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DNA damage response and DNA repair during hematopoietic differentiation and development

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DNA damage response and DNA repair during hematopoietic differentiation and development

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This dissertation is dedicated to my mother, father, and sister.

Without their unconditional love, support, and encouragement none of this work would have been possible.

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Abstract

DNA damage response and DNA repair during

hematopoietic differentiation and development

Mary Mohrin

DNA damage is deleterious to all cells, but especially to stem cells because they maintain tissue integrity by constantly self-renewing (to replicate themselves) and differentiating (to produce all mature cells of the tissue). Hence, damage occurring in stem cells can be transmitted both horizontally (to other stem cells) and vertically (to differentiating cells). To study the DNA damage response (DDR) and DNA repair mechanisms employed by stem cells, we used hematopoietic stem cells (HSCs) isolated from young adult mice. We found that quiescent HSCs have cell-intrinsic mechanisms ensuring their survival in response to ionizing radiation (IR), including enhanced pro-survival gene expression and activation of p53-mediated DDR. We show how nonhomologous end joining (NHEJ) DNA repair in quiescent HSCs is associated with acquisition of genomic rearrangements, which can persist *in vivo* and contribute to hematopoietic abnormalities. Hence, we demonstrate that quiescence, long believed to be strictly protective, actually has a dark side and renders HSCs intrinsically vulnerable to mutagenesis. It has been suggested that the decline of tissue homeostasis seen with age may be caused by impaired stem cell activity. To understand how aging affects DNA repair capability of stem cells we studied

the DDR and repair of HSCs isolated from old mice. We confirmed the previously documented numerical expansion, lineage skewing, and high basal levels of genomic stress in old HSCs. We found that old HSCs have a G1 arrest and exhibit a senescence phenotype compared to young HSCs. We show that the intrinsic genomic stress in old HSCs happens at the nucleolus and telomeres, two known fragile sites of the genome, and is potentially caused by replication stress. We demonstrate that old HSCs use NHEJ to repair IR induced DNA damage as efficiently as young HSCs. Taken together, these data indicate that old HSCs have decreased protective mechanisms and increased levels of intrinsic stress that may account for their decreased functionality. Moreover, they suggest that the life-long use of error-prone NHEJ repair in HSCs may be the driving force for the increased cancer incidence occurring with age in the hematopoietic system.

Contributions to presented work

Chapter 2 of this dissertation contains previously published material:

Mohrin M., Bourke E., Alexander D., Warr M.R., Barry-Holson K., Le Beau M.M., Morrison C.G., and Passegué E. (2010). Hematopoietic stem cell quiescence promotes error-prone DNA repair and mutagenesis. Cell Stem Cell *7*, 174-85.

This work is reprinted with permission from Elsevier. I performed the studies described in chapter 2 under the guidance of Emmanuelle Passegué (PhD). Emer Bourke (PhD) and Ciaran Morrison (PhD) aided in the design, optimization, and scoring of immunofluorescence experiments. David Alexander (PhD) assisted in the design, optimization, and scoring of COMET assays. Matthew R. Warr (PhD) performed western blots, intracellular staining for p53, 7AAD/Pyronin Y staining, and cleaved caspase 3 staining on cells isolated from H2k-*bcl2* mice. Michele M. LeBeau (MD) and Elizabeth Davis performed SKY analysis. Bryan King, Keegan Barry-Holson, and Mikhail Binneweis all provided technical assistance in these studies.

Chapter 4 of this dissertation contains unpublished material that is currently a work in progress:

Mohrin M.*, Reynaud D.*, Conroy P.C., Flach J., Royce-Tolland M.E., Schepers K., Stohr B.A., Morrison C., and Passegué E. Aging in hematopoietic stem cells is associated with increased replicative stress and senescence. (In preparation.)

I performed the studies in chapter 5 under the supervision of Emmanuelle Passegué (PhD). Damien Reynaud (PhD), Johanna Flach, and Koen Schepers (PhD) aided in cell isolation. Damien Reynaud (PhD) performed population analyses, transplantation studies, 7AAD/Pyronin Y analysis, and assisted with BrdU analysis and ATP measurements. Pauline Conroy and Ciaran Morrison (PhD) aided in the design, optimization, and scoring of nucleolar immunofluorescence and telomere FISH. Morgan Royce-Tolland (PhD) and Bradley Stohr (PhD) performed telomere length and signal analyses. Mikhail Binneweis provided technical assistance for these studies. (*These authors contributed equally to this work.)

Chapter 5 of this dissertation contains previously published material:

Blanpain C., Mohrin M., Sotiropoulou P.A., and Passegué E. (2010). DNA-damage response in tissue-specific and cancer stem cells. Cell Stem Cell *8*,16-29.

This work is reprinted with permission from Elsevier. I helped write sections of this review under the guidance of Emmanuelle Passegué (PhD).

Statement from the research advisor

I directed and supervised the work presented in this dissertation. These publications and manuscript form a body of knowledge that makes original contributions to scientific knowledge. As such, this dissertation meets the standard for a doctoral dissertation in the Biomedical Sciences Program at the University of California, San Francisco.

Emmanuelle Passegué, PhD

Table of contents

List of tables

Chapter 2

List of figures

Chapter 1

Chapter 2

Chapter 4

Chapter 5

CHAPTER 1

GENERAL INTRODUCTION

Part I: Hematopoiesis and hematopoietic stem cells

1. Stem cells

Historically, the term "stem cell" originated in the late 19th century in the context of early embryological studies on the origin of the germ-plasm and hematopoietic system (Ramalho-Santos and Willenbring, 2007). Today we define a stem cell as a self-renewing cell with the greatest developmental potential available in a certain tissue at a certain time (Morrison *et al.*, 1997). More specifically, stem cells are described as having the unique capacity to self-renew, establishing and replenishing the stem cell pool, and also to differentiate, generating mature effector cells that carry out tissue functions. Stem cells can be categorized by their developmental potential, and two such categories of developmental potency include pluripotency and multipotency. Pluripotent stem cells have the capability to differentiate into all three germ layers (*i.e.* endoderm, ectoderm, and mesoderm), while multipotent stem cells are lineage restricted (Wagers and Weissman, 2004; Seita and Weissman, 2010). Stem cells are essential for specification and morphogenesis of tissues during embryonic development (organogenesis), and are maintained throughout life in adult tissues to ensure normal cell turn-over (homeostasis) and regeneration after injury (Laird *et al.*, 2008; Blanpain *et al.*, 2011). Adult stem cell populations are found in highly regenerative tissues such as the intestinal tract, blood, and skin but are also present in non-renewing tissues such as muscle and brain.

2. Hematopoietic stem cells and the blood system

Hematopoietic stem cells (HSC) are multipotent adult stem cells that are the common ancestor of all blood cell lineages. HSCs were first discovered in the 1960s through the work of Till, McCulloch, Wu, Becker, and Siminovich, which showed the existence of a population of bone marrow (BM) derived cells that were capable of generating myeloerythroid colonies in the spleens of lethally irradiated recipient mice, and thus were clonogenic. In some cases, cells derived from these spleen-derived colonies were capable of being transferred to secondary recipients where they re-colonize the spleens (Weissman, 2000a). These discoveries established the two essential traits of HSCs: self-renewal and the ability to differentiate into all lineages of blood cells. During hematopoiesis – the process through which all the cells of the blood system are generated – HSCs self renew to generate identical daughter HSCs and/or differentiate to produce progenitor cells that become progressively more restricted in their developmental potential, and ultimately produce all the mature blood cells.

The hematopoietic system must maintain homeostasis throughout the lifetime of an organism, as well as provide for blood regeneration after stresses such as blood loss, infection, or exposure to cytotoxic agents. Most effector cells of the blood system have very short life spans and must be replenished constantly. To satisfy these needs, mature blood cells are constantly being produced and, for example in humans approximately 1.5 X 10^6 blood cells are

generated every second (Bryder *et al*., 2006). A complex network of cell-intrinsic regulation and environmental cues ensure that an appropriate number of mature blood cells are produced from HSCs (Weissman, 2000a; He *et al*., 2009; Blanpain *et al*., 2011). These mechanisms control essential cell fate decision processes and allow for maintenance and expansion of the HSC population (self renewal), production of mature effector cells (differentiation), movement of HSCs to regions when they are needed locally (migration), and involution of an expanded HSC population or clearance of damaged HSCs (apoptosis) (Weissman, 2000a).

At homeostatic conditions, HSCs predominantly reside in the BM. However, their localization is dynamic as HSCs are also capable of migration and can be found circulating in the blood and lymph as well as within tissues where they can respond locally to hematopoietic stress (immunosurveillance) (Wright *et al*., 2001; Massberg *et al*., 2007). In response to stress or differentiation cues, HSCs can produce progenitor cells which lose the ability to self renew, cannot dedifferentiate back into HSCs, and enter the cell cycle more often as they mature (Morrison *et al*., 1997; Passegué *et al*., 2005). The immediate downstream progeny of HSCs are multipotent progenitors (MPPs), which are nearly functionally identical to HSCs with full multilineage differentiation potential but can no longer self-renew (Morrison *et al*., 1997). Upon further differentiation oligopotent progenitor cells, such as common lymphoid progenitors (CLP) and common myeloid progenitors (CMP) arise from MPPs. CLPs give rise to the

lymphoid arm of the hematopoietic system, which consists of B, T, and natural killer (NK) cells that are components of the adaptive immune system. CMPs produce the myeloid arm of the hematopoietic system, which consists of granulocyte/macrophage progenitors (GMP) and megakaryocyte/erythroid progenitors (MEP) that generate the mature effector cells that are responsible for innate immunity, oxygen transport, and blood clotting (Kondo *et al*., 1997; Akashi *et al*., 1999). Currently, all these hematopoietic populations can be identified immunophenotypically using cell surface markers and monoclonal antibodies or dye efflux, and isolated using fluorescence-activated cell sorting (FACS) (Spangrude *et al*., 1988; Purton and Scadden, 2007) (Figure 1). Functionally, HSCs are defined by their ability to provide long-term reconstitution of both the myeloid and lymphoid arms of the blood system to a myeloablated recipient, and to do that over serial transplantations. HSCs are currently the only stem cell population routinely used in the clinic for treatment of human diseases such as leukemias and immunodeficiencies (Orkin and Zon, 2008; Kondo *et al*., 2003).

3. Ontogeny of the hematopoietic system

The process of hematopoiesis is highly conserved throughout vertebrate evolution and begins during fetal development. Fetal hematopoiesis must produce two types of blood cells: hematopoietic cells necessary to respond to the immediate needs of the developing organism (*e.g.* tissue oxygenation), as well as HSCs that will be specified and prepared for post-natal hematopoiesis (Gekas *et*

al., 2010). The shifting anatomic sites of hematopoiesis during embryogenesis are common in many different species and are due to the ever changing state and size of the embryo. This movement of the HSCs may allow for interactions with different niches that can provide a variety of signals to aid in their development, functional maturation, and expansion (Mikkola and Orkin, 2006; Gekas *et al*., 2010). While the specific location of the sites initially producing true de novo HSCs is still being debated, it has long been established that HSCs are derived from the ventral mesoderm. The mesoderm is one of the three primary germ layers, which are generated by the process of gastrulation that occurs early in embryogenesis (Murry and Keller, 2008). It can give rise to the tissues that will eventually become skeletal muscle, heart, vasculature, and the blood. The fraction of the mesoderm that will become the blood system becomes specified through currently unknown steps that are most likely mediated by growth factors (Orkin, 2000).

In the mouse, the initial "primitive" wave of hematopoiesis occurs around 7.5 days postcoitus (7.5 dpc) extra-embryonically in the yolk sac blood islands (Orkin and Zon, 2008). Blood islands are composed of cellular aggregates that form adjacent to the visceral endoderm in the mesodermal layer of the yolk sac, which consists of endothelial cells and developing primitive erythrocytes. They produce the first hematopoietic cell that can be detected in the embryo and are responsible for oxygenation of the developing tissues (Cumano and Godin, 2007). Although it is still unclear if the yolk sac is a direct site of de novo HSC

formation, it known to give rise to HSC precursors (Orkin and Zon, 2008; Martinez-Agosto *et al*., 2010).

The second or "definitive" wave of hematopoiesis occurs within the embryo proper at different anatomic locations and is associated with de novo emergence of true HSCs. There are four main sites of definitive hematopoiesis within the embryo known so far: the dorsal aorta hemangiogenic endothelium, the aorta-gonad-mesonephros (AGM), the placenta, and the fetal liver (Mikkola and Orkin, 2006). Studies in zebrafish and mouse embryos showed that the first de novo HSCs emerge from the dorsal aorta hemangiogenic endothelium at around 9dpc (Bertrand *et al*., 2010; Kissa and Herbomel, 2010; Boisset *et al*., 2010). It has also been shown that HSCs develop de novo in both the AGM and placenta by 11dpc. These HSCs eventually migrate via the vitelline and umbilical veins to the fetal liver, which is also seeded by cells from the yolk sac (Mikkola and Orkin, 2006). During fetal development HSCs are not produced de novo in the fetal liver, however it is the primary organ of HSC expansion and differentiation. At the peak of their expansion (~15dpc), about ~1000 cycling HSCs can be found in the fetal liver (Morrison *et al*., 1995; Mikkola and Orkin, 2006). After expansion and specification fetal HSCs and progenitor cells begin to seed sites of future adult hematopoiesis: the developing spleen, thymus, and BM. The mechanisms that influence HSC migration during fetal hematopoiesis are not fully understood, but are believed to include homing receptors, cell adhesion molecules, growth factors, and cytokines such as Steel factor (SLF) and stromal cell-derived factor

1-α (SDF 1-α) (Laird *et al.*, 2008; Christensen *et al.*, 2004; Kikuchi and Kondo, 2006).

Near the middle of mouse embryogenesis (~11dpc) the fetal thymus and fetal spleen are seeded with progenitor cells that will eventually develop into T and B cells respectively (Cumano and Godin, 2007). It is believed that once the developing bones and BM have reached a certain state of specification within the embryo and have generated the appropriate niche space for HSCs (~16dpc) the BM will begin to be seeded by HSCs, a process which will continue into the first few days of post-natal life (Mikkola and Orkin, 2006; Christensen *et al.*, 2004; Gekas *et al.*, 2005). None of these sites of definitive adult hematopoiesis are believed to keep the ability to form de novo HSCs; instead they either allow for differentiation of HSCs or promote self-renewing divisions to allow for population maintenance and expansion (Orkin and Zon, 2008). Once fetal HSCs localize and transition to the primary site of adult hematopoiesis - the BM - they begin to change their phenotypic and functional properties and continue the transition through the first three to four weeks of post-natal life (Martinez-Agosto *et al.*, 2010). In parallel fetal hematopoiesis becomes extinguished during the first two weeks of post natal life.

4. Bone marrow niche microenvironment

The adult HSC BM niche is defined as a specialized microenvironment created by supportive cells that in addition to providing physical protection also

secrete factors and express membrane-bound factors that maintain HSCs and guide their fate. Hematopoietic cells are found in bone cavities, which provide a protected space where HSCs can interact with components of the extra-cellular matrix, fat cells, stromal cells, blood vessels, and other hematopoietic cells (Suda *et al.*, 2005). Currently, the mammalian HSC BM niche is divided into two different parts, the endosteal niche (interface of bone and BM cells) and the vascular niche (interface of blood vessels and BM cells). It is still unclear if these two niches are truly separate, if they closely interact, if both potentially secrete factors that contribute to a common niche, or if they have effects on some intermediate population of cells, which then affect HSCs. In addition to providing physical protection and a space to interact with supportive stromal cells, the BM niche also contains many areas of hypoxia that can help protect HSCs by promoting low metabolic rates, slowing cell cycle entry, and by decreasing the amount of oxygen present that could contribute to the formation of reactive oxygen species (ROS) (Eliasson *et al.*, 2010; Parmar *et al.*, 2009). While the BM is highly vascularized, it also has very low oxygen tension levels, ranging from 1-6% (Eliasson *et al.*, 2010). These hypoxic regions of the BM have been shown to contain quiescent populations of HSCs, while proliferating cells were found closer to the vasculature and its associated higher oxygen tensions (Kubota *et al.*, 2008; Mohyeldin *et al.*, 2010). While the BM is the major site of hematopoiesis during homeostasis, extramedullary hematopoiesis can also occur in the spleen and/or liver during stress conditions (Kiel and Morrison, 2008). Taken together, the

structural, humoral, and cellular components of the niche combined with the metabolic milieu of the BM niche provide a specialized space where HSCs are preserved and their ability to self-renew is maintained (Lymperi *et al.*, 2010).

5. Adult hematopoiesis and HSCs

The transition from fetal to adult hematopoiesis is associated with striking changes in HSC biological properties including cell cycle status, gene expression, cell surface marker expression, developmental potential, self-renewal potential, and niche localization (Kim *et al.*, 2007; Kim *et al.*, 2006). As fetal HSCs mature, they slow their rapid cell cycling, and enter a quiescent or dormant state, which is completed during the fourth week of post-natal life (Bowie *et al.*, 2006). Quiescence is one of four types of non-dividing cell states that also includes terminal differentiation, senescence, and apoptosis. Quiescence differs in that it is reversible and is functionally unlike cell cycle arrest (Coller *et al.*, 2006). Factors that regulate entry to quiescence include lack of mitogens, lack of space (high density growth), and lack of adhesion; many of which are controlled by the niche microenvironments (Trumpp *et al.*, 2010; Li and Clevers, 2010). Maintenance of quiescence is distinct from cell cycle arrest and is regulated by suppression of differentiation and apoptosis, preservation of unlicensed replication origins, and blocking progression to G1 by cyclin dependent kinase inhibitors (CDKIs) (p21, p27, and p57) (Coller *et al.*, 2006; Sang *et al.*, 2008; Blow and Hodgson, 2002; Cheng and Scadden, 2002). Exit from quiescence is

initiated by cell cycle reentry via mitogen activation of the E2F program of gene expression and G1-Cyclin and cycling dependent kinase (CDK) activity. In genetic mouse models with increased HSC proliferation and/or loss of quiescence the result is often long term loss of HSCs and increased susceptibility to exhaustion (Orford and Scadden, 2008; Wilson *et al.*, 2009; Trumpp *et al.*, 2010; He *et al.*, 2009). The striking effect of loss of quiescence on HSCs highlights the importance of dormancy for protection and maintenance of these essential cells, and proper functioning of the blood system.

While the HSC population as a whole is quiescent, the extent to which individual HSCs exit quiescence and enter the cell cycle is still being resolved. Work utilizing bromodeoxyuridine (BrdU) incorporation showed that while mostly quiescent, all HSCs enter the cell cycle at a similar rate, with each HSC entering the cell cycle once every 1-3 months and ~6% of HSCs stochastically entering the cell cycle daily (Cheshier *et al.*, 1999; Kiel *et al.*, 2007). However, additional work using mice that express the histone 2B (H2B)-GFP reporter suggests that the cell cycle status of the HSC pool may be more complex than previously thought, with stratification of the entire population into dormant (~15%) and active (~85%) subsets. Although still controversial, it is believed that both active and dormant HSC populations are capable of self-renewal and differentiation, but are respectively responsible for the constant replenishment of blood cells (active subset) and maintenance of a reserve of an unaltered pristine HSC pool (dormant subset) (Wilson *et al.*, 2008; Foudi *et al.*, 2009). Active stem cells are

viewed as the front line of homeostasis maintenance, while dormant stem cells might be kept in reserve to fill in for exhausted active stem cells or to respond to acute injury (Li and Clevers, 2010; Cheshier *et al*., 2007).

The quiescent status of HSCs is in stark contrast to the terminally differentiated and post mitotic state of the mature cells that make up the bulk of the blood. This limitation of proliferative pressure on HSCs is believed to be beneficial because it decreases the potential hazards associated with DNA replication and passage through the cell cycle (Bryder *et al*., 2005). The localization of adult HSCs to the low oxygen - or hypoxic - region of the BM is believed to be at least in part responsible for the maintenance of HSCs in a quiescent state; this is achieved through the upregulation of hypoxia inducible factor 1-α (HIF-1α) and its target genes, which preserves HSC self-renewal capacity and prevents exhaustion (Parmar *et al*., 2007; Takubo *et al*., 2010; Simsek *et al*., 2010). Hypoxia and the quiescence of HSCs are also believed to limit the metabolic activity thereby decreasing the levels of ROS produced in the cells (Tothova, *et al*., 2007; Jang and Sharkis, 2007; Takubo *et al*., 2010; Simsek *et al*., 2010). ROS are unstable partially-reduced metabolites of oxygen, including superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-) that are most commonly generated as byproducts of normal aerobic metabolism by the mitochondria during oxidative phosphorylation and can cause oxidative damage to cellular macromolecules, such as DNA, as well as organelles (Naka *et al*., 2008). Oxidative stress and resulting DNA damage can

occur if cellular ROS are not balanced out by cellular antioxidant defenses (Naka *et al*., 2008; Simsek *et al*., 2010; Finkel and Holbrook, 2000). Maintenance of low levels of intracellular ROS is critical for proper signal transduction and to limit oxidative stress (Finkel and Holbrook, 2000). This is especially true for rare HSCs that need to be poised to respond to danger signals (*e.g.* infection, blood loss) that can be transmitted via ROS mediated mechanisms (Cheshier *et al*., 2007, Owusu-Ansah and Banerjee, 2009). HSCs are believed to be intrinsically protected from the detrimental effects of ROS, through the activity of Forkhead O (FOXO) genes and possibly through the enhanced expression of antioxidants such as glutathione (Rossi *et al*., 2005; Tothova *et al*., 2007, Diehn *et al.,* 2009). Taken together, these protective attributes of HSC quiescence are suspected to limit the accumulation of mutations and thereby to limit the potential for cancer development or tissue failure.

Part II: Genotoxic stress and DNA damage repair mechanisms

1. HSCs and cellular stress

HSCs must maintain their own fitness to preserve blood homeostasis. As mentioned in the previous section, many cell extrinsic and cell intrinsic mechanisms protect HSC integrity including: localization to a hypoxic BM niche microenvironment, limitation of ROS production, expression of ABC-transporter proteins that can efflux toxins, and maintenance in a quiescent cell cycle state (Kiel and Morrison, 2008; Tothova *et al.*, 2007; Cheshier *et al.*, 1999; Cipolleschi *et al.*, 1993; Parmar *et al.*, 2007; Zhou *et al.*, 2001). Despite these protective measures, HSCs are not immune to all insults and must recognize cellular damage and repair themselves like any other cell. Because of their function in blood maintenance HSCs are at the single cell level the most sensitive to cellular damage and at the tissue level the most important for response to cellular stress (Kenyon and Gerson, 2007). The cellular stress response is an evolutionarily conserved defense mechanism that senses damage to cellular macromolecules including lipids, proteins, and DNA, senses and regulates the redox state of the cell, and controls energy metabolism (Kültz, 2005). Stress responses ensure the survival and repair of healthy cells and removal of excessively damaged cells. Numbers of HSCs must be balanced such that there are enough cells to maintain homeostasis of hematopoietic system as well as to respond to injury. Genomic

quality of HSCs must also be maintained so that cell functions are maintained and transformation is limited.

2. Genotoxic stress (DNA damage) and DNA damage response (DDR)

It has been estimated that cells can encounter 10^5 spontaneous DNA lesions a day under normal homeostatic conditions (Hoeijmakers, 2009). These lesions can be caused by both endogenous and exogenous sources and arise in a variety of ways. Sources of intracellular damage include side products of cellular metabolism such as ROS, and examples of extracellular damage include ionizing radiation (IR), ultraviolet (UV) light from the sun, and chemical exposure (recreational, accidental, or in the context of therapy) which can cause single strand breaks (SSBs), double strand breaks (DSBs), and interstrand cross links (ICLs) (Ciccia and Elledge, 2010; Hoeijmakers, 2009). Cellular organisms have evolved multiple sensing and repair mechanisms to identify, repair, and cope with these insults to their genetic material (Lombard *et al.*, 2005). One of the most dangerous DNA lesions to a cell is DSBs because, when repaired incorrectly, they can lead to chromosomal fusions, translocations, or loss. It has been estimated that on average each cell will have 10 DSBs per day. These breaks can be intentional and part of normal cellular processes such as V(D)J recombination and class switch breaks occurring in developing lymphocytes, or can be accidental and pathological. Pathological DSBs can be caused by ROS from IR exposure or produced by cellular metabolism, mechanical stress,

erroneous nuclear enzyme activity, and replication fork collapse (Lieber, 2010; Ciccia and Elledge, 2010). The DDR is a highly conserved complex signal transduction pathway that senses damage to DNA and, through a series of tightly regulated steps, elicits cellular responses that can result in the repair and survival of a cell with low levels of damage, or the growth arrest of and/or clearance of an extremely damaged cell.

The DNA damage response (DDR) and repair components that sense and repair DNA lesions like DSBs can generally be separated into 4 functional groups: damage sensors, signal transducers, repair effectors, and arrest or death effectors (Sancar *et al*., 2004) (Figure 2). The first step of the DDR is recognition of damage; this is carried out through constant monitoring of the DNA structure by sensor proteins. In the context of DSB sensors include the Mre11-Rad50-Nbs1 (MRN) complex, which unwinds the damaged region of DNA, serves as part of the repair scaffolding, and is involved in downstream signaling, and Ku70 and Ku80, components of DSB repair that recognize the ends of DSBs, in addition to others (Ciccia and Elledge, 2010). Once DSBs have been recognized, signal transducers like ATM (ataxia-telengiectasia mutated), ATR (ATM and Rad3 related), and DNA-Pkcs (DNA-protein kinase catalytic subunit) are activated and initiate a phosphorylation signaling cascade that signals the presence of a DSB and can recruit as well as activate effector proteins that will dictate the functional response of the cell to the insult (Hakem, 2008). One of the best-characterized targets of ATM is the cell stress integrator and tumor

suppressor p53. Upon DNA damage ATM, and/or its target Chk2, will phosphorylate p53 leading to its stabilization and accumulation in the nucleus where it can initiate transcription of some of its many target genes (d'Adda di Fagagna, 2008). p53 has a multitude of cellular functions, but in the context of the DDR it can induce cell cycle arrest, apoptosis, or senescence by transcriptional upregulation and activation of its targets the CDKI *p21*, or proapoptotic genes *Bax*, *Puma*, and *Noxa* (Ciccia and Elledge, 2010). Depending upon the level of damage, the kinetics of repair, the stage of the cell cycle, and the strength and duration of p53 activation a damaged cell can have different outcomes. In the case of minor damage, the DDR results in a transient cell cycle arrest allowing for DNA repair and cellular recovery. However, in the case of extreme damage the DDR induces apoptosis or senescence hence allowing for the removal of a potentially dangerous cell (Blanpain *et al*., 2011).

Many mouse models lacking functional components of the DDR result in blood phenotypes. For example, mice with the hypomorphic mutation of one component of the MRN complex, $Rad50^{k22m}$, while mostly viable (40% die in utero), are underweight and only survive 2.5 months (Bender *et al*., 2002). These mice die of B cell lymphoma or anemia and have decreased numbers of HSCs, which highlights the function of Rad50, or the MRN complex in general, in HSC survival. In the absence of p53, $Rad50^{k22m}$ HSCs are partially rescued; which indicates that the loss of HSCs was due to a p53-mediated apoptosis response (Bender *et al*., 2002). Mice deficient for the DNA DSB damage sensors ATM and

ATR have decreased somatic growth, neurological abnormalities, decreased T cell numbers, and infertility due to loss of germ cell development (Barlow *et al*., 1996; Ito *et al*., 2004; Ito *et al*., 2006; Ruzankina *et al*., 2007). ATM deficient mice, like AT patients, are also extremely sensitive to IR. Many of these phenotypes can be accounted for by defects in stem cell functions including HSC activity (Barlow *et al*., 1996; Ito *et al*., 2004; Ito *et al*., 2006). Loss of ATM leads to an increase in intracellular ROS and an overall decrease in HSC number and function over time (Ito *et al*., 2004; Ito *et al*., 2006). Deletion of the DNA damage sensor ATR is also embryonic lethal, but conditional ablation of ATR in adult mice leads to a reduction in number and functions of a variety of stem cell populations, with phenotypes including decreased HSC and progenitor numbers, hair graying, alopecia, kyphosis, osteoperosis, and decreased spermatogenesis (Brown and Baltimore, 2000; Ruzankina *et al*., 2007). These models demonstrate that DSB sensors and signal transducers are critical for the function of HSCs, and their protection against DNA damage.

3. DNA double strand break repair mechanisms

DSBs can be repaired by homologous recombination (HR) or nonhomologous end joining (NHEJ) mechanisms. The repair mechanism used is cell cycle dependent, NHEJ can occur throughout the cell cycle, while HR can only occur during S/G2 (Sancar *et al*., 2004). NHEJ repairs DSBs by directly religating of broken ends of DNA, which can be perfect, or can result in deletions

if the nucleotides around the break are lost. HR repairs DSB using a template from the homologous chromosome (or the sister chromatid) and is usually high fidelity (Ferguson and Alt, 2001). The proper execution of DSB repair processes is critical for the development and survival of all eukaryotes. Studies characterizing mouse models with loss of or defective DSB repair components have shown the importance of these repair mechanisms for HSC function (Ciccia and Elledge, 2010; Niedernhofer, 2008).

HR restores the genetic sequence of broken DNA by using sister chromatids for repair (Ciccia and Elledge, 2010). After DSB formation, DNA around the 5' end of the break is cut away in a process called resection, stand invasion follows where the 3' end of the broken DNA enters the intact chromatid, Holliday junctions (HJ) are formed connecting the two pieces of DNA, and finally after template based repair, resolution of HJs results in crossover or non-cross over events. The key components of HR include Brca1, which is involved in resection, Rpa that binds to and stabilizes single stranded DNA and facilitates strand invasion along with Rad51 and Brca2 (Ciccia and Elledge, 2010) (Figure 2). Ablation of *Rad51*, *Brca1*, and *Brca2* in mice all lead to embryonic lethality which suggests that HR might be more important to repair DNA damage in embryonic cells than in adult tissues (Ferguson and Alt, 2001; Valerie and Povirk, 2003; Park and Gerson, 2005; San Filippo *et al*., 2008). Adult mice carrying hypomorphic alleles or conditional deletion of *Brca1* and *Brca2* are more radiosensitive, smaller in size, have improper tissue differentiation, loss of germ

cells, and often develop lymphoma (Connor *et al*., 1997; Patel *et al*., 1998; Valerie and Povirk, 2003). *Brca2* is also known as *Fancd1*, a component of the Fanconi Anemia (FA) associated complementation gene family which work together regulating cell cycle check points and repairing ICLs (Park and Gerson, 2005; Howlett *et al*., 2002; Witt and Ashworth, 2002). FA is a rare autosomal recessive cancer susceptibility syndrome characterized by congenital abnormalities, progressive BM failure, and cellular hypersensitivity to DNA cross-linking agents; FA patients often develop acute myeloid leukemia (AML), but can also develop other types of cancer (Howlett and Ashworth, 2002). The BM failure associated with *Brca1* or *Brca2* mutations in adult mice suggest a link between HR mediated repair, FA, and HSC survival.

The core components of NHEJ include the end-binding and end processing proteins Ku70, Ku80, DNA-PKcs, and Artemis; and the ligation complex Xrcc4, LigIV, and Cerrunos (Hakem, 2008) (Figure 2). As NHEJ is critical for V(D)J recombination many of the mouse models deficient for components of NHEJ have arrested development of lymphoid cells (Rossi *et al*., 2008). The only NHEJ mouse model that has a homeostatic HSC phenotype is a model with a hypomorphic mutation of *LigIV* (Nijnik *et al*., 2007; Kenyon and Gerson, 2007). Mice deficient for *LigIV* are embryonic lethal, however mice with the hypomorphic mutation $Lig4^{y288c}$ are viable but display growth retardation, immunodeficiency, and pancytopenia. Over time these mice have reduced numbers of hematopoietic stem and progenitor cells and overall reduced BM

cellularities, associated with HSC engraftment defects following transplantation (Nijnik *et al*., 2007; Kenyon and Gerson, 2007). HSCs deficient for *Ku80* also have poor multilineage reconstitution ability and decreased self-renewal in transplantation assays. In vitro assays show a markedly decreased proliferation and increased apoptosis in HSCs deficient for *Ku80* and their progeny (Rossi *et al*., 2007). Taken together, these data show that Ku80, LigV, and by extension, the NHEJ mediated repair mechanism, are essential for HSC's ability to reconstitute, self-renew, proliferate, and survive.

Part III: Implications for human health

1. Ionizing radiation

To study the molecular mechanisms of the DDR, ionizing radiation (IR) is often used as a model inducer of DSBs. IR was discovered in the 1900s and since then has been harnessed for energy generation, nuclear weapons, therapeutics (diagnostics and treatments), and scientific applications (Gudkov and Komarova, 2003). IR occurs naturally on Earth, and also comes from the sun, cosmic rays, and radioactive elements (Dainiak, 2002). X-rays and gamma-rays, which are both forms of electromagnetic radiation, as well as electrons, protons, and neutrons, which are forms of particulate radiation, are the common types of IR (Dainiak, 2002). All types of radiation injure cells the same way, but they differ in the amount of damage they can cause. IR produces ionization of atoms generating chemically reactive free radicals. IR can directly ionize DNA or indirectly damage DNA by the generation of ROS from surrounding molecules that cause SSBs and DSBs. In the aftermath of the atomic bombings of Hiroshima and Nagasaki in 1945 the effects of IR exposure on human beings became apparent. It was seen that victims of the bombing that were exposed to lower doses of IR died over a prolonged period due to failure of their hematopoietic systems (Weissman, 2000b). Following up on these observations, it was demonstrated that treating mice with X-rays could cause similar radiation syndromes (Jacobson *et al*., 1949). The study of the biological consequences of

IR has allowed for a thorough understanding and characterization of the biochemical and cellular effects of DNA damage.

2. Tissue sensitivity

Human exposure to IR from disasters such as atomic weapons, nuclear reactor accidents, and excessive exposure from medical diagnostics or therapeutics have shown that certain tissues are more radiosensitive than others, with the hematopoietic system being one of the most sensitive. Radiation accidents can result from localized or total body exposure and the clinical outcome is dictated by the type of exposure as well as the amount or dose of IR received (Mettler and Voelz, 2002). Acute radiation syndrome or sickness can result after a dose of ~1 Gray (1Gy) or more IR in a short period, having direct effects on the hematopoietic and gastrointestinal (GI) tract, the two most radiosensitive body tissues (Kirsh *et al.*, 2010). Hematopoietic syndrome is the response to IR exhibited by the hematopoietic system, and is characterized by initial nausea, vomiting, diarrhea, and anorexia, followed by a drop in circulating lymphocytes, white blood cells and platelets, and then pancytopenia at doses above 2Gy by 3 to 5 weeks. At higher doses the GI tract, and even higher doses the cardiovascular and central nervous systems are also affected by IR. When used as treatment, IR dosages are fractionated and spaced out over time to minimize the damage to healthy tissues, especially to the hematopoietic system and GI tract. This approach allows for killing of tumors while sparing healthy

tissue, and allows for greater doses to be given than could be tolerated at once (Gudkov and Komarova, 2003).

The differential sensitivity to IR seen in tissues has been suggested to be due to their proliferation status, with rapidly dividing cells being more radiosensitive than terminally differentiated non-proliferating cells. Supporting this notion, tissues with limited regenerative capacity, such as the brain, muscle, liver, lung, and kidney, are radioresistant, while highly regenerative tissues like skin, blood, and gut, or rapidly proliferating tissues like spermatagonia and hair follicles, are highly radiosensitive (Gudkov and Komarova, 2003). Additionally, rapidly proliferating tumor cells are mostly radiosensitive. However, cell cycle status alone cannot fully explain these findings as some non-dividing tissues, like the spleen and thymus, are radiosensitive and tissues at different stages of development vary in their radio sensitivities. It has also been shown that within tissues the radiosensitivity of cell types can vary. IR exposure differentially affects hematopoietic cells depending on their state of maturity, with HSCs being more radioresistant than their downstream progeny (Meijne *et al*., 1991; Down *et al*., 1995). Current works are addressing why different cell types within the same tissue respond differently to IR. One explanation is a difference in the strength or outcome of the p53 mediated DDR.

The transcription factor p53 is known to be an essential component of the DDR. Tumor cells lacking functional p53 often escape IR treatments, suggesting that p53 expression and activity could be responsible for differential responses

seen to IR treatment by cells of different developmental state or cell cycle status (MacCallum *et al*., 1996; Cuddihy and Bristow, 2004). Evaluation of radiosensitive tissues in WT and p53-deficient mice in response to IR showed that p53 was important for the induction of apoptosis (Gudkov and Komarova, 2003). Mice lacking p53 expression or mice treated with p53 inhibitors are known to have enhanced radioresistance, surviving doses of IR that would cause hematopoietic syndrome in WT mice (Westphal *et al*., 1997; Komarov *et al*., 1999). The effects of p53 in response to IR are also known to be dictated by the tissue specific expression levels of p53 target genes leading to apoptosis or arrest depending upon the context of gene expression (MacCallum *et al*., 1996; Komarova *et al*., 2000; Schmitt *et al*., 2002). It has also been shown that activation of the nuclear factor κB (NF-κB) pathway can limit the effects of IR on radiosensitive tissues, suggesting that limiting apoptosis in radiosensitive tissues is one way to decrease the negative side effects of IR treatments (Burdelya *et al*., 2008). Identifying the cell type specific effects of IR in radiosensitive tissues will allow for the targeting of tumor cells while precisely protecting sensitive tissues allowing for better treatment and over all survival.

3. Cancer treatment and consequences

Radiation therapy is a common treatment for cancer, and is usually administered with gamma or X-rays. In this context, IR is targeted at the DNA of

the cancer cells with the aim of eliminating them. However, IR intended for the cancer cells can also damage adjacent normal healthy tissue. This can cause harmful side effects and/or potentially have mutagenic and carcinogenic effects leading to the development of therapy-related cancers. Therapy-related cancers, or secondary cancers, account for one out of every six new cancer diagnosis in the United States (Allan and Travis, 2005). The most common therapy-related cancers are of hematopoietic origin, which can arise within a few years of the treatment for the primary cancer. Therapy-related myelodysplasia and acute myeloid leukemia (tMDS and AML) are the most common secondary neoplasms with about 10-20% of all acute leukemia patients acquiring their disease after a primary cancer treatment (Guillem and Tormo, 2008; Rund *et al*., 2005). Because cancer patients are being treated sooner and surviving longer, the incidence of secondary cancers is increasing (Shuryack *et al*., 2006). In the last decade there were approximately 10 million cancer survivors in the United States, accounting for 3.5% of the population; with the number of cancer survivors increasing each year by about 2%. This significant and growing population of patients is vulnerable to the development of secondary neoplasms and clearly suggests a need for better understanding of the effects of cancer treatment (Travis *et al*., 2006).

Part IV: Objectives of thesis

The effects of therapeutic or catastrophic exposure to IR and its resulting DNA damage has clear detrimental effects on the hematopoietic system and HSCs. This hematopoietic radiosensitivity coupled with the high percentage of secondary neoplasms originating in the blood system suggests that it would be beneficial to identify how HSCs respond to DNA damage. Despite extensive studies, much remains to be learned about the mechanisms by which healthy HSCs and other hematopoietic cells repair their damaged DNA and the associated consequences for their cellular functions.

