecular hallmarks of aged HSC are observed in LT-HSC i.e. an enrichment of up-regulated genes associated with an aging phenotype, and an increase in LSK circulating in the blood.

In a fully *in vivo* situation (i.e. whole body irradiation) any effect on LT-HSC numbers requires high doses of 2.5 Gy; lower doses of 20, 100 and 500 mGy have no effect; thus, the effects of HRS driven by ROS generation described in the in vitro and transplantation models are protected against by the growth of HSCs in their normal niche/microenvironment. Thus, two independent investigations on human and mouse HSC point to possible in vivo long-term effects of low doses, down to 20 mGy, on stem cell functions. Alterations to either stem cells or their niche may be postulated, and ageing effects may be implicated. The lack of effects of low doses in the fully in vivo model very clearly indicates that the microenvironment in which cells reside is important in determining cell fate after exposure to radiation at low doses.

Use of the CBA mouse model for radiation-induced acute myeloid leukaemia allowed more direct investigation of the exposure and time dependence of radiation leukaemogenesis. Analysis of 135 acute myeloid leukaemias arising in irradiated CBA mice confirmed that around 70% carry PU.1 loss, and almost all of these carry point mutations in the remaining copy of the gene. Additionally, 3% of cases carry Flt3 ITD mutations (none with PU.1 alterations), and about 2% have Kras G12 mutations, in 2 of 3 cases with PU.1 involvement. No mutations in seven further human AML-asociated genes were found. These results confirm and extend the range of mutations associated with radiation AML in the mouse.

A major drawback with standard repopulation protocols as described above is the need for high dose radiation ablation, and this can potentially be avoided through the use of immunocompromised strains such as NOD SCID Gamma (NSG). Long term transplantation of (1) bone marrow, and (2) lineage negative (Lin-) cells using *Sfpi.1/PU.1* GFP or mCherry mice as donors and non-irradiated NSG mice as recipients demonstrated that donor cells participate in the formation of blood cells as GFP positive cells were detected. Irradiation studies show a radiation dose dependent drop in repopulation capacity from 10 mGy to 3 Gy; HRS was not observed in these strains. DNA damage was observed in HSCs using the reticulocyte micronucleus assay (MN-RET) and was linearly dose-dependent over the range 10 – 300 mGy. No particular involvement of ROS has yet been observed at low doses. The differences may be in part due to strain dependant response or cell type specific responses.

The mouse model (CBA/H^{mCherry}) was used in which a fluorescent mCherry marker has been introduced on chromosome 2 in the acute myeloid leukaemia (AML) minimal deleted region (1.7 10^6 bp away from *Sfpi1/PU.1*). CBA/H^{mCherry} lineage depleted hematopoietic progenitors and stem cells (HSC/HPC) from mice irradiated with either 0 Gy, 0.01 Gy, 0.1 Gy and 1 Gy whole body exposure were transplanted into age-matched NSG mice and donor chimerism was assessed 6 months following transplantation. All transplanted NSG mice showed long-term myeloid and lymphoid engraftment. Similarly, following transplantation of irradiated HSCs, long-term multilineage engraftment was observed, although to a lesser extent. Significantly, the level of engraftment was dependent on the dose and a significantly lower

engraftment was seen already for the lowest dose studied so far i.e. 0.01 Gy. This indicates a direct effect of the low doses on HSCs, with irradiation of the host microenvironment (niche) playing a lesser role in the observed effects.

Using AML sensitive CBA mice carrying one gfp-tagged and one mCherry tagged *Sfpi1/PU.1* allele has allowed detection of deletions in the critical region for mouse radiation leukaemogenesis over time after in vivo irradiation, and time dependent accumulation of both lymphoid and myeloid lineage cells with *Sfpi1/PU.1* losses was detectable. Pyrosequencing methods allow detection of point mutations in cells prior to overt presentation of leukaemias. These detailed studies have revealed that the leukaemias arising in male CBA mice are different from those in female mice – the leukaemias in females have more lymphoid than myeloid characteristics, suggesting that the target cell for leukaemia may be gender dependent in mice, a more committed lymphoid progenitor being the target in females. Presently it is unclear why this gender difference exists.

Using a newly developed strain that carries an engineered point mutation in the *Sfpi1/PU.1* gene like those commonly found in AMLs presenting after radiation exposure, the relative effects of a maximally leukaemogenic dose of 3 Gy delivered acutely (0.7 Gy min^{-1}) or at the low dose rate of 0.042 mGy min^{-1} (2.5 mGy h^{-1}) have been compared. In this model, all unirradiated mice developed AMLs, with 100% incidence at 13 months. The mean latency to AML is 237 days in unirradiated males, 158 days in acutely irradiated mice and 199 days in low dose rate exposed mice; thus, acute irradiation reduces average life span by 79 days and low dose rate by 38 days. As it is known that AMLs in CBA mice are mainly driven by the induction of *Sfpi1/PU.1* mutations, and these 'reveal' the point mutation phenotypically, these studies indicate that the low dose rate exposure is associated with risk but is approximately 2 times less effective than the acute exposure in terms of mean days lifespan.

