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The crosstalk between cellular reprogramming and senescence in aging and regeneration

Aurélie Chiche, Cheng Chen & Han Li

Cellular Plasticity and Disease Modelling

Department of Developmental and Stem Cell Biology, CNRS UMR 3738

Institut Pasteur, 25 Rue du Dr Roux, Paris 75015, France

Corresponding author: Han LI

Email Address: han.li@pasteur.fr

Tel: (33)-1 4568 8484

Introduction:

Aging is associated with progressive functional degeneration of different tissues and a dramatically increased risk of many diseases. Moreover, it has been increasingly recognized that aging itself might be the underlying driving force for developing these diseases^{1,2}. The last 30 years of extensive research, both in model organisms and humans, enlists nine tentative hallmarks of aging reflecting potential drivers of aging process². Importantly, these studies highlight that the rate of aging is susceptible to modification³. Therefore, how to curb aging processes to improve the human healthspan is one of the most exciting challenges for biomedical research in the coming years.

Cellular senescence is one of the hallmarks of aging², a form of stress response to various stimuli that leads to a permanent cell-cycle arrest. It is well established cells with markers of senescence accumulate in tissues of aged mammals, including rodents^{4,5}, primates^{6,7}, and humans⁸⁻¹². Furthermore, recent studies demonstrated that senescence negatively impacts healthy aging¹³⁻¹⁵. Noteworthy, removing senescent cells ameliorate a wide range of aging-associated disease conditions, including atherosclerosis¹⁶, osteoarthritis¹⁷, liver steatosis¹⁸, type 2 diabetes¹⁹, improve regeneration capacity of multiple tissues^{19,20}, and extend both health and life span in mice^{14,15}. Therefore, recent studies have placed cellular senescence in the central stage of regeneration and aging^{21,22}.

Cellular reprogramming is the process of reverting terminal differentiated cells to the pluripotent state, which has tremendous potentials for regenerative medicine and aging research^{23,24}. Besides generating *in vitro* models to study aging and age-associated diseases, reprogramming has gained considerable attention recently for its rejuvenation potential^{25,26}. In particular, reprogramming the cells from aged donor to pluripotency could erase several aging hallmarks *in vitro*²⁷⁻³¹. Importantly, some rejuvenation effects preserve after re-differentiation^{28,29,31}. Intriguingly, partial reprogramming, induced by short term OSKM expression, has been shown to enhance tissue regeneration in older mice and extend the life span of the progeroid mice³². Although the underlying mechanism remains largely unknown, the tremendous potentials merit further investigation.

Interestingly, recent studies revealed that senescence has both cell-autonomous and non-cell autonomous effects in reprogramming³³⁻³⁵. At the same time, partial reprogramming could reduce certain senescent associated features on the cellular level^{32,36}, suggesting that two processes are intimately connected. Both senescence and reprogramming have been extensively reviewed respectively elsewhere^{21,37-40}. Here, we summarize the current understanding of the interplay between senescence and reprogramming, with a particular focus on their potential implication in aging and regeneration. Examining the crosstalk between cellular senescence and reprogramming will further the mechanistic understanding of both processes and devise novel anti-aging and rejuvenation strategies exploiting the potential synergistic effect²⁵.

Cellular senescence: the common cell fate within various contexts

Cellular senescence is a stable cell cycle arrest that occurs in diploid cells at the end of their replicative life span. In 1961, Hayflick and Moorhead demonstrated that human primary fibroblasts in culture could divide a limited number of times before irreversible cell cycle withdrawal⁴¹. This process is known as the “Hayflick limit” or replicative senescence, which is caused by progressive shortening of telomeres upon each cell division. Besides, there is a wide range of stimuli that could induce senescence prematurely, including DNA damage, oncogenic stress, oxidative stress, protein misfolding, and genomic/epigenomic alterations, which eventually activate the p53/p21 and p16^{Ink4a}/pRB pathways to establish and reinforce the persistent growth arrest^{37,42}. Moreover, there are many biological processes, such as tissue repair/regeneration⁴³⁻⁴⁶, and embryonic development^{47,48} rely on senescence.

