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Back and Forth in Time: Directing Age in iPSC-Derived Lineages

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Abstract

The advent of induced pluripotent stem cells (iPSC) has transformed the classic approach of studying human disease, providing in vitro access to disease-relevant cells from patients for the study of disease pathogenesis and for drug screening. However, in spite of the broad repertoire of iPSC-based disease models developed in recent years, increasing evidence suggests that this technology might not be fully suitable for the study of conditions of old age, such as neurodegeneration. The difficulty in recapitulating late-stage features of disease in cells of pluripotent origin is believed to be a discrepancy between the fetal-like nature of iPSC-progeny and the advanced age of onset of neurodegenerative syndromes. In parallel to the issue of functional immaturity known to affect derivatives of pluripotent cells, latest findings suggest that reprogramming also subjects cells to a process of “rejuvenation”, giving rise to cells that are too “young” to manifest phenotypes of age-related diseases. Thus, following the significant progress in manipulating cellular *fate*, the stem cell field will now have to face the new challenge of controlling cellular *age*, in order to fully harness the potential of iPSC-technology to advance the research and cure of diseases of the aging brain.

Keywords

iPSC-disease modeling; Neurodegenerative diseases; Aging; Rejuvenation; Maturation; Induced in vitro aging

Introduction

Median age is expected to rise significantly in the next few decades, with projections estimating approximately one fifth of the world’s population to be over 60 years of age by 2050¹. This trend will escalate the incidence of aging-associated disorders, such as Parkinson’s (PD) or Alzheimer’s disease (AD). Unfortunately, in contrast to the concrete burden that these pathologies already represent for society, effective therapies are still far

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Competing interests statement

The authors declare no competing interests.

from realization. Research on neurodegeneration has been hindered by multiple factors, such as the limitations of animal models^{2,3}, or the scarcity of patient tissue. These restrictions can now be overcome thanks to induced pluripotent stem cells (iPSC), which enable the production of virtually unlimited amounts of patient-specific cells to model disease mechanisms and test potential drug candidates in vitro. This field has witnessed an explosion of reports establishing the power of iPSC technology for modeling a wide spectrum of genetic disorders and identifying novel therapeutic targets^{4,5}. However, while the use of iPSC has proven successful in recapitulating disease phenotypes of developmental or early onset conditions^{6–10}, more modest results have emerged from studies reporting on age-related pathologies like neurodegenerative syndromes^{11,12}. Although a number of relevant disease phenotypes of PD or AD have been described in patients-specific iPSC-derived neurons, these mostly correspond to early stage biochemical markers of disease and primarily represent indicators of disease susceptibility^{13–17}. In contrast, characteristic degenerative aspects of late-onset neurological conditions are usually not observed using conventional iPSC models.

A possible explanation for this differential efficacy is the fetal nature of pluripotent-derived cells, which might impede the appearance of pathological features that only arise with advanced age. Accordingly, a recent study from our group proposes that a fundamental obstacle in recreating late-stage aspects of PD in iPSC-derived dopamine neurons from PD patients can be attributed to a re-acquired cellular youthfulness through pluripotency¹⁸. These findings show that hallmarks of biological age are lost through the reprogramming process, creating a barrier for iPSC-based modeling of age-dependent disease. In parallel, this work presents a means for bypassing this constraint using strategies to accelerate cellular aging in vitro. This novel approach allowed for the first time to elicit degenerative phenotypes in an iPSC-model of neurodegeneration and moreover, laid the foundations of the concept of *in vitro aging*.

The scope of this review is to summarize current data on the age-reversing effect of somatic cell reprogramming and its impact on stem cell applications, with a focus on disease modeling of neurological disorders. We will also discuss recent advancements in devising methods for fast-forwarding cellular maturation and age of pluripotent stem cell (PSC)-derived lineages, in particular of the neuronal type. These strategies should improve the generation of functional equivalents of human tissue for cell replacement and of more realistic platforms to study age-related diseases in vitro.

1. Maturation, Aging and Rejuvenation

The life of every metazoan organism begins with the formation of anatomic structures and cell fate *specification* during embryogenesis, followed by the attainment of full cellular and tissue functionality during *maturation* and terminates with progressive physiological decay, i.e. *aging* (Figure 1).

Although the phases of maturation and aging represent a chronological continuum during ontogenesis, they bear distinct biological features and are likely controlled by non-overlapping mechanisms. In fact, while maturation describes those processes that lead to a

gain of cell and tissue functionality during late gestation or early postnatal development, aging, in contrast, is understood as the *loss* of physiological proficiency and gradual organismal decline that follows reproductive maturity¹⁹. This deterioration renders individuals increasingly susceptible to age-dependent diseases such as cancer, cardiovascular deficits and neurodegeneration. The progression of organismal development has long been considered unidirectional and likewise has the irreversibility of aging. Nonetheless, the concepts of rejuvenation and immortality are inseparable components of life, as they embody the indefinite competence of the germline to give rise to organisms of age zero from cells of adult individuals. Thus, the information for “youth” should be preserved in the genome and be therefore potentially accessible under appropriate conditions.

In agreement with this hypothesis, growing evidence suggests that running the developmental program backwards to the initial stages of life, the pluripotent state, resets the biological clock to zero, before *aging*, but also before *maturation* (Figure 1). These related yet separate aspects of reprogramming translate into distinct traits in the stem cell progeny: first, a state of immaturity that prevents cells from functionally performing equally well to their adult counterparts, and second, the elimination of molecular traces reflecting the chronological age of the cell donor.

