Universidad Autónoma de Madrid

Programa de Doctorado en Biociencias Moleculares

IDENTIFICATION OF SENESCENCE-ASSOCIATED IL6 AS A KEY COMPONENT FOR CELLULAR REPROGRAMMING

DOCTORAL THESIS

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Madrid, 2019

Universidad Autónoma de Madrid

Facultad de Ciencias

Departamento de Biología Molecular

IDENTIFICATION OF SENESCENCE-ASSOCIATED IL6 AS A KEY COMPONENT FOR CELLULAR REPROGRAMMING

DOCTORAL THESIS

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Degree in Biotechnology
Master in Molecular and Cellular Biology

The work presented in this Thesis has been carried out at the Tumor Suppression Group in the Spanish National Cancer Research Centre (CNIO) in Madrid and at Cellular Plasticity and Disease Group in the Institute for Research in Biomedicine (IRB Barcelona) in Barcelona, under the direction and supervision of Dr. Manuel Serrano Marugán.

Madrid, 2019

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CERTIFIES:

That the Doctoral Thesis entitled "Identification of senescence-associated IL6 as a key component for cellular reprogramming" developed by Mrs. Noelia Alcázar Pérez, meets all requirements to obtain the degree of Doctor of Philosophy (PhD) in Molecular Biosciences, that will, with the aforementioned objective, be defended at the Universidad Autónoma de Madrid. This thesis has been carried out under my supervision and I authorize it to be presented to the Thesis Tribunal accordingly.

I hereby issue this certification in Barcelona on September the 18th, 2019.

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A mis abuelos

Acknowledgements

Gracias Manolo por darme la oportunidad de realizar la tesis en tu laboratorio y dejarme ser partícipe de tus brillantes, y a veces locas, ideas. Gracias por el apoyo en estos años y por transmitirme la ilusión y pasión por la ciencia.

Gracias a todos los miembros, presentes y pasados, del grupo de Supresión Tumoral y Cellular Plasticity and Disease Group, por la ayuda recibida en estos años en el laboratorio. En especial, agradecer a mis hermanos de tesis todos los momentos juntos que nos han unido en esta batalla y sobretodo, por lo que hemos compartido más allá del laboratorio. Gracias también al Grupo de Telómeros y Telomerasa por el apoyo y por formar parte de la familia. Gracias a todas la personas en el CNIO y en el IRB que son una parte de mí y han participado en esta tesis.

Gracias a mis padres por el cariño y apoyo incondicional que han creído en mí en todo momento. A toda mi familia, mi pareja y mis amigos, gracias por vuestro cariño y apoyo. Finalmente, agradecer a mis abuelos todo lo que me han enseñado y sobre todo, por el amor y cariño que desprendíais.

Summary

A major challenge of current cell biology is to understand how to change the identity of cells. A paradigmatic example of cell plasticity consists on the *in vitro* overexpression of four transcription factors (*Oct4*, *Sox2*, *Klf4* and *cMyc* (*OSKM*)) that can convert essentially any differentiated cell into an embryonic pluripotent cell in a process known as cellular reprogramming. Our group has previously demonstrated that this process can also occur in murine tissues *in vivo* using an inducible transgene expressing the same four factors (*i4F*). This demonstrates that reprogramming is possible in an adult organism. We hypothesize that the mechanism involved in this process may provide clues to understand and manipulate tissue repair and regeneration.

Senescent cells are produced in response to multiple types of damage and are characterized by a permanent cell cycle arrest and a robust secretion of factors, which are part of the senescence-associated secretory phenotype or SASP. These SASP factors have complex effects on tumor development, tissue repair or immune responses. We tested their effect on *in vitro* reprogramming to find new pro-reprogramming factors. Interestingly, conditioned medium (CM) from senescent cells favours reprogramming and its effect cannot be explained only by an increase in proliferation. Analyzing how the tumor suppressors *Ink4a/Arf* and *p53* modulate the SASP, we observed that the beneficial effect on the acquisition of pluripotency was exacerbated when *p53* was not present and impaired in the absence of *Ink4a/Arf.* Indeed, only *Ink4a* is necessary for the expression of SASP factors.

We have identified IL6 as the key paracrine pro-reprogramming factor contained in the CM. TNF was also studied however; it seems to have a detrimental effect on reprogramming. When IL6 was neutralized in the CM or directly in the reprogramming culture, the process was completely blocked. This suggests that IL6 could be secreted by damaged cells overexpressing *OSKM* or that, TNF inhibitory activity could affect reprogramming negatively in the absence of IL6. Importantly, senescent cells also promote *in vivo* reprogramming through the secretion of IL6. We confirmed the concept that IL6 is crucial during reprogramming using a model deficient for *IL6R* where IL6 signaling is completely absent. From kinetic experiments, we concluded that the IL6-IL6R axis is important at the early phases of reprogramming and then, it is replaced by the LIF-LIFR axis that coincides with the activation and maintenance of pluripotency. Accordingly, pluripotency markers, such as *Nanog* or endogenous *Oct4* and *Sox2*, as well as surface marker, SSEA1, are not induced in *IL6R* null *i4F* MEFs. Interestingly, *IL6R* deficient MEFs failed to upregulate *Lifr* in the late phases of reprogramming and the overexpression of *Lifr* rendered reprogramming independent of IL6. Therefore, we hypothesize that during reprogramming there are two phases: the first one depends on IL6-IL6R axis that induces *Lifr* which starts the second phase, which is dependent on LIF-LIFR axis.

Part of these results has been addressed also *in vivo,* reinforcing the idea that IL6 plays an important role of reprogramming. Understanding the mechanism behind will provide clues for inducing plasticity *in vivo* that could have applications in regeneration processes.

Resumen

Uno de los mayores retos de la biología celular es entender cómo manipular la identidad de las células. Un ejemplo de plasticidad celular consiste en la expresión *in vitro* de cuatro factores de transcripción (*Oct4*, *Sox2*, *Klf4* y *cMyc* (*OSKM*)), que convierte cualquier célula diferenciada en células pluripotentes inducidas en un proceso conocido como reprogramación celular. Nuestro grupo ha demostrado con anterioridad que este proceso también ocurre *in vivo* en ratones que contienen un transgén inducible para los mismos cuatro factores (*i4F*). Creemos que los mecanismos implicados en este proceso nos ayudarían a manipular la identidad de las células y podrían aplicarse en la regeneración de tejidos.

Las células senescentes son producidas en respuesta a múltiples daños y se caracterizan por una permanente parada del ciclo celular y por la abundante secreción de factores que forman el fenotipo secretor asociado a la senescencia (SASP). Estos factores secretados afectan al desarrollo tumoral, a la regeneración y a las respuestas inmunitarias. Primero, estudiamos cuál sería su efecto en la reprogramación *in vitro* para encontrar nuevos factores que favorezcan el proceso. Sorprendentemente, el medio condicionado (CM) senescente favorece la reprogramación y su efecto no se debe únicamente a un aumento de la proliferación. Analizando cómo los supresores tumorales *Ink4a/Arf* y *p53* podrían regular el SASP, observamos que el efecto beneficioso en la adquisición de pluripotencia aumentaba en las células deficientes para *p53* y se bloqueaba en ausencia de *Ink4a/Arf.* Además, observamos que solo *Ink4a* es necesario para la expresión de los factores del SASP.

Más adelante identificamos la citoquina IL6 como el factor determinante para favorecer la reprogramación. Se analizó también el efecto de TNF pero, se observó que inhibía la reprogramación. Cuando IL6 era neutralizada en el CM o directamente en el cultivo reprogramable, el proceso se bloqueaba completamente. Esto sugiere que IL6 podría ser secretada por células dañadas que expresan *OSKM* o que en ausencia de IL6, la actividad inhibitoria de TNF tendría un papel mayor. Además, las células senescentes también favorecen la reprogramación *in vivo* a través de la secreción de IL6. Por otra parte, se confirmó que IL6 es crucial usando un modelo deficiente para *IL6R* en el que la señalización inducida por la citoquina está bloqueada. En base a los resultados obtenidos sobre la cinética de reprogramación, concluimos que el eje IL6-IL6R es importante en las primeras etapas de la transición y después, es reemplazado por el eje LIF-LIFR, que coincide con la activación y mantenimiento de la pluripotencia. Marcadores de pluripotencia, como *Nanog* o los genes endógenos *Oct4* y *Sox2*, así como el marcador de membrana SSEA1, no se inducen en células deficientes para *IL6R*. Además, estas células no son capaces de expresar *Lifr* en las etapas tardías y la sobre-expresión de *Lifr* hace que la adquisición de pluripotencia sea independiente de IL6. Por lo tanto, pensamos que durante la reprogramación celular hay dos fases: la primera que es dependiente del eje IL6-IL6R e induce la expresión de *Lifr* y éste inicia la segunda fase dependiente del eje LIF-LIFR.

Parte de estos resultados se han observado también *in vivo* y refuerzan la idea de que IL6 tiene un papel esencial en la reprogramación celular. Entender el mecanismo ayudará a desarrollar estrategias para inducir plasticidad *in vivo* que podrían aplicarse en procesos de regeneración.

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Abbreviations

γ-IR	Gamma-irradiated
AKT	AK strain thymoma
AOM	Azoxymethane
ANOVA	ANalysis Of VAriance
AP	Alkaline Phosphatase
BrdU	BromodeoxyUridine
CCL5	C-C motif Chemokine Ligand 5
CDK	Cycline-Dependent Kinase
cDNA	Complementary DNA
cGAS	Cyclin GMP-AMP Synthase
CEBP/β	CCAAT/Enhancer Binding Protein Beta
CEyBA	Committee for Research and Animal Welfare
ChIP-seq	Chromatin Immunoprecipitation Sequencing
CLC	Cardiotrophin-Like Cytokine
CM	Conditioned Medium
cMyc (M)	C-Myelocytomatosis viral oncogene
CNTF	Ciliary Neurotrophic Factor
CNTFR	Ciliary Neurotrophic Factor Receptor
CT-1	Cardiotrophin 1
CXCL2	C-X-C Motif Chemokine Ligand 2
DDR	DNA Damage Response
DNMT	DNA methyltransferase
Dox	Doxycycline
DSS	Dextran Sodium Sulfate
E2F	E2 promoter binding factor
ECATs	ES cell-associated transcripts
ECM	Extracellular matrix
ELISA	Enzyme-Linked Immunosorbent Assay
En-*Oc4*	Endogenous *Oct4*
En-*Sox2*	Endogenous *Sox2*
EPI	Epiblast
EpiS cells	Epiblast-derived Stem cells
ERK	Extracellular-signal-Regulated Kinase
ES cells	Embryonic Stem cells
FACS	Fluorescence-activated cell sorting
FBS	Fetal Bovine Serum
FGF4	Fibroblast Growth Factor 4
FUW	Flap-Ub promoter WRE
Gapdh	Glyceraldehyde 3-phosphate dehydrogenase
GATA1	Globin Transcription Factor 1
gp130	Glycoprotein 130
GSK3b	Glycogen Synthase Kinase 3 Beta
Gy	Grays
HEK293	Human Embryonic Kidney cells 293
HRAS	Harvey Rat Sarcoma Viral Oncogene Homolog

i4F	Induced Four Factors
ICM	Inner Cell Mass
IFN	Interferon Gamma
IL11	InterLeukin 11
IL11R	InterLeukin 11 Receptor
IL27	InterLeukin 27
IL27R	InterLeukin 27 Receptor
IL31	InterLeukin 31
IL31R	InterLeukin 31 Receptor
IL6	InterLeukin 6
IL6R	InterLeukin 6 Receptor alpha
iPS cells	Induced Pluripotent Stem cells
JAK	Janus Kinase
JAKi	JAK inhibitor
Klf4 (K)	Krüppel-Like Factor 4
KSR	KnockOut Serum Replacement
LEF	Lymphoid Enhancer-binding Factor 1
LIF	Leukemia Inhibitory Factor
LIFR	Leukemia Inhibitory Factor Receptor
LysM	Lysozyme M
M2	Macrophages type 2
MAPK	Mitogen-Activated Protein Kinase
MCSF	Macrophage Colony-Stimulating Factor
MDM2	Murine Double Minute 2
MEFs	Primary Mouse Embryonic Fibroblasts
MEK	MAPK/ERK Kinase
MIP1-a	Macrophage Inflammatory Protein 1-Alpha
MIP2	Macrophage Inflammatory Protein 2
mRNA	Messenger Ribonucleic Acid
mTORC1	Mechanistic Target Of Rapamycin Kinase 1
MYOD	Myoblast determination protein
NEU	Neuropoietin
NFkB	Nuclear factor Kappa B
NKX3-1	Homeobox Protein NK-3 Homolog A
Oct4 (O)	Octamer-Binding Protein 4
OIS	Oncogene-induced senescence
ORF	Open Reading Frame
OSKM	*Oct4*, *Sox2*, *Klf4* and *cMyc*
OSM	Oncostatin M
OSMR	Oncostatin M Receptor
P16INK4A	Cycline-dependent kinase inhibitor 2a
P19ARF	Cycline-dependent kinase inhibitor 2a
P21CIP1	Cycline-dependent kinase inhibitor 1a
P	p-value
p53	Tumour protein p53

PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PDGFRβ	Platelet Derived Growth Factor Receptor Beta
PE	Primitive Endoderm
PG cells	Primordial Germ cells
PI3K	Phosphatidylinositol-4,5-biphosphate 3-Kinase
PIAS3	Proteins Inhibitor of Activated STAT3
PIM	Proto-Oncogene Serine/Threonine-Protein Kinase Pim-1
PIMi	PIM1 inhibitor
PTPN2	Protein Tyrosine Phosphatase Non-receptor Type 2
RAF	Rapidly Accelerated Fibrosarcoma
RAS	Rat Sarcoma Viral Oncogene
Rb	Retinoblastoma
rh	Recombinant human protein
rm	Recombinant mouse protein
ROS	Reactive Oxygen Species
rtTA	Reverse Tetracycline Transcriptional Activator
s.d.	Standard Deviation
SAHF	Senescence-Associated Heterochromatin Foci
SASP	Senescence-Associated Secretory Phenotype
SAβGAL	Senescence-Associated β-Galactosidase activity
SCNT	Somatic Cell Nuclear Transfer
sgp130	Soluble gp130
SHP2	Src Homology region 2 (SH2)-containing Protein tyrosine phosphatase 2
shRNA	Short hairpin RNA
sIL6R	Soluble IL6R
SMC-1	Structural Maintenance Of Chromosomes 1
SOCS	Suppressors Of Cytokine Signaling
Sox2 (S)	SRY (Sex-determining Region Y)-Box2
SPF	Specific Pathogen-Free
SSEA1	Stage Specific Embryonic Antigen-1
STAT3	Signal Transducer and Activator of Transcription 3
STING	Stimulator of Interferon Genes
TCF3	T Cell-specific transcription Factor 3
TE	Trophectoderm
TetO	Tetracycline Operator
TF	Transcription Factor
Thr	Threonine
Thy1	Thy-1 Cell Surface Antigen
TNF	Tumor Necrosis Factor
TS cells	Trophectoderm Stem cells
Tyr	Tyrosine
Wnt	Wingless-related integration site
WT	Wild Type
XEN cells	Extraembryonic Endoderm cells

Introduction

1. Cell pluripotency and embryonic development

1.1. Embryo Development

Mammalian development, from a zygote to an adult organism, involves a progressive restriction of cell potency. More than 50 years ago, Conrad Waddington postulated a model describing the acquisition of cell fate as a unidirectional process from an immature cell towards a differentiated one (Rajagopal and Stanger, 2016).

