

# Differences between human and rodent DNA-damage response in hematopoietic stem cells: at the crossroads of self-renewal, aging and leukemogenesis

Shahar Biechonski, Michael Milyavsky

Department of Pathology, Sackler School of Medicine, Tel Aviv University, Tel Aviv 69978, Israel

Corresponding to: Michael Milyavsky. Department of Pathology, Sackler School of Medicine, Tel Aviv University, Tel Aviv 69978, Israel.

Email: mmilyavsky@post.tau.ac.il.

**Abstract:** Life-long blood regeneration is critically dependent on self-renewing multipotent hematopoietic stem cells (HSCs). These cells' nearly unlimited self-renewal potential and lifetime persistence in the body, in contrast to the committed progenitors (CPs), signifies the need for tight control of HSC genome integrity. Indeed, accumulation of unrepaired DNA damage in HSCs is associated with bone marrow (BM) failure and accelerated leukemogenesis. Recent findings have revealed striking differences in DNA-damage response (DDR) characteristics between HSCs and CPs, especially in their DNA-repair activities and propensity for apoptosis. However, the molecular basis and physiological significance of the HSC-specific DDR characteristics are only partially understood. This review will discuss current progress in understanding the role of cell-intrinsic DDR players and the cellular microenvironment in the regulation of HSC self-renewal. In addition, we will attempt to provide a critical analysis of the differences between human and murine models employed to study the DDR in hematopoiesis.

**Keywords:** Hematopoietic stem cells (HSCs); DNA damage response (DDR); self-renewal; apoptosis; bone marrow (BM)



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*“To be, or not to be, that is the question...”*

William Shakespeare, *Hamlet*, ca. 1604

## Introduction

Hematopoiesis is a highly regulated process that supplies mature blood cells of various lineages. In an adult human, around  $10^{11}$  new blood cells are produced daily to ensure homeostasis. To meet this high regenerative demand, hematopoiesis is structured as a cellular hierarchy composed of cells endowed with different proliferative, differentiation and longevity potentials (1,2). Hematopoietic stem cells (HSCs) are located at the apex of the hierarchy and maintain blood production due to their unique ability to produce more blood stem cells (a property defined as self-

renewal), as well as to give rise to multipotent progenitors with limited self-renewal capacity (2,3). Short-lived but extremely proliferative lineage-committed progenitors (CPs), which are the progeny of multipotent progenitors, generate large numbers of differentiated cells to ensure daily homeostasis (4,5). During injury or infection, stem and progenitor compartments undergo expansion via replication to meet the increased demand for particular cell subsets, followed by a return to homeostasis.

All cells in the body, including highly proliferative and long-lived hematopoietic subsets, must constantly contend with different types of DNA damage. Most of this damage is generated by endogenous sources, such as reactive oxygen species (ROS). In addition, exogenous DNA insults, such as chemotherapeutic drugs and ionizing radiation (IR), can

induce single- and double-strand breaks (DSBs). It has been estimated that the average cell experiences approximately 100,000 spontaneous DNA lesions daily (6). To protect genome integrity, organisms have developed highly sophisticated DNA-damage response (DDR) pathways. The critical importance of the DDR in hematopoiesis is well demonstrated by the severe clinical consequences—including bone marrow (BM) failure, immunodeficiency and a high incidence of hematological cancers—observed in patients with inherited mutations in DNA damage signaling and repair components [e.g., Ataxia telangiectasia mutated (*ATM*) and Fanconi anemia (*FA*) genes respectively] (7). Analysis of human HSC DNA isolated from newborn, young and elderly individuals by whole-genome sequencing has shown that long-lived self-renewing HSC serve as a reservoir for DNA-damage accumulation and thus represent a likely cell of origin for hematopoietic malignancies (8,9). Obviously, the short life span of hematopoietic progenitors reduces the risk of leukemogenic-process initiation from this compartment.

Definitive analysis of the DDR in the early stages of human hematopoiesis was unattainable, until recently, due to our inability to isolate the distinct, functionally homogeneous cellular subsets that make up the hematopoietic tissue hierarchy. Fortunately, in the last five years, significant advances in cell-surface marker characterization, multicolor flow cytometry and functional clonal assays, which efficiently distinguish different stem and progenitor subsets, have led to the establishment of a detailed hierarchical map of human HSCs and progenitors (3-5). For example, costaining of lineage-negative ( $\text{Lin}^-$ ) hematopoietic cells with a panel of monoclonal cell-surface antibodies has identified functionally distinct populations of candidate human long-term HSCs (LT-HSCs)- $\text{CD34}^+\text{CD38}^-\text{CD90}^+\text{CD45}^{\text{RA}^-}\text{CD49f}^+$ —and various CPs that reside in the  $\text{CD34}^+\text{CD38}^-\text{CD90}^-$  immunophenotype. The self-renewal potential of LT-HSCs is most often measured by their ability to sustain multilineage hematopoiesis for at least 16 weeks upon transplantation into a properly conditioned recipient, and to reinitiate hematopoiesis upon secondary transplantation. HSCs that can only sustain hematopoiesis transiently (8 weeks) are defined as short-term HSCs (ST-HSCs) (1). Although the basic roadmap of hematopoiesis is largely conserved between mice and humans, their vastly different body masses, life spans and environmental exposures have led to several important differences in hematopoiesis, including species-specific responses to DNA damage (10).