Senescent cells are characterized collectively by several non-exclusive markers⁴². Permanent cell cycle arrest is an essential feature of senescence. Senescent cells do not resume proliferation in response to mitogenic signals, which is different from quiescence, a state of reversible cell cycle arrest. At the same time, senescent cells frequently exhibit a persistent DNA damage response (DDR) and induction of antiapoptotic genes, which separate them from post-mitotic differentiated cells. The accumulation of mitochondria and lysosomal in the senescent cells allow the detection of the β -galactosidase activity in sub-optimal pH (senescence-associated β -galactosidase, SA β Gal)¹¹. Although senescent cells do not proliferate, they remain metabolically active and robustly express a senescence-associated secretory phenotype (SASP)⁴⁹: secretion of many inflammatory cytokines, growth factors, and extracellular matrix metalloproteinases (MMPs). Therefore, up-regulation of the cyclin-dependent kinase inhibitors (CKIs), lack of proliferation, resistance to apoptosis, activation of DDR, SA β Gal, and SASP factors are commonly used markers of senescence. Noteworthy, SASP factors play a crucial role in mediating senescence non-cell autonomous functions by attracting immune cells and altering tissue microenvironment³⁷. However, the temporospatial regulation of SASP is highly heterogeneous in a cell type and stress-dependent manner⁵⁰.

Work over the last decade expanded the involvement of cellular senescence to various biological and pathological processes, including embryonic development^{47,48}, tissue repair /regeneration^{43,44,46}, tumorigenesis^{38,51}, and aging^{14,15,38}. Of note, senescence can be

either beneficial or detrimental for the organism depending on the cellular context. In the first scenario, transient and programmed senescence is initiated in the severely damaged or unwanted cells. Permanent withdrawal from proliferation is crucial for preventing the propagation of premalignant cells in the context of tumorigenesis. Then arrested cells secrete a mix of SASP factors, including cytokines, chemokines, growth factors, and metalloproteases. Besides communicating and recruiting the immune system⁴⁴, SASP could trigger the proliferation and differentiation of undamaged cells⁴³, promote ECM deposition⁵², optimize tissue remodeling^{43,52}, and induce cellular plasticity and stemness of the neighboring cells⁴⁶. Eventually, removal of the senescent cells is essential to eliminate SASP and construct/reconstruct the tissue.

In contrast, senescent cells accumulate in tissues during physiological aging³⁸ and in age-associated pathologies^{53,54}. There are several non-mutually exclusive possibilities of why senescent cells accumulate with age. Firstly, the senescent cell production rate might be increased owing to more aged cells containing a higher level of damage. Secondly, SASP mediated paracrine senescence⁵⁵ could further accelerate this process. Thirdly, the aging immune system may be less efficient in removing senescent cells. Alternatively, senescent cells may evolve in a way to directly impair the immune surveillance in the aged tissues. Noteworthy, a recent study used both experimental and mathematical models to address this question. The authors used datasets from previous longitudinal studies to identify the most suitable model describing the dynamics of the senescent cells, which was further validated by senescence induction in mice of different ages. The model suggests that the turnover rate of senescent cells decreases with age due to increased production and reduced removal⁵⁶. Surprisingly, senescent cells actively inhibit their removal. Although the mechanisms by which senescent cells use to disrupt their removal is unknown, this study proposed a provocative scheme with exciting implications in senolytic approaches.

Recent studies provided vital clues on how senescence promotes age-related tissue dysfunction in both cell-autonomous and non-cell autonomous manner. First of all, a permanent cell cycle arrest directly impairs tissue regeneration. Senescent adult stem cells (ASCs), such as hematopoietic stem cells and muscle stem cells, fail to re-enter the cell cycle and resume proliferation after exiting from the quiescence^{57,58}. Moreover, a persistent proliferative arrest could disrupt the cell turnover resulting in permanent cell loss in tissues that do not have ASCs. Besides, senescent cells can lose many cellular functions. Senescent vascular cells reduced endothelial tight junction and lessen barrier integrity *in vitro*, which might contribute to the age-related blood-brain barrier disruption⁵⁹. Senescent chondrocytes fail to secrete various ECM factors that are important for articular cartilage maintenance^{38,60}, while senescent β cells lose the cell identity and cannot produce insulin¹⁹.

