Radiation-Induced Aging and Genetic Instability of Mesenchymal Stem Cells: An Issue for Late Health Effects?

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Introduction

Adult stem cells represent a relatively small fraction of the somatic cells of an organism. For a long time their existence could only be deduced from quantitative analysis of transplantation studies or from the in vitro growth pattern of single-cell derived clones. Although it became possible now to enrich rare adult stem cells by selecting for particular protein markers expressed on their cell surface, it is still a challenge to identify and study them in their normal tissue environment.

Whereas the stem cells of the developing embryo (forming the ectoderm, endoderm and mesoderm germ layer) are extensively studied and characterized in terms of their role in the formation of the entire organism, the potential of adult stem cells for the maintenance of health or their role in the development of diseases still requires much research.

The best studied adult stem cells are those of the hematopoietic system (HSC), which produces cells of the myeloid and lymphoid lineages, forming the peripheral blood and immune system (Barnes et al. 1966). The radiation response of HSCs and the lineage-committed precursor cells derived from them have been extensively studied to better understand the factors that influence long-term success of bone-marrow transplantation after radiation accidents or after high-dose chemotherapy (Gale et al. 2014).

Although the existence of stem cells in adult solid tissues was suggested already in 1867 by J Cohnheim, their role in the regeneration of solid tissues could be studied only when histological labelling techniques using cell-type specific antibodies or autoradiography after isotope incorporation became available. The best studied non-hematopoietic stem cells of the adult organism are those that form the

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crypts of the small intestine, and their involvement in the occurrence of post-irradiation gastro-intestinal syndrome is well established (Potten 1998).

With the rise of in vivo fluorescence labelling methods for single cells and their progeny, even the involvement of specific cellular signaling pathways (such as the Wnt-pathway) in the dynamics of small-intestine stem cell regulation could be discovered.

Stem cells that contribute to adult skin regeneration are also widely studied, in part because the hair follicles in the skin form a very prominent histological pattern. Similar to the situation in the small intestinal crypts, this highly reproducible tissue pattern allows the visual identification of single stem cells, the committed precursor cells derived from them and the fate of terminally differentiated cells. The role of skin stem cells in the induction of radiation induced skin tumors (basal cell carcinoma and squamous cell carcinoma) is well understood and the involvement of specific pathways (such as the Hedgehog pathway) established.

Two other types of adult stem cells belong to organs that are usually not considered to be as radiation-sensitive: the brain and the connective tissue. Neural stem cells (NSC) and mesenchymal stem cells (MSC) are much more difficult to identify in their normal tissue context, since they don't reside at morphologically defined sites within the tissue architecture. Only by transgenic expression of SC specific fluorescence-tagged proteins, or induced expression of fluorescence proteins, followed by the analysis of stem-cell derived daughter cell lineages can the existence of these rare cells with long term repopulating potential (LTRP) in these two tissue types be analyzed. Nevertheless, NSCs and MSCs are being extensively studied because of the expectation that a better understanding of their function will help to prevent or to cure neurodegenerative diseases, age-related disabilities, cardiovascular diseases or even cancer.

There has not been much interest in the response of NSCs or MSCs to radiation exposure. This might be due to the fact that for tumors of the CNS and of connective tissue (derived from mesenchymal cells) the radiation-associated excess relative risk (ERR) among the A-bomb survivors was much lower than for carcinoma (of skin, mammary gland, lung, thyroid, and colon) or for leukemia (Preston et al. 2003). This picture looks much different, however, when cancer patients are studied who were cured from a primary malignancy by (high dose) radiotherapy and who developed a secondary tumor later in life. Here it became clear that external beam radiotherapy confers a relatively higher risk for the induction of therapy-associated secondary sarcomas (derived from mesenchymal cells Berrington de Gonzalez et al. 2012) and medulloblastoma of the brain (in the case of Gorlin syndrome patients) as compared to carcinoma or leukemia.