In chapter 2 I will present the work I performed using purified subsets of young murine HSCs and myeloid progenitors to investigate how two different blood populations respond to IR either by properly repairing their damaged DNA, which would restore normal tissue function; by misrepairing damaged DNA and accumulating mutations, which could promote cancer development; or by undergoing apoptosis and being eliminated, which could promote BM failure.

In chapter 3 I will discuss our results in the context of other studies published at the same time, which address the DNA damage response in human fetal HSCs and quiescent murine skin stem cells.

In chapter 4 I will present the work I am currently performing that investigates the effects of aging on HSC DDR and DNA repair capabilities by verifying known attributes of aging in HSCs and the blood system, by identifying

the location and source of intrinsic DNA damage in old HSCs, and by determining the ability of old HSCs to repair damaged DNA.

In chapter 5 I will present the review I helped write with the Blanpain lab to describe the role of DNA repair mechanisms in different tissue specific and cancer stem cell populations.

In chapter 6 I will discuss the questions raised by the work contained in this disseration and the future directions the research will follow.

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Figure 1. Hematopoietic development in the mouse

Model of murine hematopoietic differentiation. The cell surface markers used to separate specific hematopoietic populations by flow cytometry are shown. LT-HSC, long-term repopulating hematopoietic stem cell (HSC); ST-HSC, short-term repopulating HSC; MPP, multipotent progenitor; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; GMP, granulocyte/macrophage progenitor; MEP, megakaryocyte/erythrocyte progenitor; Gr, granulocyte (basophil, eosinophil, neutrophil).

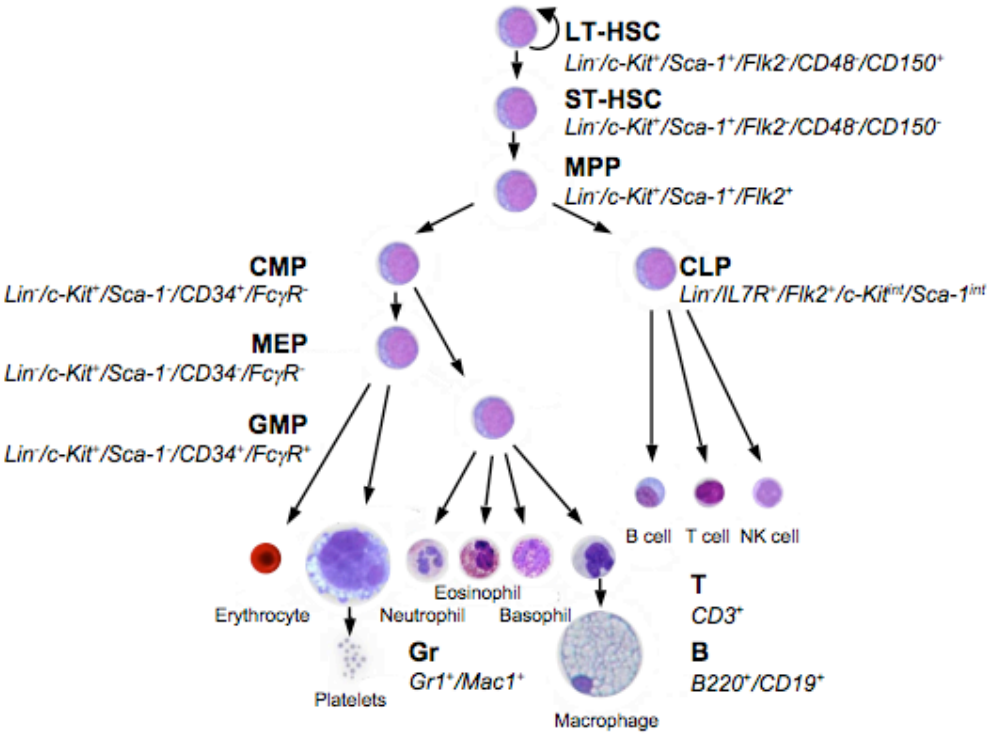
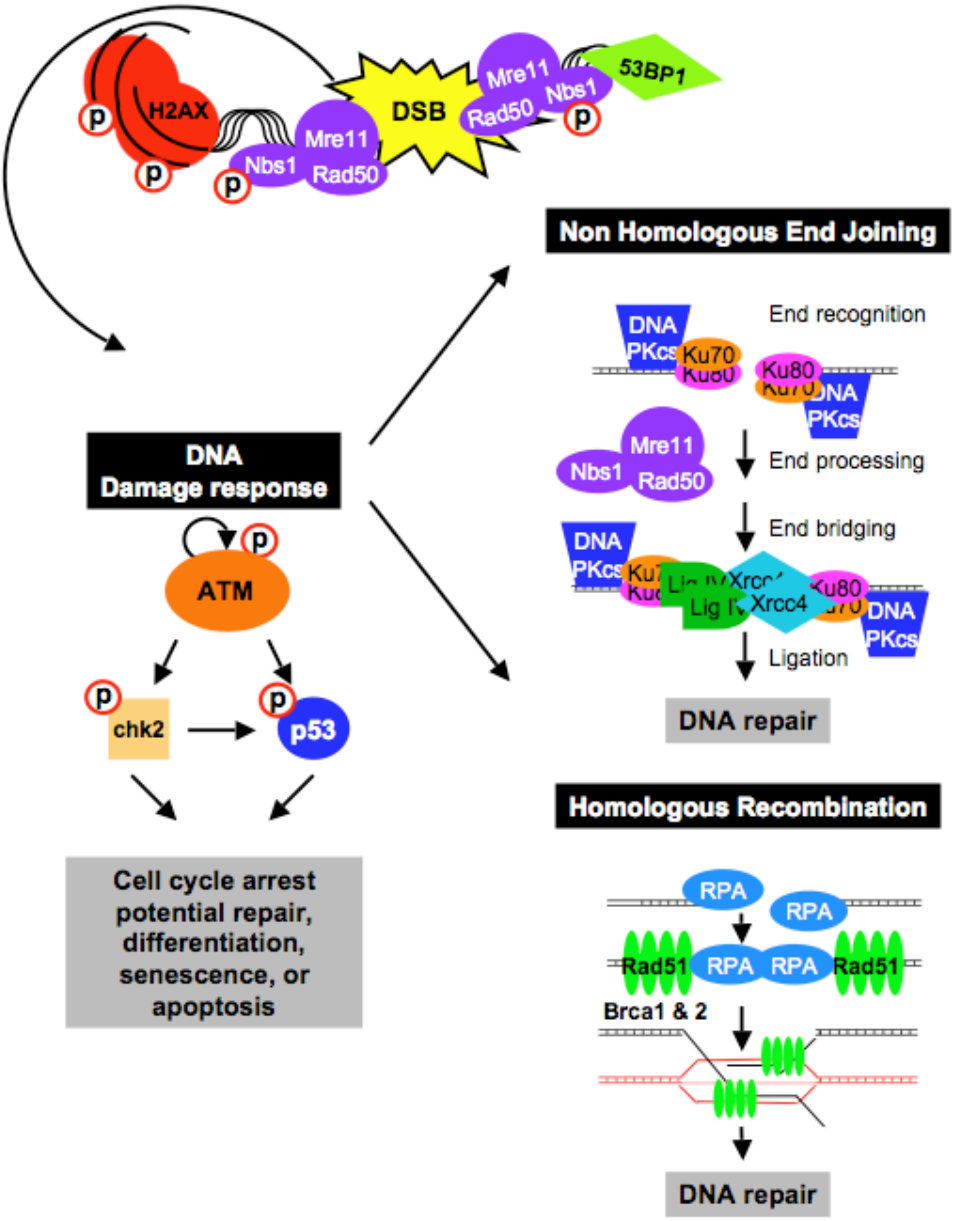

LT-HSC
Lin-/c-Kit+/Sca-1+/Flk2-/CD48-/CD150+
ST-HSC
Lin-/c-Kit+/Sca-1+/Flk2-/CD48-/CD150-
MPP
Lin-/c-Kit+/Sca-1+/Flk2+
CMP
Lin-/c-Kit+/Sca-1-/CD34+/FcγR-
MEP
Lin-/c-Kit+/Sca-1-/CD34-/FcγR-
GMP
Lin-/c-Kit+/Sca-1-/CD34+/FcγR+
CLP
Lin-/IL7R+/Flk2+/c-Kitint/Sca-1int
B cell
T cell
NK cell
Erythrocyte
Eosinophil
Neutrophil
Basophil
Platelets
Gr
Gr1+/Mac1+
Macrophage
T
CD3+
B
B220+/CD19+

Figure 2. DNA damage response and DNA double strand break repair

A simplified model of the classical DNA damage response (DDR) and DNA repair of DNA double strand breaks (DSB). DSBs can be repaired by non homologous end joining (NHEJ), an error-prone form of repair that can occur throughout the cell cycle, or by homologous recombination (HR) which is a high-fidelity repair mechanism but can only occur during the S/G_2 phases of the cell cycle.


H2AX
P
P
P
Nbs1
Mre11
Rad50
DSB
Mre11
Nbs1
Rad50
P
53BP1
Non Homologous End Joining
DNA Damage response
ATM
P
P
chk2
P
p53
Cell cycle arrest potential repair, differentiation, senescence, or apoptosis
End recognition
DNA PKcs
Ku70
Ku80
DNA PKcs
Nbs1
Mre11
Rad50
End processing
End bridging
DNA PKcs
Xrcc4
Ku80
DNA PKcs
Ligation
DNA repair
Homologous Recombination
RPA
RPA
Rad51
RPA
RPA
Rad51
Brca1 & 2
DNA repair

CHAPTER 2

HEMATOPOIETIC STEM CELL QUIESCENECE PROMOTES ERROR PRONE DNA REPAIR AND MUTAGENESIS

Abstract

Most adult stem cells, including hematopoietic stem cells (HSCs), are maintained in a quiescent or resting state *in vivo*. Quiescence is widely considered to be an essential protective mechanism for stem cells that minimizes endogenous stress caused by cellular respiration and DNA replication. Here, we demonstrate that HSC quiescence can also have detrimental effects. We found that HSCs have unique cell-intrinsic mechanisms ensuring their survival in response to ionizing irradiation (IR), which include enhanced pro-survival gene expression and strong activation of p53-mediated DNA damage response. We show that quiescent and proliferating HSCs are equally radioprotected but use different types of DNA repair mechanisms. We describe how nonhomologous end joining (NHEJ)-mediated DNA repair in quiescent HSCs is associated with acquisition of genomic rearrangements, which can persist *in vivo* and contribute to hematopoietic abnormalities. Our results demonstrate that quiescence is a double-edged sword that renders HSCs intrinsically vulnerable to mutagenesis following DNA damage.

Introduction

DNA repair is essential for cell survival and maintenance of tissue homeostasis (Lombard et al., 2005). Cellular organisms must constantly contend with endogenous DNA damage caused by intrinsic or extrinsic stresses and have evolved multiple DNA repair systems to deal with these insults (Sancar et al. 2004). DNA double-strand breaks (DSB) are considered the most cytotoxic type of DNA lesion and can arise during DNA replication or upon exposure to ionizing radiation (IR) and radiomimetic chemicals. DSB formation triggers a global DNA damage response resulting in the activation of the DNA damage sensor ATM, which in turn activates cell cycle checkpoints and phosphorylates an array of downstream targets including the tumor suppressor gene p53. This global DNA damage response (DDR) is directed toward the cells' own preservation and can lead to growth arrest and initiation of DNA repair by specialized DSB repair mechanisms, with programmed cell death being an alternative outcome of excessive or unrepaired DNA damage. The two principal and complementary mechanisms by which eukaryotic cells repair DSBs are homologous recombination (HR) and nonhomologous end joining (NHEJ) (Sancar et al., 2004). HR-mediated DNA repair uses a template for accurate repair, usually the sister chromatid, and thus can only occur in cycling cells. In contrast, NHEJ-mediated DNA repair has a limited requirement for sequence homology and can take place at any stage of the cell cycle. NHEJ-type repair is a more error-prone mechanism than the high fidelity HR-type repair, which often leads to misrepaired

DSBs that may result in chromosomal deletions, insertions or translocations and subsequent genomic instability (Weinstok et al., 2006). While defects in DNA damage responses have been associated with cancer, aging and stem cell abnormalities (Hanahan and Weinberg, 2000; Park and Gerson, 2005), much remains to be learned about the mechanism by which stem cells normally respond to DNA damage and repair DSBs.

The hematopoietic system provides a uniquely tractable model to investigate the activity of specific cell populations (Orkin and Zon, 2008). Hematopoietic development is organized hierarchically, starting with a rare population of hematopoietic stem cells (HSCs) that gives rise to a series of committed progenitors and mature cells with exclusive functional and immunophenotypic properties. HSCs are the only cells within the hematopoietic system that self-renew for life, whereas other hematopoietic progenitors cells are short-lived and committed to the transient production of mature blood cells. Under steady-state conditions, HSCs are a largely quiescent, slowly cycling cell population, which, in response to environmental cues, are capable of dramatic expansion and contraction to ensure proper homeostatic replacement of blood cells. In this context, the quiescent status of HSCs is widely considered to be an essential protective mechanism that minimizes endogenous stress caused by cellular respiration and DNA replication (Orford and Scadden, 2008). Proper execution of DNA repair processes is essential for normal HSC functions. Mice lacking components and regulators of the DNA damage response and DSB

repair mechanisms all display severe hematopoietic phenotypes and HSC defects (Ito et al., 2004; Nijnik et al., 2007; Rossi et al., 2007). Defective DNA repair has also been associated with a spectrum of human blood disorders (Wang, 2007) and the occurrence of chromosomal translocations is a hallmark of human hematological malignancies (Look et al., 1997). Previous studies have shown that genotoxic insults such as ionizing radiation (IR) differentially affect subsets of bone marrow hematopoietic cells, with HSCs being more radioresistant than their downstream myeloid progeny (Meijne et al., 1991; Down et al., 1995). This result is consistent with the low levels of intracellular oxidative species (ROS) observed in HSCs compared to myeloid progenitors (Tothova et al., 2007) and the well-established link between irradiation-induced DNA damage and ROS generation. However, limited information is currently available about the precise DNA repair capacity of HSCs and myeloid progenitor cells as well as on the mutagenic consequences of such repair for their biological functions.

Here, we use flow cytometry to isolate a highly enriched HSC-containing population referred to as hematopoietic stem and progenitor cells (HSPCs) and two distinct subsets of myeloid progenitors (MPs), the common myeloid progenitors (CMPs) and the granulocyte/macrophage progenitors (GMPs). We show that long-lived HSPCs have robust and unique cell-intrinsic mechanisms to ensure their survival in response to IR exposure, which include enhanced pro-survival gene expression and a strong induction of p53-mediated DDR leading to growth arrest and DNA repair; whereas short-lived MPs are molecularly poised to

undergo apoptosis and are predominantly eliminated in response to genotoxic stress. Most importantly, we demonstrate that HSPCs are forced to initiate DNA repair using the error-prone NHEJ mechanism due to their largely quiescent cell cycle status and the molecular composition of their DNA repair machinery. We show that this preferential use of NHEJ-mediated DNA repair renders quiescent HSPCs susceptible to genomic instability associated with misrepaired DNA, which can contribute to HSC loss of function and/or pre-malignant transformation *in vivo*. In contrast, HSPCs that have been induced to proliferate, either by *in vitro* culturing or *in vivo* mobilization treatment, undergo DNA repair using the high fidelity HR mechanism and have a significantly decreased risk of acquiring mutation(s). Taken together, our results demonstrate that HSC quiescence is a double-edged sword, which on the one hand protects HSCs against endogenous stress but, on the other hand, renders HSCs intrinsically vulnerable to mutagenesis following DNA damage.

Results

Enhanced radioresistance in HSPCs compared to MPs

We first defined the radiosensitivity of our purified hematopoietic stem and myeloid progenitor cells. We isolated HSPCs ($Lin^-/c\text{-}Kit^+/Sca\text{-}1^+/Flk2^-$), CMPs ($Lin^-/c\text{-}Kit^+/Sca\text{-}1^-/CD34^+/FcgR^-$) and GMPs ($Lin^-/c\text{-}Kit^+/Sca\text{-}1^-/CD34^+/FcgR^+$) from the pooled bone marrow of 5 to 10 wild type mice, exposed them to increasing doses of IR (0-10Gy) and performed clonogenic survival assays in methylcellulose and liquid media (Figures 1A and S1). We observed a striking difference in colony numbers at the 2Gy dose of irradiation, with HSPCs displaying significantly enhanced radioresistance compared to MPs that correlated with the differentiation status of the populations analyzed (HSPCs > CMPs > GMPs). At doses greater than 4Gy, all three populations were equally radiosensitive and did not form colonies, in agreement with the fact that the hematopoietic system is one of the first organ systems to fail after total body irradiation. To determine whether the enhanced radioresistance of HSPCs results from cell intrinsic differences in their DNA damage response, we performed a similar clonogenic survival assay with HSPCs and MPs isolated from *Atm*-deficient mice (Ito et al., 2004) (Figure 1B). In contrast to wild type cells, we found that $Atm^{-/-}$ HSPCs, CMPs and GMPs all displayed matching hypersensitivity to increasing doses of IR (0-4Gy). These results confirm that HSPCs are intrinsically more resistant to IR exposure than CMPs and GMPs, and indicate that ATM is an essential

mediator of this differential DNA damage response. We also showed that Slam-HSCs ($Lin^-/c\text{-}Kit^+/Sca\text{-}1^+/Flk2^-/CD150^+/CD48^-$), one of the most pure HSC populations characterized so far, display radioresistance similar to that of HSPCs (Figure S1), which indicate that our analysis of HSPCs may be generalized in this instance to HSC biology.

HSPCs undergo growth arrest while MPs die in response to IR treatment

We then investigated the cellular outcomes (*i.e.*, proliferation and apoptosis responses) induced by 2Gy IR in HSPCs and MPs. CFSE dilution assays uncovered a profound delay in the division rates of 2Gy-irradiated HSPCs that was still evident 3 days after IR exposure (Figure 1C). While CMPs displayed an intermediate behavior, with a recovery of normal proliferation by 3-4 days post-IR, the irradiation treatment had almost no effect on GMP proliferation rates. We then measured the apoptotic response occurring in these cells using intracellular cleaved caspase 3 (CC3) and Annexin V/7-AAD staining (Figure 1D and data not shown). We found that unirradiated MPs had significantly higher basal levels of CC3 staining compared to HSPCs after 1 and 2 days in culture (~1.3 and 8.5-fold higher in CMPs and ~ 3.7- and 10.4-fold higher in GMPs, respectively). Furthermore, we observed a robust and immediate IR-mediated apoptotic response in CMPs and GMPs but a minimal induction of apoptosis 2 days after irradiation in HSPCs. To establish the status of the apoptotic machinery in these cells, we performed qRT-PCR analysis of the expression

levels of a comprehensive panel of *bcl2* family pro- and anti-apoptotic genes in freshly isolated HSPCs, CMPs and GMPs (Figure 1E). We observed an overall deficit in pro-survival genes and a trend towards increased expression of pro-apoptotic genes in MPs compared to HSPCs. Using western blotting, we confirmed several highly significant changes ($p \leq 0.001$) found at the mRNA level including decreased Mcl-1 and increased Bid proteins in GMPs (Figure 1F). To functionally test whether a deficit in pro-survival genes contributes to the higher rate of apoptosis in MPs, we isolated cells from H2k-*bcl2* transgenic mice (Domen et al., 2000) and evaluated the effect of enhanced *bcl2* expression on their apoptotic response (Figure 1D). While HSPCs remained essentially unaffected by *bcl2* overexpression, we observed a significant decrease in the basal level of CC3 staining in MPs, especially in GMPs. However, H2k-*bcl2* MPs displayed an unchanged IR-mediated apoptotic response, which suggests that overexpressing a single pro-survival gene cannot compensate for the strength of IR-mediated death signals in MPs. Taken together, these results suggest that the short-lived, expendable MPs (especially GMPs) are poised at the molecular level to undergo apoptosis due to a deficit in pro-survival genes and are mostly eliminated in response to IR treatment. In contrast, the long-lived HSPCs predominantly survive and undergo growth arrest following irradiation.

Dual role of the p53 pathway

p53 is an important downstream target of ATM, which can mediate either growth arrest or apoptosis following DNA damage. To determine whether HSPCs and MPs engage a p53-dependent DDR, we first treated mice with 2Gy IR and measured the changes in p53 protein levels occurring in these bone marrow compartments at 12 hours post-irradiation using intracellular FACS analysis (Figure 2A). While we observed stabilization of p53 protein in *in vivo* irradiated HSPCs, no significant changes were found in irradiated MPs. We also confirmed by immunoblot a ~2-fold stronger phosphorylation of p53 (Ser15) in HSPCs compare to MPs at 1 hour post-IR and a tailing off in both populations by 4 hours post-IR (Figure S2). To directly assess p53 activity in these compartments, we then measured the induction of p53 target genes (i.e, *bax*, *bak*, *noxa*, *p21*) by qRT-PCR using purified cells grown in liquid culture for 8 and 12 hours post-IR (Figure 2B). Interestingly, the strength of the p53-mediated DDR (as measured by the levels of target gene induction) was much higher and sustained for a longer time in HSPCs compared to the limited and transient response observed in MPs, especially in GMPs. We further confirmed the functional importance of p53 in both HSPCs and MPs using cells isolated from *Trp53*$^{-/-}$ mice (Liu et al., 2009). Analysis of *Trp53*$^{-/-}$ HSPCs, CMPs and GMPs both in clonogenic survival assays and liquid culture (Figure 2C and data not shown) revealed increased radioresistance in all three populations. Furthermore, we showed that removal of p53 prevents HSPCs from undergoing growth arrest following IR exposure (Figure 2D), while in MPs, p53 deletion considerably decreased the basal level

and abrogated IR-mediated induction of apoptosis (Figure 2E). As expected, p53-mediated induction of *p21* and *bcl2* pro-apoptotic targets did not occur in irradiated *Trp53*$^{-/-}$ HSPCs and MPs (Figure 2F). Taken together, these results highlight the dual role that p53 plays in modulating opposite outcomes in irradiated HSPCs and MPs. We postulate that in HSPCs the high basal level of pro-survival genes coupled with the strong p53-mediated induction of *p21* protect against the killing effects of increased pro-apoptotic gene expression, resulting mainly in growth arrest as already observed in other cellular contexts (Abbas et al., 2009). In contrast, in MPs, the limited induction of pro-apoptotic genes that occurs in the context of very low basal levels of pro-survival genes and in the absence of or with weak induction of *p21*, results predominantly in cell death.

Ongoing DNA repair in HSPCs

To determine the extent of DSB DNA repair in irradiated HSPCs and MPs, we first used immunofluorescence microscopy to quantify γH2AX-containing ionizing radiation-induced foci (IRIF), which form at the sites of DNA damage (Figure 3A). Unirradiated HSPCs and MPs all displayed extremely low levels of IRIF and, following exposure to 2Gy IR, showed an immediate induction of γH2AX-positive DNA damage foci. By 4 hours to 24 hours post-IR, the numbers of IRIF declined in HSPCs with relatively faster kinetics than in MPs. To determine whether the loss of γH2AX foci corresponded to ongoing DNA repair or simply reflected cell elimination, we next subjected unirradiated and irradiated

cells to an alkaline COMET assay and scored the tail DNA content on a 0 (undamaged) to 4 (very damaged) scale to assess the severity of the resulting DNA damage (Figure 3B). We started this assay with identical numbers of cells for all conditions and normalized the tail DNA content score for the numbers of cells actually detected on the agarose slides, to account for the observation that dying cells are often lost during the various steps of this experimental procedure. Quantification of the results revealed that all three populations acquired equivalent amounts of DNA damage 2 hours after irradiation (Figure 3C and Table S1). By 24 hours post-IR, we observed a significant shift towards less damaged tail DNA content scores in HSPCs, which occurred without overall loss of cells thereby demonstrating active ongoing DNA repair. In contrast, in MPs, we predominantly observed cell elimination, with the persistence of only undamaged cells or a few cells undergoing DNA repair. Taken together, these results demonstrate that irradiated HSPCs survive and undergo DNA repair, and confirm that the majority of irradiated MPs are eliminated. They also highlight the fact that the decrease in γH2AX staining can be skewed due to the confounding impact of cell death (as in MPs) and, while marking the resolution of DSBs (as in HSPCs), cannot simply be equated with complete DNA repair.

Preferential use of NHEJ repair mechanism in quiescent HSPCs

We then investigated the type of DSB repair mechanisms used by irradiated HSPCs and the few surviving MPs. To assess HR activity, we

quantified IRIF containing the Rad51 recombinase protein by immunofluorescence microscopy. Unfortunately, none of the components of the NHEJ machinery that we examined (Ku70, Ku80) were detectable by microscopy in IRIF (data not shown). Therefore, as a surrogate, we quantified IRIF containing the 53BP1 DNA damage response protein as 53BP1 has been shown to function, albeit not exclusively, in NHEJ. Rad51 IRIF formation occurred rapidly in irradiated MPs, reaching its maximum (~50% of the cells) by 2 hours (CMPs) and 4 hours (GMPs) post-IR and then remaining unchanged for up to 24 hours (Figure 4A). In contrast, no significant Rad51 recruitment was observed in HSPCs until 24 hours post-IR. This staining pattern is consistent with the proliferation index of the respective populations (Figures S3), with irradiated HSPCs being mostly quiescent at the start of the culture and only initiating their first cell division by ~ 24 hours *in vitro* (Figure 1C). Conversely, recruitment of 53BP1 in IRIF occurred immediately in all three populations but then declined at a slower rate in HSPCs (Figure 4B). To support these observations, we also analyzed the basal expression level of HR and NHEJ components in freshly isolated HSPCs, CMPs and GMPs (Figure 4C). Strikingly, we found that all of the HR components and/or regulators we investigated were expressed at significantly higher levels in MPs compared to HSPCs, while the NHEJ machinery components were either dramatically decreased (*Ku80*) or unchanged in MPs. Finally, we used a reporter assay in which the re-ligation of a digested plasmid expressing the enhanced green fluorescent protein (eGFP) allowed a

measurement of the NHEJ activity present at baseline and following IR in transfected cells (Figure S4). Consistent with the predominance of NHEJ as a repair mechanism in HSPCs, we observed high basal levels of NHEJ activity in unirradiated HSPCs and a ~2-fold increase following irradiation (Figures 4D and S4). In sharp contrast, GMPs displayed extremely low basal levels and no IR-mediated induction of NHEJ activity, whereas CMPs showed intermediary levels of basal and IR-mediated NHEJ activity. Taken together, these results demonstrate that HSPCs are forced to initiate DNA repair using NHEJ-type mechanisms due to their largely quiescent cell cycle status and the molecular wiring of their DNA repair machinery. They also indicate that the few proliferating MPs that escape IR-mediated cell killing are molecularly primed to undergo HR-mediated DNA repair and do not use NHEJ-type mechanisms.

HSPC radioprotection is independent of quiescence

It has been suggested that quiescence provides HSCs with enhanced resistance to genotoxic stress (Tothova et al., 2007; Orford and Scadden, 2008). To experimentally test this assumption, we forced HSPCs to proliferate before exposing them to 2Gy IR (Figure 5A). First, we pre-cultured resting HSPCs (Rest. HSPCs) for ~ 24 hours *in vitro* (24hr preC HSPCs) to induce their proliferation and, second, we used an *in vivo* mobilization treatment (Passegué et al., 2005) to harvest proliferating bone marrow HSPCs (Mob. HSPCs) after one injection of cyclophosphamide and two days of stimulation with G-CSF. Both strategies

resulted in a net increase in HSPC cycling rates as measured after a 1-hour BrdU pulse (Figures 5B) and a loss of quiescence as measured by intracellular 7AAD/PyroninY staining (Figures 5C). Strikingly, no differences were observed in the radioresistance of proliferating HSPCs compared to resting HSPCs using either clonogenic survival assay in methylcellulose (Figure 5D) or proliferation in liquid culture (Figure 5E). At the molecular level, we found that proliferating HSPCs had decreased basal levels of pro-survival genes, varied levels of pro-apoptotic genes and constitutively higher apoptosis rates than quiescent HSPCs (Figures S5 and 5F). However, like quiescent HSPCs, proliferating HSPCs did not show a significant IR-mediated apoptotic response (Figure 5F), but unlike quiescent HSPCs, they did not undergo IR-mediated growth arrest and displayed an attenuated p53-mediated response including limited induction of *p21* expression (Figures 5G and S5). Altogether, these results indicate a major re-wiring of the DNA damage response in proliferating HSPCs to one that closely resembles the response observed in MPs. However, this does not result in an analogous loss of radioresistance suggesting that proliferative HSPCs still retain additional, yet unexplored, protective mechanism(s) that are not shared by their more differentiated progeny.

Access to HR repair in proliferating HSPCs

Next, we investigated the type of DNA repair mechanisms that were used by 2Gy-irradiated proliferating HSPCs. We observed similar kinetics of γH2AX

IRIF induction and resolution in both resting and proliferating HSPCs, indicating that they are equally efficient at repairing IR-induced DSBs (Figure S5). However, in contrast to quiescent HSPCs, proliferating HSPCs immediately formed Rad51 IRIF, which reached maximum levels by 2-4 hours post-IR and remained elevated throughout the 24-hour experiment (Figure 6A). 53BP1 recruitment to IRIF also occurred immediately in proliferating HSPCs but sharply declined thereafter and returned to basal levels by 12 hours post-IR, in contrast to the slow decline seen in resting HSPCs (Figure 6B). Consistent with the rewiring of the DNA damage response, we also found increased expression of HR genes and decreased expression of NHEJ components in proliferating HSPCs compared to quiescent HSPCs (Figure 6C). While the basal levels of NHEJ activity were not significantly different in unirradiated cells (Figure S4), we observed a complete abrogation of IR-mediated induction of NHEJ activity in proliferating HSPCs compared to resting HSPCs (Figure 6D). Taken together, these results demonstrate that quiescence dramatically restricts HSPC ability to use the high-fidelity HR-mediated repair and instead forces them to rely on the more error-prone NHEJ mechanism to repair DSBs.

Quiescent HSPCs are prone to acquire mutations

NHEJ-mediated repair can be mutagenic in many ways, most commonly by causing deletion of microhomology sequences flanking the breakpoint or insertions at the DSB joint region. Since IR can induce DSBs anywhere in the

genome, we used fluorescence *in situ* hybridization (FISH) in an attempt to determine whether NHEJ-mediated mutagenic repair could result in molecular-level deletions within a fragile chromosomal region, leading to loss of hybridization signals. Un-irradiated and 2Gy irradiated quiescent HSPCs were grown in culture for 4 to 5 days for maximal expansion, treated for 4 hours with Colcemid^{TM}, fixed for cytogenetic studies and interphase cells were then hybridized with a probe for the mouse *Fhit* locus common fragile site. No significant difference in signal intensity could be observed between unirradiated and irradiated cells using this approach (Figure S6). More sensitive techniques will therefore be required to assess these particular forms of mutagenic NHEJ-mediated DSBs repair. NHEJ has also been shown to be very proficient at mediating chromosomal translocations, whereas HR-type repair are not because of crossover suppression (Weinstok et al., 2006). To determine whether the progeny of IR-treated resting HSPCs could acquire major genomic rearrangements as the result of inaccurate NHEJ-mediated DNA repair, and whether the frequency of such mutagenic events would be decreased in HR-competent, proliferating HSPCs, we performed spectral karyotyping (SKY) analysis on the metaphase cells obtained from the same cell preparations (Figure 7A). Strikingly, we found that more than 30% of the cells derived from IR-treated resting HSPCs consistently displayed major genomic rearrangements, including reciprocal translocations, interstitial deletions and complex rearrangements, compared to unirradiated cells (Figure 7B and Table S2). Most importantly, we

showed that induction of proliferation and the availability of HR-mediated DNA repair in both 24h preC. and Mob. HSPCs significantly reduced the number and frequency of genomic aberrations occurring upon IR exposure, hence decreasing by half the risk of acquiring genomic instability in the self-renewing HSPC compartment (Figure 7C and Table S2). Taken together, these results provide a direct demonstration that IR-damaged HSPCs, which are limited to using NHEJ-repair mechanism by their quiescent status, are prone to acquire cytogenetic aberrations as a result of incorrectly repaired DNA damage.

Persistence of misrepaired HSPCs *in vivo*

We then tested whether such misrepaired HSPCs could persist *in vivo* and eventually contribute to hematological disorders. We transplanted unirradiated or irradiated quiescent HSPCs (CD45.1) immediately following IR exposure into lethally-irradiated WT (CD45.2) recipient mice and monitored them over 4 months post-transplantation for development of hematological abnormalities and genomic instability (Figure 7C). As expected, we observed a dose-dependent decrease in engraftment of IR-exposed HSPCs compared to unirradiated HSPCs, with no long-term hematopoietic reconstitution provided by 6Gy-treated HSPCs (data not shown). At 4 months post-transplantation, none of the engrafted mice developed leukemia or showed outward signs of hematological abnormalities in the peripheral blood and bone marrow (data not shown). CD45.1 donor-derived HSPCs and MPs were then isolated from pools of mice reconstituted with 2Gy-

irradiated HSPCs and used, respectively, for secondary transplantation and SKY analysis. In 3 out of 4 2Gy-treated HSPC cohorts, a significant number of donor-derived MPs displayed genomic abnormalities including the presence of the same t(16;17) balanced chromosomal translocation in ~55% of cohort I2 donor-derived MPs (Figure 7E and Table S3). This result indicates the clonal expansion of a single mutated HSPC. The complete loss of engraftment that was observed following secondary transplantation of donor-derived cohort I2 HSPCs (Figure 7E), further suggest the presence of a mutation(s) associated with HSC exhaustion and bone marrow failure. Taken together, these findings indicate that misrepaired HSPCs can survive at relatively high frequencies *in vivo*, and can contribute either to the direct expansion of aberrant clones (as in cohort I2) or, more often, to the maintenance of a background of genomic alterations (as in cohorts I3 and I4), some of which could be pre-malignant.

Discussion

Defects in DNA damage responses that cause accumulation of DNA damage and loss of DNA repair capacity, are broadly associated with organ failure, cancer, aging and stem cell abnormalities (Hanahan and Weinberg, 2000; Park and Gerson, 2005). The decline in tissue function observed with age has also been correlated with impaired stem cell activity (Chambers and Goodell, 2007; Geiger and Rudolph, 2009). However, much remains to be elucidated about the mechanisms by which DNA damage is repaired in adult stem cells and whether mutation(s) arising from aberrant repair contribute to aging and/or susceptibility to cancer in these self-renewing populations. In this study, we investigated how blood-forming HSCs respond to DNA-damaging IR exposure, determined the extent to which they use the error-prone NHEJ repair mechanism, and assessed the consequences of such mutagenic DNA repair for their biological functions. We identify some of the key molecular mechanisms that ensure HSCs resistance to IR-mediated cell killing and provide a mechanistic explanation for why HSCs are at greater risk of accumulating mutations than other cells in the hematopoietic system. Our results demonstrate that the prevalent DNA repair mechanism active in quiescent HSCs is prone to generating mutations.

Long-lived HSCs are essential for hematopoietic homeostasis and, as we show here, have unique cell-intrinsic mechanisms ensuring their survival (Figure S7). These likely include enhanced pro-survival gene expression and robust

induction of DNA damage checkpoints (*i.e.*, ATM, p53) leading to a strong p53-mediated induction of both pro-apoptotic genes (*i.e.*, *puma*, *noxa*, *bak*) and *p21* expression. We postulate that high basal levels of pro-survival factors likely limit IR-mediated cell killing in HSCs and instead favor *p21*-mediated growth arrest, DNA repair and survival as has already been observed in other cellular contexts (Abbas et al., 2009). This prominent role for *p21* in normal HSCs may explain why it has been found to have such an important function maintaining the DNA damage response and self-renewal properties of leukemic HSCs transformed by the *PML-RAR* oncogene (Viale et al., 2009). Interestingly, we show that normal HSCs that have been induced to proliferate either by *in vitro* culturing or *in vivo* mobilization treatment have decreased overall expression of *bcl2*-family pro-survival genes and display constitutively higher levels of apoptosis than quiescent HSCs. However, this rewiring of the apoptotic machinery does not result in loss of radioprotection nor in any significant increase in IR-mediated cell killing of proliferating HSCs, as observed in MPs, which indicates that additional, still unexplored, survival mechanism(s) also contribute to the specific protection of this self-renewing compartment. It is likely that maintenance of low levels of ROS (Tothova et al., 2007) and other fundamental mechanisms of cellular detoxification contribute to the enhanced survival of long-lived HSCs. It will also be interesting to confirm that endogenous HSCs in the bone marrow space display the same behavior following radiation insults than isolated HSCs *ex vivo*.

In terms of organ maintenance, it is logical to keep long-lived HSCs quiescent *in vivo* to guard them against DNA replication errors and damage associated with metabolic stress (Rossi et al., 2007; Orford and Scadden, 2008). Our *ex vivo* analyses demonstrate that a substantial limitation of HSC quiescence is reduced elimination of damaged HSCs by apoptosis and an increased likelihood of mutagenesis due to the use of error-prone DNA repair mechanisms (Figure S7). This conclusion is further supported by the observation that HR-competent proliferating HSCs have significantly decreased risk of acquiring mutation(s), which likely results from their use of a high fidelity repair mechanism. Our transplantation experiments directly demonstrate that damaged HSCs, which have undergone DNA repair and acquired mutation(s) during this process, can persist *in vivo* at relatively high frequencies and contribute either to the clonal expansion of aberrant cells or to the maintenance of cells with genomic alterations. Both events could predispose mutated HSCs to loss of function and/or cancer development and their occurrence is likely due to the stochastic combination of cell-intrinsic effects provided by the acquired mutation(s) and selection pressure *in vivo*. Although we have analyzed only a small number of transplanted cohorts thus far, we observed at least one case of each of the two possible mutagenic outcomes: mutation(s) providing either growth or survival advantages that are clonally amplified (cohort I2) and, more frequently, non-essential “passenger” mutation(s) that appear to be maintained but not expanded *in vivo* (cohorts I3 and I4). These results are consistent with the cytogenetic

pattern of human hematological malignancies, where only a handful of recurring translocations, deletions and inversions are associated with specific diseases (Look, 1997) and clonally expanded in the context of either a high or low background of genomic alterations (Radtke et al., 2009). They also extend the conclusion of a previous study performed with multipotent hematopoietic cells differentiated *in vitro* from mouse embryonic stem (ES) cells, which showed that immature hematopoietic progenitors were particularly susceptible to the formation of chromosomal rearrangements analogous to those found in human hematological malignancies (Francis and Richardson, 2007). Moreover, our findings suggest that vulnerability to mutagenesis might be a general property of all quiescent stem cell populations either normal or cancerous. They highlight why quiescent leukemic stem cells (LSCs), which currently survive therapeutic treatment in CML (Holyoake et al., 1999) and acute myeloid leukemia (AML) (Guan et al., 2003), represent a dangerous reservoir for additional mutations that is likely to contribute to disease relapse and/or evolution.

