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# Dissecting the non-cell autonomous impact of cellular senescence on cellular reprogramming

Mathieu Von Joest

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Sorbonne Université

École doctorale Complexité du Vivant

Cell Plasticity and Disease Modelling

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Présenté par Mathieu von Joest

Thèse de doctorat de Biologie Cellulaire

Dirigée par Dr. Didier Montarras & Dr. Han Li

Le 5 Décembre 2019

Devant le jury composé de :

Isabelle Petropoulos	Présidente du jury
David Bernard	Rapporteur
Manuel Collado	Rapporteur
Didier Montarras	Directeur de thèse
Han Li	Co-Directrice de thèse
Oliver Bischof	Examineur
Carl Mann	Examineur



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« I will either find a way, or make one »

Hannibal



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---

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## ABBREVIATIONS

---

2i: 2 inhibitors of GSK and MEK pathways (PD0325901 and CHIR99021)  
4EBP1: Eukaryotic Translation Initiation Factor 4Ebinding-protein 1  
AA: Ascorbic Acid  
Angpt: Angiopoietin  
APC: Adenomatous Polyposis Coli  
AREG: Amphiregulin  
AKT: Protein kinase B  
AMPK: 5' adenosine monophosphate-activated protein kinase  
ALK5: TGF $\beta$  type I receptor kinase  
ARF: Alternative Reading Frame  
ATM: Ataxia Telangiectasia Mutated  
ATP: Adenosine Tri-Phosphate  
ATR: Ataxia Telangiectasia and Rad3-Related  
BMP: Bone Morphogenic Protein  
BMPR: BMP Receptor  
BrdU: Bromodeoxyuridine  
BRCA1: Breast Cancer Type 1  
BRAF: Serine/Threonine Kinase B-raf  
BTC: Betacellulin  
BUBR1: Budding Uninhibited by Benzimidazole 3  
C/EBP: CCAAT-enhancer-binding protein  
CCL: CC Motif Chemokine Ligand  
CCN1: Cellular Communication Network 1  
CCR2: C-C chemokine receptor type 2  
CD: Cluster of Differentiation  
CDK: Cyclin Dependent Kinase  
CDKN: Cyclin Dependent Kinase Inhibitor  
CFU: Colony Forming Unit  
cGAS-STING: Cyclic GMP-AMP synthase- Stimulator of Interferon Genes  
CIP: CDK interacting protein  
CT-1: Cardiotrophin-1  
CM: Conditioned Medium  
CTGF: connective tissue growth factor  
CTX: Cardiotoxin

## ABBREVIATIONS

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CXCL: CXC Motif Chemokine Ligand  
DAPI: 4',6-Diamidino-2-Phenylindole  
DDIS: DNA Damage Induced Senescence  
DDR: DNA Damage Response  
DOT1L: Disruptor of Telomeric Silencing-1 like  
DOX: Doxycycline  
DSBs: DNA Double Strand Breaks  
E2F1: E2F Transcription Factor 1  
ECCs: Embryonal Carcinoma Cell  
EGF: Epidermal Growth Fator  
EGFR: EGFR receptor  
EMT: Epithelial to Mesenchymal Transition  
ERBB: Erb-B2 Receptor Tyrosine Kinase  
EREG: Epiregulin  
ERK: Extracellular signal Regulated Kinase  
EPGN: Epigen  
ESCs: Embryonic Stem Cells  
Exo: Exosomes  
EZH2: Enhancer of Zeste Homolog 2  
FGF: Fibroblast Growth Factor  
FOXO4: Forkhead box protein O4  
Fz: Frizzled  
 $\gamma$ H2AX: gamma H2A Histone Family  
GATA4: GATA binding protein 4  
GDNF: Glial cell-derived neurotrophic factor  
GLUT1: Glucose transporter 1  
Gp130: Glycoprotein 130  
GRO $\alpha$ : alias of CXCL1 gene  
GSK3: Glycogene Synthase Kinase 3  
HAT: Histone Acetyltransferase  
HB-EGF: Heparin-Binding EGF-like Growth Factor  
HMGB: High Mobility Group Box  
HMT: Histone Methyltransferase

## ABBREVIATIONS

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HSCs: Hematopoietic Stem Cells

HSV-TK: truncated herpes simplex virus 1 (HSV-1) thymidine kinase

hTERT: human Telomerase reverse transcriptase

i4F: induced 4 factors

ICAM: Intercellular Adhesion Molecule

IGFBP: Insulin Growth Factor Binding Protein

IGF: Insulin Growth Factor

IKK: Nuclear Factor NFkappaB Inhibitor Kinase Alpha

IL-: Interleukin

INHBA: Inhibin Subunit Beta A

INHBB: Inhibin Subunit Beta B

INK4A: Inhibitor of CDK4

INK-ATTAC:

IPF: idiopathic pulmonary fibrosis

iPSCs: induced Pluripotent Stem Cells

JAK: Janus Kinase

JNK: c-Jun N-terminal kinases

KIP: Kinase inhibitory protein

KITL: KIT ligand

Klf4: Krupper Like Factor 4

KSR: KnockOut Serum Replacement

LAKI: Lamin A/C Knock In

LEF: Lymphoid Enhancer Binding Factor

LIF: Leukemia Inhibitory Factor

LIFR: LIF Receptor

lncRNA: Long Non-Coding RNA

MafA: MAF BZIP Transcription Factor A (Pancreatic Beta-Cell-Specific Transcriptional Activator)

MAPK: mitogen-activated kinase protein 1

MDM2: Mouse double minute 2 homolog

MEFs: Mouse Embryonic Fibroblasts

MEK: MAP/ERK Kinase 1

MET: Mesenchymal to Epithelial Transition

MiDAS: Mitochondria Dysfunction-Associated Senescence

## ABBREVIATIONS

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mi-RNA: micro RNA  
MMPs: Matrix Metallo-Proteinases  
MRN: MRE11-RAD50-NBS1  
mtDNA: Mitochondrial DNA  
mTOR: mechanistic Target of Rapamycin  
myc: Avian Myelocytomatosis Viral Oncogene Homolog  
NAD: Nicotinamide adenine dinucleotide  
NADH: reduced form of NAD  
Neurog3: Neurogenin-3  
NF1: Neurofibromin 1  
NfκB: Nuclear Factor kappa light chain enhance of activated B cells  
NGF: Nerve Growth Factor  
NOTCH1: NOTCH Receptor 1  
ntPSCs: nuclear transferred PSCs  
NuRD: Nucleosome Remodelling Deacetylase  
Oct4: Octamer Binding transcription factor 4  
OIS: Oncogene Induced Senescence  
OSIS: Oxidative Stress Induced Senescence  
OSKM: Oct4, Sox2, Klf4, c-Myc  
PD: Populations Doublings  
PDGF-AA: Platelet Derived Growth Factor -AA  
Pdx1: pancreatic and duodenal homeobox 1  
PFK: Phosphofructokinase 1  
Pim-1: Proto-oncogene serine/threonine-protein kinase  
PI3K: phosphoinositide 3-kinase  
PKC: Protein kinase C  
PODXL: Podocalyxin-like protein 1  
PRC: Polycomb Repressor Complex  
PSC: Pluripotent Stem Cells  
PTEN: Phosphatase and Tensin Homolog  
p53BP1: p53 Binding Protein-1  
RAS: Rat Sarcoma  
RAF: Rapidly Accelerated Fibrosarcoma

## ABBREVIATIONS

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Rb: Retinoblastoma  
ROS: Reactive Oxygen Species  
RT-qPCR: Retro Transcriptase quantitative Poly Chain Reaction  
RPA: Replication Protein A  
SA  $\beta$ -Gal : Senescent Assay  $\beta$ -Galactosidase  
SAHF: Senescence Associated Heterochromatin Foci  
SASP: Senescence Associated Secretory Phenotype  
SCARS: DNA segments with chromatin alterations reinforcing senescence  
SCF: Stem Cell Factor  
SCNT: Somatic Cell Nuclear Transfer  
SDF: senescence associated DNA damage foci  
SEN: Senescent  
Setdb1: SET Domain Bifurcated Histone Lysine Methyltransferase 1  
SMAD: Sma- And Mad-Related Protein  
SN: Supernatant  
SNAI1: Snail Family Transcriptional Factor 1  
SOX: SRY-Box 2  
SSEA: STAGE SPECIFIC EMBRYONIC ANTIGEN-1  
STAT: Signal Transducers and Activators of Transcription  
TA: Tibialis anterior  
TF: Transcription Factor  
TGF- $\alpha$ : Transforming Growth Factor  $\alpha$   
TGF- $\beta$ : Transforming Growth Factor  $\beta$   
Thy1: Thy-1 Cell Surface Antigen  
TIFs: telomere-dysfunction-induced focus  
TIS: Therapy Induced Senescence  
Treg: T regulatory  
Twist1: Twist Family BHLH Transcription Factor 1  
VPA: Valproic Acid  
VEGF: Vascular Endothelial Growth Factor  
WT: Wild Type  
Zeb1/2: Zinc Finger E-Box-Binding Homeobox 1/2

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# INTRODUCTION



## 1. CELLULAR SENESCENCE

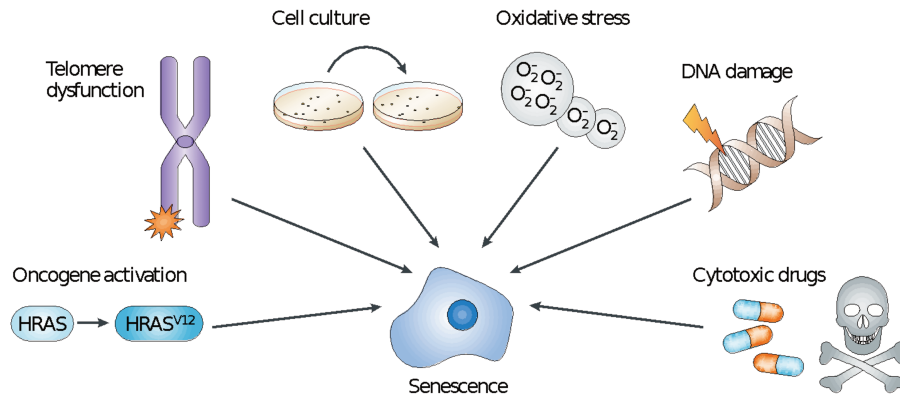
### 1.1 A brief history of cellular senescence

Cellular senescence was first described by Leonard Hayflick and Paul Moorhead (Hayflick and Moorhead, 1961). They demonstrated that human fibroblasts have a limited capacity of proliferation and could only divide up to a limited number of divisions before entering senescence, which is termed as “The Hayflick Limit”. Interestingly, the number of cell divisions has always been  $50 \pm 10$  despite the human tissue of origin, which suggests a common mechanism was responsible for this limit. It was later discovered that this mechanism is the shortening of telomeres. Nowadays, cellular senescence is defined as a cellular stress response that is characterized by a permanent cell cycle arrest, due to the increased expression of several cell cycle inhibitors (e.g. p16<sup>INK4A</sup>, p19<sup>ARF</sup>, p21<sup>CIP</sup>). For instance, the biological stresses which induce senescence can either be replication (replicative stress), DNA damage, or oncogene expression. Over the years, additional stresses have been found, such as oxidative stress or mitochondrial dysfunction. Despite the growth arrest, senescent cells remain metabolically active and secrete various molecules such as cytokines, chemokines, proteinases and growth factors, which is termed Senescence Associated Secretory Phenotype (SASP). Finally, senescent cells acquire other markers, such as increased lysosomal activity or chromatin remodeling. Importantly, these markers are not universal, depending for instance on cell type or the stress of origin triggering cellular senescence.

Since this initial discovery, cellular senescence has been mainly studied as a self-defense mechanism to prevent the proliferation of damaged cells. Recently, it has been shown that senescent cells accumulate with age and contribute to physiological aging (Baker et al., 2016; Childs et al., 2015; Dimri et al., 1995). Recent works have also showed its role in other processes such as embryogenesis (Munoz-Espin et al., 2013), tissue regeneration (Demaria et al., 2014) or pathologies (Munoz-Espin and Serrano, 2014). All these studies demonstrated the importance and multiple roles of cellular senescence during life, emphasizing the need to better understand this process.

## 1.2 Causes of Cellular Senescence

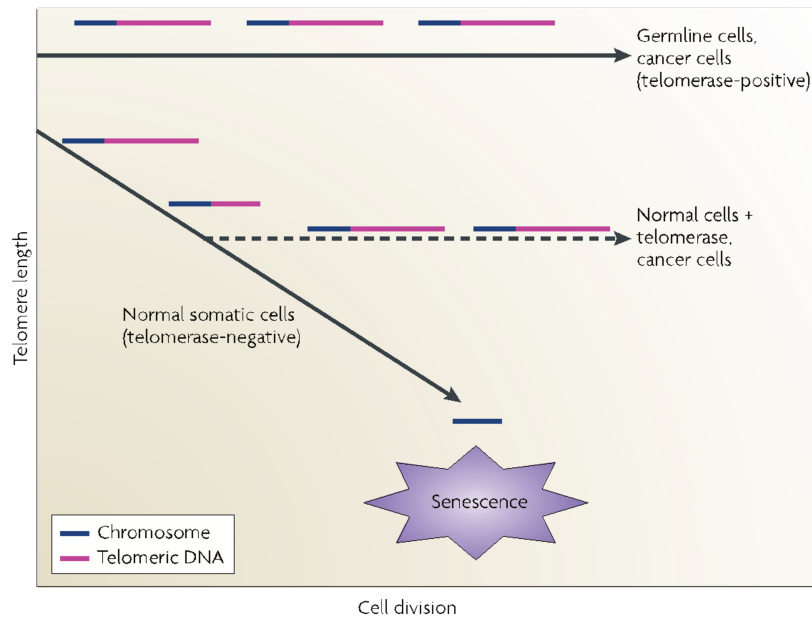
Senescence can be triggered by numerous stresses, such as replicative stress, irradiation or oncogene activation (Figure 1).



**Figure 1. Many roads to senescence.** Diverse factors can engage a common program the end point of which is the establishment of an irreversible proliferative arrest known as senescence. All of these stimuli represent stressful conditions for the cell, many of which are present in the tumor environment. Senescence functions as a self-defense mechanism to prevent the proliferation of potentially damaged cells (Collado and Serrano, 2006).

### 1.2.1 Replicative Senescence

Replicative senescence is caused by telomere shortening. Telomeres are composed of repeated DNA sequences (TTAGGG) that are present at the end of chromosomes. They can only be generated by a retro transcriptase enzyme called telomerase which is expressed in embryonic stem cells but not in somatic cells. Telomeres have specific size (depending on species) that ranges from 10kb to 20kb. At each cell division, telomeres are shortened ultimately reaching a critical size, which could be recognized as DNA breaks by the repair machinery leading to cell division arrest, and ultimately senescence induction (d'Adda di Fagagna, 2008).

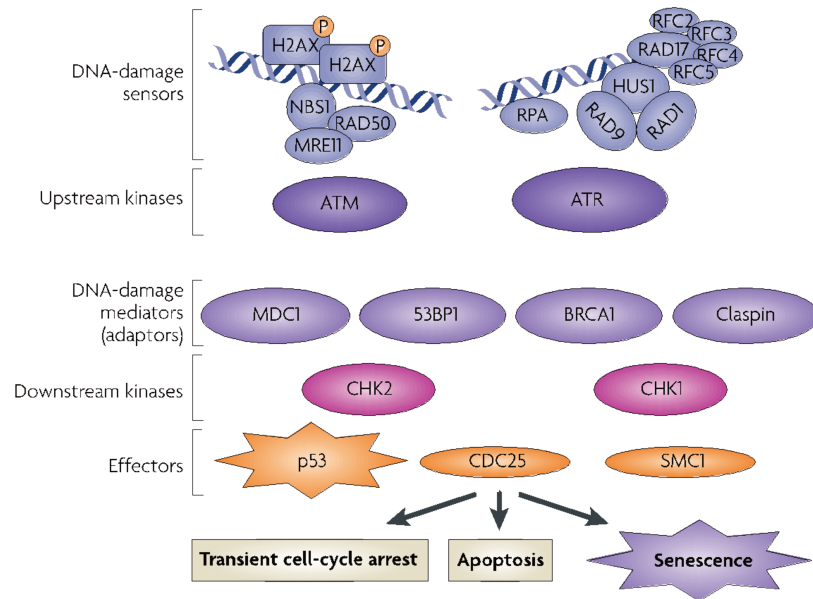


**Figure 2. Telomere-dependent senescence.** Telomeres are stretches of a repetitive DNA sequence and associated proteins (magenta) that are located at the termini of linear chromosomes (blue). The telomeric ends form a circular protective cap termed the t-loop, which may help to prevent the termini from triggering a full DNA-damage response (DDR). Telomere lengths are maintained by the enzyme telomerase, which is expressed by cells that comprise the germline as well as many cancer cells. Most normal somatic cells do not express this enzyme, or express it only transiently or at levels that are too low to prevent telomere shortening caused by the end-replication problem. In such cells, telomere lengths decline with each cell cycle. Eventually, one or a few telomeres become sufficiently short and malfunction, presumably owing to loss of the protective protein–DNA structure. Dysfunctional telomeres trigger a DDR, to which cells respond by undergoing senescence (d'Adda di Fagagna, 2008) .

### 1.2.2 DNA Damage Induced Senescence

Throughout life, DNA is continually damaged by endogenous and exogenous agents, such as metabolites, chemical reagents or physical agents (Jackson and Bartek, 2009). An exogenous source of damage to reproduce DNA damage induced by senescence (DDIS) *in vitro* is irradiation, which mainly creates DNA double strand breaks (DSBs), the most severe DNA lesions. These DNA damage trigger DNA-damage response (DDR) in order to repair them by different mechanisms (Figure 3). Unfortunately, upon DNA damage accumulation (e.g several DSBs), cells are not able to repair their DNA. Constant DDR activation induces either cell death or senescence (Beausejour et al., 2003; d'Adda di Fagagna, 2008).





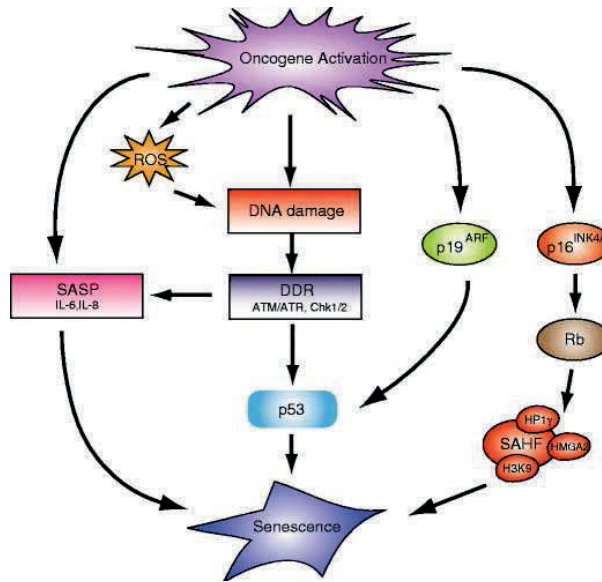
**Figure 3. The DNA Damage Response.** DNA damage in the form of DNA double-strand breaks and other DNA discontinuities is thought to be sensed by a host of factors such as replication protein A (RPA) and replication factor C (RFC)-like complexes (which contain the cell-cycle-checkpoint protein RAD17). These complexes recruit the 911 complex (RAD9–HUS1–RAD1). Damage is also sensed by the MRN complex (MRE11–RAD50–NBS1). Detection of DNA damage then leads to the activation of upstream protein kinases such as ATM and ATR, which trigger immediate events such as phosphorylation of the histone variant H2AX. The modified chromatin recruits multiple proteins. Some of these proteins augment signaling by the upstream kinases, participate in transducing the damage signal and optimize repair activities by other proteins, the identity of which depends on the nature of the damage and position in the cell cycle. Several adaptor proteins, including MDC1, 53BP1, BRCA1 and claspin, orchestrate the orderly recruitment of DNA-damage response proteins, as well as the function of downstream kinases such as checkpoint-1 (CHK1) and CHK2, which propagate the damage signal to effector molecules such as SMC1, CDC25 and the tumor suppressor p53. The effector molecules halt cell-cycle progression, either transiently or permanently (senescence), or trigger cell death (apoptosis) (Campisi and d'Adda di Fagagna, 2007).

### 1.2.3 Oncogene Induced Senescence

Oncogenic stress can also induce senescence, known as oncogene induced senescence (OIS). For instance, overexpression of oncogenic Ras can trigger cellular senescence both *in vitro* and *in vivo* (Sarkisian et al., 2007; Serrano et al., 1997). More recently, it has been found that senescence can be induced by many other oncogenes such as *BRAF*, *E2F1*, *cyclin E* and *AKT* (Courtois-Cox et al., 2008) or by disruption of tumor suppressor genes such as phosphatase And Tensin Homolog (PTEN) or Neurofibromin (NF1) (Chen et al., 2005; Courtois-Cox et al., 2006).

Upon activation of oncogenes, cells undergo a hyper proliferation phase. This massive proliferation generates replicative stress, which activates DDR and induces senescence (Kuilman et al., 2010). In the context of PTEN disruption, mTORC1 and mTORC2

phosphorylate p53 and bypass the negative regulation of MDM2, leading to the upregulation of p21<sup>CIP</sup> and induction of senescence (Astle et al., 2012). NF1 is negative regulator of RAS oncogene, thus deficiency in this gene induces senescence, through increase of ERK and Akt signaling.



**Figure 4. Mechanisms that elicit OIS.** Multiple pathways arise in response to oncogenic stress. Oncogene-driven hyper-replication causes the accumulation of DNA damage, which, in turn, activates the DNA damage response (DDR), culminating in the activation of p53, a potent mediator of senescence. The key activators of p53 in this response are ATM, ATR, Chk1, and Chk2. Oncogene activation also elicits the activation of p19<sup>ARF</sup>, an upstream positive regulator of p53. Furthermore, oncogenes may activate the p16<sup>INK4A</sup>-Rb tumor suppressor pathway, inducing the formation of senescence-associated heterochromatin foci (SAHF), which not only serve as markers of senescent cells but likely function to restrict the expression of cell cycle genes. In addition, oncogenic stress also causes the accumulation of reactive oxygen species (ROS), which, in excess, can damage DNA and generate a DDR. Finally, recent evidence indicates that oncogene activation causes the formation of a senescence-associated secretory phenotype (SASP), in which senescent cells secrete multiple factors (e.g. IL-6 and -8) that can induce and maintain OIS (Reddy and Li, 2011).

#### 1.2.4 Reactive Oxygen Species

Reactive Oxygen Species (ROS) can also triggers senescence. It has been found that culture of Mouse Embryonic Fibroblasts (MEFs) under 3% oxygen strongly increased the PDs compared to culture of MEFs under 20% oxygen (Parrinello et al., 2003). Culture in low oxygen conditions delays up-regulation of p16<sup>INK4A</sup> and p19<sup>ARF</sup> (Parrinello et al., 2003). Over time, ROS accumulation leads to high levels of oxidative DNA damage both in the nucleus and mitochondria but also protein damage (Busuttill et al., 2003). Therefore, oxidative stress is another source which can elicit cellular senescence.

### 1.2.5 *Therapy induced senescence (TIS)*

Cancer cells can still establish senescence through radiation or chemotherapy treatment (Demaria et al., 2017; Gewirtz et al., 2008). In the context of chemotherapy, mild dose of doxorubicin blocks topoisomerase II and DNA replication, creating DSBs and inducing DDR and entrance in senescence (Roberson et al., 2005). Finally, treatment with cyclophosphamide can also induces senescence and mutation in the INK4A locus generates a poor response to chemotherapy (Schmitt et al., 2002), suggesting that senescence induction is a marker for successful treatment.

### 1.2.6 *Mitochondria Dysfunction-Associated Senescence (MiDAS)*

Dysfunction of mitochondria can induce a senescent phenotype with a distinct phenotype compared to other stresses (Wiley et al., 2016). Indeed, cells display a lower NAD<sup>+</sup>/NADH ratio, which causes both the growth inhibition and prevent the IL-1-associated SASP through AMPK-mediated p53 activation. Interestingly, progeroid mouse model which accumulates mitochondrial DNA (mtDNA) mutations have a high level of senescent cells with a MiDAS SASP, which confirms a distinct senescence response.

## 1.3 Signaling Pathways Mediating Senescence

Noteworthy, most of the stresses, ranging from telomere shortening to ROS species, cause DNA damage and elicit DDR. ATM and/or ATR phosphorylate  $\gamma$ H2AX to trigger DDR (Campisi and d'Adda di Fagagna, 2007). ATM and/or ATR with the help of other mediators such as 53BP1, activate checkpoint kinase-1 and -2 (CHK1 & CHK2) which will recruit effectors, notably p53 and p21<sup>CIP</sup>. The two main pathways mediating senescence are CIP/KIP locus including p21<sup>CIP</sup> and p27<sup>KIP1</sup>/p57<sup>KIP2</sup>, and INK4 locus including p15<sup>INK4A</sup>, p16<sup>INK4A</sup> and p19<sup>ARF</sup> (p14<sup>ARF</sup> in humans). Among all these inhibitors, p16<sup>INK4A</sup> and p21<sup>CIP</sup> are considered as key effectors of cellular senescence (Herranz and Gil, 2018). Conversely, p14<sup>INK4A</sup> is more essential to induce senescence in humans while p19<sup>ARF</sup> plays a more important role in mice. These effectors inhibit cyclin-dependent kinases such as CDK4 and CDK6. Moreover, cell cycle progression relies first on CDK4/6 activation by association with

cyclin D which control the transition from G1 to S phase. Thus, their inhibition by cyclin kinases inhibitors leads to irreversible cell cycle arrest and entry in senescence.

### 1.3.1 *INK4/ARF locus*

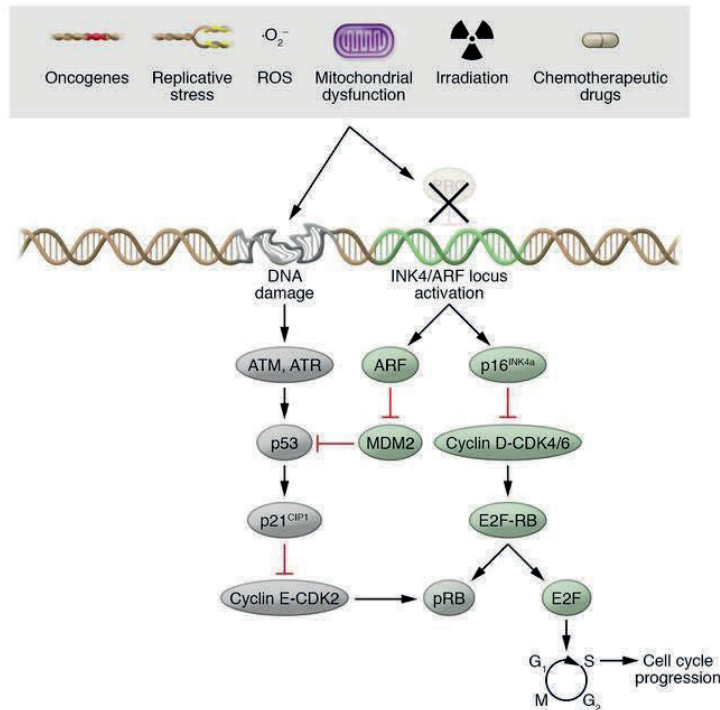
This locus encodes for three tumor suppressors, p15<sup>INK4B</sup>, p16<sup>INK4A</sup> and p19<sup>ARF</sup> (p14<sup>ARF</sup> in humans). Increased p16<sup>INK4A</sup> expression is frequently observed during RSIS or OIS (Palmero et al., 1997; Serrano et al., 1997). For instance, OIS active MAPK signaling cascade to activate p16<sup>INK4A</sup> (Lin et al., 1998). p16<sup>INK4A</sup> phosphorylates CDK4/6, preventing its binding to cyclin D, which leads to hypophosphorylation of Rb. Hypophosphorylated Rb binds to transcription factor E2F and block cell cycle progression. Rb cooperates with Suv39h1 and catalyzes histone 3 trimethylation to repress E2F (Narita et al., 2003). Thus, unreleased E2F factors is unable to activate the expression of several genes important for cell cycle progression, including cyclin A and cyclin E (Figure 5).

### 1.3.2 *p53- p21<sup>CIP</sup> pathway*

A second pathway essential in mediating senescence is (p19<sup>ARF</sup>)-p53- p21<sup>CIP</sup> signaling pathway. p19<sup>ARF</sup> represses MDM2 which subsequently leads to p53 stabilization (Pomerantz et al., 1998; Zhang et al., 1998). Indeed, p53 protein has a very short half-life expression (Giaccia and Kastan, 1998), notably due to ubiquitylation which results in its degradation by the proteasome. Expression of p19<sup>ARF</sup> sequesters MDM2, which leads to stable expression of p53 and up-regulation of p53-downstream targets. Consequently, p53 can mediate cell growth inhibition notably by activating p21<sup>CIP</sup> (Figure 5). DDR also induces p53 expression (Herranz & Gil 2018).

Finally, p21<sup>CIP</sup> establishes p53-dependent cell cycle arrest through inhibition of CDK2 (d'Adda di Fagagna, 2008). Previous findings suggest that p21<sup>CIP</sup> role may be only restricted to the onset of senescence. Unlike p16<sup>INK4A</sup>, its expression does not always persist in senescent cells (Sharpless and Sherr, 2015). In addition, p21<sup>CIP</sup> is not required for OIS in

MEFs (Pantoja and Serrano, 1999). Interestingly, mammalian embryonic development associated senescence depends on p21<sup>CIP1</sup> (Munoz-Espin et al., 2013; Storer et al., 2013).

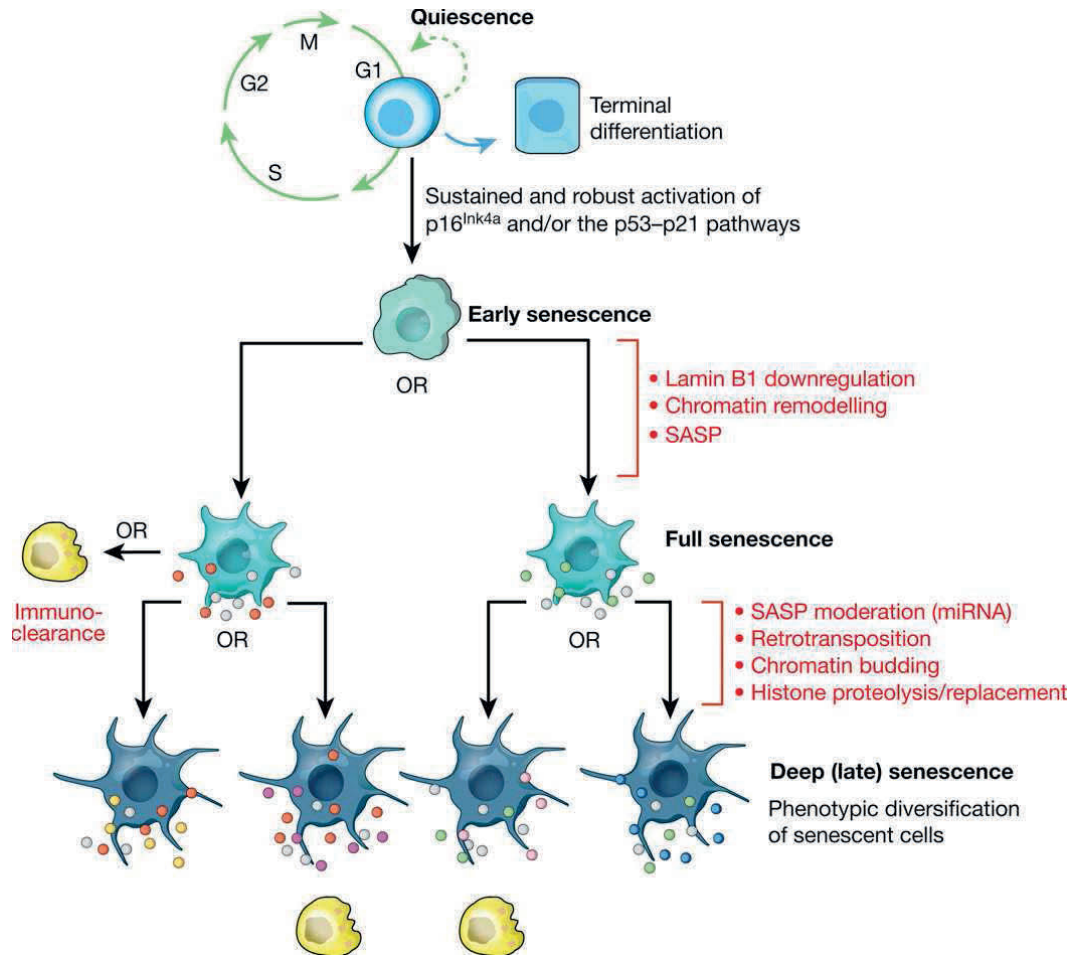


**Figure 5. Molecular pathways controlling growth arrest during senescence.** A variety of stressors induce senescence-associated growth arrest. Cell cycle exit is regulated by induction of the p16<sup>INK4a</sup>/Rb and p53/p21<sup>CIP1</sup> pathways (Herranz and Gil, 2018).

### 1.4 Markers of senescent cells

Cellular senescence is a complex phenomenon that has multiple characteristics. However, characterizing senescent cells is difficult as none of the characteristics is exclusive. Indeed, there is no universal cell marker or specific gene signature to define senescent cells as gene expression profiles vary according to the stress of origin, the senescence program, and the cell type (Hernandez-Segura et al., 2017). Moreover, the phenotypes of senescent cells evolve over time, leading to the separation of senescence process between “early” and “deep (late)” senescence (Hernandez-Segura et al., 2017; van Deursen, 2014) (figure 6). Senescence is a dynamic process that involves at least two steps. The first is “early senescence”, which is characterized by up regulation of p16<sup>INK4A</sup>, p19<sup>ARF</sup>, p21<sup>CIP1</sup>, lamin B1 down regulation, and secretion of some SASP factors. It is also referred as acute senescence, which occurs after tissue damage or when cells become senescent suddenly. The second one is “late senescence”,

which is defined as chronic senescence that appears during aging, when cells accumulate different types of damages and become senescent progressively. While chronic senescent cells maintain high p16<sup>INK4A</sup> expression, they might downregulate p21<sup>CIP</sup> (Sharpless and Sherr, 2015), exhibit chromatin budding and profound modification of SASP composition. Therefore, a combination of several markers is generally used to characterize senescent cells.



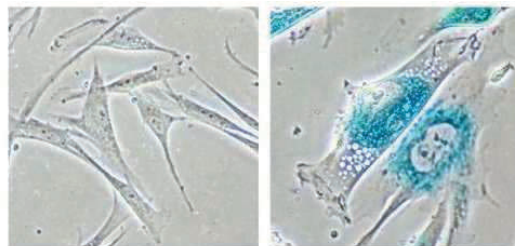
**Figure 6. Hypothetical multi-step senescence model.** Mounting evidence suggests that cellular senescence is a dynamic process driven by epigenetic and genetic changes. The initial step represents the progression from a transient to a stable cell-cycle arrest through sustained activation of the p16<sup>Ink4a</sup> and/or p53-p21 pathways. The resulting early senescent cells progress to full senescence by downregulating lamin B1, thereby triggering extensive chromatin remodeling underlying the production of a SASP. Certain components of the SASP are highly conserved (grey dots), whereas others may vary depending on cell type, nature of the senescence-inducing stressor, or cell-to-cell variability in chromatin remodeling (red and green dots). Progression to deep or late senescence may be driven by additional genetic and epigenetic changes, including chromatin budding, histone proteolysis and retrotransposition, driving further transcriptional change and SASP heterogeneity (yellow, magenta, pink and blue dots). It should be emphasized that although the exact nature, number and order of the genetic and epigenetic steps occurring during senescent cell evolution are unclear, it is reasonable to assume that the entire process is prone to SASP heterogeneity. The efficiency with which immune cells (yellow) dispose of senescent cells may be dependent on the composition of the SASP. Interestingly, the proinflammatory signature of the SASP can fade due to expression of particular microRNAs late into the senescence program, thereby perhaps allowing evasion of immuno-clearance (van Deursen, 2014).

### 1.4.1 Growth Arrest

One of the most used markers of senescence is the stable cell cycle arrest. This cell cycle arrest results from increased expression CDK inhibitors such as p16<sup>INK4A</sup>, p19<sup>ARF</sup>, p21<sup>CIP</sup> and p53 (Baker et al., 2016; Gil and Peters, 2006; Jung et al., 2010; Sharpless and Sherr, 2015). Thus, senescence can be assessed through expression of these markers. In addition, Rb phosphorylation status can be used to determine senescence (Kuilman et al., 2010; Narita et al., 2003). Absence of BrdU incorporation or Ki67 reflects the growth arrest of the senescent cells.

### 1.4.2 Senescence associated $\beta$ -Galactosidase (SA- $\beta$ -Gal)

Senescent cells have increased lysosomal compartment (Lee et al., 2006). Consequently, senescent cells have higher  $\beta$ -Galactosidase activity and can degrade X-Gal at suboptimal pH conditions (pH 5.5-6.0) whereas growing cells degrade X-Gal only in acid pH (~4.5) (Dimri et al., 1995) (Figure 7). However, SA- $\beta$ -Gal is not exclusive to senescent cells. Highly confluent cells are also positive for SA- $\beta$ -Gal (Yang and Hu, 2005). Thus, SA- $\beta$ -Gal assay needs to be used together with other analysis to identify senescent cells.



**Figure 7. SA- $\beta$ -Gal staining of proliferative fibroblasts or senescent fibroblasts.** On the left, proliferative fibroblasts are not positive for SA- $\beta$ -Gal unlike senescent cells that are on the right picture.

### 1.4.3 Morphological Changes

Senescent cells undergo several morphological changes. Senescent cells induced by hRAS<sup>V12</sup> overexpression, OSIS and DDIS become flat and enlarged (Kuilman et al., 2010; Serrano et al., 1997). Unlike previous cases, senescent cells due to BRAF<sup>E600</sup> display a more spindle-shaped morphology. Finally, a significant increase of stress vacuoles can be observed (Kuilman et al., 2010; Munoz-Espin and Serrano, 2014).

### *1.4.4 Resistance to Apoptosis*

Senescent cells upregulate several pro-survival genes and are resistant to apoptosis (Childs et al., 2014). Moreover, senescent cells overexpress BCL-2, an anti-apoptotic protein (Ryu et al., 2007). Senescent cells also down regulate proapoptotic genes such as Bax protein (Sanders et al., 2013). Moreover, p21<sup>CIP</sup> can promote cell survival in the context of chronic DDR. p21<sup>CIP</sup> prevents cell death by restraining JNK activity, which is required to induce apoptosis with caspase 3 (Yosef et al., 2017). Finally, senescent cells prevent cell death by overexpressing FOXO4, which sequester p53 in the nucleus (Baar et al., 2017).

### *1.4.5 Chromatin Remodeling*

Upon p16<sup>INK4a</sup> activation, proliferative genes are inhibited through Rb-mediated chromatin remodeling which is named Senescence Associated Heterochromatin Foci (SAHF) (Narita et al., 2003). SAHF are DNA foci constituted of reorganized heterochromatin in a core positive for H3K9me3 surrounded by rings positives H3K27me3 and H3K36me3 (Chandra et al., 2015) (Figure 8). These three histone marks are associated with repression of gene expression. These SAHF regions can be detected using DAPI counterstaining. Additionally, senescence associated DNA damage foci (SDF) and telomere-dysfunction-induced foci (TIFs) can be observed in senescent cells (Rodier et al., 2011; Takai et al., 2003). Finally, sites of chronic DDR sites are named DNA segments with chromatin alterations reinforcing senescence (SCARS) (Rodier et al., 2011). These SCARS notably contribute to SASP maintenance and reinforce cell cycle arrest.

### *1.4.6 Senescence Associated Secretory Phenotype*

Following cells cycle arrest, senescent cells establish a program to secrete numereouse factors which acts in autocrine, paracrine or juxtacrine manner (Acosta et al., 2013; Hoare et al., 2016; Kuilman et al., 2008). SASP has multiple functions, in aging, age-related diseases, tissue homeostatsis and immune surveillance (Herranz and Gil, 2018) (Discussed in section 1.5).



## 1.5 SASP (Senescence Associated Secretory Phenotype)

### 1.5.1 SASP

Following cell cycle arrest, senescent cells remain metabolically active and have profound modifications of their gene expression related to protein secretion. Many factors are secreted, including cytokines, chemokines, proteases and growth factors (Coppe et al., 2010). Importantly, SASP composition depends on the stress inducing senescence, cell types and time (Hernandez-Segura et al., 2017). Despite its heterogeneity, some factors, such as IL-6, IL1, MMP1 or CXCL8 in human cells are generally conserved between all different SASP. SASP has pleiotropic functions in aging, and age-related diseases, tissue homeostasis and immune surveillance. SASP is mainly dependent on the persistent DDR activation (Rodier et al., 2011). For instance, mechanistic Target of Rapamycin (mTOR) regulates SASP independently of DDR in DDIS cells (Laberge et al., 2015). Treatment with Rapamycin significantly inhibits secretion of several SASP factors such as IL-6, CCL8 or VEGFa but does not alter number of DNA-SCARS in senescent cells, which indicates that DDR signaling is not altered.

### 1.5.2 Transcription Factors and SASP regulation

SASP expression is regulated by critical transcription factors, notably NF- $\kappa$ B and C/EBP $\beta$ . NF- $\kappa$ B is induced by DNA damage, inflammation and the inflammasome (Salminen et al., 2012). Following DDR induction, ATM activates phosphorylation of p38MAPK and IKKg, which subsequently activates NF- $\kappa$ B (Ohanna et al., 2011). Furthermore, TGF- $\beta$  can also activates NF- $\kappa$ B by SMAD2/3 phosphorylation (Salminen et al., 2012). NF- $\kappa$ B regulates notably the expression of both IL-1 and IL-8 in human fibroblasts upon OIS, reinforcing senescence establishment and participating to senescence transmission (Acosta et al., 2013; Acosta et al., 2008; Chien et al., 2011). Repression of p65, one of the members of NF- $\kappa$ B complex inhibited the expression of IL-6, IL-8 and CXCL1 (Chien et al., 2011). Recently, several kinases have been shown to trigger senescence and trigger SASP production in an NF- $\kappa$ B dependent program (Ferrand et al., 2015). In parallel, C/EBP $\beta$  is induced in OIS and is an essential regulator of IL-6 secretion (Kuilman et al., 2008). Interestingly, C/EBP $\beta$  activity seems to be dynamically regulated by NOTCH1 during senescence. During senescence,

NOTCH expression is increased but its cleavage is dynamic with a peak of activity during early senescence phase (Hoare et al., 2016). Initially, NOTCH up-regulates TGF- $\beta$  and inhibits expression of IL-1 $\alpha/\beta$  and IL-8 via C/EBP $\beta$  repression. Then, NOTCH is deactivated and C/EBP $\beta$  repression is abolished, thus C/EBP $\beta$  is increased and drive an inflammatory SASP (Hoare et al., 2016).

### *1.5.3 Signaling pathways and SASP regulation*

Several signaling cascades have been associated with SASP secretion, including DDR, p38/MAPK, STING and mTOR. p38/MAPK pathway is activated after genotoxic stress or oncogenic stress. Its activation leads to SASP secretion, notably chemokines and cytokines (Freund et al., 2011). More recently, cGAS-STING pathway has been proposed to be a regulator of SASP production (Dou et al., 2017; Gluck et al., 2017). Loss of nuclear membrane integrity generates cytoplasmic chromatin fragments, which activates cGAS-STING pathway and promotes SASP generation. Disruption of cGAS or STING alters the secretion of several factors such as IL-1 $\alpha$  and IL-8. Furthermore, mTOR also upregulates IL-1 $\alpha$  translation, which increases the activity of NF- $\kappa$ B and consequently induces the expression of multiple SASP factors (Laberge et al., 2015). Autophagy has also been implicated in SASP regulation. Upon DNA damage, transcription factor GATA4 is stabilized by selective autophagy inhibition, which in turn activates NF- $\kappa$ B mediated SASP (Kang et al., 2015). Thus, SASP is regulated at multiple levels, by a complex redundant network of transcription factors and signaling cascades.

### *1.5.4 Roles of SASP*

#### *1.5.4.1 Immune surveillance of senescent cells*

One of SASP roles is to attract immune cells to remove senescent cells (Figure 8). In a mouse model wherein liver cells transduced with an inducible p53 shRNA and hRAS, induction of p53 triggers senescence and SASP production. SASP secretion attracts immune cells including neutrophils, macrophages and natural killer cells and removal of senescent cells (Xue et al., 2007).

Another study reported a role of the adaptive immune response and CD4<sup>+</sup> T cell response in the removal of senescent cells (Kang et al., 2011). Absence of functional CD4<sup>+</sup> T cells or macrophages leads to persistence of senescent cells and tumor development, highlighting the necessity of a complete immune response. Recently, the CCL2-CCR2 axis has been shown to be important for the clearance of senescent cells. CCL2, which is an important SASP factor, induces the differentiation of CCR2<sup>+</sup> myeloid cells into macrophages and the clearance of pre-cancerous senescent hepatocytes (Eggert et al., 2016). Deficiency in this axis leads to outgrowth of cancer cells in the liver (Eggert et al., 2016).

### 1.5.4.2 *Autocrine Effect of SASP*

SASP molecules are essential to establish and reinforce senescence in an autocrine manner. IL-6, IL-8 (in human cells), GRO $\alpha$  and IGFBP7 are particularly important to establish a complete cell cycle arrest in the context of OIS (Kuilman et al., 2008; Wajapeyee et al., 2008; Yang et al., 2006). Disruption of IL-6 or its receptor with shRNA prevents senescence induction or induces bypass of senescence induced by BRAF<sup>E600</sup> in human fibroblasts (Kuilman et al., 2008). Interestingly, blockade of IL-6 using antibody does not induce bypass of senescence, which indicates that IL-6 acts also in an autonomous fashion. Following an oncogenic stress, C/EBP $\beta$  binds to the promoter of IL-6 and IL-8, which subsequently cooperate with C/EBP $\beta$  to upregulate p15<sup>INK4B</sup> expression. Disruption of CXCR2, which binds IL-8 and/or GRO $\alpha$ , alleviates both OIS and RSIS, and attenuates the activation of DDR (Acosta et al., 2008). Neutralization of IL-8 and/or GRO $\alpha$  by using antibodies causes bypass in senescent cells (Acosta et al., 2008). Finally, repression of IGFBP7 expression results in cell proliferation maintenance and failure to enter senescence in human primary melanocytes (Wajapeyee et al., 2008).