After fertilization, the zygote is divided into equal blastomeres without increasing its size. At 4-cell stage, blastomeres of the morula are interchangeable and able to generate any type of cell, embryonic or extraembryonic, of the new organism (Johnson and McConnell, 2004). This property is called totipotency and is restricted to this stage of development. In the next divisions (from 8 to 32-cell stage), compaction and polarization starts **(Figure 1)** and the first cell fate decision is taken during embryo development. Each blastomere acquires a bias towards a particular lineage driven by its physical position. Moreover, cell to cell contacts are essential for this polarization (Leung and Zernicka-Goetz, 2015). As the blastocyst matures, a group of cells remain in the periphery of the embryo and create a one-cell layer surrounding the cells inside. Those on the outside will be the extraembryonic trophectoderm (TE) and are characterized by the expression of *Cdx2* marker. The TE is implicated in the generation of extraembryonic cellular lineages, mainly the placenta, to mediate uterine implantation and sustenance of the future fetus (Menchero et al., 2018). In contrast, inner blastomeres are predisposed to form the inner cell mass (ICM). Following the formation of the early blastocyst, which corresponds to E3.5 in mouse development, cells in the ICM acquire a specific commitment and this is the second cell fate decision. A population of cells in the ICM upregulates the transcription factor *Gata6* which commits cells to form the primitive endoderm (PE) meanwhile the other subpopulation of cells expresses *Nanog* and forms the pluripotent epiblast (EPI) (Bruce and Zernicka-Goetz, 2010) **(Figure 1)**. These two populations reallocate within the ICM following the maturation of the blastocyst prior to implantation. PE cells are precursors of a second extraembryonic lineage, the yolk sac. The EPI is the truly embryonic lineage and is coated by PE on one side and by TE on the other one **(Figure 1)**.

The mouse blastocyst implants into the uterus at E4.5-E5.0 (Wang and Dey, 2006) and initiates a cascade of morphogenetic reorganizations and changes in cell identity. The epiblast grows into a blastocoel cavity forming an elongated structure covered by the PE which is known as the visceral endoderm (VE) (Shahbazi and Zernicka-Goetz, 2018). From this moment, gastrulation is initiated at the EPI, with cells delaminating from the ectoderm give rise to the definitive endoderm and mesoderm, as well as the primordial germ (PG) cells. Finally, terminally differentiated and specialized cells generate the adult organism.

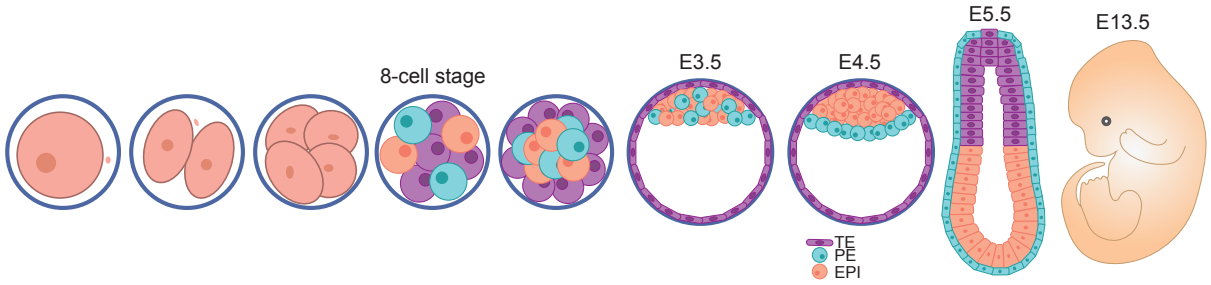

Figure 1. Overview of mouse embryo development from zygote stage to E13.5. The first cell fate decision is taken at 8-cell stage. When compaction starts, outside cells will form the TE (purple) and the inside the ICM (pink-blue). After the formation of the early blastocyst at E3.5, some cells in the ICM will commit into the PE (blue) and migrate towards the cavity side by E4.5. This is the second cells fate decision. Then, implantation takes place and the EPI (pink) is formed. Later, the developmental potential of the cells decreases and in the adult organism, multipotent stem cells are present to differentiate and repair tissues. TE: extraembryonic trophectoderm, ICM: inner cell mass, PE: primitive endoderm, EPI: epiblast.

1.2. Embryo derived stem cells

Stem cells with different degrees of pluripotency are generated during embryo development and can be isolated *in vitro* from some species (Yamanaka et al., 2006). Mouse embryonic stem (ES) cells were isolated for the first time in 1981 from the ICM of the blastocyst at E4.5 (Kaufman and Evans, 1981; Martin, 1981). ES cells are pluripotent stem cells which give rise to the three germ layers of the embryo (endoderm, mesoderm and ectoderm) (Martin, 1981). They are dependent on the expression of a combination of transcription factors, mostly *Nanog*, *Sox2* and *Oct4. Nanog* is a crucial gene for the maintenance of pluripotency in ES cells and in embryos (Chambers et al., 2003; Mitsui et al., 2003). *In vitro*, they have the capacity of self-renewal and are able to differentiate in the presence of different stimuli (Yamanaka et al., 2006). Moreover, ES cells can be re-incorporated not only into blastocysts, where they contribute partially to embryo tissues (including germline) giving rise to chimeric animals, but also into tetraploid hosts by aggregation, where the embryo derived entirely from the injected cells (Bradley et al., 1984). Therefore, ES cells represent an *in vitro* model of the early epiblast and are an important tool for generating genetically modified mouse models. At the same stage, others stem cells are present: Extraembryonic Endoderm (XEN) cells, which are the stem cell population of the PE (Kunath, 2005) and Trophectoderm Stem (TS) cells from the TE (Tanaka, 1998). TS cells can also be injected back into a host embryo and contribute to TE and placenta (Tanaka, 1998). These three stem cell lines, ES, XEN and TS cells, recapitulate the lineage of their appropriate blastocyst precursor. Interestingly, in 2018, mouse ES and TS cells were mixed *in vitro* and led to developmental structures with morphological and transcriptional characteristics similar to mouse blastocyst (Rivron et al., 2018; Sozen et al., 2018).

Another pluripotent stem cell line can be isolated from post-implantation epiblast, named epiblast-derived stem (EpiS) cells. EpiS cells, as ES cells, depend on the expression of pluripotency transcription factors, such as *Oct4*, *Sox2* and *Nanog*, and are able to generate tissues from all three germ layers. However, they cannot generate chimeric mouse after blastocyst injection *in vivo* (Brons et al., 2007; Tesar et al., 2007). Therefore, EpiSC are more lineage restricted than ES cells.

1.2.1. Naïve and primed cells

The different pluripotent states of ES and EpiS cells and their corresponding epiblast stages are distinguished by the terms naïve and primed (Nichols and Smith, 2009). Pre-implantation epiblast, at E3.5, and ES cells are defined as the naïve state and post-implantation blastocyst and EpiS cells as the primed state.

In vitro, ES cells are a heterogeneous population in terms of gene expression, morphology and functionality (Kinoshita and Smith, 2018). On one hand, ES cells express key genes that have been associated to pluripotency, called pluripotency factors, such as *Oct4*, *Sox2* and *Nanog* through the activation of LIF/Signal Transducer and Activator of Transcription 3 (STAT3) pathway which inhibits differentiation and promotes viability (Smith, 2001). On the other hand, *Oct4* and *Sox2* also induce Fibroblast Growth Factor 4 (FGF4) secretion, which triggers the entry into differentiation by activating Mitogen-Activated Protein Kinase (MAPK) pathway (Kunath et al., 2007; Yuan et al., 1995). Therefore, under standard culture conditions, ES cells oscillate between less and more differentiated depending on the relative strength of LIF or MAPK signaling, respectively. Direct blockade of MAPK signaling together with the inhibition of glycogen synthase kinase 3 (GSK3) is sufficient to stabilize and sustain an homogenous population of ES cells (Burdon et al., 1999; Silva and Smith, 2008; Ying et al., 2008). This cocktail of two inhibitors targeting MEK (PD0325921) and GSK3 (CHIR99021) is called 2i. Treatment with 2i stabilizes ES cells *in vitro* in a naïve early epiblast-like state. Naïve ES cells are characterized by a uniform expression of pluripotency markers, a global DNA hypomethylation (Lee et al., 2014), reduced levels of H3K27me3, which is a repressive histone modification, in genes and promoters (Marks et al., 2012) and the presence of two active X chromosomes in female cells (Orkin and Hochedlinger, 2011).

EpiS cells are primed pluripotent cells cultured in the presence of FGF and Activin and in the absence of LIF. Similar to naïve ES cells, they express pluripotency genes like *Oct4,* however they also expresses lineage commitment factors like *Brachyury* or *Sox17* (Tsakiridis et al., 2015). Moreover, they inactivate one of the X chromosomes in female cells and also upregulate global DNA methylation and H3K27me3 at developmental genes (Marks et al., 2012). EpiS cells can be derived also from ES cells *in vitro* directly by removing LIF and adding FGF and Activin; however, the reverse transition has not being achieved without genetic manipulation (Guo et al., 2009).

In conclusion, mouse naïve ES cells and primed EpiS cells differ in growth factor requirements, DNA methylation status, gene expression profile, morphology and X chromosome status and can be manipulated with different stimuli to facilitate their differentiation (Kinoshita and Smith, 2018).

1.3. Pluripotency regulation

ES cells are maintained in a pluripotent state *in vitro* through the activation of key pathways, being the most important ones driven by LIF. ES cells were first cultured on top of a feeder layer of damaged, non-proliferative fibroblasts (feeders) which secrete LIF into de medium (Ohtsuka et al., 2015). Once LIF was found to be the cytokine responsible for the maintenance of pluripotency, ES cells can be maintained on gelatin-coated plates, in the absence of feeders, simply with the presence of recombinant LIF and serum (Smith et al., 1988).

LIF is a member of the interleukin 6 (IL6) cytokine family. This protein family is described

in detail in the Section 4 of the Introduction. Regarding LIF activity in ES cells, this cytokine can be replaced by other cytokines of the family such as Oncostatin M (OSM), Cardiotrophin 1 (CT-1) and Ciliary Neurotrophic Factor (CNTF) which also bind to LIFR and glycoprotein 130 (gp130) (Conover et al., 1993; Pennica et al., 1995; Rose et al., 1994).