Our results provide the beginning of a molecular understanding of why HSCs are more likely than MPs to become transformed and trigger leukemia development (Bonnet and Dick, 1997). In contrast to HSCs, transformation of MPs must overcome significant self-destructive mechanisms. MPs are short-lived cells that are constantly replenished from the HSC compartment and are therefore expendable in terms of organ maintenance. Our results indicate that MPs are intrinsically poised to die and are mainly eliminated in response to DNA

damage (Figure S7). When compared to HSCs, MPs have a much-attenuated p53-mediated DDR. However, despite its limited extent, p53-mediated induction of pro-apoptotic genes is not counter-balanced by high basal levels of pro-survival factors as seen in HSCs and occurs with only a weak induction of *p21*, thereby leading mostly to cell elimination. As a consequence, mutations resulting in transformation of the MP compartment are unlikely to become established unless the cells gain substantial survival advantage(s) either by inheriting mutations from the HSC compartment (as observed with *BCR/ABL1* during chronic myelogenous leukemia (CML) progression) (Jamieson et al., 2005) or by directly acquiring leukemia-associated fusion genes with major "re-programming" activity, such as *MLL* translocations (Krivtsov et al., 2006).

Our results may also explain some aspects of the loss of function occurring in HSCs with age. Age-related defects in the hematopoietic system include a decline in the adaptive immune system called immunosenescence and the development of a broad spectrum of age-related hematological disorders (*i.e.*, myeloproliferative neoplasms, leukemia, lymphoma, bone marrow failure) that have been linked to changes in the biological function of aged HSCs (Chambers and Goodell, 2007; Geiger and Rudolph, 2009). Gene expression studies and analysis of genetically modified mice also indicate that errors in DNA repair and poorly maintained genomic stability are among the main driving forces for HSC aging (Rossi *et al.*, 2007). Our findings suggest that accumulation of NHEJ-mediated mutation(s) over a lifetime could dramatically hinder HSC

performance and be a major contributor to the loss of function observed in aged HSCs and the development of age-related hematological disorders.

Finally, our results may have direct clinical applications for minimizing the development of therapy-related cancers following cytotoxic therapy (Allan and Travis, 2005). Many solid tumors and hematological malignancies are currently treated with DNA damaging agents, which may result in therapy-related myeloid leukemia. Our work suggests that cytotoxic therapies might inadvertently mutate the patient's own quiescent HSCs by forcing them to undergo DNA repair using a mutagenic mechanism. Specifically, we show that proliferating HSCs have significantly decreased mutation rates, with no observed changes in their radioresistance, suggesting that it might be beneficial to induce HSCs to cycle prior to therapy with DNA damaging agents to enhance DNA repair fidelity and reduce the risk of leukemia development. While this possibility remains to be tested, it offers exciting new directions for limiting the deleterious side effects of cancer treatment.

Experimental procedures

Mice.

C57Bl/6-CD45.1 and C57Bl/6-CD45.2 mice were used as donor (4-8 week old) for most of the cell isolation and as recipient (8-12 week old) for cell transplantation procedures. *Atm*$^{-/-}$ mice (129/sv) were purchased from the Jackson Laboratory and both transgenic H2k-*bcl2* (C57Bl/6) and *Trp53*$^{-/-}$ (FVB/N) mice have been described (Domen et al., 2000; Liu et al., 2009). Cyclophosphamide/G-CSF mobilization of HSCs was performed as described (Morrison et al. 1997). Transplantations were performed by retro-orbital injection of purified cells. Recipient mice were either sub-lethally (9.5Gy) or lethally irradiated (11Gy), both delivered in split dose 3 hours apart, and given antibiotic-containing water for at least 6 weeks post-irradiation. Peripheral blood was obtained from retro-orbital bleeding and collected in 4 ml of ACK (150 mM NH_4Cl/10 mM $KHCO_3$) containing 10 mM EDTA for flow cytometry analysis. Donor and recipient cells were distinguished by expression of GFP or different allelic forms of CD45 (CD45.1 vs. CD45.2). All animal experiments were performed in accordance with UCSF Institutional Animal Care and Use Committee approved protocols.

Flow cytometry.

Staining and enrichment procedures for HSPC, CMP, and GMP cell sorting and analysis were performed as previously described (Passegué et al., 2005;

Santaguida et al., 2009). For regular isolation, c-Kit-enriched bone marrow cells were stained with unconjugated rat lineage (B220, CD3, CD4, CD5, CD8, Ter119, Mac-1, Gr-1) antibodies/goat anti-rat-PE-TxR and directly conjugated c-Kit-APC, Sca-1-PB, Flk2-bio/streptavidin-Cy7PE, CD34-FITC and FcgR-PE antibodies. For staining of purified cells with CFSE or FITC-conjugated antibodies (CC3, BrdU), c-Kit-enriched bone marrow cells were stained with unconjugated rat lineage/goat anti-rat-PE-TxR, c-Kit-cy7APC, Sca-1-PB, Flk2-PE, CD34-bio/streptavidin-Cy7PE and FcgR-cy5.5PE antibodies. Cells were finally resuspended in Hank's buffered saline solution (HBSS) with 2% heat-inactivated fetal bovine serum (FBS) containing 1μg/ml propidium iodide (PI) for dead cell exclusion, and sorted or analyzed on a FACS AriaII or LSRII (Becton Dickinson), respectively. Each population was double sorted to ensure maximum purity and irradiated as needed using a ^{137}Cs source (600ci^{137}Cs; J.L Shepherd & Associates Radiation Machinery M38-1 S.N. 1098).

Cell cultures.

Both methylcellulose and liquid cultures were supplemented with SCF (25 ng/ml), Flt3-L (25 ng/ml), IL-11 (25 ng/ml), IL-3 (10 ng/ml), Tpo (25 ng/ml), Epo (4 U/ml) and GM-CSF (10 ng/ml) (Peprotech). For methylcellulose assays, cells (0-2Gy/4-10Gy: 100/500 HSPCs and CMPs; 250/1000 GMPs) were plated in Iscove's modified Dulbecco's media (IMDM) based methylcellulose (StemCell Technology M3231) and colonies were counted on day 7 using duplicate plates per condition. For growth expansion in liquid culture, 500 cells were plated per well of a 96-well

plate in 200μl IMDM containing 5% FBS (StemCell Technology), 1x penicillin/streptomycin, 0.1mM non-essential amino acids, 1mM sodium-pyruvate, 2mM Gluta-Max-1, 50μM 2-mercaptoethanol. Medium was replenished and cells were expanded as needed to maintain optimal growth. Cells were counted on days 2, 4, 6 and 8 by trypan blue exclusion using triplicate wells per condition and time point. Other cell culture experiments were started with various numbers of purified (± irradiation) cells (5,000-40,000).

Apoptosis and proliferation assays.

Apoptosis levels were measured by flow cytometry using intracellular staining for FITC-conjugated cleaved caspase 3 according to the manufacturer's instructions (Becton Dickinson). For CFSE dilution assays, purified cells were washed once with PBS, incubated for 5 min at RT with 5 μM CSFE (Molecular Probes/Invitrogen) in PBS, immediately quenched with an equal volume of FBS, washed twice with complete IMDM media, eventually irradiated and then cultured as needed before flow cytometry analysis. For intracellular BrdU staining, purified cells were incubated for 1 hour with 60μM BrdU (Sigma), fixed in PBS/4% PFA for 20 min at RT, washed twice in PBS/50mM NH_4Cl, permeabilized in PBS/0.2% Triton-X100 for 5 min and washed in low PB (0.2x PBS, 3% BSA) buffer. Cells were then incubated with 50U DNAseI in low PB buffer containing 5 mM $MgCl_2$/2 mM $CaCl_2$ for 30 min at RT min, washed once with low PB buffer, incubated for 30 min with FITC-conjugated anti-BrdU antibody (Pharmingen), washed once and resuspended in low PB buffer without PI for flow cytometry analysis.

7AAD/Pyronin Y staining and p53 intracellular staining were performed as previously described (Santaguida et al., 2009).

Quantitative RT-PCR.

Total RNA was isolated using TRIzol or TRIzol-LS (Invitrogen) from equivalent numbers of purified cells (20,000 to 50,000 aliquots), digested with DNase I and used for random hexamer-based reverse-transcription according to the manufacturer's instructions (SuperScript III™ kit, Invitrogen). QRT-PCR primers were designed using Primer Express software (Applied Biosystems) and are listed in the table below. All reactions were performed in an ABI-7300 sequence detection system using SYBR® Green PCR Core reagents (Applied Biosystems) and the cDNA equivalent of 200 cells as previously described (Santaguida et al., 2009). Each measurement was performed in triplicate and expression levels of β-actin were used to normalize the amount of the investigated transcript.

Western blot.

Purified 35,000 - 70,000 cell aliquots were pelleted, washed once with PBS, lysed in RIPA buffer (50mM Tris-HCl pH7.4, 150mM NaCl, 1% NP40, 0.5% Na-deoxycholate, 0.1% SDS, 1mM EDTA, 1mM Na_3VO_4, 1mM NaF and complete protease inhibitor cocktail [Roche Diagnostics]) for 30 min at 4°C, cleared of debris by centrifugation, boiled in SDS sample buffer and entirely deposited in one lane of a 12% SDS- polyacrylamide gel as previously described (Picariello et al., 2006). Briefly, samples (≤ 5μg total protein) were resolved by electrophoresis,

transferred to nitrocellulose membranes, blocked for 1 hour at 4°C in blocking buffer (LI-COR Biosciences), incubated overnight at 4°C in blocking buffer/0.1% Tween 20 containing primary antibodies (anti-Mcl-1 [600-401-394, Rockland], rabbit polyclonal anti-Bid [gift from Dr. G. Shore, McGill University], rabbit polyclonal phospho-p53 (Ser15) [9284, Cell Signaling], anti-Actin [A2066, Sigma]), washed once in PBS/0.1% Tween 20, incubated for 1 hour at 4°C in blocking buffer/0.1% Tween 20 containing the appropriate IRDye conjugated secondary antibody (goat-anti-mouse IRDye 680 [926-32220]; goat-anti-rabbit IRDye 800CW dye [926-32211]; LI-COR Biosciences) washed 3 times in PBS/0.1% Tween 20 and visualized using an Odyssey Infrared Imager (LI-COR Biosciences).

Immunofluorescence microscopy.

Purified cells (± irradiation) grown in 5,000 (HSPCs) or 30,000 (MPs) cell aliquots were cytospun onto poly-lysine coated slides (Sigma) for 2 min at 200 rpm, fixed in PBS/4% PFA for 10 min at RT, permeabilized in PBS/0.15% Triton-X100 for 2 min at RT, blocked 1 hour at RT to overnight at 4°C in PBS/1%BSA and stained as previously described (Dodson et al., 2004). Briefly, cells were incubated for 1 hour at 37°C in PBS/1%BSA containing primary antibodies (anti-phospho-H2AX S139 [JBW301, Millipore], 53BP1 [NB100-904, Novus Biologicals], Rad51 [sc-8349, Santa Cruz]), washed twice in PBS, incubated for 1 hour at 37°C in PBS/1%BSA containing the appropriate Alexa-conjugated secondary antibody (goat-anti-mouse A488 [A11001]; goat-anti-rabbit A594 [A11037]; Invitrogen)

washed twice in PBS and then mounted in VectaShield (Vector Laboratories) containing 1μg/ml DAPI (Sigma). Microscopy imaging was performed using an Olympus BX-51 microscope (100x objective, NA 1.35 lens) driven by the OpenLab software. Deconvolved (nearest neighbor DCI) images were saved as Photoshop CS files. Cells were scored as positive or negative based on the number of foci observed by eye. All scoring was done blind and 30-50 cells were counted per time point in each experiment.

Alkaline COMET Assay.

Purified cells (± irradiation) grown in 10,000 (HSPCs) or 30,000 (CMPs and GMPs) cell aliquots were washed once in mincing buffer (Mg^{2+}/Ca^{2+}-free HBSS, 20mM EDTA, 10% DMSO, pH7-7.5), resuspended in 100μl molten (42ºC) 0.5% low melting point agarose (LMP, Promega) in PBS, and rapidly spread on slides (Superfrost Plus Micro, Fisher) coated in advance with normal melting point agarose (Sigma) without prior washing or dry cleaning. Slides were then coverslipped, allowed to solidify for at least 10 min at 4°C and processed as previously described (Klaude et al., 1996). Briefly, upon coverslip removal, slides were incubated from 4 hours to overnight at 4°C in lysis buffer (2.5M NaCl, 100 mM EDTA, 10 mM Tris/pH10 with freshly added 10% DMSO and 1% Triton-X100), rinsed once with neutralization buffer (0.4M Tris-pH7.5), incubated for 25 min on wet ice in fresh electrophoresis buffer (0.3M NaOH, 1M EDTA, pH 13) for unwinding of DNA and run for 30 min at 0.5 V/cm and 300 mA in the same buffer. After electrophoresis, slides were washed twice for 5 min in neutralization buffer,

dehydrated at RT in absolute ethanol, stained with 100 μl SyberGold (Invitrogen) and coverslipped. Observations were made at a magnification of 200X using a Nikon E800 epifluorescence microscope with FITC barrier filter. Cells were analyzed and scored by visual classification as no statistical difference between visual and automated tail moment quantification has been demonstrated (Kobayashi et al., 1995; Jalaszynski et al., 1997). For each condition, ~ 500 cells were counted and the number of visual fields required to count that number recorded. DNA damage severity was assessed on a 0 (un-damaged) to 4 (very damaged/dead) progressive scale. The COMET tail DNA content score represents the number of cells counted for each 0-4 categories. Normalization between conditions was done by dividing the COMET tail DNA content scored for each category by the total number of visual fields counted. The percentage tail DNA (expressed in arbitrary units) was calculated based on the following equation adapted from[47]: % tail DNA = (0 x n0 + 1x n1 + 2 x n2 + 3 x n3 + 4 x n4) / (En x 4), where n0-n4 represent the numbers of cells counted in 0-4 categories and En the total number of scored cells.

NHEJ activity assay.

C-Kit-enriched bone marrow cells (± irradiation) were pre-cultured for 12 hours in liquid media (full cytokine cocktail without Flt3-L) in 3-6x10^6 cell aliquots and electroporated with 2μg of BamH1-digested pcDNA-eGFP plasmid (which cleaves between the promoter and the eGFP ORF) and 2μg pcDNA-Tomato

plasmid using the Amaxa mouse macrophage nucleofactor kit following the manufacturer's protocol (Lonza, Amaxa Inc, VPA-1009). Cells were then incubated for another 24 hours in liquid media (full cytokine cocktail without Flt3-L) and labeled with cell surface markers to determine the percentage of GFP expression in transfected Tomato^{+} HSPCs, CMPs and GMPs by flow cytometry.

Cytogenetic analysis.

Purified cells (± irradiation) were grown for 3 days (MPs, 100,000 to 300,000 cell aliquots) or 4 days (HSPCs: 50,000 to 100,000 cell aliquots), treated for 4 hours with 0.01 μg/ml Colcemid^{TM} (Invitrogen), collected in eppendorf tubes and washed once with PBS. Cells were incubated for 8 min at 37°C in 0.075M KCl and then fixed in 3:1 volume absolute methanol:glacial acetic acid. Spectral karyotyping analysis (SKY) was performed as described (Le Beau et al., 2002). At least 20 metaphase cells were analyzed per case. A labeled BAC probe (9J14, 219 kb) containing the mouse common fragile site sequences within the *Fhit* gene (MMU 14A2) was prepared by direct labeling with Spectrum Orange^{TM}-labeled nucleotides (Abbott Molecular Diagnostics). Fluorescence in situ hybridization (FISH) was performed as described (Le Beau et al., 1996). Cells were counterstained with DAPI, and ~ 200 interphase nuclei were scored for each sample.

Statistics.

All the data are expressed as mean ± standard deviation (error bar) except when indicated. P values were generated using unpaired Student's *t*-test and considered significant when ≤ 0.05. N indicates the numbers of independent experiments performed.

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Wang, W (2007). Emergence of a DNA-damage response network consisting of Fanconi anaemia and BRCA proteins. Nat Rev Genet *8*, 735-748.

Weinstok, D.M., Richardson, C.A., Elliott, B., and Jasin, M. (2006). Modeling oncogenic translocations: Distinct roles for double-strand break repair pathways in translocation formation in mammalian cells. DNA Repair *5*, 1065-1074.

Figure 1. HSPCs are intrinsically radioresistant and survive IR-induced cell killing.

(**A**) Clonogenic survival assay of irradiated cells in methylcellulose (n = 9; ***p ≤ 0.001 [CMPs and GMPs *vs*. HSPCs]; •p ≤ 0.05 [GMPs *vs*. CMPs]).

(**B**) Clonogenic survival assay of irradiated *Atm*$^{-/-}$ cells in methylcellulose (n = 3; *p ≤ 0.05 [*Atm*$^{+/+}$ GMPs *vs*. *Atm*$^{+/+}$ HSPCs]; °°°p ≤ 0.001 [*Atm*$^{-/-}$ *vs. Atm*$^{+/+}$ populations]).

(**C**) Representative example of CFSE dilution assay in unirradiated (grey) or 2Gy-irradiated (color) cells grown for up to 3 days in liquid media (n = 4).

(**D**) Intracellular cleaved caspase 3 staining in unirradiated (grey) or 2Gy-irradiated WT (left side; solid colors; n = 10) or H2k-*bcl2* (right side; striped colors; n = 3) cells grown for up to 2 days in liquid media (p ≤ 0.001, *p ≤ 0.05 [unirradiated *vs*. irradiated cells]; °°°p ≤ 0.001, °p ≤ 0.05 [H2k-*bcl2 vs.* WT cells]; ns: not significant).

(**E**) QRT-PCR analysis of the basal expression level of *bcl2*-family pro-survival and pro-apoptotic genes, *Trp53* and *p21* in freshly isolated cells. Results are expressed as log2 fold expression compared to levels measured in HSPCs (n = 6; ***p ≤ 0.001, **p ≤ 0.01, *p ≤ 0.05 [CMPs *vs*. HSPCs]; °°°p ≤ 0.001, °°p ≤ 0.01, °p ≤ 0.05 [GMPs *vs*. HSPCs]).

(**F**) Western blot analysis of Mcl-1 and Bid protein levels in purified cells (protein extracted from 35,000 isolated cells per lane; b-actin is used as loading control).

See also Figure S1.

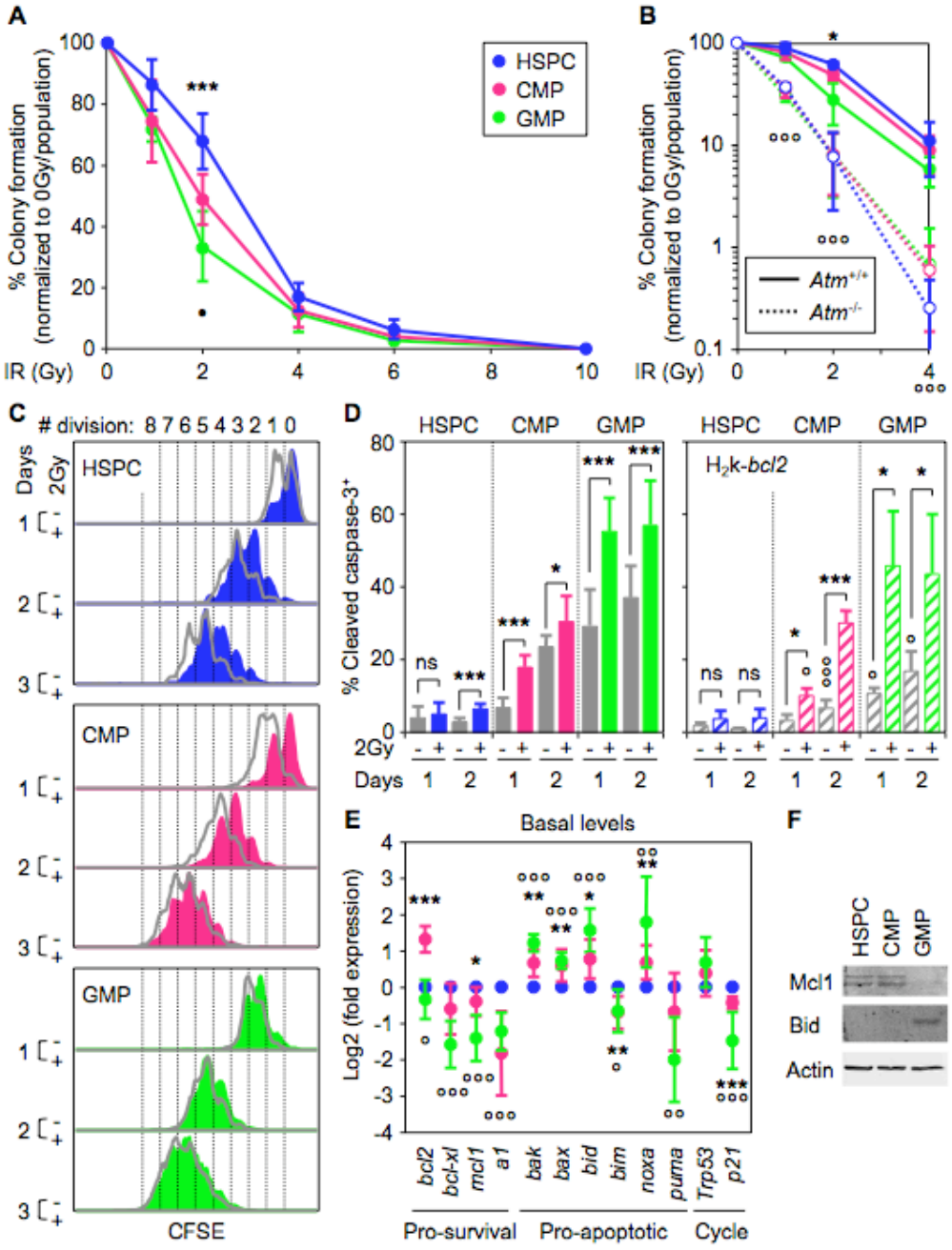

A
% Colony formation (normalized to 0Gy/population)
IR (Gy)
HSPC
CMP
GMP
B
Atm+/+
Atm-/-
C
division: 8 7 6 5 4 3 2 1 0
Days
2Gy
HSPC
CMP
GMP
CFSE
D
% Cleaved caspase-3+
H2k-bcl2
2Gy
Days
E
Basal levels
Log2 (fold expression)
bcl2
bcl-xl
mcl1
a1
bak
bax
bid
bim
noxa
puma
Trp53
p21
Pro-survival
Pro-apoptotic
Cycle
F
Mcl1
Bid
Actin

Figure 2. Dual role for p53-mediated DNA damage response in HSPCs and myeloid progenitors.

(**A**) Intracellular FACS analysis of p53 and actin protein levels in unirradiated (-IR) or 2Gy irradiated (+IR) mice 12 hours after exposure.

(**B**) QRT-PCR analysis of p53 target genes in WT cells 8 and 12 hours after 2Gy IR treatment. Results are expressed as log2 fold expression compared to levels measured in unirradiated cells cultured in the same conditions (n = 3; ***p ≤ 0.001, **p ≤ 0.01, *p ≤ 0.05) or 12 hours (n = 3; °°°p ≤ 0.001, °°p ≤ 0.01, °p ≤ 0.05).

(**C**) Clonogenic survival assay of irradiated *Trp53*$^{-/-}$ cells in methylcellulose (n = 3; **p ≤ 0.01, *p ≤ 0.05 [*Trp53*$^{-/-}$ *vs. Trp53*$^{+/+}$ cells]).

(**D**) Example of CFSE dilution assay in unirradiated (-IR: grey) or 2Gy-irradiated (+IR: blue) *Trp53*$^{+/+}$ (solid) and *Trp53*$^{-/-}$ (striped) HSPCs grown for 2 days in liquid media (n = 3).

(**E**) Intracellular cleaved caspase 3 staining in unirradiated (grey) or 2Gy-irradiated *Trp53*$^{+/+}$ (solid colors) and *Trp53*$^{-/-}$ (striped colors) cells grown for up to 2 days in liquid media (n = 3; ***p ≤ 0.001, ** p ≤ 0.01, *p ≤ 0.05 [unirradiated *vs.* irradiated cells]; °°p ≤ 0.01, °p ≤ 0.05 [*Trp53*$^{-/-}$ *vs. Trp53*$^{+/+}$ cells]; ns: not significant).

(**F**) QRT-PCR analysis of p53-target genes in *Trp53*$^{-/-}$ cells 12 hours after 2Gy IR treatment. Results are expressed as log2 fold expression compared to levels measured in unirradiated *Trp53*$^{-/-}$ cells cultured in the same conditions (n = 3).

See also Figure S2.

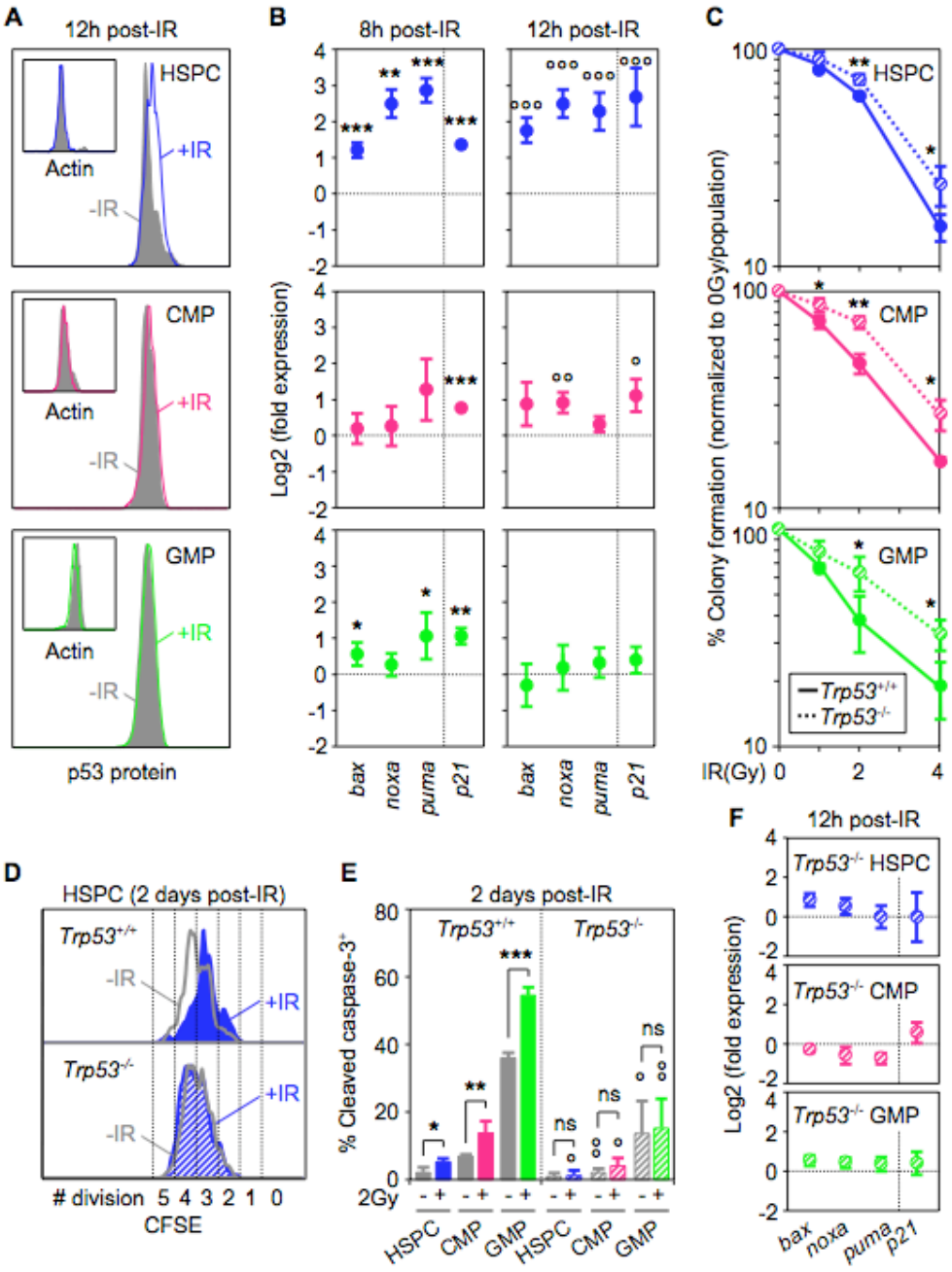

A
12h post-IR
HSPC
+IR
Actin
-IR
CMP
+IR
Actin
-IR
GMP
+IR
Actin
-IR
p53 protein
B
8h post-IR
12h post-IR
Log2 (fold expression)
bax
noxa
puma
p21
C
HSPC
CMP
GMP
% Colony formation (normalized to 0Gy/population)
Trp53+/+
Trp53-/-
IR(Gy) 0
2
4
D
HSPC (2 days post-IR)
Trp53+/+
-IR
+IR
Trp53-/-
-IR
+IR
division 5 4 3 2 1 0
CFSE
E
2 days post-IR
Trp53+/+
Trp53-/-
% Cleaved caspase-3+
2Gy
HSPC
CMP
GMP
HSPC
CMP
GMP
F
12h post-IR
Trp53-/- HSPC
Trp53-/- CMP
Trp53-/- GMP
Log2 (fold expression)
bax
noxa
puma
p21

Figure 3. Ongoing DNA repair in HSPCs versus cell elimination in myeloid progenitors.

(**A**) Immunofluorescence microscopy of ionizing radiation-induced foci (IRIF) of γH2AX in unirradiated or 2Gy-irradiated HSPCs (n = 13), CMPs (n = 8) and GMPs (n = 10). The percentage of positive cells (≥ 6 γH2AX positive foci) is shown over 24 hours (*p ≤ 0.05 [CMPs *vs*. HSPCs]; °°°p ≤ 0.001 [GMPs *vs*. HSPCs]; scale bar = 10 μm).

(**B**) Representative examples of COMET tail DNA content scoring from un-damaged (0), increasingly damaged (1-3) to very damaged (4) cells.

(**C**) Quantification of tail DNA content scores in unirradiated or 2Gy irradiated HSPCs, CMPs and GMPs after 2 and 24 hours. Results are normalized to the number of cells counted per field (n = 3; **p ≤ 0.01).

See also Table S1.

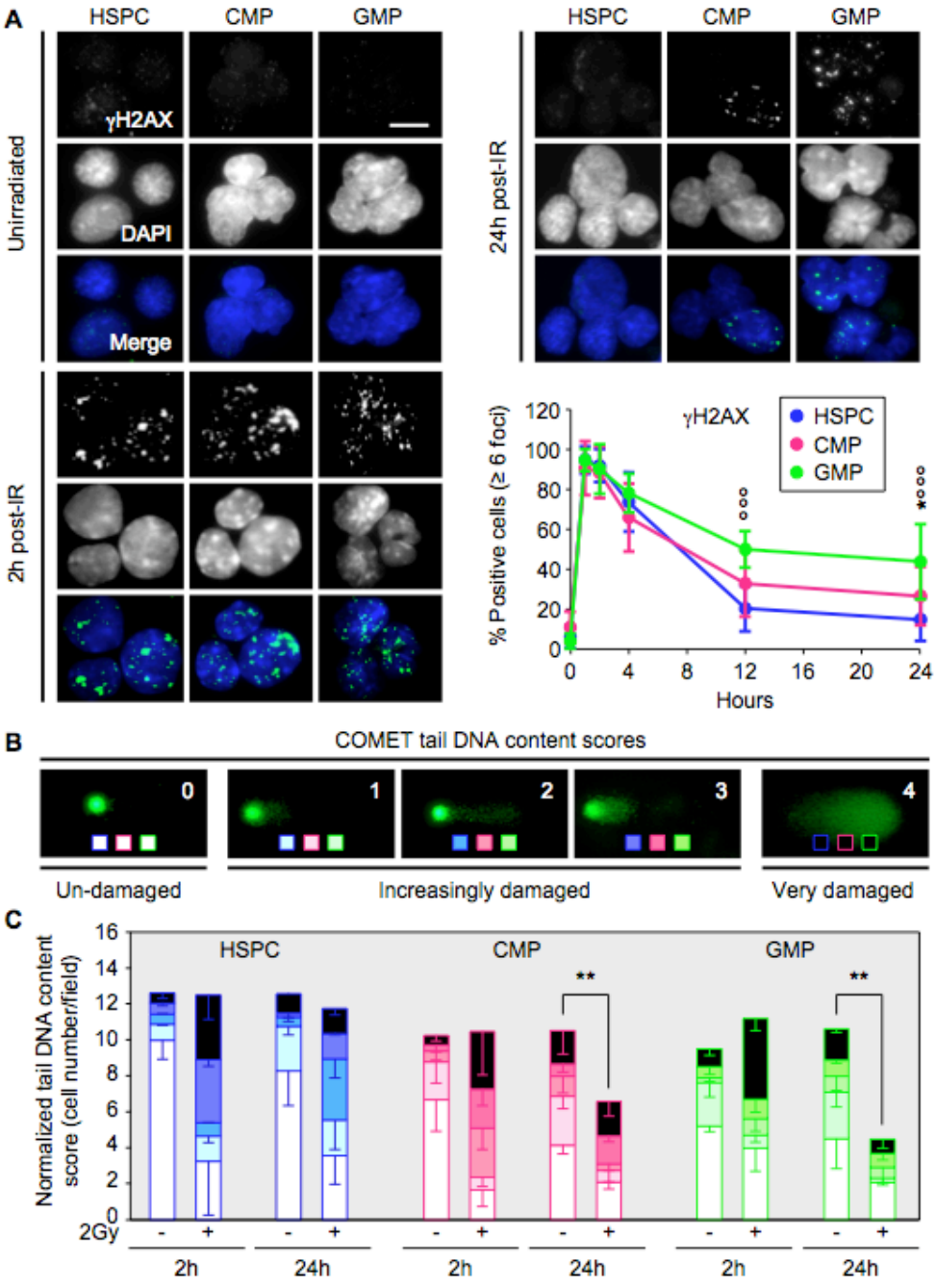

A
HSPC
CMP
GMP
Unirradiated
γH2AX
DAPI
Merge
2h post-IR
24h post-IR
γH2AX
HSPC
CMP
GMP
% Positive cells (≥ 6 foci)
Hours
B
COMET tail DNA content scores
0
1
2
3
4
Un-damaged
Increasingly damaged
Very damaged
C
Normalized tail DNA content score (cell number/field)
HSPC
CMP
GMP
2Gy
2h
24h

Figure 4. High NHEJ-mediated DNA repair mechanism in HSPCs.

(**A**) Immunofluorescence microscopy of Rad51 IRIF in unirradiated and 2Gy-irradiated HSPCs (n = 5), CMPs (n = 6) and GMPs (n = 8). The percentage of positive cells (≥ 3 Rad51 positive foci) is shown over 24 hours (***p ≤ 0.001, **p ≤ 0.01, *p ≤ 0.05 [CMPs *vs*. HSPCs]; °°°p ≤ 0.001, °p ≤ 0.05 [GMPs *vs*. HSPCs]; scale bar = 10 μm).

(**B**) Immunofluorescence microscopy of 53BP1 IRIF in unirradiated and 2Gy-irradiated HSPCs (n = 9), CMPs (n = 7) and GMPs (n = 9). The percentage of positive cells (≥ 3 53BP1 positive foci) is shown over 24 hours (***p ≤ 0.001, *p ≤ 0.05 [CMPs *vs*. HSPCs]; °°°p ≤ 0.001, °°p ≤ 0.01 [GMPs *vs*. HSPCs]; scale bar = 10 μm).

(**C**) QRT-PCR analysis of homologous recombination (HR) and nonhomologous end joining (NHEJ) DNA repair genes in freshly isolated cells. Results are expressed as log2 fold expression compared to levels measured in HSPCs (n = 3; ***p ≤ 0.001, *p ≤ 0.05 [CMPs *vs*. HSPCs]; °°°p ≤ 0.001, °°p ≤ 0.01, °p ≤ 0.05 [GMPs *vs*. HSPCs]).

(**D**) Quantification of NHEJ activity in unirradiated and 2Gy-irradiated cells. Results are expressed as fold changes upon IR-treatment (n = 5; ***p ≤ 0.001, **p ≤ 0.01).

See also Figure S3 and Figure S4.

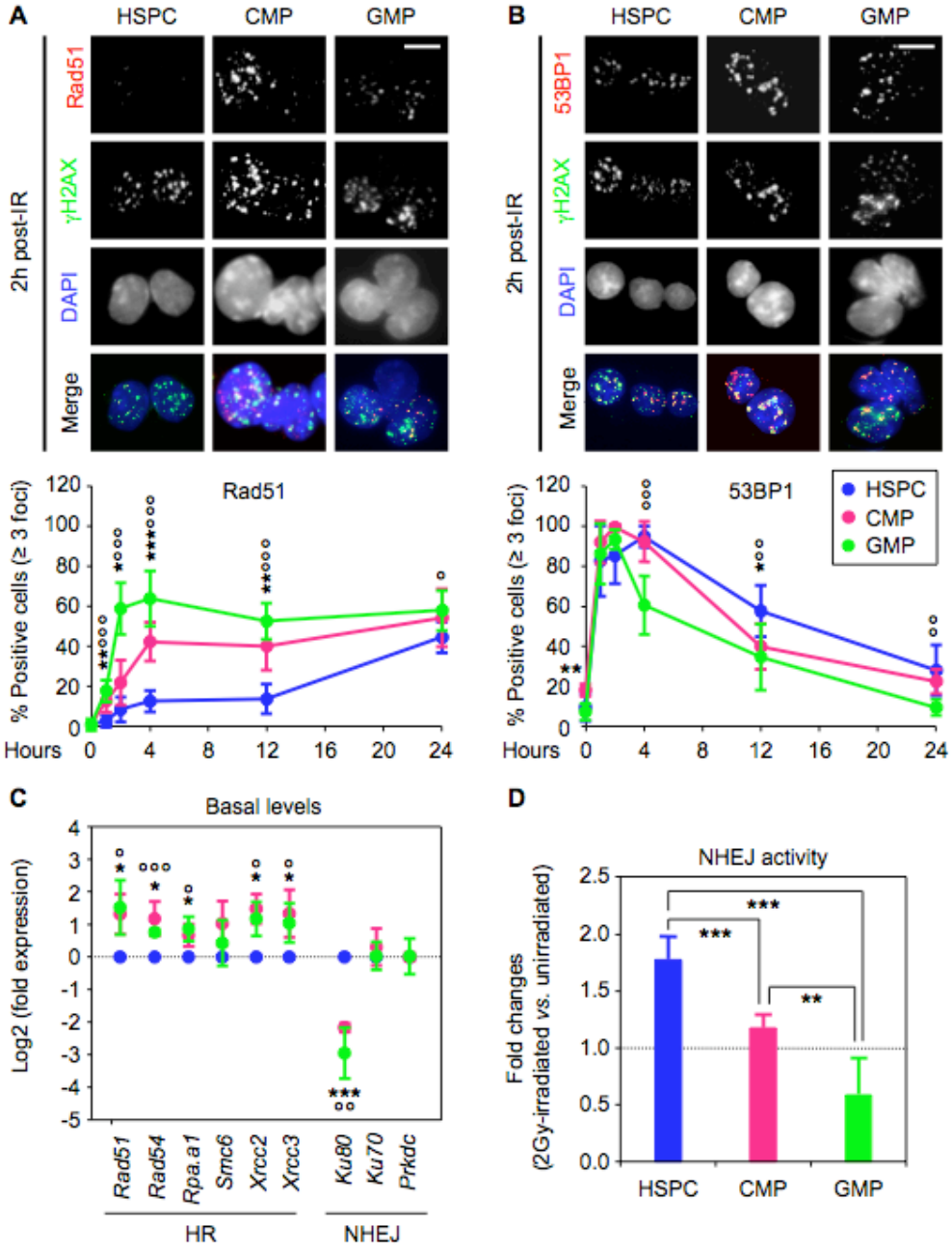

A
HSPC
CMP
GMP
2h post-IR
Rad51
γH2AX
DAPI
Merge
B
53BP1
% Positive cells (≥ 3 foci)
Hours
HSPC
CMP
GMP
C
Basal levels
Log2 (fold expression)
Rad51
Rad54
Rpa.a1
Smc6
Xrcc2
Xrcc3
Ku80
Ku70
Prkdc
HR
NHEJ
D
NHEJ activity
Fold changes
(2Gy-irradiated vs. unirradiated)

Figure 5. Similar radioresistance in quiescent and proliferating HSPCs.

