Stem Cells

From Basic Research to Therapy

Volume 2

Tissue Homeostasis and Regeneration during Adulthood, Applications, Legislation and Ethics

Editors

Federico Calegari

Claudia Waskow

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Preface

The Stem Cell Revolution

We occasionally witness fundamental changes altering the way in which we perceive the world. These sudden changes, sometimes called *revolutions*, may influence very diverse aspects of our every-day-life from politics to health care, from telecommunications to finance. Following a seemingly irreversible historical trend, changes are becoming more and more frequent, to the point that we start to get used to them and barely pay any attention anymore. Not because of that, these *revolutions* are any less important.

Stem cells are perhaps the last of a series of *revolutions* in biomedical sciences that slowly build up over time to explode in the last decade. Stem cells are, and will continue to be for years to come, a source of spectacular scientific achievements and shameful frauds, gold mine for pharmaceutical corporations and ruin for others, a charming hope to unfortunate patients and abhorrence to entire political administrations. Stem cells are the reason why an abstruse terminology limited to a few experts moved from the labs to the streets to be debated, nearly on daily basis, on worldwide media. Examples include *animal cloning, gene therapy, tissue-replacement, regenerative therapies* and so forth.

On a historical perspective, it is hard to find a subject in biology that has arisen more controversial feelings and debates at all levels of society. Perhaps the dispute over the risks of gene recombination in the '70 leading to the Asilomar Conference comes to mind as a worthy competitor but to find a clear winner we need to go back by over a century to consider the theory of evolution by Darwin and Wallace. In essence, we are witnessing here a quite remarkable *revolution*, and we should pay attention to it. To do so we first need to understand what stem cells are, which led us to consider the possibility of editing *Stem Cells*.

As it should be expected, a remarkably high number of books about stem cells are already available, in all forms and formats. As we checked the count was over 13,000; and the number is rising as a true *revolution* deserves. However, we did not need to read them all to realize that the overwhelming majority of these books are extremely specialized and of a rather narrow spectrum. Hypothetical examples in this context may range from *"Stem cells in the gastrointestinal tract"* to *"Stem cell policies in*

central Africa". Nothing bad about specialised book, we need them too, but our ambition with *Stem Cells* was to provide the reader with a solid overview about stem cells in all the relevant contexts; all in *one* book. We soon realized that the relevant contexts were very many and that a fair coverage would need to include stem cells in the most relevant tissues, in species as diverse as plants to human, during development, adulthood and disease, and in specific applications ranging from therapy to commercial exploitation, each with its specific legislations and ethical considerations; all in *one* book. The challenge was daunting and as we write this preface, we are still surprised that we did not give up our project at that point... yet the challenge motivated us and perhaps the fact that we ourselves are scientists working on basic stem cell research gave us the motivation to run this risky experiment. So here you are reading this book.

To make one point very clear, by no means we expected a single book to cover really *all* aspects of stem cells; no single book can comprehensively describe a *revolution*. Yet, we wanted to cover those aspects of stem cells that we felt were more representative of this very broad field. We felt that this can be conceptually divided in two major categories: basic research and applications although, clearly, the two are deeply interconnected. This is the reason why Stem Cells is divided in two volumes. Volume 1 focuses on basic research, starting with an historical overview to then move to a series of chapters focussed on basic stem cell biology, tissue development during development and finishing with the main model organisms that are being used in our labs for stem cell research. Volume 2 continues with a more applicative twist, including stem cells in different tissues during adulthood, disease, therapy and their commercial use, with regulations and ethics connected to them. All chapters were contributed by internationally recognized experts in the respective fields. We are extremely grateful to all of them for sharing our enthusiasm and for contributing their time, knowledge and passion to bring together the many aspects discussed in this book. The merit of *Stem Cells* is all theirs; they are among the ones who fuel the *revolution*.

As our last note, our attention while editing *Stem Cells* was primarily addressed to students approaching this field and to more advanced investigators working on any topic related to stem cells. We truly hope that our work may contribute to the formation of those readers representing the future generation of stem cell scientists. *Stem Cells* is dedicated to them; they are the ones who will carry on, and hopefully successfully conclude, this *revolution* for the benefit of society.