1.1 Functional immaturity of PSC-derived cells

The unprecedented potential of pluripotent stem cells (PSC) for regenerative medicine resulted in the rapid development of differentiation protocols for the generation of any major cell lineages *in vitro*²⁰. In spite of this early success, it soon emerged that PSC-derived cells bore more resemblance to cells of the early embryo rather than to cells from adult tissues^{21,22}. This is consistent with human ontogeny but creates a major complication for the use of this paradigm in both basic research and medical applications. In fact, incomplete cellular maturity is to date a common deficit of all the most widely studied stem cell-derived lineages, such as neurons, hepatocytes, cardiomyocytes, pancreatic beta cells and the hematopoietic system.

For instance, most types of human PSC-neurons, only acquire full functionality after months of *in vitro* culture and require additional *in vivo* maturation in order to rescue neurodegenerative phenotypes in e.g. animal models of PD²³. Moreover, hepatocyte-like cells that are generated from human PSC display considerable transcriptional and metabolic differences compared to primary adult human hepatocytes, which compromises their current utility for *in vitro* toxicology assays^{24,25}. Also the employment of *in vitro*-derived cardiomyocytes has been hindered by the scarce performance of these cells after *in vivo* transplantations, such as their poor integration capacity into the host tissue or the induction of myocardial arrhythmias^{26,27}. Furthermore, efforts around pancreatic beta cell-differentiation have only recently yielded glucose-responsive insulin-secreting cells, an essential feature for the use of these cells in both disease modeling and future therapies for diabetes^{28,29}. Finally, despite the considerable clinical interest in producing pluripotent-derived hematopoietic stem cells (HSC), a major obstacle remains the generation of cells with long-term engraftment potential, a deficit mainly attributed to the primitive hematopoietic nature of *in vitro*-differentiated HSC³⁰.

In conclusion, the realization of fully functional cells from human PSC remains a crucial issue that needs to be solved before this technology can be reliably used in the clinic or serve as a realistic “surrogate patient” for in vitro drug screenings.

1.2. Cellular rejuvenation through reprogramming

In addition to bringing cells back to a state of immaturity, somatic cell reprogramming was shown by a number of independent studies to erase molecular markers of biological age, re-establishing a youthful cellular state in cells originating from old individuals. A number of phenotypic age markers were quantified prior and after transition through pluripotency, demonstrating that the majority of these age indicators were restored to a “young” level upon re-differentiation of iPSC into various lineages^{31,32}.

Reprogramming “rejuvenates” nuclear features such as overall nuclear morphology and composition, structural components of the lamina, heterochromatin content, DNA damage and telomere length, as well as global cellular properties like senescence and proliferation, mitochondrial metabolism and related oxidative stress^{18,33–39}.

While the aspects of cellular rejuvenation have thus far mainly been explored from a phenotypic perspective, virtually nothing is currently known about the genomic dynamics that govern this phenomenon. One exception is represented by a recent study reporting epigenetic rejuvenation of mesenchymal stem cells (MSC) through reprogramming. This work describes the restoration of a youthful DNA methylation “code” at specific CpG sites associated with chronological age and cellular senescence^{40,41}. Interestingly though, the authors of this study also report that donor-specific methylation patterns are retained throughout reprogramming and subsequent re-differentiation, raising the question of the interplay between epigenetic memory of the somatic tissue and epigenomic rejuvenation. In this light, determining whether residual epigenetic marks are associated with memory of either cell fate or cell age will be particularly important. The degree of age-related epigenetic memory could be implemented as an additional parameter to assess completeness of reprogramming.

In summary, although the underlying mechanism remains elusive, these findings suggest that rewinding the program of cell fate determination is capable of reinstating several aspects of cellular youth by restoring the proficiency of biological processes that were impaired in the original cell as a consequence of chronological age. Nevertheless, in spite of the compelling implications of a possible rejuvenating effect of somatic cell reprogramming, the concomitant resetting of age along with cell fate represents a concrete obstacle to the use of PSC-derivatives for both basic research and clinical purposes.

1.3 An intrinsic cellular clock dictates the timing of cell fate acquisition

Over the last two decades differentiation techniques have been greatly refined and current protocols allow directing cell fate with extraordinary precision. However, with few exceptions, this progress has not yet succeeded in advancing beyond the fetal identity of PSC-progeny⁴². In fact, some PSC-derived lineages never reach maturity using current differentiation methods or only reach full functional features after protracted in vitro culture and, in some cases, additional in vivo maturation upon transplantation²³.

It is believed that the need for such lengthy maturation phases reflects the presence of an intrinsic clock-like mechanism that dictates the dynamics of cell fate commitment *in vitro*, like *in vivo*. This theory crystallized soon after the early observation that the sequence of events occurring during ESC differentiation mirrors the order and timing of the equivalent lineage determination during embryogenesis²⁰.

The existence of a cell-autonomous pacemaker that orchestrates the chronological program of cell fate transitions has meanwhile found robust experimental substantiation. For instance, independent studies report a temporal compartmentalization of *in vitro* neurogenesis into discrete steps, reminiscent of *in vivo* brain development, where the emergence of neural progenitors is followed by a first wave of neuronal differentiation, the subsequent birth of glial cells and finally, terminal neuronal maturation^{43,44}. In particular, directed differentiation of cortical interneurons from human PSC was shown to follow a temporal program equivalent to the timing of human fetal neurogenesis^{45,46}. Remarkably, these studies indicate that the speed of maturation is governed by intrinsic species-dependent kinetics⁴⁴, as the developmental timeline of human PSC-derived neurons is maintained even upon transplantation into mouse brains and does not adapt to a faster, rodent-specific program.

These data argue for the existence of a self-sufficient, endogenous “metronome” that defines the tempo of cell fate commitment, opening intriguing perspectives on the possibility of identifying and manipulating master regulators of this process.