In summary, the RISK-IR studies have revealed the sensitivity of HSCs in mouse and human to low dose and low dose rate exposures. These low doses can have consequences at the genetic level as indicated by the induction of micronuclei and leukaemias in the CBA mouse models. In the most primitive HSCs there appears to be a cell-intrinsic hypersensitivity to radiation that is driven by ROS production from internal sources and is associated with metabolic re-programming and altered cell adhesion properties. Broadly similar phenomena have been observed in human HSPCs. The normal stem cell niche acts to abrogate the hypersensitivity to radiation, providing strong evidence for the influence of the microenvironment on stem cell responses.

5. Epithelial/skin stem cells

Skin has a very high nominal risk of radiation cancer, but as few skin cancers are lethal, skin contributes little to radiation detriment. Through investigation of this epithelial tissue, RISK-IR aimed to gain some insights into tissue dependency of sensitivity and, due to the range of tumour types found in skin and other epithelial tissues, also specific stem cell sensitivity and the underlying determinants of that sensitivity.

RISK-IR work has demonstrated that mouse sebaceous gland (SG) stem cells (SCs) are sensitive to low dose radiation, undergoing low level apoptosis accompanied by activation of Hedgehog signaling, while bulge SCs are resistant and they do not undergo apoptosis. Further gene expression analysis has been undertaken using transcriptional arrays to

provide insights into the underlying mechanisms of low- and high- dose radiosensitivity. Results suggest that SG SCs activate mainly pathways related to apoptosis, while bulge SCs upon low dose radiation modulate pathways related to mitochondria, ribosomal activity, oxireductase activity, ATP synthesis, protein transcription and metabolism. These differences may relate to the relative 'stemness' of the populations with SG stem cells being less mature and more progenitor-like and the true stem cells being in the bulge region. Previous work form RISK-IR partner labs showed that mouse bulge SCs are resistant to high doses of irradiation (5-20 Gy). Gene expression analysis has again been used to provide insight into the underlying mechanisms. Gene set enrichment analysis (GSEA) indicates the two main pathways activated in bulge SCs upon high dose radiation (anti-apoptotic and DNA repair) are not modulated upon low dose radiation. These data revealed that indeed while bulge SCs are resistant in both low and high dose radiation, they employ distinct mechanisms to survive the different irradiation doses.

Given the indication that the resistance of bulge SCs is related to changes in the mitochondria and metabolism, and bulge SCs undergo a metabolic switch from oxidative phosphorylation to aerobic glycolysis, upregulating the glucose transporters and the effectors of the glycolytic and the pentose phosphate pathway, Hif1α signaling appears to be implicated. HIF1α was found to be localized in the nuclei of bulge SCs, while it remained cytoplasmic in SG SCs. In order to verify that the resistance of bulge SCs to low dose radiation is mediated by the HIF1α, an in vivo gain and loss of function approach was employed, using conditional transgenic mice overexpressing (K14CreER; R26StopFLHif1a) or lacking (K14CreER;Hif1afl/fl) HIF1a expression specifically in the epidermis, expecting that SG SCs would survive in the former and bulge SCs would undergo apoptosis in the latter. Indeed, no apoptosis was observed in the SG SCs of Hif1α gain-of-function mice, confirming the role of HIF1α in the resistance of epidermal SCs to low dose radiation. Importantly, Hif1α gain of function did not confer resistance of SG SCs to high dose radiation, showing the specificity of this pathway to low dose radiation-induced damage.

Long-term effects, including carcinogenic effects have been examined in epithelial tissues of animal models. As a model for basal cell carcinoma (BCC), mice carrying deletion of one allele of the Patched gene [often mutated in human BCC, the most common type of skin cancer] specifically in the skin epidermis (K14Cre; Ptchfl/+) have been used. Mice irradiated with 5 Gy, used as positive controls for cancer development, presented multiple invasive BCCs, and very few dysplastic lesions. Mice irradiated with 50 mGy presented BCCs, at comparable stage and size to the tumours of 5 Gy-irradiated mice, albeit at much lower frequency (approx. ten-fold reduced yield). This result shows that low dose radiation can induce indeed BCC in predisposed individuals, albeit with tumour incidence lower compared to high radiation doses. As expected, the progression of tumours was comparable in both conditions.

The second most common type of skin cancer is squamous cell carcinoma (SCC) and this was modelled using transgenic mouse models expressing a constitutively active form of KRas, together with loss-of-function of p53, specifically, Lgr5CreER; KRasG12D; p53f/f; RosaYFP mice, which develop multiple skin SCCs upon tamoxifen administration. Low dose radiation was found not to have an effect on the time of tumour appearance or the tumour size. However, fewer tumours were observed per mouse. The results from the two mouse

