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Chapter 5

Rejuvenation on the Road to Pluripotency

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Abstract

The technology of reprogramming differentiated cells into a pluripotent state, which can be used to derive virtually any cell type in vitro, has ignited the field of regenerative medicine. An equally revolutionary, but yet to be harnessed phenomenon, is the reset of age that occurs en route to pluripotency. This rejuvenation is clearly evident during reproduction, resulting in a young offspring from aged parental cells. Artificial reprogramming techniques built off this process, such as somatic cell nuclear transfer (SCNT) and induced pluripotent stem cell (iPSC) reprogramming techniques, are showing growing evidence for rejuvenation at the cellular level. These findings all points to an intimate relationship between reprogramming to pluripotency and the reset of age, and iPSC technology, especially, offers the possibility of a man-made intervention in the aging process. Though in vitro cell reprogramming has been studied arguably for the last three decades, this application of specifically developing a protocol to rejuvenate cells, tissues, even whole organs has only just begun to be explored. There are still many challenges to realization but this technology has already famously shown that cell differentiation is more than a one-way street, and, maybe, so is aging.

Keywords: iPSC reprogramming, aging, rejuvenation, organoids, epigenetics

In their recent *Overview on Chronic Disease*, the Center for Disease and Control (CDC) cited multiple studies in the last few years highlighting the predominance (86% in 2010) of chronic diseases in the US national health care spending. Each of these diseases has their own treatments directed by their individualized fields of research. Yet the key risk factor and fundamental correlations behind many conditions like arthritis ($128 billion in 2003), heart disease and stroke ($315.4 billion in 2010), and type II diabetes ($245 billion in 2012) are of course middle and advanced age [1]. To further quantify the core role of aging, a 2013 study did a prospective study and estimated that the economic value of delaying aging in Americans by only about 2 years would save $7.1 trillion over 50 years [2]. This study was prompted by the growing field

of aging research and longevity technologies, but now an even bolder direction is emerging. Researchers are venturing beyond just slowing age or promoting healthy aging and actually attempting to reverse the manifestations of age, from the cellular to the tissue and organ levels.

In another field of research, stem cell science has rapidly grown in the last few decades as a means of studying tissue development and maintenance and to develop methods to artificially produce cells that are either absent or dysfunctional in patients. Again the applications here are pathology specific, conditions like blood and immunological diseases, brain and spinal cord injury, and type I diabetes, as well as some of the more aging-correlated disease like Alzheimer's and heart disease [3]. A unifying approach in the field is the use of pluripotent stem cells, the crown jewel state that can produce any cell in the body. Natural and artificial processes all reach this state by starting with differentiated cell types and "reprogramming" them back to pluripotency. Clearly, this process of reprogramming represents a drastic change and powerful technology, whose potential is just beginning to be explored.

The focus of this chapter is convergence of these two fields. We will see how rejuvenation is intimately linked to reprogramming. Furthermore, we discuss how technologies that induce pluripotency may hold the key to a wholescale and stable reset of cellular age, with tissue- and organ-level consequences.

1. Natural reprogramming and rejuvenation

Rejuvenation is actually not at all an esoteric idea. It happens consistently and is crucial to the survival of virtually every species. Most would immediately think of relatively simple organisms like the hydra and the jellyfish *Turritopsis dohrnii*, which have virtually unlimited regenerative potential [4]. Yet there is a rejuvenation mechanism in much more "complicated" animals, and most importantly in humans, which hides in plain sight: the process of reproduction. The most popular and widely investigated feature of this process is attaining pluripotency, the cellular state that can differentiate into the full diversity of cells in a new organism, starting from two highly specialized and differentiated cells (i.e., the germ cells). However, rejuvenation is an equally exciting phenomenon that occurs during reproduction and is only beginning to be explored. A simple input output analysis verifies this: The inputs are the sperm and oocyte cells of aged parents, typically in their second or third decade of life, while the outputs are the myriad of different cells that make up a new, young organism, retaining none of aged aspects of the parent cells. While one could argue that germ cells have evolved special mechanisms that, unlike somatic cells, prevent them from aging, multiple studies have shown that germ cells do indeed age. Sperm show progressively accumulated DNA damage, elevated reactive ion species levels, and loss of chromatin integrity with age [5]. Oocytes show altered expression of genes implicated in DNA methylation and histone acetylation and mitochondrial dysfunction with age, as well as microniche-imposed aging, through nutrition and hormonal pathways [6]. If these manifestations of age are not erased during reproduction, each generation would progressively accumulate age and the species would no longer have the capability to produce viable offspring after just a few generations.

