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REVIEW ESSAY

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Epigenetic rejuvenation by partial reprogramming

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Abstract

Rejuvenation of cells by reprogramming toward the pluripotent state raises increasing attention. In fact, generation of induced pluripotent stem cells (iPSCs) completely reverses age-associated molecular features, including elongation of telomeres, resetting of epigenetic clocks and age-associated transcriptomic changes, and even evasion of replicative senescence. However, reprogramming into iPSCs also entails complete de-differentiation with loss of cellular identity, as well as the risk of teratoma formation in anti-ageing treatment paradigms. Recent studies indicate that partial reprogramming by limited exposure to reprogramming factors can reset epigenetic ageing clocks while maintaining cellular identity. So far, there is no commonly accepted definition of partial reprogramming, which is alternatively called interrupted reprogramming, and it remains to be elucidated how the process can be controlled and if it resembles a stable intermediate state. In this review, we discuss if the rejuvenation program can be uncoupled from the pluripotency program or if ageing and cell fate determination are inextricably linked. Alternative rejuvenation approaches with reprogramming into a pluripotent state, partial reprogramming, transdifferentiation, and the possibility of selective resetting of cellular clocks are also discussed.

KEYWORDS
ageing clock, DNA methylation, epigenetic, interrupted reprogramming, iPSC, partial reprogramming, pluripotent, rejuvenation, reprogramming, transient reprogramming

INTRODUCTION

Cellular ageing is reversed by reprogramming in induced pluripotent stem cells

Ageing is a complex process that entails continuously increasing dysfunction and degeneration, susceptibility to disease, and, ultimately, death. While this process is inevitable and associated with continuous molecular changes, it is remarkable that most effects of ageing can be reversed at the cellular level. The groundbreaking work by Takahashi and Yamanaka in 2006 demonstrated that somatic cells could be reprogrammed into induced pluripotent stem cells (iPSCs) by the overexpression of four transcription factors (Oct3/4, Sox2, Klf4, and c-Myc, now referred to as the "Yamanaka factors" or "OSKM" factors), altering the epigenetic landscape of these cells.[1] While in the pluripotent state, iPSCs can be passaged extensively, without any signs of cellular ageing. Seminal work by Marion et al. showed that even reprogramming of cells from elderly animals resulted in iPSCs with elongated telomeres, efficient telomere capping, and a heterochromatin profile similar to embryonic stem cells (ESCs).[2] Fibroblasts from very old donors and centenarians could also be reprogrammed into iPSCs by OSKM overexpression – and even more effectively in

Abbreviations: CpG, CG dinucleotide; DNAm, DNA methylation; ESC, embryonic stem cell; iMSC, iPSC-derived mesenchymal stromal cell; iPSC, induced pluripotent stem cell; MSC, mesenchymal stromal cell; OSKM, Oct3/4, Sox2, Klf4, and c-Myc; PBMC, peripheral blood mononuclear cell; RGC, retinal ganglion cell.

combination with Nanog and LIN28.[3] For example, B-lymphoblastoid cell lines derived from a supercentenarian (114-year-old donor) were reprogrammed, and the iPSCs exhibited complete erasure of the age-associated transcriptomic signature and resetting of telomeres.[4] Last but not least, iPSCs derived from elderly mice could give rise to healthy newborn mice that pursue normal ageing.[5] Aging may also involve accumulation of somatic mutations, which are certainly not repaired by the reprogramming procedure. Yet, the acquisition of mutations may rather be a side effect than the underlying mechanism of aging. Either way, there is compelling evidence that molecular features of ageing can be reversed by reprogramming into the pluripotent state.

