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ELIXIR of LIFE: THWARTING AGING with REGENERATIVE REPROGRAMMING

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Abstract

All living beings undergo systemic physiological decline following ontogeny, characterized as aging. Modern medicine has increased the life expectancy, yet this has created an aged society that has more predisposition to degenerative disorders. Therefore, novel interventions that aim to extend the healthspan in parallel to the lifespan are needed. Regeneration ability of living beings maintains their biological integrity and thus is the major leverage against aging. However, mammalian regeneration capacity is low and further declines during aging. Therefore modalities that reinforce regeneration can antagonize aging. Recent advances in the field of regenerative medicine have shown that aging is not an irreversible process. Conversion of somatic cells to embryonic-like pluripotent cells demonstrated that the differentiated state and age of a cell is not fixed. Identification of the pluripotency-inducing factors subsequently ignited the idea that cellular features can be reprogrammed by defined factors that specify the desired outcome. The last decade consequently has witnessed a plethora of studies that modify cellular features including the hallmarks of aging in addition to cellular function and identity in a variety of cell types *in vitro*. Recently, some of these reprogramming strategies have been directly employed in animal models in pursuit of rejuvenation and cell replacement. Here we review these *in vivo* reprogramming efforts and discuss their potential use to extend the longevity by complementing or augmenting the regenerative capacity.

Keywords

Aging; Longevity; Reprogramming; Epigenetics; Regeneration; Stem Cells; Basic Science Research

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INTRODUCTION

Human life expectancy has increased more than twofold in the developed world over the past two centuries, resulting in a dramatic increase in elderly population.[1] Advanced age is associated with physiological declines which ultimately lead to incapacitation of the individual and increased predisposition to diseases.[2] Among these, cardiovascular diseases are the leading cause of death worldwide. At first glance, aging could be interpreted as a regulation of lifespan emerged during the evolution of metazoans from protozoans.[3,4] In fact, longevity is used as the primary criteria in many biological models of aging, and thus, numerous studies have shown extension of lifespan upon modification of specific conserved pathways.[5–7] However, such quantitative enhancement could be considered to have a lower priority over qualitative enhancement for the human life. Therefore, alleviating the immediate consequence of aging, the physiological deterioration, is the foremost goal of aging-oriented studies in humans. In this regard, healthy life expectancy, termed “healthspan”, can be defined as the length of time an individual is physiologically competent, and able to maintain homeostasis in response to external stress; but is not necessarily equated with "lifespan".[8,9]

The fact that our healthspan is not keeping pace with the increasing life expectancy results in more years spent in physiological deficiency. Thus, there is a high socio-economic as well as medical and scientific interest to find strategies that confer optimal physiology, thereby extending healthspan.[10] However, our understanding of the biology of aging with a view towards improvement of physiological competency is still limited, thus in order to extend the healthspan, we still need to decipher and counteract the cellular triggers of aging. At the cellular level, aging can be considered as the malfunctioning of molecular mechanisms through time, causing aberrations such as telomere length shortening, increase in the production of reactive oxygen species (ROS), accumulation of toxic protein aggregates or epigenetic changes. Among these, epigenetic changes have been widely explored in the past decade.[11] Studies on a range of models spanning from yeast to humans have shown various epigenetic changes during aging [11] such as formation of senescence-associated heterochromatin foci (SAHF) [12], reduction in the bulk levels of the core histones and incorporation of non-canonical histones [13] in addition to the changes in the posttranslational histone modifications, DNA methylation pattern, and noncoding RNA profile [11,14,15]. Altogether, these molecular aberrations hamper cellular functions, which in turn manifest as systemic physiological decline that we observe as "aging" at the organismal level.[2] The physiological declines eventually result in the death of the organism once they fall below a certain threshold that sustains its life. Therefore, aging is nothing other than molecular aberrations that occur at the cellular level, which in turn perturb the composition of a biological unit or how it functions.

Aging-associated molecular aberrations are fundamentally the effect of entropy that universally acts on all matter, living and non-living. Subject to entropy, all matter tends toward disorder. Nevertheless, living beings are equipped with a vital feature that separates them from non-living matter, *the ability to actively maintain an organized state*. Regeneration refers to re-establishment of the functional units lost to deterioration or injury, and thus constitutes the major leverage of living beings against the degenerative effect of

entropy. Yet, all living beings ultimately lose the tug of war with entropy. The biological order gradually deteriorates in this struggle, manifested as aging, and eventually collapses, characterized as death. Hence, aging is characterized by systemic chronic degeneration. Furthermore, regeneration capacity declines with age, leaving the organism further vulnerable.[16] Biological units that do not have a significant regeneration capacity are the most vulnerable to the effect of entropy. Given that the integrity of the biological units is what determines one's age, interventions that counteract the damages on the biological order are expected to enhance the healthspan and longevity.

Induced pluripotent stem cells (iPSCs), launched the golden era of regenerative medicine due to their capacity to generate any cell type within the body.[17,18] This discovery cemented on the concept that cellular identity is merely a state and can be modified by inductive factors that support the state of the desired cell type. Subsequent identification of a variety of such factors that modify cellular identity allowed direct conversion of cells to another differentiated state without reaching to pluripotency. Although, the use of this technology was first directed to *in vitro* generation of cells for transplantation, its application directly *in vivo* has been recently explored for regenerative purposes.[19,20] Here, we will review the recent advances in the field of cellular reprogramming and discuss how they can be used to enhance the healthspan and longevity by complementing or augmenting the regenerative capacity.

EPIGENETICS and REPROGRAMMING

The role of epigenetics in aging has recently become a central theme. Numerous studies have demonstrated that the epigenetic profile of a cell changes during aging.[12,21–25] For instance, elucidation of age-related changes in the DNA methylation pattern have led to the term "DNA methylation clock" to be used as an accurate predictor of age at the molecular level.[26–28] Changes in chromatin structure are also correlated with aging-related phenotypes in diverse species ranging from the yeast to humans.[11] In fact, the role of epigenetic modifications in regulation of lifespan was demonstrated in the yeast long time ago due to the role of Class III histone deacetylases (HDAC III), Sirtuins, in ribosomal DNA silencing.[29] Following this line of thought, can we improve the healthspan by resetting the old epigenome to a younger state so that the cells regain their young phenotype?

The function of each cell type in the body is epigenetically programmed during its ontogeny. Nuclear transfer experiments in the frog in the mid 20th century showed for the first time that this program can be reset by the cytoplasmic factors present in the ovum and nullified the dogma that states cellular specification is irreversible.[30,31] 2006 was highlighted with the discovery of these factors that convert murine and human somatic cells to an induced pluripotent stem cell (iPSC) state.[17,18] Upon long-term combinatorial effect of 4 transcription factors (OCT4, KLF4, SOX2, c-Myc; a.k.a. 4F), any type of somatic cells dedifferentiates and acquires an induced pluripotent stem cell (iPSC) state similar to that of mammalian embryonic stem cells.[32] These studies showed that mimicking the transcriptional circuitry of the ovum in the somatic cells was sufficient to confer pluripotency, and set the substantial evidence that cellular identity can be modified by mimicking the transcriptional circuitry of the desired cell type (Figure 1).

