Mechanisms, pathways and strategies for rejuvenation through epigenetic reprogramming

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Mechanisms, pathways and strategies for rejuvenation through epigenetic reprogramming

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Andrea Cipriano[1,2,8], Mahdi Moqri[1,3,4,8], Sun Y. Maybury-Lewis[5,8], Ryan Rogers-Hammond[5], Tineke Anna de Jong[1,2], Alexander Parker[1,2], Sajede Rasouli[1,2], Hans Robert Schöler[6], David A. Sinclair[5,7] ✉ & Vittorio Sebastiano[1,2] ✉

Over the past decade, there has been a dramatic increase in efforts to ameliorate aging and the diseases it causes, with transient expression of nuclear reprogramming factors recently emerging as an intriguing approach. Expression of these factors, either systemically or in a tissue-specific manner, has been shown to combat age-related deterioration in mouse and human model systems at the cellular, tissue and organismal level. Here we discuss the current state of epigenetic rejuvenation strategies via partial reprogramming in both mouse and human models. For each classical reprogramming factor, we provide a brief description of its contribution to reprogramming and discuss additional factors or chemical strategies. We discuss what is known regarding chromatin remodeling and the molecular dynamics underlying rejuvenation, and, finally, we consider strategies to improve the practical uses of epigenetic reprogramming to treat aging and age-related diseases, focusing on the open questions and remaining challenges in this emerging field.

Aging is the primary driver of many leading causes of death, including type 2 diabetes, neurodegenerative disease and cardiovascular disease. The precise reason for why we age is not completely understood, but the progressive nature and influence of external factors suggest that the process probably stems from a failure of maintenance mechanisms that ultimately impact epigenetic regulation and gene expression[1,2]. Several studies highlight how the external environment and the genetic background of an organism have pivotal roles in the aging process by affecting events that maintain epigenetic information[2,3].

Identifying and measuring biological aging is challenging owing to its multifactorial nature. Progress in the field led to the identification of critical biological changes based on the following criteria: (1) manifestation during physiological aging, (2) accelerated aging upon aggravation and (3) improvement of the aging process and increased lifespan with intervention[1,4]. These changes, collectively referred to as the 'hallmarks of aging', include mitochondrial dysfunction, loss of proteostasis, loss of stem cell function, cellular senescence, DNA damage, telomere attrition and impaired nutrient sensing.

Among the hallmarks of aging, loss of epigenetic information has been proposed as a critical cause of aging that precedes many other aspects of age-related deterioration[2,5–8]. Perturbing these epigenetic networks leads to extensive changes in gene expression that directly

[1]Department of Obstetrics & Gynecology, Stanford School of Medicine, Stanford University, Stanford, CA, USA. [2]Institute for Stem Cell Biology and Regenerative Medicine, Stanford School of Medicine, Stanford, CA, USA. [3]Department of Genetics, Stanford School of Medicine, Stanford University, Stanford, CA, USA. [4]Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA. [5]Department of Genetics, Harvard Medical School, Boston, MA, USA. [6]Department of Cell and Developmental Biology, Max Planck Institute for Molecular Biomedicine, Münster, Germany. [7]Paul F. Glenn Center for Biology of Aging Research, Harvard Medical School, Boston, MA, USA. [8]These authors contributed equally: Andrea Cipriano, Mahdi Moqri, Sun Y. Maybury-Lewis. ✉e-mail: david_sinclair@hms.harvard.edu; vsebast@stanford.edu

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BOX 1

Key terms

Cellular reprogramming
Cellular reprogramming is the process that allows the conversion of differentiated cells back into a pluripotent state (generating iPS cells). This conversion was first achieved in 2006 by Takahashi and Yamanaka, who demonstrated that the introduction of a specific combination of transcription factors can reprogram somatic cells, such as fibroblasts, into a pluripotent state resembling embryonic stem cells[18,122,123]. This groundbreaking discovery has revolutionized the field of regenerative medicine and opened up possibilities for disease modeling, drug discovery and personalized therapies. Cellular reprogramming can also be achieved by using small molecules able to reset the epigenome[71,124], laying the foundations for developing safer regenerative therapeutic strategies.

Transdifferentiation
Transdifferentiation, also known as direct epigenetic conversion or direct cell reprogramming, is a process in which one specialized cell type is directly converted into another specialized cell type without passing through an intermediate pluripotent state[125]. This cellular reprogramming approach involves the modification of the epigenetic landscape of the starting cell, leading to the activation of a distinct transcriptional program associated with the desired cell fate. Transcription factors or other regulatory molecules are introduced into the starting cell to initiate the epigenetic remodeling and redirect its identity toward the target cell lineage. This direct conversion strategy offers a promising avenue for generating specific cell types for regenerative medicine and disease modeling, bypassing the need for pluripotent stem cells as an intermediate step.

Rejuvenation
Rejuvenation, in the context of biological systems, refers to the restoration or enhancement of cellular or organismal functions to a more youthful or healthier state, retaining their differentiated state. It encompasses the reversal or the attenuation of age-related deterioration, aiming to promote longevity and vitality[126,127]. In the context of rejuvenation research, longitudinal studies enable the assessment of the sustained effects of interventions on various parameters, including lifespan, healthspan and functional outcomes. By following individuals or model organisms longitudinally, it is possible to gather valuable data on the long-term efficacy and safety of rejuvenation approaches, as well as potential side effects or limitations[1]. Rejuvenation can be achieved through different strategies, including genetic interventions, epigenetic modifications, cellular reprogramming, regenerative therapies or modulating the organism's environmental exposure. These approaches aim to counteract the accumulation of damage, restore cellular homeostasis and enhance tissue repair and regeneration[1,128].

impact proteostasis, nutrient sensing, senescence and several other hallmarks of aging, suggesting that epigenetic networks are delicate and that their disruption probably initiates other events that cause cells to lose their function over time[8,9].

In contrast to the view of aging as an irreversible and unidirectional process akin to an increase in cell entropy, recent studies have demonstrated that multiple interventions, acting at different levels, can delay or even reverse this process[10–17]. These approaches aim to mitigate or even prevent aging-associated decline through repair or replacement strategies. Such interventions have been successful in reducing cell damage and preserving tissue health, but the long-term impact on whole organisms is still poorly understood[11–14].

One emerging strategy to intervene in the progression of biological aging is epigenetic reprogramming (Box 1), a technique first used by Shinya Yamanaka[18] to revert differentiated cells in vitro back to a pluripotent state. The landmark paper used four transcription factors, namely OCT4, SOX2, KLF4 and MYC (OSKM), to erase cell identity and reset cell-type-specific epigenetic signatures. Subsequent studies showed induced pluripotent stem (iPS) cell induction in vivo, indicating the possibility of cell de-differentiation in living organisms[19–21]. Although this discovery holds great promise for regenerative medicine, the observed frequency of tissue dysplasia and tumorigenesis implies that full cell reprogramming is not a viable anti-aging approach. Conversely, other works have shown that aging-associated DNA methylation patterns and biomarkers persisted when cells were transdifferentiated (Box 1) to different cell types, emphasizing the necessity of partial de-differentiation for rejuvenation (Box 1) to occur[22–25]. All this evidence ultimately led to the question of whether a similar, but modified, approach could be refined to rejuvenate cells in the context of age reversal. Early reports using cells derived from old donors to generate iPS cells demonstrated beneficial effects on senescence phenotypes and telomere length[26–28].

These findings raised the question of whether reprogramming strategies could be harnessed to target age-related damage by manipulating the epigenome without compromising cell identity or function.

In this Review, we describe the current state of transient reprogramming in the context of rejuvenation and discuss areas of future investigation, including fine-tuning reprogramming approaches for safety and for tailoring to specific tissues.

