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Special Issue: Aging and Rejuvenation

Review

Anti-Aging Strategies Based on Cellular Reprogramming

Alejandro Ocampo,[1] Pradeep Reddy,[1] and Juan Carlos Izpisua Belmonte[1,*]

Aging can be defined as the progressive decline in the ability of a cell or organism to resist stress and disease. Recent advances in cellular reprogramming technologies have enabled detailed analyses of the aging process, often involving cell types derived from aged individuals, or patients with premature aging syndromes. In this review we discuss how cellular reprogramming allows the recapitulation of aging in a dish, describing novel experimental approaches to investigate the aging process. Finally, we explore the role of epigenetic dysregulation as a driver of aging, discussing how epigenetic reprogramming may be harnessed to ameliorate aging hallmarks, both *in vitro* and *in vivo*. A better understanding of the reprogramming process may indeed assist the development of novel therapeutic strategies to extend a healthy lifespan.

Aging Research in the Cellular Reprogramming Era

Aging represents the major risk factor for most human diseases and can be defined as the progressive decline in the ability of a cell or organism to resist stress, damage, and disease [1]. Molecular and cellular hallmarks of aging are shared among organisms and include, for example, genomic instability, telomere attrition, mitochondrial dysfunction, epigenetic alterations, and **stem cell exhaustion** (see Glossary) [2]. Although aging is generally considered to be a unidirectional and inevitable process that affects all forms of life, recent studies have revealed a great degree of plasticity in cellular and organismal aging. First, genetic dissection of the aging process has revealed that manipulation of specific signaling pathways (e.g., insulin/IGF-1, mTOR, AMPK, and sirtuins) and environmental interventions (e.g., caloric restriction) are able to modulate the aging process in multiple organisms [3–5]. Second, experiments connecting the circulatory systems of two organisms of different ages (i.e., **heterochronic parabiosis** experiments) have elegantly demonstrated that exposing old tissues and organs to a young circulatory environment can **rejuvenate** tissue-specific **stem cells**, leading to a youthful state, which is characterized by functional and regenerative improvements [6,7] (Box 1). This form of aging plasticity has been observed in multiple tissues, including skin, liver, muscle, and bone marrow, and involves the modulation of Notch, Wnt, and TGF-β signaling pathways by young circulatory signals [8–10]. In fact, a clinical trial has been initiated at Stanford University to test the effects of young plasma on patients with Alzheimer's disease (Clinical Trial Identifier NCT02256306). The identification of circulatory signals that rejuvenate aging phenotypes is currently the focus of multiple laboratories around the world and, despite this challenge, several candidate molecules have been proposed, such as growth differentiation factor 11 (GDF11) [11–13]. However, contradictory observations have been recently made by independent groups regarding the potential of GDF11 as anti-aging molecule, indicating that further studies will be necessary to continue this quest [14,15].

Trends

Multiple experimental approaches are being developed as potential anti-aging therapies. These include the modulation of metabolic signaling pathways through small molecules, the rejuvenation of stem cells, and the elimination of senescent cells accumulating during aging.

Epigenetic changes observed during aging are being currently studied as active drivers of the aging process and are proposed as novel 'biomarkers' of biological aging.

The discovery of cellular reprogramming through forced expression of Yamanaka transcription factors has facilitated the generation of induced pluripotent stem cells (iPSCs) from patient somatic cells. Patient specific iPSCs from a wide range of diseases are being used as disease models and platforms for drug screening.

Cellular reprogramming has demonstrated that multiple aging hallmarks can be reversed to younger states, highlighting the plasticity of aging.

In vivo cellular reprogramming to pluripotency has been achieved in multiple mouse tissues including stomach, intestine, pancreas, and kidney, and is currently being studied as a potential strategy to induce *in vivo* tissue regeneration.

[1]Gene Expression Laboratory, Salk Institute for Biological Studies, La Jolla, CA 92037, USA

*Corresponding author.
belmonte@salk.edu (J.C.I. Belmonte).

Box 1. Clinician's Corner

Aging is a major socioeconomic problem for modern societies and represents the major risk factor for most human pathologies including cardiovascular diseases, neurological disorders, and cancer. Medical treatment to ameliorate the aging process will have a significant impact on human disease.

Classical anti-aging strategies recommended to patients to achieve healthy aging include the intake of a nutritionally balanced diet and regular physical exercise.

Caloric restriction, defined as a reduction in calorie intake without malnutrition, has been demonstrated to ameliorate the effects of aging and extend lifespan in multiple species. In humans, caloric restriction lowers the risk of multiple degenerative conditions and improves multiple medical markers of health.

Currently, a series of novel drugs (i.e., rapamycin, metformin, and resveratrol) as well as stem cell treatments and reprogramming strategies are being evaluated for their potential anti-aging/rejuvenating effects.

Lastly, **cellular reprogramming** has been shown to dramatically affect the aging process. Cellular reprogramming has enabled a new era of regenerative medicine, allowing the conversion of terminally differentiated somatic cells into **pluripotent cells** via **somatic cell nuclear transfer** (SCNT) (i.e., the transfer of the nucleus of an adult somatic cell into an enucleated oocytes), or forced expression of **Yamanaka factors** (Oct4, Sox2, Klf4, and c-Myc) [16,17]. In addition to altering cell fate, cellular reprogramming has the capacity to reverse cellular phenotypes associated with the aging process [18]. This has been demonstrated in reprogrammed cells taken from individuals of advanced age, as well as by using cells derived from patients suffering from premature aging syndromes. These experiments have revealed that aged cells must undergo a process of cellular rejuvenation to reach a pluripotent state where they can indefinitely self-renew. These experiments also suggest that cellular reprogramming may have a therapeutic application, and could be used to slow down or even reverse some features of the aging process. In this article we explain the biological basis for cellular rejuvenation through reprogramming, and analyze initial studies describing this phenomenon. Lastly, we discuss the potential of using reprogramming to alter the aging process at the organismal level. In summary, recent knowledge has been gained on the molecular mechanisms of aging and novel reprogramming technologies are being developed to allow the artificial manipulation of cellular epigenetic programs. As a result, novel anti-aging strategies are emerging, aiming to slow down the aging process and rejuvenate old organisms.