When LIF binds to the cell surface heterodimeric complex formed by the LIF receptor (LIFR) and the signal transducer gp130, it triggers three main intracellular signaling pathways: i) Janus Kinase (JAK) /STAT3 pathway, ii) PI3K/AKT pathway and iii) MAPK/ERK pathway **(Figure 2).**

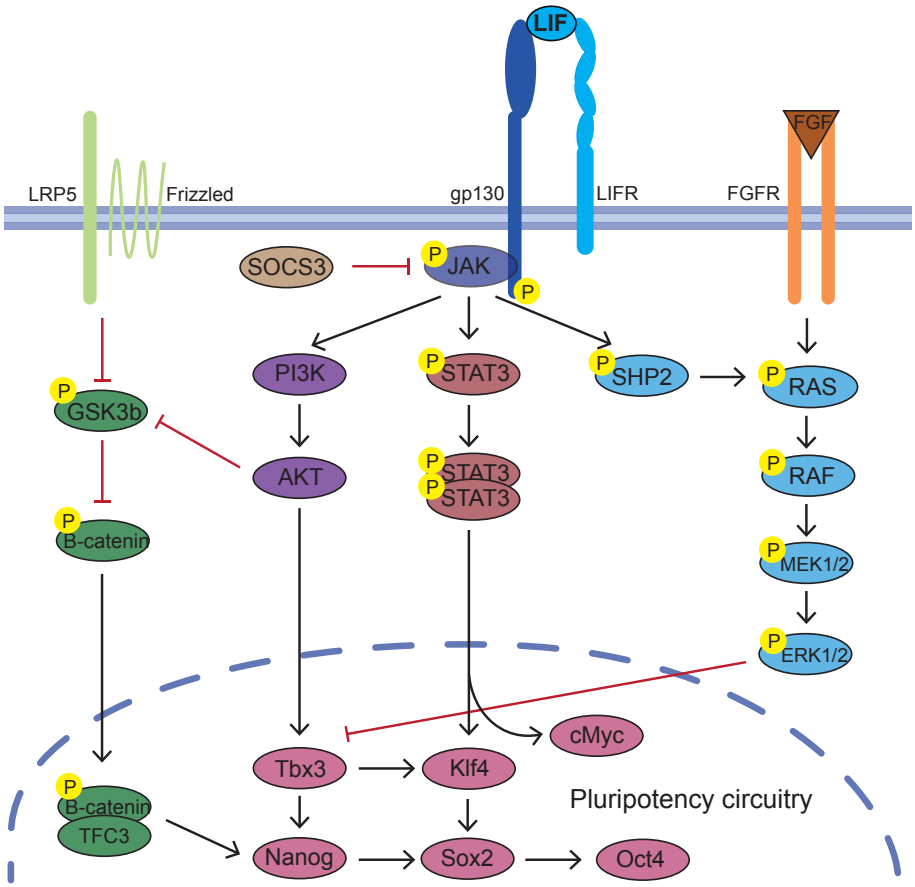

Figure 2. Signaling pathways driven by LIF. The binding of LIF to LIFR induces its heterodimerization with gp130 in the membrane. This complex phosphorylates JAKs proteins which activate three main signaling pathways, JAK/STAT3, PI3K/AKT and MAPK/ERK, to induce the expression of pluripotency genes mostly. Wnt signaling pathway acts in combination to maintain the self-renewal. SOCS3 is a negative regulator of LIF signaling.

1.3.1. JAK/STAT3 pathway

Activation of the JAK/STAT pathway is necessary and sufficient to maintain the self-renewal of ES cells, even in the absence of LIF (Matsuda et al., 1999; Niwa et al., 1998), and inhibits differentiation (Smith, 2001).

When LIF binds to its receptor, it recruits gp130 to form a heterodimer. This heterodimer, through gp130, activates JAK proteins (Ernst et al., 1996) which phosphorylate gp130 and STAT3 is recruited. Then, activated JAKs phosphorylate STAT3 on Tyr705 residue resulting in a homodimerization of STAT3 (Huang et al., 2013). Dimerized and phosphorylated STAT3 is imported into the nucleus to bind at the enhancers of its target genes. Chromatin Immunoprecipitation-Sequencing (ChIP-seq) experiments revealed that among the STAT3-binding sites, there were many pluripotency genes

including *Klf4*, *Oct4* and *Nanog* (Chen et al., 2008) and also some developmental genes such as *Gata3* (ectoderm lineage), *Eomes* (trophectoderm lineage) or *Lhx1* (mesoderm lineage) (Bourillot et al., 2009; Kidder et al., 2008). However, the net outcome of STAT3 activation is the stabilization of pluripotency and the inhibition of differentiation (Bourillot et al., 2009).

This JAK/STAT3 pathway is negatively regulated by three main mechanisms: i) phosphatases that dephosphorylate JAK and STAT proteins such as Protein Tyrosine Phosphatase Non-receptor Type 2 (PTPN2) (Zhang et al., 2018), ii) proteins inhibitor of activated STAT3 (PIAS3) which binds to STAT3 and inhibits its activity (Chung et al., 1997) and iii) suppressors of cytokine signaling (SOCS) proteins which are upregulated upon cytokine stimulation and block JAK proteins or gp130 (Nicholson et al., 2000).

1.3.2. PI3K/AKT pathway

In addition to STAT3, the activation of JAKs by LIF also results in the activation of the PI3K and AKT pathway. AKT inhibits GSK3β by phosphorylation or by facilitating its nuclear export (Bechard and Dalton, 2009) and also activates transcriptionally *Tbx3* which induces *Nanog* expression (Niwa et al., 2009). As it was mentioned previously (see section 1.2.1.), GSK3β inhibition is important for self-renewal of ES cells and a chemical inhibitor is used for maintaining them in a naïve condition (Ying et al., 2008).

GSK3β is also regulated by an independent pathway driven by Wnt. When Wnt proteins bind to its membrane receptor complex (Frizzled/LRP), GSK3β is inhibited and β-catenin is increased. Activated β-catenin enters into the nucleus and forms a complex with lymphoid enhancer-binding factor 1/T cell-specific transcription factors (LEF/TCF), being TCF3 the most abundant member in ES cells (Pereira et al., 2006). This complex form an activating complex with β-catenin and activates the transcription of pluripotency genes such as *Nanog*, *Oct4* or *Sox2* (Cole et al., 2008).

1.3.3. MAPK/ERK pathway

The third and less characterized pathway is the MAPK/ERK cascade (Burdon et al., 1999). Phosphorylation of JAKs and gp130, recruits SHP2 which is phosphorylated and interacts with Grb2-SOS complex. This complex activates the MAPK cascade RAS/RAF/MEK/ERK inducing differentiation by inhibiting *Nanog* and *Tbx3* (Hamazaki et al., 2006; Niwa et al., 2009). Inhibition of MEK by a chemical inhibitor is used to block differentiation in ES cells and is part of the 2i cocktail (Ying et al., 2008).

2. Induction of plasticity *in vitro* and *in vivo*

Briggs, King and Gurdon reported the first examples of cellular reprogramming in frogs by injecting enucleated oocytes with the nuclei from early blastocyst cells or from differentiated intestinal cells from frogs (Briggs and King, 1952; Gurdon, 1962b, 1962a). They demonstrated that cell specialization and differentiation is not irreversible and that the genetic material is not irreversibly programmed. Similar evidence was achieved using mammalian cells in 1997 by

Wilmut with the generation of Dolly the sheep (Wilmut et al., 1997). In both cases, differentiated cells were reprogrammed through somatic cell nuclear transfer (SCNT) **(Figure 3)**. This technique consists in introducing the nuclear content of a terminally differentiated adult somatic cell into an enucleated and unfertilized oocyte. This new cell proliferates and generates an organism that is genetically identical (a clone) to the organism that provided the donor somatic nucleus. This means that a totipotent cell is generated and also that the oocyte cytoplasm contains key factors able to induce the embryonic program (Yamanaka and Blau, 2010).

Later, plasticity was induced by cell fusion between somatic and pluripotent stem cells in mouse and human cells (Blau et al., 1983; Tada et al., 2001) **(Figure 3)**. The generated heterokaryons do not proliferate and contain multiple nuclei from the two initial cell types. These hybrids reactivate pluripotency genes such as *Oct4*, *Nanog* and *Sox2* and can be differentiated *in vitro* and *in vivo* into the three germ layers (Cowan et al., 2007; Do and Shöler, 2004).

These studies suggested that specific factors in pluripotent cells could be the responsible ones for inducing plasticity and gave rise to the concept of transdifferentiation. This is a direct conversion of differentiated cells into another functional somatic cell type. It is achieved by forced expression of tissue specific transcription factors and importantly, bypassed any pluripotent state. The first example showed the conversion of mouse fibroblasts into myoblasts by expressing a single muscle protein which was myoblast determination protein (MYOD) (Davis et al., 1987). Later, it was also demonstrated for myeloblasts, where GATA1 overexpression converted them into megakaryocytes (Kulessa et al., 1995), and for B lymphocytes, where CEBP/α overexpression transformed them into macrophages (Xie et al., 2004).

At the same time that plasticity was being explored, ES cells were deeply studied and ES cell-associated transcripts (ECATs) were identified. In 2006, Takahashi and Yamanaka selected 24 ECATs to examine whether those candidates could induce dedifferentiation in somatic cells (Takahashi and Yamanaka, 2006). They introduced simultaneously a mixture of the 24 factors using retroviral vectors into mouse embryonic fibroblasts (MEFs) and, surprisingly, colonies resembling ES cells were observed **(Figure 3)**. These colonies were called induced pluripotent stem (iPS) cells. Then, they narrowed down to identify the minimal cocktail of transcription factors able to reprogram and induce iPS formation. The minimal requirement was composed by *Oct3/4* (O), *Sox2* (S), *Klf4* (K) and *cMyc* (M) genes (abbreviated here as *OSKM*). These iPS cells fulfil the standard assays for pluripotency such as differentiation into the three germ layers and, after injection into blastocyst, contribution to embryo development. Moreover, they are morphologically similar to ES cells, share similar gene expression profile and cell surface markers like stage specific embryonic antigen-1 (SSEA1) (Polo et al., 2012; Takahashi and Yamanaka, 2006). One year later, iPS cells were derived from human fibroblasts using the same combination of transcription factors (Takahashi et al., 2007) and also by an alternative cocktail, *Oct4*, *Sox2*, *Nanog* and *Lin28* (Yu et al., 2007). Since the initial report, iPS cells have been derived from mouse and human cells of multiple origins (Masip et al., 2010).

This technology is a powerful tool to generate patient-specific iPS cells for studying potential treatment and pathogenesis of diseases, as well as to generate patient-specific differentiated cells with therapeutic potential.

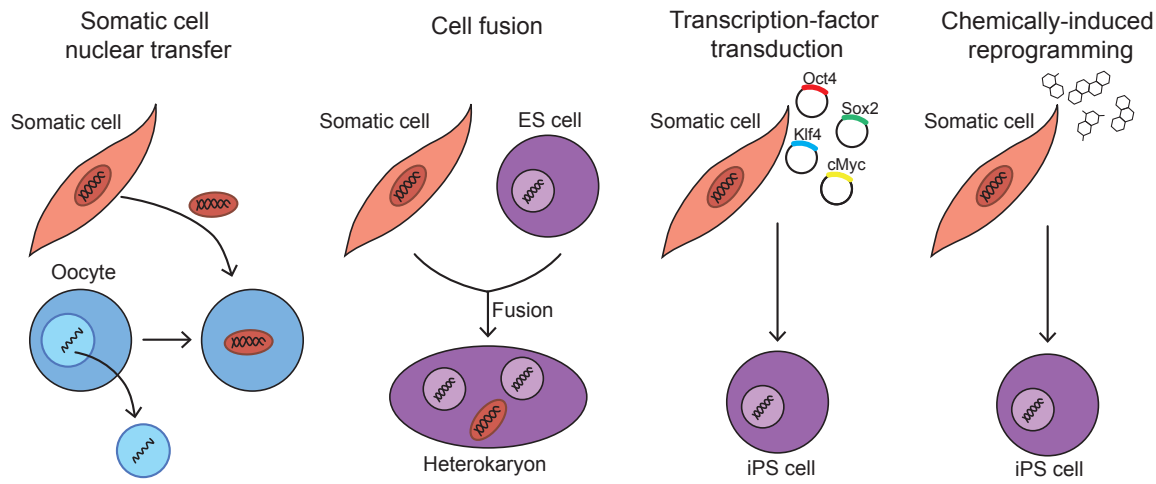

Figure 3. Four approaches to reprogram somatic cells. Reprogramming can be achieved by somatic cell nuclear transfer using the nucleus of a differentiated cell and an enucleated oocyte, cell fusion between ES cells and somatic cells, transcription-factor transduction (*OSKM*) and treatment with small molecules (from left to right).

2.1. Mechanisms of *in vitro* reprogramming

Reprogramming is a slow and gradual process where a small fraction of transduced cells become iPS cells. Typically 1 in 1000-10000 cells are successfully reprogrammed, although many variations have been reported with improved efficacy (Masip et al., 2010). In *OSKM* reprogramming, expression of the four factors has to be sustained for a sufficient time to activate the endogenous genes. Once the endogenous genes are re-expressed, the exogenous *OSKM* factors become silenced as part of a general embryonic program that shuts down retroviral promoters. The self-renewal and pluripotent state of iPS cells rely only on the expression of the endogenous genes. However, some cells are trapped in a partially reprogrammed state and undergo defective differentiation (Mikkelsen et al., 2008). The mechanism underneath reprogramming is still unclear but a stochastic model has been proposed, among others. Based on this model, reprogramming is divided in two phases. In the early phase, *OSKM* factors occupy many genomic loci (Soufi et al., 2012) disrupting the somatic identity of the cells, this includes the repression of cell identity genes such as Thy1 (surface antigen in fibroblast) (Stadtfeld et al., 2008). At the same time, cells undergo mesenchymal-to-epithelial (MET) transition (Li et al., 2010) and change their metabolism from oxidative phosphorylation to glycolysis (Nishimura et al., 2019). This phase is highly inefficient and stochastic, which could explain the low efficiency observed in most of reprogramming protocols (Masip et al., 2010). In the second phase, late pluripotency genes, such as telomerase and endogenous *OSKM*, and *de novo* DNA methyltransferases (DNMT) are activated (Brambrink et al., 2008; Polo et al., 2012; Stadtfeld et al., 2008). DNMTs together with the epigenetic remodelling machinery remodel the epigenetic code of the differentiated cells to one resembling the open chromatin of ES cells (Papp and Plath, 2013). However, a more recent study using single–cell RNA sequencing analysis established that somatic gene inactivation, upregulation of cell cycle, pluripotency and epithelial genes and the metabolic switch can take place independently (Tran et al., 2019). For example, they observed single cells expressing *Twist*, which is a mesenchymal-fibroblast marker, together with *Nanog*, which is a pluripotency marker. Moreover, they proposed a mechanism for 2i in reprogramming. In this study, they combined 2i, ascorbic acid and an inhibitor

for Dot1L to boost reprogramming. They found that 2i, in this context, promotes faster silencing of somatic genes and therefore, facilitates the formation of iPS cells (Tran et al., 2019).