Today, scientists have the tools to address the role of

single molecules and pathways in HSCs. As a result, it has become clear that DDR regulators themselves are the key players in HSC-specific processes such as self-renewal and multilineage differentiation. Several excellent reviews describing adult stem cells' responses to DNA damage are available (7,11-15). In this review, we summarize the progress made in this rapidly growing field in the last several years, placing special emphasis on the human HSC.

### Endogenous DNA-damage accrual limits HSC function

The most convincing evidence that unrepaired genomic damage results in (age-dependent) decreases in HSC regeneration is based on the careful analysis of hematopoiesis and HSC functionality in mice that are deficient in key DNA-repair genes. Loss of DNA-damage sensors (e.g., *Atm*, *Atr*), and insufficiency of DNA repair due to mutations in the homologous recombination (HR) (*Brca2*, *Fancc*, *Fancd2*), nonhomologous end-joining (NHEJ) [DNA-dependent protein kinase (*Prkdc*), *Ku80*, *Lig4*], Mismatch repair (MMR) (*Msb2*) and Nucleotide excision repair (NER) (*Ercc1*, *Xpd*) pathways largely resulted in a dramatic loss of repopulation capacity when mutant HSCs were competed against their wild-type counterparts in a BM repopulation assay (7,16-25). Interestingly, young mutant mice had normal numbers of LT-HSCs as defined by surface markers, suggesting that the reason for their functional decline was the inability to self-renew optimally under conditions of BM regeneration.

Importantly, Rossi and colleagues (19) reported greatly elevated numbers of  $\gamma\text{H2AX}$  foci, a marker for DSBs and replication stress, specifically in LT-HSCs isolated from 122-week-old mice. Of note, ST-HSCs and CPs contained similarly low numbers of  $\gamma\text{H2AX}$  foci when isolated from both young and old animals (19). Thus, mouse models overwhelmingly demonstrate the accumulation of endogenous DNA damage in the genome of LT-HSCs, which eventually impairs their self-renewal.

Recently, several groups have analyzed the relationships between genomic DNA damage and human HSC function. For example, Yahata and colleagues (26) quantitated  $\gamma\text{H2AX}$  foci in purified human HSCs (defined as  $\text{Lin}^- \text{CD34}^+ \text{CD38}^-$ ) isolated from umbilical cord blood, and the BM of elderly healthy individuals and patients that had undergone HSC transplant. They only found an age-dependent increase in  $\gamma\text{H2AX}$ -positive cells in HSCs, whereas no difference was seen in the matched progenitor compartment (defined

as Lin<sup>-</sup>CD34<sup>+</sup>CD38<sup>+</sup>). Importantly, they also found an inverse correlation between the number of  $\gamma$ H2AX foci and transplantability of human HSCs using humanized mice. Serial transplantation experiments with cord blood cells also revealed that HSCs and not progenitors accumulate signs of DNA damage that most probably originate from ROS (26).

Studies from Weissman's and Goodhardt's groups analyzed age-dependent changes in human BM primitive hematopoietic subsets (LT-HSCs, Multipotential Progenitors and different CPs), as well as in their repopulation and differentiation capacities (27,28). Interestingly, both studies found accumulation of immunophenotypic HSCs with age, with no marked decline in their reconstitution potential, which stands in contrast to the mouse models of HSC aging. Unfortunately, no analysis of DNA damage-related markers was performed, warranting further exploration. In yet another study, CD34<sup>+</sup>CD38<sup>-</sup> BM cells isolated from healthy elderly (>70 years of age) individuals contained two times more  $\gamma$ H2AX foci than the same cell subset obtained from cord blood (at birth). Of note, most of this physiological DNA damage did not colocalize with telomeres, which undergo an age-dependent decline in the same immunophenotype, arguing against telomeres as the origin of DNA damage in human primitive hematopoietic cells (29). An extreme example of the functional decline of human HSCs due to unrepaired genomic damage was recently provided by Ceccaldi and colleagues' very thoughtful study (30) using FA syndrome-derived hematopoietic cells. First, they demonstrated loss of primitive hematopoietic cells (CD34<sup>+</sup>) before the onset of clinical BM failure. Equally important, they proved that the deficient FA HSCs and progenitors exhibit strong  $\gamma$ H2AX positivity and become arrested in the G0/G1 state, thus preventing BM cell proliferation and leading to the clinical symptoms of the syndrome. Therefore, it is evident that human primitive hematopoietic cells are very sensitive to a congenital deficit in DNA repair, as in the case of FA that results in the early onset of BM failure as a result of HSC depletion and/or transformation toward Acute Myeloid Leukemia (AML). Furthermore, a gradual age-dependent increase in DNA alterations was reported in long-lived self-renewing HSCs based on next-generation sequencing analysis, supporting the notion that HSCs are reservoirs of both neutral and potentially dangerous mutations (8,9). Clearly, there must be a tradeoff between preservation of the HSC genome's integrity and maintenance of a reasonable number of HSCs to ensure homeostasis. Now, we shall discuss the molecular