In the following chapters I will try to explain why adult stem cells, in particular MSCs, should be considered an important target for the development of radiation-associated disease. This is also relevant in view of an increasing number of clinically approved MSC based therapies. These involve the collection of MSCs from various anatomical sites of a patient (including sites that might have been exposed to diagnostic, therapeutic or occupational ionizing radiation), followed by forced in vitro expansion of the cells prior to autologous re-engraftment.

Biology of Adult Stem Cell

Adult stem cells have the unique capacity of long-term repopulating tissues and organs. For this purpose they undergo self-renewal at every cell division. This implies that at least one of their daughter cells retains the long term repopulating potential (LTRP) of the parental stem cell. Depending on the tissue of origin, ASCs are multipotent in the sense that in addition to self-renewal they can produce daughter cells (committed precursor cells) that give rise to transiently amplifying cells and finally to mature cells that serve different biological functions.

The decision of stem cells to undergo symmetric cell division (only self-renewal and generation of two identical ASCs) or asymmetric cell division (self-renewal of the stem cell plus the generation of a committed precursor cell) depends on external triggers such as the presence of growth factors or their contact to neighboring cells (Fig. 1). The dynamics of stem cell proliferation and the fate of the daughter cells is a focus of current research that requires highly sophisticated methods of life single-cell analysis.

When ASCs divide asymmetrically, committed precursor cells or the terminally differentiated cells change their cellular program as compared to the parental stem cell.

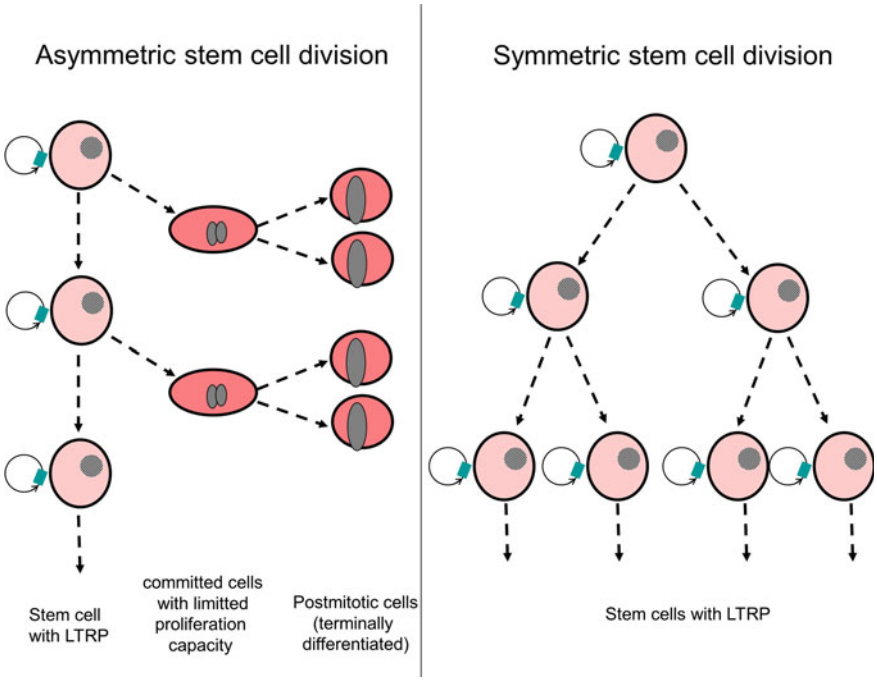

Fig. 1 Symmetric versus asymmetric division of adult stem cells determines the dynamics of self-renewal, generation of committed precursor or differentiated cells and the potential increase of the number of stem cells

This affects their metabolism (number of mitochondria, ATP production), expression of genes involved in cell signaling, DNA repair and telomere maintenance, their capability to express structural proteins and to undergo apoptosis. There is good evidence that stem cells that undergo lineage commitment or terminal differentiation do so by following epigenetic changes in large parts of their genome (affecting chromatin organization, methylation, and expression of non-coding RNA).