skin cancer models may seem contradictory. However, this is not the case taking into account the differences in the means of tumour initiation, as well as the cell of origin of each type of skin cancer. It has previously been shown that BCC initiates from cells in the junction between the interfollicular epidermis and the hair follicle or the upper infundibulum (Youssef et al, Nat Cell Biol, 2010, 12:299-305), while SCC can be initiated by distinct cell types, such as the bulge stem cells and their immediate progeny (Lapouge et al, PNAS, 2011 108:7431-6). The present data indicate that in the BCC model, low dose radiation causes loss of heterozygosity of the wild type allele, which leads to Hedgehog signalling activation and cancer initiation. Notably, loss of heterozygosity of the Patched locus has been shown upon in utero administration of 250 mGy, causing meduloblastoma (Tanori et al, Stem Cells, 2013, 31:2506-16). Conversely, in the SCC model low dose radiation appears to trigger apoptosis in a fraction of the cells that have undergone tamoxifen-induced recombination, and for this reason fewer tumours appear per mouse.
Individual humans with Gorlin syndrome are pre-disposed to skin cancers due to inheritance of mutated forms of the Patched (Ptch1+/-) gene. Sensitivity to low-dose radiation was investigated in clonogenic assays, and interestingly, toxicity was observed in mutated cells at doses between 100 to 250 mGy. Notably, the gradient of sensitivity was similar to the one observed after high doses, and significant differences between the control and the mutated cells were observed only for two cell lines, HFG 1657 and 1552. Genotyping of these cells showed that they have deleterious mutations at the beginning of the PTCH1 gene, resulting in very high deficiency in the Patched1 protein, whereas the other Gorlin cells exhibited less severe deficiency. This result extends the link between radiosensitivity and PTCH1 defects to the low-dose range. The basis of the low-dose sensitivity may reside in DNA damage response, as it has been observed that ATM and p53 responses were reduced in the Gorlin cells, and reduced γ–H2AX was also observed.

Functional consequences of radiation exposure have been analyzed in human keratinocyte precursor cells using a xenograft model. 3D human skin reconstructs were irradiated (50, 500 mGy and 2 Gy), and then grafted on nude mice. Exposure of grafts did not impair macroscopically the development of the human xenografts, even after a dose of 2 Gy. Histo-morphometric analysis of in vivo reconstructed human skin showed that 50 mGy induced both general and local defects. Firstly, in the whole epidermis exposed to 50 mGy, a trend towards epidermis hyperplasia was found, as well as a significant enlargement of intercellular spaces as compared to the controls, suggesting a global defect in intercellular junctions and adhesion proteins. The barrier to external insults function of skin might thus be impaired after low radiation doses in xenografts. The second type of defect was found locally in the xenografts, as zones with keratinopoiesis disorganization of the nucleated layers of the epidermis, observed after 50 mGy, whereas such anomalies were not observed in the controls. Both results suggest that human skin reconstitution was impaired by low doses. Immunohistochemical analysis of disorganized zones indicated a reduced expression of β-catenin and E-cadherin after exposure to 50 mGy, perhaps indicating epithelial to mesenchymal transition (EMT), a process involved in carcinogenesis. Further evidence for the 50 mGy dose leading to EMT is provided by the observation of increased Keratin 5, ZEB1 and β–catenin as well as a shift to more horizontal planes of cell division following 50 mGy exposure, and in the dysplastic areas.

Global methylome analysis of late changes induced by irradiation indicated that low- and high- dose exposures produced numerous small changes in the percentages of CpG methylation, that were generally hypermethylations. These results require further analysis and confirmation but provisionally suggest a gamma protocadherin signature of low- and high-dose exposure plus specific high (2 Gy) and low (10 mGy) dose signatures. After a dose of 2 Gy, commonly used as a daily dose in radiotherapy, 18 specific genes were found for which methylation changes were most discriminant at 16 and 23 days after irradiation: ANK1, ARID1B, BLCAP, BRE, CUX2, EHMT2, NAV2, PARD3, PRKCH, TAP1, TFDP1, VPS53, PCDHα1, PCDHα2, PCDHα3, PCDHα4, PCDHα5, PCDHα6. Six of these genes are members of the alpha super family of protocadherins located on chromosome 5q31. After 10 mGy, 15 specific genes (+ 1 miRNA) were identified, for which methylation changes were discriminant after 16 and 23 days: ANKRD11, ATF6B, CDH13, COL18A1, GSPG4, DNAH17, GNG12, ITIH5, LRRC47, mir548n, ODZ4, PEMT, PRKAB2, PTPRK, S1PR1, SMYD3. From the function of these 15 genes, it appears that the major cell responses were localized at the cell membrane, for processes involved in calcium-related cell adhesion, signaling and energy status. A strong relationship with carcinoma was also found (at least 9 genes of 15): ANKRD11, CDH13, GSPG4, DNAH17, ITIH5, PEMT, PTPRK, S1PR1, SMYD3.

Clearly then both low- and high dose radiation exposures can be seen to affect skin stem cells and potentially modify their function in a dose- dependent fashion. Long-term functional consequences of exposure on epithelial maintenance in vivo have also been studied using lineage tracking in mice. An in vivo study of C57BL/6 mouse oesophageal epithelial cell and tissue kinetics has demonstrated that detectable DNA damage is seen in these cells at 50 mGy – 2 Gy (^{137}Cs γ–radiation) as shown by γ–H2AX and 53BP1 staining, but proliferation was not significantly affected at 24 hours after exposure at doses below 400 mGy. Using fluorescence-based cell lineage tracing markers, increased cell proliferation was observed at 2-7 days after 50 mGy exposures, and the observation of an increase in the number of floating clones which represent the differentiation of proliferating cells, suggests that low dose exposure leads to cell loss through differentiation followed by increased proliferation to re-establish a complete epithelium. Similar responses have been observed in oesophageal keratinocyte in vitro cultures, thus excluding any immune system involvement in the observed effects.

