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The Emerging View of Aging as a Reversible Epigenetic Process

Micaela López-León Rodolfo G. Goya

Institute for Biochemical Research (INIBIOLP) – Histology B and Pathology B, School of Medicine, National University of La Plata, La Plata, Argentina

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Abstract
The achievement of animal cloning and subsequent development of cell reprogramming technology are having a profound impact on our view of the mechanisms of aging in complex organisms. The experimental evidence showing that an adult somatic nucleus implanted into an enucleated oocyte can give rise to a whole new individual strongly suggests that the integrity of the genome of an adult nucleus is fully preserved. Here, we will review recent experimental evidence showing that pluripotency gene-based cell reprogramming can erase the epigenetic marks of aging and rejuvenate cells from old individuals reversing most signs of aging and that when induced pluripotent stem cells are differentiated back to the cell type of origin, the rejuvenated cells share many of the features of wild-type counterparts from young donors. This evidence supports the idea that progressive epigenetic dysregulation may be the key driver of organismal aging and challenges the conventional view of aging as an irreversible process. The model of aging as an epigenetic process provides an elegant explanation of a number of age-related processes difficult to explain by conventional theories of aging.

The authors contributed equally to this article.

The Path to Cell Reprogramming

The generation of induced pluripotent stem cells (iPSCs) from somatic cells has demonstrated that somatic mammalian cells from adult animals, including humans, can be reprogrammed to a pluripotent state by overexpression of a limited number of embryonic transcription factors (TF) [1]. The achievement of induced pluripotency represents the synthesis of scientific principles and methodologies that were developed over the last 60 years. Among the pioneering efforts that paved the road to cell reprogramming, the studies of John Gurdon and collaborators in the 1960's should be mentioned. The studies demonstrated that the nucleus of a differentiated intestinal frog cell could be reprogrammed to that of a totipotent cell when transferred into an enucleated oocyte. These reprogrammed oocytes were able to give rise to a whole new frog [2, 3]. This research led, 30 years later, to the success of mammalian cloning by somatic cell nuclear transfer, achieved with the birth of Dolly, the sheep in 1996 [4]. This work was soon followed by studies reporting cloning in other species [5–7]. These results showed that the genome of even fully differentiated cells remains genetically totipotent, that is, can support the development of a whole organism. During this period, it was also discovered that a single TF, MyoD, can convert fibroblasts into skeletal myocytes, demonstrating that the fate of cells can be changed through the overexpression of specific TF [8–10]. The above achievements challenged the fundamental doc-

E-Mail karger@karger.com
www.karger.com/ger

Rodolfo G. Goya
INIBIOLP
Faculty of Medicine, UNLP, CC 455
1900 La Plata (Argentina)
E-Mail goya@isis.unlp.edu.ar

trine that cells, once terminally differentiated, are irreversibly determined in their specialization. Moreover, the success of animal cloning suggested that the somatic cell genome is highly plastic, and embryonic gene expression programs can be set in motion in terminally differentiated cells by defined factors. A second transcendental implication of the cloning studies was that in the cytoplasm of a mature oocyte there are molecules able of reprogramming a somatic nucleus by activating the developmental program for a new individual. What remained unknown in 1996 was the question of how complex would be the constellation of TF necessary for reprogramming a somatic nucleus to a totipotent embryonic nucleus. This mystery was elucidated 10 years later when Takahashi and Yamanaka [1] demonstrated in 2006 that the transfer of the 4 pluripotency genes *Oct4*, *Sox2*, *c-Myc*, and *Klf4* to adult mouse fibroblasts was able to reprogram them, taking the cells to a pluripotency stage in which they behave as embryonic stem cells. These discoveries profoundly changed the research prospects in the fields of regenerative medicine and disease modeling and have opened a horizon of up to then unimagined possibilities for the development of personalized therapeutic paradigms [11, 12].

Epigenetics and Aging: The Promise of Rejuvenation

Over the last 5 decades, it became widely accepted by mainstream gerontologists that aging was the consequence of progressive accumulation of (mostly oxidative) damage to cell macromolecules, particularly DNA [13, 14]. This phenomenon was thought to be particularly relevant in mitochondria where respiration is associated to a continuous generation of reactive oxygen species as by-products of O_2 reduction [15, 16]. It was also believed that these processes were essentially irreversible and could only be slowed down by appropriate interventions, particularly calorie restriction [17]. This framework of thought began to be challenged when animal cloning was achieved. If an adult somatic nucleus had even a small proportion of its genome inactivated by cumulative DNA damage as the above theory predicts, then it should be unable to give rise to a whole new organism after somatic cell nuclear transfer what obviously is not the case. The advent of iPSC technology further challenged the idea that cumulative DNA damage was the primary driver of aging. In light of a growing body of evidence showing that reprogramming of somatic cells from aged animals and humans rejuvenates them to their embryonic stage, the emerging view is that aging is a reversible epigenetic process [18].