(A) *In vitro* 24 hour pre-culture (24hr preC) and *in vivo* cyclosphosphamide/G-CSF mobilization (Mob.) strategies used to induce proliferation of quiescent (Rest.) HSPCs.

(**B**) Proliferation rates measured after 1 hour BrdU pulse *in vitro* (n = 3; •••p ≤ 0.001; [proliferating HSPCs *vs*. resting HSPCs]).

(**C**) Quiescence status measured by intracellular 7AAD/Pyronin Y staining.

(**D**) Clonogenic survival assay in methylcellulose (n = 3).

(**E**) Growth in liquid media (n = 3).

(**G**) Intracellular cleaved caspase 3 staining in unirradiated (grey) or 2Gy-irradiated (color) resting and proliferating HSPCs grown for up to 2 days in liquid media (n = 3; ***p ≤ 0.001, **p ≤ 0.01, *p ≤ 0.05 [proliferating HSPCs ± IR *vs*. resting HSPCs ± IR]; ns: not significant).

(**F**) Example of CFSE dilution assay in unirradiated (grey) or 2Gy-irradiated (color) quiescent and proliferating HSPCs grown for 2 days in liquid media (n = 3).

See also Figure S5.

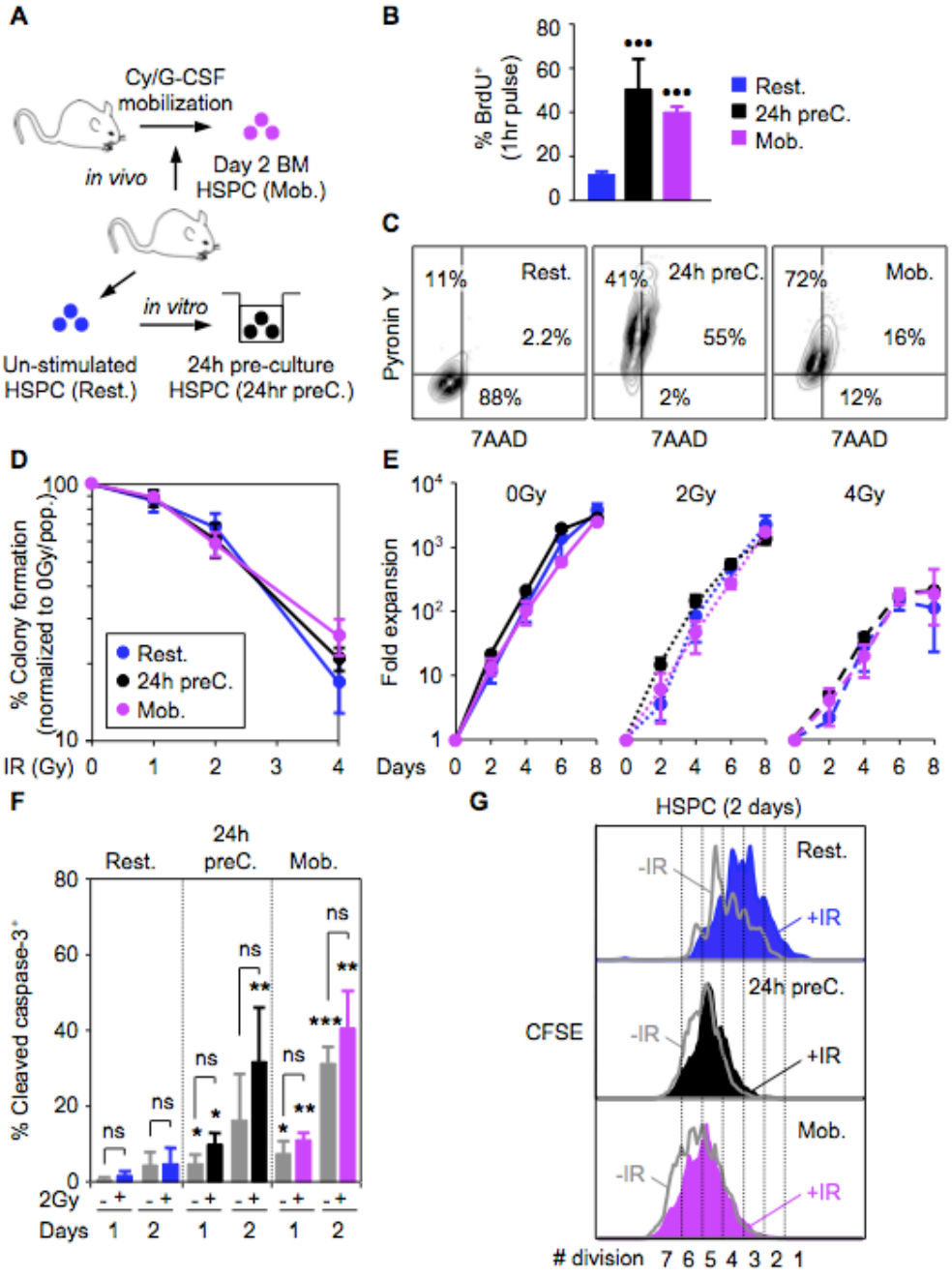

A
Cy/G-CSF mobilization
in vivo
Day 2 BM HSPC (Mob.)
in vitro
Un-stimulated HSPC (Rest.)
24h pre-culture HSPC (24hr preC.)
B
% BrdU+ (1hr pulse)
Rest.
24h preC.
Mob.
C
Pyronin Y
11%
Rest.
2.2%
88%
41%
24h preC.
55%
2%
72%
Mob.
16%
12%
7AAD
D
% Colony formation (normalized to 0Gy/pop.)
Rest.
24h preC.
Mob.
IR (Gy)
E
Fold expansion
0Gy
2Gy
4Gy
Days
F
Rest.
24h preC.
Mob.
% Cleaved caspase-3+
ns
*
**
***
2Gy
Days
G
HSPC (2 days)
-IR
+IR
Rest.
24h preC.
Mob.
CFSE
division

Figure 6. Proliferating HSPCs shift to HR-mediated DNA repair mechanism.

(**A**) Immunofluorescence microscopy of Rad51 IRIF in 2Gy-irradiated resting HSPCs (n = 5), 24h pre-cultured HSPCs (n = 3) and mobilized HSPCs (n = 5). The percentage of positive cells (≥ 3 Rad51 positive foci) is shown over 24 hours (***p ≤ 0.001, *p ≤ 0.05 [24h preC. *vs*. Rest. HSPCs]; °°°p ≤ 0.001 [Mob. *vs*. Rest. HSPCs]; scale bar = 10 μm).

(**B**) Immunofluorescence microscopy of 53BP1 IRIF in 2Gy-irradiated resting HSPCs (n = 9), 24h pre-cultured HSPCs (n = 3) and mobilized HSPCs (n = 5). The percentage of positive cells (≥ 3 53BP1 positive foci) is shown over 24 hours (***p ≤ 0.001 [24hr preC. *vs*. Rest. HSPCs]; °°°p ≤ 0.001, °°p ≤ 0.01 [Mob. *vs*. Rest. HSPCs]; scale bar = 10 μm).

(**C**) QRT-PCR analysis of HR and NHEJ repair genes in resting and proliferating HSPCs (n = 3; ***p ≤ 0.001, **p ≤ 0.01, *p ≤ 0.05 [24h preC *vs*. Rest. HSPCs]; °°°p ≤ 0.001, °°p ≤ 0.01, °p ≤ 0.05 [Mob. *vs*. Rest. HSPCs]).

(**D**) Quantification of NHEJ activity in unirradiated and 2Gy-irradiated resting and proliferating HSPCs. Results are average ± SEM (error bars) of 2 (24h preC. and Mob. HSPCs) to 5 (Rest. HSPCs) independent experiments and are expressed as fold changes upon IR-treatment (*p ≤ 0.05).

See also Figure S4 and Figure S5.

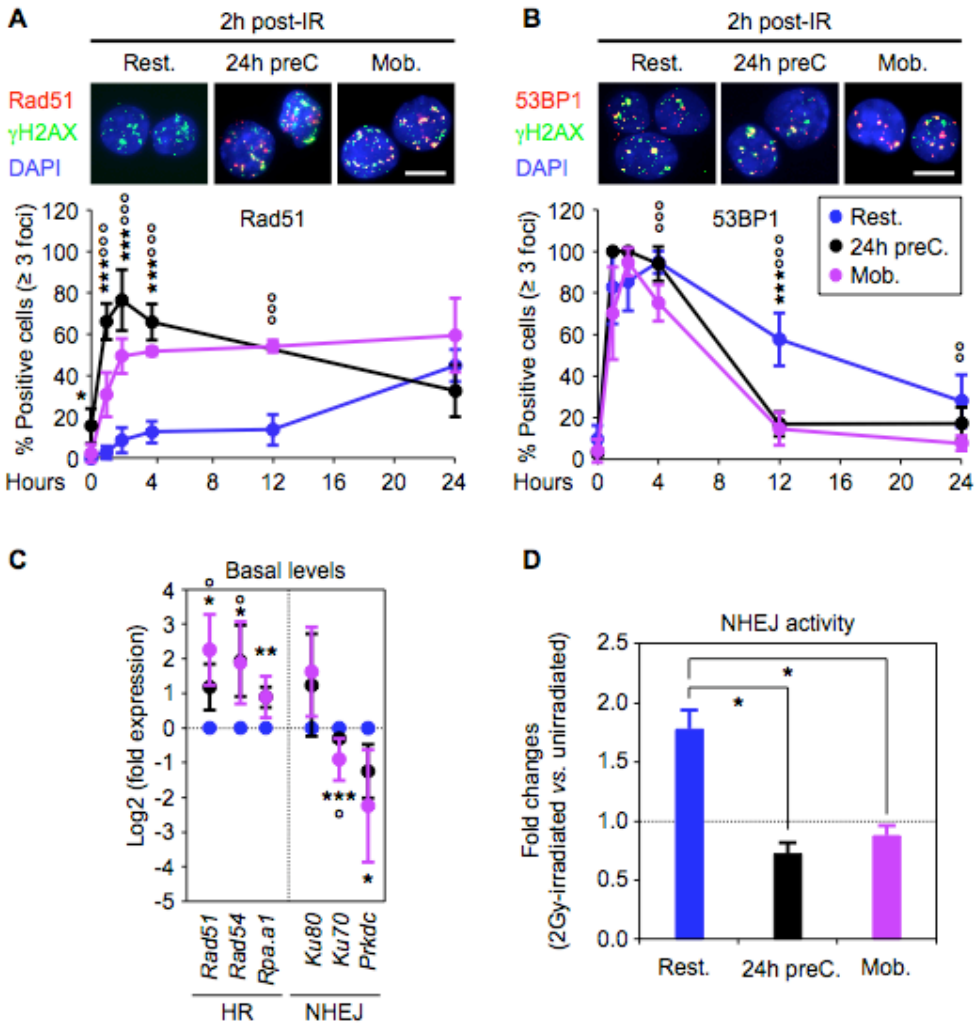

A
2h post-IR
Rest.
24h preC
Mob.
Rad51
γH2AX
DAPI
Rad51
% Positive cells (≥ 3 foci)
Hours
B
2h post-IR
Rest.
24h preC
Mob.
53BP1
γH2AX
DAPI
53BP1
Rest.
24h preC.
Mob.
% Positive cells (≥ 3 foci)
Hours
C
Basal levels
Log2 (fold expression)
Rad51
Rad54
Rpa.a1
Ku80
Ku70
Prkdc
HR
NHEJ
D
NHEJ activity
Fold changes
(2Gy-irradiated vs. unirradiated)
Rest.
24h preC.
Mob.

Figure 7. Mutagenic DNA repair in quiescent HSPCs.

(**A**) Summary of the SKY analyses performed on the *in vitro* progeny of 2Gy-irradiated quiescent HSPCs.

(**B**) Average number of genomic rearrangement (left side) and percentage (%) of aberrant cells (right side) identified by SKY analysis in the *in vitro* progeny of 2Gy-irradiated quiescent (Rest.; n = 4) and proliferating (Mob./24h preC; n = 3) HSPCs (*$p \leq 0.05$).

(**C**) Experimental design of the *in vivo* analysis of 2Gy-irradiated HSPCs assessing long-term reconstitution and genomic instability.

(**D**) Summary of the SKY analysis performed on the *in vivo* MP progeny of 2Gy-irradiated quiescent HSPCs 4 months after transplantation.

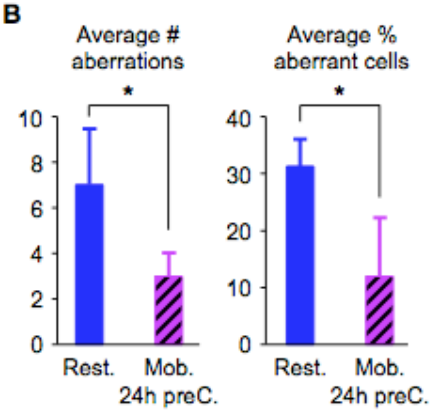
(**E**) In cohort I2, 1,500 ±IR HSPC together with 300,000 Sca-1-depleted helper bone marrow cells were transplanted per recipient (n = 5 [0Gy] and 4 [2Gy] mice per group). Long-term reconstitution was measured by sustained CD45.1 chimerism in the peripheral blood of primary transplanted mice (left graph; expressed as percent of the engraftment provided by unirradiated HSPCs) and secondary transplantation of donor-derived HSPCs re-isolated from pooled primary transplanted animals (right graph; expressed as engraftment ratio of $CD45.1^+$ cells at 4 months post-transplantation; n = 4 [0Gy] and 5 [2Gy] mice per group).

See also Figure S6, Table S2 and Table S3.

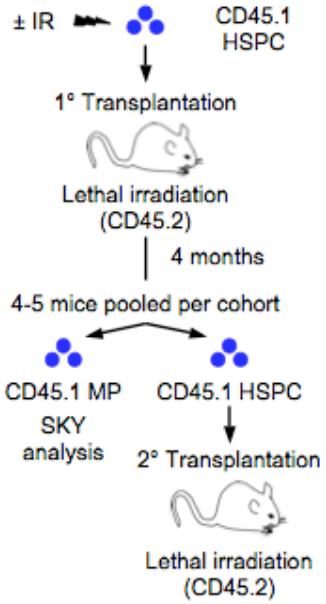
A

	in vitro progeny 2Gy-irradiated **Rest.** HSPC			
Experiments	#1	#2	#3	#4
# Cells	20	20	20	20
# Aberrations	7	4	10	7
% Aberrant cells	30%	25%	35%	35%
Mutagenic consequences	Genomic instability			

B



C



D

	in vivo MP progeny of 2Gy-irradiated **Rest.** HSPC			
Cohorts	I1	I2	I3	I4
# Cells	20	20	20	20
# Aberrations	0	1	5	4
% Aberrant cells	0%	55%	25%	20%
Mutagenic consequences	None	Clonal expansion	Genomic instability	

E

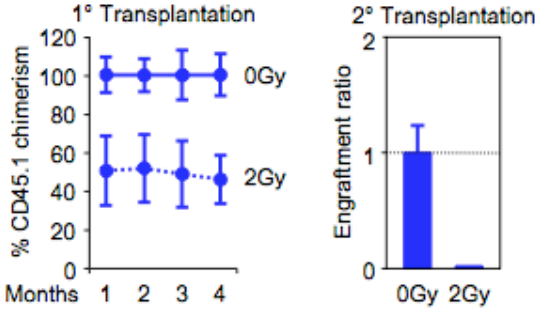


Figure S1. Effect of radiation on mouse hematopoietic stem cells and myeloid progenitors, related to Figure 1.

(A) Gating strategy used to isolate HSPCs, CMPs and GMPs from mouse bone marrow.

(B) Growth of unirradiated and irradiated HSPCs, CMPs and GMPs in liquid media (n = 5 to 9; ***p ≤ 0.001; **p ≤ 0.01; *p ≤ 0.05 [2Gy *vs*. 0Gy]; °°°p ≤ 0.001; °p ≤ 0.05 [4Gy *vs*. 2Gy]).

(**C**) *In vivo* effects of 2Gy irradiation. Mice were analyzed after 12h and 24h post-irradiation (IR) and compared to unirradiated mice (0h) for changes in the frequency of the indicated bone marrow populations analyzed by flow cytometry. The fold change is indicated for each population.

(**D**) Growth of irradiated Slam-HSCs and HSPCs in liquid media (n = 3).

(**E**) Clonogenic survival assay in methylcellulose comparing the radioresistance of the most immature Slam-HSC and HSPC (n = 3).

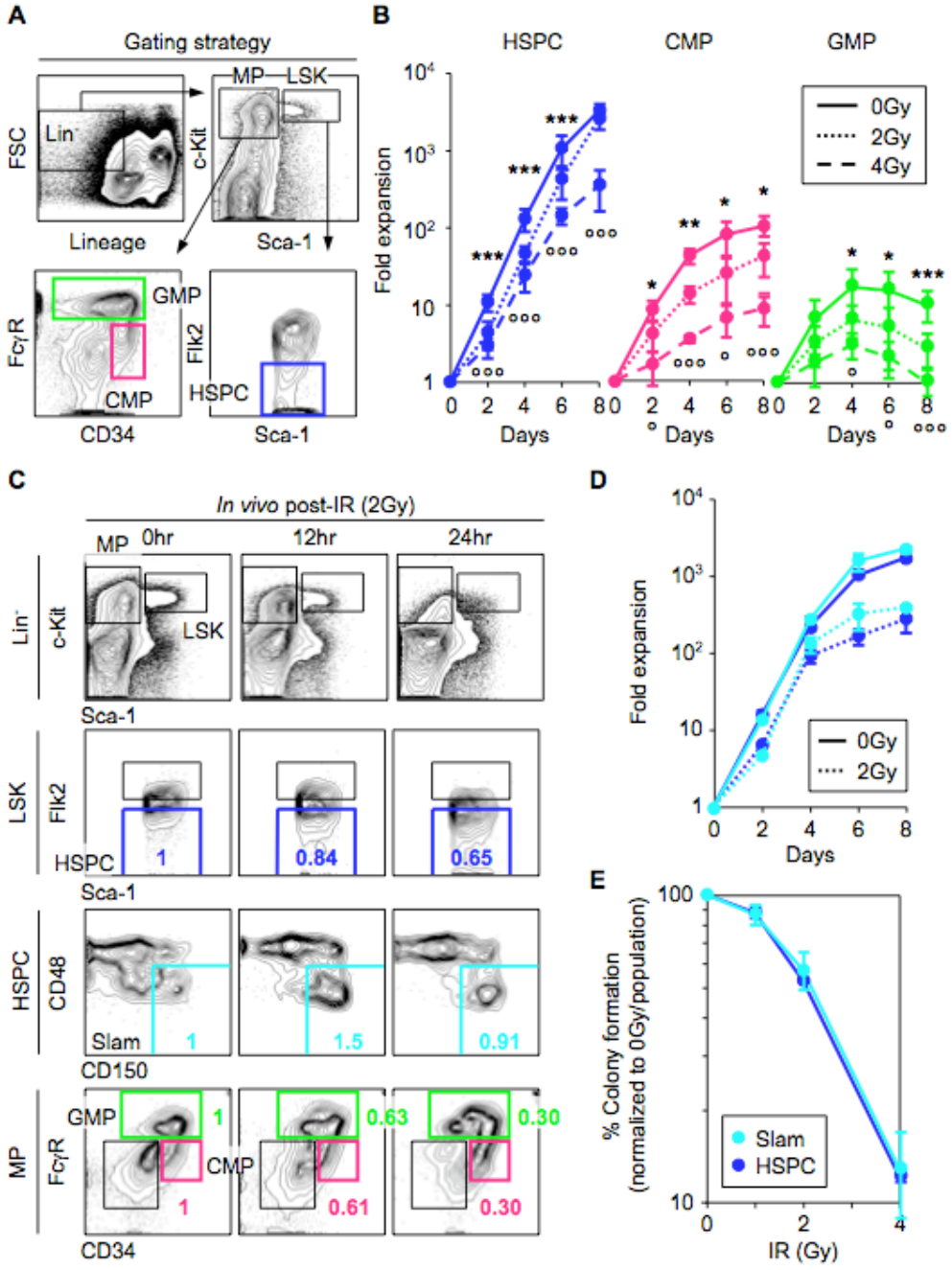

A
Gating strategy
MP
LSK
FSC
Lin-
c-Kit
Lineage
Sca-1
GMP
FcγR
CMP
CD34
Flk2
HSPC
Sca-1
B
HSPC
CMP
GMP
Fold expansion
0Gy
2Gy
4Gy
Days
C
In vivo post-IR (2Gy)
MP
0hr
12hr
24hr
Lin-
c-Kit
LSK
Sca-1
LSK
Flk2
HSPC
1
0.84
0.65
Sca-1
HSPC
CD48
Slam
1
1.5
0.91
CD150
MP
GMP
1
0.63
0.30
FcγR
CMP
1
0.61
0.30
CD34
D
Fold expansion
0Gy
2Gy
Days
E
% Colony formation
(normalized to 0Gy/population)
Slam
HSPC
IR (Gy)

Figure S2, p53 activation levels in irradiated HSPCs and GMPs, related to Figure 2.

Western blot analysis of phospho-p53 (Ser15) protein levels in unirradiated (0 hour) and 2Gy-irradiated HSPCs and GMPs 1 and 4 hours after exposure (proteins extracted from 70,000 isolated cells per lane; b-actin is used as loading control).

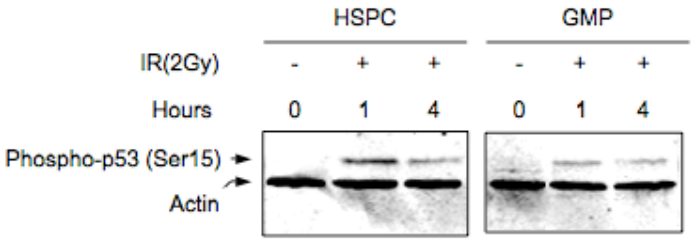

HSPC
GMP
IR(2Gy)
- + + - + +
Hours
0 1 4 0 1 4
Phospho-p53 (Ser15)
Actin

Figure S3, Proliferation and cells cycle status of HSPCs and MPs, related to Figure 4.

(**A**) Basal proliferation rates of unirradiated HSPCs (blue), CMPs (pink) and GMPs measured after 1 hour BrdU pulse *in vitro* (n = 3). The grey shapes represent BrdU staining on cells that have not been incubated with BrdU.

(**B**) Example of combined surface and intracellular 7AAD/Pyronin Y (PY) staining for analysis of cell cycle distribution in HSPCs, $Flk2^+$ LSK multipotent progenitors (MPP) and $Lin^-/Sca\text{-}1^-/c\text{-}kit^+$ MPs. Quiescent G_0 cells are $7AAD^{2n}/PY^-$ while proliferative G_1 and $S\text{-}G_2/M$ cells are $7AAD^{2n}/PY^+$ and $7AAD^{\geq 2n\text{-}4n}/PY^+$, respectively.

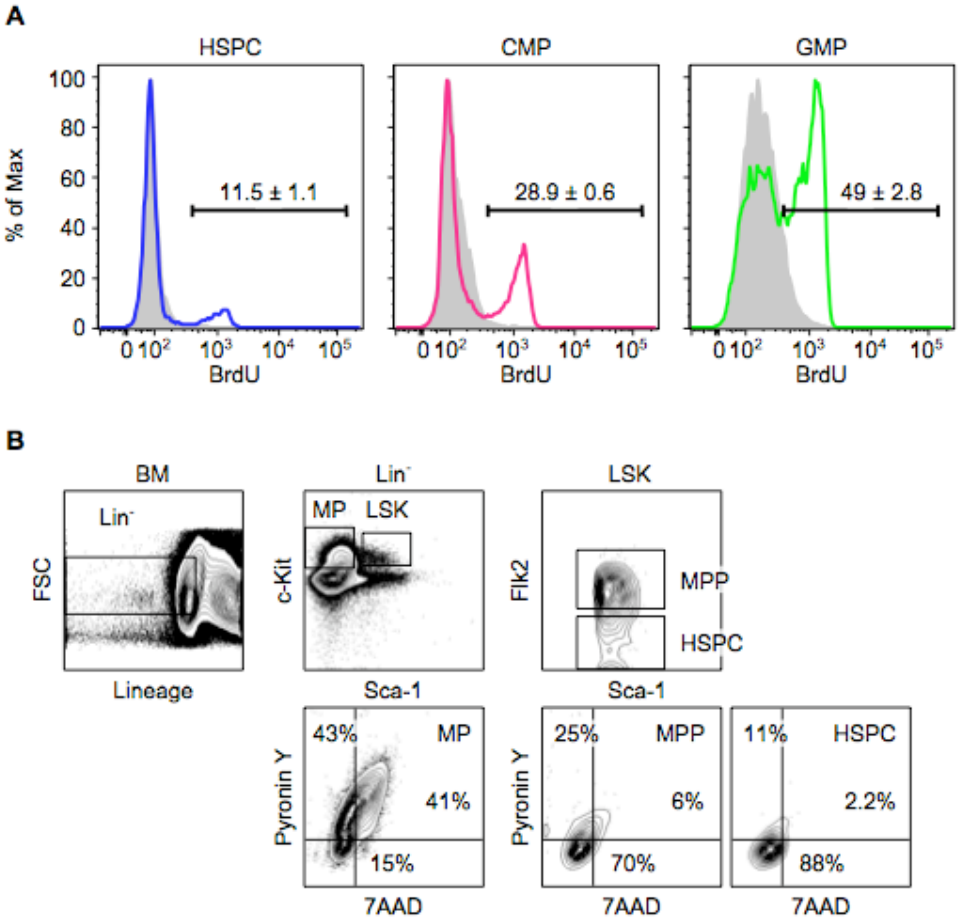

A
HSPC
CMP
GMP
% of Max
100
80
60
40
20
0
11.5 ± 1.1
28.9 ± 0.6
49 ± 2.8
0 10^2 10^3 10^4 10^5
BrdU
B
BM
Lin-
FSC
Lineage
Lin-
MP LSK
c-Kit
LSK
Flk2
MPP
HSPC
Sca-1
Pyronin Y
43%
MP
41%
15%
7AAD
Sca-1
25%
MPP
6%
70%
7AAD
11%
HSPC
2.2%
88%
7AAD

Figure S4, Assessment of NHEJ activity in HSPCs and MPs, related to Figure 4 and Figure 6.

(**A**) Experimental scheme of the NHEJ activity assay.

(**B**) Representative example of NHEJ activity in unirradiated (grey) or 2Gy-irradiated HSPCs (blue), CMPs (pink) and GMPs (green). Single measurement was performed for each condition. Fold changes upon IR-treatment were calculated as [% GFP in 2Gy-irradiated cells]/[% GFP in unirradiated cells].

(**C**) Representative example of NHEJ activity in unirradiated (grey) or 2Gy-irradiated (color) resting (blue) and proliferating (24h preC, black; Mob., purple) HSPCs.

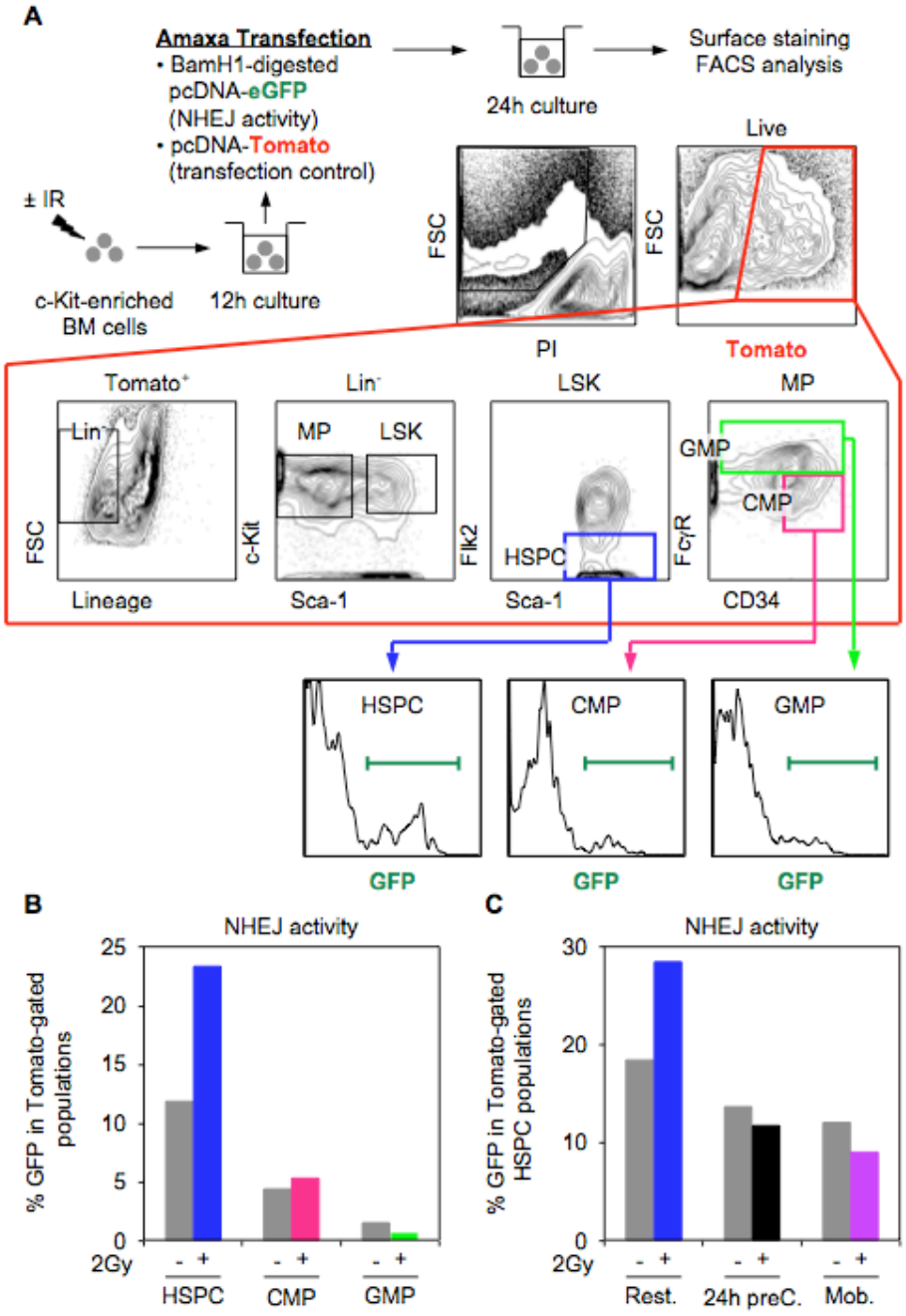

A
Amaxa Transfection
• BamH1-digested pcDNA-eGFP (NHEJ activity)
• pcDNA-Tomato (transfection control)
± IR
c-Kit-enriched BM cells
12h culture
24h culture
Surface staining FACS analysis
Live
FSC
PI
Tomato
Tomato+
Lin-
MP
LSK
GMP
CMP
HSPC
c-Kit
Flk2
FcγR
Lineage
Sca-1
CD34
GFP
B
NHEJ activity
% GFP in Tomato-gated populations
2Gy
HSPC
CMP
GMP
C
NHEJ activity
% GFP in Tomato-gated HSPC populations
Rest.
24h preC.
Mob.

Figure S5, Increased basal apoptosis in proliferating HSPCs, related to Figure 5 and Figure 6.

(**A**) QRT-PCR analysis of basal levels in resting and proliferating HSPCs. Results are expressed as log2 fold expression compared to levels measured in resting HSPCs (n = 3; ***p ≤ 0.001, **p ≤ 0.01, *p ≤ 0.05 [24h preC *vs*. Rest. HSPCs]; °°°p ≤ 0.001, °°p ≤ 0.01, °p ≤ 0.05 [Mob. *vs*. Rest. HSPCs]).

(**B**) QRT-PCR analysis of p53-target genes in resting (Fig. 2a; p ≤ 0.001 for all genes) and proliferating HSPCs 12 hours after 2Gy IR treatment. Results are expressed as log2 fold expression compared to levels measured in the same unirradiated cells cultured for 12 hours *in vitro* (n = 3; ***p ≤ 0.001, **p ≤ 0.01, *p ≤ 0.05 [irradiated *vs*. unirradiated cells].

(**C**) Immunofluorescence microscopy of γH2AX IRIF in unirradiated and 2Gy-irradiated resting and proliferating HSPCs. The percentage of positive cells (≥ 6 γH2AX positive foci) is shown over 24 hours (n = 3 to 5; scale bar = 10 μm).

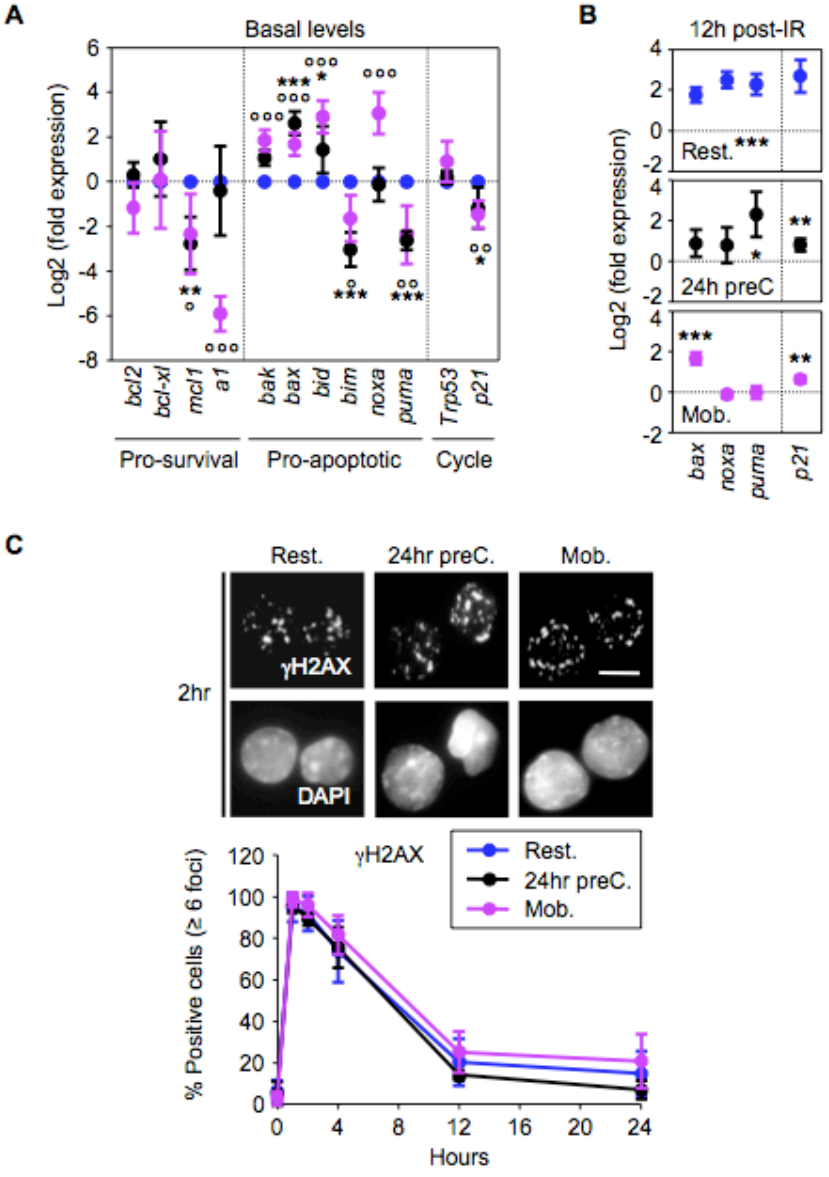
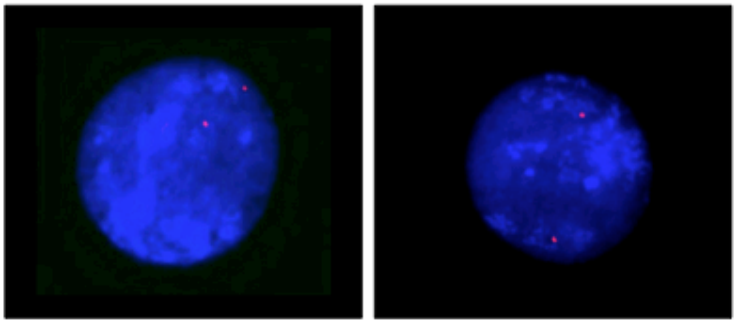

A
Basal levels
Log2 (fold expression)
bcl2
bcl-xl
mcl1
a1
bak
bax
bid
bim
noxa
puma
Trp53
p21
Pro-survival
Pro-apoptotic
Cycle
B
12h post-IR
Rest.
24h preC
Mob.
bax
noxa
puma
p21
C
Rest.
24hr preC.
Mob.
2hr
γH2AX
DAPI
γH2AX
Rest.
24hr preC.
Mob.
% Positive cells (≥ 6 foci)
Hours

Figure S6, FISH analysis of a mouse common fragile site region, related to Figure 7. (**A**) Example of hybridization with a probe for the mouse *Fhit* locus common fragile site. FISH analyses were performed on the progeny of unirradiated or 2Gy-irradiated resting HSPCs expanded in culture for 4 days. Interphase nuclei were counterstained with DAPI. (**B**) Quantification of the distribution of *Fhit* FISH signals in two independent experiments.

A



B

Treatment		1*	2	3	4	>4	# cells analyzed
Exp. I							
HSPC-derived cells	0Gy	3	190	1	7	0	201
	2Gy	4	192	1	1	2	200
Exp. II							
HSPC-derived cells	0Gy	14	180	5	2	0	200
	2Gy	8	196	9	5	0	218

Figure S7, Model describing the molecular mechanism and mutagenic consequences of DNA repair in HSCs and myeloid progenitors, related to all Figures. Long-lived HSCs have unique cell-intrinsic mechanisms ensuring their survival in response to genotoxic stress such as ionizing radiation (IR) that are independent of their cell cycle status. Quiescent HSCs have enhanced pro-survival gene expression and robust p53-mediated induction of *p21* expression, which together buffer the strong accompanying induction of *bcl2*-family pro-apoptotic genes leading essentially to growth arrest, survival and DNA repair. Proliferative HSCs, like myeloid progenitors, have decreased pro-survival gene expression and attenuated p53-mediated DNA damage response leading to limited induction of both *p21* and *bcl2*-family pro-apoptotic genes. As a consequence myeloid progenitors predominantly die in response to IR treatment, while proliferating HSCs engages additional, still unknown, protective mechanism(s) that ensure their survival. Quiescent HSCs are restricted to using the error prone nonhomologous end-joining (NHEJ) mechanism to repair DNA damage due to their cell cycle status and molecular wiring of their DNA repair machinery, which renders them susceptible to genomic instability associated with misrepaired DNA. In contrast, proliferating HSCs, like the few surviving MPs, undergo DNA repair using the high fidelity homologous recombination (HR) mechanism and have a significantly decreased risk of acquiring mutation(s). Our results suggest that quiescent HSCs (either normal or leukemic) are intrinsically

vulnerable to mutagenesis and represent an important source for leukemia development and bone marrow failure.