Gene expression analysis by microarrays of C57BL/6 oesophageal epithelium keratinocytes grown in vitro indicated a defined pattern of altered expression 24 hours following 50 mGy exposure, while at 1 hour after exposure there were few changes. By contrast, RNA sequencing analysis of expression has revealed that 50 mGy exposure upregulates anti-oxidative stress response, DNA damage and a number of other pathways. The overall patterns of change in gene expression appear to be different at 50 mGy and 2 Gy on the basis of pathway analysis. Further analysis suggests that bursts of ROS production following 50 mGy exposures may be driving the differentiation noted above, and repair pathways are not greatly affected by 50 mGy irradiation.

In addition, a dramatic effect of repeated low dose (50 mGy) exposures on the proliferation of clones carrying a mutant form of the p53 tumour suppressor has been found. Esophageal epithelial cells were tagged with a fluorescent reporter gene and a dominant negative p53 mutation at the same time. Imaging of tissues after 10 x 50 mGy irradiations showed a

substantially (3-4 fold) greater effect on clonal growth of the p53 mutant cells than a single 500 mGy exposure. The elevation of ROS in p53 mutant clones was found to be less than in wild type cells.

6. Brain/neural stem cells

The embryonic neocortex encompasses the neural stem and progenitor cells. In the ventricular/subventricular zone (VZ/SVZ), cells undergo rapid proliferation from E11 to E16.5 (Mitsuhashi, T. and Takahashi, T. 2009, Brain Dev 31:553-557; Pontious, A. et al 2008, Dev Neurosci 30:24-32; Bayer, S.A. et al 1991, J Comp Neurol 307:487-498). At E13.5, the VZ/SVZ represents the majority of the forebrain: by E14.5 the intermediate zone (IZ) occupies one half of the forebrain with the VZ/SVZ representing the remaining half. The VZ/SVZ region is located proximal to the lateral ventricle (LV). The neural stem cells initially undergo symmetric division to increase their numbers followed by a switch to asymetric cell division (Thornton and Woods, 2009 Trends Genet 25:501-510. In the adult brain, there are two stem cells regions, the SVZ and the SGZ (Ponti, et al 2013, Proc Natl Acad Sci USA, 110:E1045-1054).

The work described in this summary focuses entirely on the SVZ. The adult SVZ is more slowly proliferating than the embryonic counterpart (Ponti, et al 2013, Proc Natl Acad Sci USA, 110: E1045-1054). The SVZ is located around the LV and includes quiescent (Ki67-) Glial Fibrillary Acidic Protein (GFAP)+ stem cells, activated (Ki67+) GFAP+ stem cells, Mash1+ transit amplifying progenitors (TAPS), and Dcx+ neuroblasts (NBs) (Doetsch et al. 1999, Cell 97: 703-716). Only 5 % of GFAP+ stem cells are KI67+ (i.e. most GFAP+ cells are quiescent). The majority (~90 %) of TAPs are Ki67+, whilst 50 % of NBs are Ki67+ and 50% are Ki67-. Recent studies have subdivided the adult SVZ into four sub-domains, classified as the ventral, dorsolateral, medial and dorsal regions (Alvarez-Buylla et al., 2008, Cold Spring Harbor Symp Quant Biol 73:357-365; Azim et al., 2012, PLoS One 7: e49087; Fiorelli et al., 2015, Development 142:2109-2120).

The mouse and human embryonic brain are highly radiosensitive with microcephaly and cognitive deficits being observed after exposure to low radiation doses (Hoshino and Kameyama, 1988, Teratology 37:257-262; Hoshino et al., 1991, J Radiat Res 32:23-27). Additionally, microcephaly is a clinical manifestation observed in disorders with mutations in proteins that function in DNA non-homologous end-joining (NHEJ), the major DSB repair pathway in mammalian cells (Woodbine et al., 2014, DNA repair 16C:84-96). This suggests that neurogenesis is a sensitive stage for low dose IR exposure since the DSBs arising in the LIG4 Syndrome are equivalent to that induced by ~100 mGy (Gatz et al., 2011, J Neurosci 31:10088-10100). Furthermore, the microcephaly is present at birth but is not very progressive suggesting a sensitive window during neurogenesis (Woodbine et al., 2014, DNA repair 16C:84-96; Murray et al., 2014, Human mutation 35:76-85). Importantly, low dose IR exposure during childhood is a sensitive stage for carcinogenesis, making it interesting and important to examine the response of juvenile mice to IR (Brenner et al., 2001, Med Phys 28:2387-2388; Pearce et al., 2012, Lancet 380:499-505; Pettorini et al., 2008 Childs Nerv Syst 24:793-805).

DNA damage induction

In the embryonic neocortex and the adult SVZ, DSBs can be detected using 53BP1 foci enumeration at a number close to 1/3rd of the predicted number formed. Using mice with a hypomorphic mutation in DNA ligase IV ((Lig4Y288C), we estimated the level of 53BP1 foci in the embryonic neocortex without IR (Gatz et al., 2011, J Neurosci 31:10088-10100). We reasoned that an increased level of 53BP1 foci would reflect an enhanced level of DNA breakage in the rapidly dividing neural stem cells. Consistent with this, we observed a substantially increased level of 53BP1 foci in the neocortex in unirradiated Lig4Y288C embryos compared to WT mice, which was greater than observed in any other tissue examined. The level of breakage was similar to that induced by exposing WT mice to 120 mGy, although more DSBs were observed in the IZ compartment compared to the VZ/SVZ. Thus, we conclude that endogenous DSBs arise at high frequency in the embryonic neural stem/progenitor cell compartment, most likely due to the stage of rapid replication and require NHEJ for their repair. DSB levels became reduced at later embryonic stages and post birth as the replication capacity of the SVZ diminishes.