### 1.5.4.3 *Paracrine Effect of SASP*

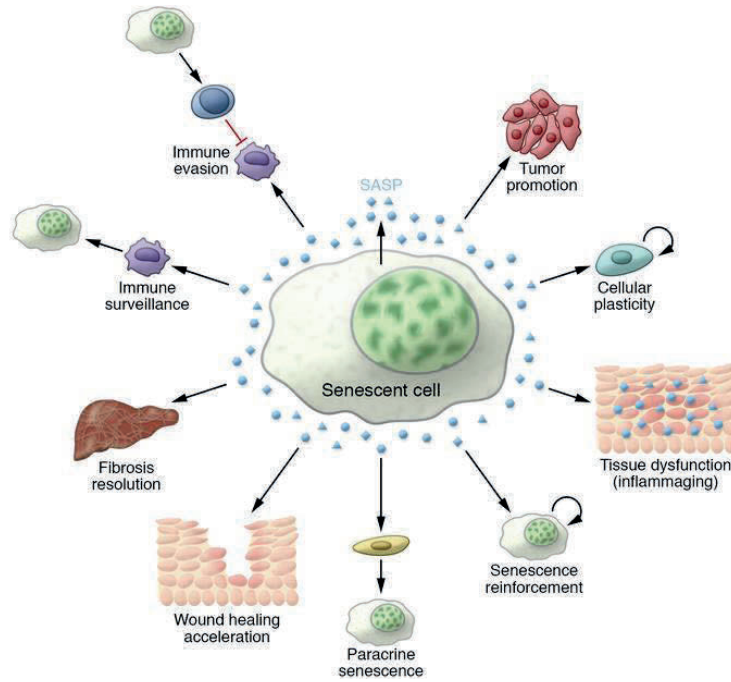
IGFBP7, IL-1 $\alpha$ , TGF- $\beta$  and VEGF are ligands which can mediate senescence establishment in neighboring cells (Acosta et al., 2013; Wajapeyee et al., 2008). Neutralization of IGFBP7 in conditioned medium from senescent cells results in failure to trigger senescence in recipient melanocytes. In contrast, treatment with recombinant IGFBP7 is sufficient to trigger senescence as indicated by SA- $\beta$ -Gal staining (Wajapeyee et al., 2008).

In a similar manner, inhibition of VEGFR2, CCR2 or TGF- $\beta$  receptor impaired senescent establishment in fibroblasts exposed to conditioned medium from senescent cells (Acosta et al., 2013). Finally, IL-1 $\alpha$  which is an important SASP factor also triggers senescence in a paracrine manner. Blocking of IL-1 $\alpha$  receptor or neutralization of IL-1 $\alpha$  in conditioned medium results in impairment of senescence establishment (Acosta et al., 2013). Indeed, IL-1 $\alpha$  upregulates expression of p53 and p21<sup>CIP</sup> in recipient cells (Acosta et al., 2013). Thus, several SASP factors are essential in the anti-tumorigenic role of senescence, both by promoting growth arrest and removal of senescent cells.

#### *1.5.4.4 SASP and tumor promotion*

Chronic accumulation of senescent cells promotes angiogenesis (Coppe et al., 2006) and tumorigenesis (Krtolica et al., 2001; Kuilman et al., 2008; Liu and Hornsby, 2007; Parrinello et al., 2005) by creating a pro-inflammatory environment via SASP. SASP factors promote tumor progression, including connective tissue growth factor (CTGF) (Yang et al., 2005), IL-8 (Schadendorf et al., 1993) and IL-6 (Kuilman et al., 2008). Recently, SASP factors secreted by senescent hepatic stellate cells have also been found to promote the proliferation and malignancy of the surrounding hepatocytes in obese mice treated with a chemical carcinogen (Yoshimoto et al., 2013). Furthermore, SASP factors may also support tumor motility and invasiveness. SASP has been shown to induce epithelial to mesenchymal transition (EMT) and enhance invasiveness of cancer cells, in particular through IL-6 and IL-8 secretion (Coppe et al., 2008). Recently, secretion of amphiregulin (AREG) has been shown to be involved in tumor resistance (Xu et al., 2019). Therefore, SASP secretion is involved in tumor development, which could partially account for the increase in cancer incidence during aging.

In conclusion, SASP is a major aspect of senescence which plays multiple roles, beneficial in the context of tissue repair or tumor formation prevention, or detrimental in the context of fibrosis, cancer growth and metastasis.



**Figure 8. Functions of the SASP.** The SASP mediates many of the cell-extrinsic functions of senescent cells. Among those, it reinforces growth arrest via an autocrine loop. The SASP also recruit immune cells and promote tissue regeneration through secretion of MMPs. Secretion of molecules such as TGF- $\beta$  mediates senescence in a paracrine manner. Finally, chronic SASP secretion can also promote tumor formation and metastasis spread, but can also lead to chronic inflammation and tissue alteration (Herranz and Gil, 2018).

## 1.6 Senescence *in vivo*

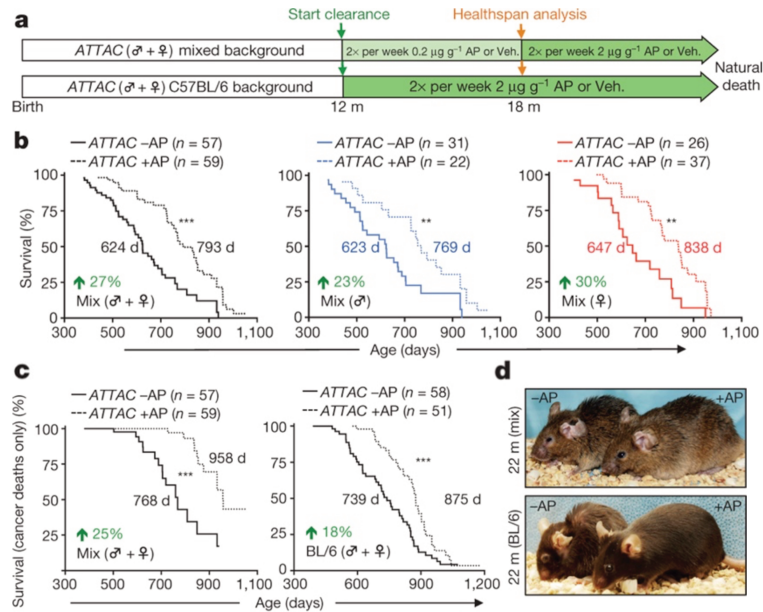
### 1.6.1 Senescence in Cancer & Aging

In 1965, Hayflick suggested that cellular senescence could be related to carcinogenesis and aging. Indeed, the transformation from a primary cell line to an immortal cell line is critical in carcinogenesis, which requires bypassing replicative senescence.

In the past decade, several studies using mouse models or human samples supported the relevance of OIS *in vivo*. For instance, studies with a mouse model that carries a conditional oncogenic *K-ras<sup>VI2</sup>* allele, thus recapitulating tumor initiation in humans, has shown that lung premalignant tumors are positive for several senescence markers, such as p15<sup>INK4B</sup> and p16<sup>INK4a</sup> (Collado et al., 2005). Conversely, malignant lung adenocarcinomas were negative for senescence. Pancreatic intraductal neoplasias (PanIN) were also positive for p15<sup>INK4B</sup> and p16<sup>INK4a</sup> whereas ductal adenocarcinomas were negative (Collado et al., 2005). BRAF<sup>V600E</sup> mutation has been found in nevi (benign skin or mucosa tumors) and has been

associated with cell cycle arrest and senescence (Michaloglou et al., 2005). Nevi show high p15<sup>INK4B</sup> and p16<sup>INK4a</sup> expression and are positive for SA-β-Gal activity. Interestingly, telomere length in nevi is equal to the ones in normal skin cells, which rules out the potential involvement of replicative senescence. A mouse model with conditional melanocyte-specific expression of BRAF<sup>V600E</sup> develops benign melanocytic melanoma (Dankort et al., 2009). Importantly, these nevi-like structures express senescence markers. Overall, these results demonstrated that OIS is an important tumor suppression mechanism.

Three decades after suggestion that aging and senescence are linked, several reports presented evidence that senescent cells accumulate *in vivo* with age in mammals. Increase in percentage of senescent cells *in vivo* has been shown in baboons (Herbig et al., 2006) and in mice (Wang et al., 2009). In line with these results, presence of cells harboring senescence markers has been found in several age-related diseases, notably degenerative diseases (i.e Parkinson's and Alzheimer's Disease) and Type 2 Diabetes (McShea et al., 1997; Naylor et al., 2013). More recently, a new transgenic model (INK-ATTAC) which allows specific elimination of p16<sup>Ink4A</sup> positive cells, has demonstrated the causative link between senescence and aging. Crossing this INK-ATTAC mouse model with BubR1 progeroid mouse model, Baker and colleagues showed that clearance of senescent cells delayed age related phenotypes. Unfortunately, the survival of these INK-ATTAC/BubR1 mice did not significantly increase (Baker et al., 2011). Following these first results, the same research team reported that removal of senescent cells extended the lifespan and healthspan of naturally aged INK-ATTAC mice (Baker et al., 2016) (Figure 9). Treated mice showed delayed tumorigenesis and reduced age-associated disorders in several organs such as kidney, heart and fat (Baker et al., 2016).



**Figure 9. Senescent cells clearances extend lifespan and healthspan.** **a**, Study design for clearance of senescent cells in mixed and C57BL/6 mouse cohorts. Healthspan analysis was done at 18 months, an age at which relatively few mice in vehicle (Veh.)- or AP-treated groups have died, and bias owing to selection for long-lived animals is unlikely. **b**, Survival curves for vehicle-treated (–AP) and AP-treated (+AP) mixed (b). **c**, Survival curves of *ATTAC* mice dying of cancer (mice that had an overt tumor at time of death; mice with lymphomas, sarcomas and carcinomas were included, mice without tumors were censored). Median survival and percentage increase are indicated. **d**, Representative images of aged mice with and without senescent cell clearance (Adapted from Baker et al. 2016 (Baker et al., 2016)).

Using a similar approach, by crossing *INK-ATTAC* mice with a neurodegenerative mouse model (tau dependent neurodegenerative pathologies), clearance of senescent brain cells delayed cognitive decline and prevented tau dependent diseases (Bussian et al., 2018). Additional studies reported benefits of senescent cells clearance, such as on aged hematopoietic stem cells (HSCs) and physical functions (Baar et al., 2017; Chang et al., 2016; Xu et al., 2018). Removal of senescent HSCs using *ABT263*, a senolytic drug, improved clonogenicity and engraftment ability of HSCs but also attenuates the myeloid skewing which is normally observed during aging (Chang et al., 2016). Use of a senolytic cocktail, composed of dasatinib and quercetin, significantly improved several physical functions including treadmill endurance, grip strength and walking speed in treated aged mice compared to untreated mice (Xu et al., 2018). Treatment with interfering peptide inducing apoptosis specifically in senescent cells restored renal function and reduced hair loss in aged mice (Baar et al., 2017). This interfering peptide blocks interactions between *FOXO4* and *p53* which prevents *p53* release and apoptosis induction in senescent cells.

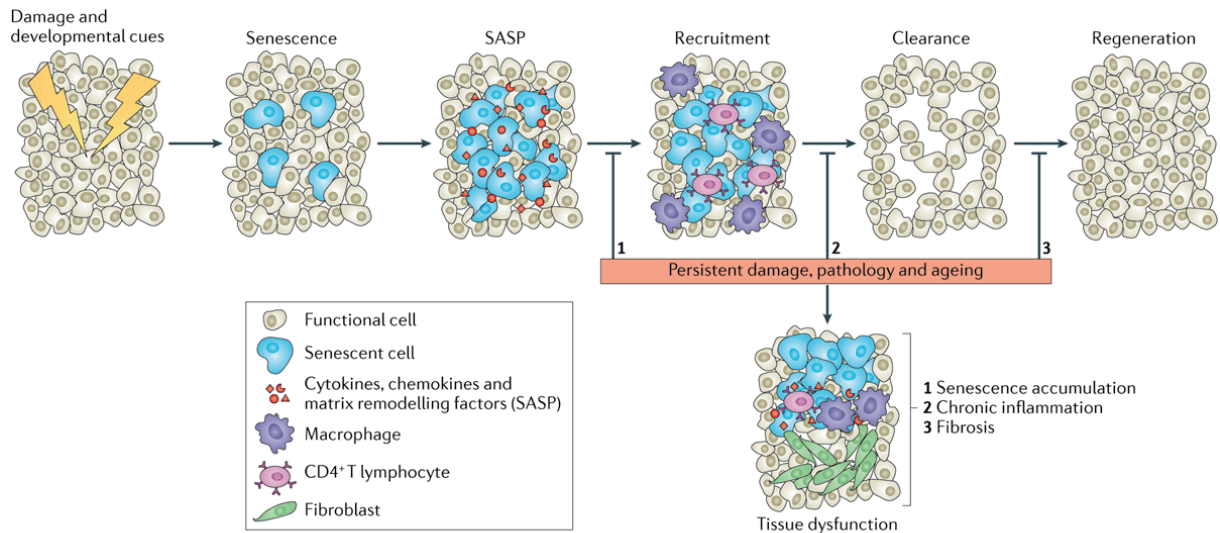
In conclusion, these recent results highlight the important role of senescence in aging process and aging related pathologies, confirming the first hypothesis from Hayflick and Moorhead. Current goal is to further understand the role of senescence in precise age-related diseases (Bussian et al., 2018) but also to develop new pharmacological treatment, called senolytic drugs, to increase life expectancy (van Deursen, 2019).

### *1.6.2 Senescence and Tissue Repair*

The potential role of senescence in tissue repair has been first suggested based on the fact that regeneration capacity decreases with age and senescent cells accumulates. Indeed, accumulation of senescent cells has been suggested to impair regeneration abilities during aging. For instance, hematopoietic tissue and skeletal muscle rely on tissue stem cells to regenerate. Early evidence of senescence has an impact on tissue regeneration came from the observation that aged individuals had reduced HSC transplantation success rate (Kollman et al., 2001). These results have been confirmed recently as clearance of senescent cells could rejuvenate HSCs (Chang et al., 2016). However, the impact of senescence on tissue homeostasis is not specific to tissue with high cell turnover. Indeed, loss of regenerative potential has been attributed to an increase in p16<sup>INK4A</sup> expression in both neurogenesis and pancreatic islet regeneration (Krishnamurthy et al., 2006; Molofsky et al., 2006). Senescence induces a decrease of progenitor cell proliferation, leading progenitor or stem cell exhaustion and an impaired tissue regeneration. Thus, senescence displays a role in regeneration through limitation of progenitor proliferation.

Stem cell exhaustion is not the only aspect of regeneration on which senescence has an impact. Cirrhotic liver, which is induced by chronic liver damaged, presents high percentage of senescent cells. In addition, following liver injury, senescent cells limit fibrosis, by expressing antifibrotic genes and controlling extracellular matrix production (Krizhanovsky et al., 2008).



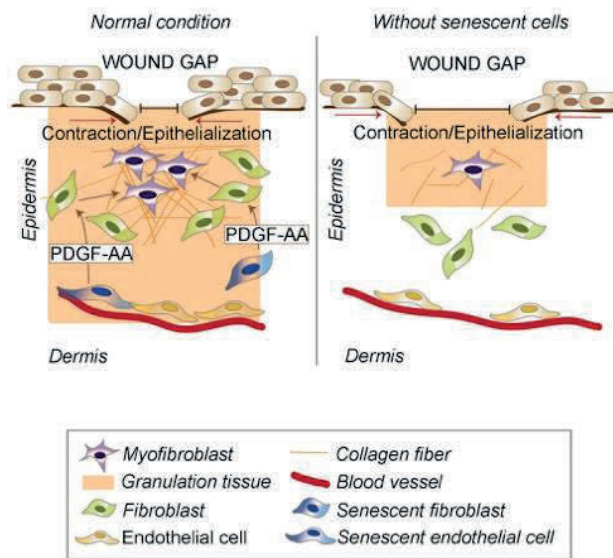


**Figure 10.** Senescence initiates a tissue remodeling process by recruiting immune cells through the senescence-associated secretory phenotype (SASP). Macrophages clear the senescent cells, and progenitor cells repopulate and regenerate the damaged tissue. This sequence of senescence–clearance–regeneration may be impaired upon persistent damage, pathological states or aging. In these cases, senescent cells are not efficiently cleared and the tissue is not fully regenerated. Resolution of the damage in these cases involves a fibrotic scar with senescent cells, inflammatory cells and fibrotic tissue (Munoz-Espin and Serrano, 2014).

Indeed, liver negative for  $p53^{-/-}$  or/and  $p16^{\text{INK4A}/-}$  display reduced SA- $\beta$ -Gal activity and stronger hepatic stellate cells proliferation, leading to an increase of extracellular matrix deposition and fibrosis. In addition, CCN1, an extracellular matrix component expressed during wound healing is required to induce senescence in skin fibroblasts and control fibrosis by activating the expression of antifibrotic genes (Jun and Lau, 2010; Kim et al., 2013). Thus, both studies reinforce the beneficial role of senescence in limiting fibrosis during tissue injury. However, recent findings in idiopathic pulmonary fibrosis (IPF) suggest that accumulation of senescent cells after bleomycin injury leads to fibrosis and impair lung function. Treatment with senolytic drugs decreased fibrosis in treated mice. In this specific context, SASP is suspected to be profibrotic in IPF and removal of senescent cells rejuvenates lung health in mice (Schafer et al., 2017).

Senescence is also involved during cellular proliferation step after inflammation during wound closure (Figure 13) (Midwood et al., 2004). In a  $p16\text{-3MR}$  transgenic mouse model which permit to selectively kill  $p16^{\text{INK4A}}$  positive cells using Ganciclovir, depletion of senescent cells delays wound closure, confirming role of senescence in tissue repair process. This transgenic mouse model contains notably a truncated herpes simplex virus 1 (HSV-1)

thymidine kinase (HSV-TK) under the promoter of p16<sup>INK4A</sup>. In the same manner, p16/p21 double KO mice shows also delay in wound closure (Demaria et al., 2014). Senescent cells secrete PDGF-AA amongst SASP factors, a factor known to play a role in regeneration (Beer et al., 1997). Indeed, application of PDGF-AA has been previously found to improve wound closure (Deuel et al., 1991). Reinforcing these results, PDGF-AA treatment of cutaneous wound depleted of senescent cells rescues the delay typically observed (Demaria et al., 2014). Moreover, CCN1 is known to induce expression of PDGF-AA in primary cell culture, supporting previous results that CCN1 induces senescence and contribute to proper tissue repair (Jun and Lau, 2010; Kim et al., 2013). Overall, this data demonstrates the positive role of senescence in tissue repair, notably through SASP.



**Figure 11. Figure illustrating senescent cells role in skin wound healing** (Demaria et al. 2014). Following skin wound, senescent cells secrete PDGF-AA amongst other SASP factors, which will turn fibroblasts into myofibroblasts and favors wound closure.

An alternative model used to study the role of senescence in tissue regeneration is salamander limb regeneration, which has impressive regenerative capacity as it can create a blastema in the injury site for complete limb regeneration. A blastema is composed of progenitors, which will contribute to the regeneration process. In agreement with observations from mouse model, senescence is rapidly triggered upon injury (Yun et al., 2013). Senescent cells are present during generation and proliferation of progenitors, suggesting potential involvement in these particular stages of regeneration (Yun et al., 2013). Then, senescent cells are cleared by macrophages. Using liposomes specifically depleting macrophages, Yun et al.

showed an accumulation of senescent cells, indicating macrophages contribute to senescent cells clearance. Interestingly, the presence of macrophages is essential for proper limb regeneration (Godwin et al., 2013), as depletion of macrophage completely disrupts limb regeneration. Moreover, these senescent cells expressed numerous SASP factors (Yun et al., 2015), suggesting potential paracrine roles. Overall, observations gathered from salamander further confirm the positive role of senescence in regeneration. Thus, senescence affects tissue remodeling in order to promote tissue regeneration.

Recently, a study reports that OIS SASP can induce cell plasticity (Ritschka et al., 2017). HRAS overexpression can promote, through SASP, adult stem cell markers after skin or liver damage. Upon injury, gene expression analysis shows that keratinocytes express adult stem cells markers including CD34 and Nestin despite becoming senescent. However, the functional consequences of the expression of stem cell markers are not completely clear. Interestingly, brief *ex vivo* treatment of newborn keratinocytes with OIS conditioned media (CM) induces “stem cell function” and enhances generation of hair follicles after skin engraftment at injury site (Ritschka et al., 2017).

While these studies indicate that senescence and particularly SASP are beneficial for tissue repair, a study shows that senescence could be detrimental for stem cell activation. In a skin induced senescence mouse model harboring an inducible p14<sup>ARF</sup> cassette, removal of senescent cells allows reentrance of resident stem cell into cell cycle and replenishment of the stem cell compartment (Yosef et al., 2016), suggesting that senescent cells could hinder proper regeneration and tissue homeostasis. It is noteworthy that senescence kinetics is different from previous studies. Senescence was induced by DOX treatment for 4 weeks in the skin and followed with senolytic drug treatment for a few days, which could be considered as chronic senescence rather than transient senescence (Yosef et al., 2016) (senescent cells are present for 5 days after skin injury). Indeed, the number of senescent cells is significantly increased one week after p14<sup>ARF</sup> activation in this mouse model (Tokarsky-Amiel et al., 2013). As indicated previously, contexts of these studies are different either in the signal inducing senescence or the time point used for analysis, which can explain many of the differences observed. Nonetheless, they highlight the need to further investigate the role of senescence in inducing plasticity in target cells.

Together, these results indicate that senescence is important for many physiological processes such as tissue repair or embryogenesis. However, the regulation of SASP is complex. Indeed, only transient SASP exposure is likely to be beneficial, whereas chronic exposure could lead to paracrine senescence or increase fibrosis, negatively affecting normal tissue repair. Thus, investigating which SASP factors are beneficial or detrimental will be helpful to design potential therapies.

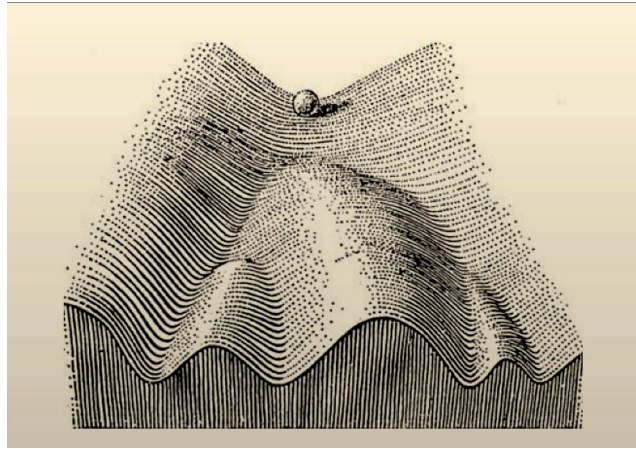
## 2. PLURIPOTENT STEM CELLS AND REPROGRAMMING

### 2.1 History and definition of pluripotency

Pluripotency is the capacity of a cell to self-renew and give rise to all the different cell types that constitute a living organism. These properties have been first attributed to epiblast stem cells, which proliferate and differentiate during embryogenesis. Then, these capacities have been discovered within other pluripotent cell types, notably in Embryonic Stem Cells (ESCs) (Martin, 1981). The first ESCs line has been obtained from the inner cell mass of blastocysts. Isolation of ESCs *in vitro* has been one of the first steps in realizing their plasticity and their potential use to study developmental and disease mechanisms. Plasticity of ESCs have been demonstrated by injection into blastocyst which led to the development of chimeric animal in more than 50% of cases. Furthermore, these chimeric animals proved to be functional chimeras, thus confirming the differentiation capacity of ESCs into multiple cell types upon different cues (Bradley et al., 1984).

However, the understanding of the developmental process and how pluripotent stem cells (PSCs) can give rise to a functional organism has proven to be complex. In 1893, August Weismann developed a first genetic theory explaining that germ cells hold heritable information while somatic cells carry genetic information of differentiated states. This author concluded that non-essential genetic information must be silenced in somatic cells (Weismann 1893). Pursuing Weismann's genetic theory, Conrad Waddington published a concept to describe how cellular differentiation is regulated during development, notably by what he called epigenetic factors. During development, pluripotent stem cells gradually become more and more restricted in their differentiation capacity until they finally become terminally differentiated cells (Waddington 1957) (Figure 12). In his picture describing epigenetic landscape, C. Waddington depicted a stem cell by a ball which had to roll down along numerous possible roads in order to specialize into the corresponding cell type. For a long time, this plasticity to become any type of cells was only attributed to pluripotent stem cells. In addition, differentiation process was considered as irreversible. Possibility that the ball could roll up or in a transversal manner has not been emitted. Despite this theory, recent

discoveries showed that differentiation could be reversed and that dedifferentiated cells are able to differentiate again and give rise to a functional organism.



**Figure 12. Waddington's Classical Epigenetic Landscape.** In 1957, Conrad Waddington proposed the concept of an epigenetic landscape to represent the process of cellular decision-making during development. At various points in this dynamic visual metaphor, the cell (represented by a ball) can take specific permitted trajectories, leading to different outcomes or cell fates (Goldberg et al., 2007).

## 2.2 *In vitro* reprogramming and transdifferentiation

### 2.2.1 *Reprogramming methods*

#### 2.2.1.1 *Somatic cell nuclear transfer (SCNT)*

The first reprogramming method to be developed has been somatic cell nuclear transfer (SCNT) or cloning. It consists in injecting nucleus from a differentiated cell into an enucleated oocyte (egg cell), resulting in the generation of nuclear transferred PSCs (ntPSCs) (Figure 13). At first, SCNT has been realized with enucleated oocytes and nuclei from early blastocysts derived from frogs (Briggs and King, 1952). Using *X. laevis* species, John Gurdon successfully reproduced SCNT using nuclei from intestinal epithelium cells and obtained adult organism (Gurdon, 1962). This was the first study to demonstrate the ability of somatic cells to be cloned and therefore their ability to be reprogrammed. Interestingly, the reprogramming process occurs very quickly. Indeed, changes in chromatin accessibility and transcriptome can be observed within a few hours after SCNT (Egli et al., 2011). However, the efficiency of SCNT is very low and abnormalities are often observed in both extraembryonic tissues and in animals (Ogura et al., 2013).

### 2.2.1.2 *Cell fusion*

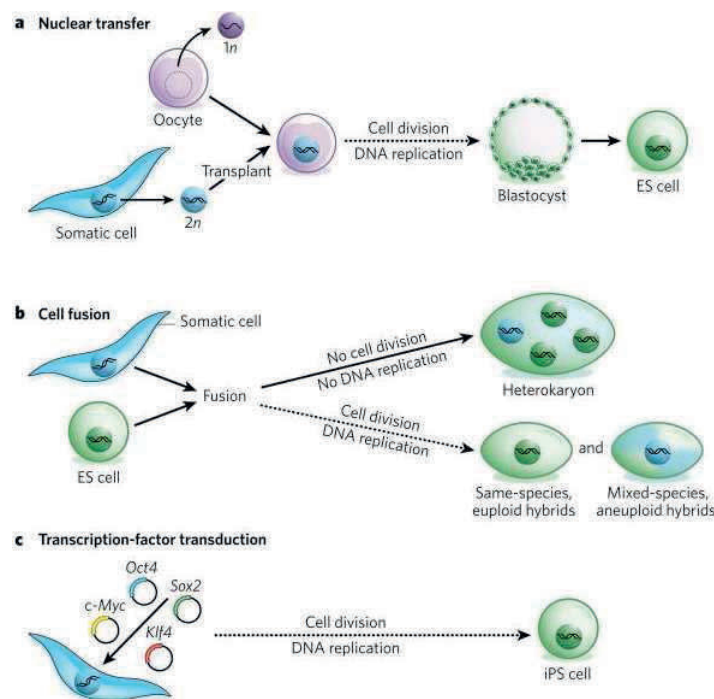
The second reprogramming method is called cell fusion. This process consists in fusing a pluripotent cell with a somatic cell (Figure 13). Resulting from this fusion, a pluripotent heterokaryon is generated and identity of somatic cell is erased. The first reprogramming which has been reported fused adult thymocytes with ESC (Tada et al., 2001). Similar to SCNT, reprogramming takes place quickly, since Oct4 is reactivated 48hrs after fusion. However, the use of these hybrid cells is difficult as they contain 4n chromosomes, one set from somatic cell and the second one from ESC. Consequently, this method has been used less than others and having 4n chromosomes also abolishes potential therapeutic applications.

### 2.2.1.3 *Cell reprogramming by transcription factors expression*

Previous studies showed that somatic cells could be reprogrammed, such as “somatic cell nuclear transfer (SCNT)” and “cell fusion”. However, these methods were both difficult to perform and to control (Briggs, 1952). Moreover, both methods need either a recipient egg cell (SCNT) or a recipient pluripotent stem cell (Fusion). In 2006, Dr. Shinya Yamanaka made a landmark discovery. He showed that murine somatic cells were plastic and could be reprogrammed back to a stage called induced Pluripotent Stem Cells (iPSCs), using a cocktail of defined transcription factors (Takahashi and Yamanaka, 2006) (Figure 13). This discovery was also reproducible using human somatic cells (Takahashi et al., 2007; Yu et al., 2007). Nonetheless, this cocktail of genes can be partially different. Klf4 and c-Myc can be replaced by Nanog and Lin28 for instance. Murine iPSCs contributed to the chimera formation when injected in blastocysts and both human and murine iPSCs were able to generate teratomas when injected into mice, indicating full pluripotency.

Thus, S. Yamanaka provided the first method to generate pluripotent stem cells directly from terminated cells. This discovery has brought new perspectives and a lot of hopes for regenerative medicine. Indeed, these results have been the first evidence that cell fate could be completely changed by using key regulatory genes. A critical step has been the identification of the essential transcription factors required to induce reprogramming process. Based on *in silico* studies, they identified 24 candidate genes that are exclusively enriched in

ESCs compared to differentiated cells. In order to test their candidates, a screening cell type (MEFs) carrying a  $\beta$ geo cassette was developed (fusion of the  $\beta$  Galactosidase and neomycin resistance genes) in the Fbx15 locus (target of Oct4 transcription factor). Thus, the activation of Fbx15 resulted in resistance to neomycin. Transfection with 24 factors generated iPSCs, then by withdrawing one factor from the pool of 24 factors, they reduced the cocktail to 4 genes. These genes are Oct4, Sox2, Klf4 and c-Myc (OSKM) and are called Yamanaka factors. Unfortunately, the process is inefficient (0.067% from fibroblasts). S. Yamanaka explained this low efficiency with a stochastic model (Yamanaka, 2009). The majority of cells starts the reprogramming process but only few go entirely through it. In addition, iPSCs generation is slow. Indeed, the whole reprogramming process needs 2 weeks to generate iPSCs colonies.

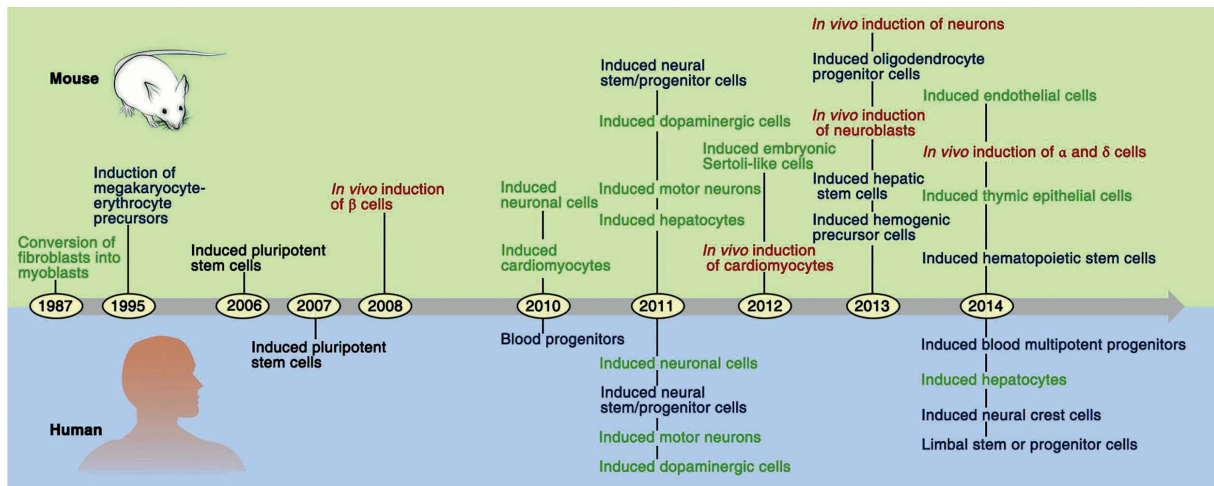


**Figure 13. Three approaches to nuclear reprogramming to pluripotency.** a, Nuclear transfer. In this approach, the nucleus of a somatic cell (which is diploid,  $2n$ ) is transplanted into an enucleated oocyte. If development is allowed to proceed to completion, an entire cloned organism is generated. b, Cell fusion. In this approach, two distinct cell types are combined to form a single entity. The resultant fused cells can be heterokaryons or hybrids. If the fused cells proliferate, they will become hybrids, and on division, the nuclei fuse to become  $4n$  or greater. If the cells are derived from the same species, their karyotype will remain euploid; however, if they are from different species, they will be aneuploid, as chromosomes will be lost and rearranged. Heterokaryons, by contrast, are short-lived and do not divide. Dashed arrows indicate slower processes (involving multiple rounds of cell division) than solid arrows (no division). c, Transcription factor transduction. This approach can be used to form induced pluripotent stem (iPS) cells, which have similar properties to ES cells and can be generated from almost any cell type in the body through the introduction of four genes (Oct4, Sox2, Klf4 and c-Myc) by using retroviruses. The pluripotent state is heritably maintained, and vast numbers of cells can be generated, making this approach advantageous for clinical applications (Yamanaka and Blau, 2010).



2.2.2 Transdifferentiation

Generation of desired cell type has been one of the main goals of regenerative medicine. Based on Yamanaka’s approach, several studies reported that terminal cells could also “transdifferentiate” (Efe et al., 2011; Vierbuchen et al., 2010) (Figure 14). Transdifferentiation is the process by which a differentiated cell can differentiate into other specialized cell type without going back to a pluripotent stage (Figure 14). Remarkably, transdifferentiation can occur between different lineages, not restricting transdifferentiation to a defined germ layer (Vierbuchen et al., 2010). For instance, fibroblasts, which originate from mesoderm, can be converted into neurons, which are derived from ectoderm (Vierbuchen et al., 2010).

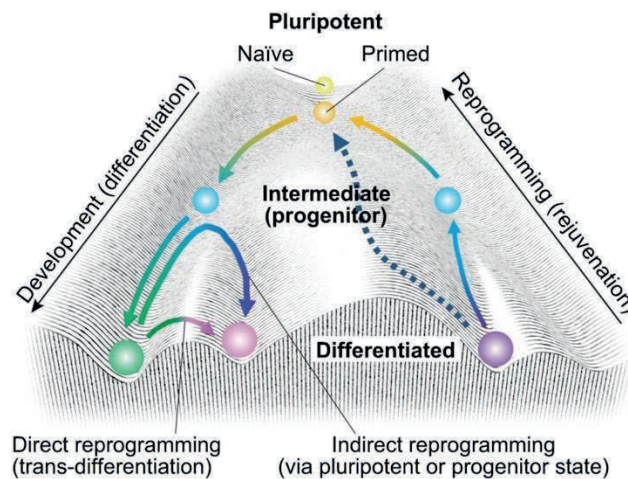


**Figure 14. Historical View of the Development of Lineage Reprogramming.** Selected advances in the development of lineage reprogramming are highlighted in different colors. Green, blue, and red indicate the induction of terminally differentiated cell types, stem cells or progenitors/precursors, and *in vivo* lineage reprogramming, respectively. Texts above the timeline indicate studies in mice, and texts below the timeline indicate studies in humans (Xu et al., 2015).

Conversion of mouse fibroblasts to other cell types have been reported, including cardiomyocytes, hepatocytes, Sertoli cells or hematopoietic progenitor cells (Buganim et al., 2013; Huang et al., 2011; Ieda et al., 2010; Sekiya and Suzuki, 2011). While most of these conversions from one cell type to another were reported *in vitro*, more recent studies report successful differentiation *in vivo*. Nonetheless, most of these *in vivo* studies have used an initial cell type close from the targeted cell type (Guo et al., 2014; Riddell et al., 2014; Torper et al., 2013). For each transdifferentiation, a defined combination of transcription factors was injected to reprogram cells. Although many master transcription factors have been identified

to induce cell type conversion, discovery of new master regulators in order to induce unseen cell type conversions remains crucial. Moreover, most of the current studies have only used epigenetic factors to induce lineage conversion. An alternative method would consist in using small chemical compounds instead of genetic factors. Even though this approach is appealing, finding molecules that can completely replace epigenetic factors and design of robust protocols remain extremely challenging.

Yamanaka's discovery demonstrates that a set of factors can modify fate of differentiated cells, highlighting their plasticity. Use of the same approach to induce direct lineage reprogramming from one specific cell type to another confirmed possibilities suggested by Yamanaka about cell fate determination. Moreover, the development of different strategies to influence cell fate provides additional evidence that cell plasticity can be manipulated with right stimulations. Consequently, theories about epigenetic stability of somatic cells have been completely rethought. Together, these recent results emphasize the plasticity of somatic cells and possibility to manipulate their cell fate through expression of key regulatory elements. Consequently



**Figure 15. Cell fate changes on Waddington's epigenetic landscape.** Pluripotent stem cells (naïve in yellow and primed in orange) can commit to any somatic lineage (green, pink, purple) via a progenitor state (blue). Direct reprogramming, or trans-differentiation, using tissue-specific transcription factors allows lineage-committed cells (green) to convert to another fate (pink), regardless of their germ layer origin, and bypasses the need for a pluripotent intermediate state. During indirect reprogramming, using a combination of OSKM expression and optimal conditions for the destined lineage, cells can be converted to another cell type via a transient pluripotent state. Finally, recently developed technologies can be used to revert mature somatic cells (purple) to pluripotency (orange or yellow) via a progenitor stage (blue) or directly (blue dashed arrow) (Takahashi and Yamanaka, 2015).

### 2.2.3 *Potential applications of iPSCs*

Several animal and pre-clinical studies have reported the use of iPSCs to treat several diseases. iPSCs have been tested to treat Parkinson's Disease (Kikuchi et al., 2017; Wernig et al., 2008) or spinal cord injury (Kobayashi et al., 2012; Tsuji et al., 2010). First clinical trial using iPSCs against age-related macular degeneration has been performed in 2014. Unfortunately, it has not improved the vision of the patient (Yoshihara et al., 2017). Nonetheless, a very recent study has reported some exciting results in regards to treatment of age-related macular degeneration in mice and pigs (Sharma et al., 2019). Even though technical progress still needs to be made in this regard, this discovery definitely demonstrates a very promising future for cellular therapy based on iPSCs. The translation of iPSCs technology from bench to bedside has taken place in less than a decade, suggesting clinical applications could take place in the near future.

### 2.2.4 *In vitro reprogramming methods*

The discovery of iPSCs created many hopes for regenerative medicine. However, the first strategies to generate iPSCs used retroviral or lentiviral vectors and led to the integration of factors into the genome of host cells. Safety issues quickly arose as it can disrupt or activate expression of important genes, such as tumor suppressors or oncogenes (Modlich and Baum, 2009). Moreover, the transcription factors could also be reactivated later. Thus, several free integration methods have been engineered, such as adenoviruses (Stadtfeld et al., 2008), Sendai viruses (Fusaki et al., 2009), synthetic mRNA (Warren et al., 2010), recombinant proteins (Kim et al., 2009) or chemical compounds (Hou et al., 2013). These techniques are preferred for clinical trials as they avoid genomic integration. For instance, clinical trial testing iPSCs applications used episomal vectors to generate iPSCs from patient derived cells (Kikuchi et al., 2017). Discovery of small molecules which can induce reprogramming back to iPSCs have been found, but it is not commonly used yet, indicating it needs to be improved (Li et al., 2013). Moreover, molecules have been shown to trigger expression of precise factors but mechanisms behind these activations have not yet been elucidated and require more investigations. Therefore, finding robust and safe methods to induce reprogramming are also important challenges for potential future clinical applications of iPSCs.

### 2.3 Characterization of Stem cells

#### 2.3.1 Colony formation

One of the classical *in vitro* assays to characterize PSCs is called colony forming unit (CFU) or clonogenic assay. This assay assesses self-renewal capacity and ability of a single cell to form a colony. Single cells seeded at a very low density will undergo cell division and form a colony, indicating that the cell can proliferate indefinitely. Obtained colonies can be detected with alkaline phosphatase staining, a marker of undifferentiated pluripotent stem cells. Additive stainings can be used for pluripotency markers such as Oct4, Sox2, Nanog or SSEA. Analysis of mRNA levels of these markers as well as Lin28 or ESG-1 can also be conducted (Bhattacharya et al., 2004; Mitsui et al., 2003).

#### 2.3.2 Teratoma formation

Teratoma formation is one of the most performed assays, with the analysis of stem cell markers *in vitro*, to characterize pluripotency. Injection of pluripotent stem cells in immunocompromised mice will lead to teratoma formation. Teratoma is a particular type of tumor that originates from pluripotent cells after a phase of proliferation and differentiation. They display differentiated cells from the three germ layers. The injection sites of choice are usually liver, sub-renal capsule, subcutaneous or intramuscular. This assay is probably the gold standard assay used in order to evaluate pluripotency of a cell clone. However, this assay requires much more time compared to other assays, and sometimes it cannot provide a clear answer on pluripotency when only differentiated cells from one or two germ layers can be observed (Li et al., 2007).

#### 2.3.3 Chimera formation

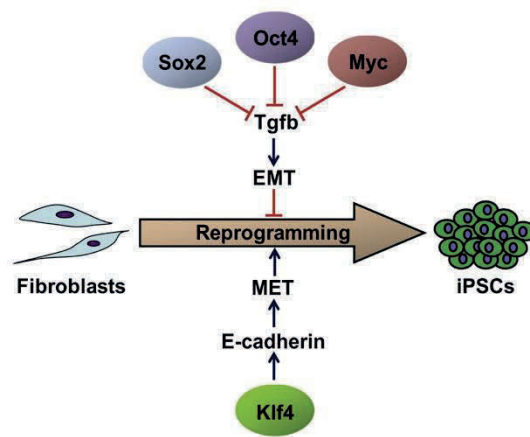
A second *in vivo* assay to assess pluripotency is by analyzing cell ability to contribute to the development of an organism when injected into host blastocysts (Nagy et al., 1990). Injected ESCs or iPSCs will theoretically colonize every tissue and participate in the development of the organism. High quality PSCs are required for proper colonization of each germ layer. Low cell line quality results in lower embryo viability and partial chimerism. An

extreme version of this assay exists in which only one ESC is injected into a blastocyst (Wang and Jaenisch, 2004).

### **2.4 Mechanisms involved in cellular reprogramming**

#### *2.4.1 Mesenchymal to Epithelial Transition (MET)*

A major event occurring during reprogramming is Mesenchymal to Epithelial Transition (MET). The reversed process, EMT (Epithelial to Mesenchymal Transition) takes place during differentiation and contributes to organism formation (Thiery et al., 2009). EMT induces loss of cell adhesion, retainment of stemness, migration and cell invasion, which are all critical characteristics for embryonic development, in particular during gastrulation and tissue formation. This transition between both states is mainly regulated by key master genes such as Snai1 and E-Cadherin (Thiery et al. 2009). Snai1 and Twist expressions appear to promote mesenchymal phenotype while E-Cadherin holds a major role in cell-cell adhesion and epithelial phenotype. Recently, studies reported that MET is required for iPSCs generation. During early reprogramming stage, inhibition of mesenchymal markers such as, Snai1 and Snai2, Twist1 & 2 and Zeb genes is required for proper reprogramming. In parallel of this repression, enhanced expression of epithelial genes is observed, including E-Cadherin and Occludin (Li et al., 2010b; Samavarchi-Tehrani et al., 2010). Blocking MET by TGF- $\beta$  treatment or E-Cadherin knockdown inhibit reprogramming process. Forced expression of Snai1 also abrogates reprogramming, indicating MET is a critical step in reprogramming. It seems that Oct4/Sox2 mediates Snai1 down-regulation and that c-Myc blocks TGF- $\beta$  secretion (Li et al., 2010). Finally, Klf4 up-regulates E-Cadherin expression (Li et al., 2010). Supporting these results, inhibition of TGF- $\beta$  during reprogramming can supplant Sox2 and c-myc requirement (Ichida et al., 2009; Maherali and Hochedlinger, 2009). Taken together, these data demonstrate the need for MET to occur for proper reprogramming process.



**Figure 16. Graphical Abstract of MET & Reprogramming** (Li et al., 2010).

#### 2.4.2 Cellular Senescence

An additional suggested barrier to reprogramming is cellular senescence. Expression of OSKM activates expression of several senescence markers, such as p53, p16<sup>INK4a</sup> and p21<sup>CIP</sup> (Banito et al., 2009). In addition, an up-regulation of JMJD3, which control INK4A locus is increased during reprogramming (Banito et al., 2009). Upon OSKM induction, both mouse and human fibroblasts display other senescent markers such as SA-β GAL activity and SAHF (Banito et al., 2009). Repression of p53, p16<sup>INK4a</sup> and p21<sup>CIP</sup> expression results in more efficient reprogramming in mouse and human fibroblasts (Banito et al., 2009; Kawamura et al., 2009; Li et al., 2009a; Marion et al., 2009a; Utikal et al., 2009), whereas transfection of an additional copy of p53 attenuates reprogramming (Hong et al., 2009). In addition, BMP-SMAD-ID signaling has been found to enhance reprogramming by suppressing p16<sup>INK4A</sup> mediated senescence during early stage of reprogramming, which resulted in increased cell proliferation (Hayashi et al., 2016). Vitamin C also improves reprogramming by reducing p53 level (Esteban et al., 2010). Finally, comparison of the reprogramming efficiency of young and aged cells indicates that cells close to the entry into senescence which present high levels of p16<sup>INK4A</sup> have reduced ability to be reprogrammed (Li et al., 2009a). Interestingly, the catalytic subunit of telomerase (hTERT), which is involved in senescence control, improves reprogramming of human fibroblasts, suggesting that senescence and reprogramming are directly connected (Park et al., 2008). Moreover, reprogramming cells under hypoxia enhances cellular reprogramming, which suggest that limitation of factors contributing to

senescence is beneficial for iPSCs generation. In conclusion, senescence appears to be an intrinsic roadblock which hinders iPSCs generation.

### *2.4.3 Signaling pathway involved in reprogramming process*

Several mechanisms are important for pluripotency acquisition. These mechanisms are notably composed of signaling pathways, epigenetic factors and molecular barriers. They cooperate to silence the somatic program of differentiated cells and activate the expression of genes related to pluripotency.

#### *2.4.3.1 LIF/JAK/STAT3 pathway*

In 1988, discovery that leukemia inhibitory factor (LIF) is the secreted cytokine which maintains pluripotency has allowed the culture of ESCs in feeder free conditions (Williams et al., 1988). It has also revealed the importance of the LIF/STAT3 pathway in regulation of pluripotency. LIF belongs to IL-6 cytokine family and binds to a complex formed by LIF receptor (LIFR) and gp130 (Zhang et al., 1998). Recently, van Oosten et al. found that JAK/STAT3 participates to reprogramming process by enabling the overcome of the pre-iPSCs blockage (van Oosten et al., 2012). Moreover, IL-6 promotes iPSCs generation through JAK/STAT3 pathway (Brady et al., 2013). IL-6 plays an early role in the reprogramming process, which corroborates observations that JAK/STAT is important at the pre-iPSCs stage. Importantly, IL-6 has an additional downstream target compared to LIF. Its target is Pim-1, a pro-survival gene (Brady et al., 2013). Overall, LIF/STAT3 plays an important role in murine ESCs, as it is essential for maintenance of self-renewal.