Studies in model organisms such as flies, worms, and yeasts have shown that aging is associated with gradual changes in chromatin structure and regulation. They will be briefly reviewed below.

Nucleosomal histones are subject to a wide variety of post-translational modifications. Each modification, either alone or in combination with others, enables regulation of the underlying DNA sequence. Among the histone modifications that are known to affect longevity, the most prominent ones are acetylation and methylation of lysine residues. Thus, acetylation at the N-terminal tail of histone H4 on lysine 16 (H4K16Ac) and acetylation present on the globular domain of histone H3 on lysine 56 (H3K56Ac) both influence replicative aging [19, 20]. Levels of H3K56Ac decrease during yeast aging [19, 20]. The trends in changed levels of histone methylation during aging have been extensively examined in recent years. Significant changes during aging have been documented for trimethylation marks on lysines 4, 9, 27, and 36 of histone H3, which are associated with a loss of heterochromatin structure. In parallel, reduction in heterochromatin protein 1 levels was evident during aging in whole flies, with a decreased level of histone H3 dimethylated at lysine 9 (H3K9me2) and altered levels of histone H3 trimethylated at lysine 4 (H3K4me3) [21]. Histone 3 trimethylated at lysine 36 (H3K36me3) has been found to promote longevity. Thus, a histone mutation screen in *S. cerevisiae* has identified mutants with altered life span showing the relevance of H3 trimethylation at lysine 36 for life span [22]. From the extensive information available, we can summarize the relevance of histone acetylation and methylation during aging as follows.

In young cells, the genome is in a relatively high state of repression effected in part by DNA methylation (particularly at promoters) and relatively high levels of histone H3 trimethylated at lysine 27 (H3K27me3) and at lysine 9 (H3K9me3) and also histone H4 trimethylated at lysine 20 (H4K20me3), all of which are associated to transcriptionally repressed chromatin, as well as low levels of H3K4me3 and H4K16ac, which are both associated with active chromatin. Aging seems to be characterized by a progressive derepression of the transcriptional activity of chromatin, which is in part the result of reduction in DNA methylation (although in specific gene groups DNA becomes hypermethylated), decrease of epigenetic repressor marks such as H3K9me3, H3K27me3, and H4K20me3 as well as an increase in activation marks such as H3K4me3 and H4K16Ac. Acetylation and methylation of histones 3 and 4 is specifically done by a number of protein groups associated with chromatin, such as the his-

tone demethylases, histone methyl transferases, and histone deacetylases (which include sirtuins), the polycomb complex, the trithorax group, and other protein complexes. A detailed description of the epigenetic mechanisms and enzyme systems involved in the regulation of chromatin is beyond the scope of this article and the reader is referred to a few comprehensive reviews [21, 23–25]. The epigenetic marks are influenced by both endogenous and exogenous environmental factors (e.g., hormones and nutrition, respectively) [25].

DNA methylation has been extensively studied and is one of the best characterized epigenetic modifications during aging [26]. In young cells, the majority of CpGs within the genome have cytosine methylation. It should be mentioned that CpG methylation within promoters induces transcriptional repression, via the formation of compact chromatin structures, such as heterochromatin. Conversely, promoters of genes that are highly expressed are devoid of DNA methylation, hence their name, CpG islands. Almost invariably, mammalian aging is more commonly associated with CpG hypomethylation, particularly at repetitive DNA sequences [27–31]. This is likely to be at least partly responsible for the loss of heterochromatin during aging. In fact, one of the earlier proposed models of aging was the "heterochromatin loss model of aging". This model suggests that the loss of heterochromatin that accompanies aging leads to changes in global nuclear architecture and the expression of genes residing in those regions, directly or indirectly causing aging and cellular senescence. Besides heterochromatin loss, aging is associated with a global loss of core histone proteins from the genome, which has been observed in multiple scenarios, and this has been shown to be a cause of aging in yeast. Thus, in budding yeast, replicative aging is accompanied by loss of approximately half of the core histone proteins [19, 20].

Nucleosomal remodeling is also an important factor during aging. Although nucleosome structure is rather rigid, cells have evolved complex machineries to enable specific nucleosomes at precise genomic locations to become very dynamic, as required, to facilitate different genomic processes. This regulated alteration of nucleosome architecture during different biological events is mediated mainly by adenosine 5′-triphosphate-dependent nucleosome remodeling complexes [32].