To maintain the capacity for long-term and high fidelity tissue regeneration, ASCs have to protect their genome from endogenous damage (by ROS or replication errors) and from cellular stress by exogenous noxae. An important role in protecting ASCs from genotoxic and cellular stress is played by supporting cells of the so-called "stem cell niche". The precise function of this niche is not completely understood yet, but it is known that cells that have a direct contact to stem cells are instrumental in directing symmetric or asymmetric stem cell division, most likely by providing a topological orientation and governing cell polarity during mitosis. When this well organized morphology of the stem cell niche is compromised, for instance by chronic inflammation or radiation-induced cell death, ASCs might respond with unscheduled symmetric cell division. This would give rise to an increased number of stem cells, and interfere with normal tissue homeostasis by so-called accelerated repopulation. One can only speculate about the potential impact of such an accelerated stem cell proliferation for their long term genetic stability.

Physiological Function of Adult Stem Cells

Adult stem cells play a crucial role in the life-long replacement of lost or worn-out somatic cells, for the tissue regeneration following trauma, injury or wound healing and for the normal plasticity of many somatic tissues. Therefore, they are instrumental in the response of a multicellular organism to environmental changes and to cellular stress imposed by exogenous factors. Adult stem cells of the hematopoietic system, for instance provide a life-long reservoir for cells of the adaptive and innate immune system, ensuring a fast responding defense against invading pathogens. HSCs also replace erythrocytes and platelets continuously. Smooth muscle stem cells (satellite cells) become activated after muscle injury, and are the source for healing of skeletal muscle. Mesenchymal stem cells also replenish various types of connective tissue in healthy and in pathological situations. They provide cells for a continuous bone remodeling, but also contribute to fracture healing of bone and wound healing of skin and connective tissue. MSC derived fibroblasts, osteoblasts and adipocytes also constitute the bone marrow niche for hematopoietic stem cells (Fig. 2). During wound healing MSCs also secrete paracrine factors that exert immune-modulating and anti-inflammatory response. This has been shown to be also an important mechanism to prevent graft-versus-host disease after allogenic bone-marrow transplantation.

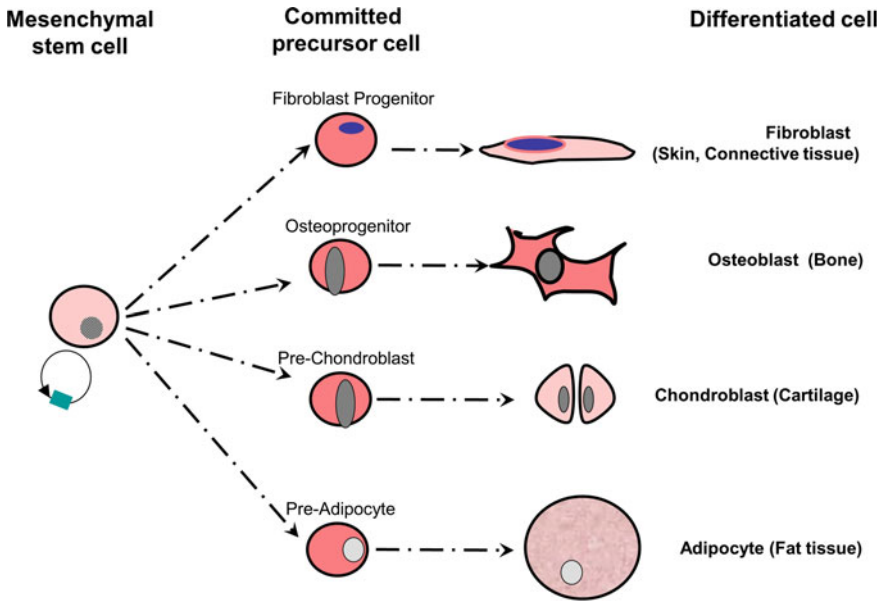

Fig. 2 Cells of the connective tissue compartment derived from MSC

Since MSCs have a multipotent differentiation capacity, they contribute to tissue plasticity under environmental changes. In response to changes in the caloric uptake, MSCs can up- or down-regulate the number of pre-adipocytes. An increase in mechanical load to bones will induce MSCs to generate more osteoblast precursor cells. Since these functions of ASCs are all associated with cell division, a stable genome in cells with a long term proliferation potential is essential.