During development, the plasticity of cells gradually declines in parallel to their specification, and this decline is accompanied by a gradual increase in the compaction of their chromatin. Conversely, the chromatin structure re-opens during 4F-induced reprogramming to the iPSC state.[33] The interplay between transcriptional factors and epigenetic modifiers eventually induces pluripotency through major epigenetic remodeling [33,34] that involves two major transcriptional waves.[35,36] The first wave is characterized by upregulation of genes involved in proliferation, and downregulation of those involved in cell adhesion and differentiation, while the second wave is characterized by upregulation of core pluripotency factors such as endogenous OCT4 and SOX2. Association of OCT4 with the H3K36me2 demethylases, KDM2A and KDM2B, activates OCT4 target genes during the first wave by decreasing H3K36me2 levels at their promoters.[37] Likewise, the interactions of OCT4, SOX2 and KLF4 with the core member of the Trithorax complex, WDR5, and the H3K27 demethylase, UTX activates the endogenous core pluripotency network during the second wave.[37–39] Therefore the components of 4F facilitate epigenetic remodeling by coordinating epigenetic modifiers during both transcriptional waves. Given this power of 4F in modulating the epigenetic topography, could it be possible to use them to reset the old epigenome?

Indeed, reprogramming somatic cells to iPSCs not only reverses their developmental clock [31,40–42] but also their aging clock [43–45] as it removes the cellular hallmarks of aging (Figure 1). For instance, senescent human cells, or those derived from centenarian individuals reset their telomere size, gene expression profiles, oxidative stress levels, and mitochondrial metabolism during this process to the levels indistinguishable from human embryonic stem cells.[43] The resulting iPSCs are able to redifferentiate into fully rejuvenated cells. For instance, while neurons directly reprogrammed from old fibroblasts retain their aged molecular profile, those derived through iPSC formation exhibit a rejuvenated phenotype.[46] These observations show that cellular identity and age are not irreversible endpoints but merely plastic cellular states dictated by the epigenetic code at a given time, and this code can be reprogrammed. However, the reprogramming to iPSCs and redifferentiation require multiple cell divisions and conversion of cellular identity through multiple states of neoplastic potential such as teratoma formation by iPSCs. Therefore, since organisms materialize from the harmonious interactions of biological units, it was doubtful whether this technology could be actually applied *in vivo.*

Remarkably, we have recently observed that transient expression of 4F is sufficient to reset the cell's aging clock without loss of its identity or cellular divisions, indicating that rejuvenation of the cell occurs much early during its conversion to iPSCs (Figure 1). Moreover, we have observed that ubiquitous induction of 4F extends the life expectancy of a mouse model of accelerated aging (Hutchinson Gilford Progeria Syndrome) in correlation with an increase in the epigenetic marks associated with youth while a decrease in those associated with old age.[47] For this study, we employed cycles of 4F expression that comprised 2 days of expression followed by 5 days of rest. This regime did not affect the expression of the fibroblast marker THY-1 or induced the pluripotency marker NANOG *in vitro*, indicating that dedifferentiation did not occur. Nevertheless, we observed evidences that the epigenetic profile was reprogrammed to the state of a young cell. For instance, transient expression of 4F restored the levels of H3K9me3 and H4K20me3, which decrease

and increase, respectively, during physiological aging [48,49] as well as in the Progeria.[50] This epigenetic reprogramming was followed by a reduction in DNA damage based on 53BP1 and histone γ-H2AX levels. The decrease in these DNA damage markers did not occur in the presence of a H3K9 methyltransferase inhibitor, indicating that the epigenetic changes are necessary for the 4F-induced protection and/or repair of DNA. Interestingly, these results are supported by a more recent publication that confirms the upregulation of H3K9me3 levels during the first 48 hours of 4F-induced reprogramming.[51] They also showed an increase of HDAC1 binding at 48 hours of induction, linked to H3K9 methylation, and did not observe significant upregulation of DNA damage-related genes during this period. Although it is currently elusive whether this approach can extend the lifespan of physiologically aged individuals, we confirmed these conclusions on late passage wild-type human and murine cells, modeling physiological aging *in vitro*. Altogether, the key concept raised by this work is that aging is a manifestation of progressive epigenetic dysregulation that can be reset by transient *in vivo* reprogramming induced by 4F expression. It should be also mentioned that works by others suggested metabolic dysfunction and telomere shortening as the drivers of aging. For instance, deletion of Pim kinases in the mouse causes premature cardiac aging in correlation with perturbed mitochondrial biogenesis and function.[52] Notably, forced expression of Pim 1 kinase in primary human cardiac progenitors removes the cellular hallmarks of aging *in vitro*.[53] Likewise, overexpression of telomerase reverse transcriptase rejuvenates murine mesenchymal stromal cells. Moreover, upon transplantation into an ischemic hindlimb model, the rejuvenated cells contributed to the tissue regeneration more efficiently than the mock control. [54] Interestingly, perturbation of epigenetic regulators correlates with telomere dysregulation [55], and telomere attrition is known to compromise metabolism and mitochondrial function through the activation of p53 [56]. Therefore, the role of epigenetic, metabolic and telomere dysregulation in aging may not be necessarily mutually exclusive.

In addition to epigenetic rejuvenation, reprogramming strategies can be potentially employed directly *in vivo* to replace the cells that deteriorate or perish during aging, thereby extending the lifespan (Figure 2). Thus, unlike epigenetic rejuvenation, this strategy is based on converting a resident cell of a tissue to another cell type.

CELL REPLACEMENT by *in vivo* REPROGRAMMING

Classically, regenerative medicine relied on transplantation for cell replacement therapies in order to alleviate physiological dysfunctions that derive from the deterioration or death of a cell population.[57] This approach has been successfully transitioned to the clinics for hematopoietic disorders.[58] However, it has not yielded satisfactory results in other cases to be considered as a generalized clinical procedure partly due to immunoincompatibility issues.[57] Since iPSCs can give rise to any cell type within the body, discovery of this technology incited the concept of *in vitro* generation of functional, rejuvenated cells for autologous replacement therapies. However, transplantation of iPSC-derived cells faces safety and functionality concerns. For instance, cells derived from iPSCs frequently display heterogeneity and immature functionality making them unsuitable for transplantation. Moreover, *in vitro* manipulation of cells bears the risk of contamination and accumulation of mutations. Additionally, there are inherent technical barriers to the transplantation procedure

itself, such as the invasiveness of the procedure, and delivery and retention of the graft. Therefore, clinical application of this *in vitro* technology has been challenging.[59]

An alternative to cell transplantation is *in vivo* reprogramming of resident cells of a tissue to generate functional cells (Figure 2). From a clinical point of view, reprogramming to a pluripotent state has the risk of tumorigenesis. However, cells can be induced to transdifferentiate without traversing a pluripotent state.[60] During this process, it is generally accepted that the cell directly switches its identity without dedifferentiation or cellular proliferation upon introduction of the inductive factors. The conversion is more efficient between cells that are developmentally closer [61,62], but can also occur between developmentally distant cells, demonstrating the possibility to cross developmental barriers. [63,64] One of the first observations of transdifferentiation was made by Davis *et al*. in 1987, who showed *in vitro* that mouse embryonic fibroblasts transform into myoblasts upon forced expression of MyoD, a master regulator of skeletal muscle.[65] Since then, various observations of transdifferentiation have been reported.[61] For instance, neurons [64,66,67], hepatocytes [68], hematopoietic cells [62,69], skeletal muscle cells [65] and endothelial cells [70] have been induced directly from other differentiated cells such as fibroblasts. Although most of these studies were performed *in vitro*, some have transitioned to *in vivo* in animal models (Table I). In this section, we will highlight some of these examples of *in vivo* transdifferentiation, as a strategy to repair damaged tissue.