Reprogramming factors

OSKM, known collectively as the Yamanaka cocktail, were used in the first successful generation of mouse iPS cells[18]. Shortly after, the same factors were used to induce pluripotency in various mouse and human somatic cell types[29,30]. Until now, cocktails containing combinations of the 'canonical' Yamanaka cocktail are frequently used for inducing partial reprogramming. In addition, partial reprogramming has also been achieved using other proteins and/or molecules. Here, we will review the structure and function of these factors in epigenetic reprogramming (below).

OCT4

Multiple studies have suggested OCT4 (Fig. 1a) as the master regulator of epigenetic reprogramming[31–33], as OCT4 overexpression alone is sufficient to induce pluripotency when other canonical reprogramming factors are endogenously expressed or are in the presence of other chromatin remodeling chemical factors[32,34,35]. Supporting this notion, optimal reprogramming requires threefold excess of OCT4 relative to the other factors[36].

During full reprogramming, OCT4 has been implicated in at least four distinct mechanisms. First, OCT4 directly recruits the BAF chromatin remodeling complex to promote a euchromatic chromatin state that enhances reprogramming factor binding[37–39]. Second, OCT4 directly binds enhancers of Polycomb group-repressed genes, which modify histones and silence target genes, to induce conversion of their associated promoters from monovalent to bivalent domains[40,41]. Third, OCT4 binds regulatory regions of pluripotency network genes to create an autoregulatory pluripotency network[42,43]. Finally, OCT4 directly binds and upregulates KDM3A and KDM4C, which demethylate H3K9Me2/3 at regulatory regions of pluripotency genes and promote their transcription by inducing a permissive epigenetic state[44].

SOX2

SOX2 is a transcription factor expressed during the emergence of the inner cell mass and is a developmentally essential gene, as its absence

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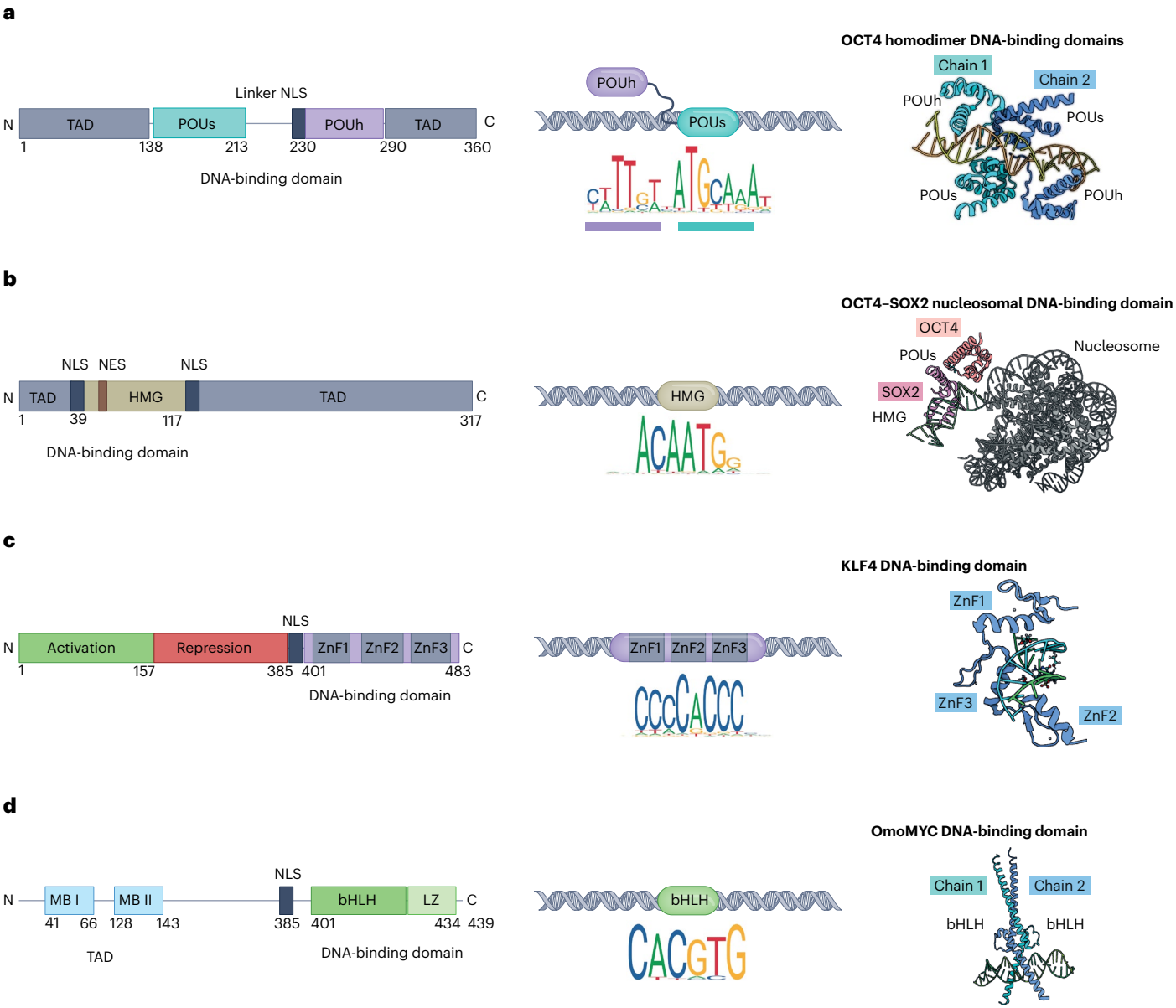

Fig. 1 | Structural diagram of each reprogramming factor. The linear domain structure (left), DNA-binding sequence (center) and DNA-binding domain structures (right) for OCT4, SOX2, KLF4 and MYC factors. **a**, OCT4 (PDB ID: 3L1P) refers to the isoform OCT4A encoded by the *POU5F1* gene, a DNA-binding transcription factor. The OCT4 DNA-binding (POU) domain consists of a POU-homeodomain (POUh) and a POU-specific (POUs) domain connected by a linker region. **b**, SOX2 (PDB ID: 6T90) contains a highly conserved, high mobility group (HMG) box DNA-binding domain comprising three α-helices, which bind the DNA minor groove and bend it by 90°. Early structural determination of the HMG POU-DNA revealed that the enhancers of target genes support the gene-specific configuration of the SOX2–OCT4 complex that binds DNA. The HMG includes two nuclear localization signals (NLS) and a nuclear export signal (NES). **c**, KLF4 (PDB ID: 5KE7) is a member of the cell-type specificity and Krüppel-like factor (SP/KLF) family, characterized by three zinc finger motifs (ZnF1, ZnF2 and ZnF3) with conserved ββα structure within the C terminus. The zinc fingers of KLF4, the second and third motifs, in particular, are responsible for contacting KLF4-specific sequences at the promoters of target genes, making them essential for KLF4-dependent reprogramming. **d**, MYC (PDB ID: 5I50) is a member of the basic helix–loop–helix zipper (bHLHZip) class of transcription factors, containing an N-terminal transactivation domain (TAD), two highly conserved sequences (known as MYC boxes), a central region containing a NLS and a C terminus containing the helix–loop–helix motif. The C terminus of MYC heterodimerizes with its obligatory partner MAX, which also contains a helix–loop–helix, and forms a stable four-helix structure, capable of recognizing specific DNA sequences (such as CACGTG) at the promoters and enhancers of target genes[75]. The leucine zipper (LZ) domain is involved in protein dimerization and DNA binding. Created with BioRender.com.

results in embryonic lethality[45]. Although SOX2 and its multifaceted roles in developmental as well as cancer biology have been well studied, most studies of SOX2 in the context of reprogramming focus on its heterodimerization and cooperative role with OCT4 (refs. 46–50) (Fig. 1b). Single-molecule imaging shows that SOX2 engages the chromatin first and primes the target site for subsequent OCT4 binding[47]. This is also supported by in vivo studies, suggesting that SOX2 alone can open the chromatin and bind target DNA sites before the arrival of OCT4 (ref. 51). Interestingly, OCT4/SOX2-shared sites have the most profound increase in accessibility during early reprogramming, and this partnership is critical for inducing pluripotency[51].