mechanisms by which DNA-damage signals regulate HSC biology.

### HSCs and DNA-repair pathways

The essential role of NER, HR, FA, NHEJ and MMR DNA-repair pathways in the regulation of murine HSC function is well documented and reviewed elsewhere (7,25). In contrast, the study of DNA repair in the human primitive hematopoietic compartment is still in its infancy. Recent studies from Dick's laboratory in collaboration with the Mein group have provided first insight into the capacity for DNA DSB repair in human HSCs and CPs (31). To investigate this pathway researchers purified Lin<sup>-</sup>CD34<sup>+</sup>CD38<sup>-low</sup>CD45RA<sup>-</sup> cells (which are enriched in HSCs) and Lin<sup>-</sup>CD34<sup>+</sup>CD38<sup>+</sup> cells (contain only CPs and are unable to self-renew) from umbilical cord blood. Cells of both fractions were exposed to IR, and kinetics of DSB rejoining was measured using the neutral comet assay. Strikingly, no detectable rejoining of DSBs occurred in the first 30 min post-IR in the quiescent HSC-enriched fraction. In contrast, the CP fraction rejoined 20% of the breaks during the same time period (31). In agreement with these findings, Zhou and his group (32) found that CD34<sup>+</sup>CD38<sup>-</sup> cells isolated from human BM are less efficient in DSB rejoining than their more mature progeny (CD34<sup>+</sup>CD38<sup>+</sup> cells). To make this observation, Shao *et al.* developed an *in vitro* cell-free assay for NHEJ based on linearized substrates to measure DNA repair (32).

The major pathway for DSB repair in quiescent human cells is NHEJ, a rapid but error-prone method that is responsible for most of the genomic structural variants and chromosomal translocations in human cancers (33). In mammalian cells, the protein components of NHEJ pathway begin to assemble at DSB sites seconds after IR, and NHEJ-mediated repair and rejoining of DSBs is detected within 10 min of DSB induction (34). In line with this rapid kinetics,  $\approx$ 50% of DSBs are rejoined within 30 min post-IR by quiescent human fibroblasts (35). Therefore, the HSC-intrinsic "deficit" in NHEJ activity relative to progenitors may indicate that a distinct DDR begins in HSCs immediately post-IR. To explain this "deficit", one can raise the hypothesis that human HSCs possess a DSB-repair machinery with higher stringency than that of their progeny to reduce the risk of mutagenesis. Alternatively, HSCs might be less efficient in DSB repair, which would explain the reported persistence of  $\gamma$ H2AX foci (26,29,31). Future experiments should be aimed at revealing the

**Table 1** DSB-repair pathway activity and expression of selected DNA-repair genes in normal HSCs and CPs. Results in the table are from (31,32,36-38)

		Mouse		Human	
		HSC	Progenitors	HSC-enriched	Progenitors
NHEJ activity		↑	↓	↓	↑
NHEJ gene expression	ATM	↑	↓	↓	↑
	Ku-70	↑	↓	ND	ND
	Ku-80	↑	↓	ND	ND
	Lig-IV	↑	↓	↓	↑
	XRCC4	↑	↓	ND	ND
HR activity		↓	↑	Not analyzed	
HR gene expression	RAD51	↓	↑	↓	↑
	RPa.a1	↓	↑	NA	NA
	XRCC2	↓	↑	NA	NA
	XRCC3	↓	↑	NA	NA
	MRE11A	ND	ND	↓	↑

Downward arrows indicate low activity/expression; Upward arrows indicate high activity/expression. ND, no difference; NA, not analyzed.

molecular mechanism underlying the distinct dynamics of the DDR in human HSCs.