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The mechanisms specifically behind the reversal of age during reproduction are largely unknown. However, there are two main categories of changes that drive embryogenesis as a whole: genetic and epigenetic. In terms of genetic, the main change is the genetic recombination of the parental chromosomes, which are gathered during zygote formation, right after fertilization, and fused during the first mitotic division into blastomeres [7]. The result is a new genome derived from the parental genomes. There are also more active DNA repair process but they can be tied to upregulation of genes for double-strand break repair and cell cycle checkpoint control that result from the epigenetic changes we will cover next [8]. The reactivated repair processes would definitely have an age-reversing effect, but there is no evidence that the recombination to produce a new genome, alone, should rejuvenate the cell. Epigenetics also undergo large shifts in the initial stage of zygote formation, characterized by a genome-wide loss of DNA methylation except in certain centromeric and parentally imprinted regions and retrovirally derived repetitive elements. In addition, there are widespread alterations to methylation and acetylation of H3 and H4 histones from the maternal genome, while the paternal genome abandons its own protamines for these modified histones as well. Increased activity of methlytransferase has also been observed during this stage; these types of enzymes preserve methylation patterns during cell division for both DNA and histones. Together, these changes further regulate overall heterochromatin organization [9]. Subsequent stages in pre-implantation development up until blastocyst formation show a more passive loss of DNA methylation with cell division, primarily due to reduced levels of methlytransferase in the cell nucleus. The blastocyst then marks the beginning of lineage specification, with an increase in methylation and additional histone modifications that are primarily correlated to differentiation rather than rejuvenation [10]. Ultimately, the bulk erasure of parental epigenetic markers, for cell type and most likely for age, is during the reprogramming of the parental germ cells into a totipotent zygote; totipotency here refers to the zygote's ability to differentiate into all embryonic and extraembryonic (supporting) tissue.

Artificial reprogramming to totipotency was further pursued in the last few decades, with the primary objective of attaining cells that could differentiate into any desired cell type. The first key deviation from the natural process was the technology of somatic cell nuclear transfer (SCNT). In fertilization, only the sperm's nuclei is transferred and it is the oocyte that provides the reprogramming environment. SCNT replaces the sperm nucleus with a somatic cell nucleus for fertilization, but the overall reprogramming process is fundamentally the same, again with large-scale genetic and epigenetic shifts to yield a viable, young offspring. [11] This method, however, established that somatic cells, in addition to germ cells, could be reprogrammed, and thus rejuvenated. The next key breakthrough, which is our main focus, is the technology of induced pluripotent stem cell (iPSC) reprogramming. This technology focuses on reprogramming somatic cells to pluripotent cells, which can specifically form only the embryonic tissues and thus is more relevant than totipotency. More importantly, the strategy here is substantially different. Instead of the reprogramming driven by the oocyte, iPSC technologies introduce exogenous pluripotency genes into somatic cells to induce reprogramming to pluripotency [12]. This process does not include a genetic recombination of two different genomes, like reproduction or SCNT. Thus, the success of this technology established that a global shift in the gene expression profile, induced by epigenetic remodeling, without

substantial genetic alterations is sufficient to execute reprogramming and, as we will see in the next section, can also implement multiple changes toward rejuvenation.

2. iPSC reprogramming: signs of rejuvenation

iPSC reprogramming was developed to artificially attain the embryonic stem cell (ESC) state, a derivative of the totipotent zygote state established by natural fertilization and SCNT reprogramming. The principle behind this technology is that forced overexpression of genes that normally maintain the pluripotent state in ESCs is sufficient to induce reprogramming in somatic cells to revert back to an ESC-like state. The most widely utilized strategy, which most of the result we discuss have applied, is the exogenous introduction of the genes Oct4, Sox2, Klf4, and cMyc, typically achieved by viral or episomal vectors [12]. In evaluating the final product of these procedures, it has been noted that the transition to pluripotency is accompanied by multiple indications of rejuvenation that, in most cases, are retained upon subsequent redifferentiation of the iPSC. As iPSC reprogramming is a cellular technology, it requires a set of cellular biomarkers by which to measure age. The search is still ongoing for a comprehensive and precise list, but the "Hallmarks of Aging" presented by Lopez-Otin et al. in 2013 provide one most generally accepted categorization of the known biomarkers [13]. We will review the multiple signs of rejuvenation through iPSC reprogramming in the context of these hallmarks.