KLF4

KLF4 is a transcription factor containing both activator and repressor domains, conferring a dual function during cell differentiation[52] (Fig. 1c). OCT4 and SOX2 are mainly responsible for increasing

chromatin accessibility during full iPS cell reprogramming, and KLF4 (as well as MYC, described in detail below) is believed to drive the first wave of transcriptional activation[42]. Co-immunoprecipitation and chromatin immunoprecipitation followed by sequencing studies reveal that OCT4–SOX2 binding increases KLF4 binding by several folds, mostly in chromatin regions that are closed in human fibroblasts[41,53,54]. We discuss the details of this process in the 'Partial reprogramming events' section.

MYC

In contrast with the OSK factors, which function synergistically as pioneer factors during reprogramming, MYC (Fig. 1d) does not exert this function[55]. Although recent studies suggest that MYC is not required to initiate reprogramming[56,57], it is considered one of the most potent amplifiers of reprogramming[58]. The presence of MYC increases OSK binding by twofold[53], and MYC binding increases by 40-fold with OSK, supporting the notion that the modulatory activities of OSK and MYC on each other are bidirectional. The strongly pro-proliferative effects of MYC underlie its potential oncogenic ability, suggesting that in vivo or in therapeutic contexts it should be used with caution[57].

Additional factors

Next to OSKM, the reprogramming potential of additional factors is being studied and might give us alternatives for therapeutic or clinical implementation. One of the most studied factors besides OKSM is NANOG, a transcription factor belonging to the pluripotency network[59] and sharing 90% of the OCT4 and SOX2 binding regions[42]. It has been shown that NANOG and LIN28 in combination with SOX2 and OCT4 can reprogram human somatic cells into iPS cells[30] by increasing reprogramming efficiency by 76-fold[60]. Shahini et al. showed that NANOG alone has a pivotal role in ameliorating of senescence hallmarks and inducing rejuvenation in myogenic progenitor cells both in vitro and in vivo[61]. In addition to cell intrinsic transcription factors for cellular reprogramming, modulators such as vitamin C[62–65], IL-6[66–68], TGFβ[69] and bone morphogenetic proteins[70] have been shown to regulate cellular reprogramming by affecting DNA methylation levels, thereby adjusting the expression of certain microRNAs or facilitating the mesenchymal-to-epithelial transition (MET). Recent studies have demonstrated the potential of small molecule stimulation in facilitating chemical reprogramming by inducing an intermediate plastic state[71–73]. These small molecules effectively target key signaling pathways involved in cellular reprogramming, benefiting both human and mouse somatic cells[71–73]. Notably, specific small molecules such as 3-deazaneplanocin A, 5-azacytidine, sodium butyrate and RG108 have emerged as potential epigenetic modifiers[72]. As chemical reprogramming does not require integration into the genome, this approach holds the advantages of increased translational potential, preservation of genomic integrity and precise control of induction.

Partial reprogramming events

As epigenetic landscapes are reset, somatic cells undergo multiple intermediate stages during transcription factor-induced reprogramming. Here, we break down the early interactions between OSKM and chromatin that facilitate the cellular rejuvenation phase of reprogramming.

Somatic silencing and transcriptional remodeling

Cellular rejuvenation through partial reprogramming requires a careful balance of epigenetic remodeling and cell identity conservation, which can be achieved using a subset of reprogramming factors. Several studies have tried to define the molecular mechanisms underlying OSKM-driven reprogramming, but most have focused on pluripotency rather than cellular rejuvenation as the end goal. Despite these different contexts, 'early transient events' evoked by OSKM factors, such as somatic silencing and chromatin remodeling while reprogramming is initiated, seem to be required for both cellular rejuvenation and full reprogramming[74–76].

Silencing the somatic gene-associated chromatin regions is the earliest event in reprogramming and is tightly regulated by OSK factors that canonically function in pluripotency[77]. As OSK factors are generally known as transcriptional activators[78,79], it may seem counterintuitive for chromatin closing to be the first step induced by these factors. There are currently two proposed models for how OSK influences somatic silencing: (1) displacement of somatic transcription factors away from their enhancers[80] and (2) downregulation of somatic factors with SAP30 through decreasing H3K27ac levels[77]. Interestingly, both models suggest that some somatic gene-associated chromatin loci that become inaccessible following OSK induction are not enriched with OSK binding motifs, supporting the notion that OSK induces somatic silencing through direct and indirect mechanisms.

OSK factors interact with the genome and each other differently in mice than in human cells[80–82], complicating the design and interpretation of studies. Specifically, in mouse embryonic fibroblasts (MEFs), OCT4 binds somatic enhancers during reprogramming and may initiate their inactivation, whereas in human fetal fibroblasts, OCT4 and SOX2 bind putative enhancers and remain bound in iPS cells. Ultimately, the 'intermediate' stages examined in these studies may reflect more of an incomplete, almost-pluripotent cell type, as opposed to the true epigenetic landscape during reprogramming. Given the differences in reprogramming duration and presumably dynamics, detangling the precise underpinnings of OSK-driven somatic silencing in different reprogramming contexts is challenging but critical.

As somatic programs are silenced, transcriptional remodeling events activate the pluripotency program. It was initially reported in MEFs that transcriptional remodeling occurs in two waves, the first driven by MYC and KLF4 in the first 3 days and the second by OSK (ref. 83). Genes associated with cell proliferation, metabolism and cytoskeleton organization were activated first, with developmental genes temporarily downregulated. Such changes were highly correlated with altered cell division, DNA replication, chromatin modification and the DNA damage response protein levels[84]. Clues on how these events relate to epigenomic rejuvenation may come from examining the mechanistic behaviors of OSKM, as discussed in the previous section. First, MYC primarily facilitates early gene induction for proliferation, whereas OCT4 and SOX2 induce chromatin opening while also binding readily open chromatin enriched with KLF4 motifs, supporting the notion that KLF4 recruits OCT4 and SOX2 to somatic chromatin that later becomes closed[83]. These findings are consistent with the proposed dual role of KLF4 in both transcriptional activation and repression.

The distinct mechanisms observed among combinations of OCT4 and SOX2 (without KLF4) versus OSK may explain the controversy regarding whether OSK mainly function as the pioneer factors or bind readily open chromatin in early reprogramming, or both[80,82,83,85]. Importantly, we must note that all of the transcriptional remodeling studies utilizing OSKM occupancy data are limited to the events that occur in the beginning (fibroblasts) or end points (pluripotent stem cells), with everything in between still up for interpretation[80,83,85]. As the early events in reprogramming are largely transient[81], we are probably missing critical transitional OSKM–chromatin dynamics during such events, leaving mechanistic questions largely unanswered.

Resetting the epigenetic landscape

Along with transcriptional remodeling of somatic and pluripotency programs, OSKM transiently induces changes to histone tail post-translational modifications and DNA methylation. Changes in DNA methylation were initially thought to happen in the late stages of reprogramming[83], but a recent study revealed that loci near *Oct4*, *Klf4* and *Nanog* are demethylated earlier and de novo DNA methylation occurs in late reprogramming[85]. This reveals a cooperative relationship between DNA methylation and histone modifications that was not previously appreciated. On nucleosomes, levels of H3K4me2, a transcriptional activation mark, change rapidly at enhancers of pluripotency and

development-related genes before gene expression changes are observed. At the same time, H3K4me3 levels at promoters remain largely stable and are correlated with transcriptional changes[86]. By contrast, repressive H3K27me3 is depleted only in enhancers that gain H3K4me2 toward a pluripotent state. Thus, alterations or additions of specific chromatin features are thought to 'prime' the transient epigenetic landscape for reprogramming before gene regulation[85]. Consistent with this notion, early loss of H3K27me3 is associated with acquiring a partially open or primed chromatin state[87].