#### *2.4.3.2 BMP/SMAD pathway*

A second pathway, which contributes to reprogramming, is the BMP/SMAD. Bone Morphogenic Proteins (BMP) are growth factors which belong to TGF- $\beta$  family. BMP/SMAD induces MET transition during reprogramming and facilitates iPSCs formation (Samavarchi-Tehrani et al., 2010). BMP4 can replace Klf4 during reprogramming process, reinforcing its key role in pluripotency (Chen et al., 2011a). Moreover, BMP/SMAD has also been shown to promote reprogramming through the inhibition of p16<sup>INK4A</sup> barrier (Hayashi et

al., 2016). In conclusion, BMP/SMAD pathway, along with JAK/STAT pathway, contributes to pluripotency acquisition.

### 2.4.3.3 *Wnt/ $\beta$ -catenin pathway*

Wnt pathway is the third pathway which contributes to reprogramming (Marson et al., 2008). The main mediator of Wnt signaling is  $\beta$ -catenin. Wnt pathway activation mediated by Wnt3a promotes reprogramming (Marson et al., 2008). It has been reported that activation of Wnt signaling has an inhibitory effect in early stage of reprogramming process whereas it has a beneficial effect in the late stage of reprogramming (Ho et al., 2013). However, contradictory results have been reported (Zhang et al., 2014). Zhang et al. found that activation of Wnt pathway during the onset of reprogramming inhibits iPSCs formation whereas activation during late stage increases reprogramming efficiency (Zhang et al., 2014). At least, both studies have found a global beneficial effect of Wnt activation in iPSCs generation. Another common fashion to activate Wnt pathway is through inhibition of GSK3, using pharmacological inhibitors. Interestingly, inhibition of GSK3 with a specific inhibitor, CHIR99021, has been shown to replace Sox2 transcription factor, reducing the required factors only to Oct4 and Klf4 (Li et al., 2009b). Together, these data show that Wnt pathway activation, either by Wnt3 or GSK inhibitors, promotes reprogramming back to the iPSCs stage.

### 2.4.3.4 *PI3K/Akt*

A fourth pathway has been linked to reprogramming enhancement. Treatment of human fibroblasts with SP48, a small compound that activates Akt has been shown to promote iPSCs generation (Zhu et al., 2010). In this context, Akt activation seems to facilitate metabolism conversion from a mitochondrial oxidation status to a glycolytic status by the up-regulation of several glycolytic related genes such as GLUT1 or PFK1 (Zhu et al., 2010). In a similar manner, a recent study showed that treatment of MEFs with IGF1 induced PI3K/Akt/mTOR pathway and increased reprogramming (Zhang et al., 2018). Intriguingly, PI3K/Akt/mTOR has also been suggested to be important during late reprogramming phase. Inhibition of PI3K/Akt/mTOR with compound BEZ-235 resulted in loss of murine iPSCs with high levels of Lin28 and Nanog markers (Zunder et al., 2015). However, another study



suggests that mTOR inhibits reprogramming, which is contradictory with previous observations. Indeed, treatment with rapamycin during the three first days of reprogramming results in increase in iPSCs generation (Chen et al., 2011b). Confirming these results, treatment with a PI3K inhibitor called LY294002 also promotes reprogramming. Interestingly, treatment with higher concentration abrogates benefits of rapamycin on reprogramming, suggesting concentration and mechanism of inhibition could explain the differences observed. Unfortunately, none of these studies explored potential mechanisms and gene targets underlying their observations, limiting our understanding of PI3K/Akt and mTOR effects on reprogramming.

### 2.4.3.5 *MAPK*

MAPK kinase family that is composed notably of MEK/ERK and p38MAPK has also been linked to reprogramming of human iPSCs. Inhibition of MEK/ERK signaling has been demonstrated to promote reprogramming (Lin et al., 2009; Shi et al., 2008). Treatment with PD0593201, an ERK inhibitor, in combination with TGF- $\beta$  inhibitors, has been shown to promote reprogramming both in MEFs and human fibroblasts (Lin et al., 2009; Vidal et al., 2014). Conversely, p38MAPK impact on reprogramming remains unclear. Genetic downregulation has been recently reported to impair reprogramming in human fibroblasts (Neganova et al., 2017). However, chemical inhibition of p38MAPK in MEFs has been shown to enhance iPSCs formation (Li and Rana, 2012). These opposite results might be due to the difference of cell origin or to the inhibition methods. Thus, role of p38MAPK in reprogramming remains unclear.

### 2.4.3.6 *NOTCH*

Recently, NOTCH repression has been shown to contribute to reprogramming (Ichida et al., 2014). Treatment with DAPT, which blocks NOTCH transduction signaling, enhances reprogramming of both mouse and human keratinocytes by suppressing p21 expression in a p53 independent manner (Ichida et al., 2014). Remarkably, treatment with DAPT permits generation of iPSCs without the use of Klf4 and c-Myc.

In summary, many mechanisms and molecular pathways influence acquisition of pluripotency and iPSCs generation. All of these studies have revealed several insights as the molecular changes and events occurring during reprogramming. Nonetheless, further understanding of the different steps and molecular pathways are necessary to better control cell fate.

### **2.5 Identification of small molecules enhancing reprogramming**

Cellular reprogramming of somatic cells opened a new era for regenerative medicine. However, the low reprogramming efficiency (less than 1% in general) for most of the somatic cell types and the time (at least 2 weeks) required to obtain iPSCs generation are hurdles for their practical use (Stadtfield and Hochedlinger, 2010; Takahashi et al., 2007; Takahashi and Yamanaka, 2006; Yamanaka, 2009). Moreover, the quality of the iPSCs generated can be heterogeneous and somatic cell reprogramming is not synchronized between somatic cell types (Buganim et al., 2012). Consequently, identifying molecules which may promote efficiency or kinetic of cellular reprogramming but also cellular reprogramming synchronization is important for future applications. Strategies used include repression of genetic or epigenetic barriers, overexpression of transcription factors and administration of small molecules and cytokines (Chen et al., 2013b; Di Stefano et al., 2014; Hasegawa et al., 2011; Rais et al., 2013; Worringer et al., 2014). Currently, several identified molecules which enhance reprogramming act on completely different targets. For instance, kinase inhibitors have been shown to promote reprogramming, such as GSK3 kinase inhibitor (CHIR99021) or ALK5 kinase inhibitor (SB431542) (Li and Rana, 2012; Lin et al., 2009). However, the mechanisms and target genes of these inhibitors still need to be determined for several of them. Similarly, several molecules targeting epigenetic factors have been identified. For instance, DNA methyltransferase inhibitors (Aza) have been shown to also promote reprogramming (Huangfu et al., 2008; Mikkelsen et al., 2008). Inhibitor of histone deacetylase called Valproic Acid (VPA) has been reported to enhance reprogramming, notably through stronger activation of ES-specific genes (Huangfu et al., 2008). Vitamin C also enhances reprogramming through TET1 repression (Chen et al., 2013a). Therefore, modulating epigenetic factors seems to be a relevant strategy to promote reprogramming. Alternatively, compounds stimulating molecular pathways can facilitate reprogramming. For

instance, BMP4 induces BMP-SMAD pathway and enhances iPSCs generation by repressing p16<sup>INK4A</sup> (Hayashi et al., 2016). Nonetheless, how small molecules trigger cell fate modifications is unclear. Currently, clinical trials using iPSCs rarely mention use of chemical compounds to enhance reprogramming, emphasizing our need to progress in this field. Finally, chemical compounds identified have mostly been tested *in vitro* only and their potential use *in vivo* still needs to be properly demonstrated. Therefore, finding new regulators of reprogramming and cellular plasticity is still of great interest for both *in vitro* and *in vivo* applications.

## 2.6 Reprogramming *in vivo* and Regenerative medicine

### 2.6.1 Limitations of *in vitro* reprogramming

*In vitro* reprogramming has been a landmark discovery for regenerative medicine field. One of the long-standing aims of regenerative medicine is to be able to replace lost cells within an organ by new cells. The use of different transcription factors to generate desired cell types has been rapidly applied *in vitro* and has allowed the generation of multiple cell types from different cell types of origin (Xu et al., 2015). However, several limits have appeared because of *in vitro* conditions. For instance, long term *in vitro* culture may cause genetic mutations. Concern about the functional maturation of cells transdifferentiated *in vitro* has also been raised. Finally, cells have to be derived from a patient to prevent future transplant rejection and have to be engrafted upon differentiation, which is laborious. Therefore, *in vitro* direct reprogramming requires many complicated steps which are cost effective, long and contain several risks.

### 2.6.2 Lineage *in vivo* reprogramming

Consequently, several attempts to perform direct *in vivo* lineage reprogramming have quickly appeared. *In vivo* reprogramming offers several advantages compared to *in vitro* reprogramming. Cell derivation, *in vitro* culture and transplantation concerns are bypassed. Moreover, the *in vivo* niche provides numerous cues to promote differentiation and functional maturation (Kroon et al., 2008). First reported case of *in vivo* reprogramming was the

transdifferentiation of pancreatic exocrine cells into  $\beta$ -cells (Zhou et al., 2008). Then, several other studies published similar results in different tissues, notably central nervous system, cardiac and hematopoietic tissue (Guo et al., 2014; Li et al., 2010a; Qian et al., 2012; Riddell et al., 2014; Torper et al., 2013; Uhlenhaut et al., 2009). Taken together, these observations indicate that *in vivo* lineage direct reprogramming might be a more simple and direct strategy to generate desired cell type in the frame of cell therapy replacement.

Currently, combinations of different transcription factors are being used to induce direct lineage reprogramming, such as in the pancreas or the brain (Torper et al., 2013; Zhou et al., 2008). In the pancreas, expression of Pdx1, Neurog3 and MafA induces transdifferentiation of acinar cells to  $\beta$  cells. Alternatively, cell type developmentally close to pancreatic cells, such as cells from the liver or the gut can be also used to obtain  $\beta$  cells (Ferber et al., 2000; Kojima et al., 2003). Nonetheless, these strategies only resulted in a partial rescue of diabetes in mice, suggesting that reprogramming was incomplete or not efficient enough. Thus, better association of different factors needs to be found to generate pancreatic cells from other tissues.

*In vivo* direct reprogramming has also been reported in central nervous system. Several reports found combination or single factors which were able to induce neuronal differentiation from astrocytes. (Guo et al., 2014; Niu et al., 2013; Torper et al., 2013). This is a promising approach for brain injury whose current treatment is transplantation, which is an invasive method. In this particular context, lineage reprogramming would have significant advantages compared to current techniques. However, direct reprogramming has been mainly performed from progenitor neural cells and direct reprogramming from non-neural cells into neurons upon massive brain injury remains an important challenge.

### 2.6.3 Lineage reprogramming milestones and tissue regeneration

A critical advantage of *in vivo* reprogramming is the ability to convert one cell type to another *in situ* to regenerate damaged tissue. However, conversion of one cell type to another could be restrained if the number of cells required for proper tissue repair *in situ* is not

sufficient. Thus, a first parameter is to have the adequate cell number for proper regeneration. Obtaining progenitor cells, such as neural stem cells from differentiated cells without going back to iPSCs stage would greatly reduce the risks associated with pluripotency and still permit to increase cell population and differentiate it into specific desired cell types. Reprogramming of fibroblasts to intermediate multipotent stages have been successfully reported *in vitro*, suggesting this strategy is possible. For instance, neural stem cells have been successfully generated and have been differentiated into neurons (Lalit et al., 2016; Ring et al., 2012). However, a recent study suggested that transient reprogramming with OSKM in combination with differentiation signals induced both iPSCs and neural stem cells, suggesting this strategy is not harnessed yet (Bar-Nur et al., 2015).

A second parameter to consider for *in vivo* reprogramming, either to generate a specific somatic cell type or progenitor cell type, is the cell of origin. The target cell type should depend on the cell type desired as many organs are made of several kinds of differentiated cells. Reprogramming method should also use the most appropriate cell type according to its plasticity degree and phenotypic proximity to the desired cell type.

A third parameter is the tissue microenvironment around recipient cells. As mentioned above, some tissues are likely more easily reprogrammed than other. This is perhaps due either to the microenvironment and extrinsic factors, or the identity of the cell of origin or a combination of both. Considering all these milestones, reprogramming of pancreas cells has been the first one to be reported and is very likely to be the most feasible (Zhou et al., 2008). Then, several research teams obtained many other differentiated cells including neuronal subtypes, cardiomyocytes and sensory cells, notably following tissue injury (Guo et al., 2014; Karow et al., 2012; Niu et al., 2013). A last parameter is also the reprogramming cocktail of transcription factors. The combination should be optimized according to the host cell and tissue microenvironment, to reduce the number of factors necessary to perform reprogramming. Finally, identification of molecules rather than transcription factors to promote cell plasticity is critical to bring lineage reprogramming closer to clinical applications. Therefore, identifying the right conditions and stimulations to control cell fate with accuracy is essential for eventual *in vivo* therapeutic applications. Multiples milestones have to be examined in each situation to obtain desired results.

### 2.6.4 *Natural in vivo reprogramming and tissue repair*

Recently, nature cell type conversion has been demonstrated following massive tissue injury to repair damaged organs, supporting that lineage reprogramming may be a relevant therapeutic strategy. Two types of reprogramming have been observed, the first one induces transient repair cell phenotypes and the second one promotes transdifferentiation to definitely replace lost cells (Arthur-Farraj et al., 2012; Chera et al., 2014; Thorel et al., 2010; Yanger et al., 2013).

Transient repairing phenotypes have been observed upon nerves injury. The distal axon degenerates and both myelin and non-myelin Schwann cells respond to this degeneration by dedifferentiation. Upregulation of c-Jun induces conversion into a specialized repair Schwann cell which promotes axon growth. Repair Schwann cells start to express several factors supporting axon and neuron regeneration such as GDNF, artemin or erythropoietin (Arthur-Farraj et al., 2012). Then, repair cells associate with regenerated axons and go back to their initial phenotype (Jessen et al., 2015).

Natural direct reprogramming has been also reported in the pancreas where  $\alpha$  or  $\delta$  cells transdifferentiate into  $\beta$  cells after near-total  $\beta$ -cells depletion (Chera et al., 2014; Thorel et al., 2010). Using a mouse model of diphtheria toxin which induce cell death, 99% of  $\beta$  cells were depleted.  $\alpha$  or  $\delta$  cells responded by conversion into  $\beta$  cells.  $\alpha$  cells directly convert into  $\beta$  cells without proliferating into young or aged adults (Thorel et al., 2010). Conversely,  $\delta$  cells have been shown to dedifferentiate and proliferate before reprogramming into  $\beta$  cells in mice before puberty (Chera et al., 2014). Therefore, two cell types can transdifferentiate through different mechanisms.

Direct reprogramming also takes place during liver regeneration (Yanger et al., 2013). Liver injury induces the conversion of hepatocytes into biliary epithelial cells through Notch signaling. Hepatocytes derived-biliary cells display several markers of biliary cells such as biliary cell polarity, or transcriptional markers (Yanger et al., 2013).

Taken together, these data provide emerging evidence of direct reprogramming as a mechanism to regenerate tissues. However, molecular mechanisms and signaling cues triggering this cell plasticity are unknown. Thus, further investigations are required to manipulate cellular reprogramming to permit tissue regeneration.

### **2.7 *In vivo* reprogramming to iPSCs as a platform to study cellular plasticity**

#### *2.7.1 Reprogramming mouse model*

Following the discovery of iPSCs formation *in vitro*, several attempts to reproduce this result *in vivo* have been realized (Abad et al., 2013; Carey et al., 2010). Moreover, in the context of regenerative medicine, current knowledge about manipulation of cell plasticity *in vivo* are limited. Both research groups created a mouse model which carries a ubiquitous OKSM cassette under a doxycycline (DOX) responsive element. More precisely, the mouse model we used carry the transcriptional activator (rtTA) within the ubiquitously-expressed *Rosa26* locus and a single copy of a DOX-inducible polycistronic cassette encoding the four murine factors Oct4, Sox2, Klf4 and c-Myc. Most of the organs developed teratomas upon DOX treatment, indicating reprogramming occurred. Thus, this mouse model can be used as a platform to study cell plasticity *in vivo*.

#### *2.7.2 Cellular plasticity regulation in vivo*

Achieving *in vivo* reprogramming is essential to gain further insights about cellular plasticity. Tissue environmental cues *in vivo* are in general favoring differentiation but some tissues are more or less permissive to reprogramming (Abad et al., 2013). Reasons underlying tissue permissiveness remain unclear. Surprisingly, pancreas is the most permissive organ to teratoma formation, whereas it is a post-mitotic organ, suggesting cellular plasticity is not necessarily associated with self-renewal. In a similar manner, kidney and intestine are very permissive tissues whereas stomach exhibits very few teratomas and skeletal muscle have never developed any teratoma. Intriguingly, based on their differentiation abilities, data suggest that *in vivo* iPSCs are both more plastic and more similar to ESCs than *in vitro* and more prone to undergo trophoblast differentiation (Abad et al., 2013). Therefore,

deciphering underlying mechanisms responsible for these differences is critical for understanding cell plasticity.

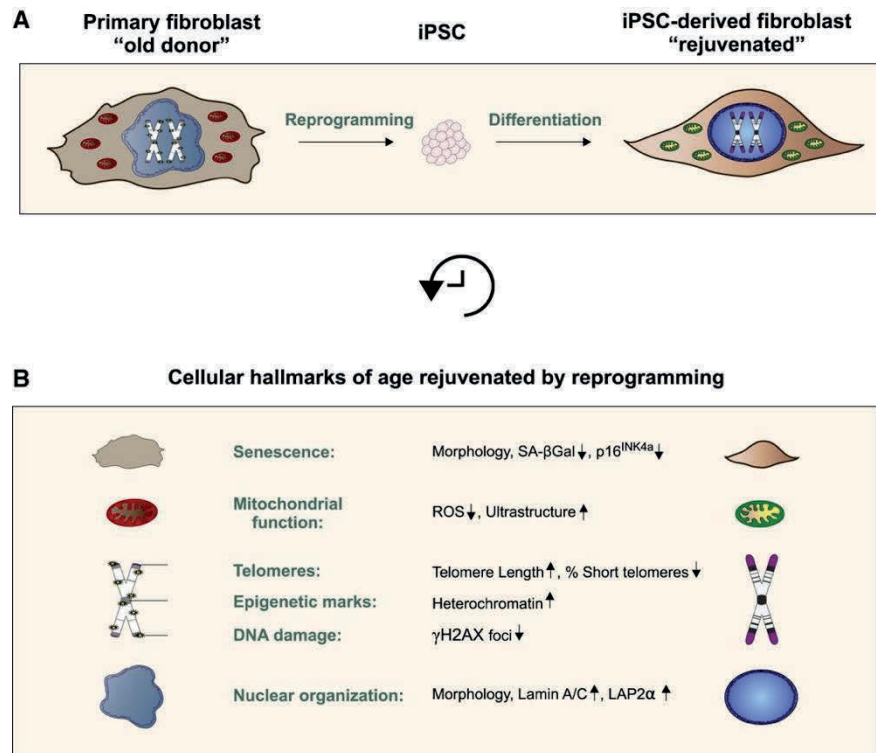
### 2.7.3 *Senescence promotes cell plasticity in i4F mice*

Recent studies relate the effects of senescence on cell plasticity using reprogrammable mouse model. (Chiche et al., 2017; Mosteiro et al., 2016; Mosteiro et al., 2018). Increase in senescence induced by tissue damage resulted in *in vivo* reprogramming in lung or skeletal muscles, two tissues where reprogramming has never been reported before (Abad et al., 2013). In these two studies, senescence induced by tissue damage resulted in production of SASP which facilitated reprogramming *in vitro* and *in vivo*. In the lung, damage inflicted by bleomycin induced senescence and promoted reprogramming, indicated by presence of Nanog<sup>+</sup> cells upon OSKM expression (Mosteiro et al., 2016). In a similar manner, muscle injury induced by cardiotoxin triggered senescence and the appearance of Nanog<sup>+</sup> cells upon OSKM expression (Chiche et al., 2017). Pharmacological removal of senescent cells reduced reprogramming efficiency *in vivo* (Chiche et al., 2017; Mosteiro et al., 2016). IL-6, a known SASP factor, has been suggested as a major mediator of this effect. (Chiche et al., 2017; Mosteiro et al., 2016). Indeed, treatment with IL-6 neutralizing antibody reduced reprogramming efficiency both *in vivo* and *in vitro*. Therefore, tissue injury creates a tissue environment supporting *in vivo* reprogramming through senescence induction. Finally, OSKM expression induced senescence in a majority of cells and reprogramming in other cells. Thus, senescent cells induced by OSKM expression facilitated reprogramming in surrounding cells, such as in the pancreas (Mosteiro et al., 2016; Mosteiro et al., 2018). This senescence induction and paracrine stimulation of reprogramming relies on p16<sup>INK4A</sup> and not on p19<sup>ARF</sup> in i4F mice. Disruption of INK4A locus results in reduced IL-6 levels and impaired dysplasia in pancreas (Mosteiro et al., 2018). Nonetheless, in mice deficient for p16<sup>INK4A</sup>, p19<sup>ARF</sup> and p53, IL-6 production become independent of p16<sup>INK4A</sup> (Mosteiro et al., 2018). Confirming previous results, mice lacking IL-6 showed impaired reprogramming efficiency, which reinforce the critical role of IL-6 in *in vivo* reprogramming (Mosteiro et al., 2018).



### 2.7.4 *Reprogramming and ageing.*

Aging is a process which affects most of the living organisms and leads to its functional decline. Like senescence, there are no universal markers to characterize the aging process. Studies suggest that many cellular and molecular hallmarks contribute to physiological aging, such as cellular senescence, genetic instability, mitochondrial dysfunction and telomere shortening (Lopez-Otin et al., 2013). One major consequence of reprogramming is not only the suppression of native cell identity but also the rejuvenation of the host cell. Upon reprogramming induction, many molecular and genetic markers are modified, leading to a global rejuvenation of somatic cells. For instance, telomere length is restored,  $\gamma$ H2AX foci are erased, senescence markers and ROS are decreased. This suggests that reprogramming may be a strategy against aging progression. Several studies report rejuvenation of specific cellular marker following reprogramming, such as telomere size and mitochondria (Marion et al., 2009b; Suhr et al., 2010). Confirming these results, reprogramming of centenarian human cells have caused their rejuvenation. iPSCs formed were identical of human ESCs and cells derived from these iPSCs were rejuvenated (Lapasset et al., 2011). Several age-related markers were reset in the rejuvenated differentiated cells, such as p16<sup>INK4A</sup> and p19<sup>ARF</sup> levels. Additional studies have reinforced these results. For instance, reprogramming also reverses the aging process of the immune system (Nishimura et al., 2013; Vizcardo et al., 2013). However, all these results have been generated *in vitro*. No evidence that this strategy could be used *in vivo* have been reported until recently.



**Figure 17. Phenotypic rejuvenation during iPSCs induction** (Studer et al., 2015).

In 2016, a report has indicated that “partial” reprogramming was also able to reverse aging *in vivo*, indicating that the aging process can be delayed *in vivo* (Ocampo et al., 2016). Transient expression of OSKM in cells obtained from aged mice display reversed cellular markers of aging *in vitro*. In addition, reprogrammable mice (i4F) have been crossed with a progeria mouse model (LAKI). These new genetic mice have been treated shortly and regularly (2 days every week) with doxycycline to induce OSKM cassette transiently while avoiding iPSCs and teratoma formation. Consequently, this short OSKM induction has been called partial reprogramming. As a result, treated mice have extended lifespan and aging phenotype have been slow down (Ocampo et al., 2016). Nonetheless, results must be confirmed in pure i4F mice to clearly demonstrate that partial reprogramming is an effective solution to delay aging. Interestingly, transdifferentiation from one differentiated cell type to another does not reset cellular aging. Cells resulting from transdifferentiation retain aging markers, indicating that going to at least a partial pluripotent stage is required to erase aging marker (Mertens et al., 2015). In conclusion, understanding the molecular mechanisms regulating this rejuvenation during reprogramming and age-related plasticity is essential in

order to control cell fate. Additionally, find the limit in the OSKM dose is critical to have the benefit of reprogramming on aging without irreversibly going to the iPSCs stage.

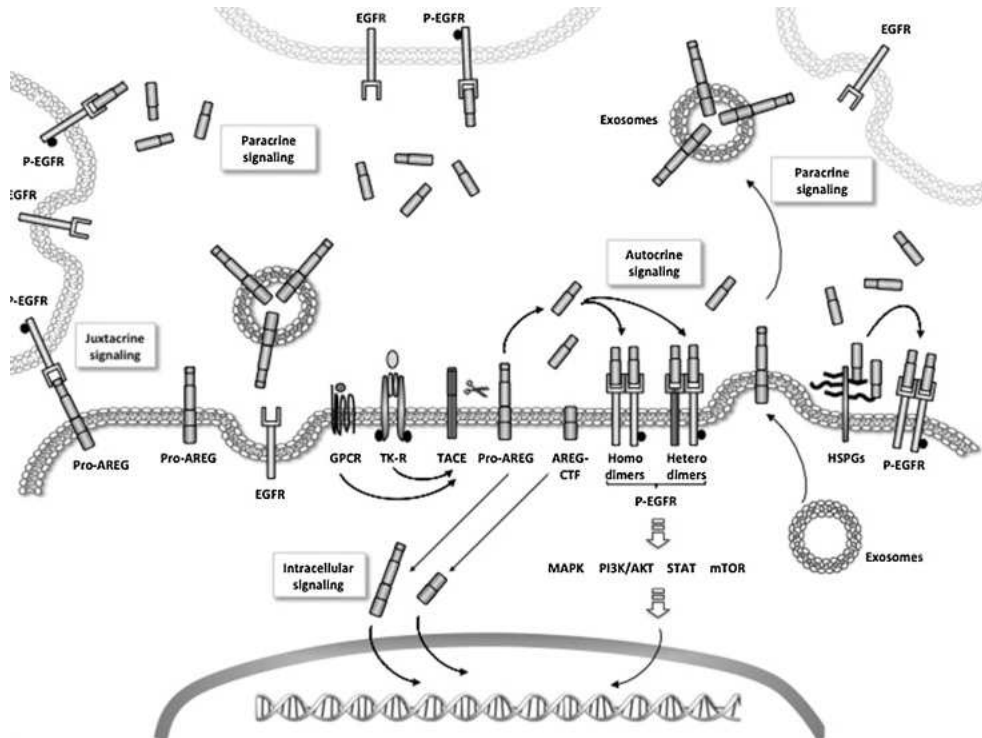
### 3. AMPHIREGULIN/EGFR PATHWAY

#### 3.1 EGFR signaling pathway

EGFR signaling pathway is involved in numerous processes, such as proliferation, apoptosis, growth or differentiation (Herbst, 2004; Yarden and Sliwkowski, 2001). Currently, seven ligands of EGFR are known, Epidermal Growth Factor (EGF), Transforming Growth Factor- $\alpha$  (TGF $\alpha$ ), Heparin-binding EGF-like Growth Factor (HBEGF), Betacellulin (BTC), Amphiregulin (AREG), Epiregulin (EREG), and Epigen (EPGN). The first four ligands of the list are considered to be high-affinity ligands, while remaining ones are low-affinity ligands. Moreover, each ligand has affinity for different subtypes of receptor, such as ERBB1(EGFR), ERBB2(HER2), ERBB3 or ERBB4, which are from ERBB family receptor. Receptor is constituted either by heterodimer (e.g. ERBB1/ERBB2) or homodimer (e.g. ERBB1/ERBB1). Upon binding, receptor is phosphorylated and activated. Each of these ligands trigger different downstream signaling notably according to which receptors they preferably bind. Phosphorylated ERBB receptor recruits specific adaptors and can trigger multiple pathways including JAK/STAT, PI3K/AKT, MAPK or MEK/ERK signaling cascades to regulate gene expression (Oda et al., 2005; Yarden and Sliwkowski, 2001). However, understanding how these ligands could promote distinct signaling pathways through the same receptor tyrosine kinase remains to be elucidated. In addition to dimer composition, dimer structures of the EGFR extracellular region upon ligand-binding is thought to be an important factor explaining these observations (Freed et al., 2017). Therefore, EGFR can trigger numerous functions depending on the ligands. Some of these functions are redundant between ligands whereas some can be specific to one ligand (Hobbs et al., 2002; Lemos-Gonzalez et al., 2007; Luetke et al., 1999; Schuger et al., 1996). Interestingly, AREG has also been shown to be part of SASP in several studies, thus we decided to focus on this particular EGFR ligand (Acosta et al., 2013; Wiley and Campisi, 2016; Xu et al., 2019).

### 3.2 Amphiregulin/EGFR axis

Amphiregulin is first synthesized as an anchored protein called pro-AREG which will be cleaved at the cell membrane by an enzyme named TNF- $\alpha$  converting enzyme (TACE). AREG can bind to neighboring cells when bound to the membrane or can be cleaved and released into the extracellular environment. Due to this particularity, it can either act in juxtacrine, paracrine or autocrine fashion. Finally, AREG can also trigger EGF pathway through exosomes (Higginbotham et al., 2011). EGF and AREG share structural similarities which permit to AREG to bind to EFR. Like EGFR, AREG activates mainly EGFR (ERBB1) homodimer but it can also activate EGFR heterodimer with ERBB2, ERBB3 or ERBB4 depending on the contexts (Berasain, 2014; Yarden, 2001). ERBB3 is particular as it is considered as a recycling receptor because it does not have any kinase activity (Baldys et al., 2009). Thus, EGF and AREG share redundant functions in mammary gland development (Luetteke et al., 1999). It is noteworthy that AREG is one of few EGF members that binds to ERBB3 and favors recycling of EGFR rather than its degradation. This particularity combined to the low affinity of AREG for EGFR (ten times lower than other ligands) have profound effects on downstream signaling (Shoyab et al., 1989). Instead of triggering EGFR transiently such as BTC, AREG induces sustained EGFR activation. Indeed, AREG fails to trigger EGFR internalization unlike other EGFR ligands and consequently can activate different downstream signaling. Ultimately, MAPK, PI3K/AKT, STAT, PKC or mTOR pathway can be triggered in recipient cells upon AREG stimulation (Busser et al., 2011). However, activation of these signaling pathways is often considered as weak comparatively to other EGFR ligands, which may also be an additional reason of AREG specific functions. Taken together, these observations likely explain the unique bi-phasic role of AREG compared to other EGFR ligands, as it either induce a mitogenic signal or a cell differentiation signal depending on the cell lines (Shoyab et al., 1988).



**Figure 18. Schematic representation of the different modes of AREG cell signaling.** TACE-mediated processing of Pro-AREG in the cell surface can be stimulated by GPCRs and TK-R ligands, leading to the secretion of soluble AREG forms. Soluble AREG can engage in autocrine and paracrine signaling through EGFR binding, and also interact with heparan sulfate proteoglycans (HSPGs) on the cell surface. Alternatively, membrane bound Pro-AREG can bind the EGFR on the surface of neighboring cells eliciting juxtacrine signals, or can be released from the cell surface in exosomes that can be internalized by recipient cells. Intracellular signaling triggered by AREG is mainly conveyed by EGFR-mediated activation of intracellular signaling pathways. Internalization and nuclear translocation of Pro-AREG, and of the AREG-cytosolic fragment (AREG-CTF) generated after TACE processing, can also signal part of AREG effects (Berasain and Avila, 2014).

Emphasizing specificities of AREG, distinct roles of EGF and AREG have been found in mouse embryonic development, such as in mammary gland development (Luetke et al., 1999). For instance, AREG and EGF have also been shown to play opposite roles in EMT transition in mammary gland epithelial cells (Fukuda et al., 2016). Moreover, AREG holds functions in other processes such as keratinocytes proliferation (Cook et al., 1991) or lung morphogenesis (Schuger et al., 1996). Mice lacking AREG present very few abnormalities under homeostatic conditions but display many impaired responses in the context of immune inflammation following infection or tissue damage (Zaiss et al., 2015). Indeed, AREG has been suggested as an important mediator of immune response, permitting interactions between numerous immune cells (Zaiss et al., 2015). Thus, AREG plays multiple roles in human pathophysiology, such as cancer, mammary gland development, immune response and tissue repair.

### 3.2.1 *Regulation of Amphiregulin*

Secretion of AREG is regulated by several factors. Activation of AREG locus can be triggered by a range of signals including prostaglandin, hypoxia or numerous cytokines under NF- $\kappa$ B control (Berasain and Avila, 2014; O'Reilly et al., 2006; Shao et al., 2003; Woodworth et al., 1995). AREG production is also induced by AREG itself in a positive feedback loop and by other EGF family members (Barnard et al., 1994; Willmarth and Ethier, 2006). Conversely, AREG is notably repressed by BRCA1, indicating that BRCA1 mutation or its loss may be involved in increased AREG expression in breast cancer cells (Berasain and Avila, 2014). Supporting observations highlighting role of cytokines in AREG up-regulation, IKK $\alpha$ , the NF- $\kappa$ B repressor, has been shown to inhibit AREG expression (Liu et al., 2008). Therefore, AREG expression can be activated through multiple mechanisms, emphasizing implications of AREG in several physiological responses.

### 3.2.2 *Amphiregulin and cancer*

AREG has been linked to several cancers since its discovery, such as colon, liver, skin, breast, and head cancer (Busser et al., 2011). AREG expression supports growth and survival of previous mentioned cancer. It has also been suggested that AREG could play a role in early cancer development and have pro-tumorigenic effects. Expression of AREG in the pancreas induces cell proliferation and pancreas presents dysplastic features similar to the ones observed in early steps of pancreatic tumorigenesis (Wagner et al., 2002). AREG overexpression also contributes to liver carcinoma by inhibiting apoptosis, promoting cell proliferation, and tumorigenic potential (Castillo et al., 2006). A second role of AREG is also to promote cancer invasiveness and metastasis (Higginbotham et al., 2011; Yamada et al., 2008), notably by promoting a mesenchymal state. Finally, AREG can also up-regulates telomerase activity in endothelial cells (Matsui et al., 2000), suggesting a potential contribution in telomerase activity frequently detected in cancer cells. Nonetheless, this activity still has to be demonstrated in cancer cells. Despite its involvement in cancer growth and survival by several mechanisms, AREG is not necessarily expressed by pre-malignant cells, thus an external source of AREG is required to initiate effects or AREG.

Initially, AREG is rarely expressed in healthy tissues. Up-regulation of AREG generally occurs upon acute and chronic inflammation (Burzyn et al., 2013; Monticelli et al., 2011). Chronic inflammation is one of the main reasons which leads to AREG expression and which sustain tumor growth. In this context, immune cells are considered as one of the prominent sources of AREG and have been shown to favor tumor progression (Bles et al., 2010), suggesting a first interplay between immune system with pre-tumor and tumor cells. A second interplay involving AREG is the regulation of Treg immune cells. AREG/EGFR pathway has been found to enhance local Treg activity whose function is to generate an immune suppressive environment. Thus, sustained source of AREG either by tumor cells or immune cells may generate an immune-suppressive environment favorable to tumor growth (Zaiss et al., 2013). Finally, AREG is also associated with drug resistance in several cancers including liver, breast and colorectal cancer (Blivet-Van Eggelpoel et al., 2012; Busser et al., 2011; Castillo et al., 2006). Therefore, AREG seems to display different functions in tumor cell plasticity and cancer development either by endowing new properties to cancer cells or by down-regulating immune reaction.

### 3.2.3 *Amphiregulin role in immune response & tissue repair*

Role of AREG in modulating immune response in the context of tissue injury has been recently demonstrated (Burzyn et al., 2013; Monticelli et al., 2011). For instance, following muscle injury, AREG is expressed by eosinophils and induces cell expansion of resident Treg population. In turn, Treg cells start expressing AREG which triggers myogenic differentiation of muscle stem cells and muscle regeneration (Burzyn et al., 2013). Injection of recombinant AREG as a possible therapeutic strategy has thus been emitted (Burzyn et al., 2013). Nonetheless, the precise effect of AREG on satellite cells requires more investigation. Different signaling can promote muscle repair by symmetric or asymmetric division depending on the context (Bentzinger et al., 2013; Le Grand et al., 2009; Wang et al., 2019). Moreover, the identity of all the cells secreting first AREG has not been investigated. In parallel of its function in modulating immune reaction following tissue damage, AREG induces proliferation signal and triggers differentiation in cells surrounding injury site (Hirota et al., 2012; Stoll et al., 2010). During *in vitro* wound healing assay, AREG promotes proliferation of keratinocytes and contributes to skin homeostasis (Liu et al., 2008; Stoll et al., 2010). In agreement, AREG has been shown to participate in tissue remodeling in asthma by



inducing proliferation and differentiation of endothelial and smooth muscle cells (Hirota et al., 2012). Recent studies in other models, such as drosophila, have shown a role of EGF pathway in expanding stem cell population and contributing to gut remodeling following infection (Buchon et al., 2010), supporting the importance of AREG in tissue remodeling following tissue damage or infection. Finally, overexpression of AREG has also been linked with fibrosis, another aspect of tissue repair (Zhou et al., 2012).

Taken together, these data indicate that AREG actively contribute to tissue repair at multiple levels. However, further analyses are required to better understand the effect of AREG during tissue regeneration. Some sources of origin have not been established and are likely to exist. Effects of AREG depending on its production duration remain also unclear. Transient presence of AREG is likely to be beneficial and participates to proper wound healing whereas prolonged secretion of AREG is probably a reason of fibrosis and development of immunosuppressive environment.

### *3.2.4 AREG/EGFR and pluripotency*

Currently, only few studies have looked at the potential function of EGFR and more particularly of AREG in pluripotency despite importance of EGF pathway in biological process. Recently, activation of EGFR pathways has been reported to act as an inhibitor of MEFs reprogramming (Tran et al., 2015). Depletion of EGFR combined with ascorbic acid (AA) and 2i further enhanced reprogramming efficiency compared to AA with 2i only. Reinforcing their results, disruption of EGFR pathway + AA improve reprogramming at similar level than AA with 2i. Thus, these data indicate that EGFR is a barrier for induction of pluripotency. Nonetheless, it is noteworthy that these results were observed using 2i, which is known to induce a specific pluripotent state, called ground state. Intriguingly, EGFR seems to be important for ESCs maintenance (Yu et al., 2019). Disruption of EGFR impairs proliferation and induces a significant decrease in mRNA levels of several markers of pluripotency. Moreover, ERBB2 has been reported to be critical for self-renewal in mESCs (Wang et al., 2007). Lack of ERBB2 reduces cell proliferation and induces massive apoptosis of ESCs in feeder free culture. Finally, RNA-seq gene analysis data showed that ERBB pathway was significantly enriched in hESCs (Abu-Dawud et al., 2018; Takashima et al., 2014). Overall, these data suggest that EGFR participates to ESCs self-renewal. However,

downstream mechanisms have not been identified and all the ligands that mediate EGFR activation in this situation has not been explored.



## **RESULTS**



The discovery of iPSCs has been groundbreaking and has led to complete rethinking of cellular plasticity, bringing tremendous hopes for regenerative medicine (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). One of the main goals of regenerative medicine is to generate different cell types to replace lost or damaged cells. Reprogramming technology provides the *in vitro* system to obtain the desired cell types, by reprogramming cells into iPSCs to expand and differentiate them in a specific cell type. In the meantime, direct reprogramming is a promising strategy as it permits to differentiate one cell type directly into another without going through the pluripotency stage. Therefore, direct reprogramming avoids concerns about potential teratoma formation due to incomplete differentiation. Importantly, several reports have found that both reprogramming to pluripotency and direct reprogramming (transdifferentiation) could be performed *in vivo* (Zhou et al., 2008).

## **1. INJURY-INDUCED SENESCENCE ENABLES *IN VIVO* REPROGRAMMING IN SKELETAL MUSCLE**

### **1.1 Context of the study**

Prior to my PhD, senescence is known as an intrinsic barrier for *in vitro* reprogramming (Banito et al., 2009). Previous reports have shown that senescence plays a role in tissue repair (Demaria et al., 2014; Yun et al., 2015). Moreover, increased *in vivo* reprogramming efficiency has been observed in multiple tissues upon injury, including liver and pancreas (Heinrich et al., 2015). Indeed, several studies reported cell identity conversion following tissue injury (Arthur-Farraj et al., 2012; Davis et al., 2012; Thorel et al., 2010; Yanger et al., 2013). For instance, conversion of  $\alpha$  cells to  $\beta$  cells has been reported in the pancreas following extreme loss of  $\beta$  cells (Thorel et al., 2010). However, the impact of senescence on cellular plasticity in the context of tissue repair has never been explored. My PhD project aims to understand the paracrine impact of senescence on cellular plasticity using both *in vitro* and *in vivo* reprogramming systems, and identify SASP factors that could promote cellular plasticity and tissue regeneration. The first part of my PhD study demonstrated that IL-6 promoting *in vivo* reprogramming, which has been published and is briefly presented here (*in the previous result section*). The main part of my project focused on

identifying novel SASP factors as cellular plasticity regulators to further our understanding on how senescence induces cell plasticity in the context of reprogramming and tissue repair.

### 1.2 Previous results

It has been reported that several tissues are permissive to *in vivo* reprogramming upon OSKM expression in reprogrammable mice (i4F) (Abad et al., 2013). However, skeletal muscle is refractory to *in vivo* reprogramming. We demonstrated that both acute and chronic muscle injury could promote cellular reprogramming in the skeletal muscle. Interestingly, it has been shown that muscle injury induces transient senescence response (Le Roux et al., 2015). Importantly, using both gain-of-function and loss-of-function approaches, we showed that injury-induced senescence promotes *in vivo* reprogramming. Moreover, using Pax7 lineage tracing mouse model, which is a marker of muscle stem cells, we demonstrated that muscle stem cell is a cell of origin for *in vivo* reprogramming in skeletal muscle.

My contribution to this study is to investigate how senescence facilitates *in vivo* reprogramming. We isolated satellite cells (SCs) and fibrogenic/adipogenic precursors (FAPs) from reprogrammable mice and reprogrammed them *in vitro* in presence of either non-senescent MEFs or senescent MEFs. Presence of senescent cells significantly enhanced the reprogramming efficiency. This result suggests that senescence promotes reprogramming via paracrine manner. Next, we performed qRT-PCR analysis on whole muscle extract and observed a significant increase of IL-6 level in injured TA compared to control non-injured TA. We focused on IL-6 given its role in enhancing *in vitro* reprogramming and muscle regeneration (Brady et al., 2013; Munoz-Canoves et al., 2013). Therefore, we hypothesized that IL-6 might enhance reprogramming in a non-cell-autonomous manner. Adding recombinant IL-6 to the media further enhance reprogramming efficiency of SCs and FAPs. Conversely, blockade of IL-6 significantly reduced the benefits of senescence on reprogramming efficiency. Oct4 and Nanog immunofluorescence staining confirmed pluripotency of iPSCs generated from SCs. Moreover, administration of IL-6 blocking antibody *in vivo* increased survival of mice and number of Nanog positive cells was decreased in TA of treated mice compared to control. In conclusion, IL-6 blockade abolished beneficial

effect of cellular senescence on reprogramming, suggesting that cellular senescence promotes reprogramming in a cell-non-autonomous fashion. In conclusion, senescent cells promote reprogramming through production of SASP, in particular IL-6.

## 2. IMPACT OF CELLULAR SENESCENCE ON CELLULAR REPROGRAMMING IN A NON-CELL AUTONOMOUS MANNER

### 2.1 Context of the project

We previously demonstrated that injury induced senescence promotes reprogramming in the skeletal muscle, notably through IL-6 (Chiche et al., 2017). Interestingly, IL-6 has beneficial effect on muscle regeneration (Munoz-Canoves et al., 2013) but its potential use as a therapeutic strategy is limited due to its pleiotropic functions (Karin and Clevers, 2016; Tanaka et al., 2014). SASP is composed of numerous factors, which have been shown to play redundant roles, such as IL-1, TGF- $\beta$ , CCL2 and VEGF in transmitting senescence (Acosta et al., 2013; Coppe et al., 2010; Hubackova et al., 2012). We hypothesized that other factor may enhance reprogramming besides IL-6. Using our i4F mouse model as a platform, our aim is to find an alternative factor to IL-6, which could successfully promote cellular plasticity and tissue regeneration. Finally, identifying other factors than to IL-6 may help also us to better understand how SASP trigger cell plasticity in the context of tissue repair.

### 2.2 Results

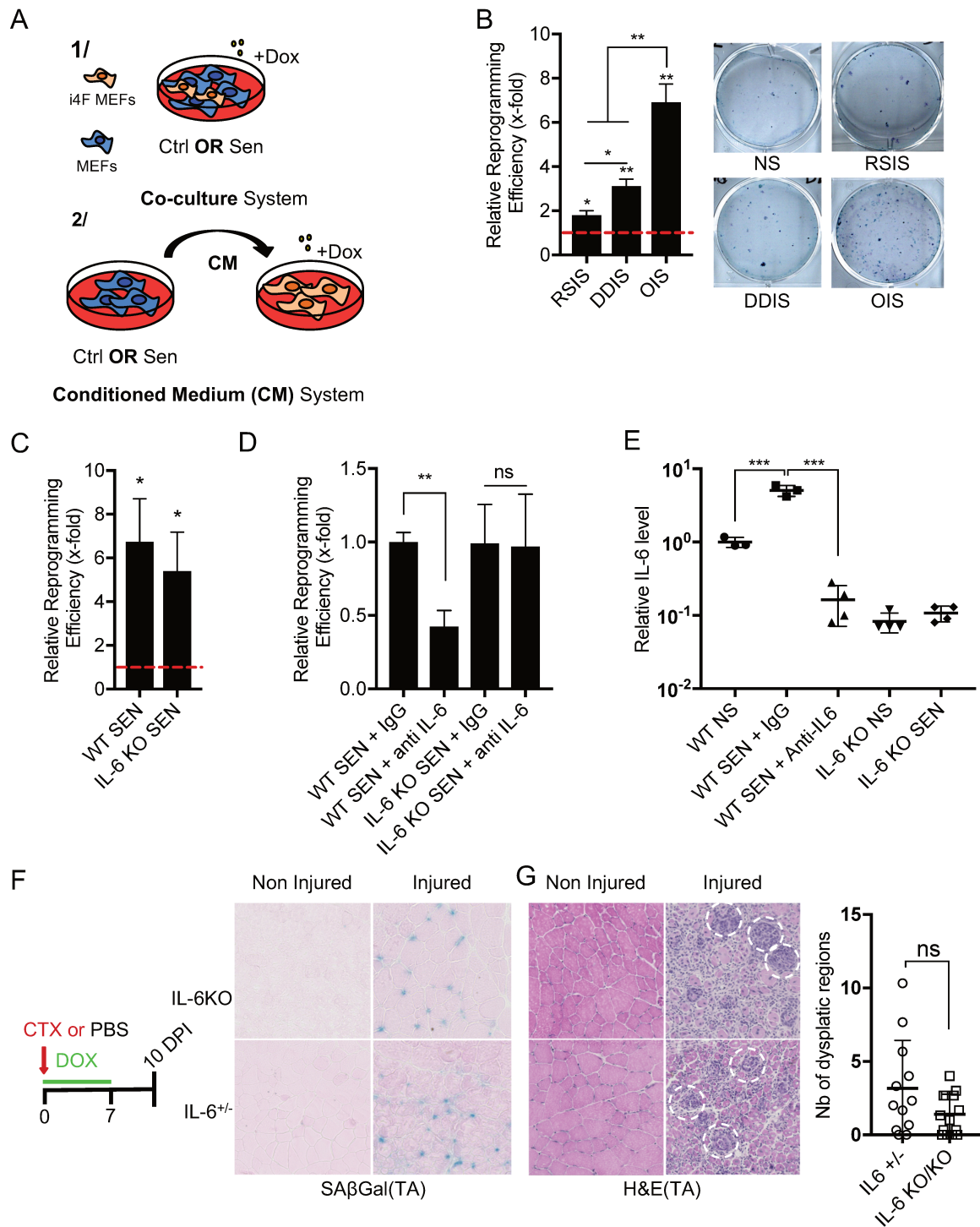
#### 2.2.1 *SASPs promote reprogramming in a stress dependent and IL-6 independent manner*

To further investigate the role of cellular senescence on reprogramming, I modified the *in vitro* assays previously established (Chiche et al., 2017). Both assays use mouse embryonic fibroblasts (MEFs) generated from i4F, where *in vitro* reprogramming could be induced by doxycycline (DOX) treatment (Abad et al., 2013). Depending on the experiments, i4F MEFs used were either heterozygous or homozygous to assess impact of senescence. Both i4F MEFs can develop iPSCs but with different efficiencies. Homozygous i4F MEFs have



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better reprogramming efficiency than heterozygous *i4f* MEFs. In the co-culture system, we co-cultured the heterozygous *i4F* MEFs in presence of either WT non-senescent (NS) MEFs or senescent (SEN) MEFs. While in the conditioned medium (CM) system, we reprogrammed homozygous *i4F* MEFs in presence of conditioned medium from either NS or SEN MEFs (Figure 1A).