In contrast to the results obtained with human hematopoietic cells, highly purified mouse HSCs showed higher NHEJ activity than committed myeloid progenitors (32,36). Several important technical aspects should be considered before interpreting these potentially important interspecies differences. First, human HSC-containing populations are more heterogeneous than those of mice and additional human HSC-specific surface markers have recently been identified (3). Second, human cord blood and adult murine BM cells might represent developmentally incomparable HSC subsets. In this respect, the role of different niche factors should also be considered and will be discussed further on. Finally, although both human and mouse HSCs are confined to the G0 stage of the cell cycle, cord blood-derived progenitors are not cycling (no cells in the S/G2/M phases of the cell cycle). In contrast, BM progenitors are highly cycling, further complicating a direct comparison. Bearing in mind these important experimental caveats, all three studies point to the existence of distinct DNA-repair characteristics in HSCs from the two species.

Differences in the basal expression of DNA-repair proteins provide important correlative evidence for the observed differences in DNA-repair activity (Table 1). For example, murine HSCs exhibited increased expression of

numerous transcripts encoding NHEJ proteins relative to their downstream progeny. The opposite pattern of expression was evident for HR-repair genes. This transcriptional analysis correlates well with the enhanced NHEJ activity of murine HSCs (32,36). Less is known about DNA-repair gene expression in human primitive HSCs (37,38). Intriguingly, lower levels of ATM and Lig4 mRNA were expressed in human HSCs than in the more mature cells (32). Whether this difference might account for the relatively less effective NHEJ in human HSCs remains to be established. It is important to bear in mind that the activity of some DNA-repair proteins is regulated post-transcriptionally and in a cell-cycle dependent manner. Furthermore, human cells typically express more Ku and DNA-dependent protein kinase (DNA-PK) proteins (discussed further on) than their murine counterparts, adding to the difference in cellular concentration and activity of repair proteins (39-41). Accurate analysis of DNA-repair protein levels and their biochemical activity in DNA-repair assays in the specific cellular contexts (HSCs and progenitors) will undoubtedly reveal the underlying mechanism and significance of the observed interspecies differences.

### DDR signaling governs HSC functions

As already discussed, the accumulation of DNA damage

in primitive hematopoietic cells threatens the organism's health. Thus, a complex network of DDR factors that include sensors, regulators and effectors has evolved to respond effectively to genotoxic stress. Activation of this network results in two primary outcomes: repair of DNA damage and genome restoration or, if damaged DNA cannot be sufficiently repaired, execution of cell death, differentiation or senescence programs (41-43).

Of the many different lesions that occur in DNA molecules, DSBs induced by IR are the most lethal for the individual cells as well as for the whole hematopoietic tissue (44). This notion is exemplified by the dramatic loss of HSC self-renewal potential as measured by competitive repopulation or serial transplantation assays, upon exposure of the mouse to even relatively low doses of IR (2-3 Gy) (31,36,45). Thus, IR is the most frequently used trigger to provoke a DDR and delineate the molecular pathways engaged.

In response to DSBs, cells mount a powerful signaling network. It starts with rapid aggregation of multiple protein "sensors" around the DNA break, which engages the activation of several kinases termed "transducers". These further amplify the DSB stress signal to numerous downstream effectors that ensure appropriate alterations in the cell's fate (41-43). It is becoming increasingly clear that many of the central regulators of the DDR play a key role in the regulation of HSC homeostasis (13,14,25,46-49).

We will first discuss the mechanisms by which upstream DDR kinases participate in HSC maintenance. Then we will integrate the tumor suppressor p53-a prototype DDR effector, and we will finish by highlighting the role of "bona fide" apoptosis and differentiation genes that become co-opted by the complex genetic network regulating HSC function under both homeostatic and stress conditions.