Adult Stem Cells as Radiation Targets

As compared to the total number of somatic cells in an organism, tissue specific adult stem cells are very low in number. Depending on the organ or tissue under consideration, between 0.1 and 0.005 % of all cells are estimated to be adult stem cells with LTRP. In some organs, such as in the hematopoietic bone marrow, the numbers of proliferative stem cells seem to shrink with age. It has been shown that from a total of $\sim$1000 pluripotent HSCs in young subjects only a few (in some studies only one single HSC) were still present in patients older than 70 years (McKerrell and Vassillou 2015). Nevertheless, despite this potential loss of adult stem cells with increasing age, the cellular life time of an individual adult stem cell can in theory be as long as the entire life span of the organism itself. Here it is important to note that adult stem cells such as pluripotent HSC seem to be rather

radio-resistant in terms of their clonogenic survival (Ploemacher et al 1992). In radiation accidents where subjects were exposed to radiation doses high enough to cause an acute complete bone marrow failure, long-term auto-transplantation and recovery of the entire blood forming hierarchy was observed sometimes after years, also suggesting that the most primitive stem cells are more radio-resistant than the committed precursors or transiently amplifying cells (Baranov et al. 1994), probably because of the long cell cycle turnover of the former.

In another well studied tissue system, the small intestinal crypt, at least a subpopulation of slowly proliferating clonogenic stem cells was found to be radio-resistant and able to repopulate the entire crypt, even after gamma doses as high as $\sim$10 Gy (Potten 1998).

To adequately weight the factors that could qualify adult stem cells as target cells of a radiation-induced malignant transformation, one can do the following comparison:

Relative abundance of target cells:

$$\text{ASC/differentiated or precursor cells} : 10^{-3}\ldots10^{-4}$$

Time window available for the accumulation of radiation-induced mutations

$$\text{ASC: 70 years}$$

$$\text{differentiated or precursor cells: 2 weeks}$$

$\rightarrow$ *Relative length of time window available for finishing carcinogenic progression*:

$$\text{ASC/differentiated or precursor cells: 1860.}$$

This means that ASCs in a 70 year old person had the chance to accumulate almost 2000 times more radiation-induced mutations in their genome than short-living committed or differentiated cells, assuming exposure to the same radiation dose-rate. In the case of the natural background radiation ($\sim$3 mSv/a in Germany), every ASC in a 70 year old person would have been exposed to a cumulative dose of $\sim$200 mSv. In the context of the multistep process of environmental tumorigenesis (Trott and Rosemann 2000) this would also mean that long-term slowly proliferating ASCs have a much higher chance to acquire a rare, complex pattern of cancer-driving mutations in their genome as compared to the faster growing, but short living precursor cells (Fig. 3).

This calculation shows that the low abundance of ASCs as cancer-forming target cells can be easily out-weighted by their higher chance of acquiring tumorigenic mutations over a long time.

Using next-generation sequencing to identify somatic mutations in single cells it was recently estimated that haematopoietic precursor cells accumulate about 10 random mutations per year, and that those mutations which by chance confer a