**Figure 1. SASPs promote reprogramming in a stress dependent and IL-6 independent manner**

## RESULTS

(A) A schematic of *in vitro* assays used to assess SASP effect on cellular reprogramming. 1/ co-culture system: heterozygous i4F MEFs were co-culture with control (Ctrl) (empty vector)/ non-senescent (NS) or Senescent (SEN) MEFs in presence of doxycycline. Control cells were cells infected with empty vector to compare with OIS cells. 2/ conditioned medium (CM) system: KSR without LIF medium was incubated with Ctrl or SEN MEFs for 48hrs then used for cellular reprogramming.

(B) *In vitro* reprogramming efficiency of i4F MEFs co-cultured on NS or Ctrl NS cells compared to SEN cells. Senescence was either induced by Replicative Stress (RSIS), DNA Damage (DDIS) or Oncogene overexpression (OIS) (left panel). Alkaline Phosphatase staining of iPSCs generated upon reprogramming with different type of senescent cells (right panel).

(C) *In vitro* reprogramming efficiency for i4F MEFs incubated in presence of either OIS WT or IL-6KO OIS MEFs in co-culture system. Control are MEFs infected with empty vector.

(D) *In vitro* reprogramming efficiency for i4F MEFs incubated in presence of WT of IL-6 SEN cells and either IgG or anti-IL-6 blocking antibody (3 $\mu$ g/mL).

(E) ELISA results measuring IL-6 concentrations from WT NS, WT SEN with IgG or anti-IL6, IL-6KO NS or IL-6KO SEN.

(F) Scheme of the experiments (left), DPI: Days Post Injury. SA- $\beta$  Gal staining of TA sections (right panel).

(G) H&E staining of TA sections (left panel). Circled regions are dysplasia. Quantification of dysplastic region in injured TA with Dox treatment (right panel). At least 12 TAs were quantified per group.

\* $p < 0.05$ , \*\* $p < 0.01$ . Mann-Whitney U test,  $n = 6$  (2 independent experiments, 3 clones). mean  $\pm$  SD.

As SASP composition vary according to stress inducing senescence (Coppe et al., 2010; Hernandez-Segura et al., 2017), we hypothesized that different inducers might impact reprogramming efficiency differently. Based on my previous experiments using SCs (Chiche et al., 2017), co-culture system detects more significant changes in reprogramming efficiency, therefore we decided to use this system to compare the impact of different stress-induced senescence on *in vitro* reprogramming. We used the most commonly methods in order to induce senescence *in vitro*, including replicative stress induced senescence (RSIS), DNA damage induced senescence (DDIS), and oncogene induced senescence (OIS). Senescence was induced by serial passages under hypoxia (5% O<sub>2</sub>), X-RAY (20Gy), and hRAS overexpression. Under normal conditions (20% O<sub>2</sub>), human fibroblasts enter into senescence because of telomere shortening, MEFs enter senescence because of oxidative DNA damage. However, MEFs passaged under hypoxia have reduced oxidative DNA damage and enter into replicative senescence because of telomere shortening. (Parrinello et al., 2003). We confirmed the senescence induction by analyzing senescence associated  $\beta$ -galactosidase activity and expression level of senescence markers p16<sup>Ink4A</sup> and p19<sup>ARF</sup> (Figure S1B & Figure S1C).

Next, we tested the effect of different types of senescence on reprogramming efficiency. Strikingly, OIS was most efficient in enhancing reprogramming (~7-fold change), whereas RSIS had the least effect (Figure 1B). To rule out the possibility that the variation in IL-6 concentration causes the differences, we quantified IL-6 by ELISA in the medium from

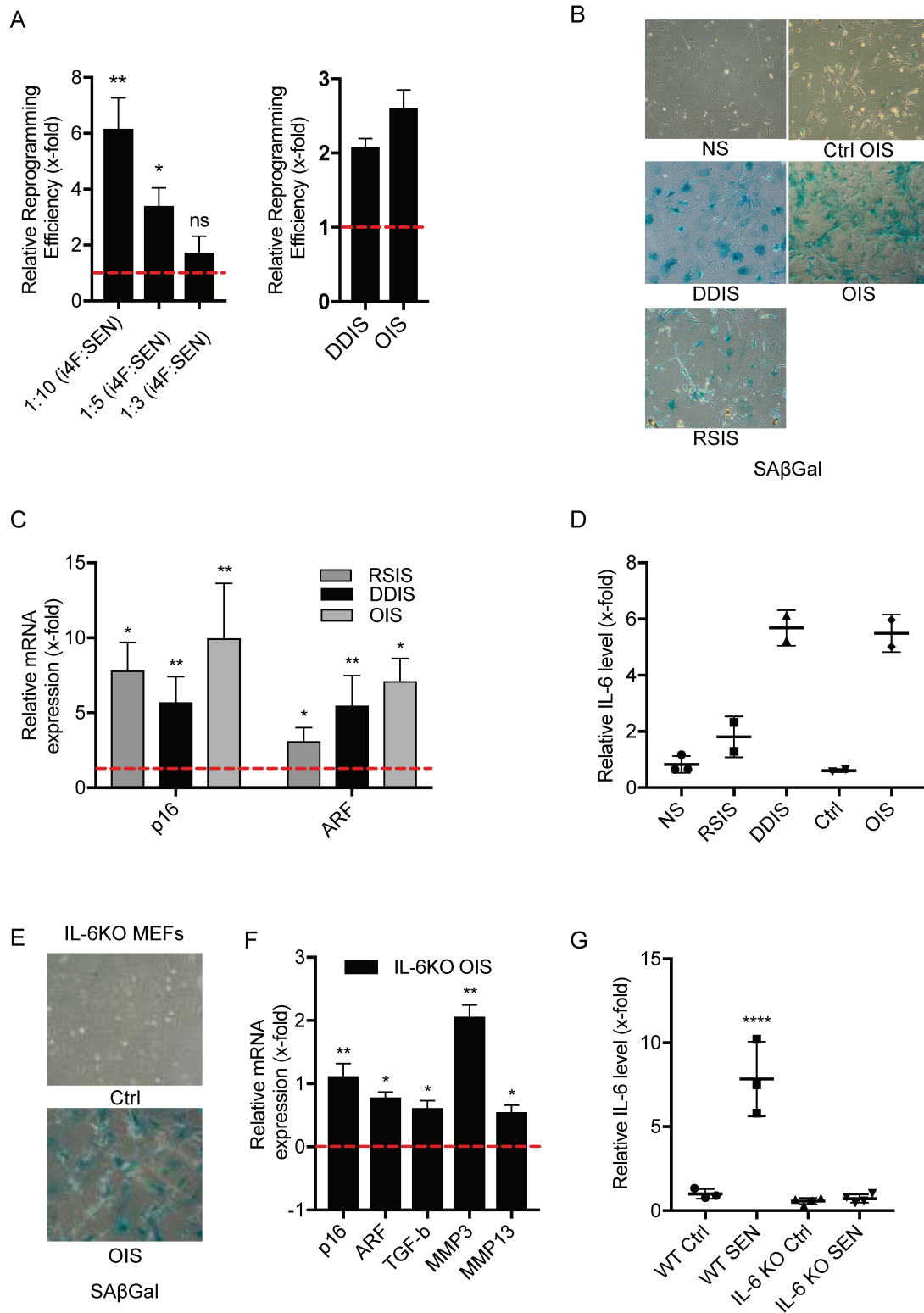
## RESULTS

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RSIS, DDIS and OIS. Levels of IL-6 were similar between DDIS and OIS but significantly higher than RSIS, suggesting other factors other than IL-6 are important for the difference (Figure S1D). Therefore, we tested whether senescence could promote reprogramming in an IL-6 independent manner. We overexpressed hRas in IL-6 KO MEFs to induce OIS senescence. Remarkably, co-culture of i4F MEFs with IL-6KO SEN MEFs enhanced reprogramming to the similar level as WT SEN MEFs (6.5 vs 5.5-fold change) (Figure 1C). Surprisingly, we observed a strong increase of SA- $\beta$  GAL activity and an induction of p16<sup>Ink4A</sup>, p19<sup>ARF</sup> and SASP factors in IL-6KO OIS SEN MEFs (Figure S1E & S1F). To ensure IL-6 protein is absent, we first measured IL-6 level in the medium of IL-6 KO SEN MEFs by ELISA following hRAS overexpression. Indeed, we did not detect significant amount of IL-6 protein in this condition (Figure 1F). More importantly, unlike WT SEN MEFs, anti-IL-6 antibody did not abolish the positive effect of IL-6 KO SEN MEFs on reprogramming (Figure 2B). IL-6 level was measured by ELISA to further confirm this observation (Figure 1E). Finally, we asked whether SASP lacking IL-6 could also trigger reprogramming *in vivo*. To address this question, we crossed i4F mice with IL-6KO mice and performed muscle injury. One of the *Tibialis anterior* (TA) of IL-6KO;i4F MEFs was acutely injured with cardiotoxin (CTX) and mice were treated with DOX in the drinking water to activate OSKM expression.. Both non-injured TA (injected with PBS) and injured TA were collected and analyzed 10 days post-injury to assess both senescence induction and *in vivo* reprogramming (Figure 1F). We stained sections with SA- $\beta$  GAL activity to identify presence of senescent cells. We found SA- $\beta$  GAL-positive cells which indicates that senescence was triggered despite absence of IL-6, which confirmed our *in vitro* observations (Figure 1F).

Expression of the OSKM upon injury generated dysplastic regions in a similar manner to the classic i4F mice, validating our *in vitro* observations (Figure 1G). Indeed there was no significant change in the number of dysplasia observed between injured TA from i4F mice and IL-6KO;i4F mice, which is inconsistent with previous study (Mosteiro et al., 2018). Taken together, these data both *in vitro* and *in vivo* suggest that SASP can also enhance reprogramming independently of IL-6.

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**Figure S1**

(A) Reprogramming efficiency in a co-culture system of different ratio of i4F MEFs with SEN MEFs (left panel). Reprogramming efficiency in a CM system.

(B) SA- $\beta$  Gal staining in vitro WT SEN MEFs induced either by RSIS, DDIS or OIS with Control.

(C) mRNA levels of genes indicated in WT SEN MEFs induced either by RSIS, DDIS or OIS. n= 4

(D) ELISA results measuring IL-6 concentrations from WT NS, RSIS, DDIS, WT Ctrl and WT OIS cells.

(E) SA- $\beta$  Gal staining of *in vitro* IL-6KO SEN MEFs induced by OIS.

(F) mRNA levels of genes indicated in IL-6KO SEN MEFs induced by Oncogene overexpression (OIS). Control is Ctrl MEFs infected with empty vector.

(G) ELISA results measuring IL-6 in WT Ctrl NS, WT SEN (OIS), IL-6KO Ctrl NS and IL-6KO SEN (OIS). *In vitro* reprogramming efficiency for i4F MEFs treated either with CM, Supernatant (SN), or purified exosomes resuspended into Ctrl SN. Control was the number of iPSCs colonies obtained with Ctrl CM only.

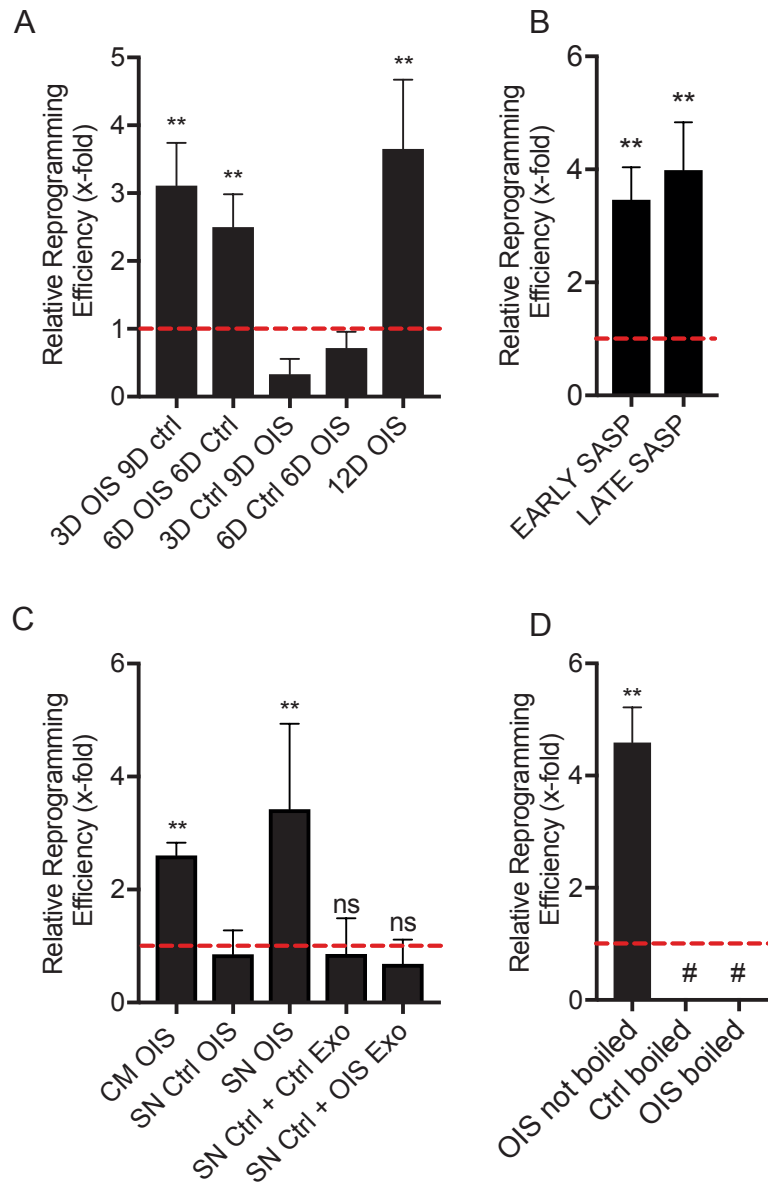
\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ . Mann-Whitney U test,  $n = 6$  (2 independent experiments, 3 clones). mean  $\pm$  SD.

### 2.2.2 *Transient exposure to SASP is sufficient to increase reprogramming efficiency*

To better understand how SASP enhances reprogramming, we switched to the CM system. We used CM from OIS cells as it was the most efficient in enhancing reprogramming. We added OIS-CM for different durations and switch to Ctrl-CM or vice versa. Addition of OIS CM, both from WT and IL-6 KO, for 3 days was sufficient to significantly increase reprogramming efficiency (Figure 2A & S2A). Conversely, initiating reprogramming with Ctrl CM and switching to OIS CM completely abolished the beneficial effect. These results suggest that senescence is particularly important during onset of reprogramming. SASP has also been reported that SASP composition fluctuates over time (Hernandez-Segura et al., 2017; van Deursen, 2014). To test if SASP timing is important for reprogramming, we collected CM from either early OIS SEN MEFs or late SEN MEFs and reprogram i4F MEFs using either only CM from either early or late SEN MEFs. We did not observe significant changes between early and late OIS CM, which suggests that either SASP effect is maintained independently from its composition or secretion of SASP factors important for reprogramming are maintained throughout senescence (Figure 2B). Of note, senescent cells also release exosomes (Lehmann et al., 2008). Recently, a study has shown that exosomes could also mediate senescence in a non-cell autonomous manner (Borghesan et al., 2019). Consequently, we tested whether senescence associated exosomes could also have an impact on cell reprogramming, we isolated exosomes from CM by ultracentrifugation and obtained either Supernatant (SN), which is soluble fraction, or Exosomes (Exo) that were directly resuspended into reprogramming culture medium or CTRL CM. To confirm our exosomes were functional, we tested their ability to mediate senescence. Addition of exosomes to i4F cells without DOX induced a decrease in BrdU incorporation and increase in both senescence markers p16<sup>Ink4A</sup> and p19<sup>ARF</sup> (Fig S2C), indicating that exosomes were functional. Remarkably, SN favored *in vitro* reprogramming at similar level that CM whereas treatment with resuspended exosomes did not increase reprogramming. Resuspending of exosomes in

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Ctrl CM did not further enhance reprogramming, suggesting effect of SASP is limited to its soluble fraction (Figure 2D). In an identical manner, resuspending of exosomes in iPSCs medium did not increase reprogramming efficiency (Figure S2B), both with WT or IL-6KO CM from senescent cells. Boiling CM abolished effect of reprogramming, further indicating that senescence enhances reprogramming through soluble factors (Figure 2E).



**Figure 2. Deciphering SASP impact on senescence**

(A) *In vitro* reprogramming efficiency for i4F MEFs incubated in different time window conditions. Cells were either first reprogrammed with OIS or Ctrl CM for different time and switched to Ctrl or OIS CM. Treatment with 3 days was sufficient to obtain the maximal colony number. Control was the number of iPSCs colonies obtained with Ctrl CM only. CM was generated using IL-6KO MEFs.

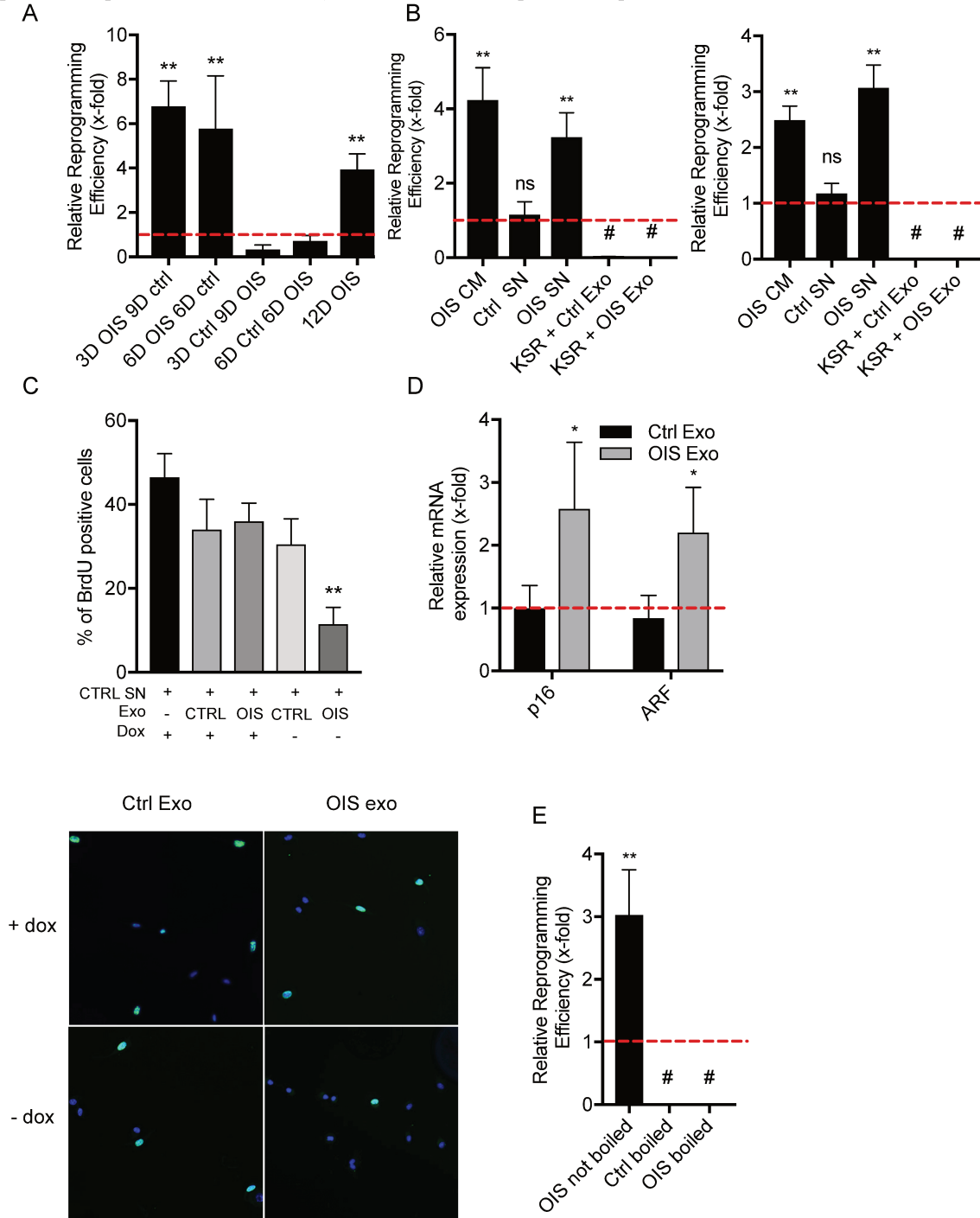
## RESULTS

(B) CM for either early senescent cells (4 days after senescence induction) or late senescence (10 days after senescence induction) was collected and use to reprogram homozygous i4F MEFs. Control was the number of iPSCs colonies obtained with Ctrl CM only.

(C) *In vitro* reprogramming efficiency for i4F MEFs treated either with CM, Supernatant (SN), or purified exosomes resuspended into SN Ctrl medium. Control was the number of iPSCs colonies obtained with Ctrl CM only. CM was generated using IL-6KO MEFs.

(D) Cells were incubated with DMEM previously incubated with Ctrl NS or OIS MEFs then boiled or not for 5 min at 95°C. CM was generated using IL-6KO MEFs.

\* $p < 0.05$ , \*\* $p < 0.01$ . Mann-Whitney U test,  $n = 6$  (2 independent experiments, 3 clones). mean  $\pm$  SD.



**Figure S2**

(A) i4F MEFs were either reprogrammed in presence of Ctrl or OIS CM. Cells were counted every day using Neubauer chamber.

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(B) *In vitro* reprogramming efficiency for i4F MEFs incubated in different time window conditions. Cells were first reprogrammed with OIS CM for different time and switched to Ctrl CM. Treatment with 3 days was sufficient to obtain the maximal colony number. Control was the number of iPSCs colonies obtained with Ctrl CM only. CM was generated using IL-6KO MEFs (left panel) or WT MEFs (right panel).

(C) Percentage of BrdU positive cells 48 hours after treatment (Top). Immunofluorescence staining of BrdU incorporation.

(D) mRNA levels of genes indicated after treatment with Ctrl Exosomes or OIS Exosomes. Control was cells treated with SN Ctrl only.

(E) Cells were incubated with DMEM previously incubated with Ctrl NS or OIS MEFs then boiled or not for 5 min at 95°C. CM was generated using WT MEFs.

\* $p < 0.05$ , \*\* $p < 0.01$ . Mann-Whitney U test,  $n = 6$  (2 independent experiments, 3 clones). mean  $\pm$  SD.

### 2.2.3 Secretome analysis of OIS SASP

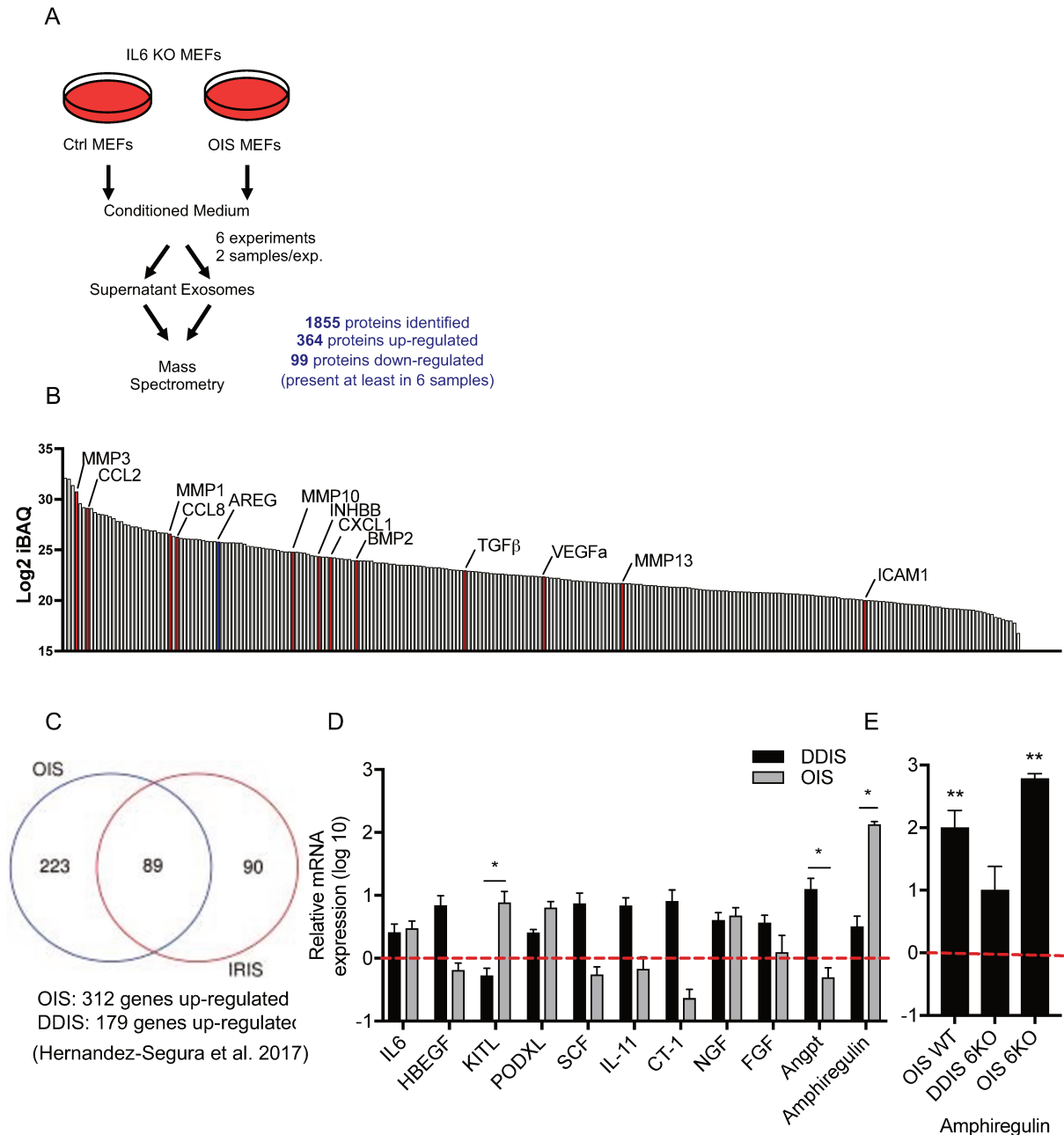
To identify potential SASP members beneficial for reprogramming, we simultaneously used two different strategies. The first strategy consisted in performing mass spectrometry to catalogue SASP components from IL-6 KO CM and discover novel factors. We aimed to explore IL-6KO CM as it can reproduce results observed with WT CM. Thus, we infected IL-6KO cells with either oncogenic or vector control to induce senescence. Cells were then selected using puromycin, seeded at proper concentration and allowed to establish full senescence for 8 days. Ctrl or SEN cells were then cultured in serum-free medium for 48 hours and the conditioned medium was collected. Finally, soluble factors and exosomes were separated by ultracentrifugation.

Quantitative proteomics offers the opportunity to directly identify proteins and quantify their change in expression. For each condition, 6 biological replicates have been generated (Figure 3A). We compared the secreted proteins from IL-6KO CTRL or SEN with a significant fold change ( $q$ -value  $< 0.01$ ) superior to 2-fold (SEN/CTRL) (Figure S3A). Overall, 1800 secreted proteins were identified and  $\sim 20\%$  were significantly upregulated ( $> 2$ -fold). Up-regulation of several known SASP factors was detected such as TGF- $\beta$ , MMPs, INHBB, VEGFa or CCL2, confirming the induction of senescence and SASP acquisition in IL-6 KO MEFs (Acosta et al., 2013; Coppe et al., 2010; Coppe et al., 2008). Remarkably, CXCL1, STC1 and MMP1 also strongly increased in IL-6KO SEN, confirming recent observations on their automatic presence in multiple SASP (Basisty, 2019). Interestingly, increase in mRNA expression levels of genes significantly upregulated only correlated for  $\sim 42\%$  of the factors detected as significantly up-regulated by mass spectrometry (Figure 3B and S3B), suggesting that many factors might be regulated at a post-transcriptional level. Due



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to its role in tissue repair (Burzyn et al., 2013), a process in which senescence significantly contribute (Demaria et al., 2014) but also cancer development and resistance (Castillo et al., 2006), we speculated that amphiregulin (AREG) may have potential positive impact on iPSCs formation. AREG is a member of the EGF family and binds to EGFR (Berasain and Avila, 2014).



**Figure 3. Secretome analysis of IL-6KO SASP**

(A) Scheme summarizing the proteomics approach used.

(B) iBAQ mean intensities from protein significantly up-regulated or exclusively present in IL-6KO OIS CM. Well known SASP factors are highlighted in red. AREG is highlighted in blue.

(C) Analysis of published RNA-Seq data to identify potential factors which could enhance reprogramming.

(D) mRNA levels of genes indicated in either WT DDIS or OIS compared to NS or Ctrl. Mean  $\pm$  SEM. n = 4. Mann-Whitney U test

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(E) mRNA levels of amphiregulin in either WT OIS, IL-6KO DDIS or OIS. Mean  $\pm$  SEM.  $n > 3$ . \* $p < 0.05$ , Mann-Whitney U test

The second strategy was to analyze previous published studies and RNA sequencing results from senescent cells (Coppe et al., 2010; Hernandez-Segura et al., 2017). Using gene annotation, we reduced our pool of genes and annotated whether these genes were specifically increased in one stress or not (Figure 3C). Then, we verified their expression level in WT SEN cells using RT-qPCR (Figure 3D). Remarkably, Amphiregulin (AREG) was strongly overexpressed (>100-fold change) in OIS cells compared to DDIS cells. AREG was also strongly and specifically upregulated in OIS IL-6-KO MEFs (Figure 3E), further suggesting it could be a relevant factor. Overall, we decided to focus on AREG role on the reprogramming process.

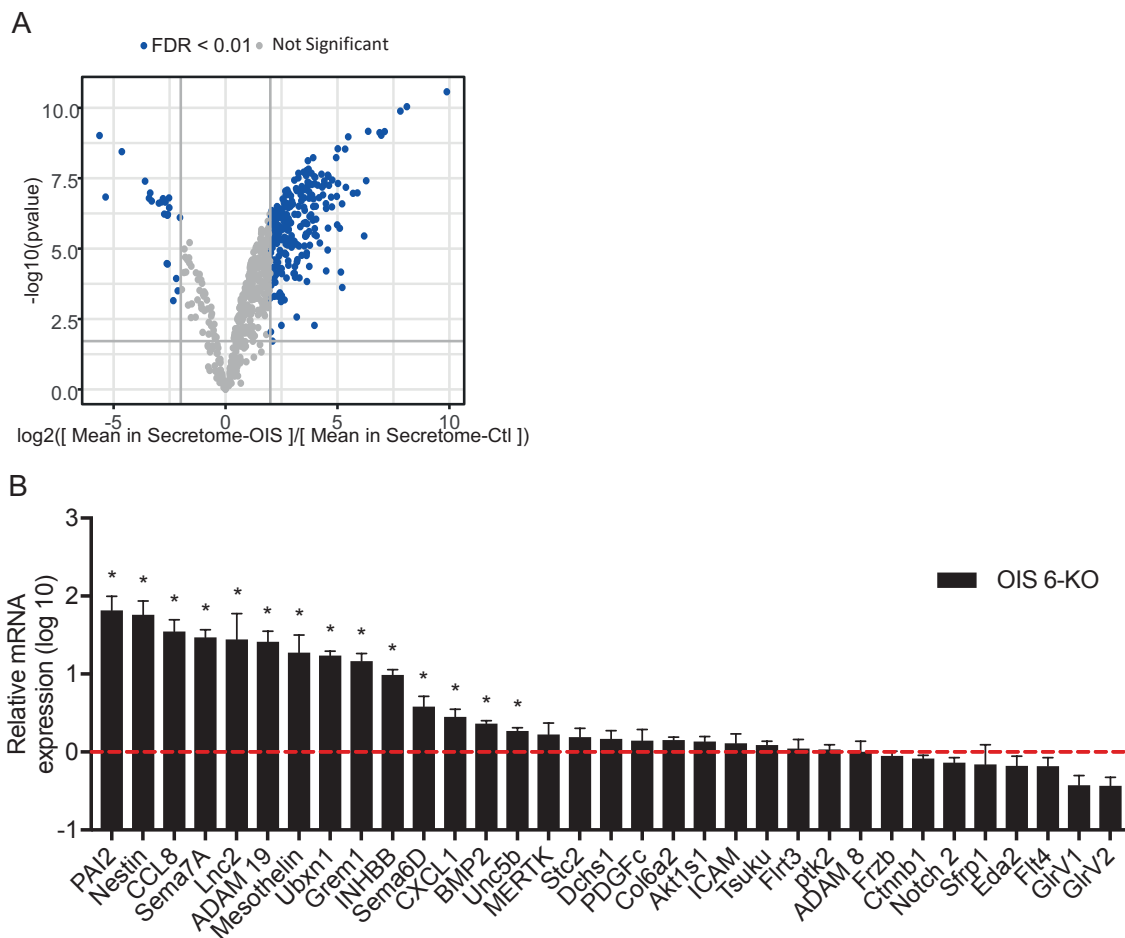


Figure S3

## RESULTS

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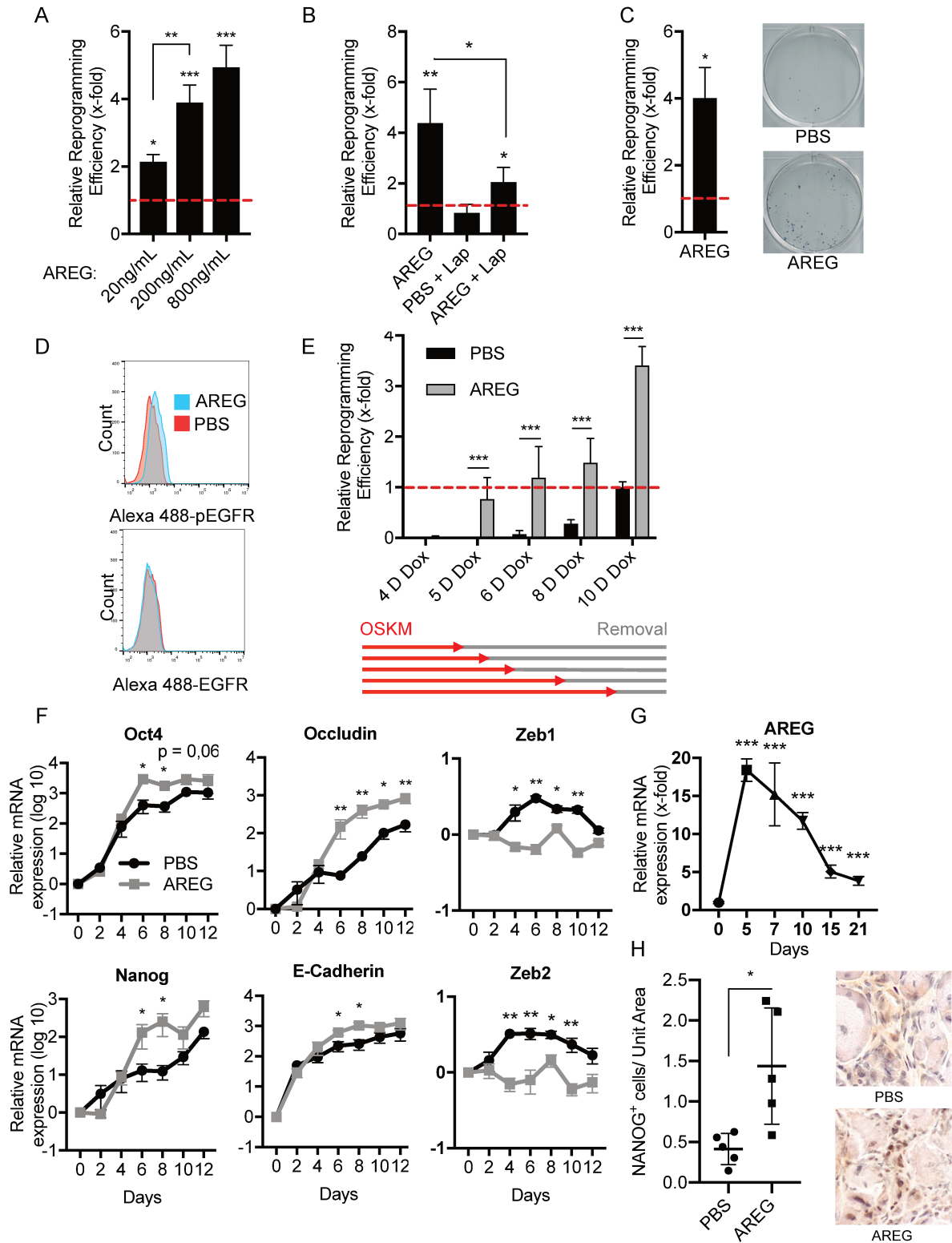
(A) Dot plot showing proteins upregulated in OIS secretome compared to Ctrl secretome. Significant changes present in at least 6 samples are colored in blue. Protein that was absent from Ctrl secretome but present from OIS secretome are not represented.

(B) mRNA levels of genes indicated in IL-6KO OIS compared to Ctrl. Mean  $\pm$  SEM. n = 4. Mann-Whitney U test \*p < 0.05

### 2.2.4 *Amphiregulin promotes both in vitro and in vivo reprogramming*

It is noteworthy that AREG is the only EGFR ligand detected in our Mass Spectrometry analysis, indicating that it is the only EGFR ligand expressed in OIS MEFs. Nonetheless, we analyzed the gene expression level of every EGF family member and found that other EGF members were increased such as Betacellulin, Epiregulin and Neuregulin, suggesting that other factors may be also upregulated but to a lesser extent (Figure S4A), explaining why we did not detect them. Based on our data, we investigated whether AREG might play a role in reprogramming. Addition of recombinant AREG to KSR medium significantly increased reprogramming efficiency whereas blocking of EGFR signaling with Lapatinib (Lap) abolished effect of AREG (Figure 4A & 4B). Moreover, administrating AREG for 3 days was sufficient to significantly promote reprogramming (~4-fold change), in a similar manner than SASP (Figure 2A & 2B). In a similar fashion, treatment with AREG promoted reprogramming of skin fibroblast obtained from adult mice (Figure 4C). Pluripotency of iPSCs generated with AREG were assessed by immunofluorescence staining for the pluripotent markers Oct4 and Nanog (Figure S4B). To ensure MEFs were responsive to AREG treatment, we analyzed the induction of EGFR signaling pathway by testing EGFR phosphorylation (Figure 4B). Next, we investigated how AREG promotes cellular reprogramming efficiency. We asked whether AREG could not only increase reprogramming efficiency but also reprogramming kinetics. MEFs were treated with AREG for 3 days and DOX was withdrawn after a precise number of days. Remarkably, adding AREG was sufficient to obtain iPSCs after only 5 days of DOX treatment (Figure 4E). Furthermore, reprogramming efficiency was identical to control only after 6 days of DOX treatment in AREG treated cells. Cell cycle analysis confirmed this increase in cell proliferation (Figure S4D). Treatment with AREG also increased expression of pluripotency markers Oct4 and Nanog (respectively ~10 and ~100-fold change at D6 and D8), but also epithelial markers Occludin (~20 and ~16-fold change at D6 and D8) and E-Cadherin (~4-fold change at D6 and D8) (Figure 4F).

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**Figure 4. Amphiregulin promotes cellular reprogramming**

(A) *In vitro* reprogramming efficiency for i4F MEFs treated with different concentrations of AREG. n=5 C<sub>1</sub>= 20ng/mL C<sub>2</sub>= 200ng/mL C<sub>3</sub>= 800ng/mL

(B) *In vitro* reprogramming efficiency for i4F MEFs treated with either PBS or recombinant AREG and Lapatinib (Lap) in KSR medium. Cells were treated for 3 days with AREG. Control was iPSCs colony number obtained with PBS. AREG Concentration: 200ng/mL

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(C) *In vitro* reprogramming efficiency for i4F Skin Fibroblasts (SF). Control was iPSCs colony number obtained with PBS. n=4 (2 independent experiments, 2 clones).

(D) Flow cytometry analysis of EGFR and phospho-EGFR (p-EGFR) protein expression in MEFs treated with either PBS or AREG.

(E) Dox withdrawal assay. OSKM was induced for the indicated time period after which dox was replaced with KSR medium until analysis at day 12. In the meantime, cells were treated with PBS or AREG for 3 days.

(F) mRNA levels of genes indicated during reprogramming in i4F MEFs treated with PBS or AREG. Control was D0. mean  $\pm$  SEM

(G) mRNA level of amphiregulin in whole muscle extract at different timepoints. Control was uninjured muscle. n>5. n = one TA per mouse

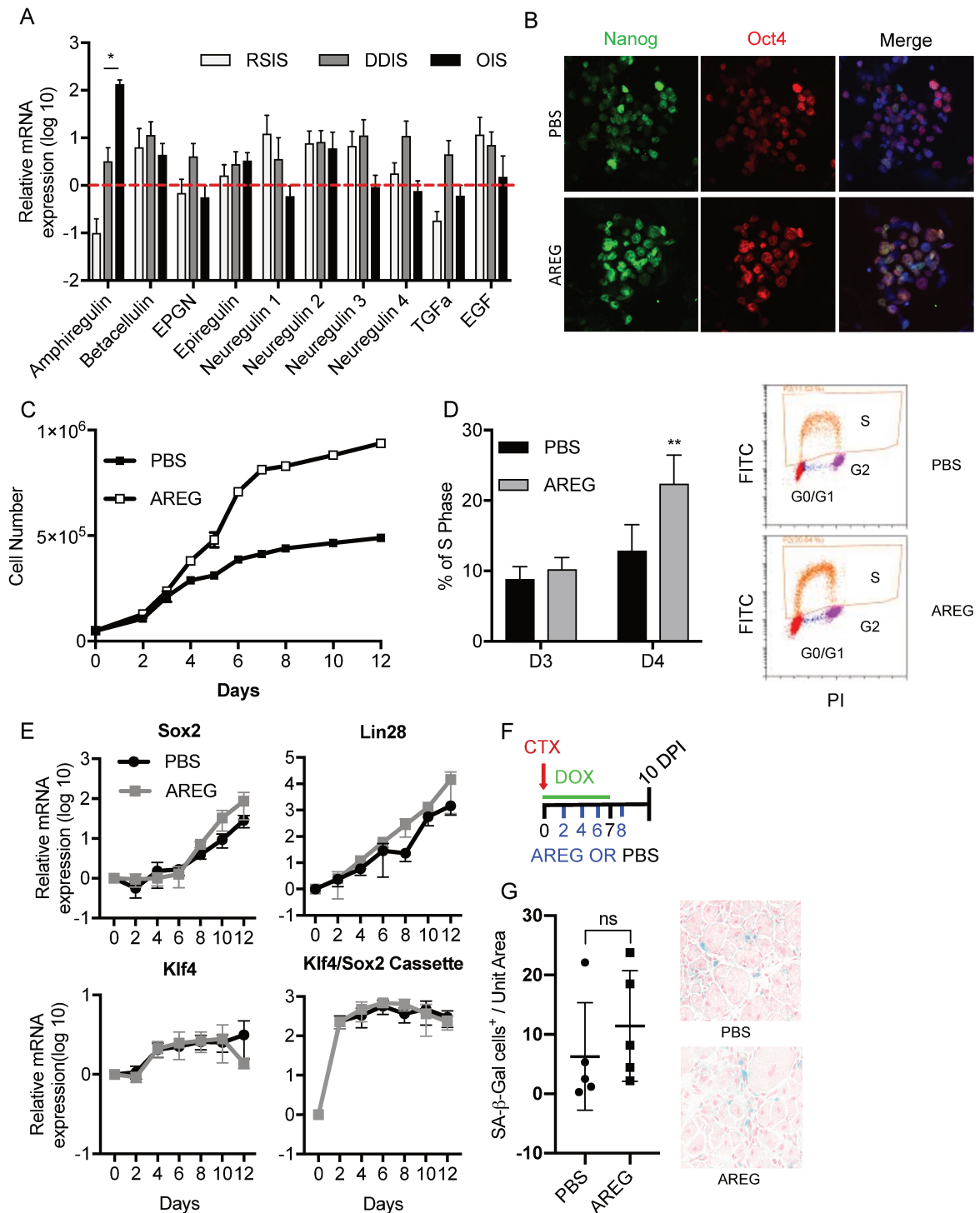
(H) Quantification of Nanog<sup>+</sup> cells in injured TA treated with PBS or AREG (left panel). Representative pictures of Nanog staining in injured TA treated with PBS or AREG (right panel). n=5 TAs.

\*p< 0.05, \*\*p< 0.01, \*\*\*p< 0.001. Mann-Whitney U test, n= 6 (2 independent experiments, 3 clones). mean  $\pm$  SD if not previously specified.

Supporting these results, Zeb1 and Zeb2, two markers of the mesenchymal states that inhibit epithelial genes were more strongly repressed in MEFs treated with AREG. Interestingly, AREG did not affect expression of Sox2 or Klf4 or Lin28 suggesting its potential effect was restricted to Oct4 and Nanog expression (Figure 4F & S4D). Finally, we tested expression of the cassette to confirm OSKM expression was not affected by AREG treatment (Figure S4D) (we used a pair of primer located at the junction between Sox2 and Klf4). Given its beneficial impact on *in vitro* reprogramming, we speculated that AREG may enhance *in vivo* reprogramming. We analyzed whole muscle extracts at different time points for the level of AREG mRNA. We found a significant increase of AREG mRNA level following muscle injury (Figure 4G). To analyze whether AREG may promote reprogramming, we then injected AREG upon injury and quantified muscle section for Nanog<sup>+</sup> cells (Figure S4E). Administration of AREG following CTX injury and DOX treatment significantly number of Nanog<sup>+</sup> cells (Figure 4H), whereas there was no significant change in the level of SA- $\beta$  Gal<sup>+</sup> cells (Figure S4F). Therefore, these data suggest that AREG facilitates both *in vitro* and *in vivo* reprogramming.

Taken together, these data indicate that additional SASP factors favors *in vivo* reprogramming besides IL-6. Moreover, it suggests that EGFR pathway also plays a role in cellular reprogramming and facilitates iPSCs generation.