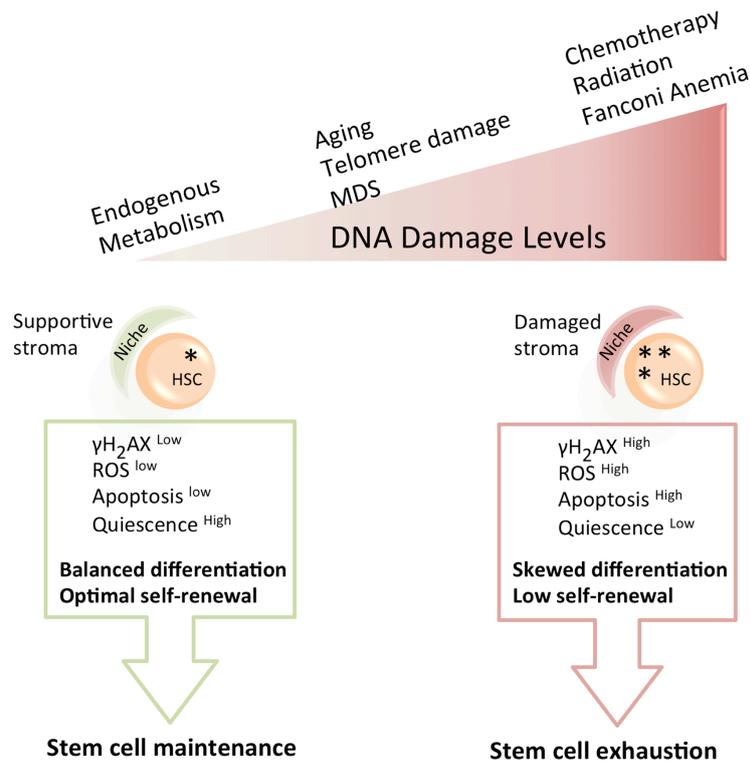
### *The upstream kinases*

The protein kinase ATM is the primary transducer of the DSB signal via phosphorylation of the numerous nuclear and cytoplasmic substrates that participate in DNA repair, apoptosis and senescence. A detailed analysis of HSC function in ATM-deficient mice by Ito and colleagues (21,47) revealed that ATM is essential to maintaining HSC regeneration, whereas it is less important for the proliferation or differentiation of CPs (21,50). Even without exogenous stress, ATM<sup>-/-</sup> animals exhibit age-dependent BM failure, due to the mutant HSC's inability to self-renew. Elevated levels of ROS were found in different primitive

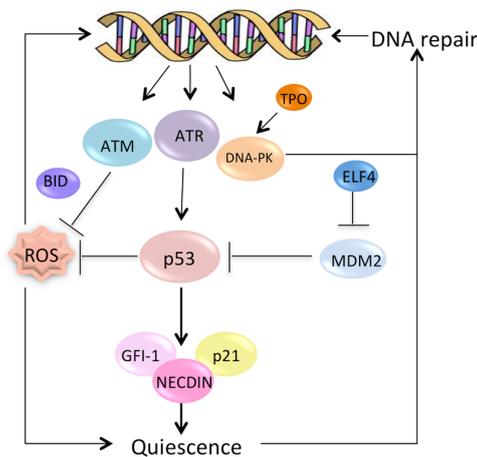
BM cells in ATM<sup>-/-</sup> mice as compared to their normal littermates, consistent with the previously described role of ATM in regulating cellular antioxidative systems (51). Crucially, LT-HSCs, in contrast to progenitors, were found to be most vulnerable to the elevated ROS levels that induced loss of HSC quiescence, increased apoptosis and diminished self-renewal. Mechanistically, HSC-specific activation of p38 mitogen-activated protein kinase (p38 MAPK) induced by ROS in ATM<sup>-/-</sup> is responsible for the loss of HSC function. These connections were proven by rescue of BM failure and restoration of HSC self-renewal by treating ATM-deficient mice with a small-molecule inhibitor of p38 MAPK or with the antioxidant N-acetyl-L-cysteine (NAC) (21,50) (*Figure 1*). A recent study from Gross's laboratory (52) provided evidence for ATM-dependent phosphorylation of BID, a BH3-only pro-death Bcl-2 family member, determining the level of ROS in murine HSCs. HSCs isolated from mice in which the ATM-phosphorylation site on the BID protein was mutated (BID<sup>AA</sup>) had elevated ROS levels, decreased quiescence, and inferior competitive repopulation potential. As in the case of ATM-knockout mice, antioxidant treatment returned HSCs to the quiescent state and restored their repopulation potential. Importantly, both human and mouse HSCs accumulated ROS during serial transplantation assay *in vivo* and increased ROS levels coincided with decreased HSC self-renewal in both species (26,53). Therefore, ROS levels inside HSCs, governed in part by the ATM-BID axis, control HSC self-renewal potential (*Figure 1*).

In addition to ATM, DSBs activate the DNA-PK complex which consists of the catalytic subunit (DNA-PKcs) and the DNA-binding Ku70/80 heterodimer. The DNA-PK complex recognizes DSBs and its function is essential for the NHEJ-repair pathway. Consistent with this notion, BM from Ku80<sup>-/-</sup> and DNA-PK<sup>-/-</sup> animals exhibited progressive loss of regeneration potential due to a decrease in HSC self-renewal (19,23). Two recent works have described HSC-specific regulation of DNA-PK function and its importance in HSC biology (23,54). To investigate the physiological significance of IR-induced DNA-PK phosphorylation, Zhang and colleagues generated a DNA-PK<sup>3A/3A</sup> mouse in which threonine residues that are subject to IR-induced phosphorylation (Thr 2643, 2634 and 2605) are replaced with alanine (23). In striking contrast to the modest HSC dysfunction observed in aged DNA-PKcs-knockout mice, DNA-PKcs<sup>3A/3A</sup> mice developed congenital hematopoietic failure caused by rapid demise of fetal liver HSCs by p53-dependent apoptosis. Compound deficiency