Although it remains to be demonstrated *in vivo*, SASP could promote a dysfunctional stem cell niche⁵⁸ and induce paracrine senescence in the neighboring cells to exacerbate the diminished stem cell functionality. Moreover, SASP factors could induce tissue degradation^{17,61}, interfere with differentiation process⁶¹, and stimulate tissue fibrosis⁶². Finally, SASP may contribute to the proinflammatory microenvironment in many

age-related pathologies^{17,53,54,63}. Work over the last decade demonstrated that selective elimination of senescent cells, either genetically or by senolytic drugs, ameliorates various age-related pathologies in mice^{5,16,17,53,54,63}, and significantly extends both the health and life span^{14,15,58,64}.

Why does the acute and chronic senescence have the opposite impact on tissue regeneration? There are two potential explanations. Cell autonomously, young ASCs are spared from acute senescence, due to either higher resistance to damage or protective stem cell niche. Therefore, their regenerative functions are preserved. On the contrary, senescence most likely occurs to all the cell types during aging. The irreversible cell cycle arrest prevents the activation of ASCs, which are indispensable for tissue regeneration. Non-cell autonomously, the SASP components might be different between acute and chronic senescence. The SASP is possibly more proinflammatory with age, which might further inhibit the functions of ASCs and impair regeneration.

In summary, there are many questions remain to be elucidated, including how senescence is induced during embryonic development and tissue repair, why senescent cells accumulate in various disease conditions, and why senescence elimination ameliorates organism aging. *In vivo* senescence program is highly heterogeneous, containing different cell types and SASP composition, which might have a significant impact on senescence phenotype and functions. Therefore, further investigation of *in vivo* senescence in different contexts will determine the shared and distinct features among various types of senescence, which might allow specifically target the detrimental effects of senescence.

Cellular reprogramming: plasticity beyond stem cells

Cell identity is established in the course of lineage differentiation during development, maintained by epigenetic memories and defined by a broad range of molecular and functional properties, which is generally stable for the terminally differentiated cells⁶⁵. Previously, it was thought that differentiation is an irreversible process. In the 1950s, Briggs and King began to test the developmental potential of differentiated cells in frogs. They transferred the nucleus from one cell to an enucleated cell (oocytes, in this case), a method known as nuclear transfer. Later, using the same methodology but a different frog species, *Xenopus laevis*, Gurdon successfully cloned sexually mature frogs using donor nuclei from cells at various developmental stages⁶⁶ and fully differentiated intestinal cells⁶⁷. Gurdon's seminal work provided the first evidence that certain factors in the oocyte cytoplasm can erase the cellular identity encoded in the nucleus of a somatic cell, strongly supported the principle of nuclear equivalence and laid the foundation for the future development of reprogramming cell identity.

Almost 50 years after Gurdon's experiments, Yamanaka demonstrated that a small set of transcription factors, Sox2, Klf4, Oct4 and c-Myc (OSKM) are sufficient to convert somatic cells into the pluripotent state, known as induced pluripotent stem cells (iPSCs)²³. Currently, there are several routes to reprogram cell identity. For example, cells can be firstly reverted to the pluripotent state, followed by differentiation to desired identities. Alternatively,

one cell type can be directly converted to another cell identity by expressing specific factors^{68,69}. The method, also known as lineage reprogramming, bypasses embryonic states altogether and eliminates the tumorigenic risk of undifferentiated pluripotent cells, an important safety issue for therapeutic applications⁶⁸. Noteworthy, cell identity can also be manipulated *in vivo* via forced expression of the same transcription factors combination as their *in vitro* counterparts³⁹. Several reprogrammable mouse models are engineered to express Yamanaka factors (OSKM) upon doxycycline treatment to induce reprogramming *in vivo*, which are evaluated by the Nanog expression (a pluripotency marker) and teratoma formation^{32,70,71}. Importantly, these studies indicate the tissue microenvironment can support full reprogramming, which raised the possibility to modulate cell fate *in situ* to promote tissue regeneration. Nowadays, direct lineage reprogramming of undamaged cells into the desired cell type using defined factors *in situ* is an emerging alternative to improve self-repair and tissue regeneration^{72,73}.