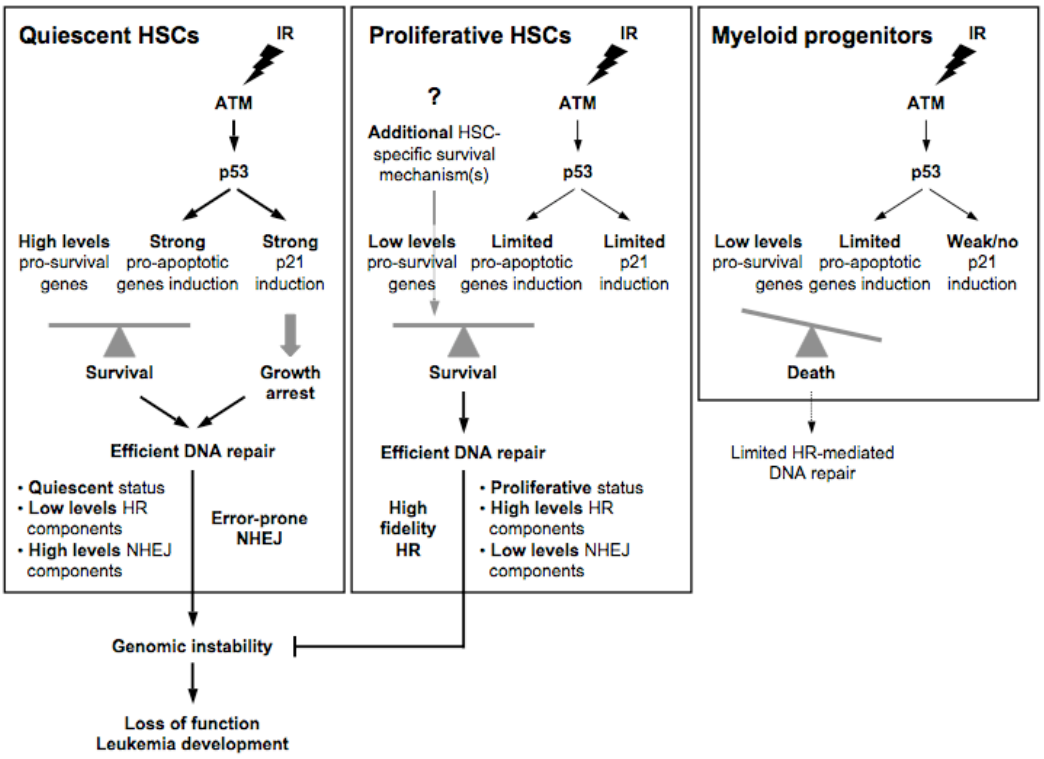

Quiescent HSCs
IR
ATM
p53
High levels pro-survival genes
Strong pro-apoptotic genes induction
Strong p21 induction
Survival
Growth arrest
Efficient DNA repair
• Quiescent status
• Low levels HR components
• High levels NHEJ components
Error-prone NHEJ
Proliferative HSCs
IR
?
Additional HSC-specific survival mechanism(s)
ATM
p53
Low levels pro-survival genes
Limited pro-apoptotic genes induction
Limited p21 induction
Survival
Efficient DNA repair
High fidelity HR
• Proliferative status
• High levels HR components
• Low levels NHEJ components
Myeloid progenitors
IR
ATM
p53
Low levels pro-survival genes
Limited pro-apoptotic genes induction
Weak/no p21 induction
Death
Limited HR-mediated DNA repair
Genomic instability
Loss of function
Leukemia development

Table S1, DNA repair following irradiation *in vitro*, related to Figure 3.

Results of alkaline COMET assays performed on HSPCs, CMPs and GMPs 2 hours and 24 hours after exposure to 2Gy irradiation. Scoring, normalization for the numbers of visual fields counted and calculation of COMET tail DNA content (arbitrary units) was performed as described in the detailed Experimental Procedures section. Results are expressed as average ± SD (n = 3 independent experiments) and *p* values are indicated when differences are considered to be statistically significant.

Table S1

	Average cell #/field	Normalized COMET tail DNA content				
		0	1	2	3	4
2Gy-irradiated HSPC						
2h post-IR	12.5 ± 1.6	3.3 ± 3.0	1.4 ± 0.4	0.8 ± 0.1	3.5 ± 0.4	3.6 ± 1.3
24h post-IR	11.8 ± 1.9	3.6 ± 1.6	2.0 ± 1.7	3.4 ± 1.1	1.4 ± 0.1	1.4 ± 0.4
p value				*p ≤ 0.05*	*p ≤0.001*	*p ≤ 0.05*
2Gy-irradiated CMP						
2h post-IR	10.4 ± 0.6	1.7 ± 1.0	0.7 ± 0.6	2.7 ± 1.2	2.2 ± 0.9	3.2 ± 2.4
24h post-IR	6.6 ± 1.1	2.1 ± 0.4	0.6 ± 0.7	0.4 ± 0.4	1.6 ± 0.4	1.9 ± 0.9
p value	*p ≤ 0.01*			*p ≤ 0.05*		
2Gy-irradiated GMP						
2h post-IR	11.2 ± 0.8	4.0 ± 1.3	0.7 ± 0.4	0.9 ± 0.7	1.1 ± 0.8	4.5 ± 0.7
24h post-IR	4.5 ± 1.6	2.1 ± 0.2	0.2 ± 0.4	0.6 ± 0.9	0.8 ± 0.4	0.8 ± 0.5
p value	*p ≤ 0.01*					*p ≤ 0.01*

Table S2, Genetic rearrangements following irradiation *in vitro,* related to Figure 7.

SKY cytogenetic analyses were performed on the progeny of 2Gy-irradiated resting HSPCs (n = 4) or proliferating HSPCs (n = 3; two mobilized HSPCs and one 24h pre-cultured HSPCs) expanded in culture for 4 and 5 days, respectively. [*]A mixture of male and female mice were used to isolate HSPCs. [§]Cells with numerical abnormalities only are not included in the percent of cells with aberrations.

Table S2

Resting HSPC (0Gy)		
Un.1:	# cells analyzed:	20
	# aberrations:	0
	% of cells with aberrations:	0%
	Aberrations:	None
	Karyotype:	40,XX[20]
Un.2:	# cells analyzed:	20
	# aberrations:	0
	% of cells with aberrations:	0%
	Aberrations:	None
	Karyotype*:	40,XX[12]/40,XY[8]
Resting HSPC (2Gy)		
Rest.1:	# cells analyzed:	20
	# aberrations:	7
	% of cells with aberrations:	30%
	Aberrations:	Reciprocal translocations (3), interstitial deletions (2), chromosome gaps/breaks (2)
	Karyotype:	40,XX[14]/40,XX,t(2;9)(C2;E3)[1]/39,X,-X,t(1;17)(H3;A2)[1]/40,XX,del(6)(B3D)[1]/40,X,del(X)(A2D)[1]/40,X,t(X;4)(F1;B2)[1]/39,XX,chrg(2)(F3),chrg(3) (F3)[1]
Rest.2:	# cells analyzed:	20
	# aberrations:	4
	% of cells with aberrations:	25%
	Aberrations:	Interstitial deletions (1), dicentrics (1) and ring (1) chromosomes, acentric fragments (4)
	Karyotype:	40,XX[15]/40,XX,del(6)(A3G2)[2]/39,XX,dic(10;18)(A2;A2)[1]/48,X,r(X)(A?1?D),+r(X)(A?1?D)x9,del(6) (A3G2),-9[1]/80,XXXX,del(6)(A3G2)x2, +4ace[1]

Rest.3:	# cells analyzed:	20
	# aberrations:	10
	% of cells with aberrations:	35%[§]
	Aberrations:	Reciprocal translocations (2), interstitial deletions (3), chromosome gaps/breaks (4), acentric fragment (2)
	Karyotype:	40,XX[13]/80,XXXX,chrb(1)(D)[1]/40,XX,chtb(12)(C2),t(17;18)(B;E1)[1]/40,XX,t(3;11)(H3;A4),del(6)(C2G2)[1]/40,XX,+der(11)(E1E2)x2,-15,+19[1]/44,XX,+X,+8,+13,+17[1]/40,XX,del(6)(A1D),der(9) chrb(9)(D)del(9)(A2D)[1]/40,XX,chrg(1)(E3)[1]
Rest.4:	# cells analyzed:	20
	# aberrations:	7
	% of cells with aberrations:	35%
	Aberrations:	Complex four-break rearrangement (2), interstitial deletion (2), reciprocal translocations (2), unbalanced translocations (1)
	Karyotype[*]:	40,XX[9]/40,XY[4]/80,XXXX[1]/40,XY,t(12;16)(12;15)(D1;B3;E;E)[1]/40,XX,t(2;16)(A2;C1)[1]/40,XY, t(10;18)(C1;E1)[1]/36,XY,-1,-5,-6,-8,t(9;10)(10;6)(D;A1;C3;B1)[1]40,XX,del(19)(BD1)[1]/40,X,t(X;8) (B;B2)[1]
Proliferating HSPC (2Gy)		
Mob1:	# cells analyzed:	21
	# aberrations:	4
	% of cells with aberrations:	23.8%
	Aberrations:	Reciprocal translocations (1), deletions (1), chromosome gaps/breaks (2)
	Karyotype:	40,XX[16]/40,XX,t(1;4)(D;D3)[1]/40,XX,del(6)(A2D)[1]/41,XX,+17[1]/40,XX,chrb(5)(D)[1]/40,XX,chrb(2)(F3)[1]
Mob2:	# cells analyzed:	14
	# aberrations:	2
	% of cells with aberrations:	7.1%
	Aberrations:	Interstitial deletions (1), unbalanced translocations (1)
	Karyotype:	40,XX[13]/40,X,del(X)(A2F3),der(19)t(8;19)(A4;D1)[1]

24h preC:	# cells analyzed:	20
	# aberrations:	3
	% of cells with aberrations:	5%
	Aberrations:	Interstitial deletions (2), unbalanced translocations (1)
	Karyotype:	40,XY[19]/41,XY,+3,del(3)(BH4),der(3)del(3)(A1A2)t(3;3)(A1;F3)[1]

Table S3, Genetic rearrangements persisting *in vivo*, related to Figure 7.

Donor-derived ($CD45.1^+$) MPs ($Lin^-/c\text{-}Kit^+/Sca\text{-}1^-$) were isolated from mice reconstituted with 2Gy-irradiated HSPCs 4 months after transplantation. SKY cytogenetic analyses were performed on the progeny of these cells expanded overnight in culture. [*]A mixture of male and female mice were used to isolate donor HSPCs. [§]Cells with random loss of chromosome are not included in the percent of cells with aberrations.

Table S3

Unirradiated HSPCs		
Cohort U1	# cells analyzed:	20
	# aberrations:	1
	% of cells with aberrations:	5%
	Aberrations:	Acentric fragment (1)
	Karyotype*:	40,XX[10]/40,XY[9]/40,XY,+ace[1]
Cohort U2	# cells analyzed:	20
	# aberrations:	0
	% of cells with aberrations:	0%
	Aberrations:	none
	Karyotype*:	40,XY[22]/40,XX[1]
2Gy-irradiated HSPCs		
Cohort I1:	# cells analyzed:	20
	# aberrations:	0
	% of cells with aberrations:	0%§
	Aberrations:	None
	Karyotype*:	40,XX[3]/40,XY[16]/35,X,-X or -Y,-5,-7,-13, -19[1]
Cohort I2:	# cells analyzed:	20
	# aberrations:	1
	% of cells with aberrations:	55%
	Aberrations:	Balanced translocation
	Karyotype*:	40,XX[3]/40,XY[6]/40,XY,t(16;17)(C1;E4)[11]
Cohort I3:	# cells analyzed:	20
	# aberrations:	5
	% of cells with aberrations:	25%
	Aberrations:	Balanced translocation (1), chromosome gaps/breaks (4)
	Karyotype:	40,XY[15]/40,XY,t(9;18)(F1;E1)[1]/40,XY, chtb(12)(F1)[1]/40,XY,chrg(5)(D)[1]/40,XY, chrg(10)(C1)[1]/40,XY,chtb(3)(F1)[1]

Cohort I4:	# cells analyzed:	20
	# aberrations:	4
	% of cells with aberrations:	20%
	Aberrations:	Balanced translocation (1), inversion (1), chromosome gaps/breaks (2)
	Karyotype:	40,XY[17]/40,XY,inv(1)(A1C2)[1]/40,XY,chtb(1)(B),t(2;10)(H3;C1)[1]/40,XY,chtb(2)(B)[1]

Table S4, QRT-PCR Primer Table.

The range of Ct values (± SD) obtained for each gene when performing qRT-PCR analysis with the cDNA equivalent of 200 cells (HSPCs, CMPs or GMPs) per reaction is indicated.

Table S4

Gene	Forward (5' to 3')	Reverse (5' to 3')	Accession	Ct range
A1	ccctgqctgaqcactacctt	ctqcatgcttggcttgga	NM_009742	31.3 ± 1.3
Bak	aatggcatctggacaaggac	gttcctgctggtggaggtaa	NM_007523.1	21.9 ± 1.2
Bax	tggagctgcagaggatgattg	agctgccacccggaaga	NM_007527	25.1 ± 0.9
Bcl2	tgggatgcctttgtggaact	acagccaggagaaatcaaacag	NM_009741	24.3 ± 0.8
Bcl-xl	ggctgggacacttttgtggat	gcgctcctggcctttcc	NM_009743	26.4 ± 0.4
Bid	gaagacgagctgcagacagatg	aatctggctctattcttccttggtt	NM_007544.3	27.8 ± 1.9
Bim	ttggagctctgcggtcctt	cagcggaggtggtgtgaat	NM_009754	27.6 ± 0.4
Ku70	ggagtcaagcaagctggaaga	agaactcgctttttggtctcctt	NM_010247.2	27.1 ± 0.3
Ku80	gacttgcggcaatacatgttttc	aagctcatggaatcaatcagatca	NM_009533	27.7 ± 0.9
Mcl1	ccctcccccatcctaatcag	agtaacaatggaaagcatgccaat	NM_008562	26.4 ± 0.5
Noxa	ggagtgcaccggacataact	ttgagcacactcgtccttca	NM_021451.1	28.4 ± 0.8
Prkdc	gcccatgagcttaggtttcaat	ctaagagctttcagcaggttcaca	NM_011159.2	27.2 ± 0.4
Puma	gcggcggagacaagaaga	agtcccatgaagagattgtacatgac	NM_133234	28.8 ± 0.4
p21	ttccgcacaggagcaaagt	cggcgcaactgctcact	NM_007669	26.2 ± 0.8
Rad51	aagttttggtccacagcctattt	cggtgcataagcaacagcc	NM_011234.4	28.0 ± 1.1
Rad54	ccaggtccaggagtgtttcc	ggccggttgagtagctgagt	NM_009015.3	26.9 ± 1.1
Rpa.a1	acatccgtcccatttctacagg	ctccctcgaccagggtgtt	NM_026653.1	27.7 ± 0.8
Smc6	ccgtgtgcttcatcctttcc	ccatatccatgtagacatcaaactca	NM_025695.4	27.9 ± 0.6
Trp53	aagatccgcgggcgtaa	catcctttaactctaaggcctcattc	NM_011640.1	22.8 ± 0.8
Xrcc2	ggaaaggcccacatgtgagt	ggatcgtttgtgacataggcatt	NM_020570.2	29.7 ± 1.0
Xrcc3	cctgaggagctgatcgagaaga	cggccgcgtgttcaat	NM_028875.2	28.6 ± 0.9
b-actin	gacggccaggtcatcactattg	aggaaggctggaaaagagcc	NM_007393	21.7 ± 0.3

CHAPTER 3

DNA DAMAGE RESPONSE IN HUMAN AND MURINE HSCS

Introduction

The hematopoietic system is both the most radiosensitive tissue and the source of the most common therapy related neoplasms, as such there has been much postulation on the importance of the DNA damage response (DDR) and repair pathways in hematopoietic cells. Specifically, there has been interest in elucidating the differential response to IR seen in HSCs and progenitor cells and in determining the mechanisms that allow HSCs and progenitor cells to maintain both cellular and tissue homeostasis, while limiting mutagenic consequences when coping with stress (Meijne *et al*., 1991; Ploemacher *et al*., 1992; Inoue *et al*., 1995). The very characteristics that define the importance of HSCs - long-lived, capable of both self-renewal and differentiation - also leave them incredibly vulnerable to accumulation of DNA mutations and the resulting loss of function and potential for transformation that could be passed along to their progeny. Thus, it has been long hypothesized that these rare and essential HSCs have specific protective mechanisms to limit the deleterious effects of DNA damage. It has been shown that with age, DNA damage accumulates, HSC function declines, and hematological malignancy rates increase, all suggesting that while HSCs may survive DNA damaging insults they do not emerge unscathed (Rossi *et al*., 2008). Our work and the recent work from John Dick's lab has begun to clarify the ways both human and mouse HSCs deal with exposure to ionizing radiation (IR) (Milyavsky *et al*., 2010; Mohrin *et al*., 2010).

Due to ethical and technical constraints, much of our knowledge about HSC biology comes from experiments using the mouse hematopoietic system. The confluence of advances in antibody and flow cytometry technologies combined with the use of congenic transplantation systems allowed for the isolation of murine HSCs in the 1990s (Huntly and Gilliland, 2005; Park *et al.*, 2008; Spangrude *et al.*, 1998). Since then, the murine HSC immunophenotype has been increasingly refined. The most pure population of murine HSCs isolatable to date are characterized immunophenotypically as $Lin^-/c\text{-}Kit^+/Sca\text{-}1^+/Flk2^-/CD48^-/CD150^+$ and can be isolated to 50% purity (Kiel *et al.*, 2005). The ease of identification and purification of murine HSC and progenitor populations immunophenotypically combined with relatively easy genetic manipulation of mice allows for their in depth characterization. While much is known about murine HSCs, the understanding of human HSCs has lagged behind. Using *in vitro* and new and improved *in vivo* techniques, human HSCs can be isolated to 10% purity (Majeti *et al.*, 2007; Park *et al.*, 2008). To date the most pure human HSC population is immunophenotypically characterized as $Lin^-/CD34^+/CD38^-/CD90^+/CD45RA^-$ (Majeti *et al.*, 2007; Park *et al.*, 2008). Immunodeficient mouse models such as NOD/SCID that lack B and T cells and $Rag2^{-/-}\gamma c^{-/-}$ mice that also lack natural killer (NK) cells are used to functionally test human HSCs, as they can accept the foreign transplantation of human cells. Human HSCs can be collected with minimal ethical concerns and risk to donors from placenta and umbilical cord blood (CB), which would normally be disposed of as medical waste

(Zhong *et al*., 2010). These CB HSCs are of fetal origin, are rapidly cycling, and are found in the circulation. They are not yet adult cells but can afford some insight into the biology of human HSCs.

DNA damage response of human and murine HSCs

Milyavsky *et al*. used CB HSCs to investigate the DDR and radiosensitivity of human HSCs (Milyavsky *et al*., 2010). They found CB HSCs to have a slower rate of DSB repair than progenitors, and increased levels of apoptosis-stimulating proteins of p53 (ASPP1) mediated apoptosis, which could be reversed if p53 expression was silenced or Bcl2 expression was enhanced. Upon primary transplantation, irradiated CB HSCs could not successfully engraft immunodeficient mice. However, CB HSCs lacking p53 or overexpressing bcl2 partially rescued this engraftment defect and the cells could be serially transplanted. The progeny of the serially transplanted p53 deficient CB HSCs were found to harbor DNA DSBs. In contrast, CB HSCs overexpressing bcl2 could serially engraft recipient mice better than p53 deficient CB HSCs and their progeny did not have DSBs. These data suggest that in human HSCs of fetal origin the main response to DNA damage is apoptosis mediated by the p53 DDR.

In contrast to the findings of Milyavsky *et al*., we showed that adult murine HSCs, which are found in a mostly quiescent state in the BM, also rely upon the p53 DDR but largely survive DNA damage. We found that quiescent HSCs utilize the error-prone NHEJ mechanism to repair DSBs, which has mutagenic consequences both *in vitro* and *in vivo* allowing for the expansion and persistence of misrepaired clones that can lose functionality or can potentially promote leukemogenesis. Since NHEJ appears to be the initial and most commonly used DNA repair mechanism in quiescent HSCs, these data help

explain why many mouse models lacking functional components of DNA DSB repair undergo hematopoietic failure upon stress (Nijnik *et al.*, 2007; Rossi *et al.*, 2007). Our results together with the work done by Sotiropoulou *et al.* on skin stem cells suggest that all quiescent stem cell populations, both healthy and cancerous, can be susceptible to the mutagenic consequences of NHEJ mediated misrepair (Sotiropoulou *et al.*, 2010). These data together suggest that that quiescent populations such as leukemic stem cells (LSCs), that often survive cancer treatments, can represent a reservoir for the additional mutations that can contribute to disease relapse or evolution. We also show that when induced to proliferate, HSCs have significantly reduced mutation rates in response to IR and have no change in their radioresistance. Our data suggest that error prone repair in quiescent stem cells can contribute to the dangerous side effects of cancer treatment that can result in the development of secondary cancers or myelodysplasias and that inducing quiescent cells to enter the cell cycle could potentially limit these side effects.

Taken together, Milyavsky *et al.* and our works show how HSCs respond to IR induced DNA damage but discover very different outcomes, which could be explained by biological differences between mice and humans or experimental differences between the studies. These include markers used to isolate stem cells and the resulting purity and homogeneity of the population studied, the dosage of irradiation given (2 vs. 3Gy), the species differences, the stage of ontogeny, the age of the cells, the use of a xenograft system, and the cell cycle

status of the cells being studied (Lane and Scadden, 2010; Seita *et al*., 2010). It is possible that HSCs from different organisms, with vastly different life spans, have potentially evolved different mechanisms to cope with DNA damage (Lane and Scadden, 2010). However, we postulate that HSCs at different stages of ontogeny have different functional goals and that their DDRs are set to ensure that their goals are met. Fetal CB HSCs are responsible for establishing the entire pool of HSCs that will go on to provide hematopoiesis throughout the life of the organism. Evolutionarily, it is essential that these cells be fully functional and free of genetic errors, as they will go on to provide all the HSCs in the adult animal. Milyavsky's findings that these cells are culled in response to damage is in line with their goal of establishment of a pristine HSC pool that will allow the animal to reach reproductive age. In contrast, the goal of adult HSCs is to maintain hematopoiesis and organism survival in response to stresses such as blood loss or infection while limiting tissue failure. The enhanced survival in response to IR is, again, in line with this hypothesis. Survival of adult HSCs, even at the cost of mutagenesis, will allow for maintenance of tissue function.

Implications of differential DNA damage response and DNA repair in stem cells

While survival and quick repair of DNA damage by adult HSCs favors both cellular and tissue maintenance in the short-term, it comes at a long-term cost: aging and potential for cancer development. Use of error-prone NHEJ repair coupled with overt cell survival by quiescent adult HSCs allows for the accumulation of mutations at the cellular level and accumulation of less functional or cancerous cells at the tissue level. Evolutionarily speaking this makes sense, and is in line with many theories of aging, as the goal of an organism is to survive long enough to reproduce (Kirkwood and Austad, 2000). Short-term repair that confers survival even if it is mutagenic will be selected for because it allows the organisms to reach reproductive maturity and reproduce. While selection pressure may allow for the accrual of mutations in HSCs and accumulation of less fit HSCs it seems that there are additional mechanisms in place to ensure maintenance of the hematopoietic system by selecting for the most competitive HSCs through the elimination of weaker cells (Marusyk *et al*., 2010; Bondar and Medzhitov, 2010). While this competition can be beneficial in selecting for the most fit cells, it can also result in the out competition of healthy normal cells when a cancerous cell has a growth or survival advantage. Together these findings show that HSCs and the hematopoietic tissue are protected by different mechanisms with strengths and weaknesses that overlap and function at many

different levels, which will require further study to understand the interplay between response to acute injury and long-term fitness.

These recent works clearly have implications both for understanding cancer development and for improving cancer treatment. Many human leukemia have now been shown to arise from LSCs, which are specific subsets of cancer cells that have stem cell-like characteristics and drive the formation and growth of tumors by giving rise to the bulk of the cancer cells (Dalerba *et al.*, 2007). LSCs have been described as arising from both transformed HSC populations and from more mature progenitor populations that have acquired the ability to self renew (Passegue *et al.*, 2003). It has been suggested that LSCs may be more resistant to current cancer therapies and that the persistence of LSCs may be responsible for disease maintenance and relapse after treatment (Elrick *et al.*, 2005; Jordan *et al.*, 2006). It seems that there are intrinsic qualities of HSCs such as a quiescent cell cycle status, increased expression of prosurvival genes, localization to a hypoxic niche, and DDR and DNA repair mechanisms that can be hijacked by leukemic cells to maintain survival (Guzman and Jordan, 2009; Blanpain *et al.*, 2010). We propose that HSC reliance upon error-prone DNA repair mechanisms could - over a lifetime - promote their transformation into LSCs. It has been shown that human acute myeloid leukemia (AML) and chronic myelogenous leukemia (CML) LSCs both use quiescence as a way to evade the effects of chemotherapy (Saito *et al.*, 2010; Elrick *et al.*, 2005). Forcing LSCs out of quiescence and then treating them with chemotherapy or radiotherapy

increases effectively of cancer elimination while sparing healthy cells (Saito *et al.*, 2010). This synthetic lethal treatment combination may also limit the generation of secondary neoplasias as we have shown that cycling healthy HSCs survive IR as well as quiescent ones but use HR to repair damaged DNA and will accumulate less mutations (Mohrin *et al.,* 2010). Another example of LSCs hijacking HSC protective mechanisms was shown using HSCs expressing AML/ETO1 or PML/RAR fusion oncoproteins. These LSCs acquired high levels of DNA damage and utilized the p21 mediated arrest to repair damage and escape apoptosis. When p21 was removed from these LSCs they were more sensitive to replicative and therapeutic stress and no longer able to transplant the disease, suggesting they had lost their LSC capabilities (Viale *et al.*, 2009). This is in line with the role of p21 in healthy HSCs, promoting cell cycle arrest that allows time for repair (Mohrin *et al.*, 2010). To truly eliminate cancers and spare healthy tissues multiple approaches to treatment may need to be combined; these early attempts at synthetic lethal treatment combinations are a start but there is much to be learned by further studying the responses of healthy HSCs to cancer treatment.

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CHAPTER 4

DNA DAMAGE RESPONSE OF OLD HSCS

Introduction

In adult animals tissue homeostasis is maintained by organ specific stem cells that can produce all the cell types necessary for tissue function, and have the ability to replace those cells when they are lost due to injury or wear and tear (Weissman, 2000). In addition to being able to generate all the mature effector cells of the tissue, stem cells also have the ability to self-renew, allowing for replenishment of the stem cell pool (Morrison *et al*., 1997). Because most of the effector cells of tissues are short-lived and are turned over regularly throughout life, stem cells must constantly function to replace them to avoid tissue atrophy or aplasia. The decreasing ability to maintain homeostasis at steady-state and to restore homeostasis after damage caused by injury or stress is a defining characteristic of aging (Rossi *et al*., 2008). As stem cells are responsible for maintenance of tissue homeostasis and repair, it is believed that their loss of number or function strongly contributes to aging.

In humans there are many well characterized age-associated changes or diminished functions of the blood system that reflect loss of hematopoietic stem cell (HSC) functionality. With advancing age the incidence of leukemia, specifically myeloid leukemia, increases. The ability to fight infections decreases in the elderly, and is referred to as immunosenescence (Linton and Dorshkind, 2004; Dorshkind *et al.,* 2009). Additionally, anemia, a decrease in red blood cell production, is more common in the elderly (Rossi *et al.,* 2008). Elderly patients are also more sensitive to the effects of cytotoxic chemotherapies, often

displaying myelosuppression suggesting a diminished HSC regenerative capacity (Sharpless and DePinho, 2007). Moreover, bone marrow (BM) transplantations done with cells isolated from older donors do not resolve disease as frequently as cells isolated from young donors (Rossi *et al*., 2008; Kollman *et al*., 2001).

There is much evidence that HSCs do indeed lose or change their function with advanced age. HSCs, along with memory B and T cells, are among the longest-lived cells of the hematopoietic system, therefore they are one of the few cell populations that actually ages. Studies in the mouse have been able to replicate many of the age-associated changes in the blood system of elderly humans.

Old mice were found to have an increase in mature myeloid cells and a decrease in mature lymphoid cells, which was due, at least in part, to the increase in number of myeloid progenitor cells and decrease in number of lymphoid progenitors (Rossi *et al*., 2005; Janzen *et al*., 2006; Rossi *et al*., 2007). Old HSCs also express higher levels of genes associated with myeloid development and differentiation and decreased levels of lymphoid associated genes (Rossi *et al*., 2005). Functionally, HSCs isolated from old mice were found to have decreased repopulating ability upon transplantation, which has been shown to be due in part to a decreased ability to home to and engraft in the BM. Upon transplantation old HSCs could reproduce the increase in HSC number and lineage skewing observed at steady state, suggesting that the changes in hematopoiesis seen with age are due to cell intrinsic changes in the HSC

population (Morrison *et al*., 1996; Sudo *et al*., 2000; Rossi *et al*., 2005). Old HSCs were also found to have decreased ability to undergo serial transplantation, due to increased levels of apoptosis and decreased proliferation suggesting that with age they lose functional capacity (Chen *et al*., 2000; Janzen *et al*., 2006).

One surprising finding about aged HSCs in mice is that while their functionality seemed to be decreased, they are actually increased in number by 3-15 fold while the overall BM cellularity remains constant (Rossi *et al*., 2008). It has been suggested that such an increase in phenotypic HSCs was due to increased proliferation; this however is still a matter of controversy. Some found old HSCs to proliferate more than young HSCs (Morrison *et al*., 1996; Yilmaz *et al*., 2006) some found them to proliferate at the same rate (Sudo *et al*., 2000; Janzen *et al*., 2006; Rossi *et al*., 2007; de Haan and Van Zant, 1999), and others found them to proliferate less than young HSCs (Dumble *et al*., 2006; Attema *et al*., 2009; Chen *et al*., 2009). It remains to be seen, but it is possible that the proliferation or quiescence status of old HSCs differs from young HSCs. Finally, Old murine HSCs have been shown to have high basal levels of DNA damage as identified by phosphorylated histone H2AX (γ-H2AX) foci formation, suggesting that these cells are undergoing intrinsic DNA damage resulting in DSB formation (Rossi *et al*., 2007). In support of this hypothesis, old HSCs were found to express increased levels of genes associated with stress and inflammatory responses, such as NF-κB targets and heat shock genes, again suggesting that

they may be under heightened stress. Old HSCs were also found to express decreased levels of genes associated with genome maintenance and chromatin remodeling, which suggests that HSCs may be less capable of recovering from genotoxic stress with age (Chambers *et al.*, 2007). Studies of mice lacking components of the DDR or DNA repair proteins have displayed their importance for HSC maintenance during homeostasis and in response to stress (Blanpain *et al.*, 2010). Additionally, studies of old mice lacking components of genome maintenance showed their near inability to cope with the stress of transplantation, exposing the importance of genome maintenance in HSC function over the lifetime of an organism (Rossi *et al.*, 2007). Human patients with progeroid syndromes (*i.e.* diseases with symptoms that mimic those associated with old age and are caused by loss of function of components of the DNA damage recognition and repair pathways) often develop cancer or failure of the hematopoietic system, further solidifying the link between DNA repair and aging (Lombard *et al.*, 2005; Ciccia and Elledge, 2010). The reduced capacity of old HSCs to survive and restore blood homeostasis could therefore be partially due to their increased levels of DNA damage. However, what is causing such genomic stress is still unknown.

We have recently shown that in young adult HSCs the use of the error prone nonhomologous end joining (NHEJ) double strand break (DSB) repair mechanism has severe mutagenic consequences for HSCs in response to extrinsic genotoxic stress (Mohrin *et al.*, 2010). We hypothesize that the use of

this mechanism and resulting misrepair over the span of a lifetime may contribute to the loss of function seen with increasing age in old HSCs. Here we investigate the role of genotoxic stress and causes of heightened DNA damage in old HSCs and how their potential misrepair by NHEJ coul dbe at the root of the greater incidence of cancer development in the elderly.

Results

Features of hematopoiesis in old mice

We began by confirming the previously described age related features of HSCs and the blood system in 18-24 month old mice. Previous results have shown that the number of phenotypic HSCs in the BM of old mice increases by 3-15 fold, while the cellularity of the whole BM (WBM) remains unchanged (Rossi *et al.*, 2008). As expected, we found a ~10 fold increase in the number of phenotypic HSCs ($Lin^-/c\text{-}Kit^+/Sca\text{-}1^+/Flk2^-/CD48^-/CD150^+$) in old wild-type (WT) C57Bl/6 mice with no change in WBM cellularity (Figure 1A and data not shown). Another established characteristic of steady-state hematopoiesis in old mice is that the percentages of mature cell populations are skewed towards the myeloid lineage when compared to young mice; which results in part from increased numbers of the granulocyte macrophage progenitor (GMPs) and common myeloid progenitor (CMPs) populations and a decrease in the common lymphoid progenitor (CLPs) population (Rossi *et al.*, 2005; Janzen *et al.*, 2006; Rossi *et al.*, 2007). We confirmed the increase in percentage of mature myeloid cells ($Mac1^+/Gr1^+$) and the decrease in percentage of mature lymphoid cells ($CD3^+$) in the blood of old mice (Figure 1B). We also confirmed a subtle expansion of the entire myeloid progenitor (MP) compartment, and a significant contraction of the CLPs in the BM of old mice (Figure 1C and D). Taken together these data strengthen the previously documented changes in steady-state hematopoiesis in

old mice. We also observed a large degree of heterogeneity between individual old mice.

Decreased repopulation abilities of old HSCs

Old HSCs are known to have decreased functionality upon transplantation. With reduced repopulation ability and persistance of lineage skewing toward the myeloid lineage and the expense of the lymphoid lineage. To confirm these observations, we isolated HSCs from the pooled BM of five to twenty young and two to three old mice and transplanted 250 young or old HSCs (CD45.1) into lethally irradiated recipient (CD45.2) mice along with 300,000 Sca-1 depleted helper BM and monitored them for engraftment and lineage output. This experiment is currently still in progress but we can already observe that at two months post transplantation old HSCs had the expected decreased engraftment compared to young HSCs (Figure 2A and B) and have skewed lineage output with increased levels of mature myeloid cells and decreased levels of lymphoid cells in the blood. However, despite having decreased functionality, old HSCs have been shown to phenocopy themselves better than young HSCs resulting in an increase in old HSC pool size in the BM of the transplanted young mice (Rossi *et al*., 2005). Our experiments are currently in progress, and we will attempt to confirm this observation once the transplantations have been stable for four months. Taken together, our results show that the changes in steady-state hematopoiesis in old mice (*i.e.* increased HSC pool size, reduced repopulation

ability, and a differentiation bias towards the myeloid lineage) are transplantable and thus intrinsic to the old HSCs.

Proliferation and expansion of old HSCs

Considering the discrepancies seen by various groups in the proliferation rates of old HSCs by various groups, we also investigated the cell cycle status of of young and old HSCs. We found that old HSCs expand in culture similarly to young HSCs and displayed slightly higher levels of ATP, which suggests that old HSCs either proliferate more than young HSCs or are more metabolically active (Figure 3A and B). To directly characterize the proliferation abilities of old HSCs we used a one hour BrdU pulse ex vivo and found that old HSCs incorporate BrdU at equal or slightly lower rates than young HSCs (Figure 3C). We also studied the quiescent status of young and old HSCs using intracellular 7AAD/PyroninY staining and found that three times more old HSCs were in the G1 phase of the cell cycle compared to young HSCs, which were mainly all in G0 (Figure 3D). Taken together, these still preliminary results suggest that both young and old HSCs progess through the cell cycle at similar rates, but that higher numbers of old HSCs are out of quiescence. This partial loss of quiescence could potentially - over time - contribute to the increase in HSC pool size and decreased repopulation abilities of old HSCs.

Basal levels of cellular stress in old HSCs

To determine if old HSCs have higher levels of intrinsic stress we began by evaluating the level of reactive oxygen species (ROS) using 2'-7'-dichlorofluorescein diacetate (DCF-DA) staining (Figure 4A). We did not observe any differences between young and old HSCs, suggesting that old HSCs are not undergoing increased levels of oxidative stress due to increased ROS. We also confirmed the higher basal levels of DSBs and γ-H2AX foci previously found in old HSCs. We used immunofluorescence microscopy to identify and score γ-H2AX foci. As anticipated, young HSCs displayed very few foci (2.59 $\pm$ 0.29 foci per cell), while old HSCs displayed ~3 times more foci, (5.22 $\pm$ 0.51 foci per cell; $p \leq 0.001$) (Figure 4B). In addition, the γ-H2AX foci observed in old HSCs were significantly larger than those found in young HSCs. To better characterize these foci we costained young and old HSCs with markers of nuclear architecture: telomeres (recognized by PNA FISH), centromeres (identified by CenPa costaining), and nucleoli (identified by UBF costaining). γ-H2AX foci did not colocalize with centromeres or telomeres in the old HSCs (data not shown and Figure 4C). In contrast, many of the large γ-H2AX foci in old HSCs colocalized with UBF staining (Figure 4D). The nucleolus is the site of ribogenesis and contains the ribosomal RNA gene repeats (rDNA) which are the most common genes in the genome and because of their repetitive structure and high level of transcription are one of the most fragile sites in the genome (Kobayashi, 2008;

Kobayashi, 2011). These results suggest that old HSCs may be undergoing DNA damage at this fragile site.

Status of telomeres in old HSCs

As telomeres are known to shorten with increased age and are also considered fragile sites of the genome, we investigated their length and integrity in young and old HSCs. Old and young HSCs were grown in culture for 4 days for maximal cell cycle entry and expansion, treated for 4 hours with colcimid to block cells in metaphase, fixed for cytogenetic studies, and hybridized with the same PNA FISH probe specific for telomeres. No decrease in signal intensity could be detected between young and old HSCs, and if anything, old HSCs had longer telomeres compared to young HSCs (Figure 5A). As mouse telomeres are extremely long compared to human telomeres (25-60kb vs. 5-15 kb) and usually do not decrease in length with age, this result is not too surprising. However upon closer inspection, we noticed that ~10% of old HSCs had multiple telomere signals which also suggests fragility at these repetative sequences (Sfeir *et al.*, 2009) (Figure 5B). These data suggest that while the telomeres of old HSCs are not decreased in length, they may be less stable. Taken together, the colocalization of γ-H2AX foci with the nucleolus and the increased fragility at the telomeres suggests that old HSCs have age-associated deterioration of fragile sites in the genome.