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Contents

PART I

TISSUE HOMEOSTASIS AND REGENERATION DURING ADULTHOOD

CHAPTER

1

Dynamics and Aging of Hematopoietic Stem Cells

*Ronald van Os** and *Gerald de Haan*

SUMMARY

Adult stem cells are responsible for tissue integrity and are able to maintain tissue homeostasis by generating sufficient numbers of functional mature cells. At the same time, stem cells maintain their own numbers to ensure tissue functionality for the lifetime of an organism. Proper functioning of tissue stem cells is of great relevance to prevent age-associated diseases, which will become more prevalent as the general population in many societies is rapidly aging. Tissue specific stem cells have been identified and isolated on the basis of cell surface markers and are functionally characterized in numerous assays (Bhatia et al., 1997; Li et al., 1998; Shackleton et al., 2006; Spangrude et al., 1988). Most tissue stem cells were shown to lose functionality upon aging of the organism. The mechanism for age-dependent stem cell decline is largely unknown, although several potential reasons have been suggested. This chapter will summarize current knowledge on aging of hematopoietic stem cells and provide some possible

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List of abbreviations after the text.

explanations for aging at the stem cell level. We distinguish between effects on the stem cells themselves; cell-intrinsic damage and mechanisms and effects on the microenvironment; cell extrinsic mechanisms.

HEMATOPOIETIC STEM CELLS

Hematopoietic stem cells (HSC) are responsible for blood cell formation and are able to produce all types of blood cells, such as erythrocytes, B-lymphocytes, T-lymphocytes, granulocytes, thrombocytes and dendritic cells. Under steady state conditions, HSC reside in the bone marrow and release mature cells to the circulation where the cells function or migrate to other organs to undergo further maturation. The hematopoietic system is organized in a hierarchical manner with the stem cells at the top of the hierarchy. These primitive cells slowly differentiate into less potent progenitors and finally mature into the functional cells in the blood. HSCs were one of the first tissue stem cells to be identified. Initially, the colony forming unit-spleen (CFU-S) was considered to be a stem cell (Till and McCulloch, 1961). The CFU-S was later found to be able to differentiate into multiple lineages but to be distinguishable from cells with long-term *in vivo* repopulation ability (Jones et al., 1990). The capacity for *in vivo* generation of all mature blood cell lineages is now required to measure hematopoietic stem cell function. In order for an organism to maintain viability, it is sufficient to generate all type of blood cells in a balanced manner to ensure proper oxygen transport, prevent bleeding and combat infections. Already early in life, the immune system develops multiple barriers against pathogens. Granulocytes and macrophages are essential for innate immunity and B- and T-lymphocytes for adaptive immunity. Adaptive immunity can provide life-long protection against antigens by generating an immunological memory. This memory may be called upon when an antigen is encountered which the organism has successfully rejected earlier. Memory B- and T-cells can rapidly divide and efficiently attack the antigen or cells carrying such an antigen.

However, during aging the output of the hematopoietic stem cell changes from a very balanced system generating all types of blood cells to a more myeloid biased blood cell production (Cho et al., 2008; Dykstra et al., 2011; Pang et al., 2011). Whether there is a change in the fate of stem cells, whether this change in output represents a decline in function and which are the mechanisms at play remains to be determined.

However, not only does the composition of mature cells change with age but also their proliferation (Mauch et al., 1982), homing (Liang et al., 2005), mobilization capacity (Xing et al., 2006) and the total number of phenotypically defined stem cells (de Haan and Van Zant, 1999; Morrison et al., 1996). Finally, in competitive transplantation assays where old stem cells

were injected into lethally irradiated recipient mice with similarly treated young stem cells, old stem cells were found to have a severely reduced capacity to generate new blood cells (Dykstra et al., 2011; Harrison and Astle, 1982; Harrison et al., 1989; Kamminga et al., 2005; Morrison et al., 1996). Until recently, most stem cell studies were performed with populations of cells, which precludes the detection and interpretation of differences at the single stem cell level. More recently, studies on single hematopoietic stem cells have been published and these showed that at the single cell level, some HSCs are prone to produce all types of blood cells (balanced), while other produce predominantly either myeloid or lymphoid progeny (Cho et al., 2008; Dykstra et al., 2007; Dykstra et al., 2011; Muller-Sieburg et al., 2004). This can be detected only when single cells transplants are performed. Alternatively, genetic barcoding may provide identification of multiple distinct stem cell clones within one recipient (Gerrits et al., 2010; Verovskaya et al. 2013). The results published from single cell transplantation studies showed that during aging more HSC with a myeloid potential are present than in young mice (Cho et al., 2008; Dykstra et al., 2011; Muller-Sieburg et al., 2004). Thus, multiple mechanisms have been proposed that may influence the functioning of hematopoietic stem cells during aging. These will be discussed below.