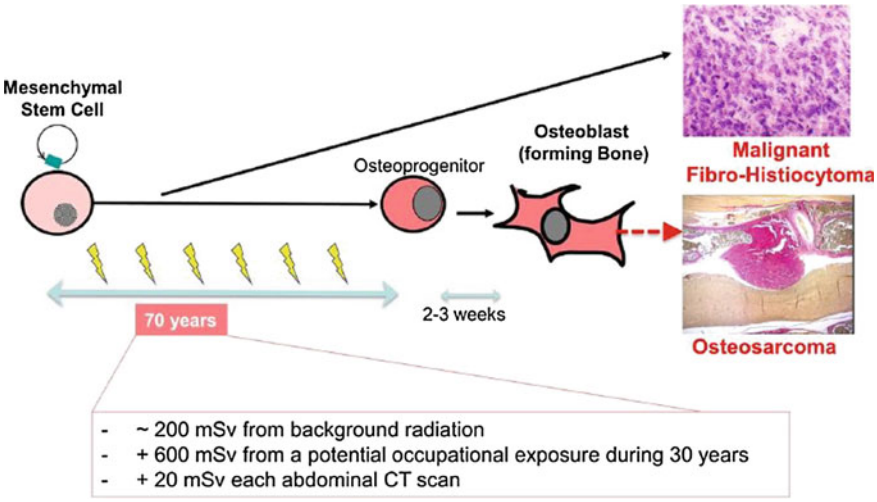

Fig. 3 Cumulative radiation dose in mesenchymal stem cells. After life-long exposure to chronic IR of various sources, MSCs in an adult person could accumulate several hundred mSv. This is in contrast to committed precursor cells, which have a proliferative life span of just a few weeks. Undifferentiated MSCs can give rise to malignant fibrous histiocytoma/pleomorphic undifferentiated sarcoma upon malignant transformation, whereas transformed osteoblasts form osteosarcoma

growth or survival advantage to the daughter cells will eventually give rise to predominant clones (Xie et al. 2014). In blood donors above the age of 70 years, a few such mutant clones were found to dominate the entire population of blood cells. The authors made the case that this is consistent with a model of long-living haematopoietic precursor or stem cells accumulating somatic mutations throughout the life span of a person. The age-associated increase of the mutational load to an organ or a tissue is also known to happen in solid tissues, such as skin (Goodell and Rando 2015), and the resulting growth of mutant clones with a concomitant loss of wildtype cells has been termed "clonal collapse". This shows clearly that throughout the entire life of an organism, stem cells accumulate more and more mutations that are passed to their progeny cells. Although most of these somatically mutated cell clones do not seem to progress to full malignancy within a life time, there is a clear association with an increased risk for myeloid-dysplastic syndrome, aplastic anaemia, leukaemia (Jaiswal et al. 2014) as well as with the frequency of other age related diseases (Bonnefond et al. 2013).

At the moment one can only speculate about the contribution of chronic background radiation (2–5 mSv/a) to this age related increase of stem cell mutations, or if repeated exposures to diagnostic X-rays ($\sim$2 mGy for a conventional X-radiography or $\sim$20 mGy for a CT) throughout life would further add to this mutation load.

Chronic Disease by Dysfunctional SC

As outlined earlier in this article, ASCs represent a life-long cell reservoir for tissue repair. Their loss or functional deprivation will therefore always cause a higher risk for chronic diseases, in particular for those characterized by tissue degeneration. They can affect neuronal tissues (Alzheimer disease, Parkinson disease, depression, stroke, dementia, hearing loss, macular degeneration or other retinopathies), the blood and immune system (aplastic anaemia, chronic inflammation, impaired immune response and increased infection risk), cardio-vascular organs (arteriosclerosis, hypertension, ischaemic infarct), the metabolic organs (metabolic syndrome, diabetes), or the musco-skeletal system (osteoporosis, osteoarthritis).

Since most of terminally differentiated cells rely on a high expression of specific proteins and/or on a sufficiently high ATP production in their mitochondria, cellular stress by endogenous radical oxygen species (ROS) and by the aggregation of misfolded proteins (or a combination of both) are likely to cause a time-limitation of their functionality. Therefore, similar to a building that requires regular maintenance and repair work in-order to ensure its long-time stability, most organs and tissues in a healthy organism can maintain their physiological function only if stem cells continuously provide fresh functional cells to replace old ones. If adult tissue stem cells are depleted or funtionally impaired, the homeostasis between newly generated cells and lost dysfunctional cells is disrupted, most likely leading to degenerative health complications.