On the other hand, if the iPSCs are re-differentiated toward mature cell types, they keep this rejuvenated phenotype. For example, when T cell-derived iPSCs were re-differentiated into $CD8^+$ T cells, they maintained high proliferation and elongated telomeres[6]; when iPSCs that were derived from skin fibroblasts of aged humans were re-differentiated into neurons[7] or fibroblasts,[8] their age-associated transcriptomic changes, nucleo-cytoplasmic compartmentalization, and oxygen consumption levels remained rejuvenated; and iPSC-derived mesenchymal stromal cells (iMSCs) also stayed epigenetically rejuvenated.[9] This aspect should be considered, because iPSC-derived cells are often used as model systems for age-associated diseases, for example, iPSC-derived neurons for research in Alzheimer's disease.[10] However, these in vitro differentiated cells somewhat resemble a fetal phenotype with regard to telomere length or epigenetic age. So far, it is impossible to accelerate these age-associated cellular changes in the iPSC-derived cells to facilitate better mimicking of the physiological disease state of the elderly.

Resetting DNA methylation clocks

DNA methylation (DNAm) is one of the central epigenetic modifications, which emerged across different kingdoms of life and revealed a range of essential biological functions.[11] DNAm was initially described in the context of cellular differentiation and development,[12] but epigenetic patterns changes also continuously with aging of the organism.[13] In the mammalian system, DNAm occurs particularly in the context of CG dinucleotides (CpG sites). So far, it is largely unclear how the tight regulation of DNAm patterns at specific genome sites is governed. While DNAm is generally considered to be associated with gene repression, it may also activate gene expression – depending on where it occurs within a gene and how it affects the binding of transcription factors and enhancers.[14] Furthermore, there is evidence that DNAm patterns reflect modifications in the histone code.[15] Even if the direct functional relevance of DNAm remains unclear, it can undoubtedly provide beneficial biomarkers for cellular composition, various diseases, and particularly for the ageing process.[16]

More than a decade ago, the first epigenetic age-predictors based on DNAm, called epigenetic clocks, were described.[17,18] Since then, numerous epigenetic clocks have been developed to predict chronological age. They were particularly trained for blood,[19–21] while others have even proven to be applicable across many tissues.[22] Notably, there is evidence that the deviation of predicted and chronological age is associated with all-cause mortality and prevalence of age-associated features, indicating that epigenetic clocks provide a measure for biological age rather than chronological age.[13,21,23]

Interestingly, upon reprogramming into iPSCs, virtually all age-associated DNAm changes are reversed.[20,22,24] The predicted epigenetic age is thus close to zero years, even if reprogramming was performed on cells from elderly donors. This resetting occurs parallel to DNAm changes in pluripotency-associated CpGs,[25,26] indicating that resetting cellular identity to a pluripotent state and rejuvenation might be directly linked.

Resetting long-term culture-associated DNA methylation changes

In addition to the ageing of the organism, long-term in vitro cell culture is also reflected by specific DNAm changes. Cells can only be propagated for a limited number of passages until they reach a state of replicative senescence, and DNAm changes are found in specific senescence-associated CpGs.[27] In fact, they are continuously acquired during cell culture and can therefore be utilized to estimate the in vitro culture time. Epigenetic clocks and culture-associated DNAm changes tick independently, albeit with some overlap in these signatures. Notably, the culture-associated epigenetic clocks can also be reversed by reprogramming into the pluripotent state.[24,25,28] However, in contrast to the epigenetic ageing clocks, the culture-associated DNAm changes are gradually reacquired as soon as the cells exit from the pluripotent state.[9] In the future, it will be interesting to understand better if specific culture conditions, co-culture, or matrices can modulate the gradual re-acquisition of culture-associated DNAm patterns.