2. Strategies for the manipulation of cellular age *in vitro* - Maturation

The molecular mechanisms underlying the kinetics of cell maturation remain largely unknown. However, the necessity of pinpointing exact regulators of maturation timing could be obviated by applying developmental cues to enhance terminal development *in vitro*. In this light, challenges in overcoming the apparent arrest at a fetal stage of PSC-progeny could be attributed to the fact that most protocols utilize molecular signals acting on early embryonic development, whereas more complex events occurring during late gestation or even postnatally are generally not implemented. This is at least partly due to our lack of understanding of the signaling cues operating in the final phases of ontogeny as well as the constraints in faithfully reproducing *in vitro* the complex environment of a developing organism¹¹. In spite of the technical difficulties in artificially recreating late stages of development, different strategies described in the following section have produced some promising results for *in vitro* maturation (Figure 2).

2.1. Accelerated cellular maturation via defined factors

One study provided an elegant solution to this problem, by screening for chemical compounds that sped up the acquisition of post-mitotic identity upon neural induction⁴⁷. This study identified three compounds which, combined to dual-SMAD inhibition⁴⁸, yielded fully mature post-mitotic pain-sensing neurons (nociceptors) from human PSC in 10 days, as opposed to the standard >30 days maturation phases of other neuronal subtypes or to the specification of the same cell type *in vivo*. Another highly effective method for fast-tracking neuronal maturation was presented in a recent work that obtained a full conversion of human

PSC to terminally differentiated neurons by forced expression of the transcription factor neurogenin-2 (NGN2)⁴⁹. This approach generated highly pure and functionally mature neurons in two weeks, with a reduction of the maturation period to approximately one third of the timing required using standard methods.

Taken together, these examples suggest that at least for certain cell types, the activation of specific cellular pathways via chemical compounds or the expression of selected transcription factors could become a viable option to accelerate cell maturation in vitro. It is however likely that in most cases more complex conditions will be necessary to obtain *bona fide* adult cells.

2.2 Improved maturation by mimicking in-vivo conditions

A common principle adopted by several strategies aimed at accelerating in vitro maturation is the recreation of physiological conditions that mimic in vivo development. This involves recapitulating the interplay of differentiating cells with their natural cellular surroundings or target tissue in co-culture, tridimensional culture systems, or by providing dynamic fluid exchange.

Co-cultures—A cell type known to support in vivo neuronal maturation especially during postnatal development, are glial cells. Astroglial cells or astrocytes are central players in the shaping of the central nervous system (CNS) after birth, mostly contributing to late neurodevelopmental events, in particular synapse formation and consolidation^{50,51,52–54}. In agreement with their in vivo function, astrocytes were shown to stimulate functional maturation of primary neurons⁵³, and subsequently iPSC-derived neurons, in vitro. In fact, when compared to standard adherent matrices such as coating with laminin, the co-culture of differentiating neurons with primary astroglia greatly enhanced both morphological and functional neuronal development. Parameters promoted by co-culture on astrocytes include differentiation efficiency, dendritic branching, electrophysiological properties and synaptic activity⁵⁵.

The importance of cell-to-cell interactions is illustrated by the growth-promoting effects of target tissues on the developing nervous system. For example, target-derived neurotrophic and axonal guidance factors are crucial in controlling neuronal survival, synaptic connectivity, synapse formation and functional maturation⁵⁶. The interaction of motoneurons with skeletal muscle⁵⁷ or cardiomyocyte innervation by sympathetic neurons⁵⁸ represent classic model systems to study non-cell autonomous factors affecting neuronal maturation. Establishing co-culture paradigms that implement target cell types may prove particularly beneficial for establishing PSC-derived, disease-relevant, functional assays to study skeletal muscle and cardiac function^{59,60}. Taken together, these findings emphasize the importance of recreating the physiological milieu and cellular interactions in guiding cell fate dynamics towards fully functional stages.

3D—cultures (organoids)—3D culture techniques aimed at generating so-called “organoids” exploits the self-organizing capacity of PSCs to reproduce in vitro the architecture of a given tissue or organ. In some of the studies, this approach couples directed

differentiation protocols with the use of artificial scaffolds enabling the creation of highly organized structures of several of organ identities^{61–63}.

Remarkable organ complexity was achieved by the creation of cerebral organoids^{64–66}, which could yield multilayered structures of considerable fidelity in morphology and neuronal subtype composition. Those features and the similarities to the developing human fetal brain earned those rudimentary in vitro organs the term “mini-brains”. Another prominent example of a 3D replica of a CNS tissue are retinal organoids. In this case the tridimensional in vitro structure was obtained through directed differentiation of PSCs into self-organizing precursors that faithfully recapitulated both the temporal and morphogenetic progression of embryonic eyecup formation. Data from those studies provided novel insight into human eye organogenesis by demonstrating the autonomous self-organizing properties of early embryonic precursors^{67,68}.

However, in spite of the striking recapitulation of tridimensional organ structure, neuronal organoids did not reach beyond early fetal stages, as suggested by the lack of six cortical layers, mature pyramidal neurons and significant contribution of glial cells in the case of the “mini-brains” or the lack of late-born neurons such as bipolar cells in the eyecup organoids⁶¹.

Nonetheless, recent implementation of 3D organoids into a stem cell model of Alzheimer’s disease allowed for a substantial breakthrough in reproducing in vitro AD features unseen in previous cellular or animal AD models, such as β -amyloid plaques and neurofibrillary tangles⁶⁹. In this study, the mutant forms of human β -amyloid precursor protein (APP) or presenilin 1 (PSEN1), responsible for familial forms of AD (FAD) were overexpressed in human neural stem cells (ReN) and differentiation was conducted in 3D by embedding cells into Matrigel (BD) droplets. This strategy allowed for the first time to detect the accumulation of multimeric β -amyloid and phosphorylated tau aggregates in a genetic model of AD.