Epigenetic Regulation of Aging

Epigenetics can be defined as changes in gene expression that do not involve changes in DNA sequence. Epigenetic regulation is orchestrated by a series of enzymes that chemically modify DNA and histones to regulate gene expression. These modifications include DNA methylation, histone methylation, and histone acetylation. Epigenetic dysregulation is a hallmark of aging and seems be a major driver of the aging process [19,20]. A variety of epigenetic alterations have been observed during aging in multiple organisms (e.g., worms, flies, mice, and humans), including changes in DNA methylation, post-translational modification of histones, and chromatin remodeling [21]. More specifically, studies in model organisms such as yeast, worms and flies have described increases in the levels of histone H3 lysine 4 (H3K4) trimethylation and H4K16 acetylation with aging, which are all associated with active chromatin, together with reductions in the levels of H3K9 trimethylation and H3K27 trimethylation, and increased H4K20 trimethylation, associated with transcriptional repression [20,22]. Collectively, these changes result in a more active chromatin state, leading to more relaxed global gene expression. On the one hand, while a global decrease in DNA methylation is observed during aging; on the other, analyses of multiple loci have revealed an increase in DNA methylation in specific regions of the genome. Although the role of DNA methylation as a 'driver' of aging remains under investigation, its analysis on specific CpG sites has allowed the establishment of an 'epigenetic clock', considered by many as one of the best biomarkers or predictors of biological age [23].

Glossary

Alopecia: condition that leads to the loss of hair.
Cellular reprogramming: conversion of a specific cell type into another.
Cellular senescence: inability of a cell to progress through the cell cycle and proliferate as a consequence of multiple mechanisms, including telomere shortening, epigenetic de-repression, and DNA damage.
Dedifferentiation: regression of a differentiated cell into a less specialized and 'simpler' cell type.
Directed differentiation: reprogramming of one cell type to another without passing through a pluripotent state.
Embryonic stem cells (ESCs): pluripotent stem cells derived from the inner cell mass of a blastocyst.
Epigenome: the collection of chemical modifications on chromatin and DNA that regulate gene expression.
Heterochronic parabiosis: surgical joining of the circulatory systems of two animals of different ages.
Induced pluripotent stem cells (iPSCs): specific type of pluripotent stem cells generated from an adult somatic cell by forced expression of transcription factors.
Lipodystrophy: abnormal or degenerative condition of adipose tissue in the body.
Mesenchymal-to-epithelial transition: conversion of mesenchymal cells to epithelial cells.
Nanog: transcription factor that is required for maintenance of stem cells in a pluripotent state and is used as a marker of pluripotency.
Osteolysis: condition that leads to the loss of bone tissue.
Pluripotent cells: cells with the capacity to differentiate into all cell types of an organism.
Rejuvenation: restoration of cellular or organismal aging hallmarks to a younger state.
Somatic cell nuclear transfer (SCNT): technique that allows the transfer of the nucleus taken from a somatic cell into an enucleated oocyte.
Stem cell exhaustion: decreased capacity of stem cells to self-renew and maintain tissues/organs.
Stem cells: undifferentiated cells that can self-renew and differentiate into other cell types.

Age-associated changes in the coordinated expression and activity of enzymes involved in epigenetic regulation, metabolism, and mitochondrial function are thought to underlie the epigenetic dysregulation observed during aging. These changes are observed both during the physiological aging of model organisms and in samples from patients with premature aging syndromes [24,25]. As with all hallmarks of aging, however, it is important to determine whether these epigenetic changes are key drivers of the aging process or instead are molecular consequences of aging. To this end, studies have shown that the elimination of histone methylation complexes (e.g., ASH-2; Trithorax complex and Polycomb repressive complex 2, PRC2) responsible for the maintenance of H3K4me3 extends the lifespan of worms and flies [26], whereas elimination of H3K4me3 demethylases reduces lifespan in these same model organisms. For example, overexpression of H3K4me3 demethylases extends the worm lifespan [26]. In yeast, overexpression of Sir2, a conserved NAD^+-dependent histone deacetylase of the sirtuin family, results in an increase in H4K16ac, leading to lifespan extension [27]. Importantly, promoting cellular rejuvenation through epigenetic remodeling may represent a more feasible therapeutic strategy against aging than other molecular approaches, including the repair of specific DNA mutations with an unknown role in aging that accumulate during a lifetime. As an example, modification of epigenetic marks by administering histone deacetylase inhibitors, acetyltransferase inhibitors, or sirtuin activators [e.g., sodium butyrate (NaB) and suberoylanilide hydroxamic acid (SAHA)] ameliorates aging phenotypes in mice such as age-dependent memory impairment and, in some instances, extends their lifespan [28,29].

Trophectoderm lineage: single layer of cells surrounding the inner cell mass of a blastocyst that gives rise to the extraembryonic tissues of an embryo.

Yamanaka factors: Oct4, Sox2, Klf4 and c-Myc are collectively called Yamanaka factors and are specifically used for the conversion of differentiated somatic cells into pluripotent cells.