DNA damage response in old HSCs

As old HSCs have heightened levels of intrinsic stress and have decreased functional capabilities as compared to young HSCs, we hypothesized that they may not be as able to cope with genotoxic stress. To test this hypothesis, we exposed young and old HSCs to 2Gy of ionizing radiation (IR) and performed clonogenic survival assays in methylcellulose. We found that non-irradiated old HSCs produced significantly less colonies than young HSCs. We also observed no change in colony numbers upon treatment with 2Gy IR, indicating that old HSCs are less radiosensitive than young HSCs (Figure 6A). To better understand the decreased basal colony formation and enhanced radioresistance of old HSCs, we started testing DDR and cellular outcomes (*i.e.* proliferation and apoptosis of young and old HSCs. We are still assessing the proliferation activity of old HSCs in response to IR treatment, but have already used intracellular staining for cleaved caspase 3 (CC3) to measure their rates of apoptosis. We found that old HSCs undergo less apoptosis compared to young HSCs, both basally and in response to IR treatment (Figure 6B). We also performed qRT-PCR analysis of the expression levels of a panel of bcl2 family pro-and antiapoptotic genes in freshly isolated young and old HSCs (Figure 6C). Overall, we observed no significant differences in the status of the apoptosis machinery of these two populations. However, we did observe constitutively higher levels of p21 and p16 expression in old HSCs (Figure 6D). Taken together with their accumulation in the G1phase of the cell cycle, these results suggest

that the outcome of the DDR in old HSCs might be senescence in contrast to the growth arrest response usually seen in young HSCs.

DNA repair in old HSCs

To understand the extent to which old HSCs repair DSBs we used immunofluorescence microscopy to quantify the formation and resolution of γ-H2AX containing ionizing radiation induced foci (IRIF). As we have previously shown, unirradiated young HSCs displayed very low levels of γ-H2AX foci and upon exposure to 2Gy IR showed an immediate induction IRIF that were mostly resolved by 24 hours (Figure 7A and 7B) (Mohrin *et al*., 2010). As expected, old HSCs displayed high basal levels of γ-H2AX foci, which mostly colocalize with the nucleolus, and upon exposure to 2Gy IR also showed an immediate induction of IRIF that were mostly resolved by 24 hours. While the high basal level of nucleolar associated γ-H2AX foci remained (Figure 7A and 7B). To investigate the type of repair mechanism used by old HSCs, we costained the IRIF with 53BP1, a DDR adaptor protein that is associated with NHEJ (Figure 7A and 7B). As we previously demonstrated, young HSCs displayed very low basal levels of 53BP1 foci but upon irradiation showed an immediate induction of 53BP1 containing IRIF that were mainly resolved by 24 hours (Figure 7A). Old HSCs showed increased basal levels of 53BP1 foci that colocalize with the nucleolar associated γ-H2AX foci, and upon irradiation also showed an immediate induction of 53BP1-containing IRIF that were resolved by 24 hours, except for the

nucleolar associated γ-H2AX foci. To support these findings we also analyzed the expression of some key genes involved in HR and NHEJ mediated DNA repair. We did not detect any significant changes in their expression levels between young and old HSCs (Figure 7C). These data indicate that young and old HSCs are equally capable of repairing IR induced DSBs using NHEJ mechanisms, but that old HSCs are undergoing persistent DNA damage at the nucleolus, which is not resolved by the DNA repair machinery that is clearing IRIF.

Discussion

Although many groups have studied the effect of old age on HSCs and the blood system, much remains to be learned about the mechanisms that cause aging. We propose that a diminution of cellular protection mechanisms and the resulting stress that ensues, over time leads to DNA damage in HSCs and is at the heart of aging and age-related loss of HSC function. In this study, we confirmed the age-related decrease in HSC function seen in old mice, investigated the cause and location of the intrinsic stress occurring in old HSCs, and followed the DDR and DNA repair mechanisms used by old HSCs to cope with IR-mediated DSBs. We identify signs of increased replicative stress and senescence in old HSCs but little difference in the DDR and DNA repair capabilities compared to young HSCs. Our findings suggest that the loss of function seen on old HSCs may be due to enhanced levels of intrinsic replicative stress leading to loss of quiescence and functional decline. We propose that such ongoing replication stress constantly generates DSBs at fragile sites of he genome (including the nucleolos and telomeres) and that the potential misrepair of these lesions by NHEJ mediated repair process contributes to HSC functional collapse and the high rates of cancerous transformation observd in the elderly.

We were able to reproduce most of the published findings regarding altered hematopoiesis and HSC function in old mice. We confirmed that old mice have an increase of HSC numbers and population skewing towards the myeloid lineage at the expense of the lymphoid lineage with both changes evident at the

progenitor and mature cell levels. Upon transplantation, we found decreased repopulation activity and lineage skewing from old HSCs. We also found that old HSCs were less quiescent than young HSCs, despite having similar expansion rates and were significantly enriched in G1 cells. Old HSCs might also be more metabolically active than young HSCs, although we did not observe changes in their ROS levels. Finally, we observed inceased levels of DSBs in old HSCs. Taken together, these results indicate that with age HSCs are less quiescent and may lose some of the protective measures that are associated with retention in the G0 phase of the cell cycle, which could contribute to their decreased functional capabilities. Cellular senescence, an irreversible cell cycle arrest, is partially defined by a G1 cell cycle block, thus it also possible that a percentage of these old HSCs are in a senescent state. Mechanisms that protect HSC function, like quiescence, appear to be diminished with age which could contribute to the increase in cellular stress and decrease in fitness seen in old HSCs.

We found that most of the DSBs spontaneously occuring in HSCs occur in the nucleolus. The nucleolus is the region of the nucleus where ribogenesis occurs and can also sense and respond to cellular stress (Pederson, 2010; Boulon *et al.*, 2010). Nucleoli are dynamic components of the nuclear architecture, they disassemble and reassemble upon cellular division and their size and morphology depend upon cell growth and metabolism; they have also been suggested to serve as a hub for ribosome biosynthesis, cell-cycle

progression, and stress signaling (Boulon *et al.*, 2010). Ribosomes are composed of ribosomal RNA (rRNA) and ribosomal proteins. Ribosomal DNA (rDNA), the genes responsible for encoding rRNA, are the most abundant genes in the eukaryotic genome, exist in tandem highly repetitive clusters, are a common site of recombination, and are actively transcribed; thus they are one of the most fragile sites of the genome (Kobayashi, 2008). Fragile sites are regions of the chromosome that challenge the DNA replication machinery, especially under conditions of limited nucleotides or inhibition of DNA polymerases (Durkin and Glover, 2007). Fragile sites are usually large and repetitive DNA sequences that often display abnormal features in metaphase chromosomes caused by replication stress – incomplete replication or improper processing of stalled replication forks (Burhans and Weinberger, 2007). These regions of the genome are believed to be where replication forks will often stall and collapse hence generating DSBs and have increased rates of recombination, deletions, and other chromosomal rearrangements (Sfeir *et al.*, 2009). Because of their fragility, rDNA genes are proposed to serve as shock absorbers of DNA damage for the whole genome, signaling to the DDR and DNA repair machineries before other more important parts of the genome are harmed (Kobayashi, 2011). However over time, these fragile rDNA regions become increasingly unstable and promote cellular senescence as their number of repeats decline or mutations accumulate (Kobayashi, 2011). It has been suggested that the way rDNA can promote senescence and genome instability is through the sequestration of DNA repair

proteins at regions of stressed rDNA such that cell cycle progression is halted to initiate repair, but that there are not enough repair proteins left to go repair other regions of the genome (Kobayashi, 2011).

Our observation of multiple telomere signals in old HSCs also suggested that other fragile sites of the genome are under stress in old HSCs. Telomeres are long arrays of TTAGGG repeats that mark and protect the ends of chromosomes by serving as binding platforms for proteins like shelterin that block a response from the DDR and DNA repair machineries that would otherwise identify the tail of the chromosome as broken DNA (De Lange, 2005). Multiple telomere signals are likely due to altered packaging of the chromatin due to extended areas of single stranded DNA resulting from replication stress (Sfeir *et al.*, 2009). DNA replication stress can lead to DNA damage and genome instability because replicating DNA found at replication forks are unwound, single stranded, and therefore much less stable than non-replicating DNA (Burhans and Weinberger, 2007).

Our results provide the beginning of a mechanistic understanding of the changes seen in old HSCs. The increase in number of HSCs and decrease in function seen at steady-state in old mice and the decreased ability to repopulate primary recipients as well as serial recipients upon transplantation can - at least partially - be explained by increased replicative stress and loss of quiescence. The combination of changes in the cell cycle status of old HSCs with the loss of quiescence and enhanced metabolic activity - suggests that they may be less

protected from the stresses associated with DNA replication and cell division. Indeed, we found old HSCs to show signs of replicative stress at two fragile sites of the genome, rDNA in the nucleolus and at telomeres. These striking observations coupled with the old HSCs decreased ability to form colonies suggests that upon proliferation signals old HSCs often fail to function, likely due to a senescence response. The increased percentage of G1 cells, decreased apoptosis, and significant upregulation of p21 and $p16^{INK4A}$ expression we see in old HSCs strongly suggest that in response to replication stress old HSCs undergo cellular senescence. Any DNA damage occurring in this old HSC population, which is still largely quiescent, will be repaired by the error-prone NHEJ DNA repair mechanism, which could promote the accumulation of mutations and contribute to the high rate of cancer development loss of function seen with age.

Aging is caused by a complex combination of acquired and genetic processes (Sharpless and DePinho, 2007). Many of the genes and proteins known to be integral in mitochondrial metabolism, oxidative stress, and dietic control of longevity have converged to play roles in the aging process. The rate of aging and appearance of age-related pathology are modulated by stress response and repair pathways which are tightly controlled by signaling pathways - insulin/IGF-1, TOR, AMPK, and sirtuins - that sense internal and external changes such as nutrient availability, temperature, oxygen levels, DNA damage, and protein misfolding (Haigis and Yanker, 2010). It has been shown that old

HSCs express increased levels of mTOR and deletion of a regulator of mTOR, Tsc1, results in activated mTOR activity that reproduces many of the age associated phenotypes in the hematopoietic system, increased expression of $p16^{INK4A}$, p21, and p19, increased number of HSCs, decreased repopulation abilities, and decreased lymphopoiesis (Chen *et al.*, 2009). Upon treatment with rapamycin, which blocks mTOR activity, old mice show a nearly complete reversion of the aging phenotype and have increased longevity (Chen *et al.*, 2009). In a similar fashion, caloric restriction (CR), a technique known to increase the lifespan of animals, results in reduced HSC cycling, and postponement of senescence (Yoshida *et al.*, 2006; Chen *et al.*, 2003). Both treatment with rapamycin and CR effectively slow the cycling of HSCs and probably limit replicative stress. These data suggest that some of the effects of aging may not be permanent and that targeting stress pathways may be a way to limit tissue function failure seen with age.

Experimental procedures

Mice.

C57Bl/6-CD45.1 and C57Bl/6-CD45.2 mice were used as young donor (4-8 week old) or old donor (18-24 months) for most of the cell isolation and as recipient (8-12 week old) for cell transplantation procedures. Recipient mice were either sub-lethally (9.5Gy) or lethally irradiated (11Gy), both delivered in split dose 3 hours apart, and given antibiotic-containing water for at least 6 weeks post-irradiation. Peripheral blood was obtained from retro-orbital bleeding and collected in 4 ml of ACK (150 mM NH_4Cl/10 mM $KHCO_3$) containing 10 mM EDTA for flow cytometry analysis. Donor and recipient cells were distinguished by different allelic forms of CD45 (CD45.1 vs. CD45.2). All animal experiments were performed in accordance with UCSF Institutional Animal Care and Use Committee approved protocols.

Flow cytometry.

Staining and enrichment procedures for HSC and analysis were performed as previously described (Passegué *et al.,* 2005; Santaguida *et al.,* 2009; Mohrin, *et al.,* 2010). For regular isolation, c-Kit-enriched bone marrow cells were stained with unconjugated rat lineage (B220, CD3, CD4, CD5, CD8, Ter119, Mac-1, Gr-1) antibodies/goat anti-rat-qdot 605 and directly conjugated c-Kit-APC-Cy7, Sca-1-PB, Flk2-bio/streptavidin-Cy7PE, CD48-APC, and CD150-PE. Cells were finally resuspended in Hank's buffered saline solution (HBSS) with 2% heat-inactivated

fetal bovine serum (FBS) containing 1μg/ml propidium iodide (PI) for dead cell exclusion, and sorted or analyzed on a FACS AriaII or LSRII (Becton Dickinson), respectively. Each population was double sorted to ensure maximum purity and irradiated as needed using a ^{137}Cs source (600ci^{137}Cs; J.L Shepherd & Associates Radiation Machinery M38-1 S.N. 1098).

Cell cultures.

Both methylcellulose and liquid cultures were supplemented with SCF (25 ng/ml), Flt3-L (25 ng/ml), IL-11 (25 ng/ml), IL-3 (10 ng/ml), Tpo (25 ng/ml), Epo (4 U/ml) and GM-CSF (10 ng/ml) (Peprotech). For methylcellulose assays, 100 HSCs were plated in Iscove's modified Dulbecco's media (IMDM) based methylcellulose (StemCell Technology M3231) and colonies were counted on day 7 using duplicate plates per condition. For growth expansion in liquid culture, 500 cells were plated per well of a 96-well plate in 200μl IMDM containing 5% FBS (StemCell Technology), 1x penicillin/streptomycin, 0.1mM non-essential amino acids, 1mM sodium-pyruvate, 2mM Gluta-Max-1, 50μM 2-mercaptoethanol. Medium was replenished and cells were expanded as needed to maintain optimal growth. Cells were counted on day 4, by trypan blue exclusion using triplicate wells per condition. Other cell culture experiments were started with various numbers of purified (± irradiation) cells (5,000-40,000).

Apoptosis, proliferation and ROS assays.

Apoptosis levels were measured by flow cytometry using intracellular staining for FITC-conjugated cleaved caspase 3 according to the manufacturer's instructions (Becton Dickinson). For intracellular BrdU staining, purified cells were incubated for 1 hour with 60μM BrdU (Sigma), fixed in PBS/4% PFA for 20 min at RT, washed twice in PBS/50mM NH_4Cl, permeabilized in PBS/0.2% Triton-X100 for 5 min and washed in low PB (0.2x PBS, 3% BSA) buffer. Cells were then incubated with 50U DNAseI in low PB buffer containing 5 mM $MgCl_2$/2 mM $CaCl_2$ for 30 min at RT min, washed once with low PB buffer, incubated for 30 min with FITC-conjugated anti-BrdU antibody (Pharmingen), washed once and resuspended in low PB buffer without PI for flow cytometry analysis. 7AAD/Pyronin Y staining and was performed as previously described (Santaguida *et al.,* 2009). ATP levlels were assessed using Cell Titer-Glo according to the manufacturer's instructions (Promega). ROS was assessed using DCF-DA (Invitrogen) as previously described (Tothova *et al*., 2007)

Quantitative RT-PCR.

Total RNA was isolated using TRIzol or TRIzol-LS (Invitrogen) from equivalent numbers of purified cells (10,000 to 50,000 aliquots), digested with DNase I and used for random hexamer-based reverse-transcription according to the manufacturer's instructions (SuperScript III™ kit, Invitrogen). QRT-PCR primers were designed using Primer Express software (Applied Biosystems) and are listed in the table below. All reactions were performed in an ABI-7300 sequence

detection system using SYBR® Green PCR Core reagents (Applied Biosystems) and the cDNA equivalent of 200 cells as previously described (Santaguida *et al*., 2009; Mohrin *et al*., 2010). Each measurement was performed in triplicate and expression levels of β-actin were used to normalize the amount of the investigated transcript.

Immunofluorescence and FISH microscopy.

Immunofluorescence staining and was performed as previously described (Mohrin *et al*., 2010). UBF antibody was used as previously described and provided as a gift of B. McStay (National University of Ireland Galway) (Mais *et al*., 2005). For immunofluorescence plus FISH, immunoflorescene staining was completed, stained cells were then fixed with 4% PFA, and FISH was completed using a PNA probe specific for telomeric repeats as previously described (Cooley *et al*., 2009). To assess telomeric length, purified HSCs were grown for 4 days in liquid culture (2,000-10,000 cells), treated for 4 hours with 0.01 μg/ml Colcemid™ (Invitrogen), collected in eppendorf tubes and washed once with PBS. Cells were incubated for 8 min at 37°C in 0.075M KCl and then fixed in 3:1 volume absolute methanol:glacial acetic acid (Mohrin *et al*., 2010). Telomere length was assessed as previously described, using FAM FISH probes and analysis was performed as previously described using a Deltavision microscope and the TFL-TELO program for analysis (Stohr *et al*., 2010; Poon *et al*., 1999).

Statistics.

All the data are expressed as mean ± standard deviation (error bar) except when indicated. P values were generated using unpaired Student's *t*-test and considered significant when ≤ 0.05. N indicates the numbers of independent experiments performed.

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Figure 1. Features of hematopoiesis in old mice.

(A) Number of HSCs in the BM of young (n = 9) and old (n = 10) mice (***p ≤ 0.001).

(B) Percentage of myeloid ($Mac1^+/Gr1^+$) and Lymphoid ($CD3^+$) cells in the blood of young (n = 10) and old (n = 10) mice (*p ≤ 0.05).

(C) Number of myeloid progenitors (CMP, GMP, and MEP) in the BM of young (n = 9) and old (n = 10) mice (**p ≤ 0.01).

(D) Number of CLPs in the BM of young (n = 9) and old (n = 10) mice (**p ≤ 0.01).

Black bars represent mean values.

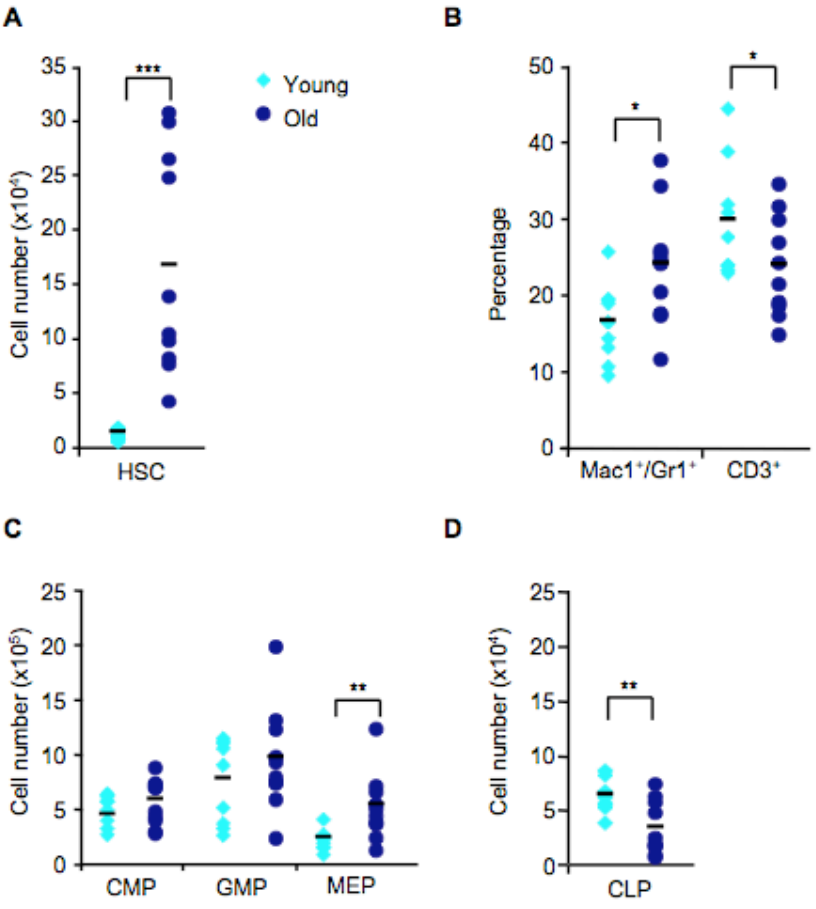

A
B
C
D
Young
Old
Cell number (x10⁴)
HSC
Percentage
Mac1⁺/Gr1⁺
CD3⁺
Cell number (x10⁵)
CMP
GMP
MEP
Cell number (x10⁴)
CLP
***
*
**

Figure 2. Functional activity of old HSCs.

Donor HSCs were isolated from CD45.1 expressing young and old mice, and 250 HSCs were transplanted into lethally irradiated (11Gy) CD45.2 young recipient mice along with 300,000 Sca-1 depleted CD45.2 helper BM cells. The percentages of donor blood chimerism and donor derived myeloid and lymphoid reconstitution were assessed by FACS analysis and are shown at 1 month **(A)** and 2 months **(B)** after transplantation (n = 5 recipient mice per cell type).

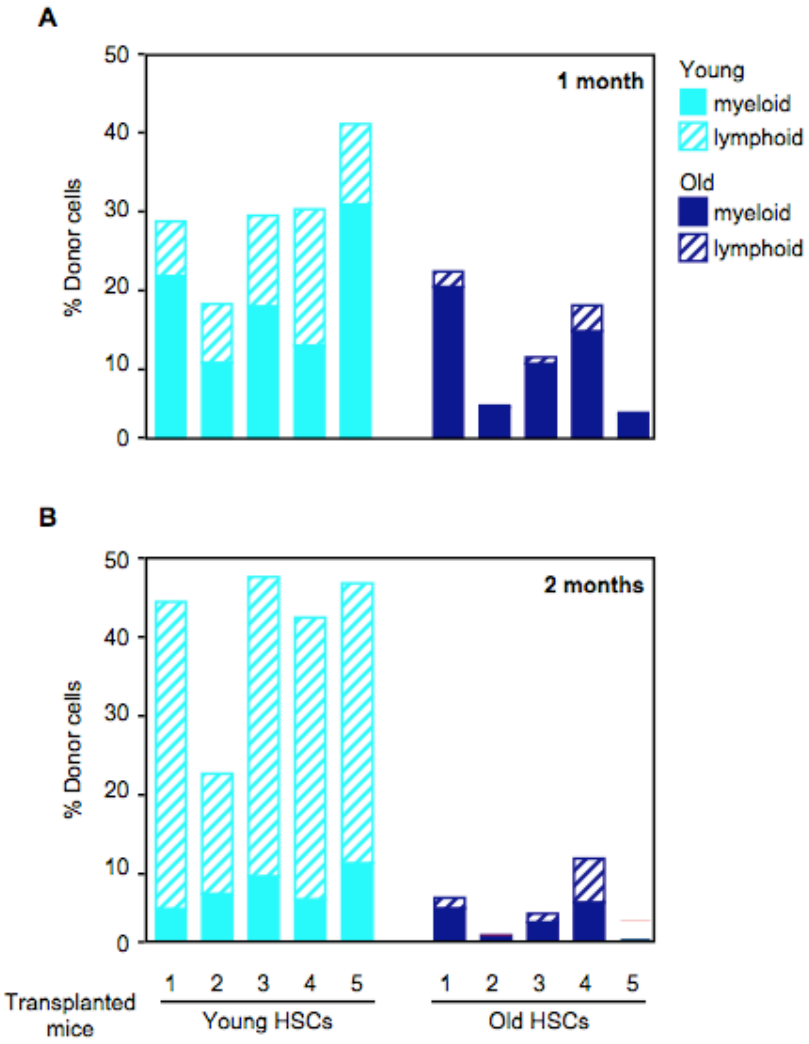

A
1 month
% Donor cells
50
40
30
20
10
0
Young
myeloid
lymphoid
Old
myeloid
lymphoid
B
2 months
% Donor cells
50
40
30
20
10
0
1 2 3 4 5
1 2 3 4 5
Transplanted mice
Young HSCs
Old HSCs

Figure 3. Proliferation and expansion of old HSCs.

(**A**) Expansion of young and old HSCs grown in liquid media for 4 days (n = 2). Results are expressed as fold expansion of original number of cells plated.

(**B**) ATP levels measured inof young and old HSCs grown in liquid media for 6 days (n = 1, experiment performed in triplicate). Results are expressed as fold change compared to base line value.

(**C**) Basal proliferation rates of young and old HSCs measured after 1 hour BrdU pulse in vitro (n = 1, experiment performed in duplicate). The FACS plot on the left shows a representative example of BrdU staining, and the histogram at the right represents a quantification of these results.

(**D**) Quiescence status of young and old HCSs measured by intracellular 7AAD/Pyronin Y staining (n = 1). The FACS plot on the left shows a representative example of BrdU staining, and the histogram at the right represents a quantification of these results.

Error bars represent standard error of the mean.

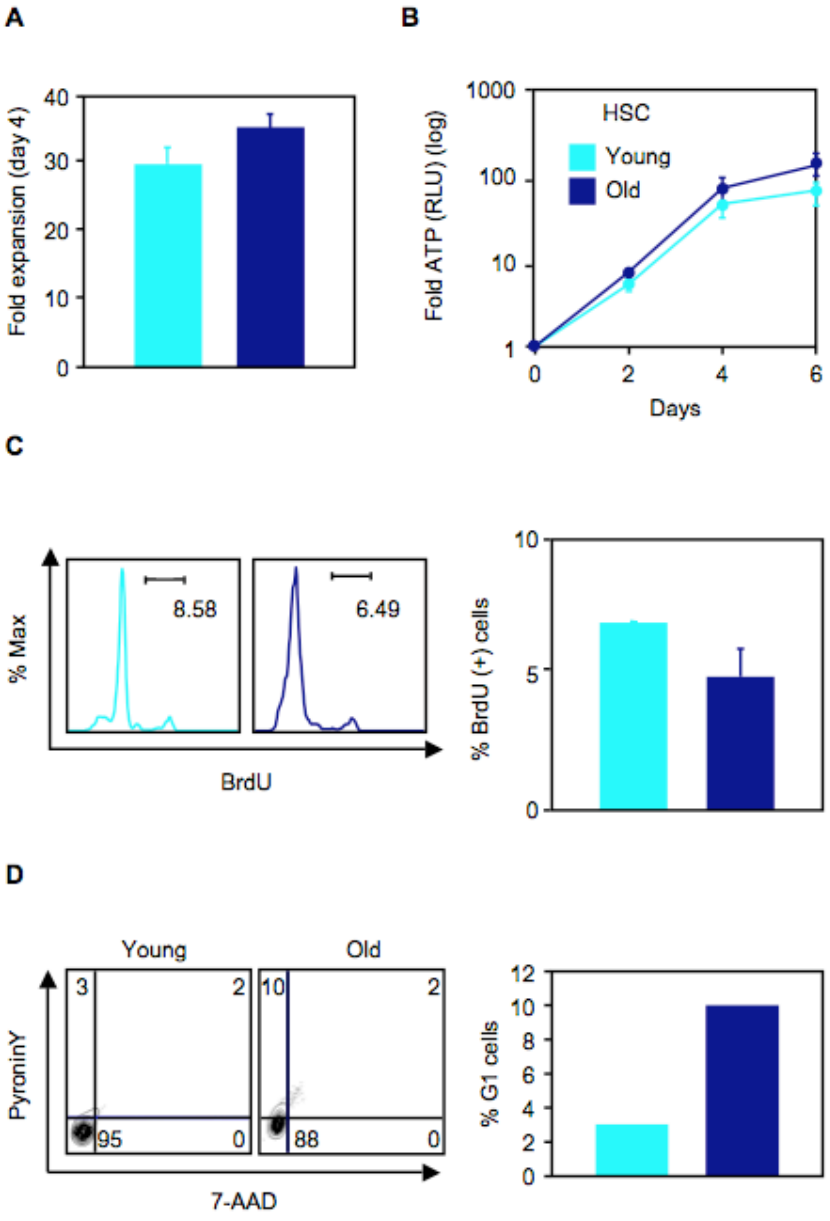

A
B
Fold expansion (day 4)
40
30
20
10
0
Fold ATP (RLU) (log)
1000
100
10
1
HSC
Young
Old
0
2
4
6
Days
C
% Max
8.58
6.49
BrdU
% BrdU (+) cells
10
5
0
D
Young
Old
PyroninY
3
2
95
0
10
2
88
0
7-AAD
% G1 cells
12
10
8
6
4
2
0

Figure 4. Increased DNA damage at the nucleolus in old HSCs.

(**A**) ROS levels in young and old HSCs (n = 1). Results are expressed as fold change compared to the levels seen in young HSCs.

(**B**) Immunofluorescence analysis of γH2AX DNA damage foci in unirradiated young and old HSCs. The number of foci per cell was scored in approximately 50 cells and results are expressed as a percent of total cells scored (n = 3).

(**C**) Representative images of immunofluorescence analysis for γH2AX DNA damage foci and telomere FISH in unirradiated young and old HSCs (n = 2).

(**D**) Representative images of immunofluorescence analysis for γH2AX DNA damage foci and nucleolar marker UBF in unirradiated young and old HSCs (n = 2).

Error bars represent standard deviation.

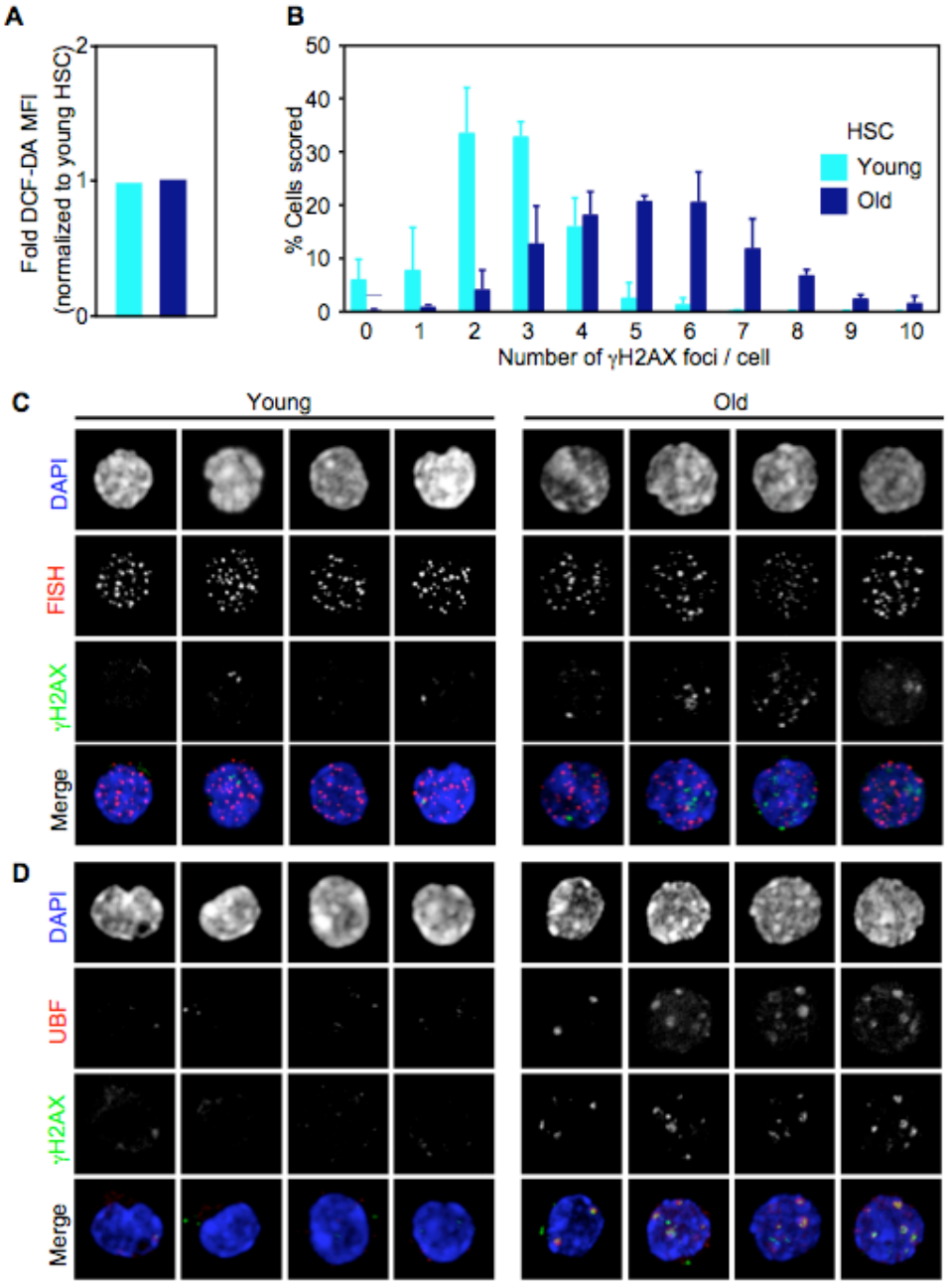

A
Fold DCF-DA MFI
(normalized to young HSC)
2
1
0
B
% Cells scored
50
40
30
20
10
0
HSC
Young
Old
0 1 2 3 4 5 6 7 8 9 10
Number of γH2AX foci / cell
C
Young
Old
DAPI
FISH
γH2AX
Merge
D
DAPI
UBF
γH2AX
Merge

Figure 5. Fragility at telomeres in old HSCs.

(**A**) Detection of telomere length in young and old HSCs using telomere FISH (n = 1; 7 young HSCs scored, and 6 old HSCs were scored) (**p ≤ 0.01, *p ≤ 0.05). p and q arms are, respectively, the short and long arms of the chromosomes. Representative images are shown on the left; quantification of the results is shown in the histograms on the right. Results are normalized to the length of the p and q arms in young HSCs (which is arbitrarily set to 1).

(**B**) Detection of multiple signals (white arrows) at telomeres in young and old HSCs using telomere FISH (n = 1; 7 young HSCs scored, 6 old HSCs scored). Representative images are shown on the left; and quantification of the results is presented in the histograms on the right. Results are expressed as percentage of HSCs scored.

Error bars represent standard deviation.

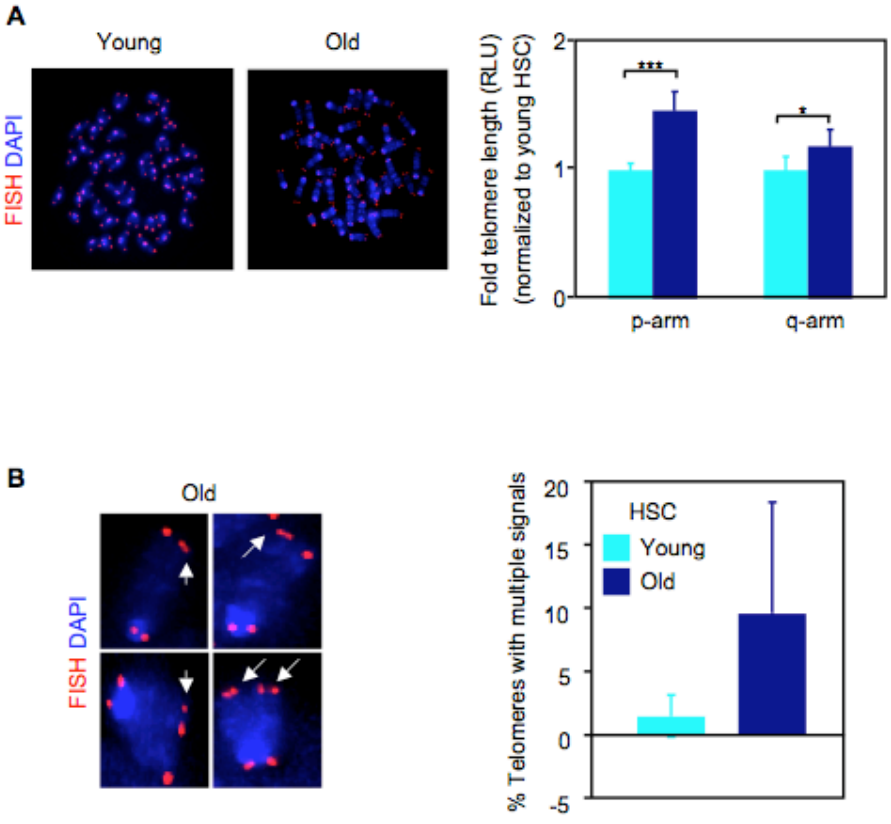

A
Young
Old
FISH DAPI
Fold telomere length (RLU)
(normalized to young HSC)
2
1
0
***
*
p-arm
q-arm
B
Old
FISH DAPI
% Telomeres with multiple signals
20
15
10
5
0
-5
HSC
Young
Old

Figure 6. DNA damage response in old HSCs.

(**A**) Clonogenic survival assay of unirradiated (0Gy) and 2Gy irradiated young and old HSCs in methylcellulose (n = 3; ***p ≤ 0. **p ≤ 0.01).

(**B**) Intracellular cleaved caspase 3 staining in unirradiated or 2Gy-irradiated young and old HSCs grown for up to 2 days in liquid media (n = 1).

(**C**) QRT-PCR analysis of *bcl2*-family pro-survival and pro-apoptotic genes, and *p21* expression levels in freshly isolated old HSCs. Results are expressed as log2 fold expression compared to levels measured in young HSCs (n = 3; ***p ≤ 0.001, **p ≤ 0.01, *p ≤ 0.05).

(**D**) QRT-PCR analysis of *p16* expression level in freshly isolated old HSCs. Results are expressed as log2 fold expression compared to levels measured in young HSCs (n = 3).

Error bars represent standard deviation.