β-Cells

Diabetes is one of the major aging-associated ailments. Over 25% of the Americans older than 65 years suffer from it and the prevalence is estimated to double in the next 20 years partly due to the aging population.[71] Diabetes is characterized by the loss or dysfunction of the insulin-producing cells, *β-cells* of the pancreatic islets. Therefore, strategies to generate β-cells have been highly explored in the field of regenerative medicine including *in vivo* reprogramming.[72] Compared to the pharmacological alternatives, this strategy has the advantage of establishing a physiological setting, whereby insulin secretion and glucose homeostasis are intrinsically harmonized. Therefore, it is not surprising that the first case of *in vivo* transdifferentiation was the conversion of pancreatic exocrine cells, *acinar cells*, into β-cells.[73] In this work, Zhou *et al* used adenoviral vectors to express three β-cell specifiers Neurog3, Pdx1 and MafA in adult mouse pancreas. The induced β-cells closely resembled primary β-cells and produced insulin. Moreover, they were able to ameliorate hyperglycemia in a diabetic mouse model. Subsequently, transdifferentiation of other type of non-β-cells into β-cells has followed. For instance, Collombat's group converted *α-cells*, the glucagon secreting cells of the islets, into β-cells in the adult mice by expressing Pax4 or inactivating Arx, the lineage specifiers of β and α cells, respectively. [74,75] Additionally, pancreatic ductal cells have been converted into α, β and δ-cells (somatostatin producers) in the adult mouse by inactivating Fbw7, the substrate recognition component of SCF-type E3 ubiquitin ligase. [76] This inactivation turns on the endocrine program by stabilizing Neurog3, which is required for the establishment of the endocrine faith during the development. Interestingly, a recent study showed conversion of acinar cells into functional β-cells by transient cytokine exposure without any genetic intervention.[77] In this study, the authors used epidermal growth factor in combination with ciliary neurotrophic factor in an adult chronic

hyperglycemia mouse model. The *de novo* generated β-cells were epigenetically reprogrammed through a process that involved reactivation of Neurog3. This strategy is especially important for clinical translation since it does not involve the use of transgenes.

Given that β-cells *per se* are the physiological units for blood glucose homeostasis and a small number of them is sufficient, extrapancreatic regions that are more accessible to manipulation than the pancreas have also been probed as a source to induce β-cells. For instance, hepatocytes and, epithelial cells of the intestine and gall bladder, have been converted to β-cells *in vivo*.[78–80] These cells share a close developmental origin with β-cells. For example, enteroendocrine progenitors that reside in intestinal crypt epithelium already express Neurog3.[81] Inactivation of the transcription factor Foxo1 in these cells induced expression of β-cell markers including insulin, suggesting that Foxo1 prevents the acquisition of β-cell features in the gut.[82] However, the induced β-cells still retained some of the intestinal properties, thus, the transdifferentiation process seem to have occurred partially. Nevertheless, a more recent study showed formation of functional β-cell islets from the intestinal enteroendocrine progenitors *in vivo* by forced expression of the transcription factors Pdx1, MafA (and Neurog3).[83]

Cardiomyocytes

Cardiomyocytes are the functional cellular units of the heart. Despite the vital role of the heart in sustaining the life of an organism, adult mammalian cardiomyocytes are not proliferative, and thus the cardiac muscle is vulnerable to injuries. Although putative cardiac stem cell population has been reported, their significance is elusive given that the turnover rate of cardiomyocytes is very low in adult mammals.[84] Therefore, injuries, such as ischemia, lead to myocardial infarction characterized by irreplaceable death of cardiomyocytes and degeneration of the cardiac tissue. In fact, ischemic heart disease is the primary cause of death worldwide according to the January 2017 report of World Health Organization (WHO). Instead of regeneration, the degenerated tissue is replaced by the formation of a fibrotic, scar tissue whose function is to seal the injury and prevent further damage to the remaining tissue.[85] However, scar tissue does not perform the physiological function such as rhythmic contraction, and furthermore restricts the function of the remaining functional tissue. Therefore, possibility of converting the scar tissue into the functional tissue by the reprogramming technologies has been widely pursued.

Direct conversion of cardiac fibroblasts into cardiomyocyte-like cells was achieved first *in vitro* [86] and later *in vivo* [87] by Srivastava's group through forced expression of cardiac lineage specifiers. By expressing Gata4, Mef2 and Tbx5 (GMT), Ieda *et al.* first showed up to 20% conversion of cardiac fibroblasts into α-MHC positive cells.[86] The generated cardiomyocyte-like cells expressed cardiomyocyte-specific markers, exhibited similar gene expression and epigenetic profiles as primary cardiomyocytes while purging the corresponding fibroblast molecular profiles. However, only a small percentage of the reprogrammed fibroblasts exhibited contraction after spontaneous maturation *in vitro*. Nevertheless, cells transplanted the day after transduction into an infarcted murine heart efficiently differentiated into cardiomyocytes pointing to the stimulatory role of the physiological environment on the reprogramming, possibly due to the presence of lineage-

specific signals in the microenvironment. Therefore, it is tempting to speculate that reprogramming *in vivo* may even be more efficient than *in vitro*. Indeed, expression of GMT directly in the infarcted mouse converted the resident fibroblasts into functional cardiomyocytes.[87] The induced cardiomyocytes (iCMs) displayed normal sarcomere assembly and a gene expression profile similar to primary cardiomyocytes, produced action potentials and responded to electrical stimuli with contraction. Notably, the reprogrammed cells exhibited electrical coupling, indicating that they successfully integrated into the tissue. Time course analyses showed that partially reprogrammed iCMs matured through time. Importantly, the animals that underwent cardiac reprogramming displayed reduced cardiac dysfunction up to three months post-injury. Altogether, these results not only indicate that cells can be reprogrammed *in vivo*, but also the native environment can augment this process and stimulate the tissue integration of the *de novo* formed cells.

Following these observations, various modifications of the GMT cocktail has been developed in order to further increase the efficiency of the reprogramming. These strategies included addition or modification of the lineage specifier transcription factors [88,89], optimizing the culture conditions [90] or supplementing with small-molecule compounds [90,91] and miRNAs [90] involved in cardiac specification. For instance, Olson's group found that addition of the cardiac transcription factor, Hand2, to the GMT cocktail (GHMT) increased the efficiency of the reprogramming *in vitro* and *in vivo.*[89] Jayawardena *et al* demonstrated that *in situ* administration of miRNAs 1, 133, 208 and 499 into ischemic murine myocardium was sufficient to reprogram cardiac fibroblasts into cardiomyocytes.[90] This is very encouraging for clinical translation because miRNAs can be more readily delivered by non-integrating transient strategies relative to transcription factors due to their small size, making them apt candidates for therapeutic purposes.

Neurons

Neurological dysfunctions comprise a wide range of disorders that cause major disabilities with high impact on the healthspan, and constitute a growing burden in the aging society. For instance, Alzheimer's disease is among the top ten causes of death worldwide (WHO, January 2017). Fetal cell transplantation trials have yielded extremely variable and unsatisfactory results in the patients with neurodegenerative diseases, calling for alternative strategies to repopulate the neurons lost in these conditions.[92–94] Given that the complex nature of the central nervous system hampers invasive procedures, therapeutic strategies based on *in vivo* approaches are greatly desirable.