## RESULTS



**Figure S4**

(A) mRNA levels of genes indicated in RSIS, DDIS and OIS compared to NS or Ctrl. n=4. mean  $\pm$  SEM.

(B) Immunofluorescence staining of Nanog and Oct4 in iPS cells generated with AREG treatment or PBS.

(C) Quantification of percentage of cell in S-phase by FACS analysis (left panel). FACS plot of cell-cycle analysis.

(D) mRNA levels of genes indicated during reprogramming in i4F MEFs treated with PBS or AREG. Control was D0. mean  $\pm$  SEM

(E) Scheme of the experiments. AREG or PBS was injected intraperitoneally at day 2, 4, 6 and 8 following muscle injury.

(F) Quantification of SA- $\beta$  Gal<sup>+</sup> cells in injured TA treated with PBS or AREG (left panel). Representative pictures of SA- $\beta$  Gal staining in injured TA treated with PBS or AREG (right panel). n=5 TAs.

## RESULTS

\* $p < 0.05$ , \*\* $p < 0.01$ . Mann-Whitney U test,  $n = 6$  (2 independent experiments, 3 clones). mean  $\pm$  SD if not previously specified.

### 2.2.5 Summary

- Senescence promotes *in vivo* reprogramming independently of IL-6
- SASP impact on senescence seems to be restricted to early phase of reprogramming and seems through soluble factors and not exosomes.
- AREG secretion promotes *in vitro* and *vivo* reprogramming

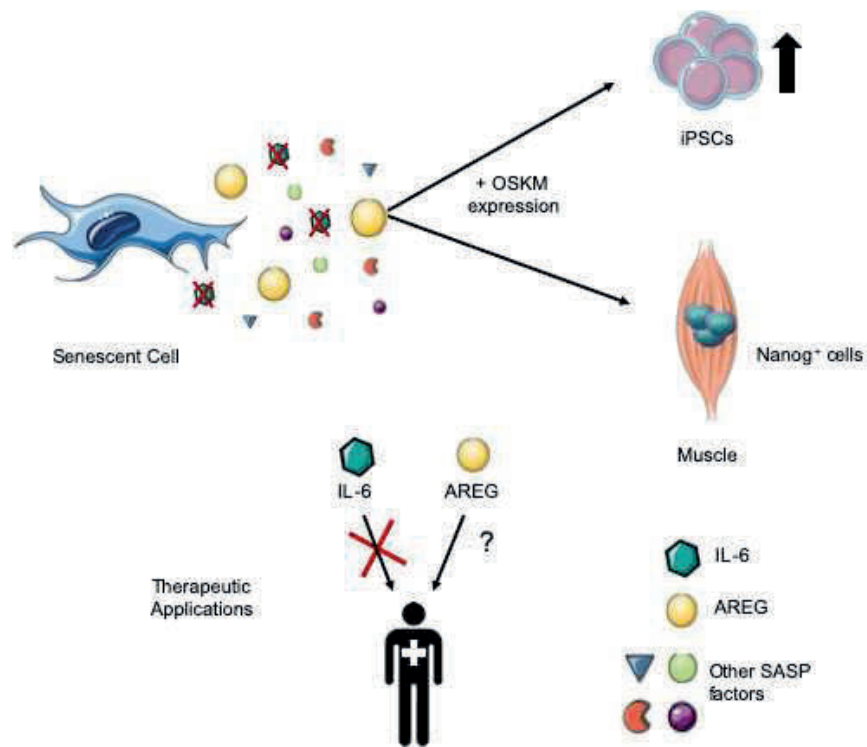


Figure 5. AREG secreted by senescent cells promotes cellular reprogramming.

## DISCUSSION





## 1. SENESCENCE REGULATES *IN VIVO* REPROGRAMMING

Cellular senescence is a biological response to a variety of stresses, which leads to permanent cell cycle arrest and phenotypic alterations, notably SASP acquisition, which mediates several of the main effects of senescence. One of the main roles of senescence is to inhibit cell growth to avoid transformation of pre-malignant cells into malignant cells. In addition, the accumulation of senescence contributes to various age-related diseases. Recently, beneficial roles of senescence in tissue repair and wound healing have been reported, highlighting the complexity of cellular senescence. Upon injury, presence of senescent cells is transient and is important for proper wound healing and tissue remodeling (Demaria et al., 2014; Jun and Lau, 2010; Krizhanovsky et al., 2008). Moreover, increased *in vivo* reprogramming efficiency has been observed in multiple tissues upon injury, including liver and pancreas (Heinrich et al., 2015). Several studies reported cell identity conversion following tissue injury (Arthur-Farraj et al., 2012; Davis et al., 2012; Thorel et al., 2010; Yanger et al., 2013). For instance, conversion of  $\alpha$  cells to  $\beta$  cells occurs in the pancreas following extreme loss of  $\beta$  cells (Thorel et al., 2010). In this context, our data suggest that senescence may contribute to cellular plasticity induction following tissue damage. Consistent to our findings, similar observations have been reported in the lung (Mosteiro et al., 2016).

Next, we observed aged i4F mice have shorter survive span compared to young i4F mice, suggesting faster reprogramming kinetic in aged mices. These results may seem surprising considering previous studies, which showed that senescence and aging negatively impact *in vitro* reprogramming, notably due to the increase of p16<sup>INK4A</sup> and p19<sup>ARF</sup> expression (Banito et al., 2009; Li et al., 2009a). We speculate that the accumulation of senescent cells during ageing might create a pro-reprogramming microenvironment. Therefore, we propose that senescence impairs reprogramming in a cell intrinsic manner by inhibiting proliferation whereas it promotes *in vivo* reprogramming in a cell extrinsic manner through SASP.

We decided to focus on SASP given its importance in senescence program. Therefore, we explored the effect of the SASP on reprogramming to understand the impact of senescence on cellular plasticity in the context of tissue regeneration. We isolated SCs and FAPs *in vitro* to confirm that SASP mediated effect of senescent cells. We focused on IL-6 as it is one of the key SASP members (Acosta et al., 2013; Kuilman et al., 2008), has been previously shown to contribute to muscle regeneration (Munoz-Canoves et al., 2013)

and to promote iPSCs generation (Brady et al., 2013). In agreement with previous report (Brady et al., 2013), adding IL-6 enhanced reprogramming of SCs whereas IL-6 blockade abolished beneficial effect of SASP on reprogramming. Therefore, our results show that senescence promote cellular through the SASP.

### **2. SENESCENCE PROMOTES *IN VITRO* CELLULAR REPROGRAMMING VIA SASP**

Interestingly, short exposure (3 Days) to SEN CM at the onset of reprogramming was sufficient to increase reprogramming at similar levels as complete exposure (12 Days). Conversely, exposure to NS CM first and switch to SEN CM later did not increase reprogramming efficiency. Interestingly, IL-6 promotes reprogramming by promoting cell survival through the pro-survival gene Pim-1 (Brady et al., 2013). Next, exposure to “early” or “late” SASP did not lead to any alteration of SASP effect on reprogramming, suggesting that the SASP factors important for reprogramming are continuously secreted by senescent cells. It would be interesting to determine the common factors produced during early and late senescence to identify more potential plasticity regulators. Finally, SASP induced cell proliferation, suggesting it could play similar function as c-Myc (Mikkelsen et al., 2008). It would be interesting to test whether SASP may also increase the generation of iPSCs in the absence of c-Myc. Taken together, we propose that SASP impacts mainly the early phase of reprogramming, potentially by facilitating the erasing of the somatic cell identity, inducing cellular plasticity and boosting proliferation.

### **3. SASP PROMOTES REPROGRAMMING IN AN IL-6 INDEPENDENT MANNER**

Our results revealed that IL-6 is a critical factor of SASP to induce cellular plasticity in vivo. However, IL-6 is a pleiotropic factor, making it very difficult to use in the clinical application (Tanaka et al., 2014). Thus, identifying other factors which could have a similar effect as IL-6 but more specificity is necessary. Therefore, we set to identify SASP factors other than IL-6 that could enhance cellular plasticity. Surprisingly, we observed senescence induction in IL-6KO MEFs. We confirmed this observation by both SAbGal staining and qPCR. Moreover, mass spectrometry analysis identified numerous classical SASP factors in

the conditional medium collected from Sen IL-6KO MEFs. These findings were somewhat surprising in light of previous studies. It has been reported that IL-6 was critical to establish senescence in human diploid fibroblasts using multiple shRNA against human IL-6 (Kuilman et al., 2008). Indeed, this study demonstrated the role of IL-6 in senescence acquisition in paracrine, autocrine and intracrine fashion. IL-6KO model used in our studies is the first and most widely used IL-6KO mouse model (Kopf et al., 1994). Therefore, we decided to verify our knockout model. Surprisingly, we detected strong induction of IL-6 mRNA in IL-6KO upon OIS. Moreover, we found IL-6 expression by IHC on injured TA from WT and IL-6KO mice. However, the staining pattern was different between WT and IL-6KO TA (data not shown). We analyzed how the mouse model has been generated in 1994, and discovered that this KO mouse model has been generated by disrupting the second exon (thought to be the first exon), according to multiple Gene Database. Thus, IL-6 can be produced within the cell, but cannot be secreted in the extracellular environment, as no IL-6 was detected in serum of IL-6KO mice and their immune response is defective (Kopf et al., 1994). Of note, IL-6 cannot be detected in the CM of SEN IL-6KO by ELISA. More importantly, IL-6 blocking antibody failed to abolish the beneficial effect of SEN IL-6KO on reprogramming. Furthermore, IL-6 was not present in Mass Spectrometry analysis on IL-6KO CM. All of these data indicate IL-6 secretion is defective in IL-6KO. Consequently, our results suggest that senescence and reprogramming can be mediated in the absence of IL-6 in a cell non-autonomous fashion. We speculate that since IL-6 mRNA can still be detected in IL-6KO MEFs upon OIS, the cell autonomous effect of IL-6 on senescence establishment and maintenance is intact in IL-6KO MEFs. Therefore, we conclude there are other SASP factors that could promote reprogramming in a similar manner as IL-6.

Interestingly, we observed dysplasia in injured TA from IL-6KO; i4F mice, similarly to IL-6HET; i4F mice, suggesting that reprogramming could occur in IL-6KO. Recent study reported reduction of both senescence and reprogramming in the pancreas of i4F; IL-6KO mice (Mosteiro et al., 2018). Firstly, we cannot exclude that the different response is tissue dependent. Muscle could simply secrete more other SASP factors upon injury, such as TGF- $\beta$ , CCL2 or VEGF (Acosta et al., 2013) (Fripiat et al., 2001; Hubackova et al., 2012; Senturk et al., 2010). Secondly, the percentage of senescent cells induced by OSKM expression in the pancreas and CTX muscle damage may be different, which could also contribute to this difference. Indeed, we previously showed that number of Nanog<sup>+</sup> significantly correlates with number of senescent cells (Chiche et al., 2017). Thirdly, we only compared IL-6<sup>+KO</sup>;i4F with

IL-6KO; i4F. We cannot rule out the possibility that WT would behave differently as IL-6 IL-6<sup>+/-KO</sup>. Thirdly, dysplasia might not be as quantitative as Nanog staining. We are performing these experiments at the moment to clarify the skeletal muscle reprogramming efficiency in IL-6KO mice.

#### **4. EXOSOMES RELEASED BY SENESCENT CELLS DO NOT AFFECT CELLULAR REPROGRAMMING**

Consistent with previous findings (Borghesan et al., 2019; Lehmann et al., 2008), we observed the exosomes isolated from senescent cells could induce bystander senescence. However, the exosomes failed to enhance reprogramming, which suggests the paracrine impact of senescence on reprogramming is mediated by the soluble fraction. Noteworthy, we resuspended exosomes in its physiological concentration for reprogramming experiments and we had to concentrate exosomes (approximately 5 to 10-fold) to observe senescence induction as described (Borghesan et al., 2019), which might not reflect the physiological condition. Therefore, it is possible that highly concentrated exosome might also have an impact on reprogramming, which will be tested in the future.

Moreover, microvesicles derived from ESCs, which contains exosomes but also bigger vesicles formed from extracellular membrane, have been shown to induce epigenetics reprogramming of hematopoietic progenitors by enhancing expression of pluripotent genes Oct4 and Rex1 (Ratajczak et al., 2006). Microvesicles enhanced cell survival and expansion by transferring both proteins and mRNAs (Ratajczak et al., 2006). Finally, similar treatment with microvesicles for mesenchymal stem cells induced a protective effect on kidney against tissue injury (Bruno et al., 2009). Therefore, both reports indicate a potential role of microvesicles in cell plasticity and tissue regeneration. Thus, it would be interesting to test whether senescent cells secreted microvesicles besides exosomes could impact reprogramming.

#### **5. MASS SPECTROMETRY ANALYSIS OF SASP**

Recent studies indicate that cellular senescence is a multi-step process and SASP is a diverse phenotype depending on the cell type, the senescence inducer, and the time

(Hernandez-Segura et al., 2017; van Deursen, 2014). Therefore, detailed analysis of specific types of SASP is essential to further unravel the function of senescent cells. SASP is composed of various proteins, which only a few of them are currently under investigation. Indeed, analysis of specific SASP factors in reprogramming and tissue repair have been limited to few factors, such as Il-6 or PDGF-AA (Chiche et al., 2017; Demaria et al., 2014; Mosteiro et al., 2016). Mass Spectrometry provides an unbiased way to identify secreted proteins exclusive from senescent cells and quantify protein expression changes. Previous SASP proteomics analysis were carried out using human cells and mainly focused on identifying SASP factors in mediating senescence phenotypes, age-related diseases and hemostasis, more particularly on coagulation and thrombus formation (Basisty et al., 2019; Wiley et al., 2019; Acosta et al., 2013).

We carried out the proteomic analysis on secretome of mouse fibroblasts (OIS) to identify novel factors that could enhance reprogramming. Based on the proteomic profiles, we identified several potential factors which might impact cell plasticity, including AREG. Therefore, a comprehensive analysis of SASP is essential to identify novel factors and decipher pathways associated with cellular plasticity.

Currently, we are performing pathway and network analysis on the mass spectrometry data to understand how SASP members could promote cellular plasticity and to explore potential signaling pathways involved in this process, which might provide additional insights in modulation of plasticity *in vivo* and how to harness *in vivo* reprogramming for therapeutic strategy.

## **6. AREG/EGFR PATHWAY PROMOTES *IN VITRO* CELLULAR REPROGRAMMING**

AREG is a low-affinity ligand of EGFR (Shoyab et al., 1988) (Jones et al., 1999) with specific functions compared to the remaining EGF family members, such as during mammary gland development and in lung morphogenesis (Luetke et al., 1999; Schuger et al., 1996). Interestingly, AREG has been previously linked to muscle repair (Burzyn et al., 2013). It has been shown that AREG secreted by T-reg cells could regulate the immune response to favors tissue regeneration. AREG has also been shown to contribute to tissue homeostasis and repair

upon lung inflammation (Monticelli et al., 2011). Therefore, we decided to focus on examining the potential effect of AREG on both *in vitro* and *in vivo* reprogramming. Of note, AREG has been shown to act through exosomes secretion (Higginbotham et al., 2011). However, our proteomic analysis did not detect AREG from the exosomes fraction, which might explain partially our observation of exosome effect on reprogramming. We showed that treatment with recombinant AREG improved iPSCs generation, while treatment with EGFR inhibitor abolished positive effect of AREG on iPSCs generation, suggesting the impact of AREG is EGFR-pathway dependent. Furthermore, the use of high concentration of EGFR inhibitor (Lap) completely abolished reprogramming (data not shown) possibly due to an inhibition of proliferation. Indeed, EGFR promotes cell growth which has been shown to be important for cell reprogramming (Nakagawa et al., 2008; Wernig et al., 2008). Interestingly, similar to conditional medium, 3 days of treatment with AREG was sufficient to significantly promotes reprogramming. Several data demonstrated that some factors were only beneficial during early phase of reprogramming, such as BMPs (Hayashi et al., 2016). Finally, we observed that treatment with AREG increases the kinetic of reprogramming. c-Myc has been previously shown to contribute to iPSC formation by increasing reprogramming kinetic (Nakagawa et al., 2008; Wernig et al., 2008). Thus, it would be relevant to test whether AREG further increase reprogramming kinetics with lack of c-Myc.

We observed a significant and specific up-regulation of both endogenous Oct4 and Nanog in AREG treated cells. Surprisingly, known role of EGFR pathway in pluripotency is largely limited to the maintenance of hESCs ((Wang et al., 2007)). It is currently unknown the downstream pathways that are stimulated by EGFR activation. Nowadays, data suggest that EGFR pathway mainly act on ESCs proliferation and self-renewal. EGFR signaling pathway has been shown to cooperate with Wnt pathway, notably in cancer and HSC development and homeostasis (Grainger et al., 2019), which has been shown previously to promote reprogramming (Ho et al., 2013; Marson et al., 2008; Zhang et al., 2014). Indeed, EGFR can activate the accumulation of b-catenin either through PI3K/Akt pathway or down-regulation of caveolin-1 in cancer cell to support cell growth and invasion (Hu and Li, 2010). In addition, Wnt signaling has been shown to promote Oct4 and Nanog expression (Faunes et al., 2013; Sato et al., 2004; Takao et al., 2007), which are also upregulated by AREG. Therefore, it is possible that EGFR might contribute to reprogramming via Wnt interaction. Thus, we will perform experiments to determine whether Wnt pathway is activated upon AREG treatment. However, other pathway susceptible to be activated by AREG, such as

STAT3, has been reported to act positively on reprogramming (Brady et al., 2013; Mai et al., 2018). Therefore, it is essential to determine whether AREG promotes through one of these pathways or an unknown one. This may help our understanding of new factors involved in physiological processes such as tissue remodeling or in pathologies such as cancer in which EGFR is involved.

Intriguingly, our observations seem to be contradictory with previous results regarding the effect of EGFR on reprogramming efficiency of MEFs ((Tran et al., 2015)). In the context of reprogramming in presence of Acid Ascorbic (AA) and 2i, disruption of EGFR by siRNA enhances reprogramming efficiency to the level equivalent AA+ 2i. It is noteworthy that these results are obtained in a culture condition (presence of Acid Ascorbic or 2i or both) different from ours, which might explain the difference. Moreover, siRNA and chemical inhibition are two different approaches. The first one completely abolishes presence of EGFR whereas the second one only block signaling activity. Finally, the impact of EGFR knockdown alone on reprogramming efficiency has not been tested. Therefore, EGFR function in reprogramming requires further investigation.

Finally, AREG is a particular EGFR ligand because of its low affinity for EGFR and the way it activates EGFR in a unique manner compared to classical EGFR ligands, potentially explaining differences observed (Shoyab et al., 1989). It would be interesting to test whether other low affinity ligands, such as EREG but also high affinity ligands, such as EGF, can reproduce beneficial effect of AREG observed on cellular reprogramming.

Intriguingly, our results suggest that AREG promotes MET transition by up-regulating activation of Occludin and E-Cadherin expression. AREG has been previously shown to down-regulate E-Cadherin in keratinocytes cells (Chung et al., 2005) and promote tumor invasion and cell motility, indicating promotion of EMT and acquisition of mesenchymal state (Busser et al., 2011; Higginbotham et al., 2011) rather than an epithelial state. Recent investigations showed that AREG secreted by senescent cells induces a gene expression change correlated with EMT in recipient pancreatic tumor cells (PC3) (Xu et al., 2019). However, AREG has also been found to be involved in MET in normal epithelial breast cell line (MCF10A). Results indicate that AREG promotes epithelial state through repression of Zeb1 by miR200c and miR205 (Fukuda et al., 2016), conversely to EGF which was shown to promote mesenchymal phenotype. In a similar fashion, our RT-qPCR analysis revealed a



stronger repression of Zeb1 and Zeb2 genes during reprogramming in treated cells, suggesting this mechanism could occur during reprogramming. Consequently, we suggest that AREG might promote reprogramming by repressing Zeb1 through the same mechanism. We will test whether miR200c and miR205 are upregulated in AREG treated MEFs. Finally, which pathways are induced upon binding of AREG remains to be elucidated. AREG has been shown to promote many different signaling cascades such as MAPK, PKz or PI3K/AKT. Deciphering which pathway is activated is essential to extend our understanding of how AREG promotes reprogramming.

### **7. AREG PROMOTES *IN VIVO* REPROGRAMMING**

Based on our *in vitro* data, we thus explored the potential effect of AREG on *in vivo* reprogramming. We observed that injection of recombinant AREG upon muscle damage and OSKM activation resulted in an increase in Nanog<sup>+</sup> cells. Thus, AREG seems to also promote cellular plasticity and *in vivo* reprogramming, which might contribute to its reported role in muscle regeneration (Burzyn et al., 2013). These results are consistent with recent observations indicating that AREG reprograms cancer cells and may induce a phenotype similar to cancer stem cells (Xu et al., 2019). EGFR signaling has been found to promote tissue repair, notably in Duchenne Muscular Dystrophy mouse model (Wang et al., 2019). It would be interesting to test whether injection of AREG favors muscle regeneration.

### **8. ADDITIONAL FACTORS POTENTIALLY INVOLVED IN REPROGRAMMING**

Interestingly, two classical SASP factors have been shown to be involved in pluripotency and reprogramming. BMP2 and BMP signaling pathways have been reported to promote cellular reprogramming (Chen et al., 2011a; Hayashi et al., 2016). Beneficial effect of BMPs effect is restricted to the early phase of reprogramming but molecular mechanisms enhancing reprogramming remain unclear. Recently, CCL2 has also been shown to promote pluripotency and reprogramming (Hasegawa et al., 2011). However, mechanistic insights underlying beneficial role of CCL2 also requires more investigations. In addition, both of these molecules have not been tested *in vivo*. Consequently, confirming their potential effect on reprogramming *in vivo* may further help to understand how SASP promotes cellular

plasticity. In addition to AREG, several others factors such as Mesothelin or Agrin, which have been detected in our mass spectrometry analysis, might also enhance reprogramming due to their role in regeneration, cell growth or cytokine regulation (Bassat et al., 2017; Bharadwaj et al., 2011; Chen et al., 2011a).

## 9. SUMMARY

My PhD project aims to understand the impact of cellular senescence on induced cellular plasticity in the context of reprogramming. At the beginning of my PhD, we demonstrated that senescence could promote cellular plasticity via SASP, and identified IL-6 as a major regulator of cellular plasticity *in vivo*. Next, I focused on elucidating SASP impact on *in vitro* reprogramming by performing proteomic analysis to identify factors that might promote reprogramming. I showed that AREG could facilitate reprogramming both *in vitro* and *in vivo* in the muscle. Together these studies helped to further understand how senescence contributes to cellular reprogramming and cellular plasticity. Further studies are warranted to elucidate the role of AREG in muscle regeneration, particularly on satellite cells and fibro/adipo progenitors. This would help to evaluate the potential of AREG as a therapeutic strategy in the context of muscle injury or genetic diseases affecting muscle integrity such as Duchenne Muscular Dystrophy.



## **MATERIALS & METHODS**



### 1. MOUSE MODEL

Animals were handled as per European Community guidelines and the ethics committee of the Institut Pasteur (CETEA) approved protocols. Both reprogrammable (JCW and JCO) mice were kindly provided by Manuel Serrano (Spanish National Cancer Research Centre, Madrid, Spain). IL-6KO mice were kindly provided by Gerard Eberl (Institut Pasteur).

To induce muscle injury, mice were anaesthetised with isoflurane. *Tibialis anterior* (TA) muscles were injured by injection 40  $\mu$ L of snake venom cardiotoxin (10 mM) (L8102, Lotaxan). Following surgery, mice were analgesic with 0.3 mg kg<sup>-1</sup> buprenorphine (Axience). In vivo reprogramming was induced by administration of Doxycycline at 1mg/mL (Sigma-Aldrich) in the drinking water supplemented with 7.5% of sucrose right after injury for 7 days. Experiments were performed indistinguishably with mice of both sexes and from 6 to 8 weeks of age. For AREG treatment, 7 $\mu$ g of recombinant AREG (989-AR, R&D Systems) was administered by intraperitoneal injection at days 2, 4, 6 and 8-post injury to each mouse. AREG was resuspended into PBS prior to injection or freeze in small aliquots.

### 2. CELL CULTURE CONDITIONS

#### 2.1 Mouse Embryonic Fibroblasts (MEFs)

##### 2.1.1 WT and IL-6KO

Primary mouse embryo fibroblasts (MEFs) were derived from wild-type C57BL/6 embryos at E13.5 following standard protocol. Embryos were chopped into small pieces in 1 ml of 0.1% Trypsin-EDTA (GIBCO) after removing the head and internal organs. Embryos were incubated at 37°C in a CO<sub>2</sub> incubator for 5 min for digestion and the suspension was collected and transferred to a 100mm tissue culture plate containing 10ml of DMEM +10% FBS and Pen/Strep. Fibroblasts were cultured for 2-3 days until reaching confluence and passed to a 150mm tissue culture plate. Upon confluency cells were frozen and considered as the first passage. Freezing media is composed of FBS with 5 %DMSO.

### 2.1.2 Reprogrammable MEFs

Reprogrammable MEFs (i4F MEFs) are obtained from either JCW or JCO mice. JCW or JCO are mice carrying the transcriptional activator (rtTA) within the ubiquitously-expressed *Rosa26* locus and a single copy of a lentiviral doxycycline-inducible polycistronic cassette encoding the four reprogramming factors *Oct4* (also known as *Pou5f1*), *Sox2*, *Klf4* and *c-Myc*. Mice used were either heterozygous or homozygous for both mutations. MEFs were cultured in Complete Medium. For cell reprogramming, MEFs are used at passage 1 or 2 as proliferation capacity is essential for proper reprogramming.

Complete Medium: DMEM+hiGlutaMAX + 10% FBS + 0,5% Pen/Strep.

## 2.2 Senescence Inductions methods

### 2.2.1 Senescent cells were generated from WT or IL-6KO MEFs by different methods

Oncogene Induced Senescence (OIS and Ctrl): MEFs were infected with hRAS viral particles or control vector particles during 2 days (see viral infection protocol below). Cells were selected with puromycin (gene resistance marker) for 4 days and seeded at proper density on tissue culture plates previously treated with gelatin 0,1% (10 min at RT) for further experiments.

DNA Damage Induced Senescence (DDIS): MEFs were trypsinized and collected into 15mL tubes. Cells were resuspended at  $5 \cdot 10^6$  cells/mL in fresh medium and subjected to  $20\gamma$  using an X-Ray Machine (Xstrahl). Then cells were seeded at proper density on tissue culture plates previously treated with gelatin 0,1% (10 min at RT). Media was changed next day then every 2 days.

Replicative Stress Induced Senescence (RSIS): MEFs were propagated every 3 days under 3% O<sub>2</sub> and 5% CO<sub>2</sub> (hypoxia conditions). Cells were seeded at 20% confluency and passed upon reaching 80-90% confluency. This protocol was performed until cells reached replicative crisis and they stopped growing. Then cells were seeded at proper density on tissue

culture plates previously treated with gelatin 0,1% for further experiments. Media was changed every 2 days.

### 2.3 Cell Reprogramming

JCW or JCO clones reprogramming efficiency was assessed prior any experiments and best clones were selected for further experiments. Reprogramming was induced by switching medium to KSR medium and by adding doxycycline (1 $\mu$ g/mL) (DOX). Medium was changed every 2 days until iPSCs colonies appeared. Reprogramming plates were stained for alkaline phosphatase activity and quantified either by eye or using ImageJ software. AP staining kit from Sigma Aldrich was used for staining and manufacturer's protocol was followed. iPSCs clones were picked, expanded and seeded at  $5 \times 10^4$  cells on coverslip coated with Poly-L-Lysine (Sigma) in 24-well plates for analyses. iPSCs were cultured in iPSCs medium described above.

#### 2.3.1 Co-Culture Experiments

Senescent MEFs were seeded at  $2 \cdot 10^5$  cells/well into 6-well tissue culture plates. Only heterozygous i4F MEFs were used. i4F MEFs were seeded on top of senescent MEFs at 20 000 cells/well into 6-well plates. iPSCs medium was changed every 2 days until iPSCs colonies appeared.

#### 2.3.2 Conditioned Medium Experiments

OIS MEFs were seeded at  $1,5 \cdot 10^6$  cells into p100mm tissue culture plates previously treated with 0,1% gelatin (10 min at RT). KSR medium (without LIF) was incubated with OIS MEFs for 48 hours and collected. Medium was centrifuged for 5 min at 500g and filtered using 0.2 $\mu$ m filter unit. Conditioned Medium was aliquoted and frozen at -20°C.

Only homozygous i4F MEFs were used. i4F MEFs were seeded at 5 000 cells/well into 6-well tissue culture plates. CM was used for reprogramming experiments and changed every 2 days until iPSCs colonies appeared.



### 2.3.3 *Amphiregulin Experiment*

JCW heterozygous (+/KI;+/Tg) MEFs were seeded at  $1.10^5$  cells/well into 6-well tissue culture plates. Following day, media was changed with KSR medium with DOX (1 $\mu$ g/mL) and Amphiregulin (200 ng/mL) (AREG) (R&D systems) to start reprogramming. Media was changed every 2 days. AREG treatment lasted 3 first days of reprogramming process. If needed Lapatinib (Tebu-Bio) was also added either at 1 $\mu$ M or 0.1 $\mu$ M.

KSR Medium: DMEM+hiGlutaMAX + 15% KSR +1% Non Essential Amino Acids + 1% Glutamax +  $\beta$  mercapto ethanol + 1% Pen/Strep and supplemented with fresh LIF (1000U/mL).

## 3. IMMUNOFLUORESCENCE

### 3.1 BrdU immunofluorescence staining

Cells were seeded on coverslip coated with Poly-L-Lysine (Sigma) in 24-well plates for analyses. Cells were incubated under different conditions for 72 hours then incubated for 1 hour with BrdU (10 $\mu$ M). Cells were washed 3 times with PBS and fixed in cold 70% EtOH (-20°C) for 20 minutes. Cells were washed with PBS and incubated with fresh 2N HCl for 20 min to denature DNA. Cells were washed 3 times with PBS and blocked into 3% BSA blocking solution for 30 min. After blocking cells were incubated with primary antibody for 2 hours at RT into humid chamber. Cells were washed with PBS three times prior incubation with secondary antibody for 45 min at RT. Finally, cells were mounted into DAPI Gold antifade mounting medium. Images were acquired with an Olympus IX83 microscope and quantified using ImageJ software.

### 3.2 Oct4 and Nanog immunofluorescence staining

$9 \times 10^3$  isolated iPSCs were seeded on poly-L-lysine coated glass coverslips covered by either MMC-treated senescent MEFs cultured in KSR medium. Cells were fixed with 4%

PFA, followed by permeabilization with a buffer containing 0.1% NaCitrate and 0.1% Triton X-100 in PBS. Slides were washed, blocked with 5% BSA for 45min at RT and incubated with respective primary antibodies overnight at 4°C. Second day, the slides were washed and stained with proper secondary antibodies for 45 min at RT. The following primary antibodies were used for immunofluorescence on fixed cells: anti-Oct3/4 (1/250, BD Biosciences, clone 40/Oct-3); anti-Nanog (1: 200, Cell Signaling, clone D2A3). Secondary antibodies: donkey anti-rabbit Dylight 488 and donkey anti-mouse Dylight 594 (1:500, ThermoFisher) were used. Coverslip were mounted using Prolong Antifade with DAPI mounting media. Images were acquired in an Olympus IX83 microscope.

### 3.3 SA-β GAL staining

#### 3.3.1 In vitro

Cells were washed with PBS twice and fixed for 15 minutes at RT in a solution containing: 2% v/v Formaldehyde; 0,2% v/v Glutaraldehyde in PBS. Cells were washed again and incubated overnight at 37°C with the following solution:

Citric acid/phosphatase buffer pH 6.0	200 mM
NaCl	5 M
K <sub>3</sub> Fe(CN) <sub>6</sub>	100 mM
K <sub>4</sub> Fe(CN) <sub>6</sub>	100 mM
MgCl <sub>2</sub>	1 M
X-Gal solution	20 mg/mL

The next morning, cells were washed with tap water three times and stored in PBS. Pictures were taken using an Axio Scan Z1.

#### 3.3.2 On skeletal muscle (TA)

TA muscles were isolated from mice and frozen directly in liquid nitrogen cooled isopentane for < 1 min and stored at 80°C. Cells were then cryosectioned in 10µm section.

Cells were fixed at room temperature for 4 min in a solution containing 1% paraformaldehyde and 0.2% glutaraldehyde in PBS. Sections were washed in PBS and incubated for 30 min in PBS pH = 5.5 and then incubated in an X-gal solution containing 4 mM  $K_3Fe(CN)_6$ , 4 mM  $K_4Fe(CN)_6$ , 2 mM  $MgCl_2$ , 0.02% NP-40 (Igepal) and 400  $\mu$ g/ml X-gal (15520-018, Sigma) in PBS pH = 5.5) at 37°C overnight. For sections, X-gal substrate was changed after 24h and stained for 48 hours in total. Samples were then washed in PBS three times and post-fixed in 1% PFA in PBS for 30 min. After washes, samples were mounted in PBS, 20% glycerol. Finally, the sections were scanned using Axio Scan Z1 or a Zeiss Observer Z1, and SA- $\beta$  GAL positive cells counted using ImageJ software.

### 3.4 H&E staining

TA muscles were isolated from mice and frozen directly in liquid nitrogen cooled isopentane for < 1 min and stored at 80°C or directly cryosectioned in 10  $\mu$ m sections. Tissue sections were dried at RT and fixed for 5 min in PFA 4%. Section were washed three times with PBS and stained for Hematoxylin for 2 to 5 min. Section were then washed three times with tap water and stained with Eosin for 2 to 5 min. Section were washed again three times with PBS. Finally, sections were dehydrated in EtOH 95% for 5 min then twice in EtOH 100%. Sections were mounted in Eukit.

### 3.5 Nanog IHC staining

Tissue sections were dried at RT and fixed for 5 min in PFA 4%. Sections were washed two times with PBS and once with PBS-T (0,3% Triton X-100). Sections were blocked with blocking buffer containing 0,2% BSA + 5%GNS + 0,3% Triton X-100 at RT for 1hr. Section were stained with primary antibody against Nanog (1/200) overnight at 4 degrees. The next morning section were rinsed with PBS-T twice and incubated with  $H_2O_2$  at RT for 15min. Sections were incubated with secondary antibody at RT for 1hr30min. Section were

finally washed with PBS-T three times and revealed with peroxidase. Slides were counterstained with Hematoxylin as mentioned above.

#### 4. QUANTITATIVE REAL-TIME PCR

Total RNA was extracted from cells using Trizol (Invitrogen) following provider's recommendations. RNA concentration was quantified using a Nanodrop and 1 $\mu$ g of RNA was used to generate cDNA. The cDNA kit used was High Capacity cDNA RT kit from Applied Biosystem (4368813). Protocol from the manufacturer was followed. cDNA was diluted 1/10. Quantitative real-time PCR was performed using LightCycler 480 (Roche) and SYBR Green Master Mix (Roche) with the standard protocol and corresponding primers. All values were obtained at least in duplicate for each samples, and in a total of at least two independent assays. Calculation for the values was made using the  $\Delta\Delta C_t$  method, as previously described (Yuan et al., 2006).

#### 5. EXOSOMES ISOLATION

Conditioned Medium incubated either with OIS or Ctrl MEFs were collected, centrifuged 10 min at 500g, then filtered through 0,2 $\mu$ m filter unit. Filtered CM was ultracentrifuged a first time at 12 000g for 30 min at 4°C. CM was kept and pellet removed. CM were then centrifuged again for 3 hours at 100 000g. CM was conserved and defined as Supernatant (SN) and was depleted of exosomes. Pellet was resuspended into PBS and ultracentrifuged again for 3 hours at 100 000g. Purified exosomes pellets were collected and resuspended into proper iPSCs conditioned Medium volume for further experiments. Samples were ultracentrifuged using an Ultracentrifuge Optima XPN-80 Ultracentrifuge from Beckman Coulter equipped with a rotor SW32Ti.

## 6. MASS SPECTROMETRY

OIS MEFs or Ctrl cells were generated as mentioned previously. Cells were seeded at density mentioned above for CM experiments. Briefly, cells were washed with DMEM twice before incubation with DMEM (without FBS and Pen/Strep) for 48 hours at 37°C under normal conditions. Conditioned medium (CM) were collected, centrifuged 10 min at 500g, filtered through 0,2µm filter unit then ultracentrifuged a first time at 12 000g for 30 min at 4°C. CM was kept and pellets (containing microvesicles) were removed. CM were then centrifuged again for 3 hours at 100 000g. CM was kept and frozen for further analysis in collaboration with Mass Spectrometry platform in Institut Pasteur. Pellets, which contain exosomes, were resuspended in PBS then ultracentrifuged again for 3 hours at 100 000g. PBS was removed as much as possible and pellets were resuspended in fresh PBS and frozen for further analysis. This procedure has been repeated six independent times.

### 6.1 Sample preparation

For each secretome, 2 technical replicates were processed. proteins from the secretome were precipitated 1h at 4°C with TCA (20% final concentration) and centrifugated 15min / 16.000g / 4°C. Then, protein pellets were washed twice with ice-cold acetone, and resuspended in Urea 8M / NH<sub>4</sub>HCO<sub>3</sub> 100mM denaturation buffer. Cysteine bonds were reduced with 50mM TCEP (#646547, Sigma-Aldrich, USA) for 1h and were alkylated with 50mM iodoacetamide (#I114, Sigma-Aldrich, USA) for 1h at room temperature in the dark. Samples were digested with Sequencing Grade Modified Trypsin (#V5111, Promega, France) ratio 40:1 (protein:trypsin) overnight at 37°C after a dilution in NH<sub>4</sub>HCO<sub>3</sub> 100mM to decrease the urea under 1M. The digestion was stop with 4% formic acid (FA) (#94318, Fluka) and digested peptides were purified with Sep-Pak C18 50mg sorbent (#WAT054955, Waters, USA). Peptides were finally eluted with 80% Acetonitrile (ACN) / 0.1% FA. Resulting peptides were dried and resuspended in 2% Acetonitrile / 0.1% FA.

## 6.2 LC-MS/MS analysis

The acquisitions were performed on a Q Exactive™ Plus Mass Spectrometer (Thermo Fisher Scientific, USA) coupled with a Proxeon EASY-nLC 1200 (Thermo Fisher Scientific, USA). 1 µg of peptides were injected onto a home-made 55 cm C18 column (1.9 µm particles, 100 Å pore size, ReproSil-Pur Basic C18, Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) and eluted with a multi-step gradient, using buffer A (0.1% FA) and buffer B (80% ACN), from 2 to 7% buffer B in 5min, 7% to 23% buffer B in 70min, 23 to 45% buffer B in 30min and 45 to 95% buffer B in 5min, at a flow rate of 250 nL/min over 132 min. Column temperature was set to 60°C. MS data were acquired using Xcalibur software using a data-dependent method. MS scans were acquired at a resolution of 70,000 and MS/MS scans (fixed first mass 100 m/z) at a resolution of 17,500. The AGC target and maximum injection time for the survey scans and the MS/MS scans were set to 3E6, 20ms and 1E6, 60ms respectively. An automatic selection of the 10 most intense precursor ions was activated (Top 10) with a 45s dynamic exclusion. The isolation window was set to 1.6 m/z and normalized collision energy fixed to 28 for HCD fragmentation. We used an underfill ratio of 1.00E<sup>4</sup> for an intensity threshold of 1.7E<sup>5</sup>. Unassigned precursor ion charge states as well as 1, 7, 8 and >8 charged states were rejected and peptide match was disable.

## 6.3 Bioinformatic analysis of LC-MS/MS data

Raw data were analysed using MaxQuant software version 1.5.5.1 (Cox and Mann, 2008) using the Andromeda search engine (Cox et al., 2011). The MS/MS spectra were searched against the mus musculus SwissProt database (53,449 entries from UniProt the 24/07/2018). Variable modifications (methionine oxidation, N-terminal acetylation) and fixed modification (cysteine carbamidomethylation) were set for the search and trypsin with a maximum of two missed cleavages was chosen for searching. The minimum peptide length was set to 7 amino acids and the false discovery rate (FDR) for peptide and protein identification was set to 0.01. At least a unique peptide per protein group was required for the identification of protein. The main search peptide tolerance was set to 4.5 ppm and to 20 ppm for the MS/MS match tolerance. Second peptides were enabled to identify co-fragmentation events and match between runs option for biological replicates of a same condition was selected with a match time window of 0.7 min for an alignment time window of 20 min.

Quantification was performed using the XIC-based LFQ algorithm with the Fast LFQ mode as previously described (Cox et al., 2014). Unique and razor peptides, including modified peptides, with at least 2 ratio counts were accepted for quantification.

## 6.4 Statistical Analysis

For the differential analyses, proteins identified in the reverse and contaminant databases and proteins “only identified by site” were first discarded from the list of identified proteins. Then, only proteins with 6 quantified intensity values in a condition are kept. After log<sub>2</sub> transformation, LFQ values were normalized by median centering within conditions (normalized function of the R package DAPAR) (Wieczorek et al., 2017). Remaining proteins without any LFQ value in one of both conditions have been considered as proteins quantitatively present in a condition and absent in another. They have therefore been set aside and considered as differentially abundant proteins. Next, missing values were imputed using the `imp.norm` function of the R package `norm` (Novo, 2013). Proteins with a fold-change under 2.0 have been considered not significantly differentially abundant. Statistical testing of the remaining proteins (having a fold-change over 2.0) was conducted using a `limma` t-test (Smyth, 2005) thanks to the R package `limma` (Ritchie et al., 2015). An adaptive Benjamini-Hochberg procedure was applied on the resulting p-values thanks to the function `adjust.p` of R package `cp4p` (Giai Gianetto et al., 2016) using the robust method of (Pounds and Cheng, 2006) to estimate the proportion of true null hypotheses among the set of statistical tests. The proteins associated to an adjusted p-value inferior to an FDR of 1% have been considered as significantly differentially abundant proteins. Finally, the proteins of interest are therefore those which emerge from this statistical analysis supplemented by those which are considered to be present from one condition and absent in another.

## 7. FLOW CYTOMETRY

### 7.1 Cell cycle analysis

MEFs were incubated under different conditions. Briefly cells were incubated with BrdU (10 $\mu$ M) for 1h30 min then washed three times with PBS. Cells were trypsinized, collected into complete medium before centrifugation at 250g for 5 min. Cells were washed in PBS, centrifuged again and fixed in cold 70% EtOH. Cells were kept at -20°C until analysis. The day of analysis, fixed cells were centrifuged at 350g for 5 minutes. Cells were washed with PBS and incubated with fresh 2N HCl for 20 min at RT to denature DNA. Cells were washed and centrifuged three times with PBS and incubated with FITC BrdU antibody (BD Pharmingen) for 45 min at RT. Cells were washed with PBS, centrifuged, resuspended in PBS containing Rnase A (100 $\mu$ g/mL) (Qiagen) and Propidium Iodide (50 $\mu$ g/mL) (Sigma Aldrich). Samples were analyzed at least 2 hours after using a Cytoflex Flow Cytometer (Beckman Coulter).

### 7.2 EGFR staining

Cells were incubated for 10min with different conditions then washed with cold PBS, trypsinized and collected in complete medium. MEFs were then centrifuged and washed with cold PBS. Finally, cells were fixed overnight in cold 90% MetOH (-20°C). The next day, cells were washed and incubated with either EGFR (D38B1, Cell Signaling) or p-EGR antibody (D7A5, Cell Signaling) for 45minutes at RT. Cells were washed and stained with a secondary FITC antibody (Jackson Immuno Research 711-545-152, 1:800) for 30 min at RT. Cells were then analyzed using a Cytoflex Flow Cytometer (Beckman Coulter).



### 8. ELISA

ELISA kit BMS603-2 from Invitrogen against murine IL6 was used. Manufacturer's instructions were followed. Plate Reader Mithras LB940 from Berthold Bio was used for absorbance measurements.

### 9. VIRAL INFECTION

$5 \cdot 10^6$  293T HEK cells were seeded on p100 tissue culture plates in complete medium. On the same day following solutions were prepared: 1/  $4 \mu\text{g}$  of plasmid pCL-ECO +  $4 \mu\text{g}$  of plasmid coding for the gene of interest in  $400 \mu\text{l}$  of DMEM and 2/  $400 \mu\text{l}$  of DMEM + PEI ( $40 \mu\text{g}/\mu\text{g}$  of plasmid). Both solutions were mixed and incubated at RT for 10min. Mix was added dropwise on 293T HEK cells. Following day, media was replaced with fresh complete medium. Four rounds of infection are done, sequentially, during the next 48 hours. Next morning, first round of infection was performed. Viral medium was collected from 293T HEK cells and fresh medium was added. Viral Medium was centrifuged for 5min at 250g and filtered through  $0,45 \mu\text{m}$  filter unit. Finally, polybrene was added ( $8 \mu\text{g}/\text{mL}$ ) and medium was used on MEFs. This second round was repeated on the evening (10-12 hours after first infection). Third and fourth rounds were performed on the next day. Last day complete medium was replaced with fresh medium containing selection marker (Puromycin  $2 \mu\text{g}/\text{mL}$ ). Selection was performed for 4 days. Eventually MEFs were trypsinized, counted and seeded at proper density.

### 10. QUANTIFICATION AND STATISTICAL ANALYSIS

The numbers of independent experimental replications, the definition of center and precision measures are reported in the figure legends ( $n$ , mean  $\pm$  sem or  $n$ , mean  $\pm$  SD). Statistical analyses were performed using GraphPad Prism v7 software. Statistical significance was assessed by Mann Whitney U test, Wilcoxon or two ANOVA Test depending on experiments.  $P$ -value  $< 0.05$  was considered statistically significant.