It has been shown that chronic low dose irradiation (that does not induce acute cell death) has the capacity to trigger premature senescence in human epithelial cells (Yentrapalli et al. 2013). In murine MSCs we observed loss of stem cell capacity by increased premature adipogenesis also at doses that do not induced cell death (Höfig et al. 2016) (Fig. 4).

This shows that despite being refractory to radiation-induced cell death, stem cells can lose their long term repopulating potential by several other mechanisms as well.

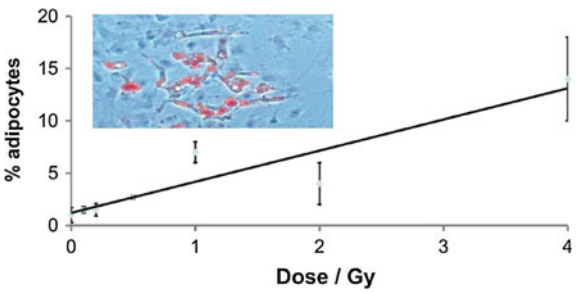

Fig. 4 Premature adipogenesis in murine MSCs after gamma iradiation. Adipogenic differentiation as detected by Oil-red staining in a culture of murine MSCs 3 weeks after gamma-irradiation

When Too Much of a Good Thing Becomes Bad: Malignant Transformation

At the opposite end of the spectrum of disturbances of the stem-cell proliferative capacity one can finds their malignant transformation. Here, a gain-of-function alteration in the cellular genome causes the stem cell to divide in an uncontrolled manner. This generates cellular progeny which escape the normal cellular program to differentiate or to undergo apoptotic cell death and which acquire proliferative immortality them self. The neoplasia that are the clinical manifestation of this transformation are an extremely heterogeneous class of disease, often require sophisticated forms of therapy depending on the genetic alterations and molecular biology the malignant cells. Despite tremendous progress in the field of personalized cancer medicine, with novel therapies using tumour-directed antibodies, tumour-targeting immune cells or therapeutic compounds directed against specific cellular pathways, on average 35 % of all malignancies will finally cause death of the patient.

In a recently published study by Tomasetti and Vogelstein (2015) an association was found between the abundance of adult stem cells in various organs and the frequency of tumours. It would be to early, however, to blame only transformed adult stem cells for each and every tumour. Hahn et al. (1999) demonstrated in various in vitro models that differentiated human cells can readily be transformed into fully malignant cells, just by the targeted genetic manipulation that affects two or three cellular signalling pathways. This clearly shows that immortality can be easily acquired by mutations in non-stem cells as well.

The Case of MSCs as Target Cells for Radiation Carcinogenesis

Post-irradiation sarcoma is a prominent late effect following therapeutic irradiation. In the case of external beam radiotherapy with healthy connective tissue in the radiation field, a large portion of sarcoma are undifferentiated malignant fibrous histiocytoma (MFH, recently termed pleomorphic undifferentiated sarcoma). When most of the radiation dose is absorbed by the bone surface (the area where pre-osteoblasts are located), as in the case of therapy with bone-seeking short-lived alpha-emitters such as Ra223, the predominant type of late arising secondary tumour is osteosarcoma. This suggests that after high-dose therapeutic irradiation the sarcoma type is determined by the type of target cell in the radiation field. On the other hand, whole body irradiated subjects that survived moderate doses (0.2–3 Gy) during the A-bomb blasts were recently found to have an increase of osteosarcoma, but not of MFH, implying that even when MSCs are in the radiation field they become more important as target cells for a malignant transformation only after higher doses. It is possible that MSCs and pre-osteoblasts (the transiently