In vivo transdifferentiation of brain-resident non-neuronal cells into neurons has been reported by several groups. For instance, pericytes and glia cells, such as astrocytes and NG2 glia have been successfully converted into neurons.[95] Unlike neurons, these cells are proliferative under certain conditions and thus they can be replaced by homeostatic proliferation when spent for the conversion.[96] For instance, forced expression of Brn2, Ascl1 and Myt1l in parenchymal astrocytes in the striatum converted them into neurons identified by the neuronal specific nuclear marker, NeuN.[97] Subsequent studies have shown that even single transcription factors can be sufficient to direct glia into the neuronal fate. For instance, forced expression of Sox2 alone, a neuroectodermal lineage specifier, transformed striatal

astrocytes into the proliferative doublecortin-positive neuroblast-like cells.[98] However, these neuroblasts were not able to differentiate without an ectopic stimulus that direct their neuronal differentiation. Similar observations were reported in the spinal cord [99] and cerebral cortex [100], where Sox2 was used to induce neuron-like cells from reactive astrocytes and NG2 glia, respectively. This is significant because these neurological sites display less plasticity than the striatum and thus they are more vulnerable to damage. In particular, the majority of the neurological disorders involve the cerebral cortex. Therefore, there is a major interest in identifying regenerative strategies that target these regions. Interestingly, the Sox2-induced neuronal conversion in the cerebral cortex required the presence of a local injury [100], supporting the idea that tissue damage and the associated inflammatory responses provide signals that boost cellular reprogramming.[101] Hence, unlike *in vitro* where the conditions are defined, *in vivo* cellular reprogramming occurs within the complex environment of the intact tissue that may necessitate conditioning, or reprogramming, of the physiological niche as well. Although the studies outlined above are proof-of-concept studies performed in the mouse, primary pericytes isolated from human cerebral cortex have also been converted to neurons *in vitro* by Sox2 and Mash1 (a neuronal specifier).[102] The converted cells fire action potentials and constitute synaptic targets for other neurons, reinforcing the possibility of the use of pericytes as the cell source in the clinics.

Additionally, Guo *et al*. found that the transcription factor NeuroD1 alone was sufficient to reprogram reactive astrocytes and NG2 glia into functional neurons in a mouse model of Alzheimer's disease.[103] In this study, the astrocytes specifically gave rise to glutamatergic neurons, while NG2 cells transformed into glutamatergic and GABAergic neurons, pointing to the importance of the origin of the cell source. However, one of the most difficult challenges in the reprogramming field is the generation of specific neuronal types perished due to neurodegenerative diseases, such as the dopaminergic neurons lost in Parkinson’s disease. Very recently, these neurons have also been induced *in situ* from striatal astrocytes. [104] Importantly, the induced dopaminergic neurons (iDANs) spontaneously matured and improved the motor symptoms in a mouse model of Parkinson's disease. Moreover, the authors demonstrated that the same strategy has been successful in the conversion of human astrocytes into iDANs *in vitro*, offering the potential therapeutic use of this technology *in vivo*.

Altogether, *in vivo* transdifferentiation strategies are therapeutic approaches that aim to undo the age-related degeneration and thus complement the intrinsic regeneration capacity. Additionally, reprogramming can potentially be used to augment the intrinsic regenerative capacity of the individuals (Figure 2).

REPROGRAMMING the REGENERATIVE CAPACITY

The importance of regenerative capacity can be appreciated by the fact that regenerative failures cause tissue integrity to decline, which is essentially the histological manifestation of aging.[105–107] Likewise, aging is associated with a decline in the regeneration capacity. [16,108,109] Therefore, the key for enhanced longevity may be enhancement of regeneration capacity. Although all living beings have the regeneration ability, their capacity ranges

broadly.[110] In mammals, regeneration ability is largely limited to the tissue level and higher order biological units do not regenerate.[111] For instance, the hematopoietic cells and mesenchyme of the connective tissue, epithelial and muscular part of the organs, and certain nervous tissue of the central nervous system can repopulate when lost. However, the organs, being made up of multiple tissue types and giving rise to the even more complex biological systems, only undergo repair upon injury to impede further functional loss and at most, to compensate for the loss.[112] On the other hand, many phylogenetically lower animals, such as planaria, hydractinia and lower vertebrates are endowed with extensive regeneration capacity, reforming organs, biological systems or even entire organisms following injury. One explanation to the phylogenetic differences in regeneration is that regeneration capacity has declined during the mammalian evolution. This is exemplified by appendage regeneration.[113] For instance, fish [114] and salamanders [115] can fully regenerate their appendages upon amputation throughout their lives, while froglets form a mere cartilaginous protrusion called "spike" lacking the digits.[116] On the other hand, reptiles, birds and mammals do not form any part of the limb amputated beyond the nail bed.[117–119] Therefore, reintroduction of the lost regenerative mechanisms to mammals may confer them with enhanced regenerative capacity observed in the phylogenetically lower animals. What makes these organisms different than mammals?

Planaria and hydractinia contain toti/pluripotent stem cells distributed throughout the body. These high capacity stem cells migrate to the injury site and give rise to the entire missing segments, thereby reestablishing the biological order.[120,121] In amphibians and fish, differentiated cells near the injury dedifferentiate to form lineage-specific progenitors and/or multipotential stem cells that compose a multipotential mesenchymal tissue called blastema.[122,123] This injury-induced plastic tissue regenerates the missing patterned structure by coordinately giving rise to all the tissues therein such as muscle, cartilage, bone and tendons during appendage regeneration via a process called epimorphosis.[124,125] In mammals, pluripotent stem cells (PSCs) and blastemas are not normally found beyond embryogenesis or re-emerge upon injury. They largely depend on stem/progenitor cells with restricted capacity for regeneration. These specialized cells give rise to the differentiated cells of only their corresponding tissue.[126] For instance, hematopoietic stem cells (HSCs) maintain the turn-over of the hematopoietic tissue during homeostasis and upon acute blood loss, while the intestinal stem cells generate the gut epithelium. This is accomplished by the asymmetric division of the stem cell, which leads to one daughter cell committing to differentiation while the other remaining as the stem cell to maintain the stem cell pool.[127] The committed cell undergoes sequential divisions that lead to the hierarchical formation of the progenitor cells of the lineage, which eventually form the cell types that constitute the corresponding tissue. While injury-induced dedifferentiation has been observed in the epithelium of multiple organs, the redifferentiation capacity is restricted to form only the epithelial tissue of the organ.[128–130] Thus, extensive regeneration capacity correlates with occurrence of highly plastic cellular states that are missing in adult mammals, and the key to the superior regeneration might be the presence of a plastic cell type and/or the ability to induce such plasticity.