## 11. REAGENTS AND PRIMERS USED

Reagents:	Reference	Source
DMEM+hiGLUTAMAX	31966-021	Gibco
FBS	10270-106	Gibco
KSR	10828-028	Gibco
GlutaMAX 100x	35050-061	Gibco
Non Essential Amino Acids 100x	M7145	Sigma Aldrich
$\beta$ mercapto ethanol:	31350-040	Gibco
Penicillin/Streptomycin 100x	15140-122	Gibco
Leukemia Inhibitory Factor (LIF)	130-095	Miltenyl Biotec
Doxycycline	9891	Sigma Aldrich
Amphiregulin	989-AR-100	R&D Systems
Lapatinib ditosylate	T0078	Tebu-Bio
Polybrene	TR-1003	Sigma Aldrich
Puromycin	P8833	Sigma Aldrich
Poly-L-lysine	P4707	Sigma Aldrich
BSA	A3608	Sigma Aldrich
Alkaline Phosphatase Kit	AB0300	Sigma Aldrich
Propodium Iodide	P4864	Sigma Aldrich
Glutaraldehyde	15090-046	Gibco
X-Gal	B4252	Sigma Aldrich
Glutaraldehyde	111-30-8	Sigma Aldrich
Paraformaldehyde	50-980-487	Electron microscopy science
NaCitrate :	18996-35-5	Sigma Aldrich

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Sodium Citrate monobasic bioextra, anhy	18996-35-5	Sigma Aldrich
Triton	93443	Sigma Aldrich
Bovine Serum Albumin	A3608	Sigma Aldrich
MgCl <sub>2</sub>	7786-30-3	Sigma Aldrich
Hematoxylin solution mayer's	MHS16	Sigma Aldrich
Eosin	380159EOF	Leica
High-Capacity cDNA RT Kit	4368813	Applied Biosystems
Trizol Reagent	15596026	Invitrogen
Mounting Media Gold Anti Fade DAPI	P36941	Invitrogen
Eukitt	25608-33-7	Sigma Aldrich
Snake venom cardiotoxin	Cat#L8102	Lotaxan
Phosphatase alkaline	Cat#AB0300	Sigma Aldrich
LightCycler 480 SYBR Green I Master	Cat#4309155	Roche

<b>Antibody:</b>		
Brdu	51-33284X	BD Pharmingen
EGFR	#4267 clone D38B1	Cell Signaling Technology
pEFGR	#3777 clone D7A5	Cell Signaling Technology
Oct3/4	clone 40/Oct-3	BD Biosciences
Nanog (Immunofluorescence)	clone D2A3	Cell Signaling Technology
Nanog (Immunohistochemistry)	clone (home made on request)	Cambridge Research Biochemicals
Anti-IL-6	MP5-20F3	eBiosciences
Rabbit FITC	711-545-152	Jackson Immuno Research

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<b>Materials:</b>		
Ultracentrifuge Tubes		Beckman Coulter
ELISA IL-6 KIT		Invitrogen
Plate Reader	Mithras LB940	Berthold Bio
Microscope	CKX41	Olympus
Microscope	IX83	Olympus
Scanner (Slide)	Axio Scan Z1 or Observer Z1	Zeiss
FACS	Cytoflex	Beckman Coulter
Rt-qPCR analyzer	Light Cycler 480	Roche

<b>Cell line:</b>		
Mouse: i4F-A		<a href="#">Abad et al., 2013</a>
Mouse: i4F-B		<a href="#">Abad et al., 2013</a>

<b>Software and Algorithms:</b>		
ImageJ software		<a href="https://imagej.nih.gov/ij/download.html">https://imagej.nih.gov/ij/download.html</a>
FlowJo software		<a href="https://www.flowjo.com">https://www.flowjo.com</a>
Graphpad – Prism software		<a href="http://www.graphpad.com">http://www.graphpad.com</a>

<b>Primers Genotyping</b>	5'- to -3'	
i4F-A		
Neto F	GCGTCAGGCAATTTATACTCTGG	Abad et al., 2013
Neto R	TTGGTGTGGGAACACAGTCC	Abad et al., 2013
OSKM R	GCACCATCCAAAGGTCAGTG	Abad et al., 2013

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i4F-B		
Pparg F	CAGCATCAAATGGCTCGGTA	Abad et al., 2013
Pparg R	CCCCATGTCCAATCCCTAGTACTAA	Abad et al., 2013
OSKM R	GCACCATCCAAAGGTCAGTG	Abad et al., 2013
rtTA		
rtTA 1	AAAGTCGCTCTGAGTTGTTAT	Abad et al., 2013
rtTA 2	GCGAAGAGTTTGTCTCAACC	Abad et al., 2013
rtTA 3	GGAGCGGGAGAAATGGATATG	Abad et al., 2013
IL6		
Il6 Common	TTC CAT CCA GTT GCC TTC TTG G	Charles River
Il6 WT Rev	TTC TCA TTT CCA CGA TTT CCC AG	Charles River
Il6 mutant Rev	CCG GAG AAC CTG CGT GCA ATC C	Charles River

<b>Primers qPCR</b>	5'- to -3'	
En-mOct4 F	TCTTTCCACCAGGCCCCCGGCTC	Abad et al., 2013
En-mOct4 R	TGCGGGCGGACATGGGGAGATCC	Abad et al., 2013
En-Sox2 F	TAGAGCTAGACTCCGGGCGATGA	Abad et al., 2013
En-Sox2 R	TTGCCTTAAACAAGACCACGAAA	Abad et al., 2013
En-Klf4 F	GCGAACTCACACAGGCGAGAAACC	Abad et al., 2013
En-Klf4 R	TCGCTTCCTCTTCCTCCGACACA	Abad et al., 2013
Nanog F	AGGGTCTGCTACTGAGATGCTCTG	Abad et al., 2013
Nanog R	CAACCACTGGTTTTTCTGCCACCG	Abad et al., 2013
mLin28a F	GAAGAACATGCAGAAGCGAAGA	Li et al. 2009
mLin28a R	CCGCAGTTGTAGCACCTGTCT	Li et al. 2009
Klf4/Sox2 cassette F	ACTGCCCCTGTGCGACAT	Chiche et al. 2017

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Klf4/Sox2 cassette R	CATGTCAGACTCGCCAGGTG	Chiche et al. 2017
mGAPDH F	TTCACCACCATGGAGAAGGC	Li et al. 2009
mGAPDH R	CCCTTTTGGCTCCACCCT	Li et al. 2009
Occludin F	TGAAAGTCCACCTCCTTACAGA	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
Occludin R	CCGGATAAAAAGAGTACGCTGG	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
Cdh1 (E-Cadherin)-F	CAG CCT TCT TTT CGG AAG ACT	Li et al. 2009
Cdh1 (E-Cadherin)-R	GGT AGA CAG CTC CCT ATG ACT G	Li et al. 2009
Zeb1 F	ACCGCCGTCATTTATCCTGAG	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
Zeb1 R	CATCTGGTGTTCGGTTTTTCATCA	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
Zeb2 F	CCACGCAGTGAGCATCGAA	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
Zeb2 R	CAGGTGGCAGGTCATTTTCTT	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
Amphiregulin F	GGTCTTAGGCTCAGGCCATTA	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
Amphiregulin R	CGCTTATGGTGGAAACCTCTC	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
Betacellulin F	AATTCTCCACTGTGTGGTAGCA	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
Betacellulin R	GGTTTTCACTTCTGTCTAGGGG	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
EGFR F	GCCATCTGGGCCAAAGATACC	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
EGFR R	GTCTTCGCATGAATAGGCCAAT	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
Epiregulin F	CTGCCTCTTGGGTCTTGACG	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
Epiregulin R	GCGGTACAGTTATCCTCGGATTC	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
EPGN F	GGGGTCTGATAGCAGTCTG	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
EPGN R	TCGGTGTGTTAAATGTCCAGTT	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
HBEGF F	CGGGGAGTGCAGATACCTG	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
HBEGF R	TTCTCCACTGGTAGAGTCAGC	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
Neuregulin F	ATGGAGATTTATCCCCAGACA	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
Neuregulin R	GTTGAGGCACCTCTGAGAC	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>

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Neuregulin 2 F	TCGACCCTAACGGCAAAAACA	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
Neuregulin 2 R	AACCAGCGATAGGAGGGCT	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
Neuregulin 3 F	TTACGCTGTAGCGACTGCATC	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
Neuregulin 3 R	GCCTACCACGATCCATTTAAGC	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
Neuregulin 4 F	CACGCTGCGAAGAGGTTTTTC	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
Neuregulin 4 R	CGCGATGGTAAGAGTGAGGA	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
TGFa F	CACTCTGGGTACGTGGGTG	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
TGFa R	CACAGGTGATAATGAGGACAGC	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
TGFb F	CACTCTGGGTACGTGGGTG	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
TGFb R	CACAGGTGATAATGAGGACAGC	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
p16 INK4A F	CGTACCCCGATTCAGGTGAT	Li et al. 2009
p16 INK4A R	TTGAGCAGAAGAGCTGCTACGT	Li et al. 2009
ARF F	GCCGCACCGGAATCCT	Li et al. 2009
ARF R	TTGAGCAGAAGAGCTGCTACGT	Li et al. 2009
P21 CIP F	GTGGGTCTGACTCCAGCCC	Li et al. 2009
P21 CIP R	CCTTCTCGTGAGACGCTTAC	Li et al. 2009
Angpt2 F	CCTCGACTACGACTCAGT	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
Angpt2 R	TCTGCACCACATTCTGTTGGA	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
bFGF F	GCGACCCACACGTCAAATA	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
bFGF R	TCCCTTGATAGACACAACCTCCTC	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
CNTF F	TCTGTAGCCGCTCTATCTGG	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
CNTF R	GGTACACCATCCACTGAGTCAA	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
DRAXIN F	CCCACGCTGTTCTGATCC	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
DRAXIN R	GCTTGGTAGCAGTGACCACA	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
HGF F	ATGTGGGGGACCAAACCTTCTG	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>

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HGF R	GGATGGCGACATGAAGCAG	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
mIL-6 F	CTGCAAGAGACTTCCATCCAG	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
mIL-6 R	AGTGGTATAGACAGGTCTGTTGG	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
KITLG F	GAATCTCCGAAGAGGCCAGAA	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
KITLG R	GCTGCAACAGGGGGTAACAT	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
MMP3 F	CCTGATGTTGGTGGCTCA	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
MMP3 R	TCCTGTAGGTGATGTGGGATTTC	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
MMP13 F	ACTTCTACCCATTTGATGGACCT T	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
MMP13 R	AAGCTCATGGGCAGCAACA	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
PODXL F	GCCACCAAAGTGCCACAAC	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
PODXL R	CGGCATAGATGGAGATTGGGTT	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
ADAM19 F	TCAGTGGCGGACTTCAGAAAG	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
ADAM19 R	GCAAAAAGGTGCTCGTTCTTC	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
ADAM8 F	TTGCCCATGTGAAACAGTATG	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
ADAM8 R	AGGTGCAGGGTGAAAACGTG	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
Akt1s1 F	CTGCTCCTAGTCCACCACCT	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
Akt1s1 R	AGAGACCTCCATTATCGCTACC	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
BMP2 F	GGGACCCGCTGTCTTCTAGT	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
BMP2 R	TCAACTCAAATTCGCTGAGGAC	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
CCL8 F	TCTACGCAGTGCTTCTTTGCC	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
CCL8 R	AAGGGGGATCTTCAGCTTTAGTA	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
Col6a2 F	AAGGCCCATTTGGATTCCC	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
Col6a2 R	CTCCCTTCGACCATCCGAT	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
Ctnnb1 F	ATGGAGCCGGACAGAAAAGC	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
Ctnnb1 R	CTTGCCACTCAGGGAAGGA	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>



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CXCL1 F	CTGGGATTCACCTCAAGAACATC	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
CXCL1 R	CAGGGTCAAGGCAAGCCTC	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
Dchs1 F	AGATCGACGAGGAACAACCAG	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
Dchs1 R	CGAGCTGTACGGACCACTC	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
Eda 2 R	GCCTTCTGGACCCGATTGA	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
Eda2 F	CACACTGCATAGTCTGCCCTC	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
Flrt3 F	CCTCATCGGGACTAAAATTGGG	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
Flrt3 R	GCAAGTTCTTCAAATCGGAAGGA	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
Flt4 F	CTGGCAAATGGTTACTCCATGA	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
Flt4 R	ACAACCCGTGTGTCTTCACTG	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
Frzb F	CACAGCACCCAGGCTAACG	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
Frzb R	TGCGTACATTGCACAGAGGAA	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
Ghr V1 F	ACAGTGCCTACTTTTGTGAGTC	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
Ghr V1 R	GTAGTGGTAAGGCTTTCTGTGG	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
Ghr V2 F	CTGCAAAGAATCAATCCAAGCC	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
Ghr V2 R	CAGTTCAGGGGAACGACACTT	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
ICAM1 F	GTGATGCTCAGGTATCCATCCA	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
ICAM1 R	CACAGTTCTCAAAGCACAGCG	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
INHBB F	CTTCGTCTCTAATGAAGGCAACC	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
INHBB R	CTCCACCACATTCCACCTGTC	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
Lcn2 F	TGGCCCTGAGTGTGCATGTG	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
Lcn2 R	CTCTTGTAGCTCATAGATGGTGC	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
MertK F	CAGGGCCTTACCAGGGAGA	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
MertK R	TGTGTGCTGGATGTGATCTTC	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
Nestin F	CCCTGAAGTCGAGGAGCTG	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>

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Nestin R	CTGCTGCACCTCTAAGCGA	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
Notch2 F	ATGTGGACGAGTGTCTGTTGC	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
Notch2 R	GGAAGCATAGGCACAGTCATC	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
PAI 2 F	GTGCTGGGGTAACACTGAAC	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
PAI 2 R	GCGAAATCACAGCCACTGAAG	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
PDGFc F	GCCAAAGAACGGGGACTCG	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
PDGFc R	AGTGACAACCTCTCATGCCG	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
Ptk2 F	CCTGCGATCAGAGGAGGTG	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
Ptk2 R	GCATTACCCCTCATCTCCAATA	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
Sema6D F	GAGAATCCAATCAGATGGTCCAC	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
Sema6D R	CATGTCACGGTAGCAGTACAC	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
Semaphorin-7A F	ACACACCGTGCTTTTCCATGA	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
Semaphorin-7A R	CCTTTGTGGAGCCGATGTTC	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
Sfrp1 F	CAACGTGGGCTACAAGAAGAT	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
Sfrp1 R	GGCCAGTAGAAGCCGAAGAAC	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
Stc2 F	CTGGGCCAGTTTGTGACCC	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
Stc2 R	ACGTCATGCAAATCCCATGTAAA	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
Tsuku F	TCAGCCTGATCCGTGTGGA	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
Tsuku R	GTTTCTAGCCGGTTGGAAGAC	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
Tsuku V1 F	CTCTGCCTTCTCCCGACTG	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
Tsuku V1 R	GGAGCTGGTGAAAATCTCTGC	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
Ubxn1 F	TCGAGGCTGCGATGGATTG	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>
Ubxn1 R	CAGGGCCAACCTGCTCTGAG	<a href="https://pga.mgh.harvard.edu/primerbank/">https://pga.mgh.harvard.edu/primerbank/</a>



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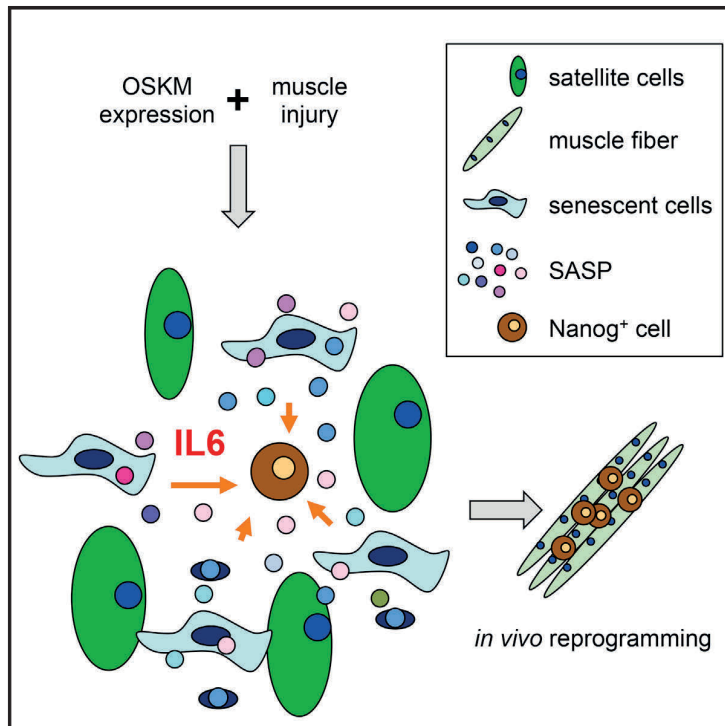
# ANNEXES



# Cell Stem Cell

## Injury-Induced Senescence Enables In Vivo Reprogramming in Skeletal Muscle

### Graphical Abstract



### Authors

Aurélie Chiche, Isabelle Le Roux, Mathieu von Joest, ..., Patricia Flamant, Shahragim Tajbakhsh, Han Li

### Correspondence

shahragim.tajbakhsh@pasteur.fr (S.T.), han.li@pasteur.fr (H.L.)

### In Brief

Li and colleagues reported that tissue damage enables in vivo reprogramming in skeletal muscle, which can arise from muscle stem cells. They also show that damage-induced cellular senescence promotes cellular plasticity through release of secreted proteins, including IL-6. Their findings could have implications for developing therapeutic lineage reprogramming strategies.

### Highlights

- Tissue damage enables in vivo OSKM-mediated reprogramming in skeletal muscle
- Satellite cells are a major cell of origin for in vivo reprogramming in muscle
- Accumulation of senescent cells after injury or in aging promotes reprogramming
- IL-6 release from senescent cells positively stimulates in vivo reprogramming



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# Injury-Induced Senescence Enables In Vivo Reprogramming in Skeletal Muscle

Aurélie Chiche,<sup>1,4,5</sup> Isabelle Le Roux,<sup>2,5,6</sup> Mathieu von Joest,<sup>1,4</sup> Hiroshi Sakai,<sup>2,4</sup> Sabela Búa Aguín,<sup>1,4</sup> Coralie Cazin,<sup>1,4</sup> Rana Salam,<sup>1</sup> Laurence Fiette,<sup>3</sup> Olinda Alegria,<sup>1,4</sup> Patricia Flamant,<sup>3</sup> Shahragim Tajbakhsh,<sup>2,4,\*</sup> and Han Li<sup>1,4,7,\*</sup>

<sup>1</sup>Cellular Plasticity & Disease Modelling, Department of Developmental & Stem Cell Biology

<sup>2</sup>Stem Cells & Development, Department of Developmental & Stem Cell Biology

<sup>3</sup>Human Histopathology and Animal Models, Department of Infection & Epidemiology

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

<sup>4</sup>CNRS, UMR3738, Rue du Dr Roux, Paris 75015, France

<sup>5</sup>Co-first author

<sup>6</sup>Present address: ICM - Institut du Cerveau et de la Moelle Épineuse, CNRS UMR 7225, INSERM U 1127, UPMC-P6 UMR S 1127, Hôpital de la Pitié-Salpêtrière, 47, Boulevard de l'Hôpital, 75013 Paris, France

<sup>7</sup>Lead Contact

\*Correspondence: [shahragim.tajbakhsh@pasteur.fr](mailto:shahragim.tajbakhsh@pasteur.fr) (S.T.), [han.li@pasteur.fr](mailto:han.li@pasteur.fr) (H.L.)

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## SUMMARY

In vivo reprogramming is a promising approach for tissue regeneration in response to injury. Several examples of in vivo reprogramming have been reported in a variety of lineages, but some including skeletal muscle have so far proven refractory. Here, we show that acute and chronic injury enables transcription-factor-mediated reprogramming in skeletal muscle. Lineage tracing indicates that this response frequently originates from Pax7+ muscle stem cells. Injury is associated with accumulation of senescent cells, and advanced aging or local irradiation further enhanced in vivo reprogramming, while selective elimination of senescent cells reduced reprogramming efficiency. The effect of senescence appears to be, at least in part, due to the release of interleukin 6 (IL-6), suggesting a potential link with the senescence-associated secretory phenotype. Collectively, our findings highlight a beneficial paracrine effect of injury-induced senescence on cellular plasticity, which will be important for devising strategies for reprogramming-based tissue repair.

## INTRODUCTION

Cellular senescence is a stable cell-cycle arrest that is induced by damage in many biological and pathological settings (Muñoz-Espín and Serrano, 2014). Recent studies have demonstrated that damage-induced cellular senescence, via the senescence-associated secretory phenotype (SASP), plays a key role in tissue remodeling (Demaria et al., 2014; Le Roux et al., 2015; Yun et al., 2015). However, the link between senescence and cellular plasticity in the context of tissue regeneration remains unexplored.

Breakthroughs in nuclear reprogramming have revealed that differentiated cells are strikingly plastic both in vitro and in vivo,

with exciting implications for disease modeling and regenerative medicine (Srivastava and DeWitt, 2016; Takahashi and Yamanaka, 2016). Many organs, such as pancreas, liver, and kidney are permissive for in vivo reprogramming both to pluripotency and lineage switching (Abad et al., 2013; Srivastava and DeWitt, 2016), while other organs and tissues—most notably, skeletal muscle—do not develop teratomas. Interestingly, it has been shown that induced in vivo lineage reprogramming in liver and pancreas is more efficient when combined with injury (Heinrich et al., 2015). Moreover, transient induction of senescence occurs in non-muscle cells during regeneration following muscle damage (Le Roux et al., 2015). In light of these observations, we hypothesized that injury can promote reprogramming in vivo in skeletal muscle and that cellular senescence might play an important role during this process.

In the present study, we demonstrate that both acute and chronic muscle damage enables in vivo reprogramming in skeletal muscle. Furthermore, using a Pax7-specific lineage-tracing model, we show that the muscle stem cell is a major cell of origin for in vivo reprogramming. Interestingly, muscle-damage-induced senescence positively correlates with in vivo reprogramming, while modulating the relative amount of senescent cells present in an organism affects the efficiency of in vivo reprogramming. Moreover, IL-6 inhibition, a major component of SASP, hinders reprogramming. Taken together, these results suggest that tissue-damage-induced senescence positively contributes to cellular plasticity via SASP. Our observations have direct implications for in vivo lineage-reprogramming-based therapies, which are currently being considered to treat a wide range of diseases such as diabetes, liver failure, and muscular dystrophy.

## RESULTS

### Tissue Injury Permits In Vivo Reprogramming in Skeletal Muscle

To evaluate the impact of injury on in vivo reprogramming in skeletal muscle, we used a previously described reprogrammable mouse model, hereinafter referred to as i4F (Abad et al., 2013),



which can be induced to express *Oct4*, *Sox2*, *Klf4*, and *c-Myc* (OSKM) when the mice are treated with doxycycline (DOX). Notably, there are two reprogrammable lines depending on the OSKM insertion site, i4F-A and i4F-B. Both lines could be induced to develop teratoma, albeit with different kinetics (Abad et al., 2013). For most experiments, we used the more efficient i4F-A line. The *Tibialis anterior* (TA) muscle of i4F-A mice was injured acutely by injection with the snake venom cardiotoxin (CTX), and doxycycline (DOX) was administered in the drinking water for 7 days to induce in vivo reprogramming (Figure 1A). We reduced doxycycline treatment time, compared to that used by Abad et al. (2013), from 2.5 weeks to 7 days to delay the onset of teratoma formation in non-muscle tissues and, consequently, early lethality. The non-injured TA (injected with PBS, internal control) and injured TA muscles were collected and analyzed at 10 days post-injury to evaluate both in vivo reprogramming and induction of senescence, as at 10 days post-injury of muscle, senescence and regeneration are readily detectable (Le Roux et al., 2015).

Expression of the OSKM cassette triggered extensive dysplasia in the injured muscle of DOX-treated mice (i4F-A), as depicted by H&E and laminin (LN) staining. This was not observed in uninjured TA muscle, nor was it observed in injured TA muscle in mice that had not received DOX (Figures 1B and S1A). To confirm that these dysplastic areas were regions undergoing reprogramming, we stained sections for the pluripotency marker *Nanog*. Strikingly, we found *Nanog*-positive cells predominantly in dysplastic regions in the muscle mass, and these were not positive for *Pax7*, a marker for muscle stem cells (Figure 1B). Notably, *Nanog*-positive cells were only found in the injured TA muscles where the OSKM cassette was induced (CTX+DOX). To rule out the possibility that enhanced reprogramming in injured muscle was simply due to increased expression of the OSKM cassette, we compared the gene expression levels of the OSKM transcript in muscle from our acute CTX injury model. There was no difference in exogenous OSKM mRNA levels (Figure S1B; using a pair of primers located in the junction between *Sox2* and *Klf4* in the cassette) (Abad et al., 2013) in CTX-injured TA muscle versus non-injured TA muscle from the same DOX-treated mice, indicating that injury did not affect expression of the OSKM cassette. This observation demonstrates that injury is required for in vivo reprogramming to occur in muscle (Figures 1B, S1C, and S1D).

Next, we asked whether our hypothesis could be applied to a more chronic, pathophysiological model of muscle injury. To do this, we generated reprogrammable mice carrying the *Dmd*<sup>*mdx-βgeo*</sup> mutation (*Dmd*<sup>*mdx-βgeo*</sup>;i4F-A). *Dmd*<sup>*mdx-βgeo*</sup> is a murine model of Duchenne muscular dystrophy that is lacking Dystrophin, an essential component of the myofiber membrane, thereby resulting in continuous degeneration of myofibers and consequent random activation of skeletal muscle stem cells for tissue repair (Grounds et al., 2008; Wertz and Füchtbauer, 1998). Six-week-old *Dmd*<sup>*mdx-βgeo*</sup>;i4F-A mice that exhibit continued myofiber degeneration/regeneration, as well as increased numbers of senescent cells (Le Roux et al., 2015), were treated with DOX for 2.5 weeks (Figure 1C). All of the *Dmd*<sup>*mdx-βgeo*</sup>;i4F-A mice developed teratomas within 4 months. Of note, there is no adverse effect of DOX treatment

on *Dmd*<sup>*mdx-βgeo*</sup> mice (Figure 1C). Remarkably, we observed fully differentiated teratomas only in the muscle mass of *Dmd*<sup>*mdx-βgeo*</sup>;i4F-A mice treated with DOX, which has never been reported in DOX-treated i4F-A mice (Abad et al., 2013) or observed in *Dmd*<sup>*mdx-βgeo*</sup> mice (Figure 1D). Interestingly, all of the muscle teratomas and dysplasias were located in the lower limbs in the region of the TA and in the diaphragm muscle (Figures 1D and S1D), muscles that are most severely affected in the *Dmd*<sup>*mdx-βgeo*</sup> mice (Grounds et al., 2008). These data show that chronic muscle injury that is associated with a naturally occurring pathological state can license full reprogramming in vivo upon DOX treatment, demonstrating that a broad range of damage stimuli can enhance cellular plasticity in vivo.

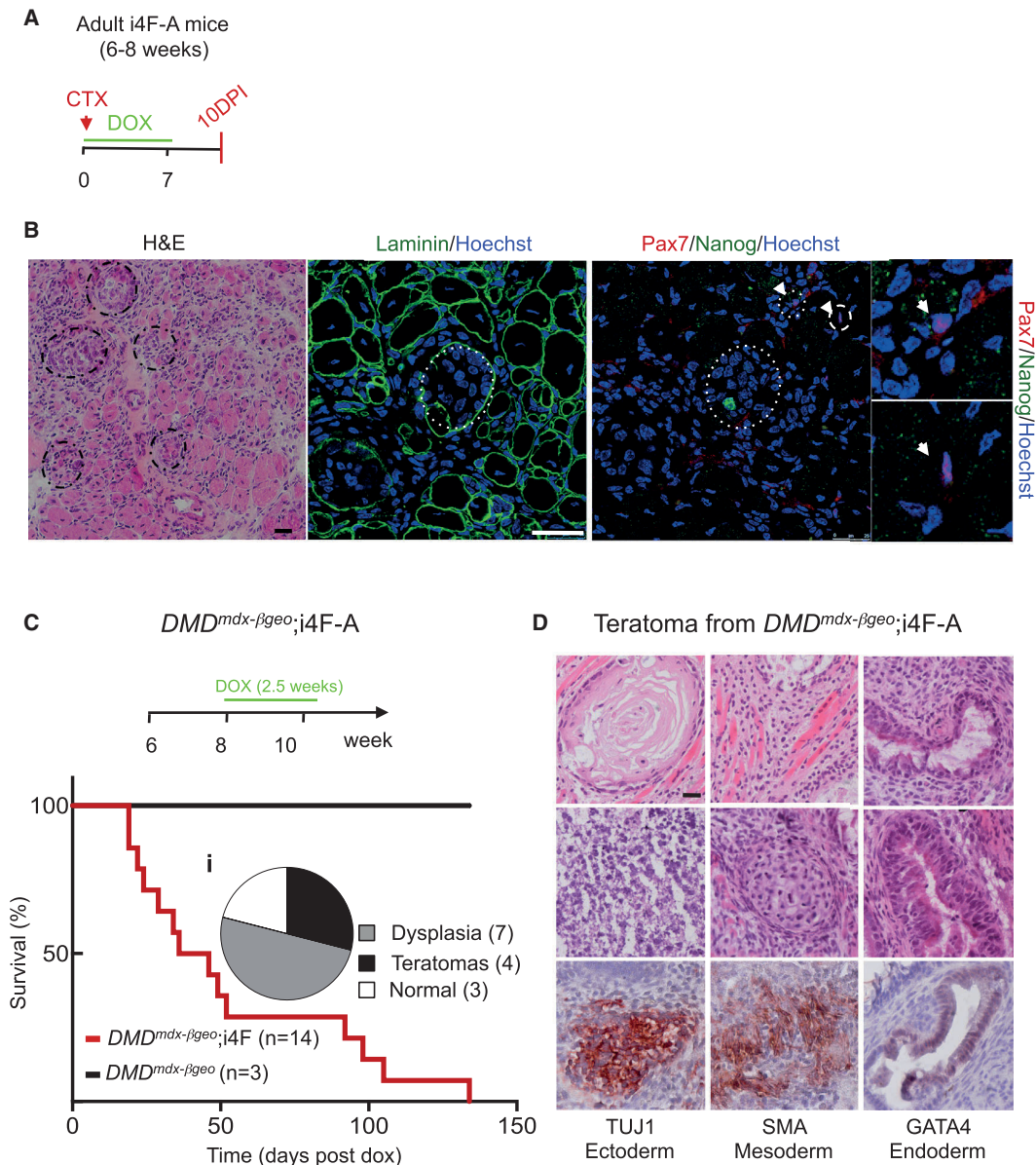
### The Muscle Stem Cell Is a Major Cell of Origin for In Vivo Reprogramming in Muscle

To date, the cell of origin for in vivo reprogramming has not been reported. This is an important consideration for future applications of in vivo lineage reprogramming if specific cell types are to be targeted. We speculated that the muscle stem (or satellite) cell (SC) might be one cell of origin in our injury models, given the more plastic state of SCs relative to more differentiated myogenic cells (Brack et al., 2007).

To test this hypothesis, we crossed i4F-A mice with previously described inducible Tg(*Pax7-cre/ERT2*);*Rosa26*<sup>*mT/mG*</sup> (hereinafter referred to as *Pax7CT2*;R26<sup>*mT/mG*</sup>) mice (Le Roux et al., 2015), where Cre-mediated recombination results in the conversion of Tomato-positive to GFP-positive satellite cells. After treatment of mice for 2 weeks with tamoxifen (TMX)-containing food, the majority of the quiescence SCs was membrane-targeted EGFP (mGFP) positive (~95% recombination efficiency; I.L.R., unpublished data) (Le Roux et al., 2015).

First, we derived SCs and fibrogenic/adipogenic progenitors (FAPs), another cell type important for muscle regeneration (Dumont et al., 2015; Murphy et al., 2011), from either *Pax7CT2*;R26<sup>*mT/mG*</sup>;i4F mice treated with TMX (induction of CreERT2) or i4F mice alone using specific cell-surface markers (Figure S2A) to examine the in vitro reprogramming capacity of these two cell types. Consistent with a previous report (Tan et al., 2011), SCs and FAPs could be reprogrammed much more efficiently in vitro than differentiated mouse skin fibroblasts (MSFs) (Figures 2A and S2A). Moreover, the induced pluripotent SCs (iPSCs) derived from both SCs and FAPs express pluripotency markers at a level similar to that of embryonic stem cells (Figures S2B and S2C).

Furthermore, most of the *Pax7CT2*;R26<sup>*mT/mG*</sup>;i4F-A mice (four out of seven) developed muscle dysplasia, some of which contained mGFP-positive cells (Figures 2B and S2D). Remarkably, most of the cells in the teratomas in the *Pax7CT2*;R26<sup>*mT/mG*</sup>;i4F-A mouse analyzed were mGFP positive (Figure 2C). We confirmed that the majority of mGFP-positive cells in the teratoma were no longer positive for endogenously expressed *Pax7*, suggesting that they had changed fate, although they were lineage derived from satellite cells. As expected, *Pax7*-positive cells were mostly observed where de novo muscle was forming (Figure 2C). Therefore, these findings demonstrate that the SC are a major cell of origin for in vivo reprogramming in muscle.



**Figure 1. Injury-Enabled In Vivo Reprogramming**

(A) Scheme of the experiments.

(B) Panels left to right represent transverse TA cryosections after histological staining with H&E and immunofluorescence with anti-laminin, anti-Nanog, and anti-Pax7 antibodies. Large dashed circle highlights Nanog<sup>+</sup> cell; arrowheads show Pax7<sup>+</sup> cells (high magnification in last panel). DPI, days post-injury.

(C) Survival curve of  $Dmd^{mdx-\beta geo};i4F-A$  mice ( $n = 14$ ) compared to  $Dmd^{mdx-\beta geo}$  mice ( $n = 3$ ) treated with DOX. (i) Analysis of TA muscles (parts of whole chart).

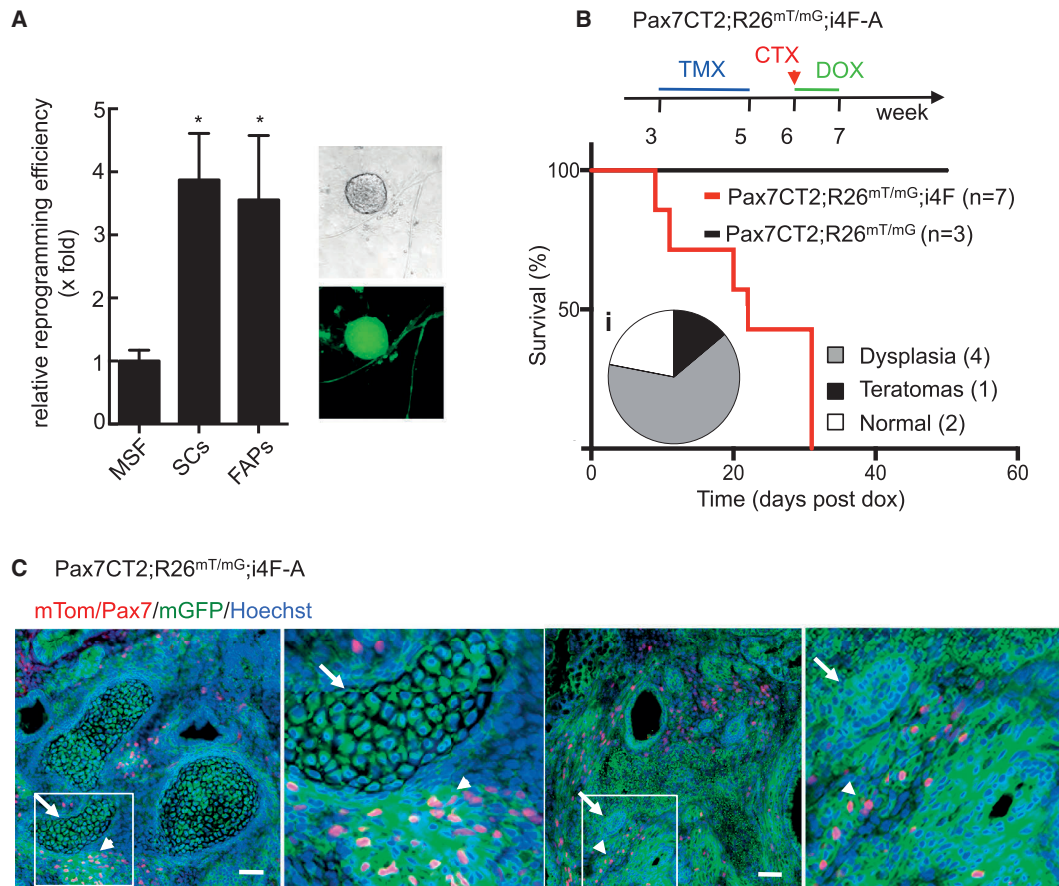
(D) Histological section of a teratoma derived from the muscle of a  $Dmd^{mdx-\beta geo};i4F-A$  mouse. H&E-stained sections revealed features of (from left to right): ectoderm (squamous epithelium with keratinization and neural tissues shown in the top and middle panels, respectively); mesoderm (striated muscle and cartilage shown in the top and middle panels, respectively); and endoderm (respiratory ciliated epithelium and columnar epithelium shown in the top and middle panels, respectively). Immunohistochemical stainings confirmed the pathological analyses (bottom row, from left to right), anti-neuron-specific Class III  $\beta$ -tubulin ( $\beta$ III) (TUJ1), SMA (smooth muscle actin), and GATA4.

Scale bars: for (B), 50  $\mu$ m for H&E image, 25  $\mu$ m for immunofluorescence images, and 20  $\mu$ m for immunohistochemistry; for (D), 25  $\mu$ m. See also Figure S1.

### Cellular Senescence Associates with In Vivo Reprogramming

Next, we set to explore the pathways that could regulate damage-induced in vivo reprogramming. We recently demonstrated

that muscle damage induces transient cellular senescence during regeneration (Le Roux et al., 2015). Interestingly, senescence is a known barrier for in vitro reprogramming (Banito et al., 2009; Li et al., 2009). Therefore, we asked whether senescence might



**Figure 2. Satellite Cells Are Major Cells of Origin for In Vivo Reprogramming in Muscle**

(A) In vitro reprogramming efficiency of SCs and FAPs compared to mouse skin fibroblasts (MSFs). Representative image of iPSC colony from SCs of Pax7CT2;R26<sup>mT/mG</sup>; i4F-A mice (n = 4 mice). Statistical significance was assessed by the two-tailed Student's t test: \*p < 0.05.

(B) Survival curve of Pax7CT2;R26<sup>mT/mG</sup>;i4F-A mice (n = 7) compared to Pax7CT2;R26<sup>mT/mG</sup> mice (n = 3). (i) Characterization of TA muscles (parts of whole chart).

(C) Immunofluorescence using anti-GFP and anti-Pax7 antibodies on TA cryosections showing teratoma formation in Pax7CT2;R26<sup>mT/mG</sup>;i4F-A mouse. White arrowheads point to Pax7<sup>+</sup> cells, white arrows show Pax7<sup>-</sup>/mGFP<sup>+</sup> cells. Scale bars, 100  $\mu$ m.

All data correspond to the average  $\pm$  SEM. See also Figure S2.

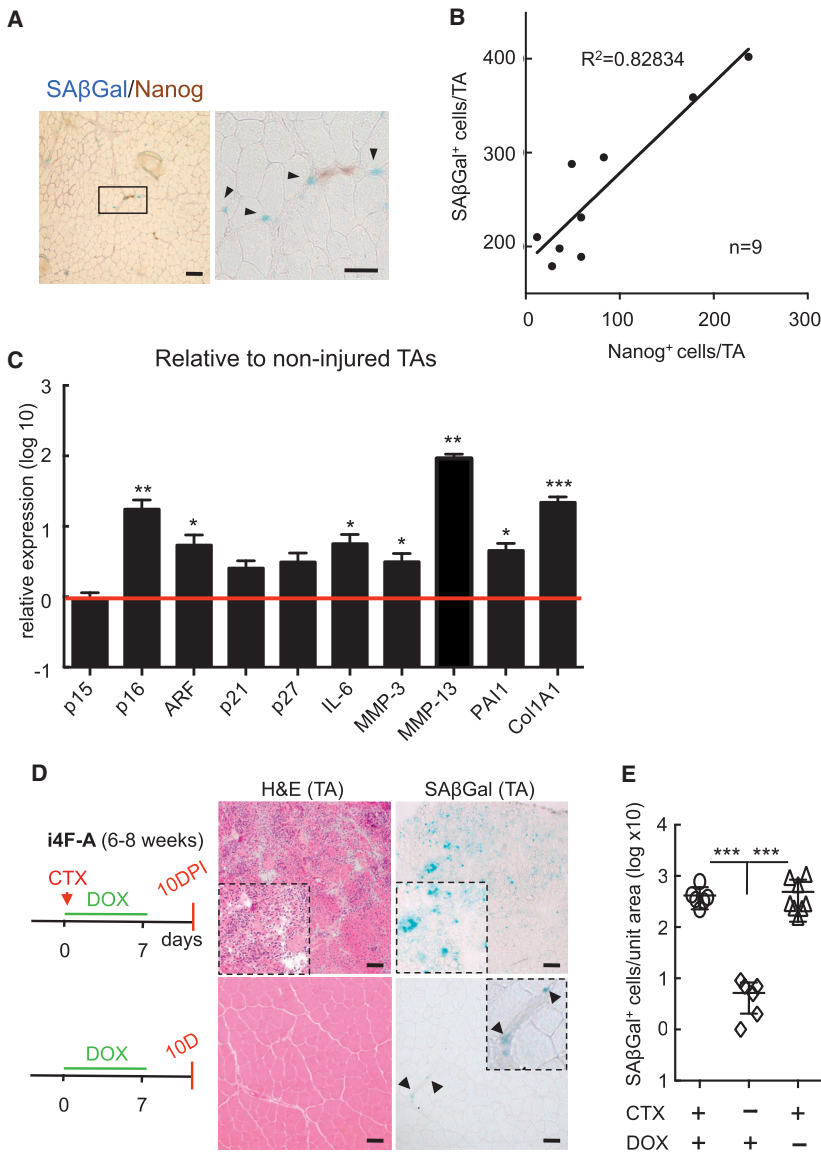
play a role in this context. To answer this question, we examined the level of senescence in injury-induced in vivo reprogramming by performing SA $\beta$ Gal (senescence-associated  $\beta$ -galactosidase) and Nanog staining on CTX-injured TA muscle compared to non-injured TA from the same i4F-A mouse treated with DOX. Notably, double staining for SA $\beta$ Gal and Nanog showed that Nanog-positive cells were frequently in close proximity to SA $\beta$ Gal-positive cells, the latter being more abundant (Figure 3A). Moreover, the number of Nanog-positive cells was strongly correlated with the number of SA $\beta$ Gal-positive cells (Figure 3B). In addition, these SA $\beta$ Gal-positive cells are non-cycling cells (Ki67 negative) and p19<sup>Arf</sup> positive, confirming that they are, indeed, senescent (Figures S3A and S3B).

To further confirm enhanced senescence in injured muscle, we analyzed whole-muscle extracts for the levels of various mRNAs associated with senescence and found an increase in the cyclin-dependent kinase inhibitors 2A p16<sup>INK4a</sup>, p19<sup>Arf</sup>, and Pai-1 and SASP factors interleukin-6 (IL-6), MMP-3, MMP-13, and Col1a1 (Figure 3C). Consistent with our previous findings (Le

Roux et al., 2015), we did not observe significant differences in mRNA levels for p21 and p27, suggesting that p16<sup>INK4a</sup> and p19<sup>Arf</sup> are the main mediators of senescence in this context.

Furthermore, in the same experimental setting, a significant increase in the number of senescent cells was observed only in the CTX-injured TA, compared to non-injured TA control (Figure 3D). The number of SA $\beta$ Gal-positive cells was equivalent in injured mice treated or untreated with DOX, indicating that injury was the main trigger of senescence in muscle (Figure 3E). To further investigate the relationship between senescence and in vivo reprogramming, we examined a tissue that is permissive for in vivo reprogramming upon DOX treatment, such as kidney. In contrast with muscle, DOX treatment alone was sufficient to induce high numbers of SA $\beta$ Gal-positive cells in the kidneys (Figure S3C). This finding is consistent with the observation that induction of reprogramming in vivo triggers senescence in various tissues (Mosteiro et al., 2016) and the notion that the in vitro reprogramming process itself can trigger a stress response similar to senescence (Banito et al., 2009).





**Figure 3. Cellular Senescence Associates with In Vivo Reprogramming**

(A) SAβGal staining combined with immunohistochemistry using anti-Nanog antibody. Low (left) and high (right) magnification panels are shown. Black arrowheads point to SAβGal<sup>+</sup> cells.

(B) Quantification and correlation of SAβGal<sup>+</sup> and Nanog<sup>+</sup> cells from the same section (n = 9 mice; value represents the average of two TAs per mouse).

(C) Expression of the indicated genes in injured muscle measured by qRT-PCR. Values are relative to the expression of these genes in the non-injured TA from the same mouse (n = 9 mice; nine TAs per condition). Data correspond to the average ± SD. For each assay, qRT-PCR values were obtained in duplicate or triplicate.

(D) Scheme of the experiments; Histological staining with H&E (left panels) and SAβGal staining on the TA muscle (right panels). Black arrowheads point to SAβGal<sup>+</sup> cells (inset, high magnification). DPI, days post-injury.

(E) Quantification of SAβGal<sup>+</sup> cells in three different experimental settings: injured TAs with DOX, non-injured TAs with DOX, and injured TAs without DOX (n = 3 per group; two TAs per mouse).

Statistical significance was assessed by the two-tailed Student's t test: \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001. For all histological, immunohistochemistry stainings, data were collected from transverse TA muscle cryosections of adult mice. Scale bars: for (A), 100 μm for the left panel and 25 μm for the right panel; for (D), 100 μm.

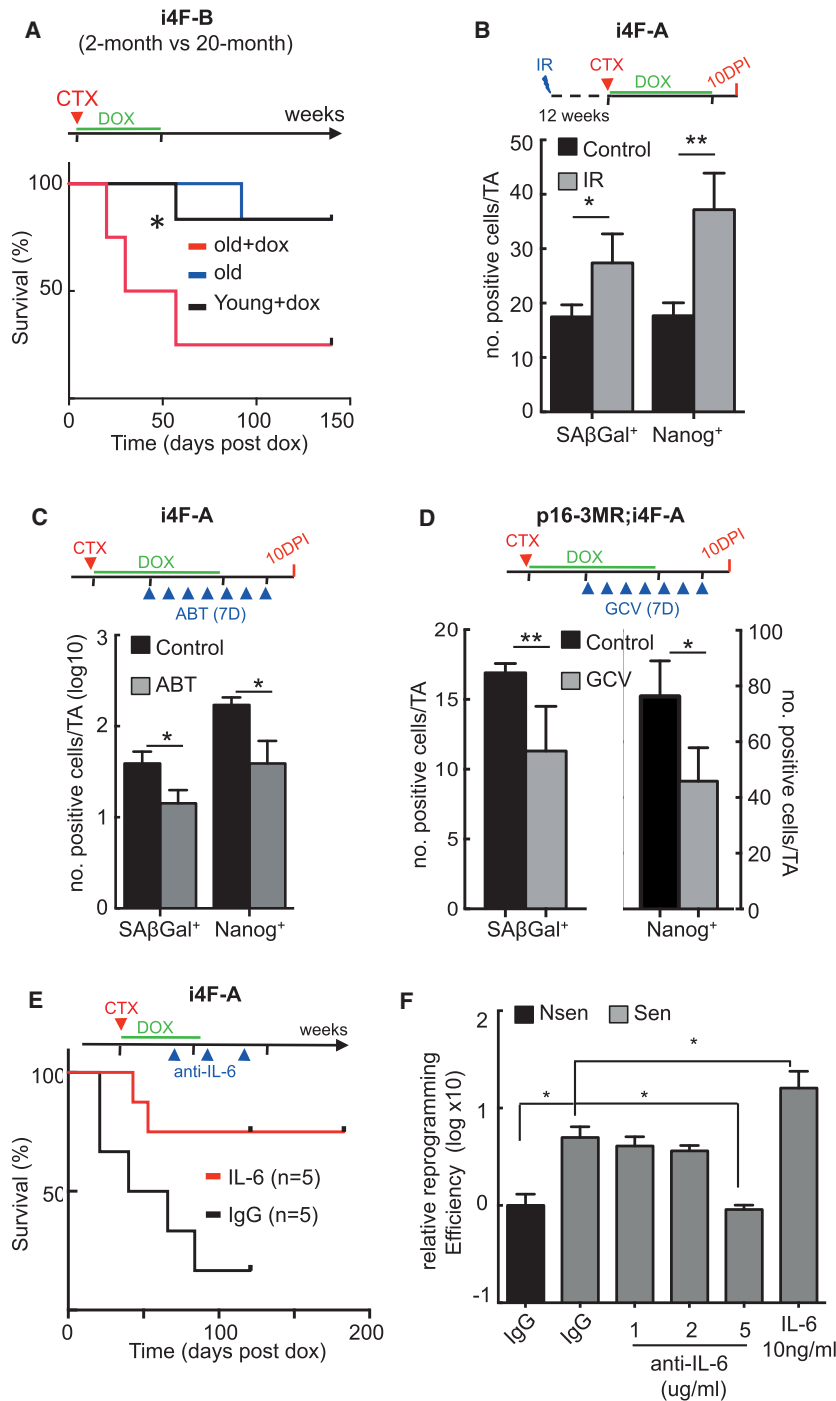
All data correspond to the average ± SD. See also Figure S3.