Resetting other types of epigenetic marks

In addition to DNAm, other epigenetic modifications have also been associated with ageing. DNA is packaged by core histone proteins that are subject to numerous post-translational modifications.[29] These histone modifications regulate gene expression by governing the accessibility of the transcription machinery. Many studies demonstrated that the histone code is also altered in an age-dependent manner.[30] These changes include a reduction in bulk histone levels, a global decrease in repressive histone modifications such as H3K9me3, H4K20me3 (reduced in ageing but increased in senescent cells),[31] and H3K27me3. Furthermore, there is age-associated remodeling of activating histone modifications such as H3K4me3 and H3K36me2/me3. Interestingly, while H3K27me3 marks reduce with age in *Caenorhabditis elegans* and human progeria model systems, they seem to increase in mouse quiescent satellite cells.[32–34] Similarly, H3K4me3 has also been shown to promote or inhibit longevity in a context-dependent manner.[35] Cells from patients with premature

ageing disorders, such as Hutchinson–Gilford Progeria and Werner Syndrome, showed reduced expression of the H3K9me3 methyltransferase SUV39H1 as well as the heterochromatin protein HP1.[36,37] Recently a novel epigenetic ageing clock was developed using chromatin accessibility profiles (ATAC-Seq) generated from peripheral blood mononuclear cells (PBMCs) isolated from young and old donors, which correlated with age-associated gene expression changes more significantly than the DNAm-based ageing clocks.[38] It would be interesting to correlate age-associated changes in histone modifications, chromatin accessibility, and DNAm, given the strong association between these regulatory pathways,[15,39] potentially leading to the development of more comprehensive epigenetic-ageing signatures.

IS IT POSSIBLE TO UNCOUPLE REPROGRAMMING AND REJUVENATION?

While, in principle, reprogramming has been shown to reverse age-related cellular phenotypes and epigenetic changes this is still not applicable for rejuvenation in the clinical setting. The process of reprogramming into pluripotent state also reverses the features of cellular differentiation – which notoriously affects functional integrity of the rejuvenated tissue. Furthermore, reprogramming toward iPSCs bears the risk of teratoma formation.[40,41] It has been suggested that transient expression of the OSKM factors can result in a de-differentiated progenitor-like state, that still maintain some of the cellular characteristics without reaching pluripotent state.[42,43] This approach of partial reprogramming gains a lot of attention for possible rejuvenation approaches. Despite the many encouraging reports, it remains to be further validated if intermediate or partial reprogramming that stably reverses epigenetic ageing over a long period while retaining cell identity can really be reproducibly achieved. If such an intermediate state is stable, it might successfully uncouple cell fate from age-associated changes and serve as a novel paradigm for understanding and applying rejuvenation through reprogramming in a clinical setting. To this end, epigenetic ageing and epigenetic modifications during cellular differentiation must also be uncoupled. Here, the following four scenarios are conceivable (Figure 1):

1. Ageing and cellular differentiation during development are directly linked and cannot be uncoupled. In this scenario, complete reprogramming and the resulting de-differentiation are essential for resetting the epigenetic clock, and further differentiation of the iPSCs is necessary to re-establish function. The observations of partial reprogramming might then instead resemble a transient state toward pluripotency or reflect heterogeneity in reprogramming. In fact, it has been suggested that the reversal of epigenetic clocks occurs simultaneously with changes in pluripotency-associated CpGs.[25]
2. Partial uncoupling of age-associated changes and cell fate determination: In this scenario, age-related epigenetic changes, cellular senescence, and cell fate would be co-regulated but not directly interlinked. This might be achieved by interrupted reprogramming, for example, by not changing to culture conditions that maintain a pluripotent state or limiting the duration of exposure to reprogramming factors. The possibility of partial reprogramming is supported by studies indicating that loss of cell-type specific gene expression, for example, for fibroblast lineage, follows different kinetics than the reversal of epigenetic clocks, suggesting a safe time window for rejuvenation without complete erasure of somatic identity.[26,44]
3. Age-associated changes can be entirely uncoupled from cellular specification and differentiation. Here, it would be possible to maintain all cellular characteristics, including epigenetic features for the specific tissue and anatomic location, while resetting epigenetic ageing. It might even be feasible to titrate the rejuvenation to a particular age without resetting all the way back to the embryonic state. However, cell-type-specific epigenetic patterns and ageing-associated patterns overlap. While it is still unclear how these exact genome-wide DNAm changes are regulated, it appears unlikely that the two processes can be entirely uncoupled.
4. Rejuvenation during transdifferentiation into other cell types: It has been suggested that direct conversion, for example, of blood cells into neurons, gives rise to specified cells with a rejuvenated transcriptomic and epigenetic state.[45] It is yet unclear if epigenetic modulation in direct reprogramming may briefly touch a pluripotency-associated state. Furthermore, there is evidence that the rejuvenated intermediate state is not stable but further declines during culture expansion (Flitch et al., unpublished findings). It will be necessary to better understand cellular heterogeneity in direct cell fate conversion and if the new cellular state is stable.