2.1. Epigenetic alterations

Epigenetics include all the changes to the structure of DNA, but not the DNA sequence itself, which control transcriptional potential and ultimately gene expression. Epigenetics most notably establishes cell type and functionality, but also includes many markers of age. DNA methylation is a key aspect of epigenetics and aging correlates with a global trend toward hypomethlyation in numerous cells types across tissues [14]. This is linked to the decline in mRNA transcription for DNA methyltransferase (DNMT), which controls the transfer of methylation patters to daughter cells [15]. Histones, which act as the "spools" around which DNA strands are wound, are another aspect of epigenetics which show age-related modifications, for instance, trimethlyation levels increase for H3K27 and H3K9 but decrease for H4K20, which are all involved in heterochromatin formation, the most densely packed form of chromatin [16]. A key driver of these alterations to histone methylations is the decreased transcription of methyltransferase proteins with age, like those of the Polycomb and trithorax groups [17]. There is an overall loss of heterochromatin with age, driven by the histone modifications but also lost during cell division itself, as recondensation processes are not perfectly efficient. These global trends lead to a loss in gene silencing with age and overall increase in transcriptional noise. iPSC reprogramming reverses of many of these changes, with a youthful restoration of the levels of HP1γ (a key marker for heterochomatin) and trimethlyated H3K9, increased heterochromatin, but there is still much yet to be explored [18]. Changes in the levels of endogenous Polycomb proteins have not been studied, but the artificial knockdown or their genes yields decreased reprogramming efficiency—hinting at a correlation between the two [19]. There is also an overall decrease in DNA methylation but this is primarily because global hypomethlyation is a characteristic of the pluripotent state, as seen

in ESCs. A better comparison, that has yet to be investigated, is between the methylation levels of the original source cells and iPSC-derived cells of the same cell type. A correlation between artificially increased DNMT and reprogramming efficiency has also been made, but no further study has been done on whether reprogramming itself stimulates DNMT transcription [20]. Ultimately, reprogramming itself is characterized by a large-scale epigenetic remodeling, most infamously for dedifferentiation but also for rejuvenation.

2.2. DNA and nuclear damage

Direct damage to DNA from natural metabolic activities, stochastic chemical (endogenous and environmental), and radiative interactions regularly occurs throughout life at rate of 10^5 total molecular lesions per cell per day [21]. There are number of enzymatic repair mechanisms in place to fix these aberrations, but these processes are not perfectly efficient and some processes, like homologous recombination (HR) and non-homologous end joining (NHEJ), which occur during cell division, also decline in efficiency with age [22, 23]. This leads to the accumulated DNA damage, which impairs cell functionality, and decreases the number of viable progeny for proliferative cells; this is the central idea in the DNA damage theory of aging that leads to tissue wide consequences in organs like brain, liver muscle, and kidney. In addition, the nuclear lamina degrades with age with conformational defects like folding and blebbing; this impairs the lamina's function as the overall structural support for the nuclear material, especially heterochromatin in the epigenetics hallmark. These nuclear lamina defects result from age-related decreases in production of laminar proteins, like lamina-associated protein 2α (LAP2α), and altered distribution of these proteins, like the localization of the laminar matrix protein lamin A/C to the nuclear rim but not the nucleoplasm [24]. iPSCs show evidence of reducing accumulated DNA damage through a marked upregulation in genes and activity in both the HR and NHEJ pathways. Further evidence for reinvigorated repair mechanism are the restored levels of phosphorylated histone H2AX which is a key biomarker for the repair of double-strand breaks, in the iPSC state and after redifferentiation. In addition, lamina structure is shown to be restored in the iPSC state which replenished levels of LAP2α. In general, reprogrammed cells also show as an increased sensitivity to extreme irradiation damage noted by a greater propensity to undergo apoptosis, to eliminate mutated cells; this is ultimately healthier for the tissue by clearing cells that may instigate cancerous growth [25].

2.3. Telomere attrition

During cell division, helicase “unzips” DNA in one direction and DNA polymerases must build off of the two template strands but can only move in the 5′→3′ direction. Thus, one polymerase can only move oppositely to the helicase, meaning it must repeatedly attach, synthesize, detach, and catch up to the helicase. Attachment sites are directed by RNA primers, which bind to DNA behind the helicase. Synthesis can only start after the primer, so the DNA by covered primer is not synthesized until the subsequent synthesis when it detaches and the polymerase starts further upstream. The problem arises at the end of the chromosome, where it is no further upstream site for polymerase to begin at and thus the end is simply not copied.

Nature's solution is to pad the end of the chromosomes with telomeres, repeats of noncoding junk DNA (TTAGGG), which are lost instead of coding segments. Telomere erosion is characteristic of cell division and thus aging itself. A number of studies have shown an elongation of telomeres, around 40%, upon reprogramming to iPSC [26–28]. This elongation of telomeres is mechanistically tied to the reactivated transcription of telomerase, a reverse transcriptase enzyme which synthesizes additional telomere repeats. Genes for telomerase are only expressed in stem cells as well as some other highly proliferative somatic cells, but they are silenced upon differentiation. Upon redifferentiation, all lines have been shown to have shorter telomeres than the original iPSC but some who have shown still have longer telomeres than the original source cell [27, 28]. This subsequent loss of telomere length is not necessarily due to accelerated telomere attrition, but just due to the lengthy redifferentiation protocols and variations in each cell line's reprogramming and redifferentiation efficiency. If these protocols can be made shorter and more efficient, the rejuvenation effects could be better retained. This is another example of how a genetic hallmark is addressed by alterations in gene expression. It has also yet to be assessed exactly when telomerase is reactivated and when it is lost during redifferentiation; this knowledge may help to further optimize the retention of this rejuvenation effect.