The ground-breaking advances in cellular reprogramming led to a paradigm shift in the biomedical research, with exciting implications for advances in disease modeling and regenerative medicine^{40,74}. Studies from the last decade have identified many combinations of factors to facilitate cell fate conversion, including transcription factors, small molecules, and microRNAs, which firmly demonstrated the remarkable plasticity of the differentiated cells both *in vitro* and *in vivo*⁷⁵. Thanks to the advance in single cell biology, cellular reprogramming has become a tractable system to study the mechanisms of cell fate conversion⁴⁰. The next challenge in the field is to understand how cellular plasticity is regulated *in vivo*, which will provide essential insights for improving tissue regeneration in a controlled manner.

Partial reprogramming: an emerging rejuvenation strategy

Cellular reprogramming offers many exciting opportunities for aging research. Generation of iPSCs from Hutchinson-Gilford progeria syndrome (HGPS) and Werner syndrome (WS) patients provided powerful *in vitro* models to unravel the molecular mechanisms of premature and physiological aging and to facilitate the drug development for these devastating rare diseases^{76,77,78}. Besides, many age-related pathologies that are associated with losing functional cells could benefit from iPSCs-based regenerative therapies. Moreover, reprogramming aged cells may gain pivotal insight into epigenetic rejuvenation⁷⁹. Lastly, *in vivo* lineage-reprogramming based tissue repair *in situ* would be particularly important in the aged organism with diminished regenerative capacity.

Recent research highlighted that reprogramming could be a rejuvenation process^{27,25}. Previously, iPSCs derived from aged donors revert several age-associated features, including elongated telomere length due to the reactivation of telomerase during reprogramming⁸⁰, improved mitochondrial quality and function, and reset of heterochromatin marks and genes expression signatures^{28,29,81}. Importantly, fibroblasts and neurons differentiated from old donors iPSCs preserved the youthful state, including the transcriptomic profile and proliferative capacity (in the case of fibroblasts)^{28,29,81}. Interestingly, neurons generated from

old donor fibroblasts via transdifferentiation (bypassing the pluripotency stage) retained the age-associated signature of the donor cells³¹. Therefore, rejuvenation might occur specifically during reprogramming to pluripotency process, where extensive proliferation is required⁸².

The impact of partial reprogramming on tissue regeneration and rejuvenation has been explored recently in both mice and human cells. Ocampo et al., showed short-term OSKM expression in the fibroblasts derived from progeria mouse model (*Lmna*^{G608G}) reduced the markers of DNA damage, features of senescence, and nuclear envelop defects. Moreover, OSKM expression reverted the levels of two age-associated heterochromatin marks, H3 (H3K9me3) and H4K20me3⁸³. These effects suggest that short-term OSKM expression could transiently ameliorate several premature aging features both on the molecular and cellular level. On the organism level, partial reprogramming could improve progeria mice's health span and life span without tumorigenesis, and enhance tissue regeneration of pancreas and skeletal muscle in reprogrammable mice³².

Next, Sarkar et al. induced transient reprogramming using a six-factor cocktail (Lin28 and NANOG, together with OSKM) in human fibroblasts and endothelial cells derived from donors of different ages. Horvath's epigenetic clocks base on DNA methylation levels, which can closely predict the biological age in a broad range of tissues and cell types⁸⁴. By applying to Horvath's epigenetic clocks, the authors showed that transient reprogramming could reduce the DNA methylation age of the cells shortly after the treatment. Moreover, treated cells exhibited a more youthful transcriptomic profile together with improved functional parameters. However, the sample size is quite small. It would be interesting to test more samples and for a longer time after the treatment to determine whether the changes would persist. Strikingly, transient reprogramming could restore the regenerative capacity of aged human muscle stem cells when transplanted in mice⁸⁵. Two recent studies showed that short-term OSKM expression could reduce scar formation⁸⁶ and muscle fibrosis⁸⁷ in young mice. Furthermore, a preprint reported OSK expression could promote axon regeneration of retinal ganglion cells to ameliorate the vision impairment in a glaucoma mouse model and naturally aged mice⁸⁸.