Response to IR exposure in the embryo

To assess the response to DSB formation, the level of apoptosis was monitored in unirradiated and irradiated WT and *Lig4*^{Y288C} embryos and Cernunnos deficience (cernu) mice (Gatz et al., 2011, J Neurosci 31:10088-10100). A low level was observed of endogenous apoptosis in WT mice but a substantially higher level in *Lig4*^{Y288C} mice, consistent with the level of endogenous DSBs i.e. the equivalent of exposing WT mice to ~120 mGy. Spontaneous apoptosis was also observed in cernu mice, equivalent to exposing WT mice to 10-20 mGy, consistent with a lower deficiency in NHEJ in these mice compared to *Lig4*^{Y288C} mice. Additionally, a dose dependent activation of apoptosis was observed in WT mice, which was linear down to 10 mGy (Saha et al., 2014, J R Soc Interface 11: 20140783). Exposure of *Lig4*^{Y288C} and Cernu mice caused a very high level of apoptosis, consistent with the response being triggered by unrepaired DSBs. The apoptosis was predominantly ATM dependent. It was concluded that the embryonic VZ/SVZ cells sensitively activate apoptosis either from endogenously arising DSBs or following low dose IR exposure. Since apoptosis can be observed after exposure to 10-20 mGy, it is likely that it can be activated, albeit infrequently, by a single DSB.

Whilst a linear dose response for apoptosis does not necessarily imply that the survival response will be linear, since cell death can arise via other processes, it is still useful to compare the response to other stem cell systems. Of the systems examined in RISK-IR, analysis of the response of HSCs provides a useful comparison. Like the neural stem cell system, HSC progenitors but not quiescent stem cells undergo apoptosis. However, the apoptotic response of progenitors has not been examined after low doses. Mouse HSCs show a low dose hypersensitivity after doses of 20-50 mGy following ex vivo radiation and assessment of survival by colony formation. This response has not been observed following in vivo irradiation however. Significantly, no evidence was found for such a hypersensitivity in the neural stem cell compartment following in vivo radiation.

Response to IR exposure in adult (3 month old) mice.

Next, the response was examined of the adult (3 month) SVZ to IR (Barazzuol et al., 2015. J Cell Sci 128:3597-3606). First, it was verified that 53BP1 foci could be detected in adult neuronal tissues with a sensitivity similar to that observed in embryos. The sensitivity of

multiple neuronal tissues was also examined, including the SVZ and SGZ to apoptosis following a range of IR doses. Sensitive activation of apoptosis was observed (detectable after exposure to 50 mGy) in the SVZ but not in other neuronal tissues (Barazzuol et al., 2015. J Cell Sci 128:3597-3606). The SGZ activated apoptosis after higher doses (500 mGy) but no significant activation was observed in any differentiated tissues examined. Using Lig4Y288C mice, A lower level of DSB formation was estimated in the SVZ compared to the embryonic counterpart, with the steady state level of DSBs in Lig4Y288C being equivalent to exposure to 25 mGy (as compared to 120 mGy in the embryo). The sensitivity for activation of apoptosis appeared similar between the adult and embryonic SVZ (see (Barazzuol and Jeggo, 2016, J Radiat Res 57 Suppl 1:i2-i10) for further discussion).

To characterise other DNA damage responses in the adult SVZ, we initially focused on assessing the response to 2 Gy. Current insight into the sub-domain structure of the adult SVZ was exploited (Alvarez-Buylla et al., 2008, Cold Spring Harbor Symp Quant Biol 73:357-365; Azim et al., 2012, PLoS One 7:e49087; Fiorelli et al., 2015, Development 142:2109-2120). Two sub-domains were noted in the SVZ containing progenitor cells whilst two other sub-domains only contained quiescent stem cells (Barazzuol et al. 2017,. PLoS Biol 15:e2001264). Three responses to 2 Gy were observed in the sub-domains containing replication progenitors: 1) apoptosis. Thus, it was concluded that progenitors (neuroblasts and transit amplifying progenitors) activated apoptosis after 2 Gy but quiescent neural stem cells were resistant to apoptosis. After 2 Gy, 50% of the neuroblasts underwent apoptosis. 2) a marked inhibition in proliferation (assessed using BudR labelling and enumeration of Ki67+ cells). 3) a marked loss of cells bearing DCX (the neuroblast marker) at 48 h post 2 Gy and increased differentiation. This was greater than could be explained by apoptosis and was shown to reflect premature differentiation of the neuroblasts into $Map2^{+}$ neurones. Additionally, quiescent stem cell activation was observed after 2 Gy uniquely in the subdomains with proliferating progenitors. These three responses were ATM dependent with no observed quiescent stem cell activation in mice lacking ATM. Thus, the neuroblasts that return after 2 Gy appear to arise from new neuroblast generation by virtue of quiescent stem cell activation (and not from transient proliferation arrest). These findings are consistent with studies using 4 Gy IR, where the reactivation of quiescent stem cells correlated with loss of neuroblasts that produce GABA, a repressor of neural stem cell activation (Daynac et al, 2013, Stem cell Research 11:516-28).