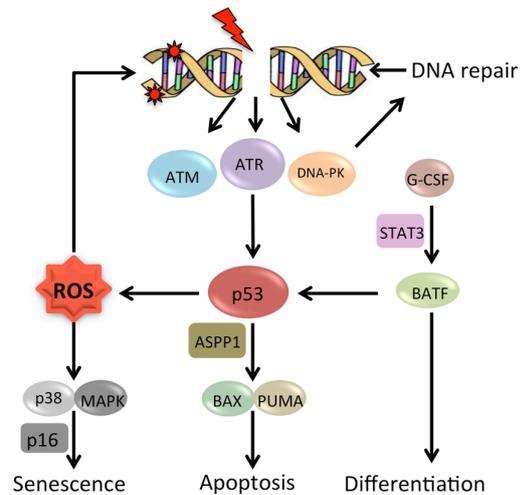
A



B Steady-state hematopoiesis



Genotoxic injury (acute or chronic)



**Figure 1** (A) Physiological and pathological conditions known to induce genotoxic stress in HSCs. According to the intensity and persistence of the DNA damage, HSCs display well-documented changes (indicated as superscripts) in cell-intrinsic DDR-related characteristics, e.g., number of  $\gamma$ H2AX foci, ROS levels, cell death and cell-cycle status. In addition, poorly defined signals from the BM microenvironment are suggested to play an important regulatory role. Integration of these different physiological changes affects HSC self-renewal and differentiation potential which is the underlying basis for HSC maintenance or exhaustion; (B) Simplified representation of the DDR molecular circuitry as discussed in the text. Left part: the DDR network operates under homeostatic conditions to resolve endogenous DNA damage and to maintain optimal HSC self-renewal by ensuring HSC quiescence. Right part: upon being subjected to genotoxic stress, the DDR machinery in HSC is activated and recruits additional regulators and effectors to cope with stress. Three possible cellular outcomes of this activation-senescence, apoptosis and differentiation-are outlined. Lines with an arrowhead indicate activation; Lines ending in a bar indicate inhibition; Thin lines reflect basal activation; thick lines signify a strong/persistent signal.

in NHEJ as well as in the HR and FA DNA-repair pathways observed in DNA-PKcs<sup>3A/3A</sup>-derived fibroblasts can explain the severity of the hematopoietic phenotype (23).

Availability of DNA repair factors in HSC and early progenitors is important for the efficient DDR and preservation of genome stability. Indeed, many DNA repair factors are differentially expressed between HSC and different committed progenitor populations (32,36-38) (Table 1). However, the molecular circuitry that governs DNA repair gene expression in primitive hematopoietic cells is not well understood. Recently, Zheng and colleagues uncovered that mTOR kinase is required to maintain basal levels of FANCD2 in murine HSC, progenitors and human hematopoietic cells (55). Intriguingly, overactivation of mTOR, caused by PTEN deletion, results in normal HSC depletion and accelerated leukemogenesis (56). How mTOR-dependent regulation of FA repair pathway contributes to its pleiotropic roles in carcinogenesis should be determined in the future.

How DNA repair in primitive hematopoietic cells is regulated by the microenvironment is not entirely known. Recently, de Laval and colleagues (54) found that thrombopoietin (TPO) and its receptor MPL, previously implicated in maintenance of HSC quiescence and self-renewal, are also important for DSB repair in mouse and human primitive hematopoietic cells. TPO/MPL-signaling stimulated DNA-PK-dependent NHEJ efficiency by a significant increase in Ser 2056 and Thr 2609 phosphorylation on DNA-PK. Intriguingly, Thr 2609 in human DNA-PK corresponds to Thr 2605 in the murine protein and, if not properly modified by the TPO/MPL axis, might contribute to HSC ablation as observed in the DNA-PKcs<sup>3A/3A</sup> model discussed above. This study highlights previously unrecognized crosstalk between a niche factor, such as TPO, and regulation of DNA repair in HSCs (Figure 1B). It is likely that more microenvironmental cues governing the HSC-specific DDR will be described in the future.

### ***p53: a double-edged sword in HSC regeneration***

The tumor suppressor p53 is a widely expressed transcription factor that plays a critical role in regulating several DDR pathways, including activation of cell-cycle arrest, induction of apoptosis, senescence, suppression of HR, and others (57). As with additional DDR genes, germline mutations in p53 increase the incidence of hematological malignancy, and somatic aberrations of p53

are frequent in leukemia, lymphoma and myelodysplastic syndrome (58,59). The importance of p53 in IR-induced apoptosis has been analyzed in detail both *in vitro* and *in vivo* and attributed mainly to its transcriptional target Puma (60) (Figure 1B).