2.2. Improvements in *in vitro* reprogramming

Numerous studies have attempted to discover molecules and transcription factors that could facilitate reprogramming, increase its efficiency or even replace the Yamanaka factors (*OSKM*).

iPS cells can be generated in the absence of *cMyc*, to avoid the use of oncogenes, but it is highly inefficient. Also, this factor can be substituted by a less oncogenic member of the Myc family, *L-Myc* (Nakagawa et al., 2008, 2010). *Klf4* can be replaced by related Klf-like transcription factors, *Klf2* and *Klf5* (Nakagawa et al., 2008) and *Sox2* by *Sox1* and *Sox3* (Nakagawa et al., 2008). Nevertheless, *Oct4* has been the factor most difficult to change since it cannot be replaced by closely related family members. Later studies described that it could be replaced by *Nr5a2* and *Tcl1a* (Heng et al., 2010; Picanço-Castro et al., 2011). On the other hand, the ectopic expression of highly expressed genes in ES cells, like *Utf1*, *Esrrb*, *Trim71*, *Tbx3* also has an impact increasing reprogramming efficiency (Feng et al., 2009; Han et al., 2010; Zhao et al., 2008).

Other studies have focused on defining the barriers that block reprogramming. Epigenetic repressors have a key role in embryo development and also in pluripotency. Hanna's group identified *Mbd3* as a molecular block of reprogramming. Nearly 100% of *Mbd3* null cells induced pluripotency and reprogrammed (Rais et al., 2013). Negative regulators of the cell cycle are also considered major barriers for reprogramming. Some of them, in particular, are upregulated by the oncogenic stress generated by the *OSKM* factors preventing the proliferation of many cells undergoing reprogramming. This is the case of the tumor suppressors *p53*, p16INK4A, p19ARF and p21CIP1 (Banito et al., 2009; Kawamura et al., 2009; Li et al., 2009; Marión et al., 2009; Utikal et al., 2009).

Some groups have found small molecules that enhance reprogramming or even replace one or several factors of the Yamanaka (Li et al., 2011; Ye et al., 2016; Yuan et al., 2011). These compounds exert a wide range of actions including the activation of transcription of pluripotency genes, self-renewal modulators, survival agents or epigenetic modifiers (Federation et al., 2014; Ichida et al., 2009; Li et al., 2012; Maherali and Hochedlinger, 2009; Masuda et al., 2013; Zhang et al., 2014). In 2013, full chemically induced reprogramming was reported in the absence of any exogenous transcription factor (Hou et al., 2013; Zhao et al., 2015) **(Figure 3)**. Mouse fibroblasts were reprogrammed using seven small molecules: Valproic acid (HDAC inhibitor), CHIR99021 (GSK3B inhibitor), 616452 (TGFB inhibitor), tranylcypromine (H3K4 demethylation inhibitor), 3-deazaneplanocin A (DZNep, methyltranferase inhibitor) PD0325901 (MEK inhibitor) and Forskolin (adenylate cyclase agonist) (Hou et al., 2013; Zhao et al., 2015). A negative aspect of this protocol is that it is comparatively slow and inefficient (40 days of treatment, 3 colonies in 100000 cells) compared to the standard *OSKM* method (10 days of induction, 100 colonies in 100000 cells).

2.3. In vivo plasticity in regeneration

The cellular strategies described in previous sections have been developed only *in vitro* but,

in vivo reprogramming has also been observed and different strategies for inducing it have been proposed. These observations provide a potential alternative for regenerative medicine, aging, rejuvenation and even cancer treatment.

Some invertebrates have the capacity to regenerate their whole body. In some species of planaria, regeneration is an integral part of their life cycle, regrowing new heads or tails or even the entire organism (Morgan, 1898; Randolph, 1897). They have dividing somatic cells (neoblasts), which maintain homeostasis in intact organisms and, upon injury regenerate (Wagner et al., 2011). Mammals, in contrast, have a limited capacity to regenerate tissues which is lost with development and aging. However, dedifferentiation examples upon *in vivo* injury have been published. In the intestine, loss of *Lgr5*$^{+}$ cells (well-known adult stem cell population) (Barker et al., 2007) triggers the dedifferentiation of quiescent cells into *Lgr5*$^{+}$ stem cells which repair the tissue (Buczacki et al., 2013; Van Es et al., 2012; Tetteh et al., 2016; Tian et al., 2011). In the stomach, *in vivo* elimination of proliferating cells by 5-fluoruracil treatment results in upregulation of *Troy* stem cell marker in differentiated cells and their subsequent regeneration of entire glands (Stange et al., 2013; Tata et al., 2013). In the retina, terminally differentiated retinal neurons, upon neuronal damage, transiently activate pluripotency factors, such as *Oct4* and *Nanog*, proliferate and differentiate to regenerate the tissue (Sanges et al., 2013). It is worth to remark that a common concept in the above examples is that injury triggers plasticity in differentiated cells.

Furthermore, transcription factor-mediated *in vivo* reprogramming has also been achieved. In 2007, a pioneer example showed that mature B cells can dedifferentiate into haematopoietic progenitors by loss of the single transcription factor *Pax5*. Therefore, this study demonstrated that B cells retain an extraordinary developmental plasticity (Cobaleda et al., 2007). Also, the direct conversion of adult pancreatic exocrine α-cells into β-cells has been achieved in mice (Zhou et al., 2008). Forced and transient expression of three transcription factors, *Pdx1*, *Neurog3* and *Mafa*, fully reprograms exocrine cells into β-cells which are functional *in vivo* and ameliorate the phenotype of diabetic mice (Zhou et al., 2008). More importantly, this has been achieved using human α-cells overexpressing *Pdx1* and *Mafa*. α-converted cells secrete insulin and, when transplanted into diabetic mice, diminish the phenotype (Furuyama et al., 2019). Furthermore, liver myofibroblast were reprogrammed into hepatocytes using adenovirus encoding for *Foxa3*, *Gata4* and *Hnf1a.* These new hepatocytes restore organ function in a model of chronic fibrotic liver injury (Rezvani et al., 2016; Song et al., 2016). More examples have been published in cardiac fibroblast, astrocytes and cortical glia cells (Guo et al., 2014; Inagawa et al., 2012; Jayawardena et al., 2012; Niu et al., 2013, 2015; Qian et al., 2012).

Finally, mice overexpressing *OSKM* factors *in vivo*, abbreviated here as *i4F* or reprogrammable mice, showed dedifferentiation in multiple tissues. Transient activation of *OSKM in vivo* for one week, led to loss of differentiation markers, like cytokeratins, and acquisition of pluripotency markers, like *Nanog*, in foci of different tissues, including pancreas, stomach, kidney or large intestine. Upon switching off the *OSKM* transgene, mice developed teratomas, which is a tumor type characteristically produced by pluripotent cells. This suggested that full reprogramming was achieved *in vivo* for the first time (Abad et al., 2013). Moreover, iPS cells were isolated from the blood of *i4F* mice. These cells not only have similar characteristics to mouse ES cells but also have totipotency features based on trophectoderm contribution after blastocyst injection,

high expression of trophectodermal markers and trophoblast differentiation *in vitro* and within teratomas. These cells are called *in vivo* iPS cells. A similar reprogrammable model was published soon after by Yamanaka and Yamada (Ohnishi et al., 2014). The transient expression of the four factors in this model led to teratoma development in multiple tissues, such as kidney and pancreas. Interestingly, partial reprogrammed cells lacked the ability to differentiate into multiple lineages and did not form teratoma, but instead formed tumors resembling Wilms tumors.

Importantly, these models have been tested in different stress/damaged contexts. On one hand, *OSKM* transient expression increases the regeneration capacity of mice after skeletal muscle injury (Chiche et al., 2017). On the other hand, short-term induction of *OSKM* (only for 2 days) in repetitive cycles ameliorates aging-associated phenotypes, elongates the lifespan of progeria mice and renders mice more resistant to subsequent muscle or pancreatic injury (Ocampo et al., 2016).

These studies are important to elucidate the molecular basis for manipulating cell fate *in vivo* and could be essential steps in developing new methods for enhancing regeneration in mammals.

3. Cellular senescence

Cellular senescence is a permanent cell cycle arrest that occurs in proliferating cells *in vitro* and *in vivo* in response to a wide variety of stressors. It was first described by Hayflick and Moorhead in 1960. They observed that primary human fibroblasts in culture proliferated efficiently until they reached a number of cell divisions, that was approximately constant and then, cells arrested permanently (Hayflick L and P, 1961). They speculated that this could be one of the causes of aging and for this reason called this process cellular senescence. Later, it was found that cellular senescence of human fibroblasts was caused by the progressive shortening of telomeres (Blasco et al., 1997; Harley et al., 1990). This type of senescence was named replicative senescence. Nowadays, it is well demonstrated that essentially all cell types can undergo senescence in response to multiple cell stressors, including telomere shortening but, extending to apparently any type of cellular damage or stress.

The roles of senescent cells have been described as beneficial and detrimental depends on the context (described in Section 3.4.). In the context of cancer, cellular senescence is considered today one of the most important cancer protecting mechanism. In fact, most of cancer cells have acquired some degree of resistance to undergo senescence; and multiple animal models with deficiencies in the senescence response are all invariably cancer prone (Collado et al., 2007). In the context of tissular remodeling, transient induction of senescence favors tissue repair upon injury and it is necessary during embryo development (Demaria et al., 2014; Muñoz-Espín and Serrano, 2014; Muñoz-Espín et al., 2013; Storer et al., 2013). On the contrary, persistent senescent cells may contribute to aging phenotypes and to multiple aging-associated degenerative (Muñoz-Espín and Serrano, 2014; Muñoz-Espín et al., 2018).

3.1. Hallmarks of senescent cells

Senescent cells do not have a single universal marker that could distinguish them from other

non-senescent cells. Therefore a combination of different biomarkers has to be checked in order to confidently define senescent cells *in vivo* and *in vitro*.

In culture, this process is accompanied by changes in morphology. Cells become flatter, larger and highly vacuolized. However, *in vivo*, this difference is not clear (Sharpless and Sherr, 2015). Permanent cell cycle arrest is a key characteristic of senescent cells and even, in a promitogenic environment, they cannot express genes required for proliferation (Dimri and Campisi, 1994). They are characterized by high expression of cyclin-dependent kinase (CDK) inhibitors, including *p16INK4A*, *p15INK4B*, *p21CIP1* and *p27KIP1*, and *p53* which results in cell cycle arrest (Campisi and D'Adda Di Fagagna, 2007; Lin et al., 1998; Muñoz-Espín and Serrano, 2014; Serrano et al., 1997). Therefore, cells are negative for proliferating markers like Ki67 and BrdU incorporation. Senescent cells have high activity of lysosomal β-galactosidase which is known as senescence-associated β-galactosidase (SAβGAL) (Dimri et al., 1995; Kurz et al., 2000; Lee et al., 2006). This probably reflects the augmented lysosomal content of senescent cells (Lee et al., 2006). In contrast to quiescent cells, senescence is also accompanied by a secretory pro-inflammatory phenotype. Senescent cells secrete a large number of chemokines, cytokines, proteases and growth factors known as senescence-associated secretory phenotype (SASP) (see Section 3.3.). Indeed, today is recognized that terminally differentiated cells, such as neurons, can also undergo senescence, which in this case is characterized by the presence of the SASP and SAβGAL. Heterochromatin foci formation is another characteristic of senescent cells due to the DNA damage at non-telomeric sites and are called senescence-associated heterochromatic foci (SAHF) (Narita et al., 2003; Zhang et al., 2005). It is a limited marker because is restricted to some types of senescence. For example, SAHF-positive cells are highly associated to oncogene induced senescence (OIS) but not to replicative senescence or aging (Kosar et al., 2011).

Other markers that can be found in senescent cells are accumulation of lipofuscin (Georgakopoulou et al., 2013), loss of lamin B1 (Shimi et al., 2011) or upregulation of Bcl-2 anti-apoptotic proteins (Yosef et al., 2016).

3.2. Mechanisms of inducing senescence

Upon extensive proliferation, cells that do not naturally express telomerase undergo telomere shortening that eventually leads to the exposure of uncapped double-strand ends. This efficiently activates an unrepairable DNA damage that results in permanent activation of the DNA-damage response (DDR) that induces senescence (Blasco et al., 1997; Harley et al., 1990). However, this is not the only inducer, premature senescence can also be activated by multiple other stresses.

A particularly relevant type of senescence-inducing stressor is the activation of oncogenes, a process known as oncogene induced senescence or OIS. An example is the overexpression of an oncogenic form of *RAS* ($HRAS^{G12V}$) in human fibroblasts which induces p53 and p16INK4A (Serrano et al., 1997). The physiological role of OIS is to inhibit tumor growth and also the progression from benign to malignant tumors. The activation of only one oncogene is not sufficient to trigger transformation, it requires the cooperation of other oncogenes and/or the inactivation of tumor suppressors (DeNicola and Tuveson, 2009; Land et al., 1983).

The absence or inactivation of tumor suppressors can also induce senescence, as it is the case

of PTEN loss (Chen et al., 2005; Toso et al., 2014), neurofibromin 1 (NF1) loss (Courtois-Cox et al., 2006) or TSC2 inactivation (Zhang et al., 2003).

Other senescence-inducing stimuli are abnormal O_2 levels (Zglinicki et al., 1995), γ-irradiation (γ-IR) (Citrin et al., 2013; Coppé et al., 2008; Le et al., 2010; Muthna et al., 2010), high intracellular ROS levels (Chen et al., 2002; Yu et al., 2002) or chemotherapy agents like doxorubicin (Piegari et al., 2013).