Lastly, it is important to appreciate that young and old cells share the same genome; however, gene expression within these cells is differentially regulated by their **epigenomes**. In this sense, cellular damage (e.g., genome instability, telomere shortening and mitochondrial dysfunction) resulting from the interactions between the environment and an organism can drive epigenomic changes ultimately leading to aging phenotypes (Figure 1). Aging plasticity observed during heterochronic parabiosis, caloric restriction, or cellular reprogramming seems to rely on reprogramming the epigenome from an older to a younger state. Importantly, aging hallmarks such as telomere attrition, **cellular senescence**, stem cell exhaustion, and mitochondrial dysfunction are in some instances regulated through epigenetic mechanisms [30,31]. During cellular reprogramming, as we discuss below, or through transitioning between organismal generations, these aging hallmarks are restored to a youthful state in a process primarily driven by epigenetic remodeling. Thus, epigenetic reprogramming may represent an effective strategy for developing anti-aging therapies.

Models of Premature Aging Based on Cellular Reprogramming: Progeroid Syndromes

The discovery that cellular fate can be reverted back to pluripotency represents one of the greatest scientific breakthroughs of recent decades. Cellular reprogramming to pluripotency by somatic cell nuclear transfer (SCNT), or by the forced expression of Yamanaka transcription factors has revealed that cell fate is malleable and that cellular differentiation is a bidirectional process (Figure 2A, Key Figure) [16,17]. In addition to these insights regarding the plasticity of cell fate, cellular reprogramming experiments have also shown that aging phenotypes in mouse and human cells can be reversed [18]. Although additional reprogramming factors are sometimes needed (e.g., **Nanog** and Lin28), fibroblasts from centenarians and even senescent fibroblasts can be reprogrammed to pluripotency [32]. Importantly, when **induced pluripotent stem cells** (iPSCs) are generated from fibroblasts from an aged individual, the induction process lowers the levels of $p16^{INK4A}$ and $p21^{CIP1}$ (which regulate cellular senescence), restores telomere length, and resets the epigenome including histone and DNA methylation marks ('epigenetic clock') to a younger state (Figure 2B) [23,32–34].

Additional characteristics of aged cells such as mitochondrial dysfunction and the associated generation of reactive oxygen species (ROS), which are both thought to play crucial roles in

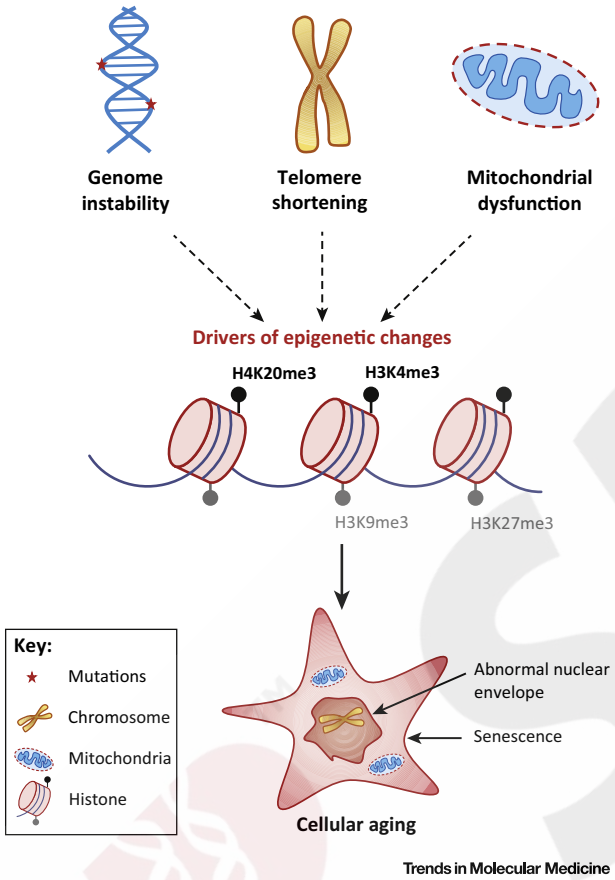

Figure 1. Aging Hallmarks as Drivers of Epigenetic Dysregulation. During aging, an increase in genome instability, shortening of telomere length, and mitochondrial dysfunction are observed, leading to changes in the epigenome and ultimately resulting in cellular aging.

aging, are also restored to a rejuvenated state upon cellular reprogramming in fibroblasts from aged individuals [32,35,36]. Importantly, reprogramming-mediated rejuvenation of aging hallmarks (e.g., telomere length, mitochondrial function, and ROS levels) has been shown to be maintained both indefinitely in pluripotent cells, and transiently in their differentiated progeny [32]. That is, the differentiation of rejuvenated iPSCs into somatic cells such as fibroblasts can lead to the generation of young cells with rejuvenated phenotypes compared to the original samples (Figure 2B) [32,35,37]. Nevertheless, despite the rejuvenation of these aging hallmarks, the accumulation of DNA damage and mutations in mitochondrial DNA cannot be erased during cellular reprogramming [18]. Moreover, cellular reprogramming or long-term cultures of iPSCs have been shown to potentially result in genomic instability [18]. Lastly, the complete rejuvenation of additional aging hallmarks, such as loss of proteostasis (processes regulating protein synthesis, folding, and degradation) and the accumulation of misfolded protein aggregates, during cellular reprogramming remains under investigation [18]. In addition, in recent years, we and others have demonstrated that somatic cells from patients with premature aging syndromes (Box 2) can be rejuvenated via reprogramming (Figure 2B) [25,38].