However, it has been difficult to establish the function of p53 in HSCs compared to other hematopoietic cells using mouse models. BM progenitors from p53-knockout mice exhibit increased resistance to DNA damage-induced apoptosis in short-term survival assays (36,60-64). Still, when wild-type murine LT-HSCs defined as Lin<sup>-</sup>Sca1<sup>+</sup>Kit<sup>+</sup>Flk2<sup>-</sup> cells were exposed to 2 or 5.5 Gy of IR, no increase in cleaved caspase-3, a marker of apoptosis, was observed at 6-24 h post-damage. At the same time, 20-60% of Common Myeloid and Granulocyte-Macrophage Progenitors underwent p53-dependent apoptosis (36,64). These observations would suggest little involvement of p53-dependent apoptosis in the irradiated murine LT-HSCs. However, meticulous analysis of BM cellularity under the even lower IR dose of 1.75 Gy revealed a dramatic, time-dependent loss of LT-HSCs cells as measured up to seven days post-damage. Analysis of cell death by Annexin V staining clearly implicated apoptosis as the reason for HSC loss (65-67). Of note, wild-type LSK cells exhibited persistent apoptosis even seven days post-IR (65). No change in cell numbers in the Annexin-positive cell fraction was found in the BM of similarly irradiated p53<sup>-/-</sup> and Puma<sup>-/-</sup> animals (49,65,68). Although the reason for this persistence of apoptosis was not examined in those studies, Wang *et al.* (69) found a sustained increase in ROS production and subsequently higher oxidative DNA damage in HSCs isolated from mice exposed to sublethal total body irradiation. Based on these pieces of evidence, murine LT-HSCs are very sensitive to IR-induced apoptosis mediated by p53 via its transcriptional target Puma. It is likely that the different apoptosis assays (cleaved caspase-3 *vs.* Annexin) and variability in the time points after IR (hours *vs.* days) employed by the different groups led to the apparently conflicting conclusions.

When human primitive hematopoietic cells derived from cord blood were exposed to 3 Gy of IR, the HSC-enriched fraction underwent significantly higher cell death relative to the downstream CPs in both short-term and long-term viability assays (31). Preferential expression of p53 pro-apoptotic partner ASPP1 in the HSCs was partially responsible for their enhanced sensitivity to p53-mediated apoptosis upon IR. Increased resistance of human HSCs and progenitors to apoptosis accounts for the enhanced

hematopoietic regeneration when irradiated HSCs in which p53 had been disabled were transplanted into conditioned recipients (31). Thus, both phenotypic and functional assays indicate that acute genotoxic injury initiates a p53-dependent apoptosis pathway that ablates primitive hematopoietic cells in both humans and mice.

Rapid transformation of BM cells isolated from p53<sup>-/-</sup> mice complicates the analysis of p53 function in normal non-malignant repopulating LT-HSCs, and precludes an examination of their self-renewal by retransplantation (70,71). When attempts were made to examine the function of putatively non-malignant HSCs from p53-deficient mice, once again, conflicting results were obtained. Although there were differences in the designs of the competitive repopulation assays, it was suggested that HSC repopulation increased, remained the same or even decreased (70,72-74). However, when the dosage of p53 was increased, HSCs exhibited reduced competitive repopulation potential (71).

Studies of human HSCs in which p53 activity had been disabled by dominant negative mutation or by means of RNA interference extended observations made in mice and provided some clarification of the role of p53 in the regulation of HSC self-renewal. Head-to-head analysis of human HSCs in which p53-dependent apoptosis was bypassed by Bcl-2 overexpression established that p53 has both positive and negative roles in the regulation of HSC function. Human HSCs with inactive p53 demonstrated increased numbers of  $\gamma$ H2AX foci in secondary transplants as compared with control and Bcl-2-overexpressing HSCs (31). Once again, engagement of the DDR machinery in HSCs resulted in reduced HSC self-renewal. Apoptosis-independent p53 effectors, such as p21 and others, might participate in HSC genome quality control coupled with self-renewal (64). In addition, positive regulation of HSC quiescence by p53 via its transcriptional targets Gfi-1 and Necdin may well contribute to the role of p53 in preserving LT-HSC function. Furthermore, fine regulation of p53 activity by E1f4/Mdm2 factors was also implicated in the maintenance of HSC quiescence (72) (Figure 1B).

Thus, p53 plays at least two distinct physiological roles to ensure optimal HSC function: apoptosis regulation and prevention of genomic damage accumulation upon HSC self-renewal. The complex network of p53 transcriptional targets and cofactors involved in the regulation of HSC quiescence, cell-cycle arrest, apoptosis and senescence determines HSC fate based on the severity and persistence

of the genotoxic stress (Figure 1).