A valuable tool to enhance reprogramming efficiency and gain mechanistic insights into epigenetic rejuvenation is the generation of pre-iPS cells. These cells, which have embryonic stem cell-like morphology[88] and SSEA-1 expression[85], may help to identify critical events occurring before cell identity programs are erased. Using this system, Chen and colleagues found that H3K9me3 inhibits full reprogramming of pre-iPS cells into stem cells, effectively functioning as a barrier to pluripotency[88]. Similarly, reducing H3K9me3 and H3K36me3 through inducible expression of a demethylase (KDM4B) improves iPS cell reprogramming efficiency[89]. How histone methylation at *cis*-regulatory elements affects transcriptional activation and repression is becoming more apparent, but the precise temporal order of epigenetic modifications and fluctuating dynamics over the course of cellular reprogramming remains elusive. Moreover, there are variations among cell-type-specific chromatin states during the early-to-intermediate phases of reprogramming that still need to be defined.

DNA methylation is another epigenetic feature that has a role in developmental programming and epigenetic reprogramming of aged cells. DNA methylation during iPS cell reprogramming facilitates chromatin remodeling while silencing differentiation-associated genes[90,91]. Despite the extensive changes in DNA methylation immediately before the final stages of MEF to iPS cell conversion, several sites in transiently accessible chromatin lack DNA methylation before OSK binding. Moreover, enhancer chromatin opening generally follows OSK binding, which suggests that chromatin accessibility establishment occurs independently of DNA methylation. There is also evidence that changes to methylation occur in parallel with the observed alteration in chromatin contacts, and active DNA methylation via TET and TDG enzymes is required for rejuvenation[92].

Because the state of chromatin features and engagement with OSKM factors have primarily been defined in the context of iPS cell reprogramming, many aspects of partial reprogramming are poorly defined. It will be interesting to decipher when, where and how the interplay among chromatin features and OSKM factors change in the context of epigenetic reprogramming.

Proliferation, metabolic switch and MET

In addition to overhauling the epigenetic landscape via somatic silencing and chromatin remodeling, reprogramming to pluripotency appears to require cell division[93] so that cells can progress through the cell cycle-associated transcriptional dynamics[91]. With full reprogramming, the kinetics can be increased through both proliferation-dependent and independent ways, suggesting that multiple mechanisms coalesce to attain pluripotency. It is worth noting, however, that full reprogramming is relatively inefficient, with ~3% of cells reaching full reprogramming in the first round[83]. However, there is evidence that continued proliferation and OSKM expression eventually convert all cells to iPS cells, although such treatment can make cells vulnerable to apoptosis and cellular senescence[85,93].

Nonetheless, cell proliferation does not seem to be always required to reach pluripotency. For example, overexpression of LIN28 increases cell proliferation and accelerates iPS cell formation, but overexpression of NANOG achieves the same goal independent of cell proliferation. As such, proliferation and pluripotency may be complementary to promote survival during development, rather than acting sequentially. Hanna and colleagues[93] point out that increased cell proliferation may promote reprogramming by amplifying transiently reprogrammed daughter cells that ultimately become stem cells. Additionally, the group notes that nuclear changes during cell division may stabilize the dynamic epigenetic landscape governed by the pluripotency network. Whether the 'transiently reprogrammed' daughter cells in this context represent epigenetically rejuvenated youthful cells remains to be investigated, as well as the molecular mechanisms underlying heterogeneity of reprogramming latency.

The earliest phenotypic and transcriptional changes following OSKM expression begin with an immediate increase in cell proliferation induced by MYC[58]. At this point, cells also transition from the mesenchymal-to-epithelial state that is actively induced during this early phase and are required to progress through the reprogramming process[94–96]. Interestingly, cells switch from oxidative phosphorylation to glycolysis during this early reprogramming phase[58]. Similar to gene expression changes, clustering analysis of proteins with temporal expression dynamics similar to gene expression changes reveals that electron transport chain proteins are downregulated in the first 3 days of reprogramming before a gradual metabolic switch to glycolysis, a characteristic that is typically seen in rapidly proliferating cells[84,97]. Moreover, multiple extracellular matrix proteins, including mesenchymal markers, are rapidly reduced, marking the initiation of MET. This process reverses the epithelial-to-mesenchymal transition in development and differentiation, characterized by downregulation of cell adhesion and motility. Interestingly, OSKM facilitates MET by suppressing the regulation of epithelial-to-mesenchymal transition through multiple mechanisms, such as OCT4 and MYC repression of TGFβ signaling[33,94], OCT4 and SOX2 activation of miR-200 family[33,98], and OCT4 and KLF4 chromatin opening and activation of epithelial genes[37].

Partial reprogramming as a strategy for epigenetic rejuvenation

Although developments in cellular reprogramming have proposed several new and promising medical avenues[19–21], continuous expression of OSKM factors in vivo is limited by substantial safety concerns including severe weight loss, the formation of circulating totipotent stem cells and teratomas in several organs[19]. Ohnishi et al.[21] provided evidence that the duration of OSKM overexpression has a pivotal role in the dedifferentiation process. These initial findings identified the factors' expression levels and duration of treatment as sensitive components of the process and helped to establish parameters for in vivo rejuvenation applications. Through careful fine-tuning of in vivo reprogramming protocols, subsequent research from Ocampo and colleagues later established the potential for the technique to modulate phenotypes of aging. We will walk through the recent landmark findings targeting aging and age-related diseases by partial epigenetic reprogramming below (Fig. 2).

Whole-organism partial reprogramming in mouse

The idea of inducing epigenetic transition to a 'youthful' state without the loss of cell identity, defined as 'epigenetic rejuvenation', was proposed for the first time in 2010 (refs. 99–101). Leveraging these findings and other works[101,102], Ocampo et al. reached an unprecedented milestone: in vivo rejuvenation through OSKM by using the LAKI premature aging mouse model engineered to carry a doxycycline (DOX)-inducible OSKM polycistronic cassette (4F) (Fig. 3a). Excitingly, pulsing OSKM expression in heterozygous LAKI 4F mice ameliorated cellular and physiological aging markers without any sign of teratomas, which were seen if the same protocol was carried out on a homozygous 4F mouse. In addition, the Ocampo reprogramming protocol improved recovery from diabetes and muscle injury in old wild-type mice, confirming their results in the context of normal physiological aging. However, the observed rejuvenation effects were transient and diminished after stopping the DOX cycle in vitro[15]. The work from Ocampo et al. opened a new chapter in the transient reprogramming field and set into motion