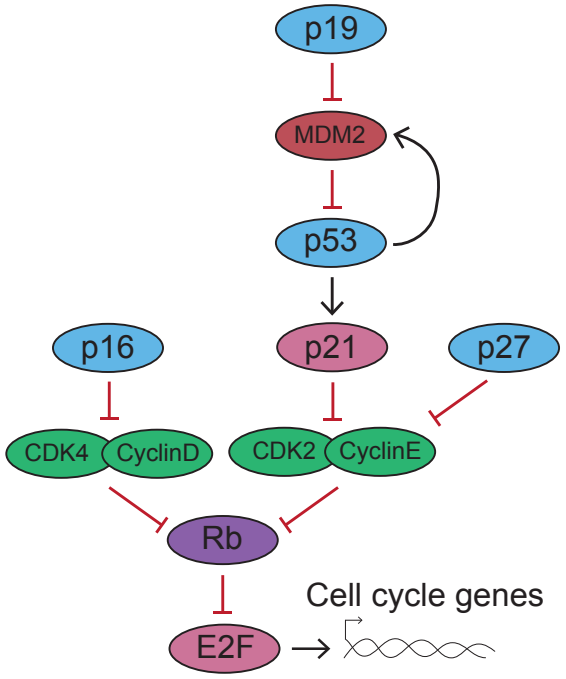

Figure 4. Senescence signaling pathways. Schematic representation of pathways activating cell cycle arrest and senescence. Damage activates *p16INK4A* and *p53* pathways. p16INK4A inhibits CDK4. p53 can be activated by p19ARF which sequesters MDM2 and stabilizes p53 which activates another CDK inhibitor, p21. Both pathways converge in the inhibition of CDKs proteins which leads to the activation of Rb and permanent cell cycle arrest.

Mechanistically, DDR signals activate and phosphorylate several cell cycle proteins like p53 (Hafner et al., 2019). Stabilized p53 activates p21CIP1 which is a CDK inhibitor, mostly of CDK2, and cell cycle is arrested (Cazzalini et al., 2010) **(Figure 4)**. A second pathway to block proliferation upon multiple type of damage, is the activation of p16INK4A encoded by CDKN2A *locus*. p16INK4A is a selective inhibitor of CDK4 and 6 (Serrano et al., 1993).The CDKN2A *locus* not only encodes for p16INK4A but also for p19ARF in mouse or p14ARF in human (Quelle et al., 1995). *p19ARF* is also a potent tumor suppressor which inactivates murine double minutes-2 (MDM2). MDM2 impairs p53 transcriptional activity and as a consequence, inactivates it (Chin et al., 1998). Therefore, both *p16INK4A* and *p19ARF* participate in arresting cell cycle via different mechanisms **(Figure 4)**.

p53, p16INK4A and p19ARF converge to block CDKs and maintain retinoblastoma (Rb) unphosphorylated which sequesters E2F factors and then, the progression into the S phase of the cell cycle is prevented (Ewen et al., 1993; Narita et al., 2003) **(Figure 4)**.

Regarding p16INK4A and p19ARF, both contribute to senescence however p19ARF is more critical in replicative senescence of mouse fibroblasts and p16INK4A in *in vitro* senescence of human cells (Evan and d'Adda di Fagagna, 2009).

3.3. SASP

SASP is a characteristic phenotype of senescence cells with autocrine and paracrine activities

however, the panel of secreted molecules is different depending on the cell type and the inducer. Among the cells that become senescent, fibroblast (Coppé et al., 2008), liver cells (Schnabl et al., 2003), endothelial cells and epithelial cells (Shelton et al., 1999) has been reported to secrete biologically active molecules.

SASP factors can be divided into: soluble signaling molecules, secreted proteases (PAI-1 and PAI-2) and extracellular matrix (ECM) components. The pro-inflammatory signaling molecules includes interleukins, chemokines and growth factors. The key components are interleukin 6 (IL6), interleukin 8 (IL8), interleukin 1α (IL1α), monocyte chemoattractant proteins (like MCP-3 or MCP-1) and TGFβ (Coppé et al., 2010). Importantly, these cytokines can create an inflammatory microenvironment which recruits cells from the immune system (Eggert et al., 2016; Kang et al., 2011; Di Mitri et al., 2014; Xue et al., 2007) and moreover, can transmit senescence to neighboring normal and tumor cells acting in a paracrine manner (Acosta et al., 2013; Hubackova et al., 2012; Nelson et al., 2012).

Most of the SASP components are regulated by several factors and signaling pathways including nuclear factor-κB (NF-κB), GATA4, CCAAT/enhancer-binding protein β (CEBP/β), STAT3, p38/MAPK and mTOR. NF-κB suppression has a limited impact on proliferating cells however, it strongly affects to gene expression of SASP components in senescent cells (Chien et al., 2013). Moreover, GATA4 acts as an upstream regulator of NF-κB and as a consequence, it induces the secretion of SASP factors (Kang et al., 2015). CEBP/β is upregulated in OIS and binds to IL6 promoter inducing its expression (Flanagan et al., 2018; Kuilman et al., 2008). Inhibition of JAK, downstream target of STAT3, results in poor SASP where secretion of many cytokines, including IL6, is decreased (Xu et al., 2015). p38/MAPK activity is sufficient and necessary for the SASP after DNA damage or in OIS. Chemical inhibition of p38 reduces SASP factors secretion, such as IL6, IL8 or GM-CSF, similar to control levels (Freund et al., 2011). Rapamycin, which is a mTOR inhibitor, decreases the secretion of some SASP components, including IL6 and IL8, while others remaine unchanged, like TIMP1 and CCL13, after ionizing irradiation meaning that it is a selective modulator (Laberge et al., 2015; Wang et al., 2017). Finally, the SASP can be also controlled by the cGAS/STING sensor pathway (Yang et al., 2017).

3.4. Role of senescence *in vivo*

Senescent cells have been identified *in vivo* based on marker analysis and reporter mice. Broadly, senescence is considered a conserved mechanism which initiates a cascade of processes to eliminate damaged cells. However, this can be corrupted and persistent senescent cells could be accumulated and aggravate tissue repair responses. Therefore, senescence has beneficial and detrimental effects.

3.4.1. Beneficial effects of senescence

During embryo development, SAβGAL$^+$ Ki67$^-$ cells are observed from E10.5 to E17.5 in some tissues including the mesonephros, neural tube, endolymphatic sac of the inner ear or interdigital webs (Muñoz-Espín et al., 2013; Storer et al., 2013). Macrophages infiltrate and eliminate those senescent cells by E15.5-E17.5. When senescence is impaired, due to p21 absence, a compensatory

process (apoptosis) is activated to prevent severe morphological defects in most of the tissues like mesonephros. Interestingly, DNA damage markers are absent. In this context, physiological senescent cells have a tissue remodeling role (Muñoz-Espín et al., 2013; Storer et al., 2013).

Senescence has also been implicated in promoting tissue repair in wound healing through a SASP factor. Fibroblasts and endothelial cells from skin become senescent after damage and secrete PDGF-AA to promote wound closure. Importantly, senescent cells were present only transiently at the beginning and when they were eliminated, wound healing kinetics was delayed. Therefore, the presence of senescence favors skin repair (Demaria et al., 2014). It has also been involved in limiting liver and skin fibrosis after injury. Senescent cells, which are induced by the matricellular protein CCN1, secrete antifibrotic proteases to degrade ECM components facilitating fibrotic resolution (Jun and Lau, 2010; Krizhanovsky et al., 2008).

In cancer, senescence prevents the expansion and progression of benign lesions to malignant tumors. In a model of lung adenomas formation, driven by oncogenic *KRAS* overexpression, *Ink4a*$^+$ and SAβGAL$^+$ cells were observed in premalignant tumors (Collado et al., 2005). These senescent cells are cleared by immune cells attracted by SASP (Eggert et al., 2016; Kang et al., 2011; Di Mitri et al., 2014; Xue et al., 2007). Recent findings have demonstrated that *CDK4/6* inhibitors, like palbociclib, are prosenescent and are being tested alone or in combination with other agents to arrest and/or eliminate senescent or tumor cells (Dickson et al., 2013; Higuchi et al., 2019; Leonard et al., 2012; Muñoz − Espín et al., 2018; Turner et al., 2018).

3.4.2. Detrimental effects of senescence

Senescent SAβGAL$^+$ and *Ink4a*$^+$ cells accumulate in aged tissues and could contribute to age-related diseases (Coppé et al., 2010; Krotica et al., 2001; Parrinello et al., 2005). The connection between senescence and aging was proven in a mouse model of progeria and in physiological aging using a suicidal transgene regulated by the p16INK4A promoter. When *Ink4a*$^+$ cells were eliminated, the phenotype of the model or the appearance of age-associated diseases was delayed respectively (Baker et al., 2011, 2016).

Senescent cells have a damaging role in lung fibrosis. In the lungs, SAβGAL$^+$ *Ink4a*$^+$ cells are observed after DNA damaging bleomycin treatment and secrete profibrotic SASP components which induce fibroblast activation in a paracrine manner. Senescent cells persist in the tissue and exacerbate the fibrosis therefore, their elimination alleviates the phenotype (Muñoz - Espín et al., 2018; Pan et al., 2017; Schafer et al., 2017).

SASP could also have a deleterious function. In the first instance, it creates a favourable environment to recruit immune cells which participate in the elimination of damaged cells (Eggert et al., 2016; Kang et al., 2011; Di Mitri et al., 2014; Xue et al., 2007). However, an inefficient clearance of senescent cells leads to an exacerbate and excessive SASP which could boost proliferation, vascularization and even tumor growth (Coppé et al., 2010).

Finally, pathological conditions are aggravated due to senescence like obesity, type 2 diabetes, sarcopenia, cataracts or tau-mediated diseases like Alzheimer (Bussian et al., 2018; Muñoz-Espín and Serrano, 2014).

All these evidences point out senescence as a novel therapeutic target by developing pro or anti-senescent treatments.

4. IL6 cytokine

The family of IL6-type cytokine represents a group of pleotropic cytokines with a four-helical protein topology (Bravo and Heath, 2000). This family includes IL6, IL11, Ciliary Neurotrophic Factor (CNTF), Cardiotrophin 1 (CT-1), cardiothrophin-like cytokine (CLC), LIF, Neuropoietin (NEU), Oncostatin M (OSM), IL27 and IL31 (Derouet et al., 2004; Heinrich et al., 1998; Pflanz et al., 2002; Rose-John, 2012; Scheller et al., 2006). These cytokines bind to specific plasma membrane complexes containing a α-receptor and a common signal transducer β-receptor which is gp130. Upon binding, gp130 induces the activation of JAK proteins and then, different pathways, including JAK/STAT3, PI3K/AKT and MAPK/ERK, are induced as it has been explained above for LIF **(Figure 2)**.

Particularly, IL6 is a pro-inflammatory cytokine which was first identified for promoting T cell activation and differentiation of B cells in 1986 (Murakami et al., 2019; Yasukawa et al., 1987). Nowadays, it represents a keystone cytokine in infection, senescence, cancer and inflammation (Jones and Hunter, 2015; Taher et al., 2018; West, 2019).

4.1. Classic signaling and trans-signaling of IL6

IL6 activates its downstream targets through two mechanisms called classic signaling via the membrane bound receptor and tras-signaling via soluble receptor. The first one is essential for regeneration and anti-bacterial activities of IL6 whereas, the second one is associated to deleterious pro-inflammatory activity.

In the classic signaling, IL6 first binds to its α-receptor (IL6R) and this causes dimerization of two gp130 receptors which initiate the intracellular signaling (Taga et al., 1989) **(Figure 5)**. The receptor gp130 is ubiquitously expressed in most cells types (Xu and Neamati, 2013) and have not affinity to IL6 or IL6R alone (Jostock et al., 2001). Therefore, IL6 can only stimulate cells which already express IL6R, and these include hepatocytes, neutrophils, monocytes, macrophages and lymphocytes (Garbers et al., 2012, 2015). Classic signaling has been demonstrated to be crucial for hepatocytes after hepatic acute damage (Wunderlich et al., 2010), glucose metabolism (Timper et al., 2017), hematopoiesis, differentiation of regenerative M2 macrophages (Chomarat et al., 2000) and regulation of the neuroendocrine system (Choy and Calabrese, 2018).

A soluble form of IL6R (sIL6R) has been found in body fluids like urine (Honda et al., 1992; Novick et al., 1989). In humans, sIL6R can be generated by two mechanism: i) limited proteolysis or shedding of the membrane bound receptor proximal to the plasma membrane by metalloproteases (ADAM17 or ADAM10) (Baran et al., 2013; Jürgen et al., 1993) and ii) alternative splicing (Lust et al., 1992). In murine cells, only shedding, and not differential splicing, has been observed (Jones et al., 2001). Shedding of the receptor can be induced by different stimuli like bacterial toxins (Walev et al., 2002), cholesterol depleting agents (Matthews et al., 2003) or high levels of C-reactive protein (Jones et al., 2002). sIL6R binds to IL6 and then, gp130 expressing cells can be activated by binding of the soluble complex (Taga et al., 1989) **(Figure 5)**. This process is termed trans-signaling and, thanks to it cells that only express gp130 but not IL6R can respond to the presence of IL6/sIL6R, as it is the case of embryonic stem cells (Yoshida et al., 1994) or neural

cells (März et al., 1999a, 1999b). This is an important process during inflammatory responses to stimulate cells that are unresponsive to the classic signaling. Shedding of IL6R has been observed in neutrophils, which are one of the first-responders upon infection, and leads to the activation of endothelial cells through IL6-sIL6R-gp130 complex. Activation of endothelial cells results in secretion of attracting cytokines, like MCP-1, which recruits the immune system to resolve the infection (Rose-John, 2012). Moreover, activation of CD4 T-cells induces shedding of sIL6R and this contributes to the status of the T-cell response (Briso et al., 2008; Dominitzki et al., 2007). Finally, trans-signaling has been associated to pro-inflammatory responses of IL6 in some chronic diseases like Crohn disease, rheumatoid arthritis or colon cancer (Rose-John, 2012) as well as in tissue fibrosis, cardiovascular diseases and infection (Jones and Hunter, 2015).