Reprogramming of fibroblasts from Hutchinson–Gilford progeria syndrome (HGPS) patients (Box 2) to pluripotency has been shown to prevent the accumulation of progerin, leading to normal nuclear envelope morphology, as well as the restoration of youth-associated epigenetic marks (Table 1) [25]. For instance, the levels of the heterochromatin mark H3K9me3 can be

Key Figure

Processes of Cellular Reprogramming to Pluripotency and Rejuvenation

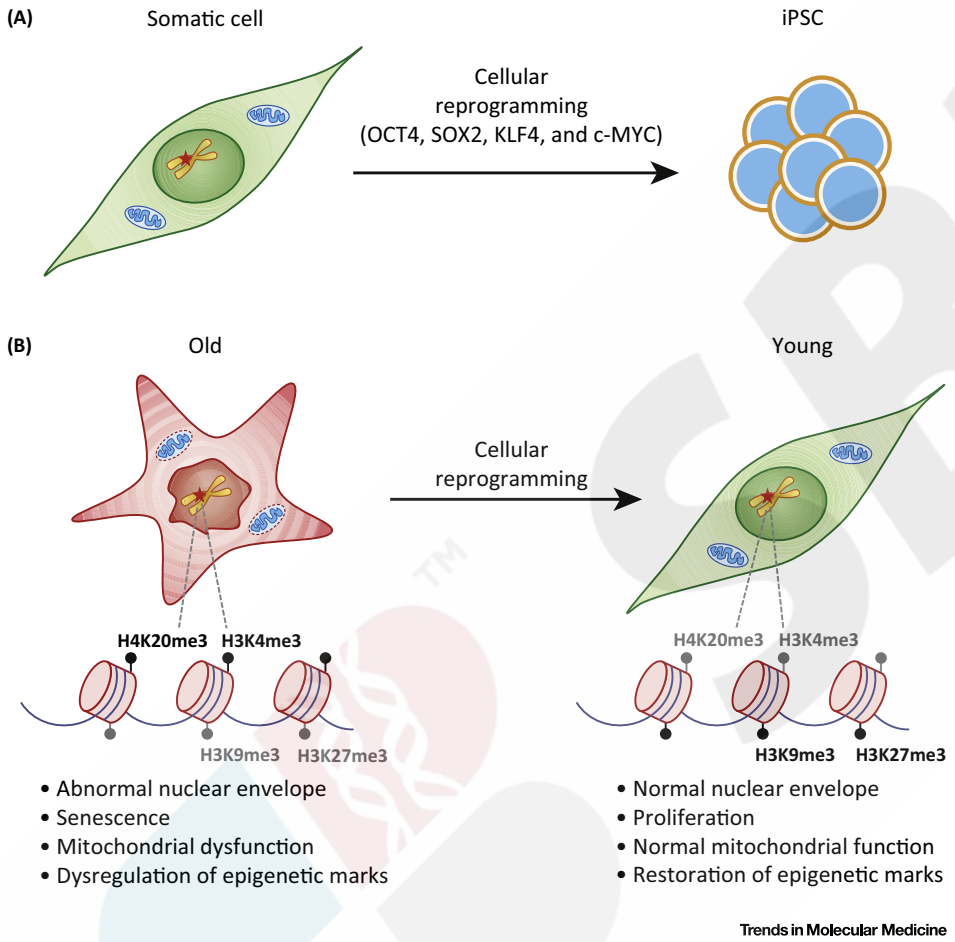

Figure 2. (A) The diagram depicts cellular reprogramming to pluripotency, in other words the conversion of terminally differentiated somatic cells into induced pluripotent stem cells (iPSCs) by cellular reprogramming through forced expression of Yamanaka factors (Oct4, Sox2, Klf4, and c-Myc). (B) The diagram depicts the rejuvenation of aged cells by cellular reprogramming. The process results in the amelioration of hallmarks of aging such as abnormal nuclear envelope, mitochondrial dysfunction, shortening of telomere length, changes in histone marks, increased DNA damage, and senescence.

restored to wild-type levels when HGPS fibroblasts are reprogrammed to pluripotency [25]. Importantly, when HGPS iPSCs are differentiated *in vitro* into vascular smooth muscle cells or mesenchymal stem cells (MSCs; cell types particularly affected in HGPS patients), premature aging phenotypes can be recapitulated (e.g., decreased proliferation, premature loss of H3K9me3, senescence-associated β-galactosidase activity, and decreased telomere length) [25]. This demonstrates that molecular changes associated with aging can be modeled *in vitro* using stem cell models of premature aging.

A recent study investigating Werner syndrome (WS) (Box 2) has shown that the degeneration of heterochromatin can drive human aging by generating WRN-null **embryonic stem cells** (ESCs)

Box 2. Human Premature Aging Syndromes

Hutchinson–Gilford Progeria Syndrome (HGPS)

A rare genetic disorder caused by a mutation in the lamin A/C (*LMNA*) gene. A G608G mutation creates a cryptic splice site, leading to a 50 amino acid deletion in the C-terminal region of the LMNA protein [59]. The lack of a proteolytic cleavage site within this region results in a permanently farnesylated pre-LMNA protein which cannot be removed from the nuclear rim [59]. The accumulation of abnormal pre-LMNA protein (progerin) perturbs nuclear morphology and affects several important nuclear functions including chromatin organization, epigenetic control, DNA replication, and mitogen-activated protein kinase (MAPK) signaling [60]. HGPS cells exhibit most features of normal physiological aging, including an abnormal nuclear envelope, telomere shortening, increased DNA damage, and early senescence [61]. HGPS patients show premature aging symptoms such as growth failure, atherosclerosis, cardiovascular problems, loss of body fat, and early death [62]. Mouse models of HGPS (*Zmpste24* knockout or $Lmna^{G609G}$) recapitulate the aging features observed in HGPS patients [63,64]. These models revealed certain molecular and physiological defects caused by the accumulation of progerin, suggesting a direction for developing therapeutic strategies to ameliorate aging phenotypes in HGPS.