**Cellular Senescence Promotes Injury Induced In Vivo Reprogramming**

To further investigate the correlation between senescence and in vivo reprogramming efficiency, we assessed teratoma formation in non-injured tissues in old versus young mice, given that the number of senescent cells is known to accumulate with age (Dimri et al., 1995; Herbig et al., 2006). Of note, for these experiments, we used the less efficient i4F-B line. Specifically, DOX was administered for 7 days to induce in vivo reprogramming in both 2-month-old and 20-month-old i4F-B mice. Interestingly, we found that aged i4F-B mice had a shorter survival curve compared to the young i4F-B mice (Figure 4A) due to increased incidence of teratomas in abdominal organs, thereby supporting the notion that naturally occurring accumulation of senescence during aging can increase cellular plasticity in vivo. Next, we

and control) were injured by CTX followed by DOX administration; there was a significant increase of Nanog-positive cells in the irradiated TA corresponding to the increased senescent cells via irradiation (Figure 4B). Therefore, these data further correlated senescence accumulated via either the aging process or local irradiation to enhanced in vivo reprogramming.

To further demonstrate that senescence fosters in vivo reprogramming, we used two methods to remove senescent cells. First, we used Navitoclax (ABT263), a potent senolytic drug, to selectively eliminate the senescent cells (Chang et al., 2016). Remarkably, we observed a significant reduction of Nanog-positive cells in the injured muscle in proportion to the depletion of senescent cells via ABT263 (Figure 4C). Notably, embryonic stem cells are extremely resistant to ABT263 compared to senescent cells (Figure S4B), suggesting that ABT263 does



**Figure 4. Cellular Senescence Promotes Injury-Induced In Vivo Reprogramming**

(A) Survival curve of young i4F-B mice treated with DOX (n = 6), old i4F-B mice treated with DOX (n = 4), and old i4F-B mice without DOX (n = 4) ( $p < 0.05$ ).

(B) Quantification of SA $\beta$ Gal<sup>+</sup> and Nanog<sup>+</sup> cells in locally irradiated (IR) TAs compared to non-irradiated control TAs of the same mouse (n = 5).

(C) Quantification of SA $\beta$ Gal<sup>+</sup> and Nanog<sup>+</sup> cells in TAs from ABT263-treated mice (n = 4) compared to vehicle control (n = 4).

(D) Quantification of SA $\beta$ Gal<sup>+</sup> and Nanog<sup>+</sup> cells in TAs from GCV-treated mice (n = 7) compared to vehicle control (n = 7).

(E) Survival curve of mice treated with IL-6 (n = 8) versus IgG (n = 6) by intraperitoneal injection.

(F) In vitro reprogramming efficiency of SCs co-cultured on non-senescent (Nsen) compared to senescent cells (Sen) and treatment of various dosage of IL-6 blocking antibody or IL-6 recombinant protein (n = 4 mice).

All data correspond to the average  $\pm$  SEM. Statistical significance was assessed by the two-tailed Student's t test: \* $p < 0.05$ , \*\* $p < 0.01$ . See also Figure S4.

(Demaria et al., 2014). Of note, the expression of  $p16^{INK4a}$  was highly upregulated in the injured TA (Figure 3C). Consistent with the ABT263 result, ganciclovir (GCV) treatment effectively removed senescent cells, which, in turn, reduced the Nanog-positive cells in the injured muscle (Figure 4D). Therefore, these data provide direct evidence that senescence is important for cellular plasticity in vivo.

We next investigated how senescence might affect the efficiency of in vivo reprogramming. Given that SASP is the major downstream mediator of cellular senescence (Coppé et al., 2010), and the observation that senescent cells appear transiently during regeneration following muscle injury, we speculated that senescence, specifically via the SASP, might provide paracrine signals to enhance in vivo reprogramming. We focused on IL-6 (Coppé et al., 2010), since it is known to play a critical role during muscle regeneration (Muñoz-Cánoves et al., 2013) and can promote

in vitro reprogramming (Brady et al., 2013). Moreover, we also observed a significant increase of IL-6 in the injured TA (Figure 3C). Administration of a neutralizing antibody against IL-6 resulted in the survival of four out of five mice (among which, one developed a teratoma), whereas five out of five mice died within 3 months when injected with control immunoglobulin G (IgG) (all mice developed teratomas) (Figure 4E). In addition, significantly

not remove Nanog-positive cells directly. Next, we used a Tg(p16-3MR) transgenic mouse model that carries a trimodal reporter protein (3MR) containing Renilla luciferase, monomeric red fluorescent protein (mRFP), and herpes simplex virus thymidine kinase (HSV-TK) under the promoter of  $p16^{INK4a}$ . Therefore, Tg(p16-3MR) mice can be used to identify, visualize, and selectively kill p16-positive senescent cells in vivo

lower levels of Nanog-positive cells were observed in IL-6-treated TA muscles compared to controls at 10 days post-injury, whereas no significant difference was noted in the number of SA $\beta$ Gal-positive cells (Figure S4C). To further confirm that senescent cells are beneficial for *in vivo* reprogramming, we isolated both SCs and FAPs from i4F-A mice and co-cultured them with either non-reprogrammable primary mouse embryonic fibroblasts (MEFs) (non-senescent) or mitomycin-C (MMC)-treated senescent cells. The co-cultured condition did not affect the OSKM expression (upon DOX) and the proliferation of the SCs (Figures S4D and S4E). However, upon DOX treatment, co-culturing with MMC-induced senescent cells dramatically enhanced reprogramming efficiency of both SCs and FAPs (Figure S4F), which could be further enhanced via adding IL-6 protein as previously reported (Figure 4F) (Brady et al., 2013). Interestingly, blocking IL-6 significantly abrogated the positive effect of cellular senescence on reprogramming (Figure 4F), suggesting that cellular senescence promotes cellular plasticity in a cell-non-autonomous manner.

Together, these data demonstrate that *in vivo* reprogramming only occurs in muscle during regeneration, indicating that senescence could facilitate cellular plasticity, which is mainly mediated by the SASP, in part via IL-6.

## DISCUSSION

Collectively, these data suggest that injury permits *in vivo* reprogramming to occur in muscle and that this process can be facilitated by the accumulation of senescent cells in the damaged tissue. Interestingly, the enhanced *in vivo* reprogramming in the aged i4F mice could, at least partially, be due to the accumulation of the senescent cells during aging. These findings are somewhat surprising in light of previous studies. It was reported that both cellular senescence (Banito et al., 2009) and aging intrinsically impair *in vitro* reprogramming (Li et al., 2009). Moreover, a recent report described the negative effects of senescence on organ functionality during aging (Baker et al., 2016). However, other recent work has identified a beneficial effect of senescence on pancreatic beta cell function (Helman et al., 2016), which could be due, in part, to enhanced cellular plasticity, as described in our study. In addition, consistent with a previous report (Brady et al., 2013), we identify that IL-6, a prominent component of SASP, is important for reprogramming both *in vitro* and *in vivo*. Therefore, we propose, in the context of cellular reprogramming, that senescence is a cell-intrinsic barrier for the initiation of the process, while, later on, it facilitates the reprogramming of neighboring non-senescent cells via cell-extrinsic mechanisms. In the context of tissue repair and regeneration, damage induces senescence and the secretion of SASP, not only to recruit macrophages for the removal of necrotic tissue but perhaps also to alter the plasticity of resident cells. More work is required to analyze the cell-intrinsic and -extrinsic effects of senescence on the cellular plasticity of the tissues during tissue repair and regeneration, particularly during the aging process.

Further understanding cellular plasticity is key to the development of emerging therapeutic strategies that seek to regenerate non-functioning tissues by *in situ* lineage conversion in affected organs. Such strategies are most prominently being considered

as treatments to increase the number of pancreatic beta cells in diabetes and hepatocytes in liver failure. Intriguingly, it has been shown that injury enhances the *in vivo* lineage reprogramming efficiency in both liver and pancreas (Heinrich et al., 2015), which, we speculate based on the findings presented here, is driven by the induction of a senescent program. It will be of great interest to see how our findings regarding senescence and specific components of the SASP in the i4F mouse, which serves as a robust readout system for reprogramming, extend to *in vivo* lineage conversion for the purpose of tissue regeneration.

It will be of additional importance to assess the relative sensitivities of different cellular populations, such as resident stem cells versus non-stem and differentiated cells, as well as different organs, to the effects of senescence on *in vivo* reprogramming. Our data involving the Pax7 lineage tracer suggest that satellite cells in the muscle are particularly sensitive to the effects of the SASP. Determination of the relative cellular susceptibility to reprogramming driven by external cues will be important to guide the efficacious and safe delivery of lineage-conversion-based therapies (Heinrich et al., 2015).

Collectively, our findings point to a beneficial role for senescence, via the SASP, in promoting cellular plasticity, especially of stem cell populations, during muscle regeneration following acute muscle injury as well as in the pathological setting of chronic muscle deterioration. These findings might have implications for the treatment of diseases such as Duchenne muscular dystrophy.

## STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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## SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and two tables and can be found with this article online at <http://dx.doi.org/10.1016/j.stem.2016.11.020>.

## AUTHOR CONTRIBUTIONS

A.C. and I.L.R. performed most of the experimental work and contributed to experimental design, data analysis, discussions, and writing the paper. M.v.J. made critical experimental contributions. H.S., S.B.A., C.C., R.S., P.F., and O.A. contributed experimentally. L.F. performed histopathological analysis. S.T. and H.L. supervised the study. In particular, S.T. guided all the muscle injury experiments, provided the muscle stem cell lineage-tracing model,

and established the key experimental protocols for detecting senescence in muscle. H.L. guided the *in vivo* reprogramming part and was responsible for the experiments of altering senescence level during the *in vivo* reprogramming. S.T. and H.L. interpreted the data and wrote the manuscript. All authors discussed the results and commented on the manuscript.

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## STAR★METHODS

## KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Antibodies</b>		
Rat anti-IL6 (clone MP5-20F3)	eBiosciences	Cat#14-7061-85; RRID: AB_468423
Rat IgG1 control (clone RTK2071)	BioLegend	Cat#400402; RRID: AB_326508
CD45-eFluor 780 (clone 30-F11)	eBiosciences	Cat#47-0451-82; RRID: AB_1548781
CD31-PE (clone MEC 13.3)	BD PharMingen	Cat#553373; RRID: AB_394819
Sca1-PECy7 (clone D7)	eBiosciences	Cat#25-5981-82; RRID: AB_469669
Itga7-649	AbLab	Cat#67-0010-10
CD34-450 (clone RAM34)	eBiosciences	Cat#48-0341-82; RRID: AB_2043837
anti-GFP	Abcam	Cat#ab13970; RRID: AB_300798
anti-Pax7	DSHB	<a href="http://dshb.biology.uiowa.edu/PAX7">http://dshb.biology.uiowa.edu/PAX7</a>
anti-laminin	Sigma	Cat#L9393; RRID: AB_477163
anti-Nanog (clone D2A3)	Cell signaling	Cat#8822S; RRID: AB_11220237
anti-Gata4	Santa Cruz	Cat#sc-1237; RRID: AB_2108747
anti-SMA	Abcam	Cat#ab5694; RRID: AB_2223021
anti-Tuj1	Biolegend	Cat#MMS-435P-0100
anti-Oct3/4 (clone 40/Oct-3)	BD Biosciences	Cat#611203; RRID: AB_398737
anti-Sox2 (clone AB5063)	Millipore	Cat#AB5603; RRID: AB_2286686
<b>Chemicals, Peptides, and Recombinant Proteins</b>		
Tamoxifen food pellets (TAMOXIFEN DIET TAM400/creER)	Harlan	Cat#TD.55125
Snake venom cardiotoxin	Lotaxan	Cat#L8102
Doxycycline	Sigma	Cat#9891
ABT-263 (Navitoclax)	Selleckchem	Cat#S1001
Ganciclovir	Selleckchem	Cat#S1878
Collagenase D	Roche	Cat#1088866
Trypsin	GIBCO	Cat#15090-046
DNase	Roche	Cat#11284932001
Collagenase A	Roche	Cat#11088793001
Dispase II	Roche	Cat#04942078001
Ultrosor G	Pall Biosepra	Cat#15950-017
KSR	Invitrogen	Cat#10828028
LIF	Miltenyl Biotec	Cat#130-095
recombinant IL-6 protein	R&D Systems	Cat#406-ML-005
Poly-L-Lysine	Sigma	Cat#P4707
X-gal	Sigma	Cat#B4252
iScript	BioRad	Cat#1708890
<b>Critical Commercial Assays</b>		
Phosphatase alkaline	Sigma	Cat#AB0300
LightCycler 480 SYBR Green I Master	Roche	Cat#4309155
<b>Experimental Models: Cell Lines</b>		
mESCs (Lex1)	<a href="#">Kim et al., 2012</a>	N/A
iPSCs	This study	N/A
<b>Experimental Models: Organisms/Strains</b>		
Mouse: i4F-A	<a href="#">Abad et al., 2013</a>	N/A
Mouse: i4F-B	<a href="#">Abad et al., 2013</a>	N/A

(Continued on next page)

**Continued**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Mouse: Tg( <i>Pax7-cre/ERT2</i> ); <i>Rosa26<sup>mT/mG</sup></i>	<a href="#">Mourikis et al., 2012</a>	N/A
<i>Dmd<sup>mdx-βgeo</sup></i>	<a href="#">Wertz and Füchtbauer, 1998</a>	N/A
Tg(p16-3MR)	<a href="#">Demaria et al., 2014</a>	N/A
Recombinant DNA		
<b>Sequence-Based Reagents</b>		
Genotyping PCR primers	Sigma	<a href="#">Table S1</a>
qPCR primers	Sigma	<a href="#">Table S2</a>
Software and Algorithms		
ImageJ software		<a href="https://imagej.nih.gov/ij/download.html">https://imagej.nih.gov/ij/download.html</a>
FlowJo software		<a href="https://www.flowjo.com">https://www.flowjo.com</a>
Graphpad – Prism software		<a href="http://www.graphpad.com">http://www.graphpad.com</a>
Other		

**CONTACT FOR REAGENT AND RESOURCE SHARING**

Further information and requests for reagents may be directed and will be fulfilled by the Lead contact, Han Li ([han.li@pasteur.fr](mailto:han.li@pasteur.fr)).

**EXPERIMENTAL MODEL AND SUBJECT DETAILS****Animals**

Animals were handled as per European Community guidelines and the ethics committee of the Institut Pasteur (CETEA) approved protocols. Both reprogrammable (i4F-A and i4F-B) mice were kindly provided by Manuel Serrano (Spanish National Cancer Research Centre, Madrid, Spain) ([Abad et al., 2013](#)). *Tg:Pax7CT2;R26<sup>mT/mG</sup>*, *Dmd<sup>mdx-βgeo</sup>* and Tg(p16-3MR) mice were described previously ([Demaria et al., 2014](#); [Mourikis et al., 2012](#); [Wertz and Füchtbauer, 1998](#)) and were crossed with i4F-A separately. Pax7 lineage tracing was performed as previously described ([Le Roux et al., 2015](#)). Briefly, all the cells from *R26<sup>mT/mG</sup>* mice are expected to express mTomato except those that have removed this gene following Cre-mediated recombination, thereby resulting in expression of mGFP+. At 5 weeks of age, the mice were given Tamoxifen food pellets for 15 days (TAMOXIFEN DIET TAM400/creER, Harlan TD.55125) to recombine the *R26<sup>mT/mG</sup>* allele. Under these condition about 95% of Pax7+ cells from *Tg:Pax7CT2;R26<sup>mT/mG</sup>* mice were GFP+ (n = 2; I.L.R., unpublished data). All the genotyping were performed by standard PCR using the listed primers ([Table S2](#)).

**METHOD DETAILS****Mice and animal procedures**

To induce muscle injury, mice were anesthetised with isoflurane. *Tibialis anterior* (TA) muscles were injured by injection 40 μL of snake venom cardiotoxin (10 μM) (L8102, Lotaxan Valence, France, <http://www.lotaxan.com>). Following surgery, mice were analgesic with 0.3 mg kg<sup>-1</sup> buprenorphine (Axience).

In vivo reprogramming was induced by administration of Doxycycline (Sigma) in the drinking water supplemented with 7.5% of sucrose right after injury or at weeks 6<sup>th</sup> for *Dmd<sup>mdx-βgeo</sup>;i4F-A*. Experiments were performed indistinguishably with mice of both sexes and from 6 to 8 weeks of age.

For IL-6 treatment, antibodies against IL-6 (eBiosciences, clone MP5-20F3) or IgG1 control (BioLegend, clone RTK2071) were administrated by intraperitoneal (i.p.) injection at 30 μg/mouse at days 5, 8 and 12-post injury. Mice were analyzed when they develop teratomas. For the quantification of in vivo reprogramming, antibodies were injected only at days 5 and 8-post injury and TAs were collected at 10 days-post injury. ABT-263 and ganciclovir treatment were performed as described previously ([Chang et al., 2016](#); [Demaria et al., 2014](#)). Briefly, mice were treated daily for 7 consecutive days starting 3 days after injury. For ABT-263 treatment, mice were treated daily by gavage with either vehicle (Phosal, PEG400 and Ethanol in the proportion 60%: 30%: 10%) or ABT263 (Navitoclax, Selleckchem S1001) at 50 mg/kg. For ganciclovir treatment, mice were treated by intraperitoneal (i.p.) injection of 25 mg/kg of ganciclovir (Ganciclovir Selleckchem S1878) or vehicle (PBS containing 5% DMSO). For IR-induced senescence, one leg of the 8-weeks old mouse was X-irradiated (10 Gy) and both TAs (irradiated and non-irradiated) were injured 12 weeks later. TAs from all the quantitative experiments were collected at 10 days post injury as described below.

**Isolating satellite cells and FAPs**

Isolation of SCs from *Tg:Pax7CT2;R26<sup>mT/mG</sup>;i4F-A* mice was performed as described previously ([Le Roux et al., 2015](#)). Briefly, muscles were chopped in cold DMEM and put into a 50 mL tube containing 30 mL of DMEM (31966, GIBCO), 0.1% Collagenase D

(1088866, Roche), 0.25% trypsin (15090-046, GIBCO), DNase 10  $\mu\text{g}/\text{ml}$  (Roche, 11284932001) at 37°C under gentle agitation for 30 min. After standing still for 5 min at room temperature, the supernatants were collected into 5ml fetal bovine serum (FBS, GIBCO) on ice. The digestion was repeated for additional 4 times allowing complete digestion of the muscle. The supernatants were filtered through a 100 $\mu\text{m}$  and then 70 $\mu\text{m}$  cell strainer (BD Falcon). Cells were spun for 15 min at 600 RCF at 4°C, the pellets were resuspended in 1 mL of DMEM containing 2% FBS and filtered through a 40  $\mu\text{m}$  cell strainer (BD Falcon) before cell sorting. Cells were isolated based on size, granularity and GFP levels using a FACSAria II Cell Sorter (BD). The isolation of SCs and FAPs from the reprogrammable mice was modified. Briefly, dissected muscles were incubated with 40ml of HBSS (24020-091, GIBCO), 0.04% Collagenase A (11088793001, Roche), 0.3% Dispase II (04942078001, Roche), DNase 10  $\mu\text{g}/\text{ml}$  (11284932001, Roche) at 37°C under gentle agitation for 90 min. Following the centrifugation, the cells were resuspended in 1 mL of HBSS with 1% BSA and were incubated with antibodies on ice for 30 min. The following antibodies were used: CD45-eFluor 780 (1/100, Clone 30-F11, eBiosciences), CD31-PE (1/50, Clone MEC 13.3, BD PharMingen), Sca1-PECy7 (1/400, Clone D7, eBiosciences), Itga7-649 (1/1000, AbLab) and CD34-eFluor 450 (1/100, Clone RAM34, eBiosciences). Five volumes of HBSS was added and cells were spun for 15 min at 600 RCF at 4°C to stop the reaction. The cells were resuspended in HBSS containing 2% FBS and filtered through a 40  $\mu\text{m}$  cell strainer before cell sorting. Cells were isolated using a FACSAria II Cell Sorter (BD) and analyzed using FlowJo.

### Cell culture

Primary mouse embryo fibroblasts (MEFs) were derived from wild-type C57BL/6 embryos at E13.5 following standard protocol. Briefly, embryos were chopped into small pieces in 2ml of 0.1% Trypsin-EDTA (GIBCO) after removing the head and internal organs. Embryos are incubated at 37°C in a CO<sub>2</sub> incubator for 5 min for digestion and the suspension is collected and transferred to a 100mm tissue culture plate containing 10ml of DMEM +10% FCS and Pen/Strep. Fibroblasts are cultured for 2-3 days until reaching confluence and considered as the first passage.

To generate MMC-induced senescent feeder cells, primary MEFs were passed for 5 times and subsequently treated with 10 $\mu\text{g}/\text{mL}$  MMC (Sigma-Aldrich) in normal cell culture media for 2.5 hr at 37°C. Cells were washed 3 times with PBS, trypsinized and stocks were frozen at 7.2X10E6 cells/mL.

SCs and FAPs were collected after sorting directly in culture media: for SCs: 20% FBS, 1% Penicillin-Streptomycin (15140, GIBCO), 2% Ultrosor G (15950-017, Pall Biosepra) in 50:50 DMEM: F12 (31966 and 31765, GIBCO) and for FAPs: 10% FBS, 1% Penicillin-Streptomycin (15140, GIBCO) in DMEM. SCs were plated at low density (3000 cells  $\text{cm}^2$ ) on regular cell culture dishes coated with matrigel and FAPs were directly plated at 5x10<sup>5</sup>/6-well plate. Two days later, SCs and FAPs were seeded over mitomycin-C (MMC) treated senescent cells on gelatin-coated plates or primary MEFs at the density of 5000 cells per well in 6-well plate. In vitro reprogramming was induced on the second day by changing to the iPSCs medium (high-glucose DMEM supplemented with KSR (15%, Invitrogen), LIF (1,000 U/ml), non-essential amino acids, penicillin-streptomycin, glutamax and  $\beta$ -mercaptoethanol) supplement with 2% Ultrosor G (for SCs only) and doxycycline (1  $\mu\text{g}/\text{ml}$ ). Medium was changed every 24h until iPSC cell colonies appeared. For the IL-6 experiment, IgG antibody (1 $\mu\text{g}/\text{mL}$ , BioLegend, purified Rat IgG1,  $\kappa$  Isotype Ctrl Antibody), anti IL-6 antibody (either 1 $\mu\text{g}/\text{mL}$  or 4 $\mu\text{g}/\text{mL}$ , eBioscience clone MP5-20F3) and recombinant IL-6 protein (10 ng/mL, R&D Systems) were used. Medium was changed every 24h until iPSC cell colonies appeared. Reprogramming plates were stained for alkaline phosphatase activity (AP detection kit, Sigma) and quantified using ImageJ software. iPSCs clones were picked, expanded and seeded at the 5  $\times$  10<sup>4</sup> cells on coverslip coated with Poly-L-Lysine (Sigma) in 24-well plates for analyses. Both iPSCs and ESCs were cultured in iPSCs medium described above.

For the survival analysis, 3x10<sup>4</sup> of either MMC-treated senescent MEFs or mESCs were seeded in duplicates onto wells of 24-well plates. 24 hr after seeding, cells were treated with the indicated doses of ABT263 for 72 hr and were counted using Neubauer chamber.

### Immunohistochemistry

TA muscles were isolated from mice and frozen directly in liquid nitrogen cooled isopentane for < 1 min and stored at -80°C or directly cryosectioned in 8- $\mu\text{m}$  sections. Tissue samples including teratomas were fixed overnight in 10% formaline, paraffine embedded and cut in 3- $\mu\text{m}$  sections, which were mounted in superfrost plus holders. For histology, sections were re-hydrated then routinely stained with Haematoxylin and Eosin (H&E). For immunohistochemistry, the muscle sections were fixed with 4% PFA in PBS and washed, the tissue sections were re-hydrated first. The following primary antibodies were used: anti-GFP (1/2000, Abcam ab13970); anti-Pax7 (1/20, DSHB); anti-laminin (1/1000, Sigma L9393); anti-Nanog (1/200, Cell signaling D2A3), anti-Gata4 (1/300, Sant Cruz sc-1237), anti-SMA (smooth muscle actin 1/200, Abcam AB5694) and anti-Tuj1 (1/500, Biolegend MMS-435P-0100). Slides were then incubated with the corresponding secondary antibodies conjugated with peroxidase from Dako.

### Immunofluorescence

For immunofluorescence on the cells, 6x10<sup>3</sup> isolated satellite cells were seeded on poly-L-lysine coated glass coverslips covered by either MMC-treated senescent MEFs (Feeders) or primary MEFs and cultured in MEF-medium: 10% FBS, 1% Penicillin-Streptomycin (15140, GIBCO) in DMEM containing doxycycline (1  $\mu\text{g}/\text{ml}$ ) for 48 hr to induce the expression of the reprogramming cassette. Cells were fixed with 4% PFA, followed by permeabilization with a buffer containing 0.1% NaCitrate and 0.1% Triton X-100 in PBS. Slides were washed, blocked with 5% BSA and incubated with respective antibodies overnight at 4°C. Second day, the slides were washed and stained with proper secondary antibodies. The following primary antibodies were used for the immunofluorescence on

the fixed cells: anti-Oct3/4 (1/250, BD Biosciences, clone 40/Oct-3); anti-Sox2 (1/250, Millipore, clone AB5063); anti-Nanog (1: 200, Cell Signaling, clone D2A3). Secondary antibodies: donkey anti-rabbit Dylight 488 and donkey anti-mouse Dylight 594 (1:500, ThermoFisher) together with DAPI (1  $\mu\text{g}$  /  $\mu\text{L}$  in PBS) were used. Images were acquired in a Olympus IX83 microscope and quantified using ImageJ software.

For immunofluorescence on the tissue section, Alexa-conjugated secondary antibodies (1/500, Molecular Probes®) together with 1g/ml of Hoechst-33342 were used. Images were acquired using a confocal Leica Spe microscope or a Zeiss Observer Z1.

#### **SA $\beta$ galactosidase (SA $\beta$ Gal) assay**

The assay was performed as previously described (Le Roux et al., 2015; Muñoz-Espín et al., 2013). Briefly, sections were fixed at room temperature for 4 min in a solution containing 1% paraformaldehyde and 0.2% glutaraldehyde in PBS. Sections were washed in PBS and incubated for 30 min in PBS pH = 5.5 and then incubated in an X-gal solution containing 4 mM K<sub>3</sub>Fe(CN)<sub>6</sub>, 4 mM K<sub>4</sub>Fe(CN)<sub>6</sub>, 2 mM MgCl<sub>2</sub>, 0.02% NP-40 (Igepal) and 400  $\mu\text{g}/\text{ml}$  X-gal (15520-018, Sigma) in PBS pH = 5.5) at 37°C overnight. For sections, X-gal substrate was changed after 24h. Samples were washed in PBS and post-fixed in 1% PFA in PBS for 30 min. After washes, samples were mounted in PBS, 20% glycerol or processed for immunocytochemistry. Finally the sections were scanned using Axio Scan Z1, and SA $\beta$ gal positive cells counted using ImageJ software.

#### **Quantitative real-time PCR**

Total RNA was extracted from cells and tissue samples with Trizol (Invitrogen) following provider's recommendations, samples were treated with DNase I before reverse transcription into cDNA following the manufacturer's protocol (iScript, BioRad). Quantitative real-time PCR was performed using LightCycler 480 (Roche) and SYBR Green Master Mix (Roche) with the standard protocol and corresponding primers (Table S1). All values were obtained at least in duplicate, and in a total of at least two independent assays. Calculation for the values was made using the  $\Delta\Delta\text{Ct}$  method, as previously described (Yuan et al., 2006).

#### **QUANTIFICATION AND STATISTICAL ANALYSIS**

The number of independent experimental replications, the definition of center and precisions measures are reported in the figure legends (n, mean  $\pm$  sem or n, mean  $\pm$  sd). Statistical analyses were performed using the GraphPad Prism v6 software. Statistical significance was assessed by the two-tailed Student's t test. *P*-value < 0.05 was considered as statistically significant.

#### **DATA AND SOFTWARE AVAILABILITY**

SA $\beta$ gal-positive cells were quantified using ImageJ software (<https://imagej.nih.gov/ij/download.html>). FACS profile analysis of SCs and FAPs were performed using Flowjo software (<https://www.flowjo.com>). All the statistical analyses were performed using the GraphPad Prism v6 software.



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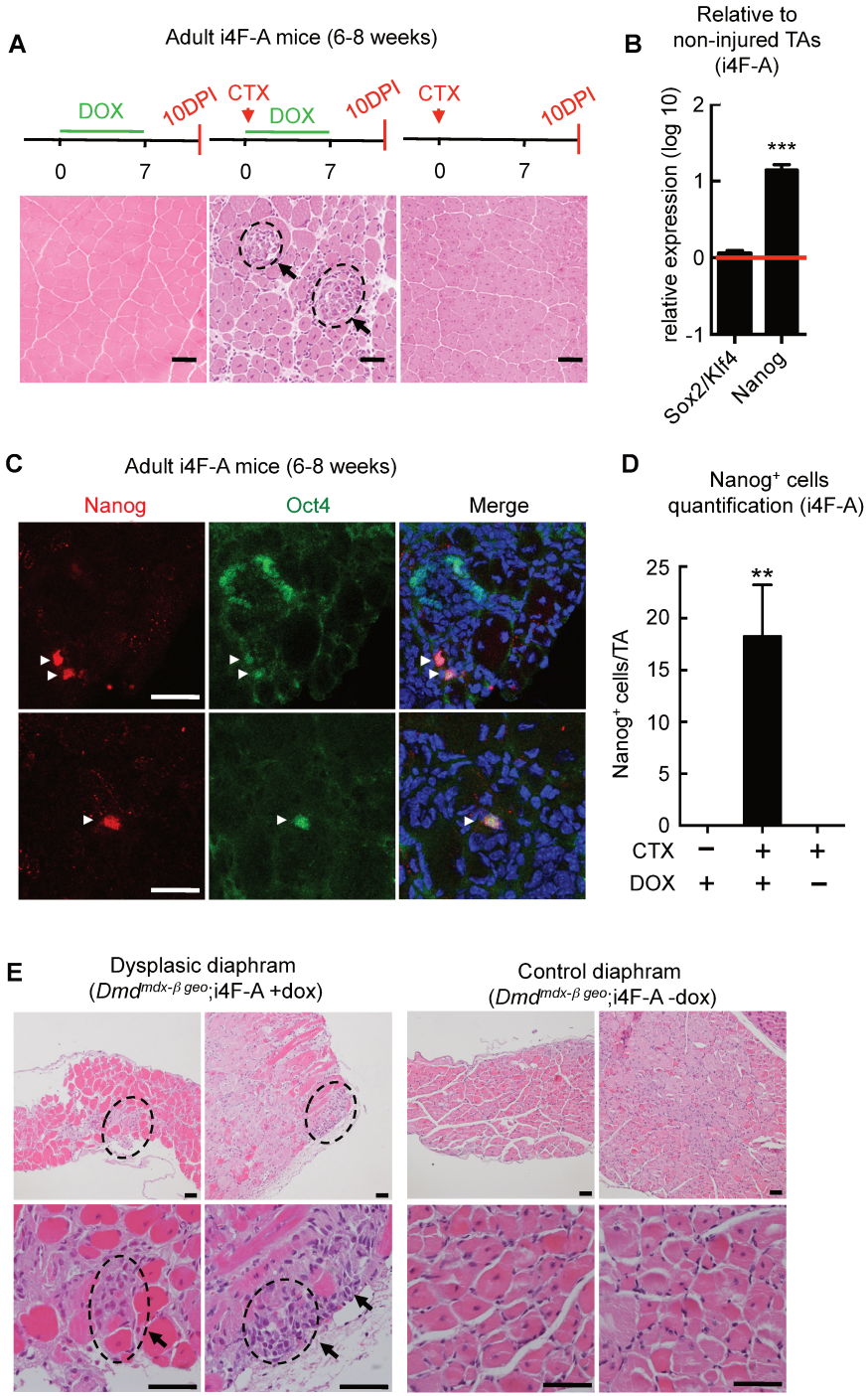
## **Supplemental Information**

### **Injury-Induced Senescence Enables**

### **In Vivo Reprogramming in Skeletal Muscle**

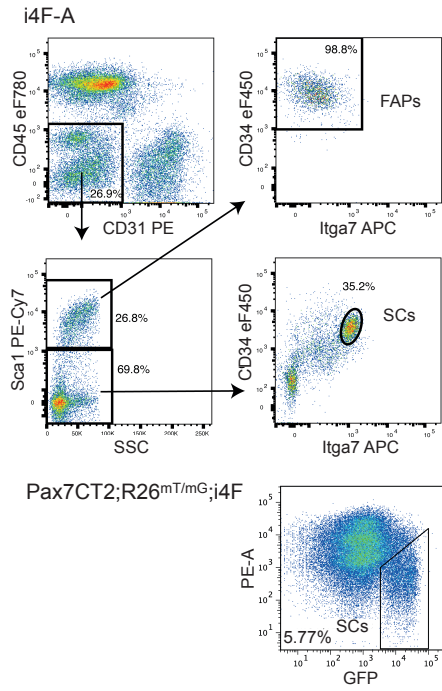
**Aurélie Chiche, Isabelle Le Roux, Mathieu von Joest, Hiroshi Sakai, Sabela Búa Aguín, Coralie Cazin, Rana Salam, Laurence Fiette, Olinda Alegria, Patricia Flamant, Shhragim Tajbakhsh, and Han Li**

**Supplemental Information**

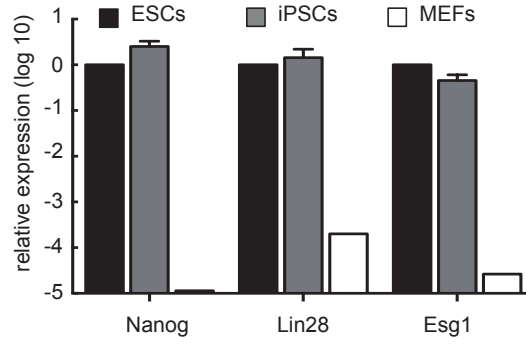


**Supplemental Figure 1 Related to Figure 1**

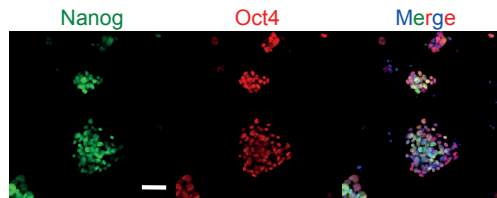
**A** FACS profile of the SCs and FAPs



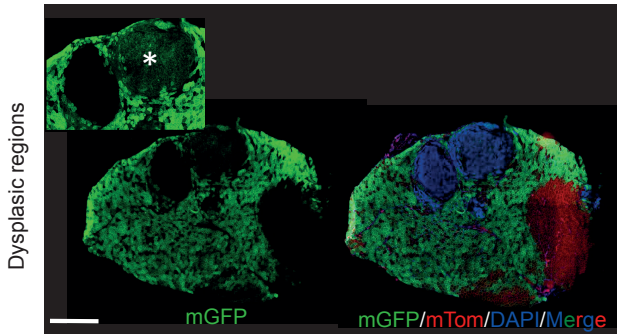
**B** iPSCs generated from SCs



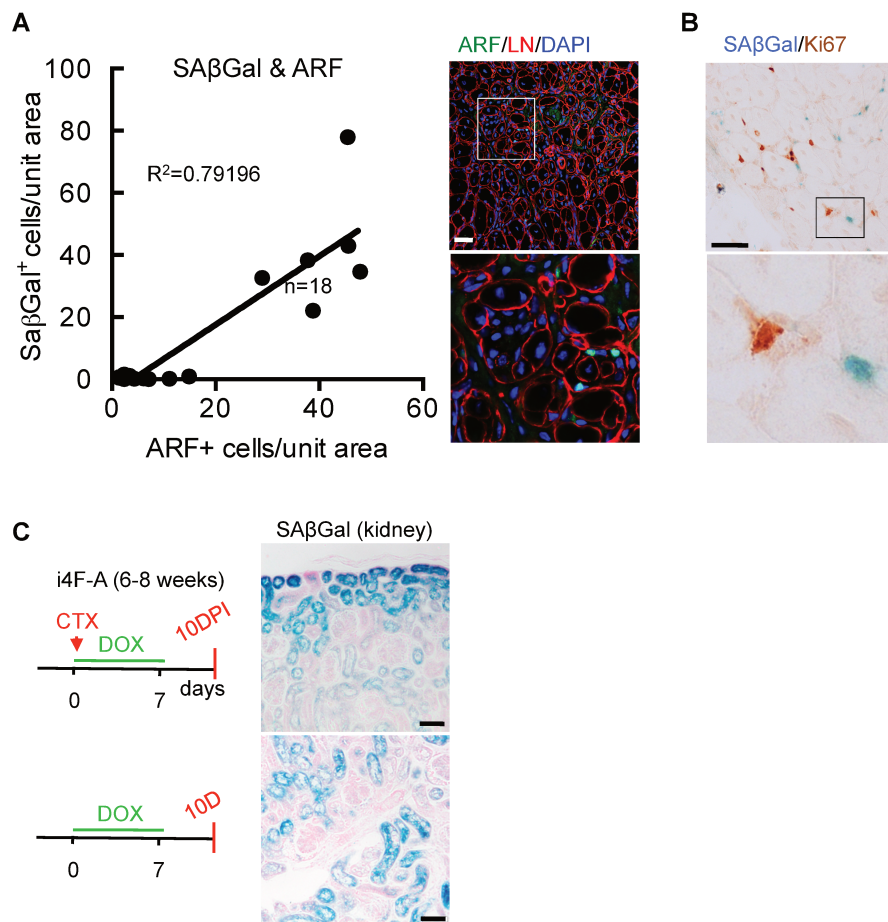
**C** iPSCs generated from SCs



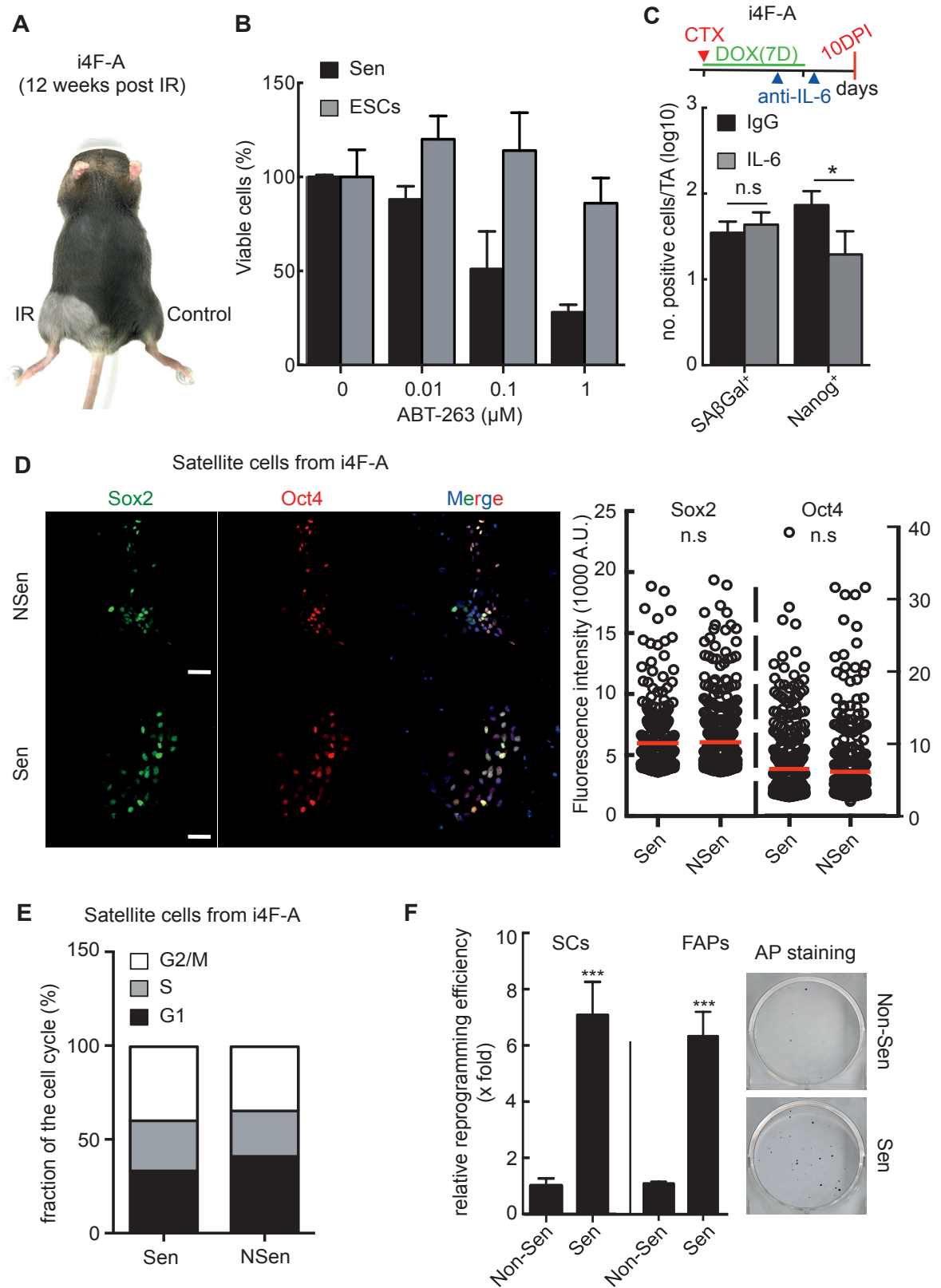
**D** Pax7CT2;R26<sup>mT/mG</sup>;i4F-A



*Supplemental Figure 2 Related to Figure 2*



*Supplemental Figure 3 Related to Figure 3*



Supplemental Figure 4 Related to Figure 4

## SUPPLEMENTAL FIGURE LEGENDS

### Figure S1. Related to Figure 1 Injury-enabled *in vivo* reprogramming

**A.** Scheme of the experiments (upper panels); Histological staining of injured TA muscles with H&E (lower panels). Arrows indicate dysplastic regions. **B.** Expression of the indicated genes in injured muscle measured by RT-qPCR. Values are relative to the expression of these genes in the non-injured TA from the same mouse (n = 9 mice, 9 TAs/condition). Data correspond to the average  $\pm$  S.D. For each assay, RT-qPCR values were obtained in duplicate or triplicate. **C.** Immunofluorescence using anti-Nanog and anti-Oct4 antibodies. White arrowheads point to Nanog<sup>+</sup> and Oct4<sup>+</sup> cells. **D.** Quantification of Nanog<sup>+</sup> cells in three different experimental settings: injured TAs with DOX, non-injured TAs with DOX and injured TAs without DOX (n = 3 per group, 2 TAs per mouse). **E.** Histological staining of diaphragms with H&E of *Dmd*<sup>mdx- $\beta$ geo</sup>;i4F-A with DOX and *Dmd*<sup>mdx- $\beta$ geo</sup>;i4F without DOX. Arrows indicate dysplastic regions. Scale bar for A: 100 $\mu$ m; B: 25 $\mu$ m; E: 200 $\mu$ m (upper panels), 25 $\mu$ m (lower panels). Statistical significance was assessed by the two-tailed Student's t-test: \*\*p<0.01, \*\*\*p<0.001.

### Figure S2. Related to Figure 2 Satellite cells are a major cell of origin for *in vivo* reprogramming in muscle

**A.** FACS profile of SCs and FAPs from i4F and Pax7CT2;R26<sup>mT/mG</sup>;i4F-A mice. **B.** Expression of the indicated genes in ESCs, iPSCs derived from SCs and MEFs by RT-qPCR. Values are relative to the expression of these genes in ESCs (n = 4 independent iPSCs clones). **C.** Immunofluorescence using anti-Nanog and anti-Oct4 antibodies on iPSCs derived from SCs. **D.** Immunofluorescence using anti-mGFP antibody on cryosections of TA muscle from Pax7CT2;R26<sup>mT/mG</sup>;i4F mice. White star shows mGFP<sup>+</sup> dysplastic region. Scale bar for C:100 $\mu$ m; D: 25 $\mu$ m.

### Figure S3. Related to Figure 3 Cellular senescence associates with *in vivo* reprogramming

**A.** The quantification and correlation of SA $\beta$ Gal<sup>+</sup> and ARF<sup>+</sup> cells from the following section (left) and the immunofluorescence using anti-ARF and anti-Laminin on injured TA (right) (n = 6 mice, value represents the average of 2 TAs per mouse). **B.** SA $\beta$ Gal staining combined with immunohistochemistry using anti-Ki67 antibody. Low (upper panel) and high (lower panel) magnification panels are shown. **C.** Scheme of the experiments; Histological staining with SA $\beta$ Gal staining on the kidney. Scale bar for A: 100 $\mu$ m; B: 25 $\mu$ m; C: 100 $\mu$ m.

### Figure S4. Related to Figure 4 Cellular senescence promotes injury induced *in vivo* reprogramming

**A.** Representative image of a locally irradiated i4F-A mouse 12 weeks post-irradiation. **B.** Survival of ESCs and senescent cells treated with different doses of ABT263 *in vitro*. **C.** Quantification of SA $\beta$ Gal<sup>+</sup> and Nanog<sup>+</sup> cells in TA muscles from anti-IL-6 treated mice (n = 4, 2 TAs per mouse) compared to IgG control (n = 4, 2 TAs per mouse). **D.** Immunofluorescence using anti-Sox2 and anti-Oct4 antibodies on SCs co-cultured with either non-senescent cells (upper panel) or senescent cells (lower panel) after 48 hours DOX administration; the quantification of fluorescence intensity (right panel) (n = 2 mice). **E.** Cell cycle analysis of SCs co-cultured with either non-senescent cells or senescent cells without dox. **F.** *In vitro* reprogramming efficiency of SCs and FAPs co-cultured on non-senescent compared to senescent cells. Representative image of plate stained with alkaline phosphatase (AP) to reveal colonies arising from reprogramming (n = 3 mice). Scale bar for D: 100 $\mu$ m. Statistical significance was assessed by the two-tailed Student's t-test: \*p<0.05, \*\*\*p<0.001.