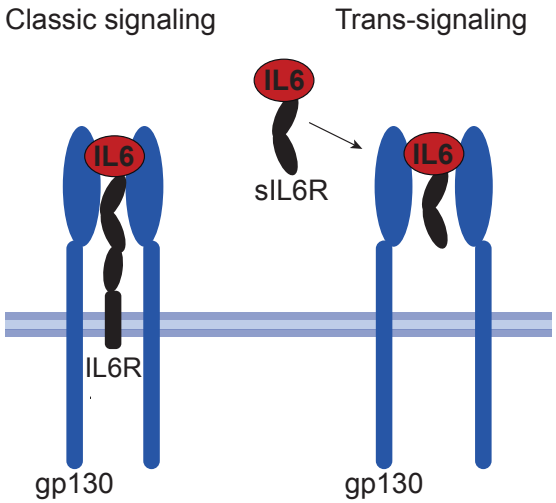

Figure 5. IL6 classic signaling and trans-signaling IL6 classic signaling involves the membrane-bound IL6 receptor (IL6R) which binds to gp130 homodimers upon IL6 binding. IL6 trans-signaling requires a soluble form of IL6R (sIL6R) which binds to IL6. This complex can stimulate cells that only express gp130.

Interestingly, several soluble forms of gp130 (sgp130) have been also found in serum. They are generated by alternative splicing and polyadenylation and bind to IL6-sIL6R complex acting as an inactivating or decoying mechanism (Jostock et al., 2001; Richards et al., 2006; Sommer et al., 2014a). In theory, all the cells expressing gp130 could be activated through trans-signaling and, therefore, a control mechanism has been developed to avoid hyper activation. This is achieved through the formation of IL6-sIL6R-sgp130 complexes in the fluids. In basal conditions, the levels of sIL6R and sgp130 are higher than IL6 and then, the activity of the cytokine is neutralized. Only, when IL6 and sIL6R levels increase, they cannot be decoyed by sgp130 and activate the trans-signaling cascade (Baran et al., 2018). sgp130 exclusively blocks trans-signaling without interfering the responses via classic signaling. This molecular tool has been used to find new therapies for diseases in which sIL6R has a central role. For example, sgp130Fc (fusion protein of sgp130 and IgG1-Fc) treatment in a mouse model of atherosclerosis lead to a significant regression of advanced states, reduced atherosclerotic plaques and lower monocyte recruitment (Richards et al., 2006; Schuett et al., 2012).

Finally, IL6 trans-signaling is mimicked by the human herpesvirus 8. This virus encodes for a viral form of IL6 which binds to gp130, but not to IL6R, and activates STAT3 and proliferation. It affects neutrophil infiltration for inducing the pathology (Adam et al., 2009; Hoischen et al., 2000).

4.2. Crosstalk of IL6 type cytokines

All the cytokines of the IL6 family, signal through the specific combination of β-receptor which is gp130 (Garbers et al., 2012) and α-receptors to increase target specificity. IL6 and IL11 are the only members which signal via gp130 homodimers and they first bind to its α-receptor (IL6R or IL11R respectively) **(Figure 6)**. The rest of the cytokines signal via formation of heterodimers gp130-LIFR, gp130-oncostatin M receptor (OSMR) or gp130-WSX1 (IL27R). Especially, CNTF and IL31 need an additional receptor (CNTFR or IL31R) (Cornelissen et al., 2012; Ip et al., 1993) **(Figure 6)**. There are differences in the binding of the cytokines to the heterodimers, LIF first binds to LIFR and then to gp130 to transduce the signal whereas, OSM binds first to gp130 and then this complex interacts with LIFR, in human cells, or OSMR, in murine and human cells (Hilton and Nicola, 1992; Miyajima et al., 1997). Importantly, IL6-type cytokine receptors can be classified into non-signaling receptors (IL6R, IL11R and CNTFR), where the signal is transduced exclusively through gp130, and signal-transducing receptors (gp130, LIFR, OSMR, IL27R), where the signal transduction involves gp130 and the specific cytokine receptor (Garbers et al., 2012) **(Figure 6)**.

Low affinity bindings have been described for IL6R. CNTF and IL27 bind to IL6R and then, gp130 is able to activate STAT3 (Crabé et al., 2009; Schuster et al., 2003). Additionally, soluble forms of other receptors, sIL11R and sCNTFR have been observed however, its trans-signaling relevance *in vivo* has not been addressed (Davis et al., 1993; Pflanz et al., 2002).

It is not clear yet whether the intracellular outcome of these cytokines is similar or it is different. All the cytokines use the signal transducer gp130, however, some of them use their specific α-receptor as signaling molecule and the intracellular consequences are not clear (Garbers et al., 2012).

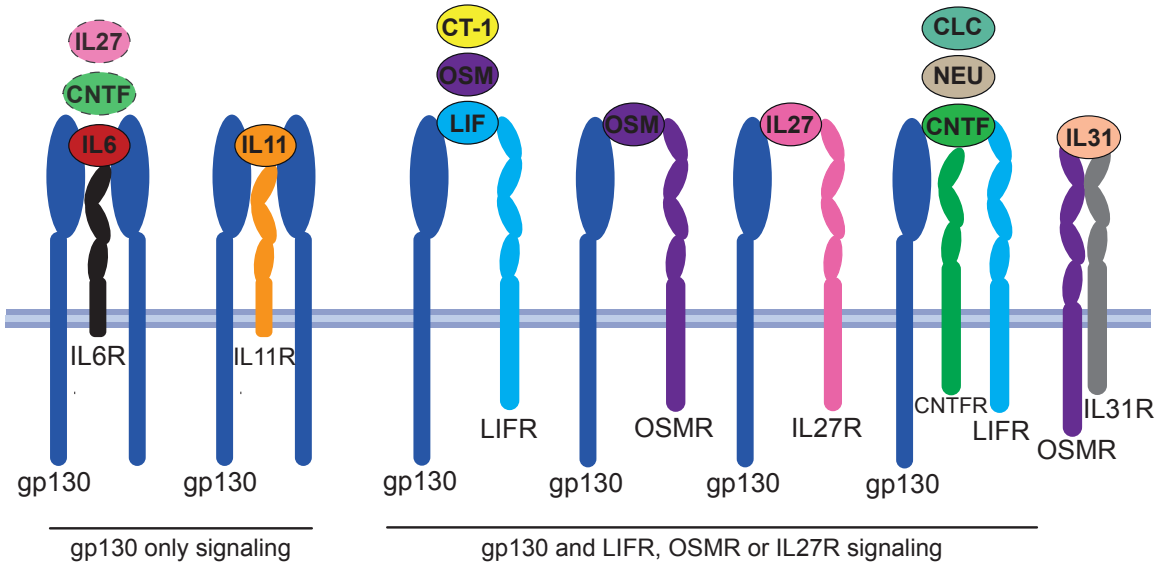

Figure 6. IL6 family cytokines and their receptor complexes. IL6 related cytokines signal via a specific α-receptor and a common β-receptor (gp130). IL6 and IL11 are the only two cytokines which bind to their receptor, IL6R or IL11R, and signal through gp130 homodimers. LIF, OSM, CT-1, CNTF, NEU and CLC signal through gp130-LIFR/OSMR/IL27R heterodimers. In the case of CNTF, NEU and CLC, an extra α-receptor (CNTFR) is required for signaling. OSM can signal using gp130-LIFR complexes in human cells or gp130-OSMR in mouse and human cells. IL31 is the exception of the family and binds to OSMR and IL31R. Low affinity bindings are depicted with dashed circles (IL27 and CNTF with IL6R). IL6: interleukin 6, IL11: interleukin 11, IL27: interleukin 27, IL31: interleukin 31, LIF: leukemia inhibitory factor, OSM: oncostatin M, CT-1: cardiotrophin 1, CNTF: ciliary neurotrophic factor, NEU: neuropoietin , CLC: cardiothrophin-like cytokine, gp130: glycoprotein 130.

4.3. Strategies to study IL6 *in vivo*

Transgenic mice overexpressing human sIL6R and IL6, since murine IL6 cannot bind to the human receptor (Dam et al., 1993), showed massive extramedullary hematopoiesis and hepatocellular proliferation (Maione et al., 1998; Peters et al., 1997; Schirmacher et al., 1998). However these phenotypes were not observed in IL6 only transgenic mice, reinforcing the idea that trans-signaling effects are different to classic ones (Woodroofe et al., 1992).

When IL6 is deleted using a knockout model, mice have less tumors and inflammation in an AOM/DSS colon cancer model (Grivennikov et al., 2009). The authors speculated that the effect could be driven by classic signaling. However, *IL6R* null mice do not have the same phenotype, these mice are comparable to wild type mice (Sommer et al., 2014b). This discrepancy has been also observed in a model of wound healing. *IL6* null mice present the most severe impairment repairing the wound whereas, *IL6R* null mice have improved wound contraction similar to wild type mice (McFarland-Mancini et al., 2010). Both studies hypothesized that possible compensatory mechanisms could be activated in the absence of the receptor or unknown receptors for IL6 or ligands for IL6R could have a role in those protocols.

In a severe inflammatory scenario mimicking human sepsis and in a more therapeutic approach, mice were treated with neutralizing IL6 antibodies. They do not survive however, when mice were treated with sgp130Fc, and trans-signaling was blocked, all the animals survived. Therefore, from these studies we can conclude that it is not only important to know which molecule is the player but also, whether classic or trans-signaling are the key drivers of the process to block specifically that part of the signaling.

All this knowledge has been applied clinically for treatment of chronic inflammatory diseases. Monoclonal antibody targeting human IL6R (tocilizumab) has beneficial effects on Castleman's disease, juvenile idiophatic arthritis and rheumatoid arthritis, where it has been approved by the FDA (Tanaka et al., 2012). Also antibodies against IL6 (siltuximab) have been proven to provide benefit in patients for Castleman's disease and are approved by FDA (Deisseroth et al., 2015). However, not all the chronic inflammatory diseases have taken advantage of these treatments. Therapies blocking IL6 signaling in multiple myeloma or metastatic renal cell carcinoma do not have effect (Rossi et al., 2015).

Objectives

The main hypothesis of this thesis was to test whether senescent cells, through their secretome, may affect the acquisition of pluripotency and, if this were the case, to study the mechanism involved. To test this hypothesis, we have addressed the following objectives:

1. Study the effect of senescent conditioned media during *in vitro* reprogramming

1.1. Identify possible pro-reprogramming molecules in the SASP of senescent cells induced by different stimuli

1.2. Determine whether *Ink4a*, *Arf* or *p53* tumor suppressors regulate this process

2. Study the role and function of IL6 in reprogramming

2.1. Explore the requirement of IL6 in reprogramming

2.2. Analyze the interplay between LIF and IL6

2.3. Identify the molecular mechanisms underlying the role of IL6 in reprogramming

Materials and Methods

1. Mouse work

1.1. Animal housing

Mice were housed at the specific pathogen-free (SPF) barrier area of the Spanish National Cancer Research Center (CNIO) in Madrid and of the Institute for Research in Biomedicine (IRB Barcelona) in Barcelona. All animal procedures were performed according to the protocols approved by the CNIO-ISCIII Ethical Committee for Research and Animal Welfare (CEIyBA) in Madrid and by the Animal Care and Use Ethical Committee of animal experimentation of Barcelona Science Park (CEEA-PCB) and the Catalan Government in Barcelona. Mice were observed daily and sacrificed when they showed signs of human end point.

1.2. Mouse models

Reprogrammable (*i4F*) mouse was generated previously in the laboratory (Abad et al., 2013). *i4F*-B strain carries an ubiquitous doxycycline-inducible transgene encoding for the four Yamanaka factors *Oct4*, *Sox2*, *Klf4* and *cMyc* (*OSKM*) inserted in *Pparg* gene and the transcriptional activator (rtTA) within the *Rosa26 locus*. These mice were crossed with null alleles for *p53* (Jacks et al., 1994) and *Ink4a/Arf* (Serrano et al., 1996) to generate reprogrammable mice deficient for those tumor suppressors. All these animals are in a pure C57BL6/J background.

i4F-B mice were also crossed with $IL6R^{lox/lox}$ mice from the Jackson Laboratory (stock number 012944) and a Sox2-Cre recombinase strain. Sox2-Cre transgenic mice (stock number 008454), kindly provided by Travis Stracker's laboratory at IRB Barcelona, express Cre recombinase under the control of *Sox2* promoter to generate full null embryos for IL6R.

1.3. Genotyping

$IL6R^{\Delta/\Delta}$, $IL6R^{lox/lox}$ and $IL6R^{+/+}$ mice were genotyped by standard PCR procedures with the primers described in Table 1.

Primer	Forward Sequence (5' → 3')	Reverse Sequence (5' → 3')
mIL6R lox wt	GGTCACGGGCACTCCTTGGATAGGTACC	CCCAGTGAGCTCCACCATCAAA
mIL6R lox del	GGGTAGGCCCTGCTACCATGAAG	CCCAGTGAGCTCCACCATCAAA

Table 1. Primers for genotyping $IL6R^{lox/lox}$ strain.

The pair of primers mIL6R lox wt amplify a band of 367 bp for wild type allele and 485 bp for lox allele. Primers mIL6R lox del amplify a band of 2000 bp for lox allele and 623 bp for delta allele.