### ***DDR-dependent HSC differentiation checkpoint***

A higher number of  $\gamma$ H2AX foci, shortened telomeres and elevated ROS levels characterize HSCs isolated from elderly humans and mice. In both species, hematopoietic stem and progenitor cells undergo alterations in lineage distribution, gene expression, epigenetics, differentiation potential and repopulation capacity (27-29,75). Decreased lymphoid cell output by aged HSCs represents one of the best-documented functional changes (76,77). An intriguing connection between the DNA-damage signal and augmented lymphoid differentiation of murine HSCs was recently provided by Rudolph's laboratory. In an elegant *in vivo* shRNA screen, Wang *et al.* (78) identified transcription factor BATF as the major culprit limiting self-renewal of HSCs with dysfunctional telomeres. IR or critically short telomeres induced an increase in BATF expression in HSCs via a G-CSF- and STAT3-dependent mechanism. Then, BATF specifically engaged the lymphoid differentiation program among CD150<sup>low</sup> HSCs, thus limiting their self-renewal potential. Intriguingly, DNA damage-induced accumulation of activated p53 (P-Ser15 p53) and of its downstream target p21 was abrogated in the BM cells expressing shBATF, positioning BATF upstream of p53 in the DDR-signaling cascade. The direct correlation between BATF mRNA level and p21 expression documented in CD34<sup>+</sup> cells derived from patients with preleukemic syndrome (MDS) warrants further investigation of BATF function in human leukemogenesis. In summary, the HSC differentiation checkpoint mediated by BATF upon DNA damage represents a failsafe mechanism that culls HSCs with accrued genomic aberrations. In concert with apoptosis, this novel differentiation checkpoint might explain the aging-associated decline in HSC function and these cells' unbalanced differentiation. Importantly, the ability of DNA damage to induce premature stem cells differentiation was also documented in another stem cell compartment in the mouse, namely, Melanocyte Stem Cells (79). In this case, IR triggered p53 and INK4A independent loss of Melanocyte Stem Cells self-renewal that led to the appearance of ectopically pigmented melanocytes and hair graying of the irradiated animals. Interestingly, murine Bulge Stem Cells that share with Melanocyte Stem Cells the same niche turned to be resistant to both irradiation induced apoptosis and differentiation (80) pointing to the distinct pathways regulating various tissue

stem cells response to DNA damage.

### Summary and future directions

An efficient DDR is one of the major evolutionary adaptations of all living organisms, ensuring the transfer of accurate genomic information from one generation to the next. Research into the DDR in the last decades has identified hundreds of proteins that form myriad functional intracellular interactions to accomplish various tasks in the injured cell, including DNA repair, cell-cycle arrest, apoptosis and others. In the context of long-lived animals, such as humans and rodents, causative relationships between the DDR, tissue regeneration, organismal aging and cancer susceptibility become established. Much of this important knowledge is derived from the study of DDR in the context of hematopoiesis—a vital process sustained by a small fraction of quiescent self-renewing stem cells (*Figure 1A*). Understanding the HSC-specific DDR has revealed important principles employed by self-renewing tissues to withstand DNA damage. Some generalizations can be made based on the available experimental evidence as outlined in this review. First, HSCs are selectively sensitive to acute and chronic genome damage, which results in a dramatic reduction in their regenerative potential. Second, elevated ROS levels and subsequent oxidative DNA damage inside HSCs are common motifs in the HSC DDR. Accordingly, antioxidant treatment is effective in reversing the ROS-induced decline in HSC self-renewal. Third, the quiescent state forces HSCs to employ the error-prone NHEJ for DSB repair. Finally, multiple genes involved in DDR pathways also regulate HSC self-renewal (*Figure 1B*). The critical differences in DDR between humans and rodents at the level of HSCs, highlighted in this review, preclude direct extrapolation of findings from one species to the other (*Table 1*). Although the driving force for the described interspecies differences is currently unknown, it is tempting to speculate that vastly different lifespan between the two species had major impact on their respective environmental adaptations, including different strategies to deal with DNA damage.

Obviously, exciting discoveries made in the last few years have presented the scientific community with even more burning questions. For instance, why is leukemia a rare disease given that  $10^{11}$  new blood cells are produced daily? Do human and rodent HSCs employ opposite strategies to deal with DNA damage? If so, what is the molecular basis and physiological significance of this interspecies

difference? Is it possible to manipulate the DDR to improve HSC fitness and delay aging of self-renewing cells? What are additional intrinsic and microenvironment-derived regulators of the DDR in HSCs? Will therapeutic targeting of DDR effectors that regulate self-renewal selectively eliminate leukemia stem cells and spare normal HSCs? The very important question asked by Shakespeare's hero, without thinking of HSC-specific DDR: "To be, or not to be..." will undoubtedly keep us busy for the foreseeable future.

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