## SUPPLEMENTAL TABLES

**Table S1. Related to STAR methods**

List of the genotyping primers

i4F-A	Neto F Neto R OSKM R	5'-GCGTCAGGCAATTTATACTCTGG-3' 5'-TTGGTGTGGAACACAGTCC-3' 5'-GCACCATCCAAAGGTCAGTG-3'	Abad et al., 2013
i4F-B	Pparg F Pparg R OSKM R	5'-CAGCATCAAATGGCTCGGTA-3' 5'-CCCATGTCCAATCCCTAGTACTAA-3' 5'-GCACCATCCAAAGGTCAGTG-3'	Abad et al., 2013
rtTA	rtTA 1 rtTA 2 rtTA 3	5'-AAAGTCGCTCTGAGTTGTTAT-3' 5'-GCGAAGAGTTTGTCTCAACC-3' 5'-GGAGCGGGAGAAATGGATATG-3'	Abad et al., 2013
Dmd <sup>mdx-βgeo</sup>	Mdx 1 Mdx 2 Mdx 3 Mdx 4	5'-CCAGATACAGAGCTAGTTAGCTAACTA-3' 5'-GCACGAGCATATGGTTGACACC-3' 5'-TAAGTTGAAAAGGTGAGGGC-3' 5'-CTCGCGGTTGAGGACAACTCTTCGC-3'	Wertz K & Fuchtbauer EM ; 1998
Pax7CT2	Cre 1 Cre 2	5'-TGATGGACATGTTTCAGGGATC-3' 5'-CAGCCACCAGCTTGATGA-3'	Le Roux et al., 2015
Rosa26 <sup>mT/mG</sup>	Rosa 1 Rosa 2 Rosa 3	5'-AAAGTCGCTCTGAGTTGTTAT-3' 5'-GGAGCGGGAGAAATGGATATG-3' 5'-GTCGTTGGGCGGTCAG-3'	Jackson 007576
p16-3MR	p16 F p16 R	5'-AACGCAAACGCATGATCAC-3' 5'-TCAGGGATGATGCATCTAGC-3'	Demaria et al., 2014

**Table S2. Related to STAR methods**

List of the primers used in the quantitative real-time PCR

mInk4a-F mInk4a-R	5'-CGTACCCCGATTCAGGTGAT-3' 5'-TTGAGCAGAAGAGCTGCTACGT-3'	Li et al, Nature 2009	PMID: 19668188
mInk4b-F mInk4b-R	5'-AGATCCCAACGCCCTGAAC-3' 5'-CCCATCATCATGACCTGGATT-3'	Li et al, Nature 2009	PMID: 19668188
mp21-F mp21-R	5'-GTGGGTCTGACTCCAGCCC-3' 5'-CCTTCTCGTGAGACGCTTAC-3'	Li et al, Nature 2009	PMID: 19668188
mp27-F mp27-R	5'- TCAAACGTGAGAGTGTCTAACG-3' 5'- CCGGGCCGAAGAGATTTCTG-3'	Li et al, Nature 2009	PMID: 19668188
mArf-F mArf-R	5'-GCCGCACCGGAATCCT-3' 5'-TTGAGCAGAAGAGCTGCTACGT-3'	Li et al, Nature 2009	PMID: 19668188
Col1a1-F Col1a1-R	5'-CCCTGGTCCCTCTGGAAATG-3' 5'-GGACCTTTGCCCTTCTTT-3'	Le Roux et al, Nat Commun, 2015	PMID: 26503169
GAPDH-F GAPDH-R	5'-TTCACCACCATGGAGAAGGC-3' 5'-CCCTTTGGCTCCACCCT-3'	Li et al, Nature 2009	PMID: 19668188
IL6-F IL6-R	5'-ATGCTCCCTGAATGATCACC-3' 5'-TCACAGATGGCGTTGACAAG-3'	Le Roux et al, Nat Commun, 2015	PMID: 26503169
MMP3-F MMP3-R	5'-CCTGATGTTGGTGGCTTCA-3' 5'-TCCTGTAGGTGATGTGGGATTC-3'	This paper	N/A

MMP13-F MMP13-R	5'-ACTTCTACCCATTTGATGGACCTT-3' 5'-AAGCTCATGGGCAGCAACA-3'	This paper	N/A
Nanog-F Nanog-R	5'-CAAGGGTCTGCTACTGAGATGCTCTG-3' 5'-TTTTGTTTGGGACTGGTAGAAGAATCAG-3'	Abad et al, Nature 2013,	PMID: 24025773
Pai1-F Pai1-R	5'-TCAGAGCAACAAGTTCAACTACACTGAG-3' 5'-CCCACTGTCAAGGCTCCATCACTTGCCCCA-3'	Kawagishi et al, Mol Cell Biol. 2013	PMID: 23508105
Sox2/Klf4-F Sox2/Klf4-R	5'- ACTGCCCTGTGCGCACAT-3' 5'- CATGTCAGACTCGCCAGGTG -3'	Abad et al, Nature 2013	PMID: 24025773





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# Genomic stability during cellular reprogramming: Mission impossible?



Mathieu von Joest, Sabela Búa Aguín, Han Li\*

Cellular Plasticity and Disease Modelling group, Department of Developmental and Stem Cell Biology, Institut Pasteur, 75015 Paris, France

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### ABSTRACT

The generation of induced pluripotent stem cells (iPSCs) from adult somatic cells is one of the most exciting discoveries in recent biomedical research. It holds tremendous potential in drug discovery and regenerative medicine. However, a series of reports highlighting genomic instability in iPSCs raises concerns about their clinical application. Although the mechanisms cause genomic instability during cellular reprogramming are largely unknown, several potential sources have been suggested. This review summarizes current knowledge on this active research field and discusses the latest efforts to alleviate the genomic insults during cellular reprogramming to generate iPSCs with enhanced quality and safety.

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## 1. Introduction

The seminal discovery by Takahashi and Yamanaka demonstrated that a small set of transcription factors, Oct4, Sox2, Klf4 and c-Myc (dispensable for acquiring pluripotency) (OSKM), are sufficient to convert terminally differentiated cells into embryonic stem cell (ESC)-like cells called induced pluripotent stem cells (iPSCs) [1]. This revolutionary breakthrough has caused an explosion in stem cell research in the last decade. It opened up numerous possibilities for disease research and regenerative medicine. Currently, patient-derived iPSCs are used as a powerful cellular system to study many diseases, which previously were difficult to investigate [2]. Furthermore, the first clinical trial using human iPSCs started in 2014. However, along with all of this exciting progress, safety concerns have been raised. The most contentious issue is the impact of reprogramming on genomic and epigenomic stability. Although the functional consequence is debatable [3], the presence of genomic aberrations in iPSCs cast a shadow over their biomedical use [4]. Genomic instability in iPSCs has been reviewed extensively elsewhere [5], therefore only a few highlights will be mentioned here. This review instead will focus on the latest efforts on understanding the source of genomic abnormalities so they might be reduced dur-

ing the reprogramming process to generate iPSCs with enhanced quality and safety.

## 2. Genomic abnormalities in iPSCs and ESCs

Pluripotent stem cells (PSCs), including ESCs and iPSCs, have two essential properties: the capacity to self-renew and the capacity to give rise to all the different cell types within an embryo [6]. Maintaining genomic integrity in PSCs is not only crucial for faithful self-renewal and accurate embryonic development, but also vital for all of their applications, such as disease modeling, drug discovery and regenerative medicine [5].

ESCs are derived from the inner cell mass (ICMs) of a pre-implantation embryo. Although the mechanism remains elusive, it is well known that ESCs accumulate genomic alterations during prolonged *in vitro* culturing [5,7]. These species-specific recurrent genomic abnormalities most likely impose a selective growth advantage, which suggests a suboptimal culturing system is potentially mutagenic. Thus, ESCs are susceptible to genomic instability that can reduce pluripotency.

iPSCs are generated directly from differentiated somatic cells through cellular reprogramming, a stochastic process accompanied by extensive rewiring of the epigenetic landscape and the gene expression network [8]. Cellular reprogramming is considered to be the ultimate proof of the nuclear equivalence theory [9] and the genomic and epigenomic properties of iPSCs have been under the spotlight since their initial discovery. Yet, we still do not

\* Corresponding author.  
 E-mail address: [han.li@pasteur.fr](mailto:han.li@pasteur.fr) (H. Li).

know if iPSCs faithfully mirror ESCs both functionally and molecularly and if they preserve the identical genome as their parental somatic cells. As elegantly enlisted in the review by De Los Angeles et al. [6], the grades of pluripotency and the quality of PSCs are assessed by multiple functional assays ranging from in vitro differentiation and teratoma formation to more stringent assays such as tetraploid complementation and single-cell chimaeras. However, the teratoma assay remains the gold standard for human iPSCs, as the most stringent in vivo methods are restricted to mouse PSCs. Lacking accurate and measurable standards impede the evaluation of human PSCs quality. Besides functional assessment, in 2011, six groups scrutinized the genome of iPSCs and revealed alarming genomic instability in these cells [10–15]. Remarkably, comparisons both to the parental somatic cells and the counterpart ESCs show that iPSCs contain a set of de novo acquired genomic abnormalities, pointing to cellular reprogramming itself as the cause of genomic instability [13].

### 3. Potential cause of genomic instability in iPSCs

Genomic instability in iPSCs could be generated in several steps [16]. This review will focus on instability generated during the reprogramming process. Although the molecular mechanism is unknown, a few clues have emerged from the growing understanding of cellular reprogramming. To endow changes in original cell identity, successful reprogramming requires reactivation of telomerase to acquire immortality, acquisition of the characteristic cell-cycle signature of PSCs [17], and induction of a metabolic reprogramming from an oxidative to a glycolytic state [8]. Thus, these processes could be mutagenic.

#### 3.1. Reprogramming methods

Yamanaka's landmark paper in 2006 used retrovirus to ectopically express OSKM. There is one obvious threat to the safety of iPSCs by employing this method, as viruses damage DNA when they integrate into the genome. The integration issue was soon overcome by several non-integrative methods [18]. Indeed, the load of genomic aberrations was reduced by the use of a non-integrative system [18,19]. However, many genomic abnormalities remained irrespective of the reprogramming methods [14,18].

#### 3.2. Replication stress (RS)

Cellular reprogramming is a rare, multi-step process, which shares many biological and molecular pathways with tumorigenesis [20]. Firstly, important tumor suppressors, p53 and Ink4a/Arf, serve as a major barrier for cellular reprogramming, most likely through regulation of proliferation, apoptosis and senescence [21,22]. Secondly, each of the four classical factors has been shown to be oncogenic in mice. c-Myc and Klf4 have well established roles in tumorigenesis, and Oct4 is an important initiator for germ cell tumors [20]. Recently Sox2 was identified as an amplified oncogene in human squamous cell carcinomas of the lung and esophagus and small-cell-lung carcinoma [23]. As oncogene activation is a major driver of genomic instability, Pasi et al. questioned the genomic status of iPSCs generated by overexpression OSKM, particularly by c-Myc. By analyzing copy number variations (CNV) in iPSCs generated with either three factors (OSK) or four factors (OSKM), Pasi et al. detected the presence of genomic abnormalities, such as deletions and amplification [10], which were much more prominent when c-Myc is included. In cancer biology, it is speculated that the cascade of oncogene-induced genomic instability is initiated by hyper-replication, which provokes the generation of replication stress (RS) [24]. RS is a type of damage defined by stalled or collapsed replication forks, which usually results in persistent

formation of single-stranded DNA (ssDNA). The pan-nuclear phosphorylation pattern of histone H2AX, reminiscent of RS [25], was observed in reprogrammed cells [26]. Noteworthy, the genomic structural variations detected in iPSCs were highly enriched at the fragile sites, a hallmark of RS [10,13,15]. In addition, acquiring iPSCs' unique cell cycle structure during the reprogramming process required increased proliferation [17], which would also generate abundant RS. Recently, Ruiz et al. further observed increased RS levels after OSK induction, by measuring  $\gamma$ H2AX expression (indirect marker of RS) and replication fork speed (direct marker of RS) [27], and the RS level was further induced with c-Myc [28]. Taken together, these studies collectively demonstrated that reprogramming factors induce RS, which contributed significantly to the de novo generation of genomic instability in the iPSCs. Moreover, they also highlighted the role of c-Myc in inducing RS and genomic abnormalities. Although c-Myc is a universal amplifier of transcriptional signals and an enhancer of cellular reprogramming processes, it is dispensable for iPSCs generation. Due to its significant impact on genomic stability of iPSCs, omitting c-Myc should become a requirement for generating hiPSCs for clinical applications.

#### 3.3. Reactive oxygen species (ROS) and oxidative stress

ROS are the natural by-products of the mitochondrial respiratory chain, which increase dramatically upon environmental stress. If they cannot be removed efficiently by the radical-scavenging system, excess ROS will cause oxidative stress and damage macromolecules like DNA and protein [29]. It is well known that ESCs have less and also immature mitochondria compared to differentiated cells [30], due to the hypoxic environment in the ICM, which corresponds to their distinct metabolic requirement [31]. Upon cellular reprogramming, cells undergo a metabolic shift from an oxidative to a glycolytic state as iPSCs' mitochondria reset back to an ESCs stage [8,31,32]. However, during the cellular reprogramming process, progressively reduced mitochondria activity cannot cope with the increased energy demand imposed by accelerated proliferation, which increases ROS production. Indeed, multiple studies detected elevated levels of oxidative stress and DNA damage highlighting the metabolic imbalance during reprogramming [33,34]. Noteworthy, hypoxic culture conditions (3–5% O<sub>2</sub>) are known to reduce oxidative stress, restrain the accumulation of DNA mutations, prevent differentiation and promote survival of multiple cell types, including PSCs [35,36]. Interestingly, hypoxia was shown to enhance the generation of iPSCs, most likely by accelerating the metabolic switch required for acquisition of pluripotency [37,38]. However, it is unknown whether hypoxia could enhance the quality of iPSCs by protecting cells from oxidative stress and DNA damage during reprogramming.

#### 3.4. Telomere maintenance

The telomere is a distinct structure consisting of repetitive DNA sequences found at the end of every chromosome. It protects chromosome ends from degradation and fusion. Due to the "end replication problem", telomere would shorten with every cell division. Telomerase is the enzyme responsible for telomere elongation, which is exclusively expressed in stem cells (including PSCs and adult stem cells) and reactivated in cancer cells. Telomere maintenance is not only important for genomic stability but also critical for cancer and ageing [39]. There are two differences between PSCs and differentiated cells in regard to telomere biology: telomere length and telomerase activity. It has been shown that telomerase is reactivated during reprogramming and both the length and epigenetic status of the telomere is rejuvenated in iPSCs similar to those found in ESCs [40]. Importantly, short telomeres

impinge both the reprogramming efficiency and the quality of the iPSCs [41,42], judged by the pluripotency tests. Therefore, proper elongation and maintenance of the telomere are essential for the genomic integrity of iPSCs.

### 3.5. DNA damage responses (DDR)

The DNA damage response is a complex signaling network that induces cell cycle checkpoints and activates DNA repair pathways once DNA damage has been sensed. DDR is an essential defense system to prevent genomic instability in the cells. ATM (Ataxia telangiectasia mutated) and ATR (Ataxia telangiectasia and Rad3-related) protein kinase cascades are the two central pathways of DDR, which sense and respond to different DNA aberrations [43]. Mainly, the ATM pathway senses double-strand breaks (DSBs) and the ATR pathway senses replication stress [44]. ATM/ATR then induces pathways that repair damaged DNA and replication forks to maintain the genome.

The impact of DDR in cellular reprogramming has been extensively studied and found to impact cellular reprogramming in a manner that is dependent on cell type. MEFs with low ATR levels are refractory to reprogramming (our own observation), while cells with one extra copy of the ATR effector, CHK1 (checkpoint kinase 1) have enhanced reprogramming efficiency [28] (more details in below). ATM-CHK2 pathway is also important for cellular reprogramming, as cells deficient in this pathway have reduced reprogramming efficiency [26]. These results suggested that both RS and DSBs are generated during the reprogramming process, and genomic integrity is important for reprogramming efficiency. Moreover, defects in DSBs repair pathways, both homologous recombination and non-homologous recombination, hindered cellular reprogramming [45–47]. Collectively, these data indicate that different types of DNA damages are generated during the reprogramming process. However, it is unknown whether they are caused by sources other than the stresses discussed above.

## 4. Recent advances in improving genomic stability of iPSCs

Clearly, genomic stability is fundamental for all of the iPSCs applications, from disease modeling and drug discovery in vitro, to cell therapy and regenerative medicine in vivo, as outlined comprehensively elsewhere [5]. Recent efforts made in improving genomic stability in PSCs could be split into three directions: (1) optimizing in vitro propagation conditions, which are important for both ESCs and iPSCs; (2) alleviating genomic instability generated during the reprogramming process, which is particularly important for iPSCs; and (3) selecting the most suitable somatic cell type. We will only discuss the latter two points, as the first point has been extensively reviewed [5]. Although the molecular mechanisms of genomic instability during reprogramming are unknown, several studies have directly tested the aspects discussed above to improve the quality of the iPSCs.

Replication stress has been linked to genomic instability in iPSCs since the initial genomic analyses of these cells in 2011 [16]. ATR-mediated checkpoint pathway is the essential replication stress response pathway. CHK1 is one of the most important ATR downstream target, which coordinates the replication stress response and cell cycle checkpoint response. Previously, it was shown that one extra copy of CHK1 protects mice from oncogene-induced RS [48]. Ruiz et al. took advantage of this model and demonstrated that genetically limiting RS generation, by CHK1 overexpression, could also improve both the reprogramming efficiency and the iPSCs quality [28], highlighting the significant role of RS in generating genomic instability during reprogramming. Insufficient nucleotide synthesis upon oncogene induced DNA replication is

partially responsible for the generation of RS [49,50]. Interestingly, nucleoside supplementation, the cell-permeable chemical form of nucleotides was shown to reduce oncogene-induced RS [49] and limit chromosomal instability [51,52]. Remarkably, cells suffered less RS when Ruiz et al. added nucleoside supplements during reprogramming. More importantly, iPSCs generated with additional nucleoside supplements contained less CNVs [28].

Nuclear transfer is another way to reprogram somatic cells into PSCs. It has been shown PSCs generated through oocyte-induced reprogramming have a better quality compared to iPSCs [53]. By screening factors highly expressed in early cleavage-stage embryos, Jiang et al. found Zscan4 dramatically increased reprogramming efficiency partially due to indirect repression of p53, an important barrier of reprogramming [54]. Moreover, iPSCs generated with Zscan4 have slightly improved in vivo potency judged by tetraploid complementation assay [54]. In ESCs, Zscan4 is critical for telomere maintenance and genomic integrity [55]. Jiang et al. further demonstrated that Zscan4 promotes telomere elongation and protects them from DNA damage during reprogramming, suggesting the positive impact of improved telomere maintenance on genomic integrity [54]. Although there is no chemical mimic to Zscan4, it is encouraging that nature has the way to actively protect ESCs fidelity in vivo. Further studies on how the genome is being protected during early embryonic development would reveal new means to improve PSCs quality in vitro.

Oxidative stress induced during cellular reprogramming is most likely intrinsic to the process. Several studies have explored using anti-oxidants to reduce ROS and oxidative stress. The first clue came from ascorbic acid, commonly known as vitamin C (Vc). Esteban et al. showed that this widely used nutritional supplement could improve reprogramming efficiency [33]. Although the following studies suggested various functions of Vc on the epigenome [56], its potent anti-oxidative activity was behind the rationale of the initial study. Subsequently, Ji et al. directly tested whether lowering ROS levels by antioxidants is sufficient to improve iPSCs quality [57]. They added two antioxidants, *N*-acetyl-cysteine (NAC) and Vc, during reprogramming. Indeed, by simply adding one of the two antioxidants, Ji et al. were able to reduce ROS levels and to generate iPSCs with less de novo CNVs. Besides supplying antioxidants, reducing O<sub>2</sub> tension in the medium during reprogramming could also improve the quality of iPSCs. As discussed briefly above, hypoxic culture condition promotes the generation of iPSCs by both classical viral-induction and synthetic modified mRNAs [37,58]. Moreover, it was also essential for direct transdifferentiation of somatic fibroblast into neuronal progenitor cells using only chemicals [59]. Although the impact of hypoxia on genomic integrity of iPSCs is unknown, it is reasonable to consider it as the routine culturing condition for iPSCs generation. Furthermore, it would be interesting to know if supplementing antioxidants in hypoxic culturing condition could further lower the genomic abnormalities in iPSCs.

All of the studies reviewed here targeted different types of stressors during reprogramming. It would be interesting to test if they have synergistic effects. Moreover, many small molecules were shown to facilitate reprogramming through different routes [60], it is highly likely that some of them would also reduce genomic abnormalities. Besides lowering the stress level, it is also important to explore the means to boost the DNA damage repair machinery, so that they could repair the genome more efficiently.

Lastly, one overlooked point is selecting the most suitable somatic cell type. It is known that different somatic cell type has different reprogramming capacity [61] and iPSCs generated from different donor cells could carry lineage-specific epigenetic memory, which limit their differentiation potential [62]. Although the molecular mechanisms that cause different reprogramming capacities among different somatic cell types are largely unknown, the

preexisting genomic instability and intrinsic DNA damage repair capacity in the donor cells might play a critical role in regulating the quality of the iPSCs. Currently, the determining factor of the somatic cell types for reprogramming is whether they can be readily obtained. It would be compelling to compare the quality of iPSCs derived from different somatic cell type and identify the most suitable cells of origin for the future iPSCs generation.

## 5. Summary

Erasing the identity of a fully differentiated cell to acquire pluripotency involves complex molecular events, which is a demanding task for genomic maintenance. There are two properties of PSCs established in the early phase of reprogramming that intrinsically threaten genomic stability. Firstly, PSCs have an atypical cell-cycle regulation with a high proliferation rate and a short G1 phase [17]. Secondly, PSCs heavily rely on anaerobic glycolysis instead of oxidative phosphorylation [38]. Hyper-proliferation and reduced mitochondria activity create an inherent conflict and result in elevated replication stress and increased ROS production. Therefore, it would be difficult to generate iPSCs completely devoid of errors. Consequently, the critical question becomes how to reduce the load of genomic instability to an inconsequential level? The methods discussed in this review summarize the current effort in reducing genomic aberrations during reprogramming. Although most of the methods alleviate the DNA damage load in iPSCs, there is no direct functional assessment of the quality of iPSCs. Therefore it would be good to know if genomic aberrations influence iPSCs function. If so then the origin and type of genomic aberrations should be evaluated to determine which are the most detrimental. Addressing these issues will largely depend on our growing understanding of the mechanisms underlying genomic instability during reprogramming. In the meantime, it is equally important to characterize and classify the genomic aberrations acquired during reprogramming; to discriminate adverse from inconsequential abnormalities and to identify their origin so they might be diminished. The discovery of somatic cells that could be reprogrammed back to pluripotent stage forever changed our restricted view on cellular plasticity. Further research in this exciting field will not only provide crucial guidelines for PSCs applications but also reveal unprecedented fundamental principles of genome maintenance.

## Conflict of interest

The authors declare no conflict of interests.

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# Chapter 1

## Detecting Cellular Senescence in Reprogramming

Coralie Cazin, Mathieu von Joest, and Han Li

### Abstract

Cellular senescence has been suggested to facilitate tissue regeneration via promoting cellular plasticity. Here, we describe multiple systems, both in vitro and in vivo, to detect senescence in the context of cellular reprogramming.

**Key words** Cellular senescence, Reprogramming, Cellular plasticity, SA- $\beta$ -Gal

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### 1 Introduction

Cellular senescence is a stable cell cycle arrest caused by stresses during various biological and pathological conditions [1–3]. Interestingly, these cells remain metabolically active and secrete a vast number of factors including cytokines, chemokines as well as growth factors, which is collectively termed as SASP (senescence associated secretory phenotype). Senescent cells have multifaceted capabilities and are involved in a wide range of physiological and pathological processes, such as development, cancer, and aging [2, 4, 5]. More recently, growing evidence indicates that senescent cells might facilitate tissue repair and regeneration [6, 7].

Cellular plasticity is the capacity of a cell to change its identity. Nuclear reprogramming presents one of the best examples of cellular plasticity. Somatic cells can be reprogrammed into a pluripotent stage via forced expression of the Yamanaka factors (Oct4, Sox2, Klf4, and c-Myc (OSKM)). The induced pluripotent stem cells (iPSCs) can be obtained both in vitro and in vivo [8, 9].

Senescence is important for cellular plasticity. It is a cell-intrinsic barrier for reprogramming [10]. However, recent studies suggest senescent cells could promote cellular plasticity extrinsically to facilitate tissue regeneration via SASPs [11–13].

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Coralie Cazin and Mathieu von Joest contributed equally and should be considered co-first authors.

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Taken together, the emerging data highlights the importance of investigating cellular senescence, particularly *in vivo* senescence, in a context-dependent manner. Here, we present various protocols to investigate the impact of cellular senescence on cellular plasticity, both *in vitro* and *in vivo*. First, we will introduce the system to study the impact of cellular senescence on *in vitro* reprogramming. Next, we will describe how to detect senescent cells in two tissues with different susceptibility to *in vivo* reprogramming: liver (permissive) and skeletal muscle (nonpermissive) [11, 12]. SA- $\beta$ -Gal assay and antibody immunostaining are used together to detect senescent cells. Nanog, a marker of pluripotency, is used to evaluate *in vivo* reprogramming in the liver and skeletal muscle.

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## 2 Materials

Prepare all the solutions using sterile water. All the reagents are prepared and stored at room temperature (unless indicated otherwise).

### 2.1 Generation of Senescent Cells

1. Mouse Embryonic Fibroblasts (MEFs) [14].
2. Mouse embryo fibroblast (MEF) Medium: Dulbecco's modified Eagle Medium (DMEM) with high glucose (4.5 g/L), 10% FBS, 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin.
3. Phosphate-buffered saline (PBS), autoclaved.
4. 0.05% trypsin-EDTA solution
5. 1 mg/mL mitomycin C (MMC) stock solution, filtered and stored at  $-20^{\circ}\text{C}$ .
6. X-ray irradiator (Optional).
7. 0.2  $\mu$ m filters
8. Tissue culture plates: 100-mm and 150-mm.
9. Conical centrifuge tubes: 15-mL and 50-mL.
10. Centrifuge.
11. Phase-contrast inverted light microscope.
12. CO<sub>2</sub> tissue culture incubator.
13. Laminar flow hood with standard tissue culture setup.

### 2.2 In Vitro Reprogramming

1. Reprogramming medium: Dulbecco's modified Eagle Medium (DMEM) with high glucose (4.5 g/L), 15% Knock-Out Serum Replacement (KSR), 2 mm GlutaMAX, 0.1 mm nonessential amino acids, 0.1 mm 2-mercaptoethanol, 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin, 1000 U/mL mouse leukemia inhibitory factor (LIF).
2. HEK 293T cells.
3. Wild-type (WT) MEFs.

4. 1 mg/mL doxycycline.
5. X-tremeGene HP DNA transfection reagent (Roche).
6. Polybrene stock solution (8 mg/mL).
7. Retroviral vectors: pMXs-c-Myc, Addgene: 13375; pMXs-Klf4, Addgene: 13370; pMXs-Sox2, Addgene: 13367; pMXs-Oct3/4, Addgene: 13366), pCL-Eco, Addgene: 12371; control retroviral vector containing GFP.
8. Syringes.
9. 0.45  $\mu$ m filters.
10. Aluminum foil.
11. Lab Rocker.
12. 4% paraformaldehyde (PFA)
13. Alkaline phosphatase detection kit.

**2.3 SA- $\beta$ -Gal Staining**

1. SA- $\beta$ -Gal fixation solution: 2% formaldehyde and 0.2% glutaraldehyde in PBS.
2. 0.4 m citric acid/phosphate buffer (pH = 6.0): resuspend sodium phosphate dibasic ( $\text{Na}_2\text{HPO}_4$ ) and citric acid monohydrate in water. Add 36.85 mL of 0.1 m citric acid to 63.15 mL 0.2 m dibasic sodium phosphate. Mix and adjust pH to 6 with citric acid if necessary (*see Note 1*).
3. X-Gal: Dissolve the X-Gal powder in dimethylformamide (DMF) and store in  $-20^\circ\text{C}$  (*see Note 2*).
4. X-Gal solution: 40 mm Citric acid/phosphate buffer, 150 mm NaCl, 2 mm  $\text{MgCl}_2$  (Store at RT.), 4 mm  $\text{K}_3\text{Fe}(\text{CN})_6$  (Store at  $4^\circ\text{C}$ ), 4 mm  $\text{K}_4\text{Fe}(\text{CN})_6$  ((Store at  $4^\circ\text{C}$ ), 1 mg/mL X-Gal in water, freshly made upon usage in a tube wrapped with aluminum foil (*see Note 3*).
5. X-Gal solution-muscle specific: 4 mm  $\text{K}_3\text{Fe}(\text{CN})_6$ , 4 mm  $\text{K}_4\text{Fe}(\text{CN})_6$ , 2 mm  $\text{MgCl}_2$ , 0.01% NP-40, and 400  $\mu\text{g}/\text{mL}$  X-Gal in PBS, pH = 5.5 in a tube wrapped with aluminum foil (*see Note 4*).
6. 0.2% (Eosin) (*see Note 5*).
7.  $37^\circ\text{C}$  incubator.

**2.4 ARF and Ki67 Staining**

1. PFA fixation solution: PBS containing 4% paraformaldehyde.
2. Permeabilization solution: 0.1% NaCitrate, 0.5% Triton X-100 in water (*see Note 4*).
3. Blocking solution: 10% FBS, 3% BSA, 0.5% Triton X-100 in PBS. Stored at  $4^\circ\text{C}$  (*see Note 5*).
4. PBS-0.5% Tween 20: PBS containing 0.5% Tween 20.
5. Antibodies: Ki67 (Abcam, ab15580); p19Arf (Santa Cruz Biotechnology, 5-C3-1).

6. 3,3'-diaminobenzidine (DAB) dilution: dilute the 3,3'-diaminobenzidine (DAB) in the buffer solution from the kit (DAB<sup>+</sup> + substrate buffer). 20  $\mu$ L of DAB for 1 mL of buffer solution.

### 2.5 NANOG Staining

1. PFA fixation solution: PBS containing 4% paraformaldehyde.
2. Permeabilization solution: 0.1% NaCitrate, 0.1% Triton X-100 in water. Store at RT.
3. Blocking solution: 5% FBS in PBS (*see Note 6*).
4. Nanog antibody (Cell Signaling, 8822S).
5. EnVision+ Kits (HRP. Rabbit. DAB+) Dako K4010.

### 2.6 In Vivo Reprogramming

1. Reprogrammable mouse model [8].
2. Doxycycline (1 mg/mL) (Sigma 24390-14-5).
3. Cardiotoxin (Lotaxan Valence, France). Stock solution (40  $\mu$ M): 1 mg in 3676  $\mu$ L of 0.9% NaCl, 50  $\mu$ L/aliquot, store at  $-20^{\circ}$ C. Working solution (10  $\mu$ M): add 150  $\mu$ L of 0.9% NaCl to 50  $\mu$ L stock aliquot on ice at the day of the injury. Inject 40  $\mu$ L /TA.
4. 0.3 mL needles: 29G  $\times$  1/2"–0.33  $\times$  12 mm.

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## 3 Methods

### 3.1 Evaluation the Impact of Cellular Senescence on In Vitro Reprogramming

Caution: All steps have to be performed in a sterile flow hood.

#### 3.1.1 Generating Senescent MEFs

1. Culture and expand MEFs: Thaw one vial of MEFs in one 100-mm tissue culture plate. Once cells are confluent, pass them into one 150-mm tissue culture plate. Pass cells again into five 150-mm tissue culture plates. When cells are confluent, induce senescence either with MMC treatment or irradiation.
2. MMC treatment induced senescence: Add 1 mg/mL MMC stock solution directly into the MEF medium to a final concentration of 10  $\mu$ g/mL. Treat the cells with MMC for 3 h in the incubator.
3. Washing the cells with PBS twice to remove MMC. Trypsinize the cells and resuspend them in MEF medium and count.
4. Seed the cells at the density of  $2.8 \times 10^4$  cells/cm<sup>2</sup> (for example seed  $1.5 \times 10^6$  cells in 100-mm tissue culture plate) and culture in MEF medium. Cells will become senescence after 48 h and can be confirmed by SA- $\beta$ -Gal staining.

5.  $\gamma$ -Irradiation induced senescence (Optional): After **step 1**, cells can also be trypsinized and resuspended in MEF medium, adjusting the concentration to  $2\text{--}6 \times 10^7$  cells/mL. Gently mix the cells and irradiate them for total 3000 rad. After the irradiation, cells can be used directly as **step 4**, or kept frozen for future use.

### 3.1.2 SA- $\beta$ -Gal Staining

1. Remove medium and wash MEFs twice with PBS. Add SA- $\beta$ -Gal fixation solution to the plate and make sure the solution covers the surface completely. Incubate at room temperature for 15 min.
2. Aspirate the fixation solution and wash three times with PBS. Incubate cells with freshly made SA- $\beta$ -Gal solution overnight at 37 °C, protected from light.
3. Remove the solution completely and wash the plates with running water. Plates can be stored in PBS at 4 °C up to 1 week, protected from light.

### 3.1.3 *In Vitro* Reprogramming with Senescence-Conditional Medium

1. Generating senescence conditional Medium (CM): Incubate senescent cells with reprogrammable medium w/o LIF (10 mL medium for 100-mm plate). Collect the CM every 24 h and replace with 10 mL fresh reprogrammable medium w/o LIF. CM can be collected for 5 days. Filter the collected CM using 0.2  $\mu\text{m}$  filter. CM can be used directly or kept at  $-20$  °C.
2. Reprogramming MEFs with retroviral infection: *in vitro* reprogramming is performed as described previously [15]. Day 1: Seed  $5 \times 10^6$  293T cells in one 100-mm plate.
3. Day 2: Transfect 293T cells using X-treme Gene HP transfection reagent and pMXs-vectors. Mix 4  $\mu\text{g}$  of individual pMXs plasmid or control vector (e.g., pMSCV Puro IRES GFP) with 4  $\mu\text{g}$  of pCLEco. Incubate the plasmids mix with 8  $\mu\text{L}$  of X-treme Gene HP transfection reagent and 594  $\mu\text{L}$  of DMEM (DNA: transfection reagent = 1:1) at RT for 30 min. Add one plasmids mix onto one plate of 293T cells.
4. Day 3: Change the medium of HEK293T cells using MEF medium. On the same day, seed  $5 \times 10^5$  WT MEFs/100-mm plate in MEF medium.
5. Day 4–5: retrovirus infection of MEFs. Collect medium from every 293T plate in separate falcon tubes and replace with 10 mL of fresh MEF medium. Centrifuge the collected medium at  $250 \times g$  for 5 min at RT. Pass the medium through 0.45  $\mu\text{m}$  filters and add Polybrene to the final concentration of 8  $\mu\text{g}/\text{mL}$ . Mix the factors (2 mL of every factor/plate) first in a falcon tube then add the mix onto WT MEFs. Perform four rounds of infection in total, 12 h interval.

6. Day 6: Seed the infected MEFs onto 35-mm plates in MEF medium. The amount of MEFs seeded should yield 20–40 clones per plate, which dependent on the infection and reprogramming efficiency. It is advised to determine these parameters prior to the experiment.
7. Day 7: Replace the medium to CM medium supplemented with LIF (1000 U/mL) to start reprogramming.
8. Change the medium every 2 days. iPSCs colonies should be clearly visible under the microscope after 2 weeks.
9. Quantification of iPSCs: Once the colonies are clearly visible, the plates are processed for alkaline phosphatase (AP) staining according to the manufacturer's protocol. Quantification can be done either manually or with image J software.

### **3.2 Evaluation the Impact of Cellular Senescence on In Vivo Reprogramming**

#### *3.2.1 SA- $\beta$ -Gal Staining on Frozen Liver Section*

1. Fix the sections for 4 min in fixation solution, at RT (*see Note 7*). Wash the sections with PBS three times, 5 min each time.
2. Incubate the sections in the X-gal solution at 37 °C overnight (*see Note 8*). Wash the sections with PBS three times, 10 min each time.
3. Post-fixed in 1% paraformaldehyde in PBS for 30 min, at RT (*see Note 7*). Wash the sections with PBS three times, 10 min each time.
4. Mount the slides with PBS containing 20% glycerol.

#### *3.2.2 SA- $\beta$ -Gal Staining on Frozen Muscle Section [16]*

The tibialis anterior (TA) muscles of reprogrammable mice are injured with cardiotoxin and treated with doxycycline (1 mg/mL) in the drinking water for 7 days to induce both senescence and reprogramming in vivo. TA muscles are harvested and prepared as described elsewhere [16].

1. Fix the sections for 4 min in fixation solution, at RT (*see Note 7*). Wash the sections with PBS three times, 5 min every time.
2. Incubate sections for 30 min in PBS pH = 5.5 (*see Note 9*).
3. Incubate sections in the X-gal solution muscle specific at 37 °C for at least 24 h protected from light (*see Note 10*). Wash the sections with PBS three times, 10 min every time.

*If only SA- $\beta$ -Gal staining is desired, continue with the next steps. If costaining with Ki67 is desired, please forward to Subheading 3.2.4. If costaining with Nanog is desired, please forward to Subheading 3.2.5.*

4. Post-fix in 1% paraformaldehyde in PBS for 30 min, at RT (*see Note 7*). Wash the sections with PBS three times, 10 min each time.

5. Counterstain with 0.2% eosin at RT. Immerse the slides in the eosin solution for 1 min and rinse them with water briefly (*see Note 11*).
6. Mount the slides with PBS containing 20% glycerol (*see Note 12*).
7. Post-fix in 1% paraformaldehyde in PBS for 30 min, at RT (*see Note 7*). Wash the sections with PBS three times, 10 min every time.
8. Counterstain with 0.2% eosin at RT. Immerse the slides in the eosin solution for 1 min and rinse them with water briefly (*see Note 11*).
9. Mount the slides with PBS containing 20% glycerol (*see Note 12*).

3.2.3 Immunostaining  
Using Anti-p19ARF

1. Fix the slides with PFA fixation solution for 10 min at RT (*see Note 7*). Wash the sections with PBS three times, 10 min each time.
2. Add 200  $\mu$ L of the permeabilization solution directly onto the slides and incubate at RT for 5 min. Wash the sections with PBS-Tween 20 twice, 5 min each time.
3. Add 200  $\mu$ L of blocking solution directly on the slides for 30 min at RT.
4. Incubate with the primary antibodies: 2  $\mu$ g/mL of Ki-67 or 0.8  $\mu$ g/mL of p19Arf overnight at 4 °C in the blocking solution (*see Note 13*). Wash the sections with PBS, 10 min each time.
5. Wash with 200  $\mu$ L PBS containing 0.25% BSA on slides at RT for 5 min (*see Note 14*).
6. Incubate with the secondary antibody in blocking solution for 1 h at RT (*see Note 15*). Wash the sections with PBS for three times, 5 min each time.
7. Mount the slides with aqueous nonfluorescing mounting medium.

3.2.4 Immuno-  
histochemistry Using  
Anti-Ki67

1. Fix the slides with PFA fixation solution for 10 min at RT (*see Note 7*). Wash the sections with PBS three times, 10 min each time.
2. Add 200  $\mu$ L of the permeabilization solution directly onto the slides and incubate at RT for 5 min. Wash the sections with PBS-0.5% Tween 20 twice, 5 min each time.
3. Add 200  $\mu$ L of blocking solution directly on the slides for 30 min at RT.
4. Adding 100  $\mu$ L of rAb-HRP from Dako kit (ready to use) for 45 min at RT (*see Note 15*). Wash the sections with PBS three times, 5 min each time.
5. Dilute DAB in the buffer solution (*see Note 16*).

6. Visualization: add 100  $\mu$ L of DAB previously diluted (*see Note 16*) on every slide up to 10 min at RT. Observe the slides under the microscope (*see Note 17*). Stop the reaction by washing with water.

3.2.5 *Immuno-histochemistry Using Anti-NANOG Antibody on Frozen Tissue Sections*

1. Fix the slides with PBS containing 4% paraformaldehyde for 10 min, at RT (*see Note 7*). Wash the sections with PBS twice, 10 min each time.
2. Add 200  $\mu$ L of the permeabilization solution directly onto the slides and incubate at RT for 5 min. Wash the sections with PBS twice, 5 min each time.
3. Wash the sections with 200  $\mu$ L PBS containing 0.25% BSA directly on slides at RT for 5 min (*see Note 14*).
4. Incubate the slides with 1.25  $\mu$ g/mL of Nanog antibody overnight at 4 °C in PBS containing 5% FBS (*see Note 15*). Wash the sections with PBS twice, 10 min each time.
5. Wash with 200  $\mu$ L PBS containing 0.25% BSA on the slides at RT for 5 min (*see Note 14*).
6. Incubate with the secondary antibody by adding 100  $\mu$ L of rAb-HRP from Dako kit for 45 min at RT (*see Note 15*). Wash the sections with PBS three times, 5 min each time.
7. Dilute DAB in the buffer solution (*see Note 16*).
8. Visualization: add 100  $\mu$ L of DAB solution on every slide up to 10 min at RT. Observe the slides under the microscope (*see Note 17*). Stop the reaction by washing with water.
9. Counterstain with Fast red solution for 20 min, at RT (*see Note 11*). Wash with water briefly.
10. Dehydrate with 95% ethanol for 5 min followed with 100% ethanol, 2  $\times$  5 min.
11. Mount the slides with quick-hardening mounting medium.

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## 4 Notes

1. The citric acid–phosphate buffer can be stored at 4 °C. Adjusting the pH is a crucial step for staining.
2. The X gal can be stored in aliquot, protected from light, at –20 °C up to 6 months.
3. The  $K_3Fe(CN)_6$  solution and  $K_4Fe(CN)_6$  solution can be stored at 4 °C but they need to be protected from light.
4. We find that this solution works better for the muscle cryosections.

5. Eosin solution can be kept at RT and reused after filtering if necessary.
6. Blocking solutions can be filtered through a 0.45  $\mu\text{m}$  filter, aliquoted and stored at  $-20\text{ }^{\circ}\text{C}$ . It can be stored at  $4\text{ }^{\circ}\text{C}$  for 6 months.
7. Perform the fixation under the hood. Do not fix longer to maintain a proper staining and let the enzymatic reactions occur for a proper SA- $\beta$ -Gal staining. It is essential to perform the post-fixation for SA- $\beta$ -Gal staining alone for a good conservation of the staining.
8. Make sure that the temperature is at  $37\text{ }^{\circ}\text{C}$  and that slides are protected from light overnight.
9. Adjusting the pH is a crucial step for staining. Use a magnetic stir bar to obtain the correct pH of the final solution.
10. The incubation requires minimal 24 h and can last for 48 h to maximize the SA- $\beta$ -Gal signal. The solution needs to be changed after 24 h incubation.
11. Eosin solution and fast red solution can be kept at RT and reused after filtering if necessary. Incubation time can be adjusted depending on the intensity wanted. Slides should be analyzed quickly after mounting for eosin counterstaining because the eosin is soluble in water and the counterstaining will be weaker with time. We choose eosin because SA- $\beta$ -Gal staining is not stable in water.
12. For a longer conservation, you can mount the slides with aqueous nonfluorescing mounting medium.
13. Incubate slides in a box with wet paper towel to prevent evaporation.
14. We find that it is best to prepare this fresh each time.
15. Incubate slides in a box with wet paper towel to prevent evaporation and protect from light.
16. Freshly prepared and the diluted DAB solution is stable up to 1 week at  $4\text{ }^{\circ}\text{C}$ .
17. The incubation time can be adjusted to minimize the background but have to be the same for all the slides.

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Cellular senescence is a physiological response to a stress, leading to a permanent and irreversible cell cycle arrest. Entry in senescence goes along with a myriad of changes, one of the major being the secretion of various factors gathered under the term Senescence Associated Secretory Phenotype (SASP). Recent studies showed the critical role of senescent cells in regeneration, in particular through SASP. However, mechanisms enabling senescent cells to influence cellular plasticity in the context of tissue repair remain unknown.

The recent discovery of cellular reprogramming, which allows the transformation of a differentiated cell into an induced pluripotent stem cell (iPSC), highlighted the plasticity of differentiated cells. This major breakthrough generated substantial hopes for regenerative medicine and understanding of diseases.

During my PhD, I contributed to decipher the mechanisms by which senescence induces cellular plasticity. As a first step, we showed that injury-induced senescence could lead to the reprogramming of skeletal muscle, in particular by IL-6 secretion. Moreover, thanks to lineage tracing experiments, we noticed that one of the major targets of the reprogramming process was the muscle satellite cell type. Secondly, I decrypted more precisely how SASP influence the reprogramming *in vitro* and showed that this process could be IL-6 independent. Finally, I performed a proteomic study to identify the set of factors secreted by senescent cells and to determine new factors that could affect cellular reprogramming, independently of IL-6. I managed to detect amphiregulin as an important factor and showed that adding this protein strengthened the SASP effect on reprogramming. I pointed out that amphiregulin allowed an increase on reprogramming both *in vitro* and *in vivo*. Overall, this study not only allows us to better appreciate how senescence influences cellular plasticity after muscular injury, but also links a new factor to cellular reprogramming.

**Key words: Senescence, in vivo Reprogramming, Cellular Plasticity, IL-6, Amphiregulin, Pluripotency.**

La sénescence cellulaire est une réponse physiologique faisant suite à un stress et qui entraîne l'arrêt permanent et irréversible du cycle cellulaire. L'entrée de la cellule en sénescence s'accompagne de nombreux changements dont un des principaux est la sécrétion de nombreux facteurs communément réunis sous le terme de SASP (Senescence Associated Secretory Phenotype). Des études récentes ont démontré le rôle crucial des cellules sénescents dans la régénération, notamment grâce au SASP. Cependant les mécanismes permettant aux cellules sénescents d'influer sur la plasticité cellulaire dans le contexte de la réparation tissulaire demeurent inconnus.

La découverte récente de la reprogrammation cellulaire qui permet de transformer une cellule différenciée en cellule souche pluripotente induite (iPSC for induced Pluripotent Stem Cell) a mis en évidence la plasticité des cellules différenciées. Cette découverte a d'ailleurs généré beaucoup d'espoirs pour la médecine régénérative et pour la compréhension des maladies.

Au cours de ma thèse, j'ai participé à la compréhension des mécanismes permettant à la sénescence d'induire la plasticité des cellules. Dans un premier temps nous avons démontré que la sénescence induite après blessure pouvait induire la reprogrammation du muscle squelettique notamment via la sécrétion d'IL-6. De plus, grâce à des expériences de traçage génétique, nous avons montré qu'une des cibles principales de la reprogrammation était les cellules satellites musculaires. Dans un second temps, *in vitro*, j'ai décrypté plus précisément la manière dont le SASP influençait le processus de reprogrammation cellulaire et montré qu'il pouvait être indépendant de l'IL-6. Enfin, j'ai effectué une étude protéomique afin d'identifier l'ensemble des facteurs sécrétés par les cellules sénescents et de déterminer quels nouveaux facteurs pouvaient affecter la reprogrammation cellulaire indépendamment de l'IL-6. J'ai réussi à identifier l'amphiréguline et j'ai démontré que l'addition de cette protéine amplifiait l'effet du SASP sur la reprogrammation cellulaire. J'ai notamment observé que l'amphiréguline permettait une augmentation de la reprogrammation *in vitro* et *in vivo*. Dans l'ensemble, ces recherches permettent de mieux comprendre comment la sénescence influe sur la plasticité cellulaire à la suite de blessures musculaires mais aussi de lier un nouveau facteur à la reprogrammation cellulaire.

**Mot clés : Sénescence, Reprogrammation *in vivo*, plasticité cellulaire, IL-6, Amphiréguline, pluripotence.**