Other genetic modifications were genotyped by Transnetyx using real-time PCR.

2. *In vitro* procedures

2.1. Cell lines and culture conditions

Primary mouse embryonic fibroblasts (MEFs) were isolated from embryos at day E13.5 of

development from wild type, *Ink4a/Arf* null, *p53* null, *IL6R* null (from Sox2-Cre strain) with and without the reprogrammable *i4F* cassette as previously described (Serrano et al., 1997). MEFs were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% heat inactivated Fetal Bovine Serum (FBS) (Gibco). MEFs were irradiated with γ-irradiation at 20 Gy to generate γ-irradiated (γ-IR) senescent cells when was required.

Induced pluripotent stem (iPS) cells or embryonic stem (ES) cells were maintained on γ-IR MEFs or on gelatin-coated plates in high glucose DMEM (Gibco) supplemented with 15% of KnockOut Serum Replacement (KSR) (Gibco), 1000 U/ml of LIF (ESGRO, Sigma), non-essential aminoacids (Gibco) and β-mercaptoethanol (Gibco). This medium is called iPS medium.

HEK293T cells were maintained in DMEM supplemented with 10% FBS.

All the cell lines were incubated at 37ºC in normoxia (20% of O_2 and 5% of CO_2).

2.1.1. Neonatal astrocyte isolation

Neonatal primary astrocytes were isolated from post-natal P1 *i4F* pups. Mouse cortex was dissected and incubated with 0.25% trypsin (Invitrogen) for 30 minutes at 37ºC. After that, cells were dissociated mechanically and seeded in T25cm^2 tissue culture flask in DMEM/F12 (Gibco) supplemented with 20% FBS. Once the cells were confluent (7-10 days), they were shaken at 250rpm overnight to remove nonadherent cells for two consecutive days. Remaining cells were treated with 6 μM of cytosine β-D-arabinofuranoside (Sigma) for one day to remove rapidly diving cells. After that, cells were trypsinized and replated on poly-L-Lysine-coated plates for reprogramming.

2.2. 3T9 assay

3T9 cultivation was done as previously described (Todaro and Green, 1963). 9x10^5 cells were plated on 10cm-diameter plates, 3 days later the cells were recounted and replated at the same density. This procedure was repeated 9 times until cells stopped proliferating. Then, 1.5x10^6 of 3T9 MEFs were plated on 10cm-diameter plates and cultured in 10 ml of iPS medium. The conditioned medium was prepared as described in Section 2.3. of materials and methods.

2.3. Conditioned Medium preparation

To produce Conditioned Medium (CM), 1.5x10^6 of senescent MEFs were plated in 10-cm-diameter gelatin-coated plates, and cultured in 10 ml of iPS medium. The medium was collected and filtered (0.22 μm) for 3 or 6 days everyday. CM preparations were used freshly or stored frozen at -20ºC.

In the case of rapamycin-treated CM, irradiated cells were plated at the previous density and treated with rapamycin (Sigma) for 5 days at 12.5 nM. Then, cells were washed twice with PBS and cultured in 10 ml of iPS medium. The medium was processed as previously described for 3 days.

2.4. Retroviral and lentiviral transduction

HEK293T cells (ATCC number CRL-11268) were transfected with TetO-FUW-*OSKM* (Addgene 20321), FUW-M2rtTA (Addgene, 20342), pMXs-*Oct4/3* (Addgene, 13366), pMXs-*Sox2* (Addgene,

13367), pMXs-*Klf4* (Addgene, 13370), pMXs-*cMyc* (Addgene, 13375), pBabe-H-*ras*V12 (Serrano et al., 1997), pBabe-h*PIM1*, LV-m*Lifr* (VectorBuilder, pLV[TetOn]-Bsd-TRE>mLifr[NM_013584.2]), vectors expressing mouse sh*RNA* against *Ink4a*, *Arf*, *Ink4a/Arf* and *Il6* (Sigma, TRCN0000067552) and their corresponding packaging vectors using Fugene6 transfection reagent (Promega). *Ink4a/Arf* sh*RNAs* vectors were kindly provided by Scott Lowe (Dickins et al., 2005) and pBabe-h*PIM1* by Amancio Carnero.

One day after transfection, viral supernatants were collected twice a day every two days and used freshly or stored at -80ºC.

MEFs were plated the day after the transfection at the desired density and infected two consecutive days. Viral supernatants were supplemented with Polybrene (Sigma) at 8 μg/ml before adding to the cells. When MEFs were transduced with shRNAs (*Ink4a*, *Arf*, *Ink4a/Arf* or *Il6*), pBabe-h*PIM1* or pBabe-H-*ras*V12, puromycin (Sigma) was added at 2 μg/ml to select the positive population. In the case of LV-m*Lifr*, MEFs were selected with blasticidin at 10 μg/ml.

2.5. In vitro reprogramming

MEFs were reprogrammed following Yamanaka's protocol as previously described (Takahashi and Yamanaka, 2006) in the experiments in which not all the four factors were present in the cocktail. $3x10^5$ cells were transduced with combinations of pMXs-*Oct3/4*, pMXs-*Sox2*, pMXs-*Klf4* or pMXs-*cMyc* retrovirus and were cultured in iPS medium for approximately 10 days until iPS colonies appeared.

When *OSKM* reprogramming factors were overexpressed, $3x10^5$-10^5 *i4F* MEFs or MEFs transduced with lentiviruses encoding for Tet-O-FUW-*OSKM* and FUW-M2rtTA were reprogrammed. Cells were treated with doxycycline (Sigma) at 1 μg/ml in iPS medium for approximately 10-14 days until iPS colonies appeared.

For co-culture reprogramming experiments, $3x10^5$ γ-IR MEFs (20 Gy) were plated in 35-cm-diameter plates and, on top of them, $1x10^5$ wild type, *Ink4a/Arf* null, *p53* null *i4F* MEFs were plated. Cells were treated with doxycycline at 1 μg/ml in iPS medium for 10 days until iPS colonies appeared.

For *in vitro* reprogramming with CM from γ-IR MEFs, $2x10^5$-10^4 *i4F* MEFs were cultured in iPS medium or CM with doxycycline at 1 μg/ml for 10-17 days.

2.6. In vitro treatments

To block IL6 during *in vitro* reprogramming, anti-IL6 antibodies (BioXCell, BE0046) or control IgG (Santa Cruz Biotechnology sc-2027) were added at 0.1 mg/ml or 0.02 mg/ml. When IL6 was neutralized in CM, medium was preincubated with anti-IL6 antibodies at 0.1 mg/ml or 0.02 mg/ml for 30 minutes before adding to reprogrammable cells.

iPS medium was supplemented with rmIL6, rmCNTF, rmNEU, rmCT-1, rmIL11, rmIL27 or rhOSM (Peprotech) at 100 ng/ml during the process of reprogramming when was required.

Inhibitors for JAK (JAKi) (Merck, 420099) and Pim1 (PIMi) (Merck, 526520) were used for time course experiments at 1 μM and at 100 nM respectively.

2.7. Time course experiments

$3x10^5$ WT or *i4F* MEFs were plated in 6-well culture plates and treated with rmIL6 (Peprotech) at 100 ng/ml, JAKi (Merck, 420099) at 1 μM or PIMi (Merck, 526520) at 100 nM in DMEM medium or iPS medium (in the case of simultaneous induction of *OSKM*). Cells were treated once and then, samples were harvested at different time points for mRNA and protein analysis.

2.8. Alkaline phosphatase staining

Cells were fixed with 4% paraformaldehyde, washed with PBS and incubated from 30 minutes to 1 hour with the alkaline phosphatase (AP) staining solution (AP Blue Membrane Substrate Solution, Sigma). After washing with distilled water, plates were scanned and positive colonies were counted.

2.9. Crystal violet staining

Cells were fixed with 4% paraformaldehyde, washed with PBS and incubated for 30 minutes with the staining solution (0.5% crystal violet and 6% glutaraldehyde in distilled water). After washing with distilled water, plates were scanned.

3. Reprogramming efficiency analysis

Reprogramming efficiency was calculated based on AP staining at day 10-17 of reprogramming. AP^+ colonies were scored and divided by the number of cells plated at the beginning to calculate the reprogramming efficiency.

4. Cytokines analysis

Cytokine levels in CM were analyzed by Mouse Cytokine Array (ProteomeProfiler mouse Cytokine Array Panel A, R&D Systems) following the manufacturer's instructions. The pixel density was determined by ImageJ Software.

5. DNA analysis

For genotyping mice, DNA was extracted from mouse tails. Tissue was incubated with proteinase K (Roche) at 400 μg/ml overnight and isolated following standard phenol:chloroform:isoamyl alcohol (25:24:1) (Sigma) extraction protocol. Up to 100ng of DNA was used to perform a genotyping PCR as specified in Section the 1.3. of materials and methods.

6. mRNA analysis

Total RNA was isolated from cells or tissue samples with Trizol (Invitrogen), chloroform and isopropanol following manufacturer's instructions. 1 μg of total RNA was retrotranscribed into cDNA using iScript™ cDNA Synthesis kit (BioRad 170-8891) according to the manufacturer's

protocol. Quantitative real time-PCR (qPCR) was performed using GoTaq® qPCR Master Mix (Promega A6002) in a QuantStudio 6 Flex thermocycler (Applied Biosystem). Samples were analyzed in triplicate and normalized to *Gapdh*. Calculations were made using the ΔΔCt method.

Primer sequences for mRNA analysis are described in Table 2 for mouse (m) and human (h) genes.

Primer	**Forward Sequence (5' → 3')**	**Reverse Sequence (5' → 3')**
Aldh1l1 (m)	CAGGAGGTTTACTGCCAGCTA	CACGTTGAGTTCTGCACCCA
Aqp4 (m)	GACCCGCAGTTATCATGGGAA	CACTTGGCTCCGGTTGTCC
Arf (m)	GCCGCACCGGAATCCT	TTGAGCAGAAGAGCTGCTACGT
CCL5 (m)	GCCCACGTCAAGGAGTATTTCTAC	AGGACTAGAGCAAGCGATGACAGG
E2A-cMyc (m)	GGCTGGAGATGTTGAGAGCAA	AAAGGAAATCCAGTGGCGC
Endogenous *Oct4* (m)	TCTTTCCACCAGGCCCCCGGCTC	TGCGGGCGGACATGGGGAGATCC
Endogenous *Sox2* (m)	TAGAGCTAGACTCCGGGCGATGA	TTGCCTTAAACAAGACCACGAAA
Gapdh (m)	TTCACCACCATGGAGAAGGC	CCCTTTTGGCTCCACCCT
Gfap (m)	CCCTGGCTCGTGTGGATTT	GACCGATACCACTCCTCTGTC
gp130 (m)	TCCCATGGGCAGGAATATAG	CCATTGGCTTCAGAAAGAGG
Il6 (m)	GTTCTCTGGGAAATCGTGGA	GGTACTCCAGAAGACCAGAGGA
Il6ra (m)	GGTGATCATTCAGGGAGCAT	GGCTCACAAAACAGAGAATGG
Ink4a (m)	TACCCCGATTCAGGTGAT	TTGAGCAGAAGAGCTGCTACGT
Lif (m)	GGCAACCTCATGAACCAGATC	GTCTGTCATGTTAGGCGCAC
Lifr (m)	AGCTCTGACCCTCCTGCAT	TGGGTGACAAGAATGGAACCT
MIP-1α (m)	CTCCCAGCCAGGTGTCATTTT	CTTGGACCCAGGTCTCTTTGG
MIP-2 (m)	CTCAAGGGCGGTCAAAAAGT	TTTTTCTTTCTCTTTGGTTCTTCC
Nanog (m)	CAAGGGTCTGCTACTGAGATGCTCTG	TTTTGTTTGGGACTGGTAGAAGAATCAG
NKX3-1 (m)	ATGCTTAGGGTAGCGGAGC	TGCGGATTGCCTGAGTGTC
PIM1 (h)	CGAGATCGCCATATTTGGTGTCCCCGAG	CCAGCTTGGTGGCGTGCAGGTCGTTGCA
Sox2-Klf4 (m)	ACTGCCCCTGTCGCACAT	CATGTCAGACTCGCCAGGTG
Stat3 (m)	GTCCTTTTCCACCCAAGTGA	TATCTTGGCCCTTTGGAATG
Tnf (m)	GCCTCTTCTCATTCCTGCTT	CTCCTCCACTTGGTGGTTTG
Cntf (m)	TCTGTAGCCGCTCTATCTGG	GGTACACCATCCACTGAGTCAA
Il27 (m)	CTGTTGCTGCTACCCTTGCTT	CACTCCTGGCAATCGAGATTC
Il11 (m)	TGTTCTCCTAACCCGATCCCT	CAGGAAGCTGCAAAGATCCCA
Osm (m)	CAGTATGCAGACACGGCTTC	TGATTCTGTGTTCCCCGTGA

Table 2. Primers used for mRNA analysis.

7. Protein analysis

Samples were homogenized in RIPA lysis buffer (10 mM TrisHCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS and 5 mM EDTA) supplemented with protease inhibitor cocktail (Sigma) and sonicated for 10 minutes each. In the case of tissue extracts, lysates were homogenized using FastPrep-24 5G (MP Biomedicals). Up to 30 µg of total protein per sample was loaded in NuPAGE 4 - 12% Bis-Tris Gel 1.0 mm (Invitrogen) and immunoblot analyses were performed according to standard procedures. Membranes were incubated with the primary antibodies described in Table 3 overnight at 4ºC. Then, membranes were washed three times with PBS-Tween and incubated for 1 h with the LICOR secondary antibody **(Table 3)**. Membranes were analyzed using Odissey Fc Imaging System (LICOR).