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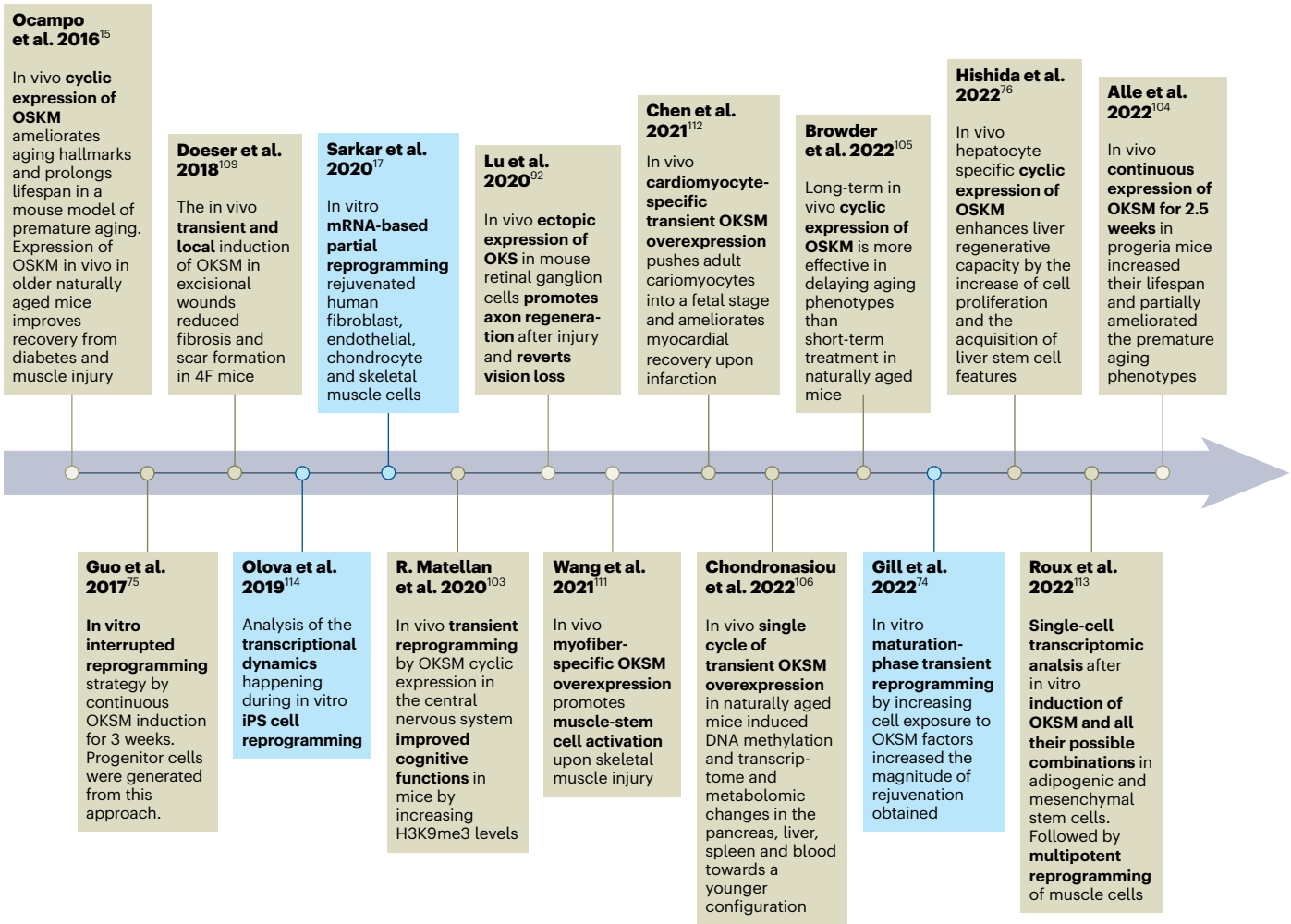

Fig. 2 | Timeline highlighting publications on partial reprogramming in mouse and human models. Important takeaways from in vitro and in vivo partial reprogramming experiments with the aim of relieving aging phenotypes. Brown and blue boxes indicate works performed in mice and humans, respectively.

experiments attempting to find a way to reprogram, prove age reversal and define optimal parameters.

Mechanistic studies were later complemented from Rodríguez-Matellán et al., who demonstrated that the cyclic expression of OKSM improved cognitive functions in mice by increasing H3K9me3 levels in dentate gyrus adult neurons. Migration, survival and, by consequence, synaptic plasticity (Fig. 3b) were all improved[103], suggesting that cell physiology could be enhanced through partial reprogramming without affecting cell replication.

As more insight was garnered regarding experimental approaches to achieve partial reprogramming, dynamics of four-factor expression were identified as a primary variable that needed to be addressed in reprogramming efficacy. A recent paper published by Alle and colleagues demonstrated that rejuvenation outcomes might be more consistent by modifying OSKM methods (Fig. 3c)[104]. Specifically, they compared continuous overexpression of OSKM with a lower dosage of DOX (0.2 mg ml^{-1}) in LAKI/+;4F/+ mice to the standard protocol of Ocampo and colleagues and observed a near-identical increase in the lifespan of mice treated with both protocols. It is noteworthy that, when the same continuous induction protocol was applied by Abad et al., it resulted in teratoma formation and mice death, indicating possible intrinsic genetic differences between the different strains (probably due to the different 4F insertion and the different genetic background of the mice R26rtTA/+;Col1a14F2A/+;LmnaG609G/+ versus R26rtTA/+;Col1a14F2A/+;) that could account for the divergent outcomes observed. Furthermore, a single 2.5-week period of continuous overexpression increased lifespan by 15% and partially ameliorated the premature signs of aging in mice, despite having no noticeable difference in the median age of death. Interestingly, when the same protocol was applied to nonprogerice mice, the effects were markedly less pronounced, with lifespan only being extended by 18 weeks. This finding led authors to speculate that healthy animals may be less sensitive to partial reprogramming early in life (Fig. 3c).

Additionally, work from the Serrano and Belmonte groups has further examined reprogramming in naturally aged mice, using single-shot and cyclic OSKM, respectively[105,106]. In particular, Chondronasiou and colleagues demonstrated that a single pulse of continuous OSKM induction for 7 days (using 0.2 mg ml^{-1} of DOX) in naturally aged mice can trigger epigenetic, transcriptomic and metabolomic rejuvenation patterns (Fig. 3d) (interestingly, no effect on the number of senescent cells was detected). The study demonstrated that the rejuvenation effect was more prominent in the pancreas than the spleen, liver and blood[106]. In addition to this, the authors, in a related study, monitored the roadmap of reprogramming in the pancreas and identified intermediate reprogramming states by using a single-cell transcriptomic, which could be functionally relevant for tissue regeneration and rejuvenation[107]. This study also highlights how OSKM overexpression has drastically different effects on different cell types within the same organ. This is probably due to the different plasticity or susceptibility of the different cell types to reprogramming, further stressing the

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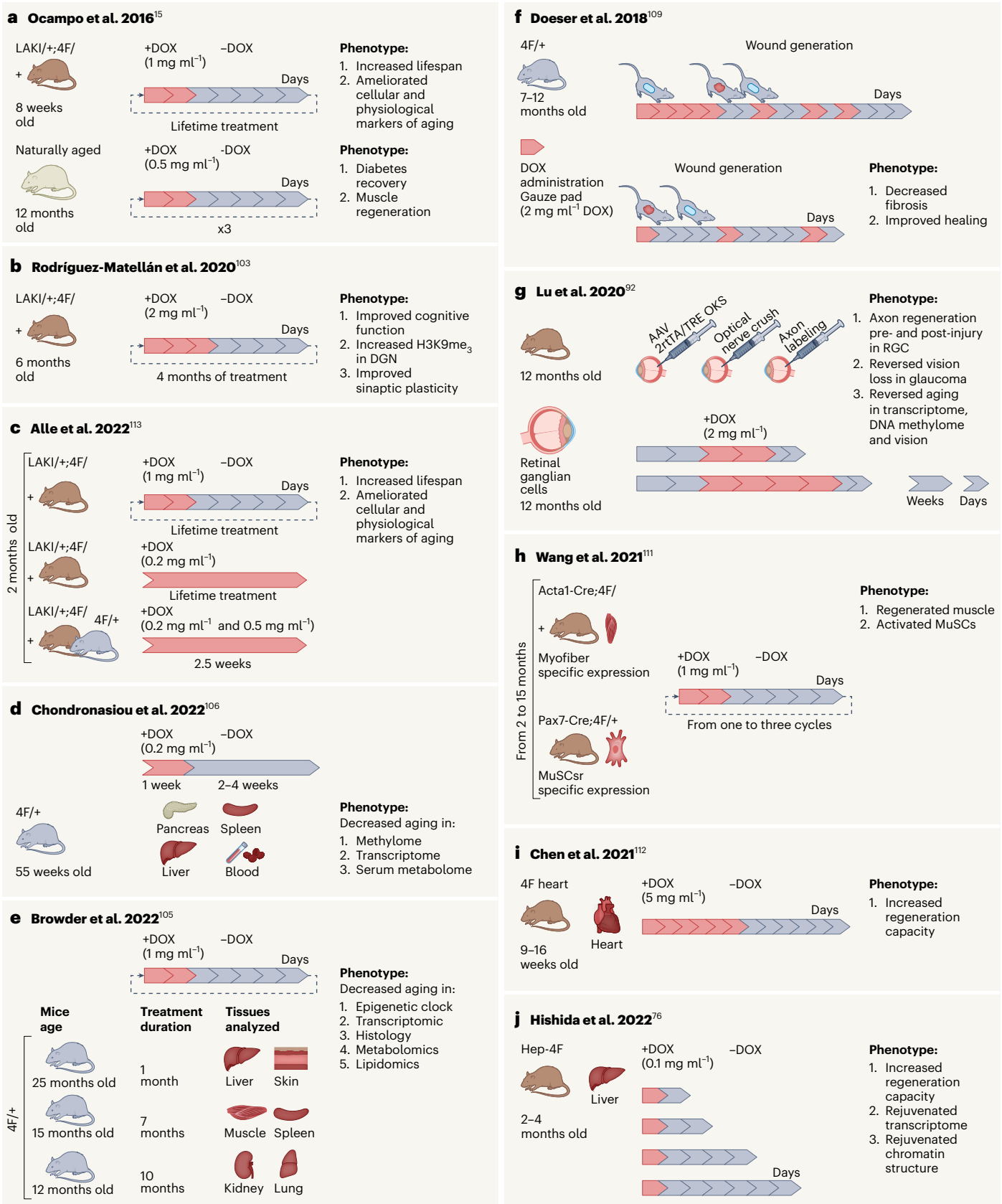