Noncoding RNAs (ncRNAs) are the most recent players in the epigenetics field, influencing seemingly all biological processes in virtually all organisms. The occurrence of ncRNAs has been highly conserved through evolution; even budding yeast is now appreciated to have many ncRNAs. ncRNA transcription from the ribosomal DNA (rDNA) locus, which is silent under normal conditions, serves as a life span determinant of this unicellular organism through its function to regulate rDNA stability [33]. Micro RNAs (miRNAs) are short ncRNAs that negatively control their target gene expression post-transcriptionally and have been implicated in aging. Despite the fact that miRNAs do not modify chromatin structure, these molecules are considered mediators of epigenetics because they lead to heritable changes in gene expression without altering DNA sequence. The best-characterized examples of roles of miRNAs during aging come from studies in *Caenorhabditis elegans*. For a comprehensive review on chromatin and epigenetic changes during aging, see Pal and Tyler [34].

Focusing back on rejuvenation, let's begin by stating that a core of recent studies strongly suggests that when somatic cells from old animals or humans are reprogrammed to the iPSC stage, all epigenetic marks of aging are erased. Moreover, if iPSCs derived from old individuals are redifferentiated to the cell type of origin, the rejuvenated cells show structural and functional features of young wild-type counterparts. Thus, it has been reported that reprogramming skin fibroblasts from aged humans to the iPSC stage and differentiating them back to induced neurons (iNs) rejuvenates their transcriptomal profile and their nucleocytoplasmic compartmentalization to that of wild-type fibroblasts from young donors [35]. iNs were also generated by transdifferentiation, a procedure that bypasses the pluripotency stage. Transdifferentiated iNs retain the transcriptome signature of old fibroblasts and show a disrupted nucleocytoplasmic compartmentalization as old fibroblasts do [35]. The authors conclude that dedifferentiation to the iPSC stage is necessary to fully erase all epigenomic marks of aging. In another study in healthy humans, skin fibroblasts from centenarian and old donors were dedifferentiated to the iPSC stage and then redifferentiated into fibroblasts. In the rejuvenated fibroblasts, the authors found that telomere length was restored and that oxidative stress levels, gene expression profiles, and mitochondrial metabolism was comparable to that of fibroblasts from young individuals [36]. In an interesting study with skin fibroblasts from healthy aged individuals, it was observed that the lower oxygen consumption typically observed in the mitochondria of cells from old individuals was restored to youthful levels after the old cells were dedifferentiated to iPSCs and subsequently differentiated back to rejuvenated fibroblasts. The DNA damage in mitochondrial DNA was comparable in fibroblasts from young donors and rejuve-

nated fibroblasts from old individuals. It was also found that in the old fibroblasts, there was no detectable damage to the nuclear *GCAT* gene, which codes for glycine C-acetyltransferase, an enzyme involved in glycine synthesis. Rather, the gene was downregulated in old cells. Rejuvenation reversed this repression and restored normal glycine levels in the mitochondria of rejuvenated fibroblasts [37].

In an interesting study, Nishimura et al. [38] reprogrammed clonally expanded antigen-specific CD8+ T cells from an HIV-1-infected patient to pluripotency. The T cell-derived iPSCs were then redifferentiated into CD8+ T cells that had a high proliferative capacity and elongated telomeres. These rejuvenated cells possessed antigen-specific killing activity and exhibited T-cell receptor gene-rearrangement patterns identical to those of the original T-cell clone from the patient, thus demonstrating that cell reprogramming does not reverse rearrangements (or damage) in DNA sequences.

More recently, rejuvenation of dysfunctional hematopoietic stem cells (HSCs) from old mice was achieved by genetically barcoding them and reprogramming to the iPSC stage. These old mouse-derived iPSCs were redifferentiated in vivo by implanting them in the blastocyst of normal mice possessing different genetic markers, thus obtaining chimeric mice. The bone marrow of these chimeras had HSCs from the host and from iPSCs derived from old rats. Their genetic barcode labels allowed identification of the HSCs coming from the old mice. When bone marrow from these chimeras was implanted in lethally irradiated mice, the chimera HSCs colonized the immune system of the irradiated mice. It was found that the capacity of both HSCs form the normal young mice that provided the host blastocysts and HSCs derived from iPSCs generated from the old mice had the same functional performance concerning production of different immune and erythroid cell lineages (peripheral B-, T- and granulocyte/myeloid cells, as well as bone marrow erythroid progenitors). Additional characterization confirmed that old HSCs converted to iPSCs and redifferentiated back to HSCs had been fully rejuvenated [39].

Another study reported that overexpression of the pluripotency factor NANOG in progeroid senescent or myogenic progenitors reversed cellular aging and fully restored their ability to generate contractile force. This was mediated by the reactivation of the ROCK and TGF-β pathways [40].