THE KINETICS OF REJUVENATION DEPENDS ON THE ADMINISTRATION OR REPROGRAMMING FACTORS

In the following sections, we want to highlight some relevant studies that support the general feasibility of partial reprogramming. For a historic time line of major discoveries, we would like to refer to other review articles.[46–48]

Evidence for partial reprogramming from mouse in vivo studies

In a landmark study, the group of Carlos Izpisua Belmonte[49] induced partial in vivo cellular reprogramming in a Hutchison-Gilford Progeria Syndrome mouse model. They expressed the OSKM factors cyclically (2 days on and 5 days off) using a doxycycline-inducible reprogramming cassette. This method resulted in the reversal of multiple hallmarks of ageing, such as reduced accumulation of DNA damage and cellular senescence, demonstrating the potential of partial reprogramming in rejuvenation. At the organism level, cyclical expression of OSKM led to an extended lifespan in progeroid mice, which was closer to a normal murine life span. Furthermore, it improved age-associated histological changes in multiple organs such as skin, spleen, kidney,

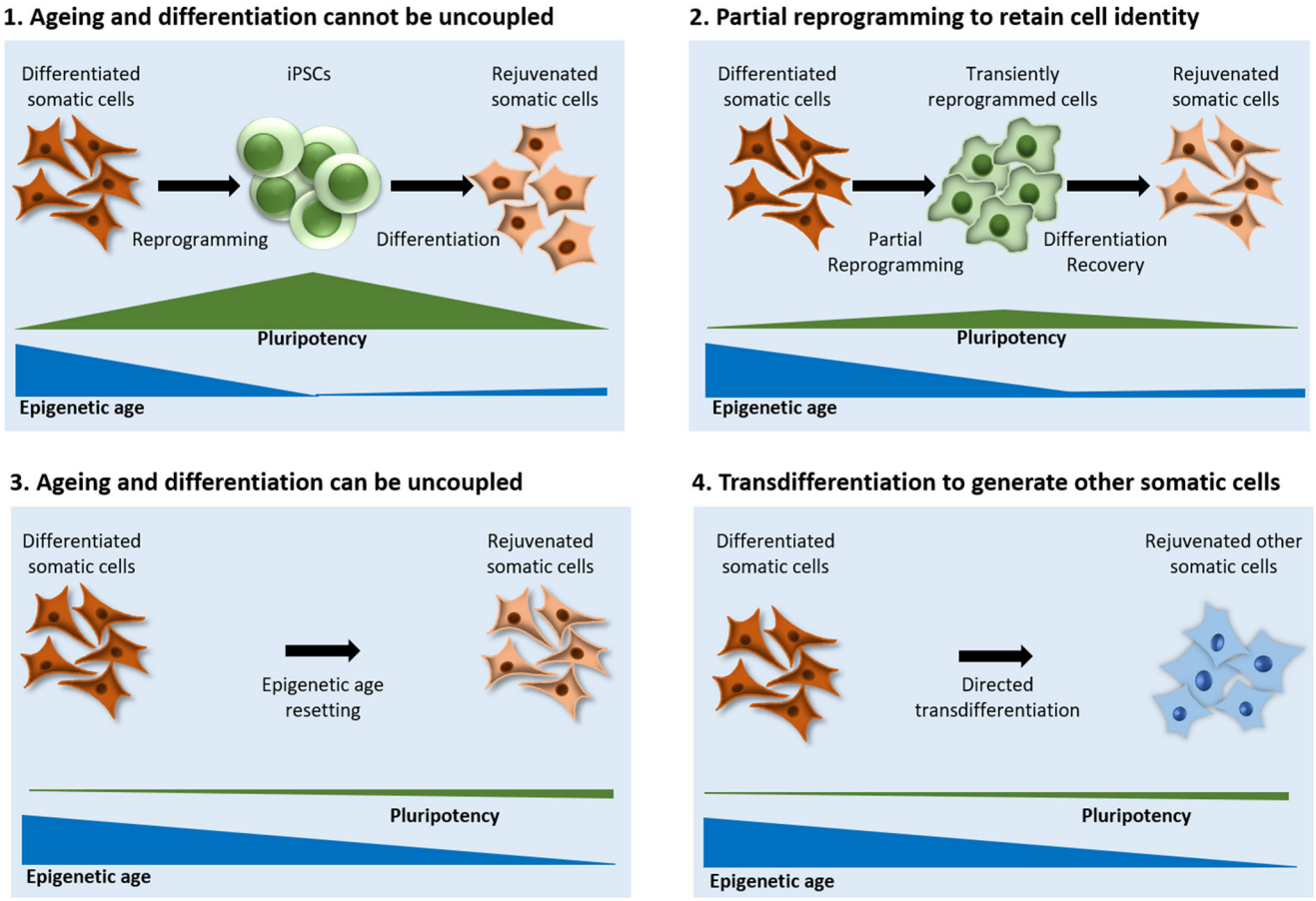

FIGURE 1 Possible scenarios for uncoupling epigenetic ageing and cell fate determination. (A) Ageing and differentiation cannot be uncoupled: this scenario assumes that age-associated epigenetic changes cannot be uncoupled from cell fate determination. Hence, differentiated somatic cells would need to be completely reprogrammed into induced pluripotent stem cells (iPSCs), followed by redifferentiation. (B) Partial reprogramming to retain cell identity: in this scenario, somatic cells can be partially reprogrammed to achieve significant reversal of age-associated modifications while still not reaching a fully pluripotent state. (C) Ageing and differentiation can be uncoupled: in this case, age-associated epigenetic modifications could be reversed without altering the cell identity. (D) Transdifferentiation to generate other somatic cells: here, older somatic cells could be transdifferentiated into other somatic cells, associated with epigenetically rejuvenation, but without entering a pluripotent state