Conversion of differentiated cells to iPSCs suggests that plastic states can be now induced in mammals by 4F. Indeed, the evidence indicates that 4F induces dedifferentiation of mature

cells sequentially through time leading to stepwise formation of progenitors. This gradual dedifferentiation is coupled with progressive gain in redifferentiation potential before the pluripotency state is reached. For instance, conversion of fibroblasts, a mesoderm derivative, into iPSCs occurs within 3–4 weeks *in vitro* [131] and during this process, the somatic features are lost first, which is followed by the reactivation of the developmental patterns (e.g., mesendoderm markers) before the epiblast-like pluripotency features emerge.[35,132,133] Interrupting this process after 8 days converts fibroblasts to an intermediate, mesoderm-like state without reaching pluripotency.[134,135] Likewise, 4F expression *in vivo* causes the formation of tissue-specific developmental progenitors in multiple organs before iPSCs emerge.[132]

Interestingly, 4F or related pluripotency factors are naturally expressed in some of the organisms that show high regeneration capacity, either in the resident plastic cells that mediate the regeneration or upon dedifferentiation into such state during the regeneration.[136–138] Given that 4F induces epigenetic changes at the molecular level that convert cellular characteristics to a more plastic state, the outcome at the tissue level might be enhancement of regeneration, which eventually leads to deceleration of aging at the organismal level. If this is the case, this will imply that regeneration capacity and longevity can be reprogrammable by inducing the cellular plasticity through epigenetic reprogramming. Indeed, we have observed that the ability of 4F to reprogram the epigenome correlates with histological improvements in multiple organs in the Progeria mice such as the skin, stomach, spleen and kidneys in parallel to deceleration of aging. Notably, aging-related cardiovascular failure, also the leading cause of death in the Progeria [139], is partially rescued as evidenced by an increase in the number of nuclei in the medial layer of the aortic arch (Figure 3a vs. 3b). These observations suggest that 4F expression improves tissue homeostasis by suppressing degeneration and/or improving regeneration. However, given that Progeria is a systemic disease associated with symptoms of aging rather than a model for natural aging, one can argue that the observed histological improvements may be an indirect effect of 4F expression on a diseased mouse (Figure 3). As such, any improvement in the physiology of a biological unit within the organism may affect the physiology of the other units. For instance, 4F-induced cardiovascular improvement can restore the systemic balance, thereby invoking a global physiological improvement in the body and thus extending the life expectancy of the Progeria mice. Therefore, although it is still elusive whether 4F can extend the life span of wildtype mice, we have tested its effect on the recovery of aged wildtype mice from acute injuries.[47] We have observed that 4F treatment correlated with better histological response in the pancreas and muscle at a rate similar to the young suggesting that their regenerative capacity has been rejuvenated. Notably, the muscle injury experiments were based on local activation, suggesting that the effect of 4F is direct.

How can the reprogramming effect of 4F at the cellular level materialize as enhanced regeneration at the tissue level? Since stem cells are the major drivers of the tissue integrity, 4F might be counteracting the exhaustion of the stem cell pool that occurs during aging.[140] Aging-associated stem cell exhaustion can be due to a diminish in stem cell number and thus repopulating the stem cell pool may in turn rejuvenate the tissue. For instance, muscle stem cells (MuSCs) [141] and neural stem cells (NSCs) [142] decrease in number during aging in correlation with a decline in muscular and cognitive functions, respectively. Intriguingly,

forced expression of 4F increases the number of MuSCs although the physiological outcome of this expansion is elusive.[47] Similar to the quantitative decline, the exhaustion of the stem cell pool might derive from a decline in the stem cell potential [143], and reverting this potential to the young state may in turn elicit tissue rejuvenation. For instance, old bone marrow contains more cycling, activated HSCs, which display functional defects such as inefficiency in homing to the hematopoietic niche [144] and myeloid-bias at the expense of the lymphoid lineage.[145–147] The decline in the lymphoid potential of the HSC pool is one of the reasons underlying the immunodeficiency observed in the elderly. These aging-associated phenotypes are correlated with various epigenetic changes in HSCs. For instance, the promoters of key transcription factors involved in the hematopoietic lineage-specification and targeted by the histone methyl-transferase PRC2 are hypermethylated during aging in parallel to the development of the myelolymphoid imbalance.[148,149] In correlation, perturbation of the regulators of DNA methylation such as DNMTs [150] and Tet2 [151,152] in the mouse causes myeloid bias, and these enzymes are differentially expressed between young and old HSCs.[21,25] Likewise, MuSCs lose their reversible quiescence during aging in parallel to the decline in muscle regeneration.[153] This phenotype is in part due to the progressive loss of bivalent Histone 3 domains (H3K4me3 and H3K27me3) in the promoters of stem cell maintenance genes.[154] Interestingly, forced expression of the epigenetics-associated enzymes, Sirtuins 3 and 7, in aged HSCs re-equilibrates their myelolymphoid potential, leading to a re-balanced hematopoietic tissue composition as in the young.[155,156] In addition, repletion of the Sirtuin cofactor NAD+ improves the function of MuSCs, NSCs and melanocyte stem cells in the old mice in parallel to physiological improvements and extension of the lifespan.[157] These observations suggest that aging-associated epigenetic dysregulation of the stem cells impedes tissue homeostasis. Thus, 4F may exert a regenerative effect by resetting the epigenetic clock of the stem cells. Additionally, certain mammalian tissues have been recently shown to regenerate through cell fate conversions following an acute injury. For instance, renal epithelium recovers from minor acute injuries through dedifferentiation, proliferation and redifferentiation to repopulate the nephrons however nephrons lost to injury do not reform.[130,158,159] Upon extreme damage to pancreatic β cells, α [160] and δ cells [161] give rise to β cells in the mouse. Similarly, parenchymal astrocytes in the striatum of the brain spontaneously acquire neural stem cell-like characteristics following a stroke [162] or stab wound [163]. Augmenting such cell fate conversions that naturally occurs in mammals by 4F expression may enhance the regenerative capacity of the corresponding tissue, thereby extending the longevity.

It should be also noted that factors other than 4F have also been inquired for their regenerative potential. The major strategy behind these factors is to repopulate the tissue by inducing proliferation of the resident differentiated cells. For instance, Hippo pathway controls organ size by regulating cell proliferation and apoptosis, and it is involved in the regeneration of the fly eye disc [164] and zebrafish fin [165]. Moreover its modulation can promote mammalian cardiac regeneration by inducing the resident cardiomyocytes to re-enter mitosis.[166] Likewise, we have identified a microRNA-regulated program that naturally induces dedifferentiation of the zebrafish cardiomyocytes during cardiac regeneration, and shown that its forced activation in the infarct murine heart induces the regeneration through cardiomyocyte de-differentiation.[167] A similar phylogenetic comparison has shown that

urodele amphibian limb regeneration is mediated by a homeodomain protein, Msx1. This myogenic transcription factor is expressed in the limb bud during the development but also activated during the limb regeneration [168] where it appears to be necessary for the de-differentiation of the myofibers.[169] Hence, urodele amphibians reactivate their developmental program upon limb amputation. Expression of Msx1 is limited to the limb development in mammals [170], but its forced expression in murine myotubes also induces their de-differentiation.[171] Altogether, these examples highlight the significance of studying phylogenetically lower organisms that have high regeneration capacity.

FUTURE PROSPECTS

Although the past three decades have shown tremendous insight into the nature of a cell and how it can be manipulated *in vitro*, translation of the findings to *in vivo* reprogramming is still at its dawn. There are around two hundred cell types in the human body [172] and we have experimented to induce only a handful of them. There are multiple barriers that need to be overcome to translate the transdifferentiation studies into the clinics. First, the cell source that will be targeted for reprogramming to the desired cell type must be identified wisely as it will lose its function. The cells of connective tissue, such as fibroblasts, appear to be the best candidates. They are numerous and common in every organ, and their role is supportive for the essential physiology of the organ. Fibroblasts are proliferative and thus can replenish their population lost for the reprogramming. Their contractile form, myofibroblasts, are essentially what causes formation of the non-functional and maladaptive scar tissue when they are not eliminated after repairing an injury.[173] Second, effective functionality of induced cells is still a concern despite the microenvironment within the tissue appears to promote functional maturation of the induced cells relative to *in vitro* reprogramming.[174] As we have seen, each study adds on the previous one, and *in vitro* platform still proves to be the best setting to pinpoint the factors to start with. Therefore, it is only a matter of time and more screening that will provide the optimum cocktail of factors necessary for each cell type. Third, integration of the induced cell into the tissue function is challenging, especially in a diseased setting where the tissue composition has already been disrupted due to the default maladaptive repair that causes accumulation of the scar tissue. Reconstructive approaches supplementing the *in vivo* reprogramming may be pursued in such circumstances. For instance, the diseased area can be removed surgically or conditioned by matrix metalloproteinases, allowing the reprogrammed cells to populate over the excised area. Such reconstructive approaches have proven successful to make the targeted area more receptive in cell transplantation studies [175–177] and thus can likewise promote the tissue integration of the *in situ* induced cells. Additionally, a biodegradable scaffold that mimics the natural extracellular matrix (ECM) can be transplanted into the excised area.[178] The scaffold is expected to degrade as the reprogrammed cells lay their natural ECM.