Microfluidics – organ on a chip—An even more sophisticated approach to simulate physiological conditions are so-called microfluidics systems, based on microscale devices for highly controlled liquid exchange. This “organ on a chip” technology greatly improves the delivery of nutrients and growth factors, creating a more comprehensive niche for the support of cell differentiation and maturation. This strategy was capable of recreating dynamic multi-tissue structures such as e.g. the blood-brain-barrier⁷⁰ and neurovascular units⁷¹. Finally, microfluidic chips enclosing polymeric scaffolds have been utilized to generate in vitro the layered organization of primary cortical neurons, resulting in enhanced neuronal connectivity⁷².

To date only few studies have incorporated these devices into PSC-differentiation models, probably due to the challenges in reproducing the organ microenvironment as well as the cost and laboriousness of this technique. Nevertheless, early results indicate that increasing the complexity of the in vitro niche could hold great promise for building more realistic replicas of human tissue for basic research and pharmacological purposes.

In conclusion, the generation of functionally mature PSC-derivatives still represents a bottleneck for the broad applicability of stem cell technology to both disease modeling and cell replacement. Yet, recent efforts to reproduce the complex biochemical and three-dimensional surroundings of a developing tissue have not only pushed in vitro maturation, but have also enabled breaking barriers of iPSC disease-modeling. These results highlight the importance of recreating a physiological environment for terminal cell fate determination aimed at modeling disease etiology in vitro.

3. iPSC models of neurological diseases

3.1 Neurodevelopmental and juvenile diseases

iPSC-technology represents a game changer for the study of human genetic disease, in particular for those fields of research that have long been paralyzed by the lack of primary tissue, e.g. specimen from neurological disorders⁷³. The possibility of producing in a dish unlimited amounts of patient-specific cells of disease-relevant lineages circumvents this obstacle and furthermore allows for personalized in vitro drug-screenings. In this light, a long series of studies have established the power of iPSC for efficiently modeling human diseases, discovering mechanisms of pathogenesis and identifying novel therapeutic compounds. These works encompass a large number of pathologies inside and outside of the CNS, but for the scope of this review we will only focus on milestone studies of iPSC modeling of neurological conditions.

The first ever iPSC model of a human disease described the generation of motor neurons from iPSC of spinal muscular atrophy (SMA) patients¹⁰. After short periods of in vitro culture, mature iPSC-derived motor neurons from SMA patients showed disease-specific degeneration and cell death, recapitulating the clinical observation that SMA patients display correct embryonic development of motor neurons, but show first signs of muscle degeneration 6 months after birth.

Shortly after, a study from our group presented a first example of iPSC disease modeling coupled to successful in vitro drug screening and identification of therapeutic compounds^{7,8}. In this work, iPSC from Familial Dysautonomia (FD) patients were differentiated into neural crest (NC) precursors and the NC-specific splicing defects of the *IKBKAP* gene responsible for the disease were confirmed in vitro. Mis-splicing, cell differentiation and migration defects could be rescued through a known candidate drug and later by a newly identified molecule⁸.

In a separate report, a novel mechanism of pathogenesis involving non-hematopoietic immunodeficiency was discovered using iPSC derived from patients affected by Herpes simplex encephalitis (HSE)⁶. While infection with Herpes Simplex virus (HSV) is ubiquitous and harmless for the vast majority of the population, it can lead to the development of life-threatening encephalitis in children carrying a mutation for Toll-like receptor-3 (*TLR-3*) or other crucial regulators of innate immunity. Differentiation of iPSC with *TLR-3* mutations into CNS lineages revealed neural-specific deficits in HSV immunity, caused by impaired cell-intrinsic defense through interferon-signaling (IFNs). These results established the role of cell-autonomous, non-hematopoietic immunity as a primary defense

against infective diseases and highlighted the value of iPSC technology in uncovering novel pathogenic mechanisms.

3.2 Modeling neurodegeneration with iPSC

The promising results obtained by these studies suggested that the same approach could yield valuable insight also for the etiology of other classes of neurological diseases with a genetic component, such as neurodegenerative syndromes. Accordingly, several groups set out to create iPSC models of most familial forms of late-onset neurological conditions, like AD, PD, Huntington's Disease and Amyotrophic Lateral Sclerosis (ALS).

Parkinson's disease is one of the most common forms of aging-dependent neurodegeneration and is caused by a progressive loss of neuronal cell types, in particular dopaminergic (DA) neurons in the substantia nigra pars compacta. Although the majority of PD is sporadic and hence has no known genetic origin, specific mutations have been identified as the cause of familial PD. Dominant mutations in the α -synuclein (*SNCA*) or leucine rich repeat kinase 2 (*LRRK2*) genes or recessive loss-of function mutations in the PTEN-induced putative kinase 1 (*PINK1*) or Parkin (*PARK*) genes are the most prominent causes of inherited PD⁷⁴. Parkinsonian brains from familial or sporadic forms of PD are characterized by the appearance of distinctive cellular inclusions known as Lewy bodies, which contain aberrant aggregates of α -synuclein and ubiquitin^{75,76}. Given the high impact of PD on the health status of an increasingly aging population and the poorly understood pathogenesis of this disease, the introduction of iPSCs prompted a wave of studies aimed at reproducing PD in a dish. Models were derived for all major genetic forms of PD and the resulting studies described a number of PD-related biochemical features as well as hypersensitivity to certain stressors^{13,14,18,77–88}. Among the most commonly observed cellular PD phenotypes were elevated α -synuclein expression, mitochondrial dysfunction and hypersensitivity to oxidative stress or other toxic compounds.

Similarly to PD, a large effort was devoted to the creation of iPSC models of AD, which accounts for 70% of dementia cases in the aging population⁸⁹. AD is characterized by neuronal and synaptic loss in cortical and subcortical brain regions. For both PD and AD, protein misfolding is considered the primary culprit of AD pathogenesis, the major players being plaques of β -amyloid aggregates and neurofibrillary tangles composed of hyperphosphorylated Tau protein. The most prevalent hereditary forms of AD, caused by mutations in amyloid precursor protein APP or presenilin 1 and 2 genes (*PSEN1/2*) have been modeled using iPSC, shedding new light onto early stages of the disease such as increased β -amyloid levels, Tau phosphorylation and enhanced GSK3 β activity^{15,16,90,91}.