Interestingly, transient expression of reprogramming factors could reduce several biomarkers of senescence *in vitro*, including the expression of senescence-related genes, such as p16, p21, and various SASP factors^{32,36}; and SA β Gal positive cells. However, reprogramming factors could induce robust senescence response³³, which might due to much higher expression of OSKM. It would be informative to compare the OSKM expression quantitatively between full reprogramming and partial reprogramming. Besides, the impact of partial reprogramming on senescence remains to be tested *in vivo*. Nonetheless, these observations suggest that senescence suppression might be one of the mechanisms mediating the effects of partial reprogramming.

Therefore, partial reprogramming can be defined broadly as short term OSKM expression, which could revert various age-associated features on the molecular and cellular level, and induce functional improvement without promoting cancer development. Mechanistically, partial reprogramming could induce epigenetic rejuvenation, such as histone

modifications³² and DNA methylations^{85,88}. Although it has been proposed that epigenetic rejuvenation could occur faster than erasing the somatic identity^{85,89}, it is currently unknown how to control this stochastic process to uncouple rejuvenation from dedifferentiation. Determining the key epigenetic changes that drive the rejuvenation effect while maintaining the somatic cell identity remains an important future direction, which could also help to identify means, other than OSKM-mediated *in vivo* reprogramming, to promote tissue regeneration via epigenetic changes. Besides, it is currently unknown which cell types are amenable for partial reprogramming to mediate its beneficial effects on the organismal level. Partial reprogramming has been shown to increase numbers and improve the function of muscle stem cells^{32,85}, a cell type intrinsically susceptible to *in vivo* reprogramming³⁵. Therefore, partial reprogramming might directly enhance the cellular plasticity of ASCs. Noteworthy, partial reprogramming might facilitate the removal of senescent cells^{32,85}. However, the impact of partial reprogramming on senescence remains to be tested *in vivo*.

Cell-autonomous and non-cell autonomous effects of senescence in cellular reprogramming

The involvement of senescence on reprogramming was first assessed *in vitro*. The Ink4/Arf locus encodes three potent tumor suppressors, namely p16(Ink4a), p19(Arf), and p15(Ink4b), which are crucial senescence mediators. Genetic inhibition of the Ink4/Arf locus enhances both reprogramming kinetic and efficiency. Interestingly, reprogramming culture condition significantly induces the expression of the Ink4/Arf locus. While silencing the locus via epigenetic remodeling is necessary for the successful reprogramming⁹⁰. Concordantly, knockdown of *p53* and *p21^{CIP1}* could also enhance reprogramming efficiency⁹¹⁻⁹⁴.

These studies raised the question of whether senescence is critical for reprogramming (**Table 1**). Overexpression of OSKM in human primary fibroblasts triggers a stress response with characteristics of senescence, including up-regulation of p53, p16^{INK4a}, and p21^{CIP1}, increased SA β Gal staining, impaired proliferation, and forming senescence-associated heterochromatin foci (SAHF)³³. Mechanistically, overexpression of OSKM triggers replicative stress and DNA damage to induce senescence, known as the reprogramming-induced senescence (RIS)³³. Besides, several pathways have been identified to affect reprogramming efficiency via modulating RIS, including histone demethylase JMJD3^{33,95}, the BMP-SMAD-ID pathway⁹⁶, and the mechanistic target of rapamycin (mTOR)^{97,98}.

Although the molecular mechanisms of *in vitro* reprogramming have been extensively characterized⁴⁰, little was known about reprogramming *in vivo*. Recently, two relevant publications revealed the paracrine effect of senescence in promoting reprogramming *in vivo*^{34,35}. Mosteiro and colleagues found, similar to *in vitro* reprogramming, expression of OSKM induces widespread senescence response in multiple tissues. Interestingly, Nanog⁺ cells appear in close proximity to senescent cells, and there is a positive correlation between reprogrammed cells and senescence induction. To further understand the role of senescence on *in vivo* reprogramming, the authors examined the effect of genetic ablation of *Ink4a/Arf* or *p53* on *in vivo* reprogramming. Surprisingly, the deletion of the *Ink4a/Arf* locus, but not *p53*,

largely abolished *in vivo* reprogramming accompanied by the diminished senescence response. Importantly, modulating senescence levels by various small molecules can affect reprogramming efficiency accordingly, demonstrating a causative link. Mechanistically, authors find SASP, in particular IL-6, promotes *in vivo* reprogramming in a non-cell autonomous manner. Moreover, tissue damage-associated senescence triggered by bleomycin treatment is sufficient to induce *in vivo* reprogramming in the lung. Finally, accumulation of senescence during accelerated or physiological aging could enhance *in vivo* reprogramming³⁴.