2.4. Mitochondrial dysfunction

Older cells exhibit a greater percentage of mitochondria in the less efficient “condensed” and “ultra-condensed” configurations than in the healthier “orthodox” configuration; the former have their inner and outer mitochondrial membranes further apart, promoting a smaller matrix and larger cristae. This condensed structure leads to a lower transmembrane potential, which is correlated with higher, less regulated respiratory activity. iPSC's and their redifferentiated lineages exhibit a reversal of this trend, with a higher proportion of orthodox mitochondria as well as higher membrane potentials when compared with the aged source cells. In addition, reprogramming and redifferentiation studies have also shown decreased mitochondrial mass, increased levels of adenosine triphosphate (ATP), and decreased reactive oxygen species (ROS) levels, which are all characteristic of a more youthful phenotype [29]. The lowered ROS levels are especially promising, as elevated levels are a key player in the free radical theory of aging. This idea holds that the electrically active by-products of metabolic reactions, like ROS, can aberrantly ionize the biomolecular machinery of cells, ultimately impairing the functionality of mitochondria and other cell compartments. These youthful changes can be fundamentally tied to upregulated mitochondrial biogenesis genes like PGC-1 alpha and antioxidant genes like GPX1 during reprogramming [30]. This upregulated biogenesis may directly explain the noted increase in youthful mitochondrial morphology and functionality, while the antioxidant genes explain the loss of ROS and further damage to mitochondria. The time course of these changes during reprogramming has not been documented, with the exception of mitochondrial mass, which has been noted to decrease as early as 3 days after the induction of reprogramming [31]. Interestingly, during SCNT reprogramming, cells began exhibiting higher membrane potential in the first week of reprogramming and were linked to increased expression of Glut1, Pfkm, Hxk2, and Ldha which drive glycolytic activity over oxidative phosphorylation [32]. This transition would explain the decreased ROS levels despite the observed

increase in ATP, as glycolysis also produces ATP but without the production of ROS. This initial week, however, does not show the onset of pluripotency, as marked by the expression of pluripotency genes like Nanog and Oct4, suggesting a temporal segregation of the gene expression changes driving rejuvenation from those that drive differentiation.

2.5. Loss of proteostasis

Proteostasis is regulated by modulating levels of both protein transcription/stabilization, to ensure the cell has the proteins it needs, and proteolytic activity, to prevent proteotoxicity. The former, of course, is a direct result of gene expression dynamics. The latter is implemented through the ubiquitin-proteasome and autophagy-lysosome systems; the activity of both notably diminishes with age. This leads to an aggregation of macromolecules as well as degraded organelles that contribute to other aging hallmarks—like mitochondrial dysfunction and senescence [32, 34]. Reprogramming studies have explored relatively little in regard to rejuvenated proteolytic activity. The key finding so far has been an elevated level of autophagy upon induction of reprogramming, peaking on day 2 then relaxing to basal levels from day 3 on until pluripotency is reached [35, 36]. This transient activity has been linked to increased mitophagy, a key role of autophagy, which is responsible for the clearing of degraded mitochondria. This works in tandem with the aforementioned increased mitochondrial biogenesis to create the noted skew in the mitochondria distribution to the younger, orthodox configuration [26]. It also postulated that the additional autophagy is responsible for eliminating cell type-specific protein complexes early on, thus promoting dedifferentiation. This effect may be transient, but the additional degraded proteins and organelles that are removed during the interim definitely contribute to cell rejuvenation. It is crucial to note that these studies were on murine cells, so translation of these effects to human systems also needs to be investigated. Proteasomal activity has yet to be studied in the context of iPSC reprogramming, but increased activities have been noted in human ESCs, linked to elevated expression of the PSMD11 gene [38].

2.6. Senescence

Senescence is defined as the state of full mitotic arrest, relevant primarily for proliferative cells. In vitro, this is linked to the Hayflick limit—the point at which cells have undergone enough cell divisions to deplete their telomeres. In vivo, senescence is also linked to the accumulated DNA damage with age and triggered by epigenetic (chromatin) remodeling at the INK4a/ARF locus. Senescence is likely a programmed biological mechanism for preventing the propagation of cells with damaged DNA, which is more prevalent in aged cells as characterized by shortened telomeres. This prevents mutation-induced cancerous growth and aberrant functionality, but it also arrests tissue growth and repair in general. iPSC reprogramming has been achieved on completely senescent populations, propagated for 51 population doublings then maintained for 2 months, and show revived proliferation in the pluripotent state as well as upon redifferentiation [39]. In addition, expressions of cell cycle-inhibiting proteins like p16 and p21, which initiate the INK4a/ARF remodeling, are known to increase with age but

diminish after reprogramming and redifferentiation. Crucially, the redifferntiated progeny can once again be passaged into senescence, thus verifying that no cancerous growth was induced during the entire process.