and heart, expansion in muscle satellite cells, and an improvement in muscle regeneration after injury in naturally aged and progeroid mice. While the findings of this study led to crucial insights into partial reprogramming and rejuvenation, the authors did not quantify epigenetic age.

Using the same OSKM expression system, a more recent study indicated that a single cycle of transient OSKM expression in naturally aged mice could partially reverse epigenetic and transcriptional changes in the pancreas, liver, spleen, and blood cells that are maintained after the termination of the OSKM expression.[50] Consistent with this data, analysis of serum metabolites revealed a reversal of the metabolomics profiles toward a younger profile. Specifically, 4-hydroxyproline and thymine, that declined with age, showed an increase upon OSKM treatment. This study provides evidence that low levels of OSKM are sufficient to induce transcriptomic and epigenetic rejuvenation, thus minimizing the risk of teratoma formation associated with long-term OSKM treatment. The authors also argue that the recovery period after the OSKM expression was critical as they detected many rejuvenation changes a week after stopping of OSKM expression, indicating that rejuvenation events continue even during recovery period post-OSKM termination. In analogy, another recent study demonstrated that single short reprogramming induction is sufficient to prevent musculoskeletal deterioration in mice when applied in early life.[51] These authors also suggested the reversal of age-associated epigenetic changes but did not test established epigenetic clocks.