The epigenetic model of aging provides an elegant explanation as to why complex animal life has remained viable over hundreds of million years. According to the accumulating DNA damage theories of aging cells, even germ cells, are likely to sustain some accumulation of DNA damage despite the fact that DNA is particularly well-protected in this type of cells. Therefore, if one takes into account that complex animal life emerged during the era of life evolution known as the Cambrian Explosion, some 550 million years ago [41], the hundreds of thousand generations of germ cells that took place through the successive millennia during which many complex animal species thrived and evolved, should have accumulated increasingly large amounts of DNA damage causing complex animal species to progressively weaken and eventually become extinct, what obviously has not been the case as otherwise complex life would no longer exist on our planet. If, on the other hand, we assume that at the time of fertilization, all epigenetic marks of aging are erased, the resulting zygote becomes vital and young again, thus allowing that complex animal species flourish and diversify.

Although early animal subcloning approaches failed to achieve more than 2 or 3 generations [42–45], when a histone deacetylase inhibitor was included in the cloning protocol, mice could be serially recloned for at least 25 generations without showing any symptoms of disease or degeneration. In all 25 generations, mice displayed a life span comparable to wild-type counterparts, and the recloned female mice showed preserved fertility [46]. This achievement strengthens the robustness of the above hypothesis.

Rejuvenation by cell reprogramming is one of the new frontiers opened by iPSC technology. Interestingly, rejuvenation by partial reprogramming has recently been achieved in whole animals. In effect, a highly promising report has recently been published [47], showing that partial cyclic reprogramming in transgenic progeric mice carrying a Tet-On regulatable cassette harboring the 4 Yamanaka pluripotency genes whose expression is activated in cycles consisting of a 2-day period of transgene overexpression (effected by administration of doxycycline via the drinking water) followed by a 5-day period or repression (doxycycline removal from the drinking water) can prolong their survival time by 30%. Analysis of internal organs revealed that many signs of senescence disappeared or were attenuated in adult (senile) mice after a few cycles of partial reprogramming [47]. An important implication of this study is that epigenetic aging marks can be erased, sparing the differentiation marks, which, in turn, suggests that both types of epigenetic marks are not necessarily the same. To our knowledge, this is the first report documenting that in vivo rejuvena-

tion can be achieved by partial reprogramming. It is likely that follow-up in vivo studies trying to achieve in vivo rejuvenation by partial cell reprogramming will bloom during the coming years. Although the above report provided a strong proof of concept that in vivo rejuvenation by partial cell reprogramming is achievable, transgenic mice were used, a procedure that is not applicable to humans. A possible alternative to achieve cell reprogramming in vivo in nontransgenic individuals comes from the evidence that reprogramming occurs in vivo. In effect, many organs, such as the pancreas, liver, and kidney, are permissive to in vivo reprogramming [48, 49]. Recent studies have demonstrated that damage-induced cellular senescence, via the senescence-associated secretory phenotype (SASP), plays a key role in tissue remodeling [50]. The effect of senescence appears to be, at least in part, due to the release of interleukin 6, suggesting a potential link with the SASP. Chiche et al. [51] demonstrated that in vivo reprogramming only occurs in muscle (a tissue refractory to in vivo reprogramming) during regeneration, indicating that senescence could facilitate cellular plasticity, which is mainly mediated by the SASP, in part via interleukin 6.

Therefore, considering the ever-accelerating pace of cell reprogramming research and the emergence of powerful technologies such as genome and epigenome editing [52], there is a clear promise that in the not-too-distant future, reprogramming-based medical technologies will be developed not only to treat so far incurable age-related diseases but also to reverse the functional declines that occur in humans during normal aging.

Concluding Remarks

The advent of animal cloning and iPSC technology has allowed scientists to gain new insights into the mechanism of aging. The observation that implanting the nucleus of a somatic cell from an adult animal into an enucleated oocyte can reprogram that nucleus so that it becomes able to give rise to a whole animal as well as the seminal studies of the Yamanaka group showing that a limited number of pluripotency factors can reprogram a somatic cell, taking it to the state of an embryonic cell, has two transcendental implications. First, that every cell harbors a genetic program able of resetting its genome so that it can become an iPSC, and second, that a limited number of master factors can set in motion that genetic program, apparently in most cell types. Some of these master factors are present in the cytoplasm of a mature oocyte, and as far as we know, nowhere else in an adult organism. This fact suggests that at the time of fertilization, the epigenetic aging clock of the new zygote is reset to zero. The epigenetic model of aging elegantly explains a number of observations difficult to account for by other theories of aging.

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