Werner Syndrome (WS)

A rare recessive condition caused by genetic mutations in WS syndrome RecQ-like helicase (*WRN*), encoding an essential protein for DNA replication, transcription, repair, and recombination, as well as for the maintenance of telomere length [65]. The loss of WRN causes genomic instability, increased DNA damage, and loss of p53 function, resulting in premature cellular aging. Patients with WS syndrome present short stature, premature grey hair and aged face, wrinkles, and **lipodystrophy** [66]. The pathology causes the emergence of aging symptoms during early adulthood. WS is also known as adult progeria.

Dyskeratosis Congenita (DC)

A disease caused by mutations in multiple genes, including *DKC1*, *TERC*, *TERT*, *TCAB1*, or *CTC1*, that lead to shortening of telomeres and accelerated cellular senescence [67]. This genetic disorder leads to the inability of bone marrow cells to produce fewer blood cells. DC patients present a wide range of clinical phenotypes including abnormal skin pigmentation, cancer predisposition, hepatic fibrosis, premature greying of the hair, osteoporosis, testicular atrophy, nail dystrophy, and bone-marrow failure [68].

Nestor–Guillermo Progeria Syndrome (NGPS)

A recently identified progeroid syndrome caused by mutations in *BANF1* (barrier to autointegration factor 1), a gene encoding a member of the BAF family of highly conserved DNA-bridging proteins with roles in nuclear assembly and chromatin organization [69,70]. NGPS patients present an aged appearance with growth retardation, decreased subcutaneous fat, **osteolysis**, and **alopecia**. Unlike HGPS patients, no signs of cardiovascular impairment are observed until early adulthood. Fibroblasts from NGPS patients exhibit reduced levels of BANF1, abnormal nuclear morphology, and an altered distribution of emerin, a member of the nuclear lamina-associated protein family, important for nuclear membrane attachment to the cytoskeleton [69].

(Table 1) [39]. Specifically, when human WRN-null ESCs were differentiated into MSCs, premature aging phenotypes were recapitulated (e.g., global loss of the repressive mark H3K9me3, with dramatic loss of H3K9me3 clusters in the sub-telomeric and sub-centromeric regions) [39]. These global epigenetic changes led to heterochromatin degeneration and an increase in transcription from these regions of the genome [39]. Moreover, using a similar approach, results showed that, upon deletion of SUV39H1, the major H3K9me3 histone methyltransferase in human ESCs, the differentiation of SUV39H1-null ESCs to MSCs *in vitro* led to decreased levels of H3K9me3 and, more importantly, to premature aging phenotypes that included cell growth defects and cellular senescence, as indicated by decreased proliferation rates and increased senescence-associated β-galactosidase activity. Thus, epigenetic dysregulation was found to drive human aging via altered levels of H3K9me3 in hESCs [39]. Other studies have reported that iPSCs derived from fibroblasts of patients with WS exhibited restored telomere lengths as well as downregulation of senescence-associated genes such as $p16^{INK4A}$, $p21^{CIP1}$, and *IL6* compared to WS fibroblasts [40,41]. In addition, WS syndrome-derived iPSCs displayed normal proliferation, genomic stability, and differentiation capacity after being cultured for over 2 years.

Table 1. iPSC models of Premature Aging Syndromes and Age-Related Diseases[a]

Disease	Genetic Mutation Modeled from Patient Cells (Fibroblasts and Neurons)	Clinical Phenotypes	Refs
HGPS	*LMN* (G608G)	Growth failure Atherosclerosis Cardiovascular problems Loss of body fat Early death	[25,38,44]
WS	*WRN* (deletion) *WRN* (R368X/Q748X/F1074L) *WRN* (SNP at 5′ end of exon 26)	Short stature Premature grey hair Premature aged face, wrinkles Lipodystrophy	[39–41]
DC	*DKC1* (del-L37) *DKC1* (L54V/del-L37) *TCAB1* (H376Y/G435R) *TERT* (P704S/R979 W)	Abnormal skin pigmentation Cancer predisposition Hepatic fibrosis Premature greying of the hair Osteoporosis Testicular atrophy Bone-marrow failure	[42,43]
NGPS	*BANF1* (A12 T)	Growth retardation Decreased subcutaneous fat Osteolysis Alopecia	[44]

[a]Abbreviations: DC, dykeratosis congenital; HGPS, Hutchinson–Gilford progeria syndrome; NGPS, Nestor–Guillermo progeria syndrome; SNP, single-nucleotide polymorphism; WS, Werner syndrome.

Previous studies from two independent groups have also described the generation of iPSCs from dyskeratosis congenita (DC) patients (Box 2 and Table 1). One of the studies found that reprogramming DKC1 del-L37 patient mutant fibroblasts *in vitro* induced telomere extension through upregulation of telomerase RNA (TR) [42]. By contrast, the second study found that the molecular defects observed in patient fibroblasts carrying the *TCAB1* (H376Y/G435R), *TERT* (P704S/R979 W), or *DKC1* (L54 V/del37L) mutations were still present in DC-hiPSCs [43]. These defects included diminished telomeres and reduced telomerase activity [43]. The discrepancy between these two studies could be attributed to cell-to-cell variability in telomerase activity and also clonal differences during reprogramming to iPSCs.