Response to IR exposure in juvenile mice.
Given the sensitivity to IR induced neuronal carcinogenesis during childhood, these three DNA damage responses were examined in neonatal (P5) mice (Brenner et al., 2001, Med Phys 28:2387-2388; Pearce et al., 2012, Lancet 380:499-505; Pettorini et al., 2008 Childs Nerv Syst 24:793-805; Barazzuol et al. 2017,. PLoS Biol 15:e2001264). A similar sensitivity to activate apoptosis was observed (indeed since there were more proliferative cells in the neonatal mice, there was a high overall level of apoptosis although the level per neuroblast appeared similar to that in adult mice). However, substantially less marked and more transient arrest of proliferation was observed in the neonatal SVZ compared to in the adult. Importantly, no quiescent stem cell activation was observed following 2 Gy in neonatal mice. Thus, it appears that in neonatal mice neuroblasts transiently but not permanently arrest proliferation and the recovery of neuroblast proliferation precludes quiescent stem cell activation. It was unable to quantify the DCX marker in neonatal mice.

Finally, the response was examined of the adult SVZ to low doses of IR (50 and 100 mGy compared to 200 mGy--2 Gy). As stated above, a linear activation of apoptosis was observed down to 50 mGy. However, the loss of proliferation arrest was much reduced after 50 and 100 mGy compared to higher doses. Indeed, there appeared to be a threshold for this response at around 200 mGy. Additionally, loss of the DCX marker was only observed following exposure to 500 mGy but not at lower doses. Importantly, these results suggested that whilst apoptosis appears to be activated by IR in a linear dose-response manner, the other two responses (proliferation arrest and neuroblast differentiation) do not appear to be activated after exposure to low doses. Thus, it is likely that after low dose IR, exposed neuroblasts can regain proliferative potential. This potentially provides the possibility for cells with translocations to persist and a window whereby low dose exposure can cause carcinogenesis.

Cancer Risks

Based on the data on IR induced effects in in vitro cultured stem and differentiated cells together with the results on the effects of IR on mouse in vivo brain, LDIR related cancer risk can be speculated. A threshold in checkpoint induction that is observed after low dose IR (in repair proficient cells and mutants with NHEJ deficiency) indicates that stem and differentiated cells can pass through mitosis with unrepaired DNA damage. This is further strengthened by a linear induction of cancer relevant genetic damage (micronuclei) and apoptosis down to 20 mGy despite efficient repair pathways. NHEJ deficiency resulted in a significant increase of LDIR induced micronuclei frequencies.

Neonatal mice and humans are sensitive to radiation-induced carcinogenesis in the brain. In newborn mice, cells in the SVZ show a greater level of proliferation but less prolonged proliferation arrest after IR compared to adult tissue and replenishment without quiescent neural stem cell (NSC) activation. Thus, the neonatal SVZ progenitor pools after HDIR and LDIR are derived from surviving irradiated progenitors, most likely due to checkpoint induction thresholds. It is proposed that progenitor cells in juvenile mouse brain after LDIR can proliferate in the presence of unrepaired DSB and thereby generate cancer relevant damage (MN) with potential risks for carcinogenesis. In contrast, the adult mouse brain prevents the proliferation of the irradiated progenitor cells. The quiescent status of NSCs allows them to repair DSBs before proliferation and subsequently to replenish the progenitor pool, thereby diminishing the likelihood of genetic damage and carcinogenesis.

Potential impact and main dissemination activity and exploitation of results (10 pages)

As noted earlier, health risk estimates for ionising radiation have as their main basis evidence from epidemiological studies. However, there are several areas where the epidemiological evidence requires support from experimental studies. This is particularly the case in situations of low dose and low dose rate exposures, where the size of epidemiological study cohorts constrains statistical power. The estimation of risk at low doses and dose rates is based currently on a linear extrapolation of risk estimates obtained in studies at higher doses, applying a dose and dose-rate effectiveness factor of 1.5-2. Judgements on risk at low doses/dose rates and the appropriate risk extrapolation model to use are based in large part on UNSCEAR reviews of relevant science, review and evaluation

work of the US National Council on Radiation Protection and Measurement, and ICRP evaluations of evidence and subsequent recommendations. It is expected that many of the published outputs from RISK-IR will be cited in relevant reports of UNSCEAR, ICRP, NCRP as well as other national and international review bodies. RISK-IR project members are directly involved in two important activities in this respect. They are involved in an UNSCEAR review of the biological mechanisms of radiation carcinogenesis and an ICRP Task Group on low dose risk inference. These involvements will help ensure RISK-IR results are recognised and taken into consideration in these important international evaluations.

One of the main justifications for use of a linear non-threshold extrapolation model for low dose/dose-rate risk estimation is the so called 'biophysical argument'. This holds that ionising radiation damage is caused in cells in direct proportion to energy absorbed, and thus when the delivered dose is sufficiently low damage will be caused in single cells by single tracks. While it is well recognised that mammalian cells have systems to repair and correct damage to DNA, none of the repair systems known and described allows completely accurate and faithful repair, and so there is a finite probability of mutations to be induced even at the lowest levels of damage (i.e. lowest doses). This argument clearly depends upon acceptance that this holds for the target cells that become cancer cells eventually after exposure to radiation and that DNA mutations are the main driver of carcinogenesis in humans. The latter is generally held to be true though there are now indications that there are a wider range of epigenetic alterations that are stable and may contribute to the cancer phenotype. This model therefore needs critical examination in the target cells for radiation carcinogenesis, i.e. stem and early progenitor cells.