2.7. Deregulated nutrient sensing

This hallmark concerns poorer regulation in gene expression pathways that regulated cell metabolism based on the availability of nutrients. The mTOR family of genes is a key glucose- and amino acid-sensing pathway, while the AMPK pathway is route triggered by adenosine monophosphate (AMP) levels, a by-product of ATP synthesis. Artificial downregulation of mTOR and upregulation of AMPK genes have both led to increased longevity in model organisms, while the inverse (as occurs naturally) has been linked with many age-related pathologies like diabetes, hinting at epigenetic alterations to these pathways with age [40, 41]. During reprogramming, mTOR was downregulated in the same pattern that autophagy increased and AMPK activity was upregulated in the first 6 days; both pathways are drivers for autophagy [31, 36]. In addition, downregulation of mTOR is also involved in mitochondrial biogenesis [36]. Thus, the deregulated nutrient-sensing hallmark further links the loss of proteostasis and mitochondrial dysfunction hallmarks. The two remaining FOXO and Sirtuin pathway have yet to be studied for their role in reprogramming.

There are two remaining hallmarks. The hallmark of *stem cell exhaustion* is defined by age-associated losses in both proliferative capacity and stem cells reserves, like in hippocampal neural stem cell populations, as well as altered epigenetics for differentiation potential, for instance, aged hematopoietic stem cells bias toward myeloid lineages over lymphoid lineages [42, 43]. The hallmark of *altered intracellular communication* results from increased expression and secretion of pro-inflammatory cytokines by senescent and pre-senescent cells as well as proteotoxicity from the loss of proteostasis. This leads to a larger, immune system phenotype of "inflamaging," an age-induced propensity to sustain a chronic low-grade innate immune response in multiple tissues, which ultimately interferes with single cell function as well as cell-to-cell interactions [44]. To our knowledge, no iPSC reprogramming studies have really examined rejuvenation of these hallmarks, primarily because both truly need be assessed within aged tissue. The organoid technology we will discuss in the next section may offer the platform to evaluate the rejuvenation of these hallmarks as well through iPSC reprogramming.

These rejuvenated biomarkers are summarized pictorially in **Figure 1**, but there are still many other known biomarkers within these hallmarks that must be assessed for iPSC- and iPSC-derived cells and, as new ones are being discovered, they must also be evaluated. In addition, the results are primarily in fibroblasts, the most common source material for iPSC reprogramming, so the generalization of the rejuvenation effect to other cell types still needs to be assessed. Still, these studies provide initial evidence that iPSC reprogramming can reset age to some degree. In the next section, we will see where this technology stands as a possible rejuvenation procedure.

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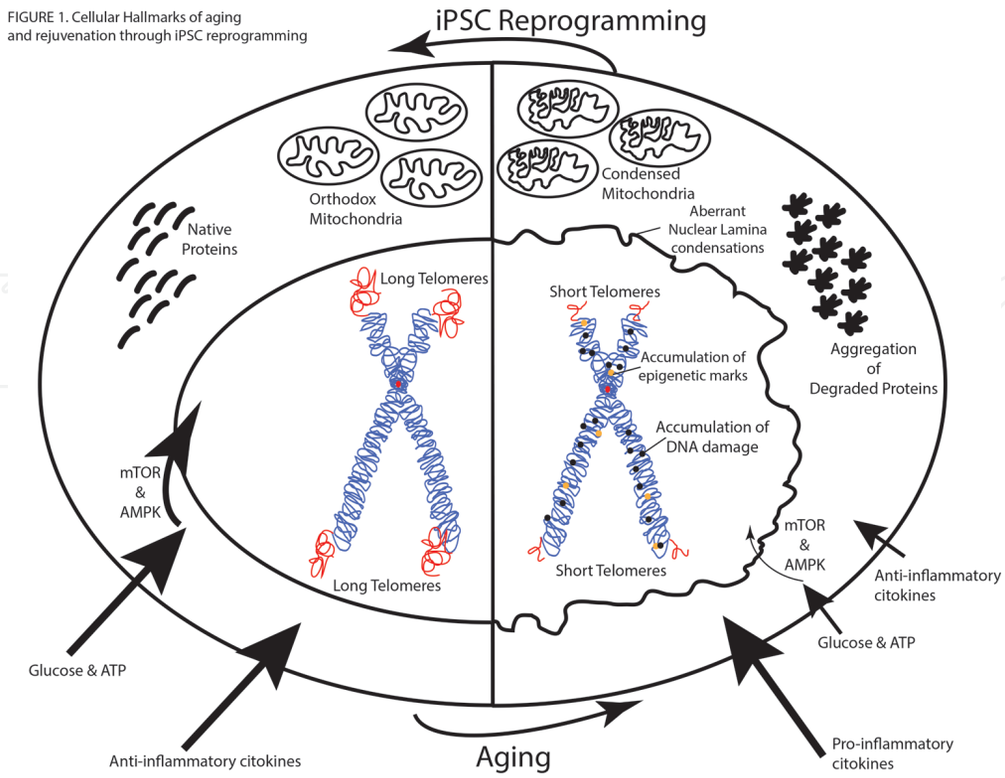