Interestingly, some organs, such as the pancreas and liver, are far more permissive for *in vivo* reprogramming than others. To identify potential tissue-specific barriers of *in vivo* reprogramming, Chiche and colleagues focused on skeletal muscle a tissue refractory to *in vivo* reprogramming. Using the same reprogrammable mouse model, they showed that both acute and chronic injury enables reprogramming in the skeletal muscle, which triggers cell proliferation and transient senescence response in the same time⁴⁵. In sharp contrast to the tissues permissive for *in vivo* reprogramming, OSKM expression alone failed to induce senescence response in the resting skeletal muscle, which might partially account for the lack of reprogramming. Next, authors showed elevated senescence level, either by local irradiation or aging, could enhance reprogramming efficiency. While specific elimination of senescent cells, either by genetic ablation of p16 positive cells or senolytic drug ABT263, could reduce reprogramming efficiency proportionately. Consistently, IL-6 is an important mediator for this process. Moreover, they demonstrate that Pax7⁺ muscle stem cells are a cell of origin of *in vivo* reprogramming using the lineage tracing system, which is critical for future applications of *in vivo* reprogramming that require to target specific cell types³⁵.

The seemingly contradictory effects of senescence on reprogramming could be reconciled by the cell-autonomous and non-cell autonomous effects of senescence (**Figure 1**). Intrinsically, senescence is a barrier for reprogramming via the upregulation of the Ink4/Arf locus, activation of the p53 signal pathway, and prevention of proliferation. However, extrinsically, it could promote the plasticity of neighboring non-senescent cells via SASP factors. Importantly, tissue injury could enhance *in vivo* lineage reprogramming efficiency in the liver and pancreas³⁹. Therefore, it would be interesting to examine whether injury-induced senescence could promote cellular plasticity in the lineage reprogramming setting. Therefore, further investigation of SASP produced by RIS might identify novel factors that could promote cellular plasticity, which is relevant to all reprogramming-based tissue repair strategies and physiological regeneration.

Future perspectives: Exploring the synergistic effect of senescence elimination and reprogramming on rejuvenation

Although it remains to be further explored, both senescence elimination and reprogramming may use distinct mechanisms to rejuvenate overlapping targets. For example, reprogramming might directly revert aged ASCs to a more youthful state via epigenetic reprogramming. Eliminating senescent cells, including ASCs and other cell types within the

stem cell niche, could reduce inflammatory factors in the microenvironment and indirectly improve the functionality of ASCs and tissue regeneration.

Therefore, a key question is whether we could combine senescence eradication and partial reprogramming for more effective rejuvenation with less potential detrimental effect. For example, reprogramming might increase the proliferation capacity of cells containing mutations to promote tumorigenic risk. Excessive elimination of senescent cells might interfere with their beneficial role during tissue repair. However, as discussed above, the relationship between senescence and reprogramming is complicated. The opposing effects of reprogramming factors on senescence response (between full reprogramming and partial reprogramming) might be due to their induction level and the duration. For future studies, it is crucial to determine the impact of partial reprogramming on the accumulation of senescent cells in the tissue, the difference between reprogramming induced and physiologically accumulated senescence, and the possibility of combining both strategies in a timely order.

Both senolytic and reprogramming based rejuvenation are still in their infancy. Further elucidation of the modes of action for both strategies and the interplay between them will provide crucial information for understanding human aging and aging-associated diseases.