PROLIFERATIVE STRESS IN HEMATOPOIETIC STEM CELLS

Hematopoietic stem cells need to proliferate continuously to generate new blood cells. However, the hierarchical organization of the system allows HSCs to remain quiescent for long periods of time, resulting in a division rate that was estimated to be once per 2 months for phenotypically defined HSCs (Cheshier et al., 1999). More recently, it was found that two populations of HSCs may exist that differ in proliferation kinetics; one with the previously reported turnover time of approximately 2 months and the other population turning over only once every 5 months (Wilson et al., 2008). The latter population was considered to be a particularly dormant stem cell population that could be activated to proliferate in response to infection (Baldridge et al., 2010; Essers et al., 2009; Trumpp et al., 2010). Whether this is truly a functionally distinct hematopoietic stem cell population remains to be determined, but what may be a more important question is why there would be a physiological need for two distinct HSC populations? Rapidly proliferating stem cells would be required to provide steady-state blood cell production. It is possible that the need for (very) slow cycling cells is to avoid accumulation of DNA damage, which increases with each cell division. Such quiescent stem cells could thus provide a reservoir of cells that is infrequently called upon. However, deeply quiescent cells rely on an error-prone mechanism for DNA repair (Milyavsky et al., 2010; Mohrin et al., 2010).

Quiescence and reactivation of cells is tightly regulated by cyclin-dependent kinases (Cdk's), and their inhibitors (Cycline-dependent kinase inhibitors, Cdkn's). The role of Cdkn's in hematopoietic stem cells has been studied extensively. First, it was reported that deletion of the cell cycle inhibitor Cdkna1 or p21, enhances exhaustion of HSCs (Cheng et al., 2000b), Thus, lack of a proper cell cycle control prohibits HSCs from maintaining quiescence and optimal functioning during life. Similar effects were seen when mice deficient for other Cdkn's, such as p18 (Cdkn2c, $p18^{INK4c}$) and p27 (Cdkn1b, $p27^{Kip1}$), were evaluated (Cheng et al., 2000a; Yuan et al., 2004). However, the effects of deleting cell cycle inhibitors was also found to be mouse strain-dependent, suggesting the presence of modifier genes (van Os et al., 2007). More recently, expression of Cdkna1c, p57, was shown to be a hallmark of hematopoietic stem cells (Matsumoto et al., 2011; Yamazaki et al., 2006). Maintaining quiescence is nature's way to to prevent damage to DNA as a consequence of replication and by reducing cellular metabolism, thus requiring less transcription.

CELL-INTRINSIC DAMAGE TO HEMATOPOIETIC STEM CELLS

All cells within the body of an organism are susceptible to damage, but when a damaged cell can be replaced by another cell during tissue turnover, this will likely not result in persistent tissue dysfunction. When, however, stem cells, that normally replenish each tissue, are damaged, there is a risk of irreversible damage to an organ or tissue.

Fortunately, many types of damage such as damage to RNA or proteins, can be handled by the cell, because these molecules can be replaced by newly formed molecules. Damaged or misfolded proteins are degraded by proteases or by the proteasome and destructed in lysosomes (Ciechanover, 2005). Cells can accumulate damage that is caused by oxidative (free radical) stress, which could lead to cross-linking of proteins and DNA, and DNA mutations. All this can lead to exhaustion. Stem cell exhaustion, usually caused by extensive proliferation can, among others, lead to telomere shortening (Allsopp et al., 1995). Telomeres are the ends of chromosomes that protect the chromosomes from being recognized as damaged DNA. Telomere shortening has been suggested as the main cause for limiting cell proliferation and subsequent senescence (Wright and Shay, 1992). Senescence could be a mechanism to avoid proliferation of cells with dangerously short telomeres or other damage. The cell cycle inhibitor p16 (Cdkn2a, $p16^{INK4a}$), has been identified as a marker for senescent cells (Alcorta et al., 1996). At the same time, expression of p16 prevents excessive cycling of cells and in the hematopoietic system it was shown to cause HSC repopulating defects, apoptosis and improved stress tolerance (Janzen et al., 2006). However, recently it was shown that p16 expression in aged HSC is a very rare event