Fig. 3 | Summary of detailed in vivo whole-organism partial reprogramming protocols. a–**e**, Whole-organism partial reprogramming for Ocampo et al. 2016 (ref. 15) (**a**), Rodríguez-Matellán et al. 2020 (ref. 103) (**b**), Alle et al. 2022 (ref. 104) (**c**), Chondronasiou et al. 2022 (ref. 106) (**d**) and Browder et al. 2022 (ref. 105) (**e**). **f**–**j**, Tissue-specific partial reprogramming for Doeser et al. 2018 (ref. 109) (**f**), Lu et al. 2020 (ref. 92) (**g**), Wang et al. 2021 (ref. 111) (**h**), Chen et al. 2021 (ref. 112) (**i**) and Hishida et al. 2022 (ref. 76) (**j**). Each panel shows the strain of mice used and the respective ages (left), the tissues analyzed and the protocol of partial reprogramming (center) and the phenotype analyzed (right). Pink and grey arrows indicate days or weeks (specified below or above the arrow) of DOX administration and DOX removal, respectively. Created with BioRender.com.

importance of studying these molecular dynamics in individual cell types to be able to design cell-type-specific rejuvenation protocols.

Browder and colleagues have instead increased the duration of partial reprogramming to up to 10 months using the 4F/+ mouse model at different ages with a cycling DOX regimen[105] (Fig. 3e). In this study, a short-term cycling partial reprogramming cohort was compared with two different cohorts of long-term partial reprogramming and subsequently examined for markers of rejuvenation (Fig. 3e). They did not observe teratoma formation during the long-term treatment. Most importantly, they found that an extended treatment regimen leads to more pronounced rejuvenation effects as compared with short term when examining epigenetic clocks, transcriptomics and metabolomics in several different organs and tissues (skin and kidney in particular). It is important to highlight that, whereas previous studies have predominantly focused on the premature aging LAKI model, these two studies both implemented wild-type, natural aging models, thus providing more physiologically relevant insights. In addition, it was demonstrated in these studies that cells can be effectively rejuvenated by employing different regimens of overexpression. These findings are consistent with Macip and colleagues who reported in 2023 that adeno-associated viruses encoding an inducible OSK system extended the median remaining lifespan of extremely old mice by 109% compared with untreated mice and improved several health parameters, including frailty[108].

Tissue-specific partial reprogramming in mouse

Despite making substantial progress and providing clear evidence that controlled induction of reprogramming factors could improve cell function and extend lifespan without forming iPS cells in mice, many questions remained. Why rejuvenation was temporary, how systemic induction prevented loss of cell identity and whether benefits were due to cell autonomous or nonautonomous events remained poorly understood and required further analysis. Doeser et al. took advantage of well-characterized wound healing protocols to fill in the gaps. Instead of adding DOX to drinking water, they applied DOX directly to wounds to induce local overexpression of OSKM and measured the effect on wound healing (Fig. 3f). Results from these experiments uncovered an essential element of OSKM rejuvenating potential, showing that local and transient factor exposure to cutaneous wounds significantly reduced fibrosis[109]. Analysis of treated tissues revealed that the observed effects were due to diminished fibroblast-to-myofiber differentiation, promoting healing and concomitantly reducing scar formation. It is essential to note here that despite the induction being local, the cell types responsible for the rejuvenation cannot be uniquely identified because of the heterogeneous composition of the skin at the cellular level.

The demonstration that the DNA methylation clock can be reversed in vivo by reprogramming and that long-term reprogramming can be achieved using a specific subset of reprogramming factors came from Lu et al. in 2020 (ref. 92). The authors showed that the ectopic and constant expression of OSK in retinal ganglion cells in vivo is able to restore vision by promoting axon regeneration in aged and glaucomatous mice without increasing cell proliferation (Fig. 3g). Interestingly, induction of the factors both before and after the optical nerve injury led to increased regeneration capability of retinal ganglion cells. The authors demonstrated that the observed regenerative phenotype is mediated by the active contribution of TET1 and TET2 DNA demethylases and the TDG DNA glycosylase, indicating a critical role for dynamic DNA methylation during partial reprogramming. Similar results were also obtained in human neurons, suggesting an evolutionarily conserved mechanism[92]. Of note, when OSK were co-delivered as monocistronic vectors, no axon regeneration was observed, further supporting the idea that specific levels of OSK expressions of the elements in addition to cell identity are essential to exert optimal function. Importantly, this study is also the first to show that cellular damage accelerates epigenetic aging, a change that OSK counteracts to allow cells to regain youthful gene expression patterns and functions. Interestingly, whole-body overexpression of OSK for a year was found to have no observable negative effects, suggesting that MYC might be responsible for some of the toxicity. However, it is crucial to note that continuous ectopic overexpression of OCT4 also resulted in dysplastic growth in epithelial tissues[110], providing additional evidence for the tissue-specific nature of these effects.

Similarly, Wang and colleagues focused on muscle stem cells (MuSCs) and employed a MuSC-specific OSKM inducible mouse model to investigate OSKM's effects on muscle regeneration. They showed increased MuSC activation and proliferation only when the factors are overexpressed before the injury (both in old and young mice) without affecting their self-renewal capability. This increase in MuSC proliferation upon OKSM overexpression was induced via p53–p21 signaling, which inhibits Wnt4 and contributes to the activation of MyoD and YaP in MuSCs, suggesting that manipulation of specific pathways may be critical to achieving rejuvenation[111] (Fig. 3h). Interestingly, the specific overexpression of the factors in MuSCs did not improve muscle regeneration, arguing that the rejuvenation effect on MuSCs is cell nonautonomous and induced by myofibers[111]. Shortly after, similar work showing that partial reprogramming of a specific cell type can revert aging by promoting cell regeneration came from Chen and colleagues[112] (Fig. 3i). The authors were able to partially reprogram in vivo adult mouse cardiomyocytes, bringing them from an adult to a fetal stage where the cells could proliferate without losing their identity. Cardiomyocytes were then allowed to regenerate after myocardial infarction. Interestingly, similar to prior reports, the effects induced were almost wholly reversed after 6 days of DOX withdrawal[112], and partial de-differentiation results correlated with the cardiomyocyte developmental stage, confirming the dependence of the phenotype observed on the epigenetic state of the cells.