ALS is a devastating neurodegenerative condition typically arising between the ages of 50 and 60 and defined by a rapid decay of motor neurons and consequent muscular paralysis. Known familial variant of ALS are due to mutations in the genes superoxide dismutase 1 (*SOD1*), *TDP-43*, *VAPB* and expansion of the *C9orf72* locus. iPSC have been generated from patients affected by any of the above mentioned genetic ALS forms as well as from patients with sporadic ALS⁹². Those studies reported on varying degrees of pathological phenotypes^{92–97}. Again, markers of disease predisposition comprised biochemical features such as high levels of mutant proteins or transcripts. Late disease features such as plaque

formation or cell death were either not detected or only induced upon exposure to toxic compounds, respectively.

While the previous pathologies mostly occur in a sporadic form and only a minor portion is hereditary, Huntington's disease (HD) in contrast is a predominantly familial disease caused by the expansion of the CAG triplet in the gene huntingtin (*HTT*). HD affects medium spiny neurons in the basal ganglia, whose extensive loss in HD leads to a broad spectrum of symptoms ranging from psychiatric to cognitive and motoric deficits⁹⁸. iPSC modeling of HD from patients' cells with variable lengths of expanded repeats revealed a range of transcriptional and metabolic phenotypes indicative of underlying disease-related deficits^{99–101}.

Such studies demonstrate how iPSC-based models of neurodegenerative disorders can shine light on early phases of disease development, previously inaccessible to investigation in humans. Recreating initial stages of disease pathogenesis is an essential step in understanding the biochemical events leading to the subsequent degeneration. Identifying early cellular aberrations present already in pre-symptomatic individuals will be key for defining disease biomarkers and developing novel therapies that may counteract disease progression.

However, although these studies provide precious insight on the initiating cellular events of disease pathogenesis, the absence of typical late-stage features of disease remains a major shortcoming of in vitro models of neurodegeneration. A common denominator of late neurodegenerative traits across different syndromes are e.g. the formation of macromolecular protein aggregates (plaques) and the progressive deterioration of neuronal structures resulting in cell death. Neither of these conventional models of late-onset neurological conditions succeeded in recapitulating these advanced features and in most cases selective cell death was only achieved upon acute treatments with ectopic stressors.

4. Strategies for the manipulation of cellular age in vitro - Aging

4.1 Toxic stressors

The limited efficacy of using iPSC to recreate advanced phenotypes of late-onset diseases illustrates the fact that genetic predisposition is often not sufficient to trigger the emergence of a given pathology, neither in vivo nor in vitro. In fact, most conditions require a synergistic action of genetic and environmental or biological factors. For certain disorders such as PD, exposure to pesticides or dysfunctions in mitochondrial pathways have been robustly implicated in disease pathogenesis¹⁰². Accordingly, several iPSC models of genetic PD have sought to promote the appearance of disease phenotypes by challenging the cells with common pesticide toxins or reactive oxygen species (ROS)^{13,78,81,83,85,86} (Figure 3).

The rationale behind an acute exposure of cells to toxic factors is to compress the time period required for accumulation of DNA- and other types of molecular damage that in a patient occurs over an entire lifespan, within an experimentally appropriate timeframe. This strategy has considerably facilitated the manifestation of intrinsic cellular and biochemical hypersensitivities likely brought about by genetic background. Nevertheless, such treatments

consistently failed to recapitulate key aspects of disease such as high order protein aggregation, consequent formation of plaques or inclusion bodies and gradual degeneration of cellular structures. These late cellular phenotypes represent the stage at which neurodegenerative diseases are most frequently diagnosed and therefore dominate disease pathophysiology and manifestation of clinical symptoms in actual patients. Hence, in order to create representative in vitro models of neurodegeneration with a real utility for the identification of novel therapies, it is of utmost importance to be able to reproduce degenerative hallmarks in vitro.

4.2 In-vitro Aging

Aging is the single most important risk factor for the onset of neurodegeneration. In fact, even for familial forms of PD or AD, patients carry the pathogenic mutations throughout their lives, but only develop the disease with old age. Thus, in the same way that age is required to trigger symptoms of late-onset diseases in predisposed individuals, it is conceivable that age needs to be implemented in iPSC models in order to elicit the appearance of late-onset pathological traits in vitro (Figure 3). Manipulating cellular age represents a considerable experimental challenge and ideally would require a mechanistic understanding of the aging process. However, the highly pleiotropic nature of this process renders it seemingly impossible to discern between causative events and secondary effects. Accordingly, whether aging is to be considered a passive accumulation of damage over time or rather an actively regulated state, is still debated¹⁰³. In favor of a programmed nature of aging is the robust evidence that its pace can be bi-directionally modulated by numerous interventions and in several model organisms or even experimentally reversed in contexts such as parabiosis or, as detailed in this review, reprogramming to pluripotency^{104–106}.

Because of its profound implications for human life, the biology of aging has never ceased to be the object of intensive study, leading to the accumulation of a wealth of information on its endless physiological manifestations and molecular players. This knowledge can now be exploited to devise methodologies to *measure* and *induce* cellular age in an experimental setting.

4.2.1 How to measure age in vitro—Distinctive aging marks appear at the level of tissues as well as single cells and give rise to the global physiological decline that characterizes an aging organism. Systemically, aging is accompanied by the exhaustion of tissue-specific stem cells, an increased ratio of senescent cells and a global inflammatory state, also known as “inflammaging”¹⁰⁷.