With regard to Nestor–Guillermo progeria syndrome (NGPS) (Box 2), reprogramming of NGPS fibroblasts has been shown to lead to the generation of NGPS iPSCs that, although obtained with low efficiency, are morphologically indistinguishable from controls, and do not display alterations in either nuclear envelope structure or epigenetic marks (Table 1) [44]. Similarly to HGPS- and WS-derived iPSCs, the differentiation of NGPS iPSCs to MSCs recapitulates premature aging phenotypes, including increased senescence-associated β-galactosidase activity, expression of $p16^{INK4A}$, and decreased lamin B1 levels, as well as impaired differentiation into cartilage and adipose tissue [44].

Collectively, these studies have independently verified that the process of reprogramming cells from aged individuals or from patients with premature aging is able to suppress various molecular phenotypes associated with aging (i.e., rejuvenation) and, furthermore, that this youthful state is maintained in pluripotent cells and their derivatives.

Cellular Rejuvenation by Partial Reprogramming

Because epigenetic dysregulation is a main driver of the aging process, it is logical to hypothesize that epigenetic reprogramming may be an effective strategy for manipulating the aging process. At the organismal level, sexual reproduction demonstrates the plasticity of aging. Indeed, during fertilization the chronological age of the two germ cells involved is reset to 'zero', resulting in the generation of an organism with a normal lifespan. Resetting the aging clock allows each generation to begin life fully rejuvenated, thereby preventing species extinction. Similarly, cloning experiments based on SCNT have used cells from old mice to create an animal with a normal lifespan, demonstrating once again the bidirectionality of the aging process [45]. In addition, and as described in the previous section, reprogramming cells from an aged individual, or from a patient with a premature aging syndrome, provides additional examples of the capacity of epigenetic reprogramming to alter aging-associated phenotypes and restore cells to a younger state (Table 1) [18,46].

It is important to understand that, independently of the different methods described to date, cellular reprogramming through forced expression of the Yamanaka factors occurs through major epigenetic remodeling (Table 2). In recent years, several independent reports have described molecular mechanisms of cellular reprogramming at single-cell or bulk-cell population levels [47–49]. According to these reports, two distinct transcriptional waves or phases can be distinguished during cellular reprogramming: the first phase is described as the stochastic or probabilistic phase, characterized by the differential expression of genes involved in the cell cycle (e.g., *Ccnb1* and *Cdkn2b*), a **mesenchymal-to-epithelial transition** (e.g., *Snai1* and *Cdh1*), as well as downregulation of genes associated with cell adhesion and differentiation (e.g., *Col1a1, Fbln5*, and *Mmp14*) [48]. These initial changes are followed by a second phase, the deterministic or hierarchical phase, characterized by progressive activation of markers of pluripotency (e.g., *Nanog, Oct4, Sox2*, and *Dnmt3L*) [48]. Importantly, major epigenetic remodeling is observed during both phases of cellular reprogramming. For instance, changes in active and repressive histone marks such as H3K4me3 and H3K27me3 respectively, are detected in the first phase; changes in microRNA (e.g., let-7, miR34c, miR-294 and miR-106a) expression are observed during both phases; lastly, alterations in DNA methylation in genes such as *Nanog, Oct4* and *Rex1* occur during late stages of the process. These observations are important in that they may help us understand the capacity as well as the nuances of reprogramming cells to manipulate the aging process, which in our view is mainly driven by epigenetic dysregulation.

Based on the fact that reprogramming is a progressive process, rejuvenation of cells during cellular reprogramming may occur through two theoretical modes. One possibility is that cells undergo progressive and continuous rejuvenation as the epigenome is remodeled during the reprogramming process. Alternatively, rejuvenation may occur all at once upon reaching a final pluripotent stage. It may also be possible that some aging hallmarks are rejuvenated during reprogramming, whereas others are only restored to a youthful state once cells have reached pluripotency. At the moment, experimental data that would allow us to distinguish between these possibilities are lacking. As an example, although telomere length is elongated upon reprogramming and *in vitro* culturing of iPSCs, specific details about the timing of telomerase re-expression and the dynamics of telomere elongation during reprogramming are still missing [34]. Nevertheless, a progressive and continuous rejuvenation of aging hallmarks during cellular reprogramming would suggest that partial rejuvenation could be achieved by transition through an intermediate reprogramming state. Our laboratory has reported that brief exposure of cells to reprogramming factors allows their entrance into an intermediate state, characterized by partial **dedifferentiation**, that can be subsequently reversed by **directed differentiation** [50]. Using this approach, human fibroblasts have been successfully converted into angioblast-like progenitor cells that give rise to endothelial and smooth muscle lineages [50]. Therefore, partial reprogramming through brief or cyclic expression of reprogramming factors might allow the

Table 2. Current Methods of Cellular Reprogramming

Method	Cell Types	Advantages	Disadvantages	Refs
Type: Integrating				
Retroviral	Mouse and human fibroblasts, stomach cells, liver cells, keratinocytes, blood cells, adipose stromal cells	Good efficiency Easy to implement	Genomic integration Slow kinetics	[17,71,72]
Lentiviral	Mouse and human fibroblasts	High efficiency Applicable to dividing and non-dividing cells	Genomic integration	[73,74]
Inducible lentiviral	Mouse and human fibroblasts and human keratinocytes	Very high efficiency Controlled expression	Genomic integration	[75–78]
Type: Excisable				
Lentiviral floxed transgene	Human fibroblasts	Relatively good efficiency	Intense screening of excised lines	[79,80]
Transposon	Mouse and human fibroblasts	No genomic integration	Intense screening of excised lines	[81,82]
Type: Non-Integrating DNA-Based				
Adenoviral	Mouse fibroblast and hepatocytes and human fibroblasts	No genomic integration	Low efficiency Technically challenging	[83,84]
Episomal plasmids	Human and mouse fibroblasts	Low genomic integration	Low efficiency	[85,86]
Minicircles	Human adipose stem cells and fibroblasts	No footprint	Low efficiency	[87]
Type: Non-integrating DNA-Free				
Sendai virus	Human fibroblasts	No genomic integration Good efficiency Commercially available	Challenging viral production High commercial cost	[88]
Protein	Mouse and human fibroblasts	No footprint	Low efficiency Slow kinetics	[89,90]
Modified mRNA	Human fibroblasts	No footprint High efficiency Fast kinetics	Technically challenging High cost	[91]
microRNA	Mouse and human adipose stromal cells and fibroblasts	No footprint Fast kinetics	Low efficiency	[92,93]
Small molecules	Mouse embryonic fibroblasts	No footprint	Validated only in mouse Low efficiency	[58]