Following this work, Hishida et al. showed other evidence of the importance of the level of expression of OSKM factors, depending on the targeted cell type. They examined the effects of partial reprogramming, specifically on liver regeneration capacity, using an Alb–Cre-transgene 4F mouse model (Hep-4F), and applied an optimized DOX treatment (0.1 mg ml^{-1})[76] (Fig. 3j). One day of DOX treatment resulted in increased cell proliferation in the liver and loss of mature hepatocyte markers, indicative of successful partial reprogramming to a progenitor state and enhanced liver regeneration capacity. In addition, the global transcriptomic analysis showed that chromatin accessibility was actively changed by 4F, suggesting that reprogramming is dependent on chromatin reshaping. Furthermore, they showed that expressing only MYC, known as a hepatocyte proliferation accelerator, could not induce the loss of hepatocyte markers. This suggests that cell proliferation is not the only driver of partial reprogramming[76].

In vitro partial reprogramming in mouse

The possibility of pushing partial reprogramming further to promote cell regeneration via partial de-differentiation by a temporal and partial loss of cell identity came from the work of Guo and colleagues[75]. The authors generated 'induced progenitor-like cells' in vitro by expressing OSKM for 3 weeks in fully differentiated bronchiole secretory cells from 4F mice (Fig. 4a). Proliferative capacity was restored and accompanied by a shift toward a transcriptionally intermediate state, characterized by a minimal expression of embryonic stem cell-related genes and the constitutive expression of tissue-specific profiles. Interestingly, 2 weeks after DOX withdrawal, cells returned to their fully differentiated stage by preserving their lineage commitment both in vitro and in vivo, as shown by their ability to repopulate injured airway epithelium in vivo[75].

Roux and colleagues obtained a similar partial de-differentiation event in adipogenic and mesenchymal stem cells from old and young mice[113] (Fig. 4b). Using a single-cell approach to account for potential heterogeneity, the transcriptome of each cell population revealed

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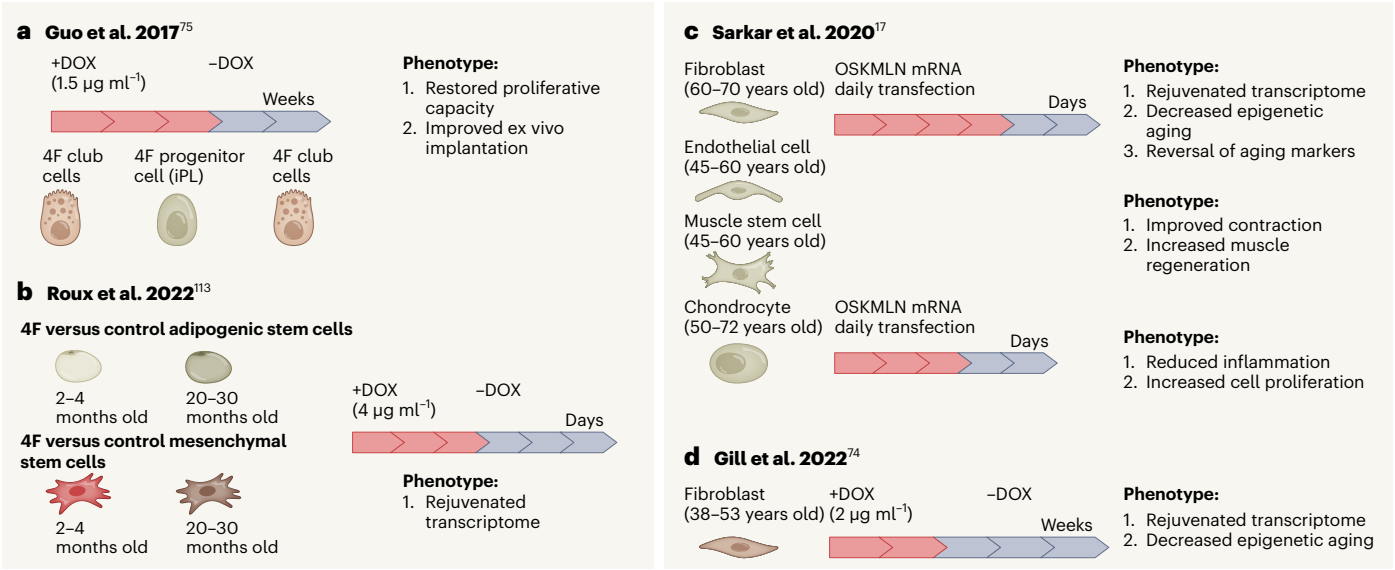

Fig. 4 | Summary of detailed in vitro partial reprogramming protocols in mouse and human. a,**b**, Mouse summary of reprogramming protocols for Guo et al. 2017 (ref. 75) (**a**) and Roux et al. 2021 (ref. 113) (**b**). **c**,**d**, Human summary of reprogramming protocols for Sarkar et al. 2020 (ref. 17) (**c**) and Gill et al. 2021 (ref. 74) (**d**). Each panel shows the cell type used with the respective donor or mice ages (left), the protocol of partial reprogramming (middle) and the phenotype analyzed (right). Pink arrows indicate days or weeks (specified above each induction protocol) of DOX administration (**a**,**b**,**d**) or mRNA transfections (**c**), and purple arrows indicate days or weeks upon DOX removal (**a**,**b**,**d**) or after mRNA transfections (**c**). Created with BioRender.com.

suppression of somatic cell identity genes in both cell types while inducing hallmark pluripotency programs following 3 days of OKSM DOX exposure and 3 days of DOX withdrawal (Fig. 4b). These findings echoed previous observations using bulk approaches[75,114], but the resolution of single-cell RNA sequencing here showed that reprogramming events are consistently partial. Interestingly, based on RNA velocity analysis, they forecasted that partially reprogrammed cells tended to transition back toward their original gene expression states[113]. Further analysis will be required to fully understand the role of identity suppression and retrieval during partial reprogramming. Interestingly, Roux and colleagues used the same induction protocol to repeat their analysis and overexpressed all the possible combinations of the four factors in both cell types in a pooled screen. They found that all combinations of the Yamanaka factors at least partially suppressed the cell identity program and restore youthful gene expression at a magnitude that correlated with the number of factors used rather than their identities. This was a critical finding because it provided evidence of a synergistic function between the factors that directly impacted reprogramming potential. Finally, to prove that partial reprogramming can be achieved by also overexpressing cell-type-specific regenerative factors, they tried a new partial reprogramming approach in skeletal MuSCs by overexpressing the *Msx1* gene (which is involved in digit and limb regeneration[115–117]). They found that this approach partially restores youthful gene expression in myogenic cells and promotes differentiation without inducing any partial loss in cell identity[113], further supporting the idea that other transcription factors might represent a safer alternative to induce epigenetic rejuvenation as also reported by other groups[61,118].

In vitro partial reprogramming in human cells

Although experiments in mice provided foundational evidence that epigenetic reprogramming can reverse aspects of aging, it is an open question of how these effects will translate into human biology. Early experiments in human cells were somewhat encouraging as they provide evidence that some of the biomarkers of aging (that is telomere lengths) were responding to the epigenetic reprogramming, but protocols relied upon rejuvenation through re-differentiation after iPS cell formation[20,26,28]. The first attempt to optimize the process in humans and eliminate the need to start from a blank slate was made by Manukyan and colleagues in 2014, nearly 2 years earlier than the landmark publication from Belmonte's group[15]. LIN28, an RNA-binding protein best known for its role in promoting pluripotency via regulation of the microRNA let-7, was added to the OSKM cocktail and used to treat senescent fibroblasts. After 9 days of reprogramming, HP1b mobility, an indicator of active cell cycling, was restored to the same levels found in young fibroblasts without sacrificing cell identity[100].