At the cellular level, aging affects all compartments of the cell, ranging from impaired nuclear regulation which leads to DNA damage, altered nuclear morphology, loss of heterochromatin, transcriptional noise and telomere attrition, to compromised biochemical processes such as mitochondrial dysfunction, reduced proteostasis and macromolecular turnover. These aging-associated cellular alterations ultimately result in an overall metabolic deficit and cumulative damage of both structural and catalytic cellular components¹⁰⁷.

Based on these commonly accepted hallmarks, our recent study employed a set of seven criteria for the in vitro quantification of cellular age: nuclear morphology, levels of

heterochromatin-specific marks (H3K9me3, H3K27me3, HP1 γ), DNA-damage (γ H2AX) foci, nuclear lamina components (Lamin A/C&B1, LAP2 α), mitochondrial ROS production, telomere length and cellular senescence measured by the activity of senescence-associated β -galactosidase (SA- β -Gal)¹⁸. These parameters are readily measurable in an experimental setting and collectively define a “phenotypic aging signature”.

In parallel to phenotypic aging markers and in accordance with the profound effect of biological age on nuclear functions, a robust “genomic aging signature” has been described. Generally, aging is associated with a global loss of epigenetic silencing, reflected both by a decrease of repressive histone modifications as well as a widespread state of DNA hypomethylation^{31,107}. This is in contrast to the simultaneous acquisition of local hypermethylation preferentially at promoters and CpG-islands¹⁰⁸. In agreement with the frequently reported changes in DNA methylation patterns with aging, a series of independent studies claim the existence of specific CpG sites, whose methylation state very strictly correlates with chronological age in a tissue-specific or -invariant manner. According to the authors, these methylation “clocks” could be effectively utilized in forensics or in some cases as predictors of individual longevity^{41,109,110}. Although these criteria would likely require higher cost and -complexity assays to be assessed compared to phenotypic markers, they could potentially provide a more accurate means to monitor the dynamics of cellular age in vitro.

4.2.2. Induced aging strategies—In spite of the ancient ambition of mankind to understand and control aging, no consensus has yet been reached on the nature or even the existence of a universal cause of aging. Nevertheless, decades of intensive investigation have revealed a number of evolutionarily conserved hallmarks that both coincide with aging and at the same time are capable of modifying the pace of its progression, thereby establishing causative significance for this process. Virtually any of these known aging regulators has been experimentally manipulated in different model organisms, achieving varying degrees of lifespan extension or reduction^{104,107}. However, a major problem for understanding and therefore manipulating the biology of aging is the deep interconnection and reciprocal control of its molecular components, which renders the establishment of a regulatory hierarchy extremely difficult.

Ideally, strategies for inducing (or reversing) age should be aimed at targeting effectors on top of the hierarchy of the aging program. Assuming that such a well-defined hierarchy actually exists, such a trigger would subsequently induce a broad and likely multifaceted downstream cascade mimicking physiological aging.

Aging-dependent transcriptional networks: In light of the extraordinary success of manipulating cell fate transitions by forced expression of defined factors, key transcriptional regulators could be employed to convert *age* instead of *identity*. However, the long quest for identifying master regulators of aging, driven by the hypothesis of aging being a programmed state, has to date only yielded ambiguous results. In fact, numerous comparative gene expression analyses of young and old tissues have so far failed to identify conserved transcriptional networks underlying the aged state¹¹¹. In contrast, aged tissues display a general increase in transcriptional noise and deregulation^{112,113}. One possible

exception is the consistent age-dependent transcriptional change detected throughout different studies involving hyperactivation of inflammatory pathways¹¹¹. In particular, signaling downstream of the ubiquitous N- κ B transcription factor has been repeatedly implicated in physiological aging^{114–116}. Moreover, interventions targeting this pathway have been shown to exert an instructive role on promoting or preventing aging at the organismic level^{115,117}. Based on these data, N- κ B activation by genetic or pharmacological means could represent a viable option as a potential age-inducing approach.

Signaling pathways involved in aging: In agreement with the importance of studying systemic conditions and niche factors in determining the biological age of tissues and organs, broad experimental evidence indicates that exposure to a differentially aged (heterochronic) environment is capable of affecting aspects of physiological age of cells and tissues. This is achieved by heterochronic parabiosis, the surgical connection of blood circulation between an old and a young animal¹⁰⁵. A series of studies employing this technique showed that not only does young blood have a rejuvenating effect on old tissues, vice versa young organs acquire a more aged physiology when subjected to old blood^{118,119}.

Among the circulating factors responsible for bi-directionally affecting tissue age, members of the Notch-, Wnt- and TGF β - pathways have been implicated^{105,120,121}. In light of their central role during embryonic development and cell differentiation, the involvement of these pathways in systemic aging opens the intriguing possibility of aging being a potentially developmentally controlled process. Furthermore, these pathways represent key morphogenetic circuits commonly manipulated in current in vitro differentiation protocols of human PSCs. Hence, a broad spectrum of recombinant factors or small compounds has been developed for targeting specific branches of these signaling pathways. These compounds could be screened for a potential pro- or anti-aging effect on terminally differentiated cells mimicking the concept of heterochronic parabiosis in vitro. To study the impact of such signaling pathways on age-related cellular phenotypes it may be important to implement more complex cultures systems. For example studying non-cell autonomous factors and cell-to-cell interactions, as discussed above in the context of maturation (Section 3.2), might facilitate the appearance of pathological phenotypes. Of particular interest for in vitro models of neurological disease is the co-culture with patient-specific astroglia, which may contribute to both neurodevelopment and neurodegenerative disorders¹²².

Aging the epigenome: Aging is accompanied by a wide spectrum of alterations to the epigenome, which involve changes in histone marks, direct DNA modifications and tridimensional chromatin architecture^{123–125}. As for most cellular and physiological changes associated with aging, it remains difficult to determine whether these alterations are to be considered as cause or effect. However, the apparent reversibility of an aged cellular state through pluripotency opens the distinct possibility of aging being the result of active or passive fluctuations in the epigenetic landscape, which is subject to profound remodeling and possible restoration to a youthful state as a consequence of reprogramming.