Figure 1. Cellular Hallmarks of aging and rejuvenation through iPSC reprogramming.

3. iPSC rejuvenation: mechanism, comparisons and outlook

The field of iPSC reprogramming was developed to attain and harness the power of pluripotency. The rejuvenation effects that accompany the process were secondary discovery, though one with great potential as well. As we now move to capitalize on this effect, we must evaluate it in the broader field of aging research and technologies. In this section, we will first explore how the mechanistic principle behind this iPSC rejuvenation fits in with unifying theories of aging. Then we will assess the reprogramming strategy in the context of other technologies for healthy aging and rejuvenation. Finally, we will discuss the future perspectives and outlook of this technology in terms of scalability and clinical application.

3.1. iPSC reprogramming and programmed aging

The common theme we note from all the hallmarks are that multifaceted changes in gene expression that are linked to and propagate the aging phenotype. We similarly saw that the rejuvenation effects of iPSC reprogramming could be traced to alterations in gene expression. We noted a few examples, yet these are but a small sample of the full gamut of changes that drive rejuvenation along with dedifferentiation. The core driver of these changes in iPSC reprogramming is the massive overhaul of the epigenetic landscape, which as previously noted, alters the transcription potential throughout the genome. Additionally, the noted

changes in nuclear lamina influence epigenetics and further regulate the distribution of transcription factors as well as the transport of mRNA for translation. Furthest downstream, the increased proteolytic activity, though transient, also clears out gene products—another form of gene regulation. The key concept this strategy has tapped into is the so-called programmed aging hypothesis [45]. This idea holds that the aging phenotype is driven in large part by deterministic and programmed changes, and thus is reversible. At the cellular level, this directly points to changes in gene expression, which is reversible especially through the modes of epigenetic and nuclear lamina modifications. So if age is programmed, iPSC technology could possibly reprogram age—truly apt naming in hindsight. The dual theory is the damage-induced aging hypothesis, which holds that aging is driven by the stochastic degradation of multiple cellular components. This damage is driven by environmental interactions as well as internal degradation as a result of metabolic processes [46]. We discussed manifestations of this in previous section, such as DNA damage, ROS damage, and proteotoxicity from accumulated macromolecules. Unlike programmed aging, the results of this damage are random and thus are not inherently reversible. iPSC reprogramming cannot directly oppose damage but it can help to mitigate some of this damage. As we have seen, it can boost expression for natural repair mechanisms, like homologous repair of DNA damage; it can promote the synthesis of new organelles. From an evolutionary perspective, the two hypotheses may be fundamentally linked. The continued accumulation of age-related damage and the resulting loss in functionality may make retaining older individual less beneficial from a species-level perspective. Older individuals would be less fit and less capable of performing their role in a communal society, more susceptible to and may further transmit pathogens, more likely to produce mutated or dysfunctional offspring, and still take up resources that could go to the younger generations. Thus, species may have evolved programmed mechanisms to further the decay with age and thus increase the mortality and clearing of the older individuals. This could explain why a natural rejuvenation exists but only occurs in the production of the next generation, instead of somehow being applied to retain the youthful phenotype [6], like in the case of mitochondrial biogenesis, and it can boost the clearing of damaged components by transiently increasing proteolytic activity. At the same time, there are age-induced damages that iPSC reprogramming cannot counter (for instance, genetic mutations or damage) without an intact reference template. So in regard to damage-induced aging, there are definitely inherent limitations to this gene expression level technology.