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FIGURE LEGENDS

Figure 1. Cell autonomous and non-autonomous effects of senescence in cellular reprogramming

Summary of recent findings describing the roles of senescence during cellular reprogramming. Reprogramming factors (OSKM) induced senescence is an important cell intrinsic barrier for reprogramming. While SASP could promote cellular plasticity and reprogramming in the neighboring non-senescent cells.

Table 1. Summary of studies on the impact of senescence on reprogramming in vitro

Figure 1: Cell-autonomous and non-cell autonomous effect of senescence on reprogramming

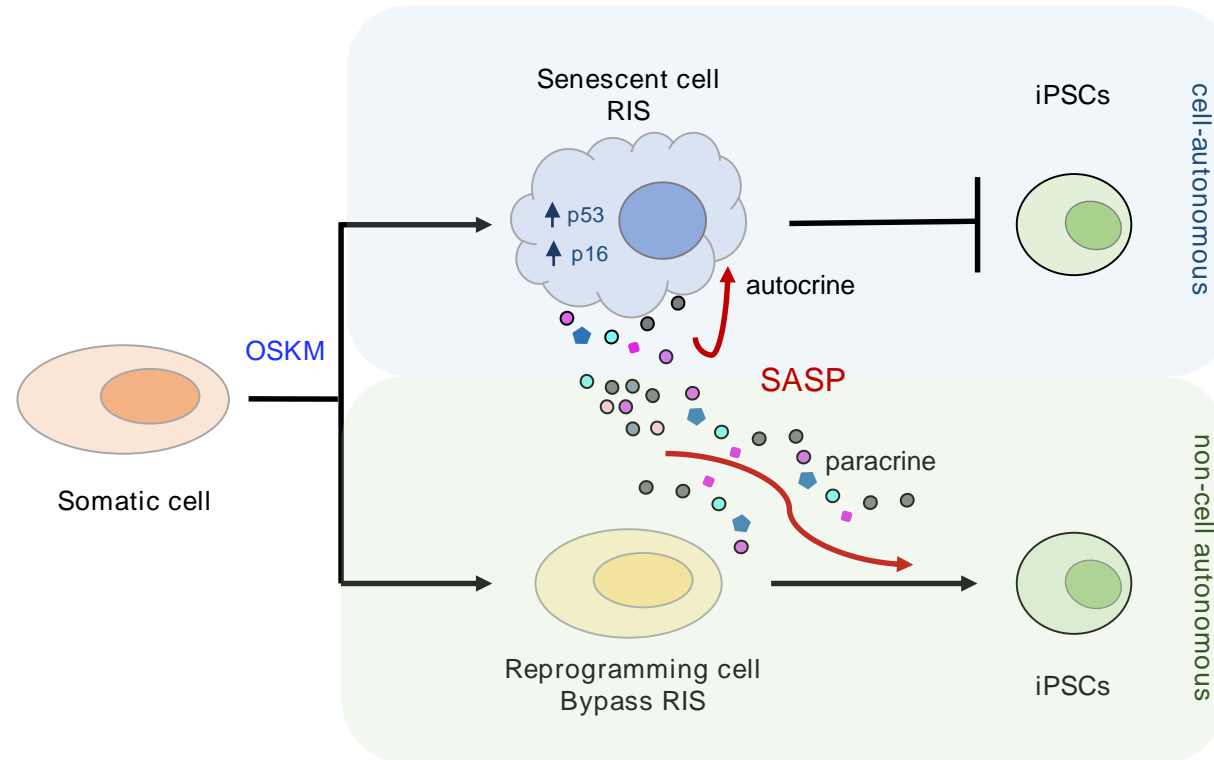


Table 1: Summary of studies on the impact of senescence on reprogramming in vitro

Species	Gene/pathway involved	Impact on RIS	Effect on reprogramming	Mode of action	Re.
Human	P53, p16, JMJD3	Mediating RIS	Inhibition	Cell-autonomous	33
Mouse & Human	JMJD3	Induction	Inhibition	Cell-autonomous	33, 95
Human	BMP-SMAD-ID	Suppression	Promotion	Cell-autonomous	96
Mouse	mTOR	Suppression	Inhibition	Cell-autonomous	97
Human	mTOR	Suppression	Dual effects	Cell-autonomous & Non-cell autonomous	98