The RISK-IR project brought together for the first time a unique combination of stem cell researchers and those with a background in radiobiology and related to radiation protection to examine issues of stem cell radiobiology at low doses in relation to carcinogenesis. The key scientific achievements include:

- Demonstration of stem cell sensitivity in vitro and in vivo to doses in the range 10-100 mGy (i.e. a dose range including the dose from a single CT scan) in a range of tissue types with strong indications that free radical responses dominate in adult haematopoietic and skin/epithelial stem cells, and DNA damage response has little impact. At the low doses several studies also suggest a 'metabolic shift' occurs that in itself may be mediated by ROS. Oxygen radicals/reactive oxygen species (ROS) are also found to contribute to the generation genetic damage (micronuclei) in cultured embryonic stem cells and contribute to longer term effects in low dose exposed skin and haematopoietic stem cell populations. This emphasises the importance of ROS in stem cell effects, particularly in the low dose range. The killing of stem cells would likely reduce cancer risk, but may lead to an increased risk due to replication-associated mutation on repopulation.

- The added value of integrative approach manifested by the key role of the NHEJ repair pathway in LDIR response at the level of stem cells in vitro, as well as in tissues and the organism. Studies of the phospho-proteome suggest that ESC responses to high (1 Gy) and low (100 mGy) doses can be similar in terms of the affected proteins but different in the magnitude of response, suggesting that low- and high- dose response may differ either in terms of the affected genes/proteins or the magnitude of effect, or both. The impact of these

findings for risk are not entirely clear as they relate to early post-irradiation response, however they suggest a linear response with increasing dose.

- Skin stem cells: indications of stem cell population specific sensitivity to radiation induced cancer, where, in experiments using specifically pre-disposed mouse models in some populations low (50 mGy) doses increase cancer incidence and in others they reduce cancer incidence. The differential sensitivity appears to relate differential apoptotic response of the target stem cell populations to low dose exposure. This highlights the complexity and stem cell specificity of response to low dose exposures. These results serve to highlight the variation in response between stem cell types, and suggest that low doses may serve either to increase or decrease risk in particular situations.

- Hematopoietic stem cells: indication of non-linear dose responses characterised by a dose range showing hypersensitivity in terms of cytotoxicity; these responses appear to be very lineage-stage dependent and it remains unclear the impact these may have on cancer risk in vivo as the stem cell niche appears to protect against the hypersensitivity phenotype. The deletion of damaged cells due to cell killing might be expected to reduce risk, but bring potential risk of replication associated mutation.

- Generation of quantitative evidence of radiogenic leukaemia risk in an engineered model system that revels specific chromosome deletions at the low dose-rate of 2.5 mGy per hour. At this dose rate, single track events in single cells predominate; in effect modelling the very lowest exposures, lower dose rates will only reduce the number of cells traversed by radiation tracks. The low dose rate has an approximately two-fold lesser effect on lifespan shortening than an acute exposure.

- Brain/neural stem cells: A more complete understanding of the age/developmental stage dependence of brain/neural stem cell sensitivity to apoptosis and how this may contribute to observed differences in cancer risk at differing ages/developmental stages and potentially impact on other neurological conditions. Age dependant responses for a range of endpoints in mesenchymal stem cells have also been observed.

- iPS cells: Development of unique models of iPS in which to explore in more detail the role of radiation reprogramming. Results to date suggest a negative impact of high doses (>6 Gy) on reprogramming frequency but the secretion of IL6 on induction of senescence by radiation being able to promote reprogramming. The impact of lower doses is not yet fully understood, but some results suggest that 90 – 500 mGy may reduce the frequency of induced reprogramming. Radiation itself does not substantially affect the expression levels of the key ‘Yamanaka’ stem cell transcription factors. The implications for risk of these experiments that are at an early stage are difficult to assess; however, any impact of low doses on reprogramming either directly or through secretion of factors would need careful consideration in terms of risk.

- Indications that even low dose exposures can contribute to stem cell exhaustion and affect long term repopulation in mesenchymal stem cells and haematopoietic stem cells. Stem cell exhaustion per se may not have specific risk implications, but any increased proliferation to allow repopulation could bring risk of replication associated mutation.

- Early proof of principle work on in vivo esophageal cell fate tracking models to investigate radiation effects demonstrate sensitivity at low (50 mGy) doses that triggers cell

loss through differentiation and repopulation by proliferation and identification of pathways that respond to low dose exposures. Low doses also lead to the preferential growth of clones carrying p53 mutations, suggesting that low doses could act as promotors of carcinogenesis.

Returning to the stated project objectives, the work undertaken addresses each issue as summarised:

1. The identification and enumeration of stem/progenitor cells at risk of developing into cancers.

The nature of the cells at risk for radiation leukaemia in mouse has been defined and found to differ between males and females. In the mouse skin two different stem cell populations have been shown to respond differently to low doses in terms of cancer induction. The numbers of relevant target cell populations are available in the scientific literature. In the skin, different stem cell populations are targets for basal cell carcinomas and squamous cell carcinomas, and differing response of these stem cell populations likely underlie differing responses to low dose exposures.