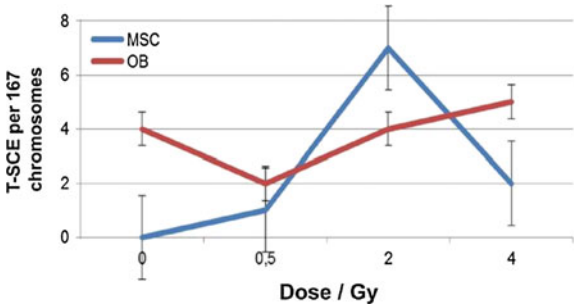

Fig. 5 Telomere sister-chromatid exchange in murine MSCs and osteoblasts. Metaphase chromosomes were differentially stained for 3′ and 5′ telomeres after gamma-irradiation and BUdR incorporation. For each experiment, 167 chromosomes were scored for the occurrence of 3′–5′ telomere exchange

amplifying cells that after malignant transformation give rise to osteosarcoma) have different mechanisms to respond to the genotoxic effect of ionizing radiation. Analyzing the impact of gamma radiation onto the degree of telomere sister chromatid exchange we found, that although non-irradiated MSCs had a much lower level of this cytogenetic abnormality than osteoblasts, the frequency went up sharply after 2 Gy gamma-irradiation (Kilinc et al., unpublished) (Fig. 5).

Telomere stability in osteoblasts has been found to be impaired by loss of Rb1 expression (Gonzalez-Vasconcellos et al. 2013), and this is assumed to contribute to the high risk of Rb1 mutation carriers for the development of radiation-induced osteosarcoma (Rosemann et al. 2014). In contrast to this, MSCs derived from the same Rb1 ±mice as those that yielded the osteoblasts did not show an impaired telomere stability. The telomere length during extended proliferation was always higher in MSCs than in osteoblasts, and it was independent on the Rb1 gene status (Fig. 6).

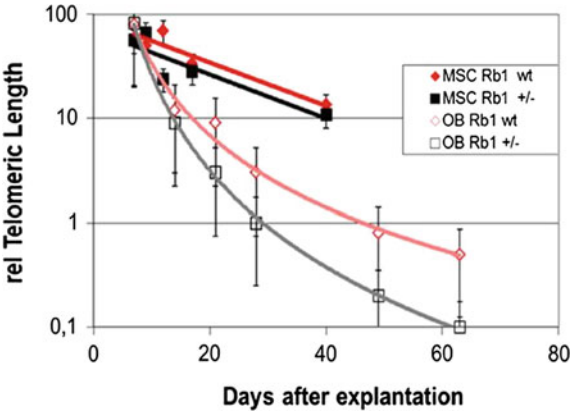

Fig. 6 Telomere length in murine MSCs and osteoblasts of different Rb1 gene status. DNA extracted from in vitro growing cells of Rb1 ±knockout mice was used measure average telomere length by qRT-PCR

This suggests that the radiation-risk for these two histological types of bone sarcoma can differ significantly, depending on whether the target cell that undergoes transformation is a stem-cell or a committed precursor cell.

A similar picture was seen in the case of myeloid leukaemia, where acute myeloid leukemia (AML) (characterized by mutations in genes such as Aml1, Flt3 or Pou1) exhibit a high excess relative risk in IR exposed cohorts, whereas chronic myeloid leukemia (CML) incidence (characterized by the recurrent Bcr-Abl translocation) remains almost at the background level as seen in unexposed cohorts (Gale et al. 2014). This suggests that cells which belong to the same stem cell lineage, but differ in their differentiation stage, can vary dramatically in terms of their potential to undergo a radiogenic transformation.

Conclusion

Adult stem cells represent only a small portion of somatic cells. This, together with earlier findings showing that ASCs are relative radio-resistant, led to the assumption that they might not be important for the expression of radiation-induced late effects. With the availability of sophisticated single-cell assays to identify and analyse stem cells, however, it now becomes more and more obvious that rare ASCs can be important target cells for different forms of health impairment following acute or chronic radiation exposure. Since it has been shown that ionizing radiation to ASCs can impair their genetic stability and their stem-cell capacity, their very distinct regulation of cellular and molecular processes should be considered as a potential factor that modulate the radiation risk for late health effects in various organs.

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