At the organ level, Chen et al. expressed OSKM in cardiomyocytes of mice for 6 or 12 days and saw that the functional, morphological, and gene expression profile reversed to neonatal stages.[52] Transient OSKM expression also extended the regenerative window of juvenile murine hearts and enabled adult heart repair through the proliferation of preexisting cardiomyocytes, indicating a strong potential for partial reprogramming in achieving functional cardiac recovery in a clinical setting. Similarly, Lu et al. used Tet-Off AAV2s carrying OSK as a single polycistron injected into the vitreous body to induce OSK in murine retinal ganglion cells (RGCs) of the central nervous system.[53] They observed significant regeneration, survival, and sprouting of RGC

axon fibers upon induction of OSK, and this was reversed upon the suppression of OSK. Furthermore, OSK induction also reversed global transcription and DNAm changes caused by injury or natural ageing, specifically at light detection and synaptic transmission genes. Similar effects were seen in differentiated human neurons where OSK expression reversed the axonal loss and DNAm age after injury. OSK expression was also shown to reverse vision impairment in glaucomatous eyes and during natural ageing. However, this restoration of visual acuity was not seen beyond a certain age, indicating a temporal cut-off.

While the above studies provide important insights suggesting that limited exposure to OSKM can successfully rejuvenate cells and organs without alteration in cell identity, it must be noted that the premature ageing murine genetic model does not fully recapitulate the complexity of natural ageing. Additionally, these studies do not account for cellular heterogeneity or analyze the long-term rejuvenation effects to determine stability. Notably, this murine model's doxycycline-inducible OSKM expression system can hardly be translated for clinical application. It is challenging to examine efficacy and safety in human patients based on animal studies. This necessitates alternative approaches and further work on understanding the role of reprogramming in rejuvenating cells derived from human donors.

Attempts for partial reprogramming in human cells

Recently, Sarkar et al.[54] indicated that transient expression of reprogramming factors could reverse ageing hallmarks without erasing cell identity in naturally aged human cells. Their experiments in fibroblasts and endothelial cells showed that OSKLMN expression promoted activation of a more youthful gene expression profile, heterochromatin state, autophagy levels, and mitochondrial membrane potential. Epigenetic clock analysis indicated that transient reprogramming had a moderate effect on methylation age, which was more pronounced in endothelial cells (average age difference = −4.94 years) than in fibroblasts (average age difference = −1.84 years). Expression levels of cell identity genes and telomere length remained unaltered, indicating that the cells did not de-differentiate. This study was also extended to aged chondrocytes from osteoarthritic human donors as well as murine skeletal muscle stem cells and showed a partial reversal of gene expression and cellular physiology to a more youthful state in chondrocytes and improved regenerative potential in MuSc. By identifying day four of transfection as the point of no return during cellular reprogramming when age-associated changes are reversed before epigenetic reprogramming of cellular identity occurs, this study provides insights into the temporal sequence of partial reprogramming. However, further validation may be required since the epigenetic rejuvenation was only very moderate, and there is notorious offset between such experiments. Either way, a rejuvenation of only 4 years might not be sufficient for many clinical applications. It is also unclear if the rejuvenation remains stable or if a subset of de-differentiated cells might ultimately outgrow the other cells.

Gill et al. used a novel strategy where OSKM factors were expressed using a doxycycline-inducible polycistronic cassette encoding OSKM and GFP in fibroblasts isolated from middle ages donors by lentiviral transduction.[44] This expression was terminated after the maturation phase of reprogramming at different lengths of time (10, 13, 15, or 17 days). They observed robust transcriptional rejuvenation (approximately 20 years), partial functional rejuvenation, and substantial reduction of DNAm age (approximately 30 years) whilst retaining overall cell identity. Interestingly, the authors reported that 13 days of reprogramming was optimal for rejuvenation and lower or higher reprogramming periods led to reduced rejuvenation. It should be noted that this rejuvenation was based on DNAm clocks, which may be affected by the heterogeneity within the samples and the presence of outliers among the age-associated CpGs. Also, the rejuvenation was calculated based on the Horvath clock,[22] which is a multi-tissue clock trained for 353 CpGs. It is difficult to determine whether the change in DNAm occurs over all the CpGs or rather in a subset of these CpGs. Analyzing the changes at individual CpGs would better elucidate the mechanism of epigenetic rejuvenation. Interestingly, transient reprogramming did not lead to telomere elongation,[44] indicating that the senescence pathway is not reversed. It would be interesting to compare this strategy with the cyclical expression of reprogramming factors, as described by Ocampo et al.,[49] to narrow down the temporal window of partial reprogramming to permit efficient rejuvenation without loss of cell function.