2. Understanding the low dose radiosensitivity of stem cells and tissues and dose-response relationships.

The sensitivity of adult stem cells in bone marrow, skin, brain and mesenchymal tissue has been assessed for a range of endpoints, and responses to low doses of 10 – 100 mGy have been documented. Embryonic stem cell effects at 20 - 100 mGy have also been observed. Each stem cell population has its characteristic response relationship. In skin, differential response to low dose apoptosis may underlie a differential response to low dose in terms of cancer. In bone marrow, the sensitivity of stem cells can clearly be substantially modified by the niche/microenvironment in which cells are growing. Cell cycle checkpoint activation has been examined and the repair of DNA breaks over a wide dose range; double strand break repair in stem cells is inefficient following doses of 10 mGy, but can be improved by exposure be exposure to free radical damaging agents such as hydrogen peroxide.

3. Improving the understanding of mechanisms of age-dependent cancer risk

In mesenchymal stem cells an age-dependence of in vitro transformation has been observed. In skin, age influences the magnitude of apoptotic response to 50 mGy in the bulge stem cell population. In the esophageal epithelium system, the effect of low dose exposure leading to preferential growth of cells carrying p53 mutations indicates a promotional activity of low doses that will be greater in at older ages due to the accumulation of mutations over time and through replication and other DNA damage. In the brain, higher cancer risk is apparent in neonatal animals by comparison with adults. The basis for this difference is proposed to be due to the observations that progenitor cells in juvenile mouse brain after LDIR can proliferate in the presence of unrepaired DSB and thereby generate cancer relevant damage (such as micronuclei, and likely other forms of damage) with potential risks for carcinogenesis. In contrast, the adult mouse brain prevents the proliferation of the irradiated progenitor cells, thereby reducing the risk of cells proliferating with un-repaired damage.

4. Improving the understanding of mechanisms contributing to tissue specific differences in cancer risk

In skin, the differential apoptotic response of bulge and sebaceous gland stem cell populations appears to contribute to differential effects of low dose exposure on cancer induction observed; thus, there are differences within tissues in terms of risk of specific cancer types.

5. Identification of key events and individual susceptibility factors associated with cancer development

The genetic event driving mouse radiation leukaemogenesis have been further characterised, and at low dose rate (2.5 mGy h^{-1}) the same deletion-driven mechanism has been observed to operate. Multiple susceptibility factors have been identified as low dose risk modifying factors, notably in skin and epithelial tissues. The possibility of the modulation of stem-ness of differentiated cells by radiation as revealed in the experiments using iPS models raised the possibility that exposure modulates the numbers of cells at risk. This area needs considerably more work before being taken into consideration in risk assessment frameworks.

Many of the phenomena described relate to the 'hallmarks of cancer' as described by Hanahan and Weinberg, most notably (i) genetic instability and mutation, (ii) enabling replicative immortality, (iii) deregulation of cellular energetics and (iv) inflammation. The findings are therefore of direct relevance to carcinogenesis and so will impact on radiation cancer risk assessment. The RISK-IR results indicating that radiation even at low doses may affect stemness do not relate simply to the hallmarks of cancer but are nonetheless likely to be relevant. Of the results obtained to date, effects on mutation/genome instability and on deregulation of cellular energetics generally indicate a cancer risk enhancing action of low dose exposures; effects on proliferative signalling and inflammation are harder to interpret simply but may act to reduce cancer risk. We would stress though that RISK-IR results also indicate that each stem cell population may respond in its characteristic fashion, and uniformity of response across all stem cell populations should not be assumed. Drawing definitive conclusions therefore on the overall impact of low dose radiation effects on stem cells in terms of risk will require further investigation. RISK-IR studies have however demonstrated the value and feasibility of stem cell effects studies at low doses. Further work could usefully utilise in vivo models, possibly with manipulation of oxygen conditions at dose rates relevant to occupational and public exposures. Such occupational and public dose rates are considerably lower than any used within RISK-IR and would lead to a much wider spatial and temporal distribution of damage amongst cells.

The dissemination strategy adopted within RISK-IR reflects a reasonably standard model used throughout science. The primary output is in the form of publications in the peer reviewed scientific literature. Reviews were undertaken to place RISK-IR findings in context and to consolidate results more widely, further review articles are planned to help consolidate and communicate the findings of RISK-IR partners. The use of open access publication was encouraged within RISK-IR by provision of a specific budget held by the co-ordinator for open access publication fees. All partners were encouraged to request funds to

help ensure wide dissemination accessible to all. Project findings have been presented in many scientific meetings ranging from basic science meetings through to more general radiation protection audiences (see tabulation below). Important amongst the presentations was that at the September 2016 Radiation Protection Week that served as the key presentation to stakeholders in radiation protection. The meeting had some 350 attendees form over 30 countries drawn from a wide range of research communities and radiation protection areas, including from influential bodies such as UNSCEAR, ICRP, IAEA, WHO and NCRP.

Further dissemination will continue with additional open literature publications anticipated. The main route for impact of this type of work is through the consideration of RISK-IR publications in international reviews and consensus building activities. As noted above, several members of the RISK-IR project group have roles in bodies such as UNSCEAR and ICRP, and are thus well placed to bring relevant RISK-IR publications to the attention of these organisations involved in radiation risk assessment and radiation protection. Specifically, RISK-IR partners have roles in the UNSCEAR work on Biological mechanisms of radiation carcinogenesis, and the ICRP Task Group on low dose risk inference. These activities will ensure the recognition and consideration of RISK-IR results in these international reviews.