Antibody	Isotype	Reference	Dilution
anti-SMC-1	Rabbit IgG	Bethyl, A300-055A	1:2000
anti-STAT3 total	Mouse IgG2a	Cell Signaling, 9139	1:1000
anti-pSTAT3 Tyr705	Rabbit IgG	Cell Signaling, 9145	1:1000
anti-pNFκB (p65) Ser536	Rabbit IgG	Cell Signaling, 3033	1:1000
IRDye 800CW Goat anti-Rabbit	Goat	LICOR, 926-32211	1:20000
IRDye 680RD Goat anti-Mouse	Goat	LICOR, 926-68070	1:20000
SSEA1-BV647	Mouse IgM	Biolegend, 125607	1:400
Thy1-FITC	Rat IgG2a	eBioscience, 11-0902-85	1:1000
PDGFR-PECy7	Rat IgG2a	Biolegend, 135912	1:400

Table 3. Antibodies used for immunoblotting and FACS analysis.

8. FACS analysis

Cells were trypsinized at different time points of reprogramming (day 0, 3, 5, 7 and 11) and blocked in Fc blocking solution (CD16/CD32, eBioscience 25-0161-82) diluted 1:200 in FACS Buffer (0.5% BSA and 5 mM EDTA in PBS) for 20 minutes at 4ºC. Then, cells were incubated with DAPI and the conjugated primary antibodies for 30 minutes at 4ºC. We analyzed the following combinations: i) Thy1-FITC and SSEA1-BV647 and ii) PDGFR-PECy7 and SSEA1-BV647. Cells were analyzed in a Gallios Flow Cytometry System (Beckman Coulter) and all data was analyzed using FlowJo v10 and GraphPad Prism softwares.

9. Statistical analysis

Data are expressed as mean ± SD and the differences were considered significant based on *P* value (* $P<0.05$, ** $P<0.01$, *** $P<0.001$). Data were obtained from independent biological replicates (n values correspond to independent mice or MEFs) specified in each figure. Technical replicates were not considered in the n value.

Statistical significance was assessed using two-tailed unpaired Student's t-test with Welch's correction and one-way or two-way ANOVA with Bonferroni post-hoc test. These statistical analyses were performed using GraphPad Prism software.

Results

1. INK4A DEPENDENT SASP FACTORS PROMOTE REPROGRAMMING

The results described in this part have been done in close collaboration with Lluc Mosteiro and Cristina Pantoja at CNIO and have been published in 2016 (Mosteiro et al., 2016).

1.1. Impact of senescent conditioned media on *in vitro* reprogramming

Regarding reprogramming, many studies have identified alternative transcription factors, small molecules or systems to overcome some limitations of the process such as inefficiency and safety. On the other hand, the role of senescent cells in cancer (Collado et al., 2005), embryo development (Muñoz-Espín et al., 2013) or fibrosis (Muñoz-Espín et al., 2018) have been studied deeply in our laboratory. However, the potential involvement of senescent cells on reprogramming was unexplored. We wondered whether senescent cells could facilitate reprogramming in a paracrine manner through their highly secretory phenotype (SASP), which includes the production and secretion of numerous cytokines and growth factors. First, we generated senescent cells using different inducers and then, conditioned medium (CM) was collected every 24 hours for three or seven days. We tested oncogene-induced senescence (OIS) by overexpression of $HRAS^{G12V}$, replicative senescence using serially passaged 3T9 MEFs, which had exhausted their proliferative capacity, and DNA damaged senescent cells by γ-irradiation (γ-IR) at 20 Gy. We added the three types of CM during the reprogramming of mouse embryonic fibroblasts (MEFs) carrying a doxycycline-inducible *OSKM* cassette from our reprogrammable mice, here abbreviated as *i4F* MEFs (Abad et al., 2013). iPS colony formation was evaluated with alkaline phosphatase (AP) staining. AP^+ colonies were scored and only CM from γ-IR cells increased reprogramming efficiency significantly after 10 days of treatment **(Figure 7)**.

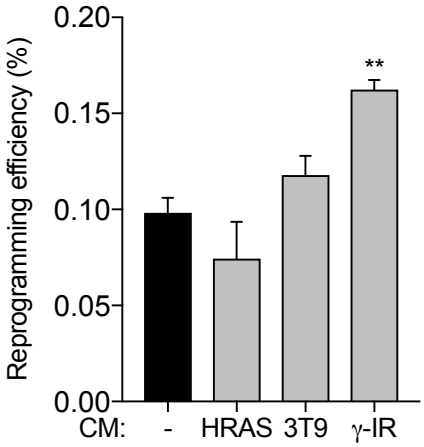

Figure 7. γ-irradiated senescent conditioned medium increased *in vitro* reprogramming efficiency. Conditioned medium from different senescent cultures was added into *i4F* MEFs together with doxycycline at 1 μg/ml for 10 days. Reprogramming efficiency indicates the number of iPS colonies positive for alkaline phosphatase (AP) activity at day 10 of reprogramming divided by the initial number of MEFs ($2x10^5$). The values represent the average ± SD of a total of three independent MEF clones for each treatment (n=3). Statistical significance to untreated control was assessed using unpaired Student's t-test with Welch's correction. ** $P<0.01$.

We next wondered whether this beneficial effect of the CM from γ-IR cells could also favor *in vitro* reprogramming in non-optimal conditions, decreasing up to 20 times the initial cell density

(from $2x10^5$ to 10^4 cells). Positive colonies for AP were observed in all γ-IR CM-treated *i4F* MEFs at day 10 reprogramming however, untreated cells were not reprogrammed at the lowest densities **(Figure 8A, 8B and 8D)**. We also observed that CM-treated cells proliferated more during the process based on crystal violet staining performed at day 10 **(Figure 8C)**. This could be one of the reasons for the improvement since stimulation of the cell cycle has been associated with highly efficient *in vitro* reprogramming (Ruiz et al., 2011).

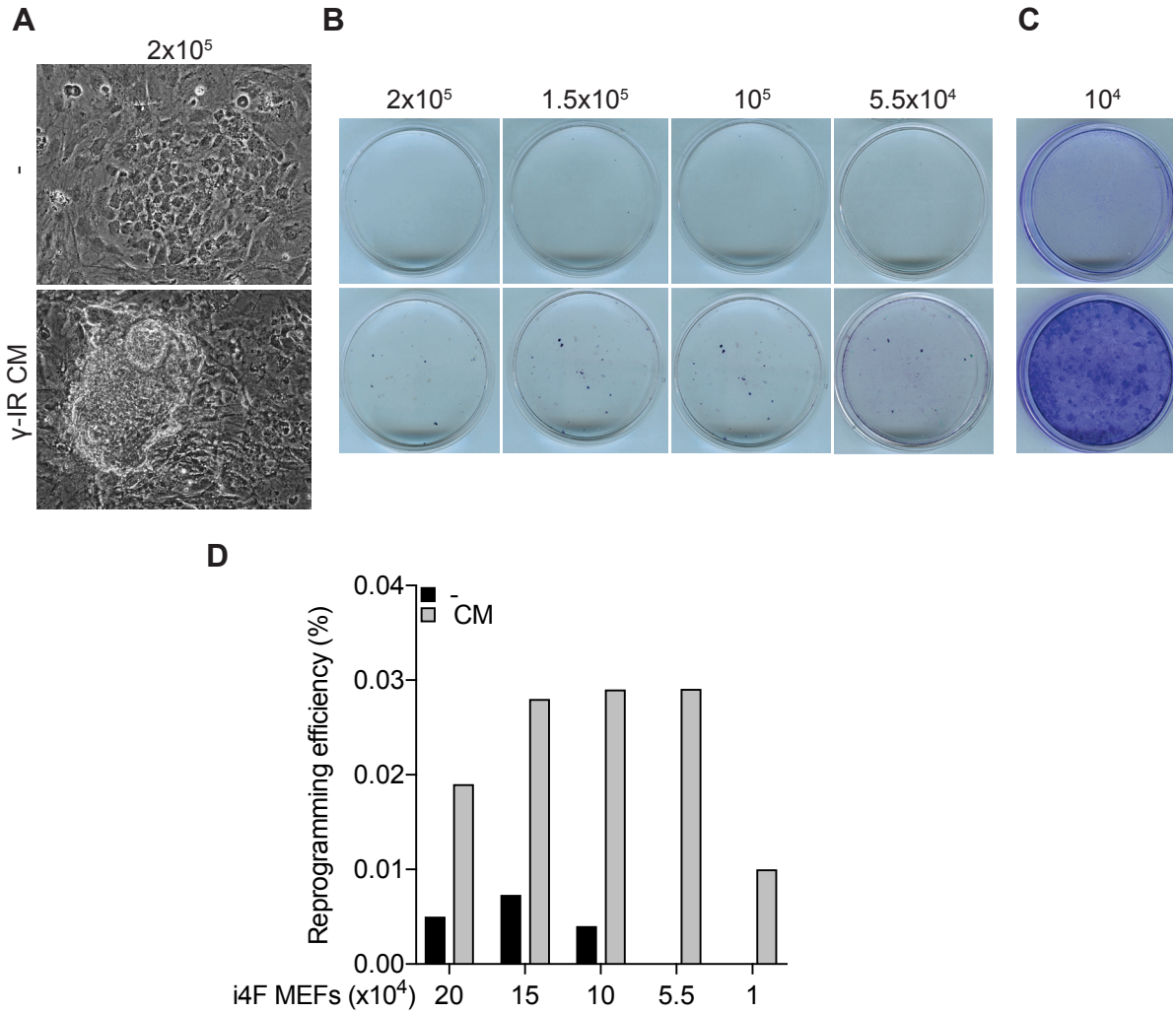

Figure 8. Effect of γ-irradiated senescent conditioned medium in non-favorable reprogramming. (A) Representative bright field pictures of untreated (no iPS colony) and γ-IR CM (iPS colony) *i4F* MEFs at 10 day of *in vitro* reprogramming. **(B)** Plates stained for alkaline phosphatase at day 10. **(C)** Crystal violet stained plates at the lowest cell density. **(D)** Reprogramming efficiency indicates the number of iPS colonies positive for alkaline phosphatase activity at day 10 of reprogramming divided by the initial number of MEFs. Statistical analysis was not performed because it was done with only one biological replicate.

On the other hand, we also checked whether our CM from γ-IR cells could have an effect on cells with high proliferation and high efficiency of reprogramming. *Ink4a/Arf* and *p53* null MEFs have been reported to reprogram *in vitro* with a higher efficiency than wild type (WT) cells (Banito et al., 2009; Kawamura et al., 2009; Li et al., 2009; Marión et al., 2009). Interestingly, we also observed an increase in reprogramming efficiency, although the magnitude of the effect was lower than in WT MEFs **(Figure 9)**. These observations suggest that the beneficial effect of the senescent CM cannot be explained exclusively by an increase in proliferation.

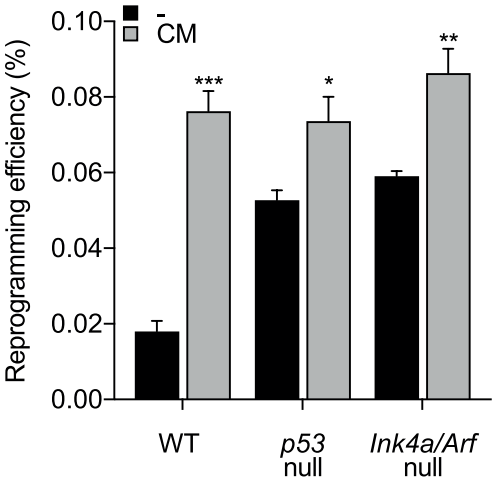

Figure 9. Reprogramming efficiency of *p53* null and *Ink4a/Arf* null *i4F* MEFs is increased with γ-irradiated senescent conditioned medium. Reprogramming efficiency was calculated counting positive iPS colonies for alkaline phosphatase at day 10 of reprogramming divided by the initial number of MEFs ($2x10^5$) of the different conditions. The values represent the average ± SD of a total of three independent MEF clones for each condition (n=3) Statistical significance to the corresponding untreated control was assessed using two-way ANOVA and Bonferroni post-hoc test. * P<0.05, ** P<0.01, *** P<0.001.

Together, these data indicate that the CM from γ-IR senescent cells has a beneficial effect on *in vitro* reprogramming.

1.2. Role of the tumor suppressors *Ink4a, Arf* and *p53* in the production of pro-reprogramming CM upon damage

It is well characterized the involvement of the tumor suppressors *Ink4a, Arf* and *p53* in senescence, as it was explained in the Introduction (Section 3.2.), as well as in the regulation of SASP (Coppé et al., 2008, 2011). Based on this, we decided to evaluate whether the absence of *Ink4a/Arf* or *p53* could affect the beneficial effect of the CM described previously. For that, we first prepared CM from irradiated MEFs of three different genotypes: WT, *p53* null and *Ink4a/Arf* null as it was done in previous experiments. The CMs were tested on WT *i4F* reprogramming. WT and *p53* null derived CMs increased *in vitro* reprogramming efficiency however, γ-IR *Ink4a/Arf* null derived CM lacked a positive effect and it was comparable to the untreated condition **(Figure 10A)**. To confirm these results, WT *i4F* MEFs were co-cultured with γ-IR MEFs of the three genotypes (WT, *p53* null and *Ink4a/Arf* null) in a 3:1 ratio. γ-IR WT and *p53* null MEFs increased the formation of iPS colonies from *i4F* cells achieving the highest efficiency in the absence of *p53*. On the contrary, *Ink4a/Arf* null senescent cells had little effect on *i4F* reprogramming, similar to the untreated condition **(Figure 10B)**. Interestