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Review

Stem Cells and Aging: A Chicken-Or-The-Egg Issue?

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ABSTRACT: Aging is a process that involves all organs and tissues of the human organism. Cells and tissues are impacted by aging in differing degrees, depending on their regenerative potential and sensitivity to outside stimuli. In this review, we discuss the potential role of adult stem cells in the aging process, and the new results that support the role of stem cells in the aging process. Finally, we discuss new evidence from progeroid syndromes that supports the stem cell hypothesis of aging.

Key words: Stem cells; Aging; Progeroid syndromes; Microenvironment

Stem Cell Hypothesis of Aging

Aging is the process of accumulating changes in an organism over time. Despite its significance for the well-being of individuals and the population as a whole, aging is a poorly understood process. Multiple hypotheses were proposed to address the biological basis for aging. These include: shortening of telomeres, DNA damage, epigenetic alterations of transcriptional regulation, and others [1-3]. Very recently, a stem cell hypothesis of aging was proposed, which states that a major reason of aging is either depletion, or failed differentiation of stem cells.

Stem Cells

Stem cells are undifferentiated cells that that are capable of self-renewal and differentiation. Most human tissues are composed of a majority of differentiated cells with a limited life span. These cells die and the tissue shrinks, unless replenished by new cells. A crucial source of these new cells is tissue stem cells, which compose only a small minority of a tissue's cells. These cells are termed adult stem cells and are multipotent (capable of differentiation into several cell types, but not all three germ layers). Nevertheless, they are required for the maintenance of adult tissues.

A subspecies of stem cells are embryonic stem (ES) cells, which can be obtained from early stage embryos (blastocyst). ES cells are pluripotent, which means they can differentiate into all three primary germ layers [4, 5]. ES cells are critical to organism development. A defect in ES cell differentiation may have a pleiotropic effect on the organism.

ES cells recently gained tremendous attention due to the reprogramming of adult somatic cells into induced pluripotent (iPS) cells [5]. iPS cells posses ES cell properties and were developed with the long-term objective to gain a new therapeutic tool. Since they are pluripotent, iPS cells could hypothetically be used to develop any desired cell type to replace cells that are abnormal or absent in patients, from whose cells the iPS cells originated (e.g. insulin-producing cells for the treatment of diabetes).

Stem cells in an Aging Organism

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From the standpoint of aging, adult stem cells attract the most attention of all stem cell types. Since they are critical for the renewal of many tissues, it appears to make sense that loss of these cells or their function would contribute to aging. Yet, the issue is not that simple. Human tissues have varying regenerative potential and their cells have varying life spans. For example, the brain contains post-mitotic neurons whose life span may be as long as the life span of the whole organism. Yet aging occurs even in these tissues, which do not require replenishment by the stem cell pool. In contrast, in some tissues, replenishment by stem cell differentiation is continuous and occurs during the whole body existence. An example of this type of tissue or organ is the hemopoietic system. Finally, in some tissues, the differentiated cells retain proliferative potential and can regenerate the organ as needed. An example of this type of organ and tissue maintenance is liver, which can be regenerated by hepatocytes themselves. Thus, for practical purposes, aging is sometimes divided into replicative, which requires the presence of dividing cells, and chronological, which occurs in nondividing cells, and can, of course, also occur in cells that are capable of dividing, such as stem cells. In order to understand the role of stem cells in aging, the first question to be answered is: do adult stem cells change during the aging process?

Evidence for Stem Cell Aging

The function of stem cells in an aging organism has been explored for a relatively short time. One of the best-studied stem cell types in this respect are hemopoietic stem cells. Hemopoietic stem cell differentiation is ongoing until death, as is hemopoietic stem cell renewal. However, there are distinct changes as an organism ages. Bone marrow decreases in cellularity, anemias are more common, lymphopoiesis declines, and the incidence of myeloid abnormalities, such as malignancies, myelodysplastic syndromes and myeloproliferative disorders, increases [6]. These changes are associated with alterations in the hemopoietic stem cell pool [6-9]. Hypothetically, hemopoietic stem cells could be affected into two ways. They can either decrease in number, or they cannot properly differentiate. I reality, the number of hemopoietic stem cells increases with age in humans [6, 10-13]. However, there appear to be aberrations in differentiation. Specifically, the stem cells appear to be myeloid-biased, and exhibit less inclination to differentiate into lymphoid lineages [6-9]. This would at first sight suggest and problem in differentiation. However, it has been recently shown that there is a loss of a specific hemopoietic stem cell subpopulation that prefers to form lymphoid progeny [14-17]. In addition, aged hemopoietic stem cells show aberrant engraftment [6]. Similar findings were reported from mouse studies. Thus, the picture of hemopoietic stem cell aging is complex and involves both loss of cell numbers and loss of the ability to differentiate.

The other major type of adult stem cells is mesenchymal stem cells. They are present in bone marrow and other tissues, such as adipose. These are rare cells, representing ca. 0.001 to 0.01% of the nucleated cells in bone marrow. They are multipotent and can differentiate into mesodermal and nonmesodermal tissues [18-22]. Studies in mice demonstrated that the number of bone marrow mesenchymal stem cells decreases only marginally as the organism ages [23]. However, their self-renewal ability and bone formation ability dramatically drop. Thus, both major features of stem cells are affected by aging in this stem cell type [24-26].

A third type of adult stem cells to be discussed in this review is skin-derived precursors. These cells reside in dermal papillae of human hair follicles anda re not to be confused with epidermal stem cells [27-29]. Studies on aged human subjects demonstrated that human SKP abundance and differentiation potential sharply decreases with age [30]. They are thus affected, similarly to mesenchymal stem cells, in both major features of stem cells. In contrast, the above mentioned epidermal stem cells show little response to aging. They do not appear to decrease in number and functionality with age [31].

Finally, stem cells are involved in tissue repair, which is known to decline with age. One example is the age-related endothelial dysfunction [32]. It has been shown that bone-marrow-derived endothelial progenitor cells (EPCs) play a role in maintenance of endothelial function by contributing to re-endothelization and neovacularization [33, 34]. EPCs functionally decline with age, however, and this decline correlates with impairment of the endothelium [32].

A similar situation occurs in muscles. The muscle stem cell (termed the satellite cell) is usually quiescent and mobilized when required for repair, for example when an injury occurs. The numbers of satellite cells do not appear to change much with age [35, 36]. However, their repair, i.e. differentiation ability is dramatically reduced [37, 38]. Thus, the satellite cells are altered in at least one of the major features of stem cells.

In summary, adult stem cells, with an exception of epidermal stem cells, undergo age-related changes. The age-related decline is mainly functional, but in some cases, decline in stem cell numbers can be observed (see above). What is the molecular mechanism underlying the observed stem cell deficits in the aged organism?

Molecular changes underlying the aging process and stem cells

In general, mechanisms that are involved in aging of somatic cells are also involved in aging of adult stem cells. These include telomere shortening, oxidative stress, epigenetic dysregulation and miRNAs. During DNA replication, cellular DNA has to be replicate faithfully. It had been suggested first by the Russian scientist Olovnikov in 1970s that DNA replication may result in chromosomal shortening, due to difficulties when replicating chromosomal ends, and this shortening may be related to aging [39]. The chromosomal ands are protected by telomeres, which are ribonucleprotein complexes that consist of tandem DNA repeats (TTAGGG) and several proteins, together termed shelterin [40]. Conventional DNA polymerases can not replicate the chromosomal ends. To prevent shortening of these ends (telomeres) with each cell cycle, cells posses the enzyme telomerase, which can add the TTAGGG repeats to the chromosomal ends. The activity of telomerase is known to decline with age [41]. The shortening of chromosomal ends can eventually lead to the loss of telomere protection. The naked chromosomal ends than may trigger a DNA damage response, with resulting growth arrest and/or apoptosis [42]. Adult stem cells express high levels of telomerase [43]. The proposed role of telomere shortening in aging is supported by evidence from telomerase-deficient mice, which exhibit premature aging [44-47]. This finding is accompanied by dramatic impairment of adult skin stem cells [48, 49]. This phenomenon can be rescued by suppression of p53, which is a key protein of cellular DNA damage response [48, 49]. This latter data thus support the hypothesis that naked chromosomal ends trigger DNA damage response. Consistently, p53 overexpression leads to premature aging in mice [41]. In addition to skin stem cells, late generation telomerase-deficient mice have also limited hemopoietic reserve, which is accompanied by short telomeres in the stem cell population [41].

The DNA damage response can be also triggered by other mechanisms than telomere shortening. The DNA damage theory of aging postulates that the main cause of the functional decline associated with aging is the accumulation of DNA damage and ensuing cellular alterations [50, 51]. From this point of view, telomere shortening is only a part of the picture. Cells are exposed to many types of DNA damaging agents and sources, both extrinsic and intrinsic, including reactive oxygene species [52]. Accordingly, they posse multiple DNA repair systems, which can repair the damage. Function of many of these systems declines with age [50, 51]. The hypothesis that cumulative, unrepaired DNA damage may play a role in aging is supported by some premature aging (progeria) syndromes. These include the Werner syndrome, trichothiodystrophy, Cockayne syndrome and ataxia telangiectasia. Individuals that suffer from these syndromes have significantly shortened life span and exhibit signs on premature aging [36, 50]. Significantly, mutations and genes in which these mutations occur were identified for each of these syndromes. The Werner syndrome protein is a RECQ-related DNA helicase that is known to be involved in DNA repair, in addition to other cellular processes as outlined below [53]. Trichothiodystrophy is caused by point mutation in the XPD gene, which is involved in nucleotide excision repair (NER,[54]). Similarly, Cockayne syndrome is caused by mutations in either CSA or CSB gene, both of which are required for nucleotide excision repair [54]. Finally, the gene mutated in ataxia telangiectasia is involved in double strand break DNA repair [55]. Thus, this link between DNA repair and aging strongly supports the DNA damage hypothesis of aging. Similar results were obtained with transgenic animals that carry mutations in DNA repair genes. Mutations in Ligase 4, Ku80 and Ku70 genes, which are involved in double-strand DNA break (DSB) repair, lead to poor hemopoietic reconstitution due to a hemopoietic stem cell defect in repopulation ability [8, 56, 57]. Similarly, a deficiency in another component of the DSB repair, Rad50, results in hemopoietic failure due to ablation of hemopoietic stem cells [58]. In addition, a repopulation defect was observed in mice carrying a mutation in the NER gene XPD [8]. Finally, mice carrying a mutation in the ataxia telangiectasia gene (ATM) have decreased stem cell numbers, loss of repopulation ability and increased apoptosis of hemopoetic stem cells [59]. These results raise a question whether this type of stem cell defects is due to a decreased DNA repair ability of hemopoietic stem cells. In support of this hypothesis, it had been demonstrated that antioxidants reduced the oxidative stress and maintained stem cell function in ATM-deficient mice [59]. Finally, in aged humans, aged hemopoietic stem cells from normal individuals were shown to accumulate DNA damage, which appears to contribute to their functional decline, and exhibit increase in the numbers of □H2AX foci, which are markers of double strand DNA breaks [60]. Taken together, the presented results suggest that DNA damage may play a significant role in aging and loss of functionality of stem cells.

Another phenomenon associated with aging is changes in epigenetic modifications of histones and DNA and consequent dysregulation of gene expression. The epigenetic modifications that were observed to change during aging are related to histone acetylation, histone methylation and DNA methylation. In mice, it has been observed that the level of the histone deacetylase SirT1 decreases with age [61]. Significantly, decrease of SirT1 expression correlates with premature aging in mice with

increased p53 activity [62]. Inactivation of fhe yeast homologue of SirT1, Sir2, decreases the replicative lifespan of yeast [63-65]. Interestingly, the well known SirT1 activator, resveratrol, prolongs the life span of mice when added to the diet [66]. However, it is not yet possible conclude that SirT1 controls aging by controlling histone acetylation, although histones are a major SirT1 target [67]. The second observed epigenetic change is increased methylation of H4K20, and decreased methylation of H3K27 and corresponding decrease in the EZH2 histone methyltransferase [68, 69]. These changes were observed in senescent human cells [66]. Senescence is also associated with the presence of senescence-associated heterochromatin foci (SAHFs, [70]). The third aging-associated epigenetic phenomenon is a global decrease in DNA methylation [71]. However, at the same time, there is an increase in DNA methylation at certain loci. Among these are tumor suppressor genes [72-74]. DNA methylation generally suppresses expression, and inactivation of tumor suppression genes may increase risk of cancer in aged individuals. Finally, aging may involve miRNAs. Expression of these transcriptional regulators significantly varies with aging and may predict the onset of aging-related diseases [75]. One recently described example is miRNA targeting p16. This miRNA increases with age (average 16 fold decrease over the human lifespan), and suppresses expression of the tumor suppressor gene p16 [75]. It is not yet clear if all described epigenetic changes occur in aging stem cells. However, it has been demonstrated that the levels of some DNA methyltransferases (DNMTs) control the self-renewal and differentiation of stem cells. For example, DNMT1 is essential for self-renewal of hemopoietic stem cells [76]. Epigenetic dysregulation of H3K9 and H3K14 acetylation also occurs in aging mesenchymal stem cells [77]. In summary, the presented data suggest that aging of stem cells is associated with epigenetic changes, and these changes may affect either self-renewal or differentiation of these essential cell types.

Aging and Stem Cells: cause or consequence?

Given the key role of stem cells in maintenance of many tissues, it is easy to assume that they play a central role in the aging of the said tissues. This model would assume that the somatic cells can be easily replaced, and, as long as stem cells are intact, tissue aging does not occur or is reversible. This was first outlined as so-called disposable stroma theory of aging [78, 79]. Yet, several factors need to be considered. First, not all somatic cells, even in tissues or organs with high overall cell turnover, have a short life span. One example could be memory T cells, which have a very long life span, particularly when compared to erythrocytes, which originate from the same stem cell. Thus, aging can occur differently in long-lived subset of these cells. Another aspect to consider is that the potential aging of differentiated cells can lead to exhaustion of stem cells, which are then driven to continual effort to replace the somatic cells. A third aspect to consider is the stem cell environment, in other words, the niche the stem cell reside in, as well as more distant interactions, mediated by circulating proteins and growth factors. The possible role of the niche was has been addressed using heterochronic approaches, where young stem cells were transplanted into an old niche and vice versa [6, 36]. What was demonstrated is that, at least in hemopoietic stem cell case, the bone marrow environment significantly affects stem cell aging, and a young environment can actually rejuvenate the old stem cells [6, 36]. This is consistent with results from studies with induced pluripotent stem cells, which demonstrate that reprogramming of somatic cells into stem cells leads to lengthening of telomeres, and thus indicates that the rejuvenation and reversibility of the aging process is indeed possible [80]. Conversely, young stem cells fail to efficiently repopulate an old niche [6, 81]. The second possibility, i.e. a possible role for circulating, systemic factors, was addressed in a recent paper published in Nature, which used surgically joined young and old mice to demonstrate that young environment can rejuvenate the hemopoietic stem cell niche, and consequently, the stem cells themselves [82]. This was attributed to circulating soluble factors that control local insulin-like growth factor1 (IGF-1). Unfortunately, the paper was very recently retracted by 3 out of 4 authors, so the role of systemic factors in stem cell aging is still unclear. In conclusion, stem cell aging is influenced by the aged microenvironment they are in. Thus, stem cell and organism aging is an interconnected process.