(1 in 308 aged HSC) and it was proposed to play a minor role in the reduced functionality of aged HSC (Attema et al., 2009). Furthermore, senescent cells (p16 expression by $Lin^{-}IL\text{-}7R^{-}Sca^{+}Kit^{+}CD150^{+}$ cells) do not qualify as HSC by definition because they are no longer able to proliferate. On the other hand, clearance of p16-expressing senescent cells was shown to delay age-related pathologies (Baker et al., 2011) which may be because senescent cells secrete proteins that negatively affect neighboring cells (Coppe et al., 2008). Mammalian cells have the means to cope with or overcome DNA damage and do so by activating a machinery that senses damage, stalls cell cycling, repairs the damage or activates apoptosis (Mallette and Ferbeyre, 2007; Reinhardt and Schumacher, 2012). Deficiencies in this machinery often lead to accelerated aging or cancer predisposition.

Bone marrow progenitor cells have long been considered to be very sensitive to chemotherapeutic drugs and radiation (Mauch et al., 1995) thereby limiting the dose for clinical treatment of malignancies. However, where progenitors have been found to be radiation sensitive, more primitive stem cells are relatively more resistance to radiation and have a higher capacity for radiation damage repair (Meijne et al., 1991; van Os et al., 1993a; van Os et al., 1993b). In general, stem cells have been found to be more resistant to most ablative therapies due to their quiescent state (Blanpain et al., 2011; Mandal et al., 2011). On the other hand, as stem cells rely on the error-prone mechanism of non-homologues end joining (NEHJ) for their repair (Milyavsky et al., 2010; Mohrin et al., 2010), residual DNA damage may render stem cells more likely to acquire tumorigenic aberrations. The role of DNA damage in aging of murine hematopoietic stem cells has been studied in various models of DNA damage repair deficiencies. The most comprehensive study was performed by Rossi et al. (Rossi et al., 2007) who showed expansion of phenotypically defined LT-HSCs with reduced self-renewal and *in vivo* repopulation potential. Absence of proper DNA repair leads to increased stem cell apoptosis and accelerated exhaustion and other aging-like phenotypes (Table 1.1) (Rossi et al., 2007). These data suggests that accumulation of damage is a physiological mechanism contributing to aging.

Thus, DNA damage is thought to play a pivotal role in the functional decline of stem cells. Therefore, several groups have investigated the role of molecules involved in the DNA damage response in stem cell aging. These studies focused mainly on enzymes involved in DNA repair. DNA replication is a complex process, which often leads to errors and to base damage, single strand breaks or ultimately DNA double strand breaks (DSBs). Several enzymes have been investigated in HSC aging. These include Msh2, Ercc1, Ku80, Brca1 and many others. Msh2 is involved in mismatched repair and was found to have only minor effects on HSC function (Reese et al., 2003). Ku80, encoded for by the *Xrcc5* gene and involved in non-homologous

Table 1.1 DNA damage response genes and HSC aging phenotypes.

Gene	Observed aging phenotype	Mechanism	Ref
$Ku80^{-/-}$	1, 2	Residual DNA damage	(Rossi et al., 2007)
$TR^{-/-}$	1, 2	Residual DNA damage	(Rossi et al., 2007)
XDP^{ITP}	1, 2	Residual DNA damage	(Rossi et al., 2007)
$Atm^{-/-}$	2, 3	Elevation of ROS	(Ito et al., 2004; Ito et al., 2006)
$Ercc1^{-/-}$	4	Severe progeroid syndrome	(Prasher et al., 2005)
$Msh2^{-/-}$	2*	Residual DNA damage	(Reese et al., 2003)
$Brca2/Fancd1^{-/-}$	2	Residual DNA damage	(Navarro et al., 2006)
$FancC^{-/-}$	2, 4	Residual DNA damage	(Haneline et al., 1999)