A consistent age-dependent epigenomic change observed across diverse species and tissues is a drift towards epigenetic de-repression, both reflected by the decrease in global levels of silencing histone modifications (see previous chapter) as well as DNA methylation. In

parallel, aged cells and tissues also acquire hypermethylation at selected loci, in particular around gene promoters and CpG-islands^{108,126}.

A direct causative link between aging and this epigenomic drift has not been explored, while its significance has been clearly established for tumorigenesis^{127–129}. Cancer cells are in fact characterized by a very similar set of epigenetic alterations as observed in aged tissues, which might not be coincidental given that age is the main risk factor for most cancers. It is in fact conceivable that epigenetic erosion with age could have profound repercussions on various aspects of nuclear metabolism eventually resulting in the dysfunctional genomic state of aged and tumor cells. Therefore, exploring the possibility of inducing local or global chromatin alterations by direct manipulation of chromatin modifiers or by newly developed techniques for targeted epigenetic engineering^{130,131} might shed new light on the mechanisms of aging and open interesting avenues for directing genomic aging.

Premature aging syndromes: The lack of molecular understanding of the aging process and the corresponding difficulty in pinpointing a pathway that best reproduces the complexity of the aged phenotype, might be overcome by taking advantage of naturally occurring shortcuts. Obvious examples include genetic disorders leading to premature aging in humans. Among the most prominent progeria- and progeroid syndromes are Hutchinson-Gilford Progeria- (HGPS), Werner- (WRN), Cockayne Syndrome (CS) and Ataxia Telangiectasia (AT). Molecular defects underlying these pathologies have been identified, most of which affect pathways involved in broad (as in the case of HGPS) or specific pathways of nuclear function, in particular DNA repair. Although the direct relevance of premature aging syndromes for physiological aging is still debated, these diseases undeniably recapitulate to varying degrees aspects of human aging, such as shortened lifespan, cardiovascular and skeletal problems, loss of hair and subcutaneous fat tissue or other-aging related aspects of physiological decay¹³². Moreover, with the goal of selecting effective and technically feasible strategies to phenocopy aging in a dish, these diseases represent a very attractive entry point.

Progerin-induced in vitro aging: The potential of the above mentioned approach is illustrated by a recent study from our group, which took advantage of the genetic mutation responsible for the most aging-relevant and deleterious form of progeria, namely Hutchinson Gilford Progeria (HGPS)¹⁸. This disease is caused by a silent point mutation in the human Lamin A (*LMNA*) gene, which leads to defective splicing and subsequent aberrant post-translational processing of Lamin A, giving rise to a truncated variant known as Progerin¹³³. Lamins are integral components of the nuclear envelope, involved in virtually any aspect of nuclear metabolism¹³⁴. Accordingly, accumulation of the mutant protein Progerin in the nucleus of HGPS patients interferes with many aspects of nuclear physiology and leads to abnormal nuclear shape, genomic instability and aberrant epigenetic landscape. These nuclear defects also have downstream repercussions on cellular physiology such as mitochondrial dysfunction and increased production of ROS¹⁸.

Since among the known progeroid syndromes HGPS pathophysiology best reflects the broad range of aging-associated manifestations¹³² and expression of progerin is the only known disease-causing mutation of HGPS, we reasoned that expression of progerin in iPSC-derived

cells might represent a viable means to accelerate cellular aging in vitro. In further support of our hypothesis, we could assess that cells from HGPS patients share a wide range of cellular hallmarks that are characteristic of cells from old donors¹⁸.

Our prediction on the pro-aging effect of progerin expression proved correct, as the introduction of progerin in iPSC-derived cells successfully triggered the expression of the known cellular aging hallmarks. The main scope of our endeavor was to induce aging in iPSC-derived cells of neuronal lineages, aimed at eliciting age-dependent disease phenotypes of neurodegeneration in iPSC disease-models. However, HGPS patients do not display neurological deficits or premature neurodegenerative traits, which is attributed to the expression of mir-9, a CNS-specific micro-RNA that targets Lamin A and progerin¹³⁵. Strikingly though, treatment with progerin was capable to prompt the appearance of a subset of aging markers also in postmitotic iPSC-derived midbrain dopamine neurons (mDA) and additional, cell-type specific phenotypes associated to neuronal aging such as gene expression changes associated to neuronal aging or, upon in vivo transplantation, accumulation of neuromelanin.

Finally, we tested whether progerin expression would reveal age-related pathological phenotypes in iPSC-models of PD. Expression of progerin in iPSC-mDA neurons from two independent familial forms of PD resulted in the appearance of cellular PD-phenotypes not detected in previous iPSC models, such as dendrite degeneration, activation of pro-apoptotic signaling or impairment of the PD-specific AKT pathway¹⁸.

These results argue for the synergistic action of genetic predisposition and age in the manifestation of late-onset phenotypes in iPSC models of disease.

Direct conversion (Transdifferentiation): Finally, cellular immaturity and age erasure of iPSC-progeny is generally believed to stem from the transition through the pluripotent state and consequently, the reversal of cell fate to a very early developmental stage. Reprogramming to the embryonic state can however be avoided by direct conversion of mature lineages into different mature cell types, bypassing a pluripotent intermediate. Transdifferentiation techniques have been explored for various lineages, including the generation of disease-relevant neuronal subtypes^{136,137}. It will therefore be highly relevant to investigate whether transdifferentiation retains cellular age and accordingly, whether patient-specific cells obtained via this method display age-dependent disease phenotypes without requiring any age-inducing intervention.

Discussion

In less than a decade the stem cell field has taken giant leaps in the manipulation of cellular *fate*, making it possible to soon recreate virtually any known cell type from pluripotent stem cells in a dish. Now, a new challenge has emerged, which is controlling the cellular *age* of differentiated cell lineages, both developmental and postnatal, in order to achieve realistic in vitro replicas of human tissue.