The studies reviewed here, especially those pertaining to *in situ* induced cells in an injury setting, demonstrate the potential of *in vivo* reprogramming to enhance the healthspan, and in turn, the lifespan of humans. Yet, bench-to-bedside translation has the set-back of species-specific differences. This was clearly illustrated during the turn of the century by gene therapy trials, the holy grail of the time. Despite the promising data in the preclinical studies that involved mice and even non-human primates, the clinical trial resulted fatal for the

patient as well as the much explored gene therapy field due to the different reaction the human patients elicited to the procedure relative to the animal models.[179,180] Could it be possible that the preclinical studies are merely the steps of the regenerative medicine field to its doomsday in the clinics? This is especially important given that almost all the reprogramming events involve genetic interventions. The technical barrier of delivering the inductive factors will eventually be overcome with the advancements in the fields of gene therapy [181,182] and nanoengineering [183]. Additionally, cell-intrinsic differences may also exist between different species. This is very well illustrated by the induction of cardiomyocytes from fibroblasts. Although GHMT induces cardiomyocytes with functional properties from murine fibroblasts [89], this combination was ineffective on human fibroblasts and required additional factors and/or further modification.[184–187] Therefore, inductive factors might differ between the animal models and humans. Confirmation or optimization on human cells or organoids, or in humanized animal models may bridge the preclinical studies to the clinical trials. On the other hand, the reprogramming power of 4F or its derivatives appear to be universal as they have been shown to be effective on the cells of frogs [188], fish [189], birds [189,190], flies [189] and a range of mammals.[191,192] Therefore, induction of a plastic cellular state by transient expression of 4F or analogous inductive factors can be a universal approach among all the species for *in vivo* reprogramming of every cell type.

Cellular plasticity is a double-edged sword. While high plasticity correlates with high regeneration capacity, it also brings a higher chance of tumorigenesis. For instance, overdue maintenance of pluripotent cells beyond gestation causes teratomas in mammals.[193] Transplantation of pluripotent cells or their *in vivo* induction by long-term expression of 4F [194] also causes teratoma formation in the mouse indicating that uncontrolled expression of reprogramming factors can be catastrophic. Nevertheless, temporal control of 4F expression may refine the induced plasticity and restrict the risk of tumorigenesis. Thereby, the somatic cell gains the plasticity of its developmental precursor, and the potential of this plasticity is proportional to the degree of induction. For instance, while longer than 8 days of *in vivo* 4F expression induces teratoma formation, 4–7 days of expression causes tissue-specific dysplasias [132], and 2 days of induction is sufficient for the epigenetic rejuvenation without any neoplasm formation.[47] Additionally, lineage-specifiers can be applied *in situ* as driver factors to direct redifferentiation of the induced progenitors.

It is still elusive how the molecular profile of a cell, including the epigenetic landscape, is affected during *in vivo* reprogramming. Elucidation of the molecular roadmap of *in vivo* 4F-induced reprogramming in the mouse can enhance our understanding of the possibilities and risk factors of using this technology towards regenerative medicine. For instance, comparison of the molecular dynamics of different cell types, such as the derivatives of different germ layers, undergoing reprogramming can indicate the molecular mechanisms underlying *in vivo* reprogramming irrespective of the cell type as well as what gene circuitries might be stimulating or antagonizing it. Such molecular signature can be used to predict the outcome of *in vivo* reprogramming of other cell types as well as to identify markers and the timing for the intermediate plastic states *en route* to the pluripotent state. It can also help identify any associated risk of oncogene activation. Identification of the molecular pathways and their dynamics during *in vivo* reprogramming will ultimately allow

us to control these circuitries for safer, more robust and efficient *in vivo* reprogramming strategies.

Although the therapeutic effect of 4F on the Progeria mice and acute injuries discussed here are very encouraging, we have not seen the best of it yet. We do not know yet whether 4F or similar reprogramming factors can extend the life span of wildtype animals. Likewise, we do not know the mechanistic details of 4F-induced histological improvements in pancreatic and muscular injury models or whether 4F can be effective in injury settings other than pancreas and muscle. Next few years are bound to see the effect of 4F on the life span of wildtype models and on the injury settings that involve regeneration mechanisms mediated by stem cells or cell fate conversions. Given the recent progress in identifying chemicals that can boost [195] or even replace [196,197] 4F *in vitro*, we envisage that findings related to 4F will also eventually lead to safe chemical-based therapeutic strategies in regenerative medicine that will shift the focus from invasive replacement therapies to regeneration-oriented self-healing. Thereby, winning the tug of war with entropy offers significant clinical implications in alleviating the need for organ transplantation and thus will have a direct impact on the aging society.

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Non-standard Abbreviations and Acronyms

ROS	Reactive Oxygen Species
SAHF	Senescence-Associated Heterochromatin Foci
PSC	Pluripotent Stem Cell
iPSC	Induced Pluripotent Stem Cell
iCMs	Induced Cardiomyocytes
iDAN	Induced Dopaminergic Neurons
HDACIII	Class III Histone Deacetylases
4F	4 Yamanaka Transcription Factors OCT4, KLF4, SOX2, c-Myc
GMT	Gata4, Mef2 and Tbx5
GHMT	Gata4, Hand2, Mef2 and Tbx5
HSC	Hematopoietic Stem Cell
MuSC	Muscle Stem Cell

NSC	Neural Stem Cell
ECM	Extracellular Matrix
WHO	World Health Organization

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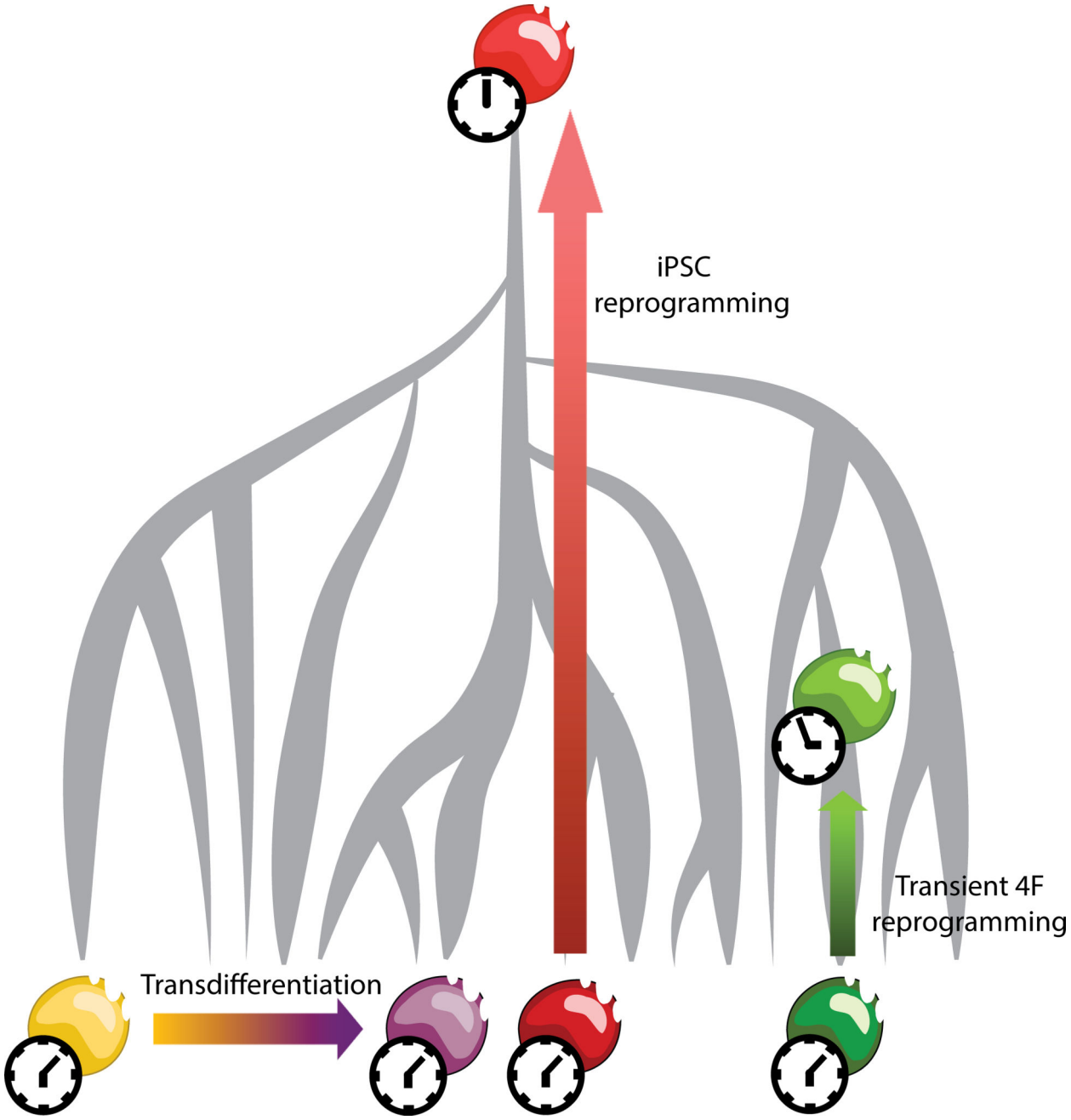

Figure 1. Cellular Reprogramming

A cell can be induced to trans-differentiate into another type or to de-differentiate into a progenitor state by inductive factors. De-differentiation by 4F induces epigenetic rejuvenation unlike transdifferentiation. The risk of teratoma formation hampers any strategy that involves dedifferentiation to the iPSC state *in vivo.* However, temporal modulation of 4F expression can be used to induce epigenetic rejuvenation without identity change or with dedifferentiation into plastic states.

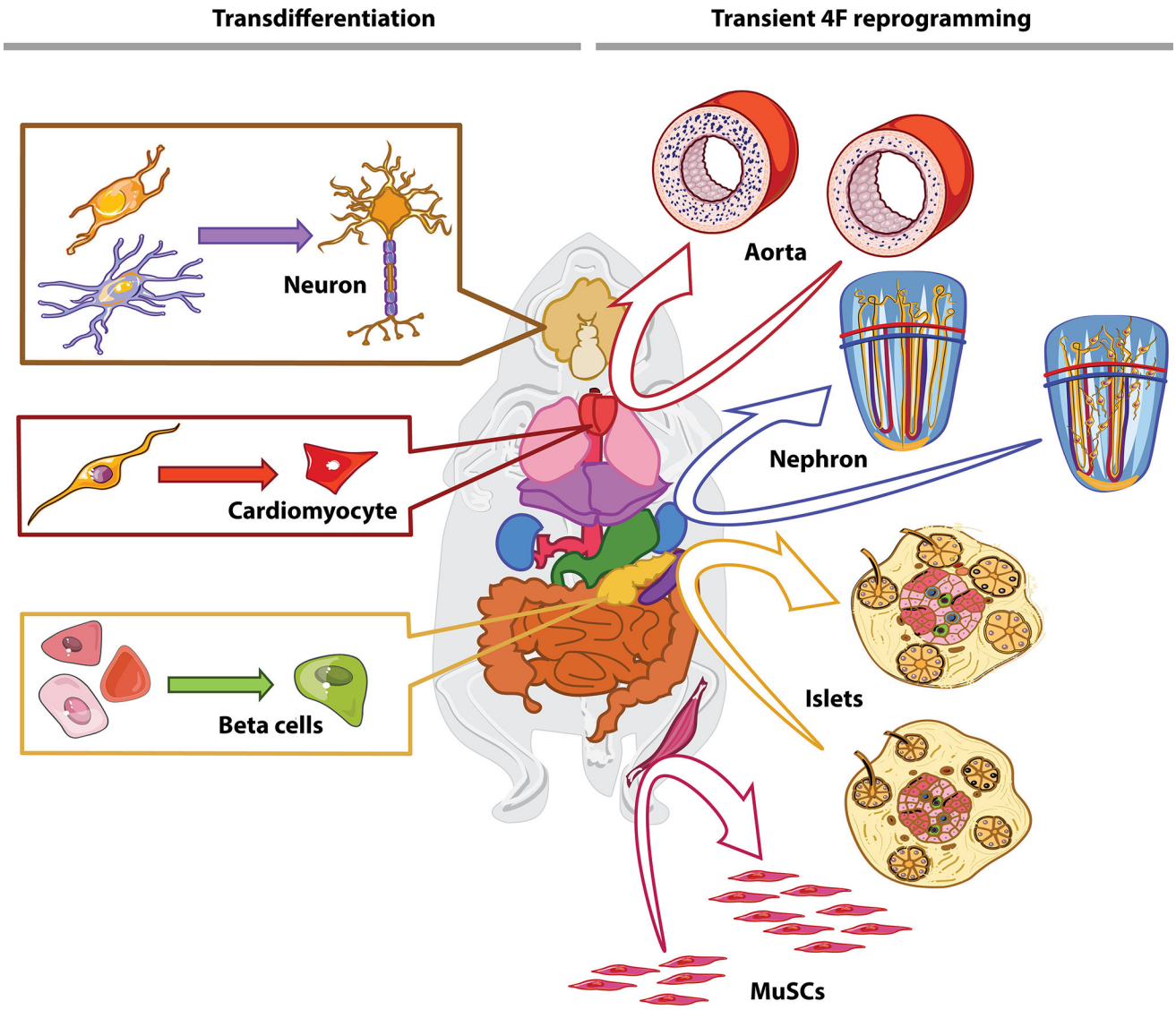

Figure 2. Regenerative Reprogramming Approaches

In vivo induction of transdifferentiation can be used to repopulate the cells lost during aging as an alternative to transplantation, complementing the intrinsic regenerative capacity. For instance, neurons lost to neurodegenerative diseases can be replaced by transdifferentiating resident glia or astrocytes; cardiac fibroblasts can be the cell source for induced cardiomyocytes; alpha, ductal and acinar cells can be used for beta cells. Alternatively, transient 4F expression can be used to rejuvenate cells. This in turn can decelerate degeneration of biological units that have low regeneration capacity (e.g; aorta) or augment regeneration capacity by counteracting stem cell exhaustion (e.g; muscle), or by enhancing the plasticity of organs that intrinsically undergo cell conversions during regeneration (e.g; trans-differentiation in the pancreas and de-differentiation in the kidney).

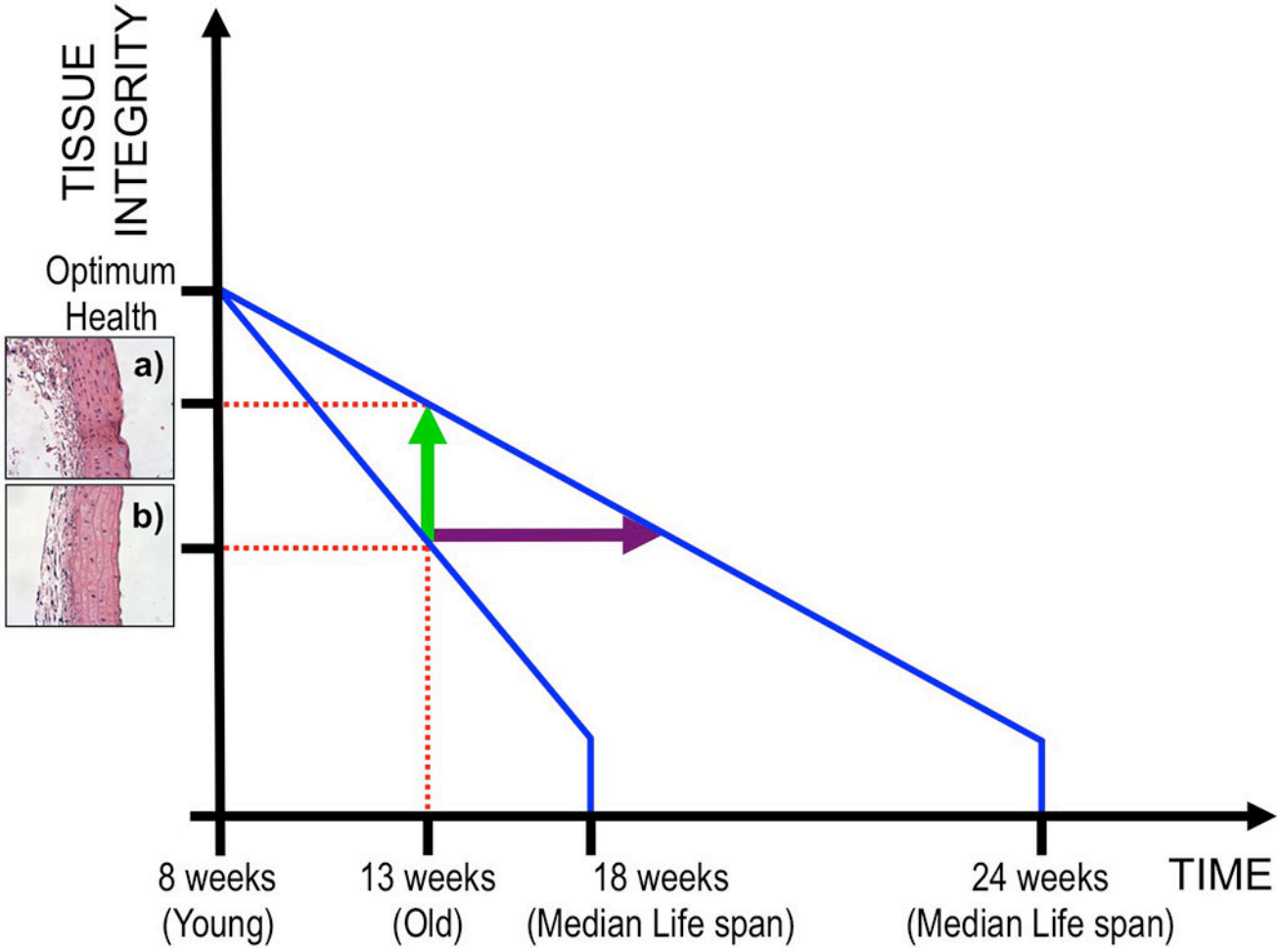

Figure 3. Inverse correlation between tissue integrity and age

Intermittent expression of 4F on the Progeria background from 8 weeks onwards increases the median life span from 18 weeks to 24 weeks. Healthier tissue morphology is observed under the 4F regimen at 13 weeks (**a**), which is considered to be aged for the Progeria background mice (**b**). The improvements on the life span and tissue integrity can have two explanations: The 4F regimen improves tissue integrity (green arrow) by enhancing regeneration, and thereby attenuates aging. Alternatively, it extends the life span (purple arrow) by affecting other aspects of aging such as senescence-associated inflammatory responses, metabolism, neuroendocrinological rhythm, protein homeostasis, free radicals and DNA damage, and thereby attenuates tissue deterioration.

Table I

In vivo transdifferentiation cases

Cell source	Induced Cell	Environment	Inductive Factors	Funtional Outcome	References
Acinar cell	β-cell	Pancreatic acinus	+Ngn3/Pdx1/MafA	Amelioration of diabetes	Zhou et al., 2008
Acinar cell	β-cell	Pancreatic acinus	+EGF/CNTF	Amelioration of diabetes	Baeyens et al., 2014
α-cell	β-cell	Pancreatic islet	+Pax4	Reversal of diabetes	Al-Hasani et al., 2013
α-cell	β-cell	Pancreatic islet	−Arx	Reversal of diabetes	Courtney et al., 2013
Ductal cell	β, α, δ cells	Pancreatic duct	−Fbw7	Glucose-responsive insulin release	Sancho et al., 2014
Enteroendocrine progenitor	β-cell	Instestine crypt	−Foxo1	Amelioration of diabetes	Talchai et al., 2012
Enteroendocrine progenitor	β-cell	Instestine crypt	+Ngn3/Pdx1/MafA	Amelioration of diabetes	Chen et al., 2014
Cardiac fibroblast	Cardiomyocytes	Cardiac muscle	+Gata4/MEF2/TBX5	Amelioration of ischemic injury	Qian et al., 2012
Cardiac fibroblast	Cardiomyocytes	Cardiac muscle	+Gata4/MEF2/TBX5/Hand2	Amelioration of ischemic injury	Song et al, 2012
Cardiac fibroblast	Cardiomyocytes	Cardiac muscle	+miR 1, 133, 208	n.d	Jayawardena et al., 2012
Astrocytes	NeuN+ neurons	Striatum	+Brn2/Ascl1/Myt1l	n.d	Torper et al., 2013
Astrocytes	DCX+ neurons	Striatum	+Sox2	Excitability	Niu et al., 2013
NG2 glia	DCX/NeuN+ neurons	Injured cortex	+Sox2	Excitability	Heinrich et al., 2014
Astrocytes	Neuroblast	Injured spinal cord	+Sox2	n.d	Su et al., 2014
Astrocytes	Glutamatergic neurons	Cortex/Alzheimer's disease	+NeuroD1	Excitability	Guo et al., 2014
NG2 glia	GABAergic/ glutamatergic neurons	Cortex/Alzheimer's disease	+NeuroD1	n.d	Guo et al., 2014
Astrocytes	DA neurons	SN/Parkinson's disease	+NeuroD1/Ascl1/Lmx1A/miR 218	Excitability, improvement of motor behavior	Rivetti di Val Cervo et al., 2017

n.d : Not determined.
SN : Substantia Nigra
DA : Dopaminergic Neurons
DCX : Doublecortin
All of the studies were performed *in vivo* in the mouse.