Stem Cell Aging and progeroid syndromes

As outlined above, the molecular basis of many progeroid syndromes support the DNA damage theory of aging. However, two syndromes point to the role of stem cells. The Hutchinson-Gilford progeria syndrome (HGPS) is a disease in which patients develop symptoms of premature aging during their first year of life. The average lifespan is 13 years [83, 84]. HGPS is caused by a mutation in the LMNA gene, which encodes lamin A proteins. The mutation activates a cryptic splicing site, which results in production of a truncated protein, known as progerin. Since lamin A associates with the nuclear lamina, it presumably affects multiple cellular functions [85-87]. However, a model has been proposed, supported by *in vitro* studies on mesenchymal stem cells, in which the premature aging of HGPS patients is caused by

premature exhaustion of stem cells [88]. This hypothesis was recently given support by data from a mouse model of HGPS, which demonstrated initial epidermal hyperplasia followed by epidermal hypoplasia in mice expressing the HGPS mutation [89]. This phenotype was accompanied by downregulation of the epidermal stem cell maintenance protein p63 with accompanying activation of DNA repair and premature senescence of adult stem cells [90]. Thus, the HGPS results suggest a key stem cell role in the pathogenesis of this progeroid syndrome.

New evidence for a potential stem cell role in aging also comes from our recent studies of the Werner syndrome (WS). WS is an autosomal recessive disorder. Its hallmark is premature aging, which is associated with the early onset of degenerative and neoplastic diseases [91]. Gene expression in WS closely resembles that of normal aging and supports the use of WS as a model of aging [91]. The gene, whose mutation underlies the WS phenotype, is called WRN. Mutations in WRN result in the instability of WRN mRNA, as well as truncation of the protein with loss of the nuclear localization signal (NLS) and all or some enzymatic domains of the protein. The protein encoded by the WRN gene, WRNp, has helicase activity [92]. WRNp is a member of the RecQ DNA helicase family, which in humans includes four other members (RecQ1, Bloom Syndrome Protein (BLM), RecQ4 and RecQ5, [93]). Several functions were suggested for WRNp. Since WS cells exhibit high sensitivity to the topoisomerase I poison camptothecin, it has been proposed the WRNp plays a role in DNA replication and DNA repair [91]. Interestingly, WRNp deficiency does not accelerate aging in mice, which occurs only in double knock-out WRN and telomerase-deficient animals [94]. Overall, WRNp function is not yet fully understood. It has been noted that the RecQ helicases, including the WRN protein, engage in dynamic subnuclear relocalizations in response to different conditions of cell growth [95]. Very recently, it has been shown that altered chromatin structure, even in the absence of DNA breaks, recruits the WRN protein into chromatin [96]. We have studied the WRNp function in NCCIT cells, which are a teratocarcinoma cell line somewhat resembling embryonic stem cells in their ability to differentiate into all three major embryonic layers [97]. These cells express a transcription factor called Oct4 [98]. The fundamental role of Oct4 is to maintain pluripotency of ES cells and primordial germ cells. The Oct4 gene is expressed at high levels in ES cells, but undergoes rapid heterochromatinization and silencing upon the addition of retinoic acid (RA, [99]).

This process involves a cascade of epigenetic histone and DNA modifications. First to occur is deacetylation of lysines 9 and 14 of histone H3, which occurs within several hours after the addition of RA. This is accompanied by a loss of trimethylation of lysine 4 of H3 (H3K4). Approximately 12 hours after the addition of RA, trimethylation of lysine 9 of histone H3 (H3K9) can be observed. The histone methyltransferase (HMT) responsible for this event is G9a. In contrast, the majority of H3K9 trimethylation in pericentric heterochromatin is performed by Suv39h HMT [100]. The process of heterochromatinization of the Oct4 promoter thus possesses certain specific characteristics. H3K9 methylation reaches its peak at 24-48 hrs after RA exposure. Trimethylation of H3K9 sets the stage for local heterochromatinization via binding of the heterochromatin protein 1 (HP1) to the trimethylated H3K9 (H3K9me3). Finally, Oct4 promoter DNA is methylated by the *de novo* DNA methyltransferase Dnmt3b.