1. Increased LT-HSC frequency
2. Reduced *in vivo* repopulation; 2* after serial transplantation
3. Myeloid skewing
4. Reduced HSC proliferation *in vitro*

end-joining, repairs DNA DSBs and its deficiency was shown to have an aging-like phenotype (Rossi et al., 2007). *$Ku80^{-/-}$* mice show a faster increase in phenotypically defined stem cells and diminished *in vivo* repopulation ability with age. Deficiency of the *Brca2/Fancd1* gene, a pivotal gene for homologous recombination, caused a proliferation defect resulting in a severe repopulation disadvantage of hematopoietic (stem) cells (Navarro et al., 2006). Chromosomal instability was suggested as the cause for the defect. The most severe effect has been observed with Ercc1 deficiency where hematopoietic stem and progenitor cell function was rigorously affected and extensive progeroid phenotypes were found (Prasher et al., 2005). In addition to genes involved in DNA repair, maintenance of telomere length was also found to affect HSC function. Telomeres are chromosome ends that require the action of a complicated mechanism revolving around the telomerase enzyme, to maintain telomere length upon cell division. However, despite the presence of this machinery, a gradual decline in telomere is observed *in vivo* and *in vitro* with age (Allsopp et al., 1995; Allsopp and Harley, 1995; Allsopp et al., 1992; Aubert et al., 2012). The relationship between telomere shortening and DNA damage response remains uncertain, but it has been shown that critically short telomeres elicit a DNA damage response (Hewitt et al., 2012). The DNA damage response may lead to dysfunctional proliferation or even senescence, which will result in reduced stem cell functioning. Telomerase deficiency lead to accelerated telomere shortening and was shown to reduce HSC functioning (Allsopp et al., 2003a; Rossi et al., 2007). These studies showed increased HSC numbers by phenotype but reduced repopulating ability (Rossi et al., 2007) and reduced functionality after serial transplantation (Allsopp et al.,

2003a). On the other hand, telomerase overexpression did not increase the repopulation capability of HSCs (Allsopp et al., 2003b). Since telomerase is expressed in HSCs, albeit at low levels, this may be sufficient to maintain telomere length in this model.

In summary, it has been shown that accumulation of DNA damage hampers the functionality of HSC. Inefficient repair of DNA damage accelerates HSC aging, resulting in loss of *in vivo* repopulation and in some cases a myeloid skewing of HSC output. Not in all cases an increase in stem cells as measured by phenotype was observed. This may be caused by distinct mechanisms of stem cell loss with age. We propose that during the lifespan of an organism there is a gradual increase in phenotypically defined stem cells, accompanied by a gradual loss in function (Figure 1.1A and B). However, during "normal" aging there is a large variation in this increase and it is not clear whether this correlates with loss of function in individual old mice (Figure 1.1C). It has been suggested that there is no correlation (Dykstra et al., 2011) between stem cell numbers and functionality but this may require more data. What would happen after the last measurement remains a mystery. Two options seem possible of which the first could be a further growth in stem cell numbers followed by a sudden drop when the stem cells become exhausted. The second possibility is a relative gradual decline in stem cell numbers after the rise in the first two years (Figure 1.1D). On the other hand, when HSC turnover is affected by for instance a deficiency in DNA repair, another gene involved in HSC maintenance, a rapid increase early in life may be followed by a steep decline in phenotypically defined HSC (Figure 1.1E). The inability to measure stem cell numbers sequentially in individual mice renders it impossible to draw definitive conclusions on the fluctuation in stem cell numbers in individual mice during aging.

Three main HSC phenotypes are considered to be associated with aging. Firstly, an increase in phenotypically defined stem cells has consistently been determined (Dykstra et al., 2011; Morrison et al., 1996; Rossi et al., 2007). Secondly, on a per stem cell basis, old stem cells have a competitive disadvantage over young stem cells (Dykstra et al., 2011; Morrison et al., 1996; Rossi et al., 2007; Chen et al., 2003). Finally, a skewing to a more myeloid output as compared to a more balanced output (equal lymphoid and myeloid output) is observed when old stem cells are transplanted (Cho et al., 2008; Dykstra et al., 2011; Muller-Sieburg et al., 2004). In some studies these three parameters have been observed in combination with a reduced proliferative potential and reduced peripheral blood cell counts in one or more lineages.

In Table 1.1., several genetic defects are listed that have hematopoietic stem cell aging-like phenotypes. In fact, these mice also typically show major aging-like syndromes in non-hematopoietic tissues. However, one