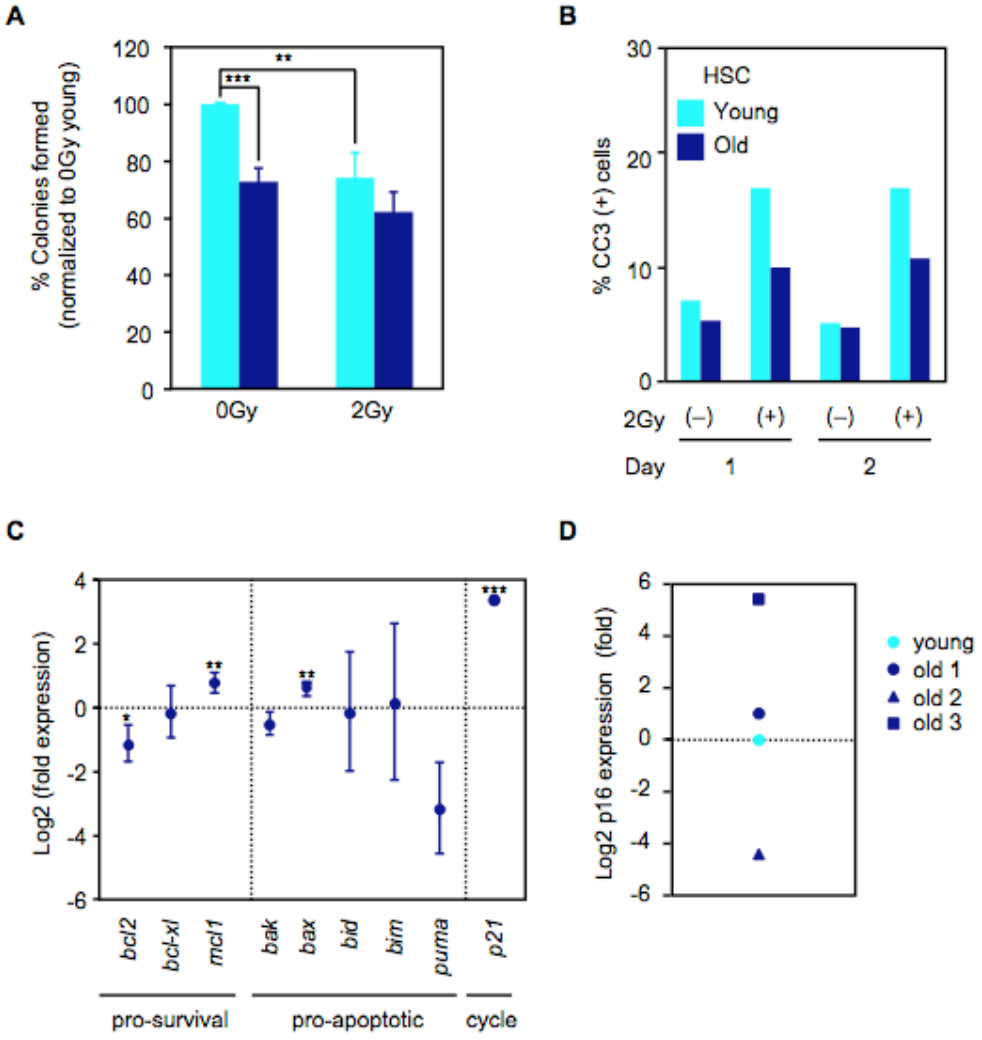
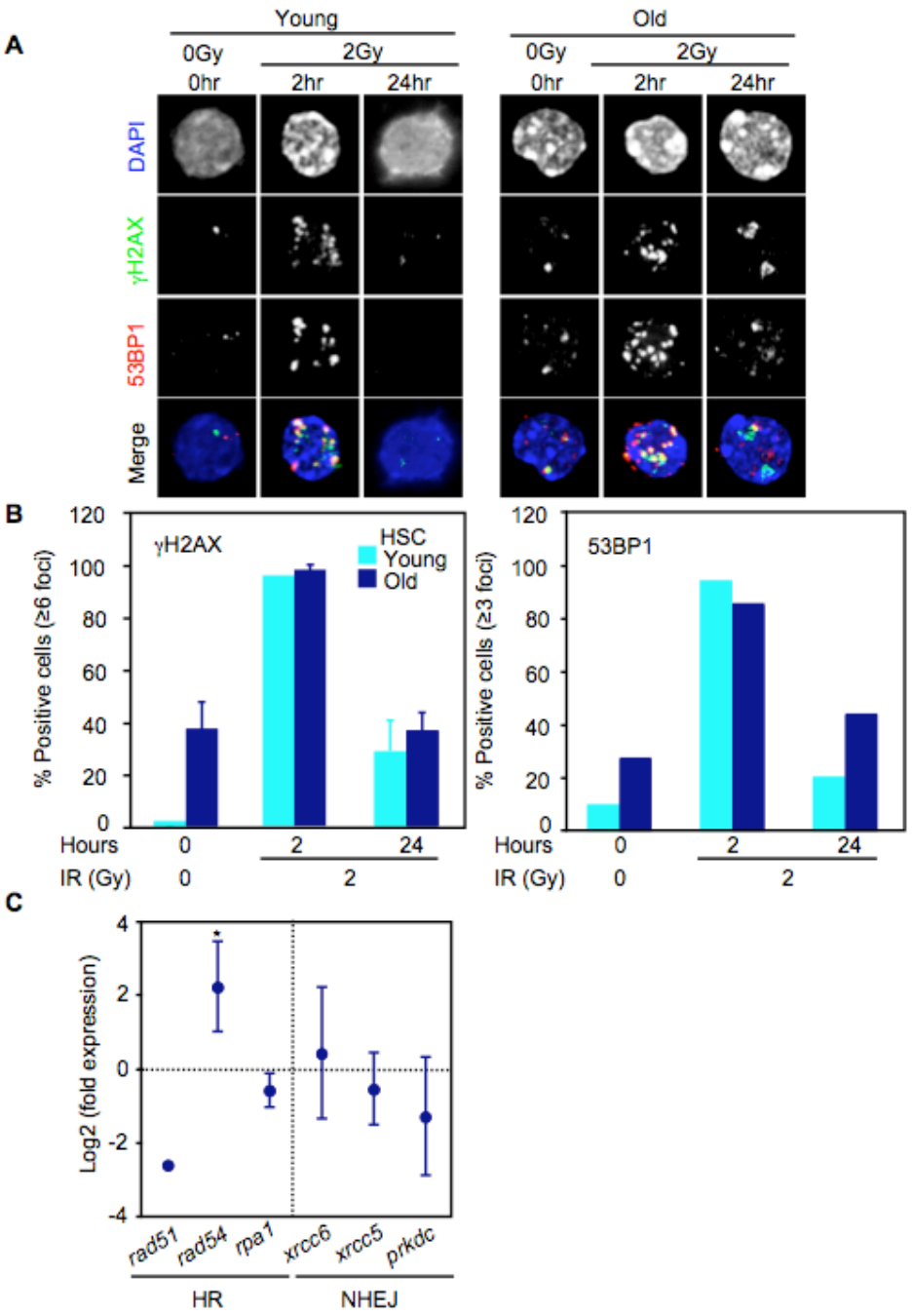

A
% Colonies formed (normalized to 0Gy young)
120
100
80
60
40
20
0
**
***
0Gy
2Gy
B
% CC3 (+) cells
30
20
10
0
HSC
Young
Old
2Gy (–) (+) (–) (+)
Day 1 2
C
Log2 (fold expression)
4
2
0
-2
-4
-6
bcl2
bcl-xl
mcl1
bak
bax
bid
bim
puma
p21
pro-survival
pro-apoptotic
cycle
D
Log2 p16 expression (fold)
6
4
2
0
-2
-4
-6
young
old 1
old 2
old 3

Figure 7. DNA repair in Old HSCs.

(**A**) Representative images of immunofluorescence analysis of 53BP1 containing γH2AX IRIF in unirradiated and 2Gy-irradiated young and old HSCs.

(**B**) Quantification of foci shown in A. The percentage of positive cells (≥ 6 γH2AX positive foci and ≥ 3 53BP1 positive foci) is shown over a 24 hour period (n = 1, γH2AX duplicate). (Left graph n = 2, right graph n = 1).

(**C**) QRT-PCR analysis of the basal expression level of some key HR and NHEJ DNA repair genes in freshly isolated old HSCs. Results are expressed as log2 fold expression compared to levels measured in young HSCs (n = 3; *p ≤ 0.05). Error bars represent standard error of the mean (B) and standard deviation (C).


A
Young
Old
0Gy
2Gy
0hr
2hr
24hr
DAPI
γH2AX
53BP1
Merge
B
γH2AX
53BP1
HSC
Young
Old
% Positive cells (≥6 foci)
% Positive cells (≥3 foci)
120
100
80
60
40
20
0
Hours
0
2
24
IR (Gy)
0
2
C
Log2 (fold expression)
4
2
0
-2
-4
rad51
rad54
rpa1
xrcc6
xrcc5
prkdc
HR
NHEJ

CHAPTER 5

DNA DAMAGE RESPONSE IN TISSUE-SPECIFIC AND CANCER STEM CELLS

Abstract

Recent studies have shown that tissue-specific stem cells (SCs) found throughout the body respond differentially to DNA damage. In this review, we will discuss how different SC populations sense and functionally respond to DNA damage, identify various common and distinct mechanisms utilized by tissue-specific SCs to address DNA damage, and how these mechanisms can impact SC genomic integrity by potentially promoting aging, tissue atrophy and/or cancer development. Finally, we will discuss how similar mechanisms operate in cancer stem cells (CSCs) and can mediate resistance to chemo- and radiotherapy.

Introduction

Stem cells (SCs) are often referred to as the mother of all cells, meaning they sit at the apex of a cellular hierarchy and, upon differentiation, give rise to all the mature cells of a tissue (Rossi et al., 2008). More specifically, SCs are described as having the unique capacity to self-renew, in order to establish and replenish the SC pool, and also to differentiate, thereby generating progeny that carry out specific tissue functions. SCs are essential for specification and morphogenesis of tissues during embryonic development (organogenesis), and for the maintenance and repair of adult tissues throughout life by replacing cells lost during normal tissue turnover (homeostasis) or after injury. Although tissue-specific SCs are found in many highly regenerative organs, such as blood, skin and the digestive tract, they are also found in non-renewing organs like muscle, where they allow repair after tissue damage.

Like every other cell in the body, SCs must constantly contend with genotoxic insults arising from both endogenous chemical reactions, such as reactive oxygen species (ROS) generated by cellular metabolism, and exogenous insults coming from their surrounding environment (Sancar et al., 2004). It has been estimated that every cell undergoes about 100,000 spontaneous DNA lesions per day (Lindahl, 1993). As SCs ensure the lifetime maintenance of a given tissue, any misrepair of DNA damage can be transmitted to their differentiated daughter cells thereby compromising tissue integrity and function. Consequently, mutations that diminish the renewal and/or differentiation

potential of SCs can result in tissue atrophy and aging phenotypes, whereas mutations providing a selective advantage to the mutated cells can lead to cancer development (Rossi et al., 2008).

As such, a delicate balance must be struck to prevent exhaustion and transformation of the SC pool, while maintaining the ability of SCs to preserve homeostasis and to respond to injury when necessary. To fulfill these demands, the numbers of SCs and their functional quality must be strictly controlled through a balance of cell fate decisions (self-renewal, differentiation, migration or death), which are mediated by a complex network of cell-intrinsic regulation and environmental cues (He et al., 2009; Weissman, 2000). Specific protective mechanisms also ensure that SC genomic integrity is well preserved and include localization to a specific microenvironment, resistance to apoptosis, limitation of ROS production, and maintenance in a quiescent state (Orford and Scadden, 2008); Rossi et al., 2008). Altogether, these attributes of SCs ensure tissue maintenance and function throughout the lifetime of an organism, while limiting atrophy and cancer development.

DNA Damage Response

All living cells, including tissue-specific SCs, must constantly contend with DNA damage (Sancar et al., 2004) (Figure 1). Due to its chemical structure, DNA is particularly sensitive to spontaneous hydrolysis reactions which create abasic sites and base deamination. Furthermore, ongoing cellular metabolism generates ROS and their highly reactive intermediate metabolites, which can create 8-oxoguanine lesions in DNA as well as a variety of base oxidations and DNA strand breaks that are all highly mutagenic and can lead to genomic instability. DNA is also constantly assaulted by mutagens present in the external environment. UV light from the sun, as well as various chemical reagents, can react with DNA and induce nucleotide chemical modifications. Ionizing radiations (IR) generated by the cosmos, X-rays, and exposure to radioactive substances, as well as treatment with certain chemotherapeutic drugs, can induce base modifications, interstrand crosslinks, single and double strand breaks (DSBs), which can all lead to genomic instability.

Consistent with the wide diversity of potential DNA lesions, eukaryotic cells exhibit many highly conserved DNA repair mechanisms that can recognize and repair different types of DNA damage with varying fidelity and mutagenic consequences (Lombard et al., 2005) (Figure 1). For instance, base modifications induced by spontaneous chemical reactions and ROS-mediated DNA lesions are repaired by base excision repair (BER), whereas nucleotide

modifications induced by chemicals and UV light are repaired by the nucleotide excision repair (NER) pathway. The pathways that mediate the repair of DSBs vary depending on the cell cycle status of the damaged cells. During the G_0/G_1 phase, DSBs are repaired by the non-homologous end-joining (NHEJ) pathway, while, during the S-G_2/M phase, these lesions are repaired by the homologous recombination (HR) pathway. These two modes of DNA repair are not equally faithful. HR is an error-free DNA repair mechanism due to the use of the other intact strand as a template, while NHEJ is an error-prone repair mechanism, which may result in small deletions, insertions, nucleotide changes or chromosomal translocations due to the absence of an intact template for repair. Lastly, replication errors leading to insertion, deletion and base misincorporation resulting in base mispairing are corrected by the mismatch repair (MMR) pathway.

Irrespective of the type of lesion and the repair mechanism, DNA damage is rapidly sensed and activates evolutionarily conserved signaling pathways, known collectively as the DNA damage response (DDR), whose components can be separated into 4 functional groups: damage sensors, signal transducers, repair effectors, and arrest or death effectors (Sancar et al., 2004) (Figure 2). Ultimately, activation of DDR leads to the phosphorylation and stabilization of p53, inducing its nuclear accumulation and upregulation of its target genes (d'Adda di Fagagna, 2008). Depending upon the extent of DNA damage, the type of cell undergoing DNA damage, the rapidity of DNA repair, the stage of the cell

cycle, the strength and the duration of p53 activation, and the genes transactivated by p53, cells can either undergo transient cell cycle arrest (through induction of the cyclin-dependant kinase inhibitor *p21*), programmed cell death (through induction of the pro-apototic *bcl2* gene family members *bax*, *puma* and *noxa*) or senescence (through induction of the cyclin-dependant kinase inhibitor *p16/Ink4a* and the tumor suppressor gene *p19/ARF*).

Diversity of DNA repair mechanisms in tissue-specific stem cells

The critical role of the different DNA repair mechanisms for overall tissue integrity and function is well illustrated by the severe clinical consequences observed in both humans and mice for mutations in genes regulating these pathways (Hakem, 2008). The involvement of tissue-specific SCs in mediating such symptoms, and the role of the diverse DNA damage recognition and DNA repair mechanisms in maintaining tissue-specific SC function is now starting to emerge (Kenyon and Gerson, 2007).

Defects in DSB recognition machinery lead to premature aging, neurodegeneration and increased cancer susceptibility. ATM (ataxia-telengiectasia mutated), ATR (ATM and Rad3 related) and DNA-PKs are DNA damage sensing protein kinases that, through a series of phosphorylation events, signal the presence of DNA lesions and initiate DNA repair or cell cycle arrest (Figure 2). Patients with mutations in ATM present with blood vessel abnormalities, cerebelar degeneration, immunodeficiency and increased risk of cancers (Hoeijmakers, 2009). Mice lacking *Atm*, like ATM patients, are extremely sensitive to IR exposure and have decreased somatic growth, neurological abnormalities, decreased T cell numbers, and exhibit premature hair graying and infertility (Barlow et al., 1996). Many of these phenotypes can be linked to defects in SC function, which highlights the critical role of this DDR component for the survival and preservation of various SC compartments. *Atm*-deficient

hematopoietic SCs (HSCs) harbor increased ROS levels and display an overall decrease in number and function over time, leading to eventual hematopoietic failure (Ito et al., 2004; Ito et al., 2006). *Atm*-deficiency also sensitizes mice to IR-induced premature melanocyte SC differentiation, resulting in hair graying (Inomata et al., 2009). Germ cell development is also altered in *Atm*-deficient mice, and mutant animals experience a progressive loss in germ SCs (spermatogonia) and become infertile (Takubo et al., 2008). Mutations in ATR also cause developmental defects in mice (pregastrulation lethality) and humans (Seckel syndrome) (Hakem, 2008; Hoeijmakers, 2009; Seita et al., 2010). Conditional deletion of *Atr* in adult mice leads to the rapid appearance of age-related phenotypes, such as hair graying, alopecia, kyphosis, osteoporosis, thymic involution and fibrosis, which are associated with SC defects and exhaustion of tissue renewal and homeostatic capacity (Brown and Baltimore, 2000; Ruzankina et al., 2007). The MRE11, RAD50, and NBS1 (MRN) complex senses DSBs, unwinds the damaged region of DNA, serves as part of the repair scaffolding, and induces downstream signaling including ATM activation (Figure 2). Deletion of any component of the MRN complex results in embryonic lethality in mice (Hakem, 2008). However, mice bearing a hypomorphic $Rad50^{k22m}$ mutation are viable but die around 2.5 months from of B-cell lymphoma or bone marrow failure due, in part, to p53-dependent DDR-mediated apoptosis and loss of HSC function (Bender et al., 2002). Moreover, mutations in *BRCA1* and *BRCA2*, two DSB mediators that trigger DNA repair through the HR pathway

(Figure 2), lead to a major increase in the risk of developing breast and ovarian cancers in women, which, at least in the breast, has recently been linked to the accumulation of genetically unstable mammary SCs (Liu et al., 2008).

While no spontaneous mutations in NHEJ pathway components have been reported so far in human syndromes associated with premature aging or increased risk of cancers, the inactivation of various NHEJ genes in mice has demonstrated their essential function in lymphocyte development and prevention of lymphoma. The core components of the NHEJ repair pathway include the end-binding and end processing proteins Ku70, Ku80, DNA-PKcs, and Artemis, as well as the ligation complex XRCC4, LigIV and Cerrunos (Lombard et al., 2005). As NHEJ is critical for V(D)J recombination during lymphocyte maturation, many of the mutant mouse models deficient in particular NHEJ components exhibit arrested lymphoid development. Mice carrying a $Lig4^{y288c}$ hypomorphic mutation also display growth retardation, immunodeficiency, and pancytopenia associated with severe HSC defects (Kenyon and Gerson, 2007; Nijnik et al., 2007). Mice lacking the end binding and end processing components of NHEJ, Ku70 and Ku80, have stress-induced HSC self-renewal defects associated with poor transplantability, increased apoptosis, decreased proliferation and impaired lineage differentiation (Kenyon and Gerson, 2007; Rossi et al., 2007).

Mutations in NER pathway components induce human syndromes known as Xeroderma Pigmentosum (XP), Cockayne syndrome (CS) and Trichothiodistrophy (TTD), which are characterized by premature aging,

neurodegeneration and extreme photosensitivity, especially in XP syndromes (Hoeijmakers, 2009). XP patients often completely lack NER repair activity and have increased incidence of skin cancer, while CS and TTD patients have defects in transcription-coupled repair, which has little mutagenic effect since it only deals with lesions in the transcribed strand. Mice expressing XPD^{TTD}, a mutated form of an essential NER component, have decreased HSC function with reduced self-renewal potential and increased apoptosis levels (Rossi et al., 2007). Mice deficient in *Ercc1*, a component of both NER and intrastrand crosslink (ICL) repair, die within four weeks of birth, have multilineage hematopoietic cytopenia due to progenitor depletion, HSC senescence, and a defective response to DNA cross linking by mitomycin C (Hasty et al., 2003; Prasher et al., 2005).

Mutations in MMR pathway components induce hereditary non-polyposis human colorectal cancer known as Lynch syndrome, which presents with about an 80% lifetime risk of developing colorectal cancers as well as other malignancies (Hoeijmakers, 2009). Mice mutant for genes important for the MMR pathway, including *Msh2* and *Mlh1*, also display higher frequencies of hematological, skin and gastrointestinal tumors, consistent with a critical role of the MMR in preventing accumulations of oncogenic mutations (Hakem, 2008). In addition, mice lacking *Msh2* exhibit defective HSC activity, with enhanced micro satellite instability observed in their progeny (Reese et al., 2003).

Other human conditions associated with defects in DNA damage recognition and repair pathways include Fanconi's Anemia (genetic defects in the FANC family of proteins), Bloom's or Werner's syndromes (both caused by mutations in DNA helicases) and a range of diseases associated with telomerase dysfunction and telomere instability (Kenyon and Gerson, 2007). These diseases are not specifically reviewed here, but their complex pathologies involve defects in various tissue-specific SCs.

DNA damage response in tissue specific SCs

While tissue-specific SCs share the same purpose of maintaining organ functionality, recent studies have shown that the mechanisms of their responses to DNA damage, the outcome of their DDR, and the consequences of DNA repair for their genomic stability vary greatly between tissues.

Hematopoietic SCs.

The hematopoietic (blood) system is one of the best-studied adult tissues in terms of its hierarchical development, in that all blood cell lineages derive from a small number of quiescent HSCs via a highly proliferative amplifying progenitor compartment (Orkin and Zon, 2008). Being a highly regenerative compartment, it is also one of the most radiosensitive tissues in the body (<4Gy), and one of the first organ systems to fail after total body irradiation. IR exposure differentially affects hematopoietic cells depending on their state of maturity, with HSCs being more radioresistant than their downstream progeny (Meijne et al., 1991). By comparing the way HSCs and their differentiated progeny respond to low doses of IR (2-3Gy), recent work has begun to clarify the ways in which HSCs at different stages of ontogeny deal with DNA damage, and the mutagenic consequences of different DNA repair mechanisms in this tissue-specific SC population (Figure 3A).

HSCs are specified in the aorta-gonad-mesonephros (AGM) region of the developing fetus, are actively expanded in several anatomic locations, including the liver and placenta, during fetal development, and are finally seeded in the bone marrow cavity during late embryogenesis. In the bone marrow, HSCs progressively mature after birth to become the quiescent adult HSCs that are maintained during the lifetime of the organism. Fetal and adult HSCs differ in many aspects of their biological regulation including cell cycle status and transcriptional control (Orkin and Zon, 2008). Using human umbilical cord blood (CB)-derived HSCs, which are highly proliferative, circulating cells that are still considered to be of fetal origin, Milyavsky and colleagues found that irradiated (3Gy) CB-derived HSCs had a slower rate of DSB repair than more mature progenitors, and increased levels of apoptosis mediated in part through the ASPP1 protein, which could be reversed if *p53* expression was silenced or *bcl2* expression was enhanced (Milyavsky et al., 2010). Upon primary transplantation, irradiated CB-derived HSCs could not successfully engraft into immunodeficient mice. In contrast, irradiated cells with disabled *p53* or *bcl2* overexpression could be serially transplanted, albeit with decreased efficiency compared to non-irradiated normal cells. In this context, transplanted CB-derived HSCs with disabled *p53* reconstituted even less well than cells with *bcl2* overexpression, and their progeny harbored high levels of DSBs that were not observed in the progeny of *bcl2* overexpressing cells. This study emphasizes the role of p53-mediated DDR and the Bcl2 family of pro-survival genes in HSC function (Asai et

al., 2010; Seita et al., 2010)(Weissman, 2000), and indicates that the main outcome of the DDR in fetal HSCs is induction of apoptosis and overt cell elimination (Figure 3A). On the other hand, using adult mouse HSCs that are kept mostly quiescent within the bone marrow cavity, Mohrin and colleagues showed a very different response to irradiation, with overt cell survival and DNA repair being the main outcomes of the DDR (Mohrin et al., 2010). Adult HSCs, either quiescent or induced to proliferate by cytokine pre-treatment, engage specialized response mechanisms that protect them from low doses of IR (2Gy). In quiescent HSCs, these mechanisms include enhanced pro-survival gene expression (*bcl2*, *bcl-xl*, *mcl1*, *a1*), which inhibits cell death induced by p53 pro-apototic genes (*bax*, *noxa*, *puma*), likely allowing p53-mediated induction of *p21* to engage a transient growth arrest response and to permit DNA repair. While the exact mechanisms of the survival response in proliferating HSCs is less clear, they were found to be as radioresistant as quiescent HSCs (Mohrin et al., 2010). Dictated by their cell cycle status, proliferating HSCs use the high-fidelity HR pathway to repair DSBs, while quiescent HSCs employ the error-prone NHEJ pathway. Irradiated quiescent HSCs display high levels of chromosomal abnormalities when compared to proliferating HSCs, and their progeny show persistent genomic instability associated with misrepaired DNA and engraftment defects in secondary recipient mice. Since NHEJ appears to be the initial and most commonly used DNA repair mechanism in quiescent HSCs, these results help explain why most mouse models lacking functional components of DSB

recognition and repair pathways undergo hematopoietic failure upon genotoxic stress (Hakem, 2008). Moreover, this study indicates that while adult HSCs, in contrast to fetal HSCs, may survive DNA-damaging insults, they do not emerge unscathed (Figure 3A), which might have direct implications for aging and cancer development. It may also explain why cancer patients treated with radiotherapy or chemotherapy may develop leukemias and lymphomas (blood cancer) or myelodysplasias (bone marrow failure) as the use of error-prone DNA repair in quiescent HSCs may be at the heart of these dangerous side effects of cancer treatment.

Taken together, these two studies (Milyavsky et al., 2010; Mohrin et al., 2010) unveil some striking differences in the outcome of irradiation-induced DDR in HSCs from different species and at different developmental stages. While it is possible that different organisms with vastly different lifespans have evolved distinct strategies to cope with DNA damage, it is tempting to speculate that these differences reflect an adaptation in the stress response mechanisms used by HSCs at distinct stages of ontogeny to ensure optimal function of the blood system. During embryogenesis and until birth, the goal is to expand the SC population while protecting its genomic integrity, in order to establish a pool of pristine HSCs that will ensure blood homeostasis for the lifetime of the organism. In this context, the efficient elimination of irradiated human CB-derived HSCs described by Milyavsky and colleagues fulfill this demand by eliminating damaged fetal HSCs that could be detrimental to the organism and its

reproductive purpose. Conversely, in adults, the main function of the HSC compartment is to preserve blood homeostasis and to quickly respond to hematopoietic needs (blood loss, infection, etc). The fact that adult HSCs reside in hypoxic niches in the BM cavity and are mostly kept in a quiescent phase of the cell cycle contribute to their overall maintenance (self-renewal) and protect their genomic integrity (fitness) by minimizing DNA damage associated with ROS production, cellular respiration and cell division (Orford and Scadden, 2008; Rossi et al., 2007). In this context, the survival and efficient DNA repair of irradiated mouse adult HSCs described by Mohrin and colleagues fulfills the same purpose by protecting the most important cells of the tissue. Since both quiescent and proliferating mouse adult HSCs show similar radioresistance, it is likely that the radiosensitivity displayed by human CB-derived HSCs reflect cell-intrinsic differences in transcriptional programs or chromatin states between HSCs at various stages of development. Additional investigations are clearly needed to fully understand the mechanisms underlying these differences in DDR outcomes between fetal and adult HSCs.

However, the short-term survival strategy used by adult HSCs likely comes at a cost for their long-term genomic integrity. While quiescence is one of the very mechanisms that protects adult HSC function, it also renders damaged HSCs intrinsically vulnerable to mutagenesis since it forces them to use the error-prone NHEJ pathway to repair DSBs, thereby increasing the risk of creating mutations in this self-renewing population. In fact, the accrual of chromosomal

translocations resulting from unfaithful DNA repair following DSBs is a hallmark of human blood malignancies (Look, 1997). Such accumulation over time of NHEJ-mediated mutations may hinder cellular performance and could be a major contributor to the loss of function occurring with age in the HSC compartment, and to the development of age-related hematological disorders (Rossi et al., 2007).

Epidermal SCs

The skin epidermis is composed by the juxtaposition of the many pilosebaceous units consisting of a hair follicle, its associated sebaceous gland and its surrounding interfollicular epidermis. Different classes of SCs ensure homeostasis of the skin epidermis (Blanpain and Fuchs, 2009). Multipotent hair follicle bulge SCs (BSCs) contribute to the cyclic regeneration of the hair follicle and to the repair of the interfollicular epidermis following wounding. In the absence of injury, the interfollicular epidermis can self-renew independently of BSCs through the presence of unipotent progenitors scattered throughout the basal region of the epidermis. Specialized SCs and progenitor cells are also found in the infundibulum and sebaceous glands (Blanpain and Fuchs, 2009).

Since the epidermis serves as a barrier between the body and the external environment, it is constantly assaulted by genotoxic stress such as UV irradiation. As discussed earlier, UV radiation causes the formation of thymidine dimers, (6-4) pyrimidine photoproducts and ROS-induced DNA lesions that are repaired by the NER, NHEJ or HR pathways, depending on the type of damage

and the state of the cell cycle. Upon UV irradiation, basal epidermal cells exhibit sustained p53 activation compared to the more differentiated suprabasal cells (Finlan et al., 2006). Following chronic administration of UV radiation, slow cycling SCs and progenitor cells of the infundibulum and sebaceous glands also retain UV-induced photoproducts longer than more differentiated cells of the epidermis, suggesting a decrease in the repair activity of these cells (Nijhof et al., 2007). Recently, *Nrf2* has been shown to regulate the expression of critical regulators of oxidative stress (such as several enzymes of the glutathione metabolism) and to protect the epidermis from UV-induced apoptosis. The gradient of apoptosis levels observed between basal (high) and suprabasal (low) cells following UV irradiation is inversely correlated with *Nrf2* expression. Surprisingly, while Nrf2 overexpression protects basal cells from UV induced apoptosis, it does not decrease the proportion of cells that harbor thymidine dimers. In addition, suprabasal expression of *Nrf2* offers some protection from UV-induced apoptosis to basal cells through a paracrine mechanism (Schafer et al., 2010). These data indicate that proliferative cells of the interfollicular epidermis are more sensitive to UV mediated apoptosis relative to their more committed progeny.

While the skin epidermis is more radioresistant than the blood system, acute administration of more than 5Gy results in severe skin reactions consisting of inflammation (erythema) and loss of differentiated skin layers (desquamation) that rapidly appear following IR, whereas hair loss and chronic ulcerations appear

with a delay of 2 to 3 weeks after IR administration. The sensitivity of the epidermis to IR is also illustrated by the common side effects of radiotherapy, which include acute and chronic dermatitis and an increased incidence of skin cancer (Goldschmidt and Sherwin, 1980). While the field is still in search of specific cell surface markers that will allow high purity isolation of interfollicular epidermal progenitors, a combination of markers including α6 integrin and CD71 have been used to enrich SCs from the mouse and human interfollicular epidermis (Li et al., 1998; Tani et al., 2000). Following exposure to low doses of IR, rapidly cycling human epidermal progenitor cells (α6H high/CD71+) undergo apoptosis and display decreased in vitro colony forming efficiency, whereas slow cycling human epidermal SCs (α6H/CD71-) were resistant to IR-induced cell death (Rachidi et al., 2007). The enhanced survival of human epidermal SCs upon IR exposure has been linked to a higher secretion of FGF2 following DNA damage, which increases DNA repair activity in epidermal SC by autocrine/paracrine mechanisms (Harfouche et al., 2010). While these studies have been performed *ex vivo*, Sotiropoulou and colleagues have recently investigated how epidermal cells respond to DNA damage within their native niche, and showed that multipotent hair follicle BSCs, like HSCs, are more resistant to DNA damage induced cell death compared to the other cells of the epidermis (Sotiropoulou et al., 2010). At least two important mechanisms contribute to the higher resistance of BSCs to IR-mediated DNA damage (Figure 3B), both which are independent of the relative quiescence of these cells and of

the induction of premature senescence. First, BSCs express higher levels of the anti-apoptotic protein Bcl2 and the proportion of BSCs undergoing apoptosis is increased in *bcl2* null mice, demonstrating that similar to HSCs, a higher expression of pro-survival factors contributes to the resistance of BSCs to apoptosis. The other contributing mechanism is the transient nature of DDR activation in BSCs. Soon after IR exposure, p53 is expressed in the nuclei of almost all epidermal cells, including BSCs, and is required for DNA damage-induced cell death in the epidermis (Botchkarev et al., 2000; Song and Lambert, 1999; Sotiropoulou et al., 2010). However, unlike other cells of the epidermis, the number of BSCs expressing p53 is greatly decreased by 24 hours following irradiation and mutant mice exhibiting sustained expression of p53 show increased IR-induced apoptosis in BSCs. This indicates that the short duration of IR-mediated p53 activation promotes BSC survival following DNA damage. Interestingly, BSCs also display accelerated DNA repair and enhanced NHEJ repair activity. In SCID mice, which have a mutation in *DNA-PK* and thus exhibit decreased NHEJ activity, BSCs are radiosensitive, suggesting that accelerated NHEJ-mediated DSB repair contributes to their protection against IR exposure. The importance of DDR in BSCs is also illustrated by the SC exhaustion and progressive alopecia that occurs in mice where *Atr* has been deleted in hair follicle BSCs and their progeny (Ruzankina et al., 2007).

Since NHEJ is an error-prone DNA repair mechanism, the higher resistance of BSCs to DNA damage-induced apoptosis and the accelerated

NHEJ-mediated DNA repair activity could be, like in HSCs, a double edged sword that promotes short-term survival of BSCs at the expense of their long-term genomic integrity, and could potentially allow for the accumulation of cancerous mutations (Figure 4). Consistent with this notion, SCID mice and mice deficient for Bcl-X_L, a pro-survival gene show decreased susceptibility to chemical carcinogenesis (Kemp et al., 1999; Kim et al., 2009), which has been attributed to the elimination of mutated BSCs by apoptosis.

Melanocyte SCs

Melanocytes are neural crest-derived cells responsible for the pigmentation of skin and hair. The mature melanocytes responsible for hair color are derived from melanocyte SCs (MSCs), which reside in the same niche as hair follicle BSCs. At each cycle of hair regeneration, MSCs are stimulated to proliferate and give rise to transit amplifying cells, which will expand in the lower hair follicle before undergoing terminal differentiation, which results in the integration of their pigment into the new hair. At the end of each hair cycle, mature melanocytes undergo apoptosis and are eliminated with the rest of the follicle, to be subsequently replenished by the renewal and differentiation of MSCs during the next cycle (Robinson and Fisher, 2009). Hair graying, which is one of the most common signs of aging, results from the depletion of MSCs from the hair follicle. The onset of hair graying in mice and humans is accompanied by the presence of ectopically pigmented melanocytes, suggesting premature differentiation of MSCs within their niche (Nishimura et al., 2005). Premature hair

graying can also result from a hypomorphic mutation in *Mitf,* the main regulator of MSC differentiation, that results in a downregulation of *bcl2* and in premature differentiation of MSCs in the hair follicle (McGill et al., 2002). Bcl2 is critical for MSC maintenance as *bcl2* null mice lose their coat pigmentation after the first hair cycle due to massive MSC apoptosis (Nishimura et al., 2005). Premature hair graying and progressive MSC loss also occur following administration of DNA damaging agents such as IR, mitomycin C or hydrogen peroxide (Inomata et al., 2009). While the mechanisms underlying the DDR in MSCs are not yet fully understood, p53, p16 and $p19^{ARF}$, although transiently activated by DNA damage, are not responsible for the premature differentiation and loss of MSCs. Indeed, mice deficient for *p53* or the *Ink4a* locus (p16 and $p19^{ARF}$) are not protected from DNA damage-induced hair graying, contrasting with the requirement of p53 in mediating DNA damage-induced cell death in other tissue-specific SCs. In contrast, DNA damage induces prolonged activation of the canonical differentiation program of MSCs including sustained upregulation of *Mitf*, a key regulator of melanocyte differentiation and melanogenic enzymes, which in turn stimulates the premature and ectopic differentiation of MSCs within their niche. The ATM checkpoint regulator also exerts a protective function in MSCs since *Atm* null mice and ATM deficient patients exhibit premature hair graying (Hakem, 2008), and loss of *Atm* sensitizes mice to IR induced premature MSC differentiation (Inomata et al., 2009).

Despite being located in the same hair follicle niche, BSCs and MSCs

respond very differently to DNA damage. Both types of SCs do not senesce or commit apoptosis upon DNA damage, but while BSCs repair their DNA rapidly and express high levels of anti-apoptotic molecules in order to avoid programmed cell death, MSCs are eliminated by premature differentiation (Figure 4). These different outcomes imply that cell intrinsic properties are more important than the local microenvironment in controlling DDR in skin SCs. It is interesting to note that melanoma, a malignant tumor of melanocytes, does not arise from hair follicle MSCs but rather from skin melanocytes. These cells are located along the interfollicular epidermis, suggesting that the premature differentiation of MSCs following DNA damage may serve to eliminate pre-cancerous MSCs residing in the hair follicle.

Intestinal SCs.

The intestinal tissue is very sensitive to DNA damage. Acute whole-body irradiation (<6Gy) induces considerable damage to the intestine, resulting in severe diarrhea and electrolyte imbalances, which can be lethal in extreme cases. The intestinal lining is a simple epithelium composed of a single layer of cells that can be divided into two compartments: the proliferative base of the intestine, called the crypt, and the differentiated intestinal cells forming the villi that face the intestinal lumen. The intestinal SCs (ISCs) are localized at the bottom of the crypt, where they proliferate to give rise to transit amplifying cells, which are found along the crypt, and divide faster and migrate to the upper part of the crypt where they undergo cell cycle arrest and terminal differentiation

(Barker et al., 2010; Casali and Batlle, 2009; Marshman et al., 2002). Although the exact position of the ISCs within the crypt is still under intense debate, it has long been suggested that ISCs reside at the +4 position from the base of the crypts and that these SCs are more quiescent compared to the other crypt cells. Consistent with that notion, Bmi1, which is preferentially expressed in +4 crypt cells, induced long term labeling of the crypto-vilus unit in *Bmi1CRE^{ER}* reporter mice, consistent with the labeling of long-lived multipotent ISCs (Sangiorgi and Capecchi, 2008). A second population of ISCs expressing Lgr5, a leucine-rich orphan G protein-coupled receptor and Wnt pathway activated gene, has recently been identified (Barker et al., 2007). Lgr5+ cells cycle more frequently than the +4 cells and are located at the bottom of the crypt intercalated between the paneth cells. Lineage tracing experiments using *Lgr5-GFP-IRES-Cre-ERT;;RosaLacZ* reporter mice demonstrated that Lgr5+ cells give rise to all intestinal cell lineages and result in the long-term labeling of the cryptovilus unit, also consistent with the labeling of long-lived multipotent ISCs.

ISCs are extremely sensitive to DNA damage, and undergo massive apoptosis upon low doses of irradiation (1Gy). Interestingly, while it is generally assumed that radiosensitivity is correlated with cell cycle status (Gudkov and Komarova, 2003), the apoptosis sensitivity of intestinal crypt cells is inversely correlated with their relative quiescence. The most quiescent ISCs located at +4 position are the most sensitive to IR-induced cell death, followed by the more active Lgr5+ ISCs, whereas the rapidly cycling transit amplifying cells appear to

be the most radioresistant (Barker et al., 2007; Potten et al., 2002; Wilson et al., 1998). Different mechanisms are responsible for the extreme sensitivity of ISCs to DNA damage, including an enhanced activation of the p53 pathway, lower expression of the anti-apoptotic protein Bcl2 (Merritt et al., 1995) and general lack of DNA repair activity (Potten, 2004). Upon irradiation, expression of p53 and its downstream target genes *p21* and *puma* increases throughout the crypts, but the frequency of p53 positive cells and the levels of expression of its target genes are higher at the base of the crypt, and progressively decrease along the crypts towards the vilus (Merritt et al., 1994; Qiu et al., 2008; Wilson et al., 1998). Furthermore, IR does not induce apoptosis in the intestine of *p53* null mice (Merritt et al., 1994; Qiu et al., 2008; Wilson et al., 1998). IR-induced ISC apoptosis is also blocked in *puma*-deficient mice and ISC survival is prolonged after administration of *puma* antisense nucleotides thereby demonstrating that Puma is the main pro-apoptotic target of the p53-mediated DDR in ISCs (Qiu et al., 2008). In contrast to other SC populations described above, *bcl2* expression is not detected in ISCs and irradiated *bcl2* null mice only show a modest increase in ISC apoptosis, suggesting that Bcl2 does not play a critical role in protecting ISCs from DNA damage-induced cell death (Merritt et al., 1995). Finally, the absence of an irradiation dose response of crypt degeneration suggests that quiescent ISCs lack DNA repair capacity, thereby increasing their propensity to undergo apoptosis following DNA damage (Hendry et al., 1982; Potten, 2004).