The maturity gap between PSC-derivatives and their adult in vivo counterparts is an issue long known to the field and considerable progress has been made to improve cell maturation.

Examples include the targeted activation of key pathways or the adoption of new culture strategies that implement tissue complexity. In contrast, it has only lately become clear that loss of biological age that accompanies reprogramming might hamper the use of stem cell technology for modeling conditions of old age. These findings indicate that *age* might therefore be yet another component of cell identity that at least in some cases will need to be incorporated into the ensemble of differentiation signals. However, manipulating cellular age represents an exceptionally bold endeavor, particularly in light of the fact that the biology of aging remains one of the least understood fields of science. Hence, a scenario in which it will suffice to add just one more compound to the differentiation cocktail to yield *aged* cells might seem naïve. Yet, sometimes processes can be manipulated even without complete insight into their mechanisms. As shown by the example of progerin-induced aging, the employment of a molecular shortcut provided by nature can prove surprisingly efficacious in manipulating even a poorly understood phenomenon such as aging. This one-factor strategy allowed not only to re-establish markers of cellular age in iPSC-derived cells that had been “rejuvenated” through reprogramming, but also to elicit degenerative cell phenotypes in iPSC-neurons from PD patients.

While it remains to be determined to which extent progerin is capable of recapitulating true physiological aging and whether this strategy is applicable to the modeling of other late-onset diseases, it remains to date the first proof of principle that cellular aging can be manipulated in vitro and that such an intervention may be necessary to bridge the gap between the age of iPSC-derived cells and the onset of age-associated diseases. Although this field is still in its infancy, the wealth of knowledge accumulated on the molecular pathways involved in aging will hopefully prove useful in devising novel and accurate strategies to accelerate cellular age. Such “*induced aging*” paradigms may contribute to building faithful iPSC models of human disease.

Finally, these findings shine new light on one aspect of reprogramming that has only received limited attention since the discovery of iPSC, namely the phenomenon of *rejuvenation* through reprogramming. In fact, breaking the dogma of the irreversibility of cell fate determination has spurred a wave of efforts directed at understanding the molecular pathways leading to the reacquisition of *potency*. In contrast, much less emphasis was put on understanding the mechanisms of reacquiring *youth*.

The notion that cellular age might be a reversible process, even if coupled to the reprogramming of cell identity, opens novel avenues for understanding the mechanisms that dictate aging and provides unprecedented opportunities for “directed cell rejuvenation” in the future.

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Highlights

- Reprogramming cells to pluripotency rewinds the biological clock of cell *maturation* and *age*
- Immaturity and rejuvenation hinder both basic and clinical applications of iPSC-derived cells
- We discuss current and future methodologies for improving cell maturation
- iPSC disease-modeling presents limitations for age-dependent diseases due to cell rejuvenation
- We introduce the concept of “*induced in vitro aging*” for modeling late-onset disorders using iPSC

Figure 1.

Waddington Landscape depicting the continuity of the developmental phase of specifying cell identity with the later stages of ontogenesis including cell maturation and aging. Reversibility of all three steps can be achieved through the reprogramming process. Cells are represented as spheres and the subsequent temporal dynamics of for a given type through maturation and age are reflected by their changing colors.

Inducing Maturation

Figure 2.

Techniques to promote cellular maturation of hPSC derived lineages. A) 2D strategies include the identification of optimized cocktails of directed differentiation, co-cultures with developmentally relevant cell types such as astroglia, or directed conversion of somatic cells to induced neurons through forced expression of defined factors such as *NGN2*. B) Accelerated maturation has also been achieved through three-dimensional approaches such as the in vitro generation of organoids with highly complex structures including cerebral (mini-brains) or retinal organoids. Improved connectivity and network formation was

achieved by culturing primary neurons in microfluidic devices (organ-on-a-chip), however this approach has not yet been fully implemented for PSC-derived neurons (dashed line).

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Induced Aging

Figure 3.

Inducing cellular age in hPSC-derived lineages for improved modeling of late-onset disorders. Somatic cells are isolated via biopsy from a patient (as illustrated here for PD). These primary cells are reprogrammed to iPSC and re-differentiated into disease-relevant neuronal subtypes for in vitro disease modeling and drug-screening. Cell fate transitions in vitro (upper part of the figure) represent matched developmental and ontological stages in vivo (lower part of the figure). For example, the iPSC stage is equivalent to the pre-implantation embryo (blastocyst/early epiblast), while mature iPSC-derived neurons correspond to brain cells of young adults. In fact, neurons differentiated from iPSCs appear rejuvenated and apparently healthy in spite of the presence of a disease-causing genetic mutation (purple nuclei), similarly to their in vivo equivalents in young, pre-symptomatic patients. The onset of neurodegenerative symptoms in patients requires the synergistic action of genetic background, environmental factors and importantly, *age*. Accordingly, manifestation of late-onset disease-phenotypes in iPSC-derived neurons is dependent on challenging cells with either toxic compounds (ROS, pesticide toxins) or age-inducing

genetic factors (progerin). While the exposure to toxic agents has proven successful in revealing early biochemical indicators of disease susceptibility and hypersensitivity to metabolic stress (red neurons with intact structures, cell death represented as faded neurons), treatment of cell with progerin has succeeded in triggering typical age-dependent degenerative phenotypes such as dendrite shortening and neuromelanin accumulation (grey neurons with dark spots). However, it remains to be determined whether alternative approaches based on known molecular aging mechanisms (transcriptional networks, signaling pathways, epigenetic manipulations or other progeroid syndromes) can further improve on currently available patient-specific models. Alternatively, it will be important to assess whether induced neurons established via direct conversion will prove efficient in faithfully inducing physiological age in vitro (dashed arrow lines) and modeling age-dependent disorders.

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