OPEN QUESTIONS

Reprogramming toward iPSCs, particularly partial reprogramming, seems an attractive rejuvenation mechanism. Yet, this research is still in its infancy, and the following questions need to be addressed:

1. How to define partial reprogramming? Is there a difference between partial, interrupted, and transient reprogramming? It is yet unclear if such definitions should include alteration of transcription profile, cellular morphology, cellular function, telomere length, or epigenetic markers.
2. Is a partially reprogrammed state a stable intermediate state between somatic cells and iPSCs? It would be interesting to determine whether epigenetic rejuvenation is retained in partially reprogrammed cells over multiple passages. Notably, rejuvenation has hardly been demonstrated on a single-cell level, and heterogeneous effects within a given tissue may hamper bulk analysis.
3. The different studies that establish partial reprogramming follow different protocols. For efficient uncoupling, does OSKM alone work, or are additional factors needed? How important is the duration of reprogramming, and do these conditions vary with cell type?
4. Is it possible to identify and develop targeted strategies to induce rejuvenating marks on chromatin without altering function and cell identity?
5. Does partial reprogramming altogether avoid the tumor formation risk as seen with longer OSKM overexpression?[40,41] Our previous work has also indicated that interrupted reprogramming

with episomal plasmids might result in immortalization with karyotypic alterations.[55] These safety aspects need to be very critically investigated.

6. A discussion on partial reprogramming as an anti-ageing treatment paradigm is incomplete without addressing the ethical considerations of anti-ageing research. These include basic questions such as whether ageing itself can be considered a disease to warrant medical intervention or should these treatments be limited to only age-associated debilitating disorders; questions of justice and equity in the accessibility of these interventions; and, more urgently, the cost of extended lifespan in an already highly populated world with limited resources. Hence, assessing the ethical feasibility of anti-ageing treatments requires critical discussions and policy decisions taking into account effects on individuals and society.[56]

CONCLUSIONS AND PERSPECTIVES

Reprogramming toward iPSCs, particularly partial reprogramming, is an exciting approach for rejuvenation. Yet, there are still many open questions. Unequivocal and stable rejuvenation by partial reprogramming remains to be demonstrated and such protocols need to be validated. Until then, studies should be assessed with the necessary caution and it is important to publish negative studies as well. In an attempt to determine the role of partial reprogramming, our lab previously reprogrammed human mesenchymal stromal cells (MSCs) with episomal plasmids and switched back to standard MSC culture conditions at various time points.[55] This interrupted reprogramming did not result in epigenetic rejuvenation, which might be attributed to lower levels of reprogramming factors or insufficient time. Notably, in one experiment, we observed immortalization and karyotypic aberrations after the reprogramming procedure, which points to the possibility that partial reprogramming might also favor genomic instabilities. Further research and critical discussion regarding the definition of common standards, efficacy, long-term stability, safety aspects, and ethical considerations need to be addressed before a potential clinical application of partial reprogramming for age reversal.

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CONFLICT OF INTEREST STATEMENT

RWTH Aachen University Medical School has applied for patent applications for DNAm changes during ageing and long-term culture. Wolfgang Wagner is involved in the company Cygenia, which provides services for epigenetic analysis to other scientists (www.cygenia.com). Apart from this, the authors have nothing to disclose.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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