3.2. iPSC reprogramming vs other longevity/rejuvenation technologies

The predominant strategy in the field of aging is to identify individual gene expression pathways that maintain the youthful phenotype or advance aging and artificially control their expression to promote healthy aging. Treatment involves either controlling the expression of endogenous genes through cues like DNA-binding agents and small molecules or introducing exogenous genetic material into the cells through vectors (primarily viral and episomal). The strategies have been applied to genes for telomerase, autophagy, mTOR, AMPK, p16, and p21 to name a few of the aging biomarkers we discussed [47]. Additionally, many pharmacological strategies have been developed to induce these pathways with more exogenous control, based on administration and dosage of the drug. Notable examples, specifically in regard to nutrient

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sensing, include rapamycin, an mTOR inhibitor, metformin, an AMPK activator, and fisetin and resveratrol, both are situin activators [48]. Both the genetic and pharmacologic approaches are fundamentally lacking in two respects. First, these approaches are too narrow in scope. These strategies target one or a handful of genes, but aging is a complex phenotype propagated by a full array of genetic pathways. iPSC reprogramming, in contrast, is more comprehensive with a whole scale epigenetic remodeling that instigates global changes in gene expression which encompass many of the candidate pathways for gene/pharmacological therapies as well as others for aspects like mitochondrial function and gene repair. Even though neither the pathways of aging nor all the gene expression changes in reprogramming are known, the signs of iPSC rejuvenation already discussed and the intuition that iPSC reprogramming mimics natural reprogramming during reproduction both suggest that iPSC reprogramming may offer a more holistic reset of age. The second failing of the traditional genetic/pharmacological approaches is that they are too simplistic in application. These approaches are primarily static over/under expressions of their target genes, but outside of the direct rejuvenation effects, these changes may have detrimental consequences on other cellular processes. For instance, genetic knockout or knockdown of p16 genes may prevent senescence and promote tissue repair, but they increase the chance of spontaneous cancer development [49, 50]. Another example is rapamycin's induction of immunosuppressant pathways, which would outweigh its benefit in inhibiting mTOR [51]. A more viable solution would be a dynamic sequence of changes to gene expression which can respond and compensate for each other to produce a stable, youthful homeostasis. Reprogramming also meets this criterion. iPSCs are very similar to the stable, naturally occurring pluripotent state of ESCs, in terms of both epigenetics and gene expression [52]. In addition, redifferentiation of iPSCs yields viable somatic cells which do not show signs of aberrant functionality or advanced aging. In addition, they regain the aged and senescent phenotype after extensive passaging, having avoided cancerous mutations, as noted earlier.

Another direction in the aging field has gained attention in recent years: heterochronic parabiosis, the surgical joining of circulatory system between old and young mice. This promotes more than just longevity or healthy aging; it rejuvenates whole tissue in multiple organs, like muscles, heart, and brain, in the aged mouse. The mechanism behind this is the replacement of old organism's aged extracellular milieu—distributed through the bloodstream—with that of a youthful organism [53]. This milieu contains cytokines, hormones, and other signaling molecules which influence and bias gene expression at the single cell level expression through the intracellular communication and nutrient-sensing hallmarks. Programmed changes to the immune and neuroendocrine systems with age drive the production of this old milieu, and thus this technology also hinges on the programmed aging hypothesis, specifically its extracellular manifestation. The drawback of parabiosis, and immune and hormonal therapies in general, is that the “youthful” milieu is a dynamic and complex mixture, again a homeostatically maintained youthful state. This would require continuous transplantation of youthful blood from another patient, which is not feasible. The stand in would be artificial synthesis and constant administration of the youthful factors in the blood, which is not completely known and would be extremely difficult to develop and maintain. The same is true for immune and hormonal therapies, which also have detrimental side effects; a clear example

is the use immunosuppressors would reduce chronic inflamaging but also weaken the response to pathogens. Ultimately, this extracellular rejuvenation approach would require rejuvenation of the entire immune and neuroendocrine systems to regulate and maintain. iPSC reprogramming is still primarily a single cell technology, and an entire rejuvenated system is still far beyond its scope. Yet the technology can still have extracellular effects since every somatic cell secretes signaling molecules that influence the cells in their local microniche. Thus, rejuvenated iPSC-derived cells, with sufficient numbers, can start to propagate the youthful phenotype in the tissue in which they are transplanted. In addition, multiple groups are working on producing rejuvenated iPSC-derived lymphoid lineages and T cells. As part of immunoaging, hematopoietic stem cells tend to differentiate more to the myeloid rather than the lymphoid lineage, thus artificial iPSC derivation of the latter can help compensate for this shift [54]. In addition, adaptive immunity to counter specific pathogens diminishes with age due to weakened response by T cells. In vitro production of rejuvenated T cells from iPSCs and transplantation could restore this functionality [55]. The key target that is being explored in iPSC endocrine rejuvenation is the production of insulin producing β-islet cells, which deplete with age [56]. If iPSC-derived cells can be used to holistically rejuvenate just these two systems, then it may capture or surpass the effects seen in parabiosis and subsequently rejuvenate the rest of the somatic cells.