Although the process of Oct4 promoter suppression was thus described in some detail, fundamental questions remain. One question is the identity of players that suppress the promoter. Currently, only G9a, HP1 and Dnmt3b have been identified (see above). Another question relates to the relationships among these proteins, since we do not know how they are recruited and how they interact with each other. In our study, we demonstrated that WRNp localizes to the Oct4 promoter during retinoic acid-induced differentiation of human pluripotent cells, and associates with the *de novo* methyltransferase Dnmt3b in the chromatin of differentiating NCCIT cells. Depletion of WRNp does not affect demethylation of lysine 4 of the histone H3 at the Oct4 promoter, nor methylation of lysine 9 of H3, but it blocks recruitment of Dnmt3b to the promoter and results in reduced methylation of CpG sites within the Oct4 promoter. The lack of DNA methylation was associated with continued, albeit greatly reduced, Oct4 expression in WRN-deficient, retinoic acid-treated cells, which resulted in attenuated differentiation. The presented results revealed a novel function of WRNp, and demonstrated that WRNp may play a role in stem cell differentiation. In addition to stem cell differentiation, our data also tie WRNp to regulation of DNA methylation, dysreguation of which is also associated with aging (see above). Our data are also consistent with studies of the Chua's lab showing that WRN is not recruited to the telomeres of Sirt6-/- mice, which lack an apparent H3K9 deacetylase [101]. Finally, we note that NCCIT cells are related to ES cells, which raises another question, when the process of aging actually begins, before it manifests phenotypically. It has been shown extremely recently that aging may begin as early as embryogenesis, when IGF-1 switches cells from pluripotency to differentiation and its levels decline and

miR-675 levels increase [102]. Our results are also consistent with this hypothesis.

The presented results from progeroid syndromes thus strengthen the hypothesis that stem cell aging is a key event in the overall aging of the organism. However, several caveats should be noted. First, NCCIT cells are not true stem cells. Thus, it will be necessary to reinvestigate the WRNp role in normal adult stem cells. The second caveat applies to all progeroid syndromes and it is that it is not yet clear that the premature "aging" we observe in these diseases is a faithful model of and recapitulates normal aging. Nevertheless, the stem cell link observed in these studies should focus our attention on the stem cell role in aging of normal organisms.

Perspectives

The new findings regarding the role of stem cells in aging give rise to new important questions. One critical, still unresolved issue, is the degree to which aging of stem cells contributes to the overall aging process of humans. The second question is what molecular mechanism underlies stem cell aging. What is the contribution of epigenetic changes? Cellular levels of telomerase? Status of DNA repairs in aged stem cells? A third question is how do the niche stem cells that reside in influence aging of stem cells, and vice versa. Finally, are there systemic factors that could delay or accelerate stem cell aging? Regenerative medicine, which aims to restore organ function, puts a lot of hopes into stem cell approaches. Yet, the questions outlined above and answers to them will largely determine the success or failure of these approaches. One example is a transplantation of young stem cells into an aged niche. Since the status of the niche may impact the success of this approach, it may be necessary to determine the overall suitability of the niche prior to the transplant. Conversely, a potential role of epigenetic modifications in stem cell aging may lead to development of approaches where these modifications can be altered in the aged stem cells *in situ*, and these cells regenerated, without a need for stem cell transplant. In summary, new stem cell findings provide new perspectives and should stimulate new research on stem cells and aging, which can potentially result in breakthrough treatments and lifespan extension.

Conflict Of Interest Statement

Authors declare no competing interests.

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