rejuvenation of particular aging hallmarks. In addition, partial reprogramming could induce cellular rejuvenation without loss of cellular identity and function. That is, partial dedifferentiation achieved through short expression of reprogramming factors may allow cells to maintain their identity and to spontaneously revert back to their terminally differentiated state after reprogramming. Thinking ahead, a separation between reprogramming of cellular identity and aging might be possible in the future if reprogramming factors are discovered which can specifically act on aging marks without affecting cellular identity. Of note, reprogramming of cellular identity without rejuvenation has been demonstrated by expressing factors that drive direct conversion of one

cell type to another (transdifferentiation). This strategy has been used to directly convert human fibroblasts into neurons by expressing neuronal transcription factors *Ngn2* and *Ascl1* and by applying small-molecule inhibitors targeting TGFβ/SMAD and GSK3β signaling, as well as enhancers of intracellular cAMP [51]. Modeling aging processes *in vitro*, induced neurons have thus been found to retain molecular signatures in the process, including the age-specific transcriptional profiles of a specific donor's age [51].

Implications for Regenerative Medicine: Successes and Limitations of *In Vivo* Reprogramming

Regenerative medicine is a multidisciplinary area of medicine that aims to maintain, improve, or restore cell, tissue, or organ function by using methods mainly related to cell therapy. At the moment, thousands of clinical trials are underway to test the safety and efficacy of cell therapies to treat numerous medical conditions. Most of these therapies involve adult stem cells [52]. Because age affects the quality and quantity of adult stem cells in an organism, *ex vivo* anti-aging strategies based on cellular rejuvenation could have an enormous impact on the outcomes of autologous cell therapies for patients of advanced age [53,54]. Alternatively, cellular reprogramming could be used *in vivo* to induce the rejuvenation of tissues and organs and to ameliorate age-associated phenotypes (Figure 3). Ultimately, *in vivo* rejuvenation through cellular reprogramming could dramatically extend organismal lifespan.

Interestingly, *in vivo* reprogramming by forced expression of Yamanaka factors has been recently demonstrated in mice [55]. Induction of Oct4, Sox2, Klf4, and c-Myc was documented to lead to the detection of Nanog-positive cells (i.e., pluripotent cells) in multiple tissues, including stomach, intestine, pancreas, and kidney [55]. This indicates that reprogramming to pluripotency can be achieved *in vivo*. There is also evidence from mouse bone marrow transplantation experiments and the detection of circulating iPSCs that the hematopoietic system may be reprogrammed *in vivo* [55]. Moreover, the authors demonstrated that mouse iPSCs generated *in vivo* could contribute to the **trophectoderm lineage** upon blastocyst injection, suggesting that iPSCs can drive cell differentiation and development, and that their pluripotency state is more primitive than ESCs, which cannot contribute to this lineage [55].

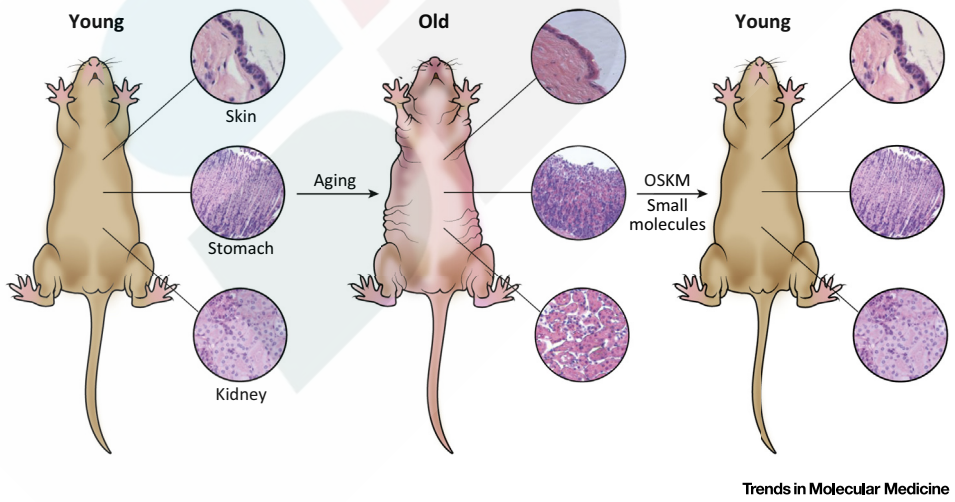

Figure 3. Amelioration of Aging Phenotypes in Old Mice by *In Vivo* Reprogramming. Distinct changes in tissues are observed during organismal aging, including loss of the epidermal layer in the skin, reduced number of parietal cells in stomach, and increased interstitial space in kidney. Owing to the driving role of epigenetic changes occurring during organismal aging, partial *in vivo* reprogramming of tissues using Yamanaka factors (OKSM: Oct4, Sox2, Klf4, and c-Myc) or small molecules without loss of cellular identity could ameliorate some of these aging hallmarks and restore tissue homeostasis.