3.3. Future perspectives

Outside of single rejuvenated cell production, iPSC technology is showing some progress in developing rudimentary tissue through the use protocols for the derivation of organoids. These protocols are methods of guided differentiation that direct the iPSC to a somatic stem cell fate, instead of a fully differentiated cell. By mimicking the environmental signals during embryo development and providing 3-D development infrastructure (air-liquid interface or Matrigel embedding), pluripotent cells can be directed to form a rudimentary organ bud. The cells at this state can be further proliferated or instructed to differentiate into multiple cell types by switching to a differentiation-promoting media. The final product resembles a fragment of in vivo tissue in its diversity and arrangement of the different cell types; successful examples include intestine, liver, and thyroid [57]. This is still a very nascent technology, with the primary focus of using organoids to study normal and cancerous development. There have not been any studies assessing to what degree the rejuvenation effects of iPSC reprogramming are retained in the final product, especially since there are now two phases of differentiation to reach the initial organ bud and then to attain the fully differentiated tissue. One encouraging sign is that these organoids can be maintained up to and some times longer than 6 months in culture, despite most of the primary cell donors being of advanced age (50s–70s). This has been noted to surpass the Hayflick limit, so at least telomere attrition and senescence may be reversed in the derivation of these organoids. Future studies can investigate the same aging hallmarks in the cells of organoids, but also explore the two additional hallmarks yet untouched in previous studies. They would be able to assess the intercellular communication—how the interactions of different cell types maintain or degrade the youthful phenotype. In addition, the differentiated organoid also retains some somatic stem cells, so these studies can also look at the stem cell exhaustion in maintaining and regenerating the organoid. The

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organoid platform also provides the opportunity to look at an additional dimension of aging in addressing the rejuvenation of tissue functionality. This moves beyond the Hallmarks of Aging, which are fundamentally at the cellular level, to focus on the emergent function of specific tissues which degrades due to the hallmarks. So far, very little work has been done on assessing organoid functionality. One notable example is a 2015 study that show albumin and bile acid production, ammonia elimination, CYP3A4 activity, and midazolam metabolism in liver organoids, all of which decline with age [58]. These organoids were formed directly from somatic stem cells of the patient, another method for organoid development, and thus show levels of functionality in these areas similar to in vivo. Future studies can use the iPSC derivation and compare this functionality with the in vivo counterpart for a possible rejuvenation effect.

Even if iPSC technology progresses to the point where generating whole rejuvenated tissue or organs becomes feasible, transplantation still presents a major hurdle. iPSC-derived transplant do have the benefit of being autologous, thus avoiding issues like rejection and graft-vs-host disease. However, they still require extensive surgical procedures, which would be especially detrimental to aged patients. In addition, heterochronic transplant studies, specifically transplanting muscle from a young organism into an aged one, show that the young tissue succumb to the aged environment within a few months [59]. Multiple aged tissues and organs must be replaced together, possibly even entire systems (like immune and neuroendocrine mentioned earlier), to change the aged environment and get a lasting rejuvenation effect. These obstacles necessitate a procedure that can scale and rejuvenate multiple tissues simultaneously (which even organoids could not eventually do). Ideally, all of this would be in vivo itself. In vivo reprogramming to iPSC has been achieved in mice, using transgenic specimen but also through the injection of exogenous transcription factors. The immediate drawback is that the subsequent differentiation of the iPSCs cannot be guided because they invariably form teratomas. This actually captures the core problem behind both scalability and in vivo use: the undesired dedifferentiation. The technology iPSC reprogramming was developed to replace cells that were depleted or deficient in a patient. The rejuvenation effect of this process was discovered, essentially, as a side effect. Now with the explicit goal of rejuvenation, we can assume that the cells are already present in the patient but they be made younger, so dedifferentiation is unnecessary. This dedifferentiation necessitates complicated, lengthy, and cell type-specific redifferentiation protocols that can at best generate very rudimentary tissue. They cannot generate multiple tissue types simultaneously, cannot be carried out in vivo, and may erase some of the rejuvenation effects. Reprogramming, itself, is just a series of epigenetic changes so a subset of these must be for dedifferentiation and another subset (possibly overlapping) must be for rejuvenation. The optimal solution, now, would be to identify and execute the epigenetics for rejuvenation without those for dedifferentiation. This may be the best hope for one day utilizing the rejuvenation potential of iPSC reprogramming as a clinical treatment.

Ultimately, rejuvenation is realized on the road to pluripotency. Harnessing these mechanisms to reset the age of cells in an organism is indeed an audacious goal, but not more audacious than artificially achieving pluripotency itself. One can argue that the true scientific leap was

developing the iPSC technology, that made reprogramming controllable and generalizable to any cell type, and this new direction is just building upon, optimizing, and repurposing the technology for the ulterior motive of rejuvenation—comparably a less daunting task. We believe that as our understanding of aging become clearer, and hard evidence for age reversal becomes more prevalent, the dogma that aging is an immutable, irreversible process will be shattered. The field of medicine is fundamentally about challenging these limitations and revolutionizing the human condition. We believe the next revolution is upon us, as rejuvenation goes from mythology to gerontology.

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