The architecture of the colon resembles that of the small intestine. Similar

to ISCs, colonic SCs (CoSCs) are also localized at the bottom of the crypt and express Lgr5, although CoSCs exhibit a longer cell cycle time than ISCs. Interestingly, the DDR of CoSCs differs significantly from that of ISCs, with CoSCs being considerably more radioresistant than ISCs (Figure 4). It is estimated that CoSCs require eight times the dose of irradiation needed by ISCs to reach similar levels of apoptosis (Barker et al., 2007; Potten and Grant, 1998; Pritchard et al., 2000). The greater radioresistance of CoSCs has been attributed to a lower expression of *p53* (Hendry et al., 1997; Merritt et al., 1994) and higher expression of *bcl2* (Merritt et al., 1995; Qiu et al., 2008). Furthermore, in contrast to ISCs, CoSCs from *bcl2* null mice show a much greater increase in DNA damage-induced apoptosis, demonstrating that *bcl2* expression in CoSCs does contribute to their higher relative radioresistance. The altruistic suicide of ISCs in response to DNA damage could decrease the acquisition of pre-cancerous mutations in these cells, and potentially explain the rarity of intestinal neoplasia compared to the higher frequency of colonic cancers, despite the higher cellular turnover of the intestine.

Germline SCs

Primordial germ cells (PGCs) are transient precursors of germ SCs (GSCs), which upon meiosis give rise to the gametes (sperm and egg), which are the only cells capable of transferring genetic information from one generation to the next (Chuva de Sousa Lopes and Roelen, 2010; Laird et al., 2008; Richardson and Lehmann, 2010). PGCs are specified in the embryo, migrate to

the gonadal ridges were they undergo sex determination and give rise to the female (oogonia) or the male (spermatogonia) GSCs. The spermatogonia exhibit an almost unlimited life span, remaining quiescent until puberty, at which point they re-acquire the ability to self-renew, undergo meiosis and produce mature male gametes for the lifetime of the organism. In sharp contrast, the pool of oogonia is established during embryogenesis and consequently females are born with a finite number of oogonia.

The generation of haploid chromosomes during meiosis requires many of the proteins involved in DNA repair (Sasaki et al., 2010). During PGC maturation, genome wide DNA demethylation occurs in order to erase genomic imprinting. DNA demethylation in mouse PGCs is initiated by the appearance of single strand breaks and activation of the BER pathway, which may be linked to deamination of methylcytosine or to other yet to be discovered mechanisms (Hajkova et al., 2010). Mutations in the germ line can be extremely dangerous and can either directly lead to sterility (Loft et al., 2003) or transmission of heritable genetic diseases by the gametes. Genetic aberrations in GSCs may occur upon radiation exposure, such as radiotherapy and radiological examination, or after exposure to teratogenic or mutagenic chemicals, but the main source of DNA damage is their normal metabolic activity and ROS production (Kujjo et al., 2010). Microarray analysis uncovered that DNA damage sensors and multiple components of the NHEJ, BER, NER and MMR pathways are expressed in human oocytes (Menezo et al., 2007), with a similar high

expression of DNA repair proteins found in human sperm (Galetzka et al., 2007), which suggest that GSCs and gametes are well equipped to respond to DNA damage. Accordingly, spermatogonia in *Atm*-deficient mice are progressively lost, undergo meiotic arrest, accumulate DNA damage and lose their self-renewal potential in a *p21*-dependent manner (Takubo et al., 2008). Mice expressing the hypomorphic mutation of $Rad50^{k22m}$ also show severe attrition of spermatogonia, which could be minimized by loss of p53 (Bender et al., 2002).

The cell cycle duration of human spermatogonia is estimated to be around 16 days, with male GSCs being mostly kept in the G_0/G_1 phase of the cell cycle. Consequently, NHEJ is the first line of DNA repair in these cells. Interestingly, *in vitro* studies in mice showed that spermatogonia are more sensitive to IR when they are quiescent than when they are proliferating (Forand et al., 2009; Moreno et al., 2001). In oogonia, the homologous chromosomes are close to each other and female GSCs preferentially repair their DNA using HR (Baker, 1971). Mutations in the HR repair pathway render female GSCs more susceptible to DNA damage-mediated cell death as shown by the increase sensitivity to doxorubicin-induced apoptosis in oocytes from mice deficient in *Rad51* (Kujjo et al., 2010). Contrary to most SC populations and somatic cells, the DDR in female GSCs does not depend on p53. Instead, TAp63, an isoform of the p63 gene and a p53 homolog, is constitutively expressed in oocytes and is rapidly phosphorylated following DNA damage. Deletion of *TAp63* in mice results in a

major increase in oocyte radioresistance, consistent with the notion that TAp63 is the primary mediator of DDR pathway in oocytes (Suh et al., 2006).

Mammary SCs

The mammary gland alternates between cycles of growth and degeneration in relation to the estrus cycle. Mammary stem cells (MaSCs) are responsible for homeostasis of the breast tissue and for the massive tissue expansion and remodeling that occurs during pregnancy and lactation (Visvader, 2009). MaSCs have been isolated from mice and humans and represent multipotent SCs that have the ability to self renew as well as to differentiate into ductal, alveolar, and myoepithelial cell lineages (Ginestier et al., 2007; Shackleton et al., 2006; Stingl et al., 2006). Breast cancer is the most common form of malignancies in women. Mutations in genes involved in DNA repair such as *BRCA1* and *BRCA2* are found in the majority of patients with hereditary breast cancers, demonstrating the importance of the HR-repair pathway in preventing the occurrence of mammary tumors (Bradley and Medina, 1998). Mice deficient for *Brca1* are embryonic lethal, but mice with a conditional deletion of *Brca1* in the mammary epithelium are viable, display severe abnormalities in mammary morphogenesis and develop undifferentiated breast cancers (Hakem, 2008). Knock down of *BRCA1* in human MaSCs leads to a decrease of differentiated luminal cells and an increase in cells with SC characteristics, which suggests that BRCA1 is required for normal MaSC differentiation and that *BRCA1* loss may

result in the accumulation of genetically unstable MaSCs that are susceptible to cancer development (Liu et al., 2008).

While the role of DNA repair in mammary development, maintenance and prevention of breast tumors is well established, the mechanisms underlying the DDR in MaSCs have only just begun to emerge (Figure 4). Mouse MaSCs are more radioresistant than their differentiated progeny, and their numbers increase following IR (Woodward et al., 2007). Interestingly, MaSCs present less DNA damage and rapidly activate the Wnt/ß-catenin signaling pathway following IR. Furthermore, increasing ß-catenin signaling by overexpression of Wnt1 or stabilized ß-catenin increases the survival of MaSCs following DNA damage, indicating that Wnt/ ß-catenin signaling is an important component of the DDR in MaSCs that may promote MaSC survival through upregulation of survivin, a direct Wnt/ ß-catenin target gene (Chen et al., 2007; Woodward et al., 2007). It would certainly be interesting to determine whether the selective activation of Wnt/ß-catenin pathway observed in MaSCs also occurs in other tissue-specific SCs and promotes their survival following DNA damage. Another mechanism that might promote MaSCs resistance to DNA damage is their low level of ROS compared their differentiated progeny (Diehn et al., 2009).

DNA damage response in cancer stem cells

A number of human cancers including leukemia, glioblastoma, breast and skin cancers contain cells with higher clonogenic potential that are capable of reforming the parental tumors upon transplantation. These cells functionally resemble tissue-specific SCs, albeit with aberrant self-renewal and differentiation abilities, and have been collectively referred to as cancer SCs (CSCs) despite their variable developmental origin (Clarke and Fuller, 2006; Jordan et al., 2006). It has been suggested that CSCs are responsible for disease progression and tumor relapse after therapy. Recent studies indicate that CSCs may take advantage of the mechanisms of DNA repair used by tissue-specific SCs to mediate resistance to chemo- and radiotherapy.

CSCs in leukemia

Leukemias are cancers of the blood system, which often arise due to deregulated HSC functions or acquisition of extended self-renewal capabilities by more mature progenitor cells (Passegue, 2005). Leukemia CSCs exist in acute myeloid leukemia (AML) and chronic myelogenous leukemia (CML) and have been shown to be more resistant to cancer therapies than the bulk of the leukemia cells, indicating that their survival may be responsible for disease persistence and cancer relapse (Elrick et al., 2005; Jordan et al., 2006). Leukemia CSCs also use to their advantage some protective mechanisms of HSCs, including quiescent cell cycle status, localization to a hypoxic niche and

DDR mechanisms, to specifically escape chemo- and radiotherapy that kill the bulk of the tumor cells (Guzman and Jordan, 2009).

CML is a two-stage blood disease caused by the acquisition of the chromosomal translocation fusion product BCR/ABL in HSCs, which can be separated into chronic and acute phases. The transition from chronic to acute disease is still poorly understood, but the presence of DNA damage and the acquisition of additional chromosomal aberrations resulting in overall genomic instability in both HSCs and their downstream progeny is believed to play a critical role in this transition (Burke and Carroll, 2010). BCR/ABL expression increases intracellular ROS levels, which in turn enhances oxidative stress and DNA damage, and deregulates DNA repair mechanisms thereby promoting unfaithful and/or inefficient DNA repair leading to mutations and chromosomal aberrations (Perrotti et al., 2010). Malfunctioning MMR, mutagenic NER, and compromised DSB repair (both HR and NHEJ) are all hallmarks of cells expressing BCR/ABL (Burke and Carroll, 2010; Deutsch et al., 2001; Slupianek et al., 2002; Slupianek et al., 2006). Once DNA damage occurs, BCR/ABL-mediated signaling can also inhibit apoptosis, thereby allowing cells to survive DNA damage with which they normally would not be able to cope (Burke and Carroll, 2010; Deutsch et al., 2001; Slupianek et al., 2002; Slupianek et al., 2006). The genomic instability induced by BCR/ABL has major implications for the pathogenesis and treatment of CML since it can facilitate disease progression from chronic to acute phase, and promote the acquisition of resistance against

the current drugs used to treat CML (tyrosine kinase inhibitors such as imatinib). Indeed, evolution from HSC-derived CSCs to myeloid progenitor-derived CSCs has been observed during the transition to myeloid blast crisis in human CML, and linked to activated mutations in the Wnt/b-catenin pathway and acquisition of aberrant self-renewal activity in HSC progeny (Rice and Jamieson, 2010). Preventing oxidative stress and correcting defects in DNA repair pathways in BCR/ABL-expressing CSCs at all stages of the disease may therefore be beneficial to limit the acquisition of drug resistance and slow down CML progression (Koptyra et al., 2006; Perrotti et al., 2010).

Leukemia CSCs maintain some of the same protective mechanisms as normal HSCs. CSCs in both CML and AML have been found to be quiescent (Elrick et al., 2005; Guan et al., 2003; Ishikawa et al., 2007), suggesting that cell cycle restriction is one of the protective mechanisms that leukemia CSCs utilize to their advantage (Guzman and Jordan, 2009). Indeed, human AML CSCs transplanted into immunodeficient mice use quiescence as a protective mechanism against chemotherapy (Saito et al., 2010). When these cells are induced to exit quiescence and to enter the cell cycle by treating the mice with the cytokine G-CSF, AML CSCs become more sensitive to chemotherapy and are effectively eliminated *in vivo*. Leukemia CSCs are also able to co-opt other mechanisms used by normal HSCs for their protection, such as p53-mediated induction of *p21* and resulting growth arrest that has recently been found to be critical in protecting adult HSCs from IR (Mohrin et al., 2010). Expression of the

PML/RAR or AML1/ETO fusion oncoproteins in murine HSCs induces high levels of DNA damage and activates a *p21*-dependent cell cycle arrest in AML CSCs, which allows them to repair excessive DNA damage and to escape apoptosis, thereby maintaining their leukemic self-renewal capacity (Viale et al., 2009). While it may seem paradoxical that a leukemia-initiating oncogene promotes cell cycle arrest instead of proliferation, the hijacking of such a protective mechanism provides a strong selective advantage to the CSCs. In the absence of *p21*, AML CSCs were more sensitive to replicative and therapeutic stress, and *p21*-null HSCs expressing PML/RAR or AML1/ETO were unable to transplant the disease into recipient mice, indicating a failure to maintain CSC activity (Viale et al., 2009).

CSCs in Breast Cancer

The first evidence that solid tumors also contained cells with CSC properties came with the demonstration that in human breast cancer, $CD44^{+}CD24^{-/low}$ cells are more clonogenic and, when transplanted in immunocompromized mice, are able to generate tumors that recapitulate the parental disease (Al-Hajj et al., 2003). Transcriptional profiling of murine mammary gland CSCs revealed increased expression of many DDR and DNA repair associated genes (Zhang et al., 2008), suggesting that mammary gland CSCs might be more resistant to chemo- and/or radiotherapy. Comparison of tumor biopsies before and after neoadjuvant chemotherapy showed an increase in the proportion of mammary gland CSCs with mammosphere-forming capacity

following chemotherapy, hence confirming that mammary gland CSCs are more resistant to chemotherapy (Li et al., 2008; Shafee et al., 2008). Like normal MaSCs, mammary gland CSCs harbor lower levels of ROS compared to the rest of the tumor cells, due to increased levels of genes regulating free radical scavenging systems, such as those of the glutathione metabolism. Mammary gland CSCs from human xenografts (Phillips et al., 2006) or MMTV-Wnt1 tumor bearing mice (Diehn et al., 2009) exhibited higher survival upon IR treatment. Consistent with the fact that ROS levels control IR-induced DNA damage and apoptosis in CSCs, inhibition of glutathione metabolism decreased the clonogenic potential and sensitized mammary gland CSCs to IR (Diehn et al., 2009). Furthermore, *p53*-deficient mammary gland CSCs show accelerated DNA repair activity as well as high Akt and Wnt signaling activity, which promotes CSC survival following IR treatment (Zhang et al., 2010). Interestingly, administration of an Akt inhibitor inhibits □-catenin signaling and sensitizes mammary gland CSCs to radiotherapy.

Understanding the role of DNA repair genes in the pathogenesis of breast cancer has been exploited for the development of novel anti-cancer strategies. Tumors derived from *Brca1*-deficient cells are extremely sensitive to the inhibition of PARP, which plays an important role in the repair of single strand breaks by the BER pathway. In the absence of *Brca1* and HR-mediated DNA repair, persistent single strand breaks need to be repaired by the BER pathways, and as a consequence inhibition of PARP blocks this alternative pathway of DNA repair,

inducing cell death preferentially in cancer cells. A PARP inhibitor prolonged disease free survival when administered alone or in combination with chemotherapeutic drugs in a mouse model of *brca1*-deficient mammary gland tumors (Rottenberg et al., 2008), and also exhibits clinical efficacy in human breast cancers (Fong et al., 2009).

CSCs in glioblastoma

Glioblastoma multiform (GBM) represents the most aggressive type of brain tumor. The standard treatment combines surgery and radiotherapy, but still most patients relapse after therapy, with a median survival of less than 12 months (Prados and Levin, 2000). CSCs from human glioblastoma have been isolated based on the expression of prominin (CD133) (Singh et al., 2004). Irradiation of human GBM xenografts led to increased proportions of CD133+ cells, indicating that CSCs may be responsible for tumor relapse after radiotherapy (Bao et al., 2006). CSCs from GBM are more resistant to IR induced cell death compared to non-CSCs, and show more robust activation of DNA damage checkpoint proteins, including ATM, Chk1 and Chk2, as well as more efficient DNA repair activity. Importantly, treatment with inhibitors of Chk1 and Chk2 kinases sensitizes CSCs to IR induced cell death, suggesting that inhibition of DNA damage checkpoint in CSCs may improve the efficiency of radiotherapy in GBM (Bao et al., 2006). However, this increase in DNA repair activity was not observed in all glioma derived cell lines (Ropolo et al., 2009) and loss of Chk2 instead potentiates GBM radioresistance in mice (Squatrito et al., 2010),

indicating that this characteristic may be related to certain glioblastoma subtypes. Moreover, glioma stem cell-like cells have been shown to exhibit elevated levels of the anti-apoptotic protein Mcl1 that contributes to their radioresistance (Tagscherer et al., 2008). Temozolomide, the most commonly used chemotherapy in the treatment of GBM that induces cell death by triggering the methylation of guanine at position 6, which can be removed by the methylguanine DNA methyltransferase (MGMT), induced CSC depletion in MGMT negative but not in MGMT positive GBM (Beier et al., 2008).

Future directions

The study of DDR in different types of tissue-specific SCs has clearly highlighted the existence of common mechanisms acting in certain adult SC populations to limit the amount of DNA damage, to restrain them from undergoing massive apoptosis and being exhausted following DNA damage, and to preserve overall tissue function. These protective mechanisms may have a cost for these tissue-specific SC populations, such as blood HSCs and hair follicle BSCs, as they preserve immediate survival at the expense of long-term maintenance of genomic integrity, which may lead to aging, tissue atrophy and/or cancer development. Further studies are required to fully understand and ultimately prevent the long-term deleterious consequences of these protective mechanisms. In contrast, some tissue-specific SCs such as intestinal SCs are not well protected and undergo massive death after DNA damage. More studies are needed to better understand why some SCs prefer to commit suicide after DNA damage while others decide to survive, as well as to understand how altruistic suicide might provide a selective advantage to overall tissue function, and what molecular mechanisms dictate these very different outcomes.

Most of the studies on DDR in tissue-specific SCs have been performed in adult animals during normal, or homeostasic, conditions. Since the activity and relative quiescence of SCs varies considerably during organogenesis, adult homeostasis and tissue repair following injuries, the consequence of DNA

damage might be very different in SCs at different ontogenic stages or levels of activity, as it has now been shown for fetal and adult HSCs. During organogenesis and tissue regeneration, SCs divide more frequently, whereas during homeostasis SCs are more quiescent. Since different mechanisms of DNA repair are used depending on the cell cycle stage of the damaged cells, are HR and NHEJ repair pathways differentially important to preserve SC fitness depending on their activation state? Are DNA repair-associated genes differentially activated during morphogenesis, homeostasis and regeneration? Do mice with defective NHEJ or HR repair genes present different phenotypes when these genes are ablated during embryonic development compared to adult life? Future investigations are needed to fully comprehend the role of these different DNA repair mechanisms in SC biology.

In addition to the conserved set of genes that act in DDR and DNA repair pathways, some miRNAs have recently been shown to be induced by p53 in response to DNA damage, and play an important role in DDR outcomes of survival *vs.* apoptosis by interacting with key tumor-suppression networks (He et al., 2007). Irradiation of cultured cells uncovered the involvement of miR-34a in promoting apoptosis (Chang et al., 2007), and of miR-192 and miR-215 in cell cycle arrest induction (Georges et al., 2008). Moreover, miR-34a is lost in several cancer cell lines (Chang et al., 2007). Future studies will determine whether DNA damage and repair-associated miRNAs are differentially expressed in tissue-specific SCs compared to their differentiated progeny, and whether these

miRNAs modulate the DDR in different types of tissue-specific and cancer SCs. Another important question is whether CSCs from different types of cancer also exhibit a survival advantage following chemo- and radiotherapy? If so, is this resistance related to enhanced DNA repair mechanisms or higher expression of anti-apoptotic factors? Do CSCs retain the DNA repair properties of the SCs of their tissue of origin, or do they acquire functionally similar characteristics during cancer progression through a selective pressure? Do DDR abnormalities in CSCs *vs*. bulk cancer cells account for the vast genomic instability present within the bulk of the tumors? Progresses in next generation whole genome sequencing and further studies of defined CSC populations will be needed to assess how defects in their DDR contribute to cancer evolution and associated genomic or base-pair level changes.

Addressing these open questions will have profound implications for our understanding of how tissue-specific SCs respond to DNA damage and maintain the integrity of their genome, how deregulation of these mechanisms leads to cancer and aging, how CSCs respond to chemo- and radiotherapy, and how these characteristics may be exploited to increase the efficacy of current anti-cancer treatments.

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Figure 1. DNA repair pathways in mammalian cells.

Each type of DNA assault results in a different type of lesion, which can be repaired with different fidelity by distinct and highly specialized repair pathways.

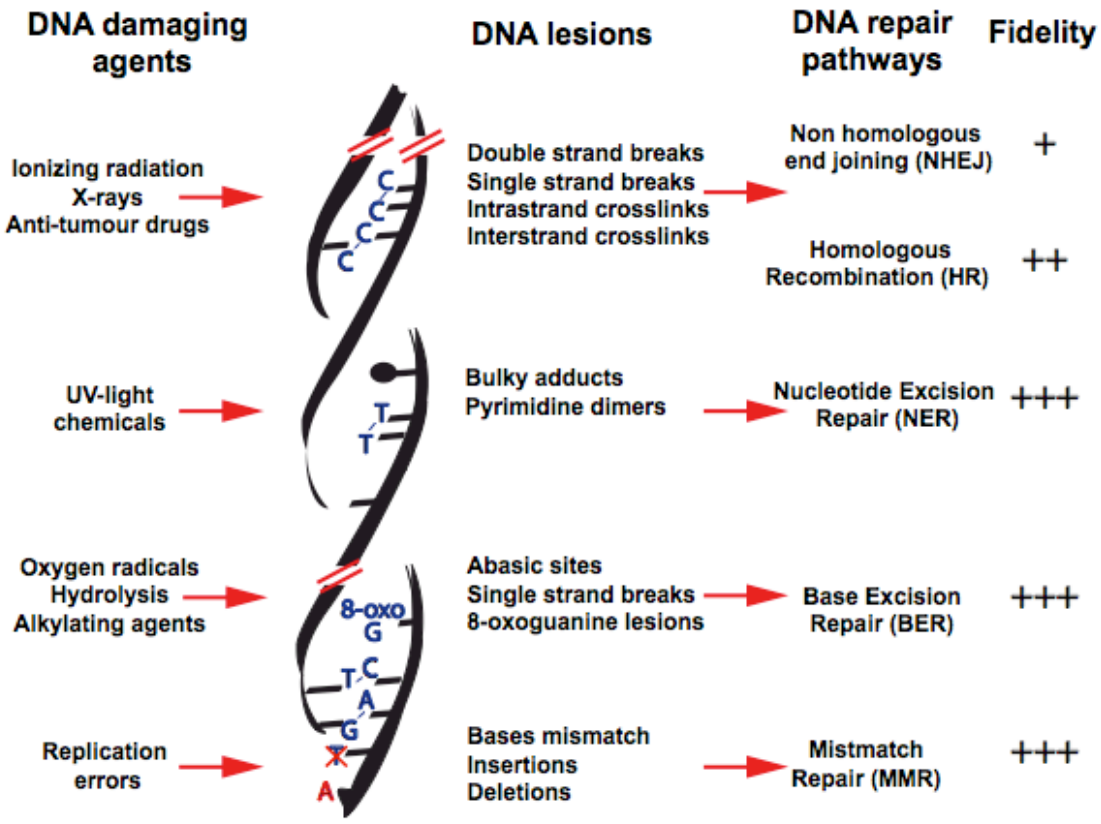

DNA damaging agents
DNA lesions
DNA repair pathways
Fidelity
Ionizing radiation
X-rays
Anti-tumour drugs
Double strand breaks
Single strand breaks
Intrastrand crosslinks
Interstrand crosslinks
Non homologous end joining (NHEJ)
+
Homologous Recombination (HR)
++
UV-light chemicals
Bulky adducts
Pyrimidine dimers
Nucleotide Excision Repair (NER)
+++
Oxygen radicals
Hydrolysis
Alkylating agents
Abasic sites
Single strand breaks
8-oxoguanine lesions
Base Excision Repair (BER)
+++
Replication errors
Bases mismatch
Insertions
Deletions
Mistmatch Repair (MMR)
+++
8-oxo G

Figure 2. DNA damage response pathways.

Upon DNA damage, distinct factors detect, transmit and amplify the DNA damage signal. DNA double strand breaks can be repaired by homologous recombination (mediated among other factors by the MRN complex, ATM and Brca1) or by non-homologous end joining (in which the Ku70/Ku80/DNA-PKcs complex plays a major role). This DNA damage response converges upon p53 which, depending on the target genes activated, regulates different cellular outcomes.

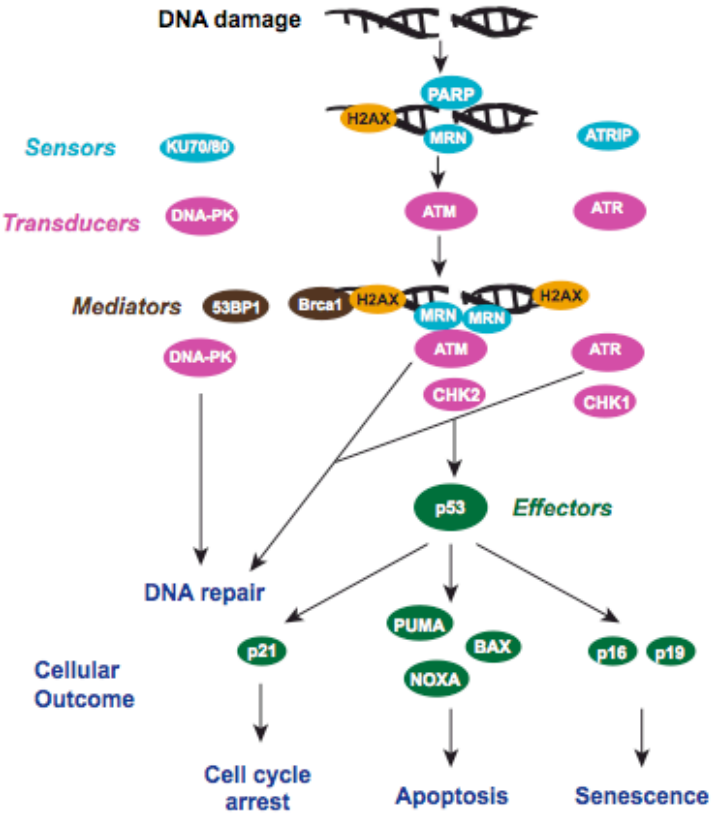
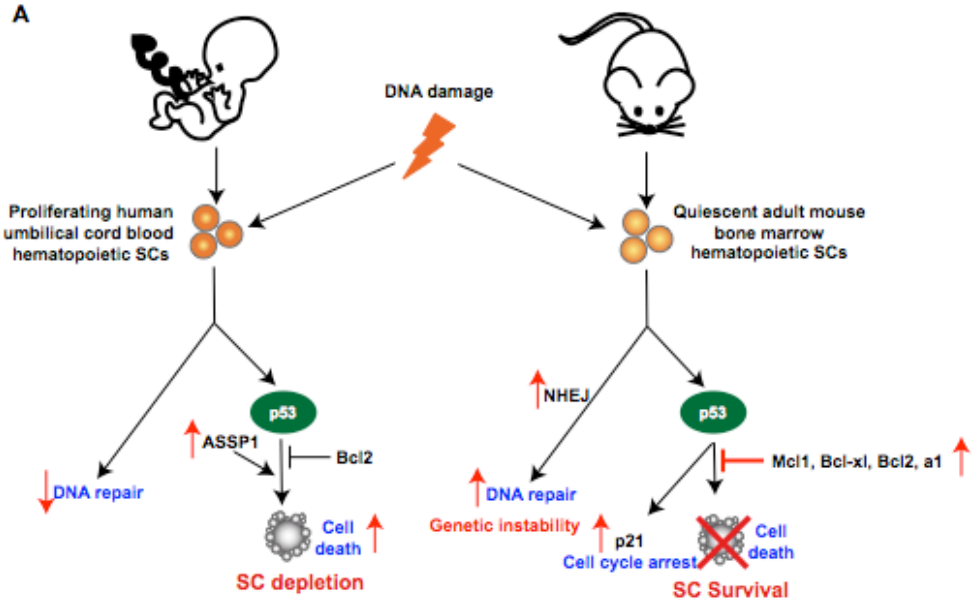

DNA damage
PARP
H2AX
MRN
ATRIP
Sensors
KU70/80
Transducers
DNA-PK
ATM
ATR
Mediators
53BP1
Brca1
H2AX
MRN
MRN
H2AX
ATM
ATR
DNA-PK
CHK2
CHK1
p53
Effectors
DNA repair
PUMA
BAX
NOXA
p21
p16
p19
Cellular
Outcome
Cell cycle
arrest
Apoptosis
Senescence

Figure 3. DNA damage response in hematopoietic and hair follicle bulge stem cells.

(**A**) Human umbilical cord blood-derived HSCs and mouse bone marrow-derived HSCs exhibit opposite outcomes following irradiation-induced DNA damage, with different consequences for their overall maintenance and genomic integrity.

(**B**) Upon irradiation, mouse hair follicle bulge stem cells exhibit transient p53 activation due, in part, to high levels DNA-PK-mediated NHEJ repair, and strong induction of Bcl2 expression that block apoptosis, resulting in enhanced survival.


A
DNA damage
Proliferating human umbilical cord blood hematopoietic SCs
Quiescent adult mouse bone marrow hematopoietic SCs
p53
ASSP1
Bcl2
DNA repair
Cell death
SC depletion
NHEJ
p53
Mcl1, Bcl-xl, Bcl2, a1
DNA repair
Genetic instability
p21
Cell cycle arrest
Cell death
SC Survival

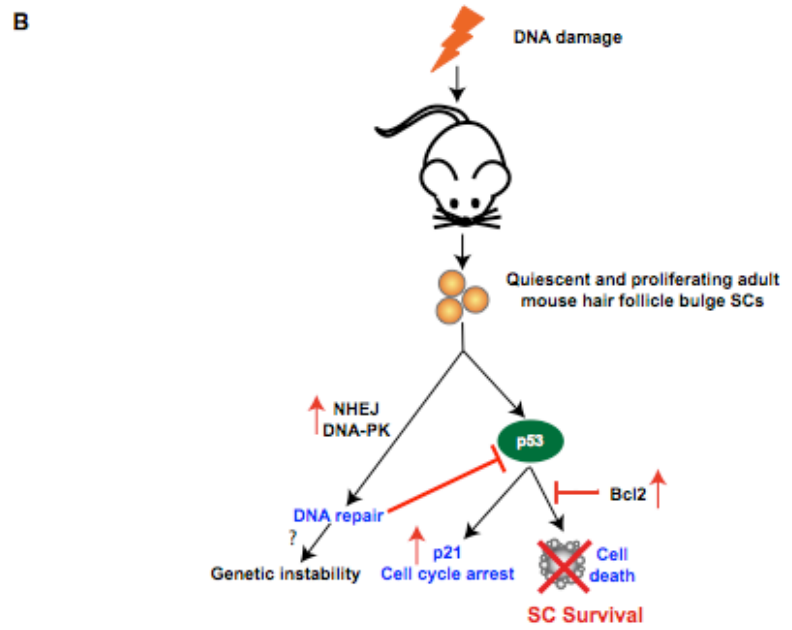
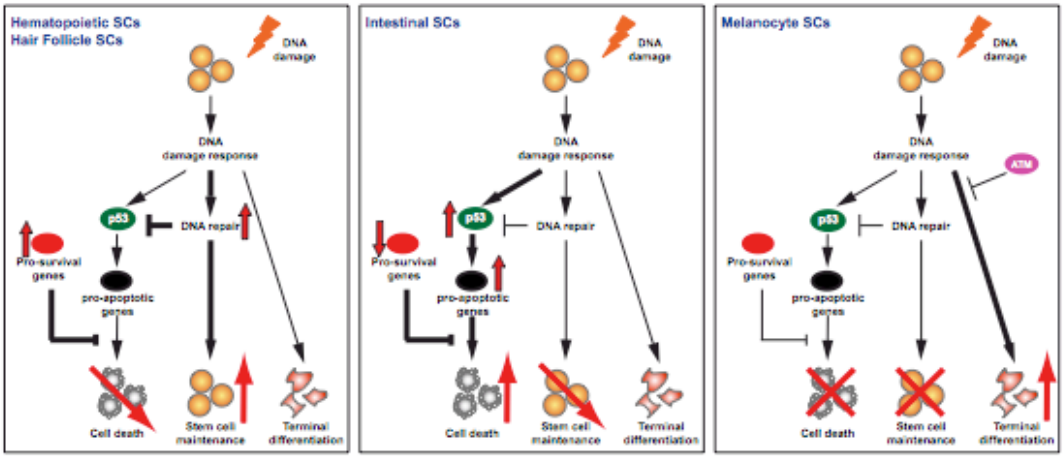

B
DNA damage
Quiescent and proliferating adult mouse hair follicle bulge SCs
NHEJ
DNA-PK
p53
Bcl2
DNA repair
?
Genetic instability
p21
Cell cycle arrest
Cell death
SC Survival

Figure 4. DNA damage response in tissue-specific stem cells. Common and distinct pathways of DNA damage response in different types of tissue-specific SCs.


Hematopoietic SCs
Hair Follicle SCs
DNA damage
DNA damage response
p53
DNA repair
Pro-survival genes
pro-apoptotic genes
Cell death
Stem cell maintenance
Terminal differentiation
Intestinal SCs
DNA damage
DNA damage response
p53
DNA repair
Pro-survival genes
pro-apoptotic genes
Cell death
Stem cell maintenance
Terminal differentiation
Melanocyte SCs
DNA damage
DNA damage response
ATM
p53
DNA repair
Pro-survival genes
pro-apoptotic genes
Cell death
Stem cell maintenance
Terminal differentiation

CHAPTER 6

FUTURE DIRECTIONS

The work presented in this dissertation establishes the mechanisms by which young adult hematopoietic stem cells (HSCs) respond to DNA damage and begins to identify how the outcomes and consequences of DNA repair can contribute to aging and cancer development (chapter 2). We found that HSCs have unique cell-intrinsic mechanisms ensuring their survival in response to low doses of ionizing radiation (IR) (2Gy). These mechanisms include enhanced pro-survival gene expression and a strong activation of p53-mediated DNA damage response (DDR), which includes an upregulation of the cyclin dependent kinase inhibitor p21 that promotes cell cycle arrest. Both quiescent and proliferating HSCs are equally radioprotected but use different types of DNA repair mechanisms. The quiescent status of HSCs forces them to use nonhomologous end joining (NHEJ)-mediated DNA repair and is associated with acquisition of genomic rearrangements, which can persist *in vivo* and contribute to hematopoietic abnormalities. In contrast, proliferating HSCs have access to homologous recombination (HR) and have less chromosomal aberrations than quiescent HSCs. Our results demonstrate that quiescence, while exerting a protective function by limiting cellular metabolism and DNA replication errors can also have a dark side for HSCs by rendering them intrinsically vulnerable to mutagenesis following DNA damage. These findings highlight the importance of cell cycle arrest for survival in HSCs, as well as the use of NHEJ repair and the mutagenic consequences of this error-prone DNA repair mechanism.

One striking difference in the DDR of HSCs and more mature progenitor populations was that HSCs undergo a clear cell cycle arrest following IR exposure, while progenitors do not. HSCs also had a strong and prolonged induction of p21 expression in response to IR compared to progenitor cells. We suspect that enhanced p21 expression in HSCs promotes their cell cycle arrest and allows HSCs time to repair their damaged DNA. To assess the importance of p21 in the DDR of HSCs, we will use mice that lack p21 expression (Deng *et al.,* 1995; Brugarolas *et al.,* 1995). By analysing the DDR and DNA repair abilities of cells isolated from $p21^{-/-}$ mice, we should be able to determine if HSCs rely upon a p21 mediated cell cycle arrest for survival in response to DNA damage.

Since NHEJ appears to be the initial and most commonly used DSB repair mechanism in quiescent HSCs, we are interested to assess their reliance upon this type of DNA repair mechanism. We will use mice that lack NHEJ-repair activity and are deficient in the NHEJ components Ku70 and DNA-PKcs (Gu *et al*., 1997; Gao *et al.,* 1998). By analysing the DDR and DNA repair abilities of cells isolated from $Ku70^{-/-}$ and $DNA\text{-}PKcs^{-/-}$ mice, we should be able to establish the direct dependancy on NHEJ-repair mechanisms for the functionallity of the HSC compartment. The preferential use of NHEJ mediated DNA repair and its associated misrepair activity can also over time potentially lead to the acquisition of mutations that could promote loss of function associated with aging. To assess the contribution of NHEJ in age dependent mutations in HSCs, we will isolate cells from old $Ku70^{-/-}$ and $DNA\text{-}PKcs^{-/-}$ mice. Additionally, we are also interested

in investigating the full extent of mutagenic potential of NHEJ-mediated repair in HSCs and will use cells isolated from transgenic mice carrying a reporter cassette that encodes a site for the rare cutting enzyme I-SceI (DR-GFP I-SceI) (Dr. Maria Jasin (MSKCC, New York)). We will isolate hematopoietic cells from these mice, and express the I-SceI enzyme through lentiviral delivery. The I-SceI mediated cut in the DNA will introduce a single DSB, and by sequencing the DNA around the break, we will be able to directly measure the fidelity of NHEJ-mediated DNA repair. Taken together these mouse models should allow us to understand the extent to which NHEJ is essential for HSC survival as well as to address the mutagenic consequences of NHEJ mediated repair.

As accumulated DNA damage and loss of DNA repair capacity have been implicated in cancer development and evolution, and many cancers have been shown to arise from a cancer initiating cell, or cancer stem cell, we will study the DDR and DNA repair capabilities of leukemic HSCs (Passegué *et al.,* 2003; Jordan *et al.,* 2006). Our goal will be to understand how transformed HSCs cope with additional genotoxic stress and how efficient their DDR and DNA repair mechanisms are. We will use HSCs that either expressing the oncogene BCR/ABL or HSCs deficient for the tumor suppressor JunB, which both have been shown to have leukemic stem cell (LSC) properties (Koschmieder *et al*., 2004; Passegué *et al*., 2003). This approach should determine how dysregulated DNA repair mechanisms in LSCs contribute to disease evolution and cancer progression.

Finally, we will address differences in how fetal human and adult murine HSCs respond to DNA damage. In contrast to our findings, work from John Dick's lab studying the DDR and DNA repair of human cord blood (CB) HSCs found fetal human HSCs to be more radiosensitive than progenitors and less able to repair DSBs (chapter 3) (Milyavsky *et al.,* 2010). In addition to the species difference, one major difference between our work and the work of Milyavsky *et al*. is the developmental age of the HSCs studied. Milyavsky *et al.,* isolated HSCs from human CB that are of fetal origin, while we isolated HSCs from the bone marrow (BM) of adult mice. To understand whether the discrepancies between our results are due to the stage of development of the cells, and the associated cellular differences (cell cycle status, etc) we will need to study the DDR of fetal mouse HSCs. We will isolate HSCs from the fetal livers of embryonic day 14.5 (e14.5) mouse fetuses. We hope that this approach will resolve the controversy between our two studies.

In summary, these future directions will provide a better understanding of the mechanisms involved in the DDR and DNA repair capabilities of HSCs. Unveiling how healthy HSCs respond to DNA damage will pave the way for the development of treatments that specifically target cancerous cells, while sparing healthy cells. This work will also minimize the development of secondary cancers while providing possible targets to limit the deleterious consequences of DNA damage for HSC function.

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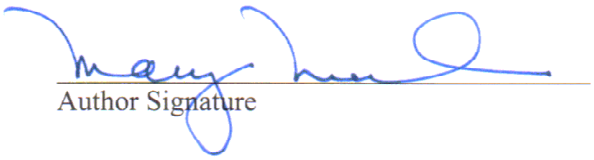

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