Unfortunately, using Yamanaka factors to induce reprogramming *in vivo* is associated with high mortality in mice [55,56]. These animals often die as a result of teratoma development (cancer) in multiple tissues, but for others the cause of death is unclear. It is reasonable to hypothesize that loss of cellular identity upon dedifferentiation could impair organ or tissue functionality, resulting in organismal death. Although this result may represent a significant hurdle for rejuvenation strategies based on *in vivo* reprogramming, other strategies such as partial reprogramming or the reprogramming of specific organs or tissues might represent viable alternatives. In these cases, the extent or duration of partial reprogramming must be tightly regulated, and potentially adjusted for a specific target cell, tissue, or organ.

An important point to emphasize is that premature termination of reprogramming *in vivo* has been shown to lead to cancer development through altered epigenetic regulation [56]. For example, the induction of Yamanaka factors for only 4 days in mice was found to result in the proliferation of abnormal dysplastic cells in multiple tissues, including kidney, pancreas, and liver [56]. Moreover, the fate of these dysplastic cells depended on the duration of *in vivo* reprogramming. Following short-term (4 day) *in vivo* reprogramming, all dysplastic cells reverted to cells with normal morphologies, whereas this was not the case following a 7 day induction with Yamanaka factors, even if the expression of the latter had been terminated. This study also showed that, in the kidney, *in vivo* reprogramming led to cancers resembling Wilms tumors, a common form of pediatric kidney cancer [56]. These cells exhibited an altered epigenetic status that resembled ESCs, including the aberrant expression and DNA methylation of imprinted genes such as *Nnat, Impact*, and *Meg3* [56]. Based on these observations and on the known molecular mechanisms of cellular reprogramming, it is plausible to speculate that *in vivo* reprogramming converts terminally differentiated cells into a dedifferentiated or progenitor-like state. Although progenitor-like cells induced by *in vivo* reprogramming may have a beneficial effect in maintaining tissue homeostasis during aging, lack of differentiation signals or appropriate niches may fail to lead these progenitor cells to terminally differentiate into a functional state. Consequently, from these findings it can be concluded that there is a fine line delineating beneficial from detrimental effects during *in vivo* reprogramming.

How can some of these detrimental effects be overcome? *In vivo* reprogramming with different combinations of factors may help to prevent the complete loss of cell fate and tumor formation by inducing mild, partial reprogramming. In support of this idea, *in vivo* reprogramming in the absence of c-Myc, a well-known oncogene, requires longer induction periods before persistent tumors develop [56]. Similarly, ectopic expression of Oct4 alone in mice induces dysplastic growth, but such Oct4-induced tumors revert back to normal tissue when Oct4 expression is terminated [56,57]. Nevertheless, additional studies will be necessary to test whether rejuvenation of aging-associated phenotypes can be achieved by different combinations of Yamanaka factors. It is important to emphasize that these are the first studies to demonstrate the potential of *in vivo* reprogramming in modifying homeostasis of adult tissues. Additional studies focusing on the *in vivo* reprogramming of specific tissues (e.g., those most sensitive to the effects of aging), or of specific cell populations (e.g., progenitor cells or adult stem cells), may offer alternative ways of preventing the detrimental effects of *in vivo* reprogramming. Lastly, these studies could facilitate an understanding of whether amelioration of aging phenotypes in specific tissues or organs could lead to systemic improvement of organismal health. Along these lines, it is exciting to speculate that specific tissues or organs, owing to their physiological role, are capable of driving organismal aging during a lifetime, and therefore ought to be considered as potential targets of anti-aging strategies.

Concluding Remarks

Aging represents the inevitable and progressive decline of cells, tissues, and organisms over time. This process is primarily driven by epigenetic changes that regulate gene expression. Expression of Yamanaka factors in differentiated cells induces cellular reprogramming by

Outstanding Questions

What is the role of epigenetic dysregulation in aging? How does the environment alter our epigenome? Could we delay the aging process by restoring our epigenome to a younger state?

Do all tissues and organs age at the same rate? Do specific tissues and organs drive organismal aging? Will it be possible to restore the regenerative capacity of an organ through cellular reprogramming?

Could cellular reprogramming slow down or reverse the aging process? Will this rejuvenation apply to all tissues and organs? Could cellular reprogramming extend the lifespan of an organism?

What specific epigenetic programs are restored by cellular reprogramming during rejuvenation? Could epigenetic reprogramming rejuvenate cells without altering their cell identity or function?

Will rejuvenation of tissues and organs lead to increased cancer rates? Could organismal rejuvenation be repeated through life?

remodeling the epigenome, and has the capacity to rejuvenate cells to a younger state. For these reasons, cellular reprogramming methods are currently being devised and tested to develop novel *ex vivo* or *in vivo* anti-aging strategies. At this point in time the *in vivo* overexpression of Yamanaka factors in humans may seem to be an unrealistic approach for treating age-associated conditions. However, we believe that studies such as these will demonstrate that the aging process can be manipulated through cellular reprogramming (Box 1 and Outstanding Questions). *In vivo* reprogramming with alternative factors (including factors yet to be discovered) or small molecules such as valproic acid, CHIR99021, tranylcypromine, and forskolin among others (recently used for *in vitro* reprogramming of mouse cells) may represent more viable therapeutic strategies toward rejuvenating human specific organs (Table 2) [58]. In this exciting and fast-moving field, learning how to artificially control and manipulate the epigenome and to modulate the aging process may hold the key for extending lifespan. Although many broader questions remain–for example: what is the cause of cellular and organismal aging; what are the molecular drivers of aging; and how does our environment influence how we age?–the approaches outlined here may one day help to ameliorate aging symptoms, reduce the prevalence of age-associated diseases, and ultimately improve human health and lifespan.

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