A detailed description of reprogramming kinetics in human cells was provided by Olova and colleagues in 2019 (ref. 114), who analyzed epigenetic and transcriptomic data to define the time course of human dermal fibroblast rejuvenation[119]. Using Horvath's clock[120], they found that epigenetic rejuvenation started between days 3 and 7, continuing until day 20 when the treated cells reached an epigenetic age of zero[114]. Transcriptomic analysis of fibroblast gene signatures showed an overall reduction in expression with comparatively stable levels until day 15. By contrast, pluripotency genes showed an inverse relationship that steadily increased over time until reaching an apparent threshold around the same time. These observations led to the conclusion that this time point represents an optimal point for achieving epigenetic rejuvenation without losing cell identity. The rationale for this conclusion lies in the fact that many aging-associated epigenetic marks are shed without the initiation of actual pluripotency programs. Maintenance of somatic profiles makes these cells more probable to revert toward the original and previously established cell fate when the expression of reprogramming factors is stopped.

Although this work established a benchmark for reprogramming human cells, it was not possible to consider transcriptional heterogeneity during reprogramming as bulk-based analyses were used, which (as previously discussed) were later shown to exert mosaic-like expression patterns in tissues[113]. Exploiting this caveat has led to single-cell-based transcriptomic approaches and a better understanding of reprogramming potential in specific cells and tissues. Leveraging directives from foundational publications, Sarkar et al. obtained the first evidence of partial reprogramming-induced rejuvenation in old human cells in vitro[17]. A new, noninvasive messenger RNA-based delivery strategy was used for the transient expression of six reprogramming factors, OSKM, LIN28 and NANOG (OSKMLN), in human cells (Fig. 4c). Transcriptomic analysis of the three cohorts of cells showed that OSKMLN

treatment induced a more youthful and cell-type-specific gene expression profile without affecting the expression of cell-specific genes. Importantly, the extent of OSKMLN reprogramming was multifaceted and far-reaching, as epigenetic hallmarks of aging (H3K9me3, HP1g, LAP2a and SIRT1), functional parameters (autophagosomal, mitochondrial and proteasomal activities) and epigenetic clocks were significantly impacted, with treated cells more closely resembling younger cells compared with their age-matched old counterparts. Interestingly, rejuvenation was retained 6 days after interrupting the treatment. Similar results were also obtained in chondrocytes and human MuSC cell types, confirming that this method works in different cell types. These findings validate a nonintegrative clinical method for reprogramming and open avenues for in vitro and in vivo rejuvenation strategies.

Although substantial progress was made concerning developing an efficacious reprogramming cocktail, the timing and duration of induction was still open for optimization. Fortunately, work in mice identified a window, known as the maturation phase, during reprogramming where cells were most probable to experience rejuvenation without crossing the threshold and become iPS cells[96]. Gill and colleagues took advantage of this window and induced extended overexpression of the reprogramming factor cocktail for 10–14 days before withdrawing for an additional 4 weeks to allow for fibroblast re-differentiation (Fig. 4d)[74]. With this protocol, defined as 'maturation phase partial reprogramming', they achieved more substantial rejuvenation than had been described before at levels comparable to Guo et al.'s work in mice[74,75]. Specifically, analysis of the epigenetic clock after in vitro partial reprogramming showed approximately 30 years of rejuvenation. Surprisingly, rejuvenation only occurred when the factors were overexpressed for 13 days, and no significant effects were observed at the other time points, suggesting that day 13 is a critical time point in the rejuvenation process, though, notably, later time points (days 15 and 17) failed to show an epigenetic rejuvenation effect. Moreover, rejuvenation effects were maintained for at least 4 weeks after treatment, representing the most extended retention of youthful status following reprogramming. A possible explanation for these interesting outcomes may be that fibroblast-specific enhancers remained demethylated during partial reprogramming[74], which may indicate retention of epigenetic memory required for reversion to the proper somatic state.

Concluding remarks and future perspectives

The ability to re-shape the chromatin into a more youthful state without inducing irreversible changes to cell identity has pioneered a new area of aging research. In this Review, we have focused on elucidating the biological mechanisms and pathways involved in reprogramming initiation, as well as the connection between reprogramming and epigenetic rejuvenation. Collectively, the works described suggest that timing and the identity of each factor and their relative stoichiometry require fine-tuning in different cell types to achieve a rejuvenated phenotype. For these reasons, they may need to be tailored for each organ or tissue to promote rejuvenation without inducing a complete loss in cell identity.

In this direction, tissue-specific overexpression studies paired with single cell-based analyses will be invaluable to better consider the unique identity of the cells and of their initial chromatin state, as well as the co-existence in vivo of cell autonomous and nonautonomous functions.

An important aspect to consider is whether a partial 'de-differentiation' is necessary for effective rejuvenation. Although some studies suggest that rejuvenation of the epigenome can occur without a de-differentiation step and involve 're-differentiation'[121], others indicate that partial de-differentiation has a crucial role. These diverse perspectives underscore the complexity of cell-type-specific rejuvenation mechanisms and highlight the need for further investigation to determine the precise requirements in different cellular contexts. In addition to this, the use of a naturally aged system has important strengths over premature genetic models, as the latter may introduce confounding factors that can alter the outcome of the results.

Despite being useful to validate feasibility in human cells and perform molecular studies, partial reprogramming in vitro poses additional challenges as cultured cells often lose some aging-related features observed in vivo and might exhibit confounding environment-induced phenomena.

Another limiting factor in advancing the field is the lack of suitable and universal molecular biomarkers for measuring age reversal. Existing epigenetic clocks, originally developed for different purposes, have not been thoroughly demonstrated to provide accurate readouts for anti-aging interventions.

Lastly, safety remains a critical aspect of partial reprogramming. Although some studies have not seen any detriment to tissues after more than a year of overexpression[92], this issue remains unresolved. Although RNA-based approaches or the replacement of OKSM genes with potentially safer genes or small molecules acting as chromatin modulators hold promise, comprehensive evaluation and mechanistic investigation will be necessary to establish safety and efficacy in humans.

The convergence of these diverse sets of evidence underscores the necessity for additional strengthening and validation by the scientific community to establish acceptance and credibility regarding certain findings. The resulting perplexity serves as a stark reminder of the ongoing challenge in developing clinically applicable rejuvenation protocols. However, it also highlights the vital importance of comprehending the intricate molecular dynamics that underlie this process. Such understanding is indispensable for paving the way toward the development of effective and safe strategies that hold the potential to be applied to humans in a clinical setting.

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Author contributions

A.C., M.M. and V.S. designed and conceived the Review. The manuscript was written by A.C., M.M. and S.M.L. M.M. wrote the reprogramming factors section, S.M.L. the partial reprogramming event section and A.C. the partial reprogramming sections. T.A.J. contributed by writing the additional factor section. A.C. generated the figures, with help from S.R. R.R.H. provided a major contribution to the editing process with help from A.P. and S.R. H.R.S. provided suggestions and contributed on the reprogramming factor section. The Review was supervised and edited by V.S. and D.A.S.

Competing interests

V.S. is a co-founder, SAB Chairman, Head of Research and shareholder of Turn Biotechnologies. D.A.S. is a consultant, inventor, board member and, in some cases, a founder and investor in Life Biosciences (a reprogramming company), EdenRock Sciences (Cantata, Metrobiotech), InsideTracker, Fully Aligned, Zymo, Galilei, Immetas, Animal Biosciences, Tally Health and others. For full information, see Supplementary File 1. The other authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to David A. Sinclair or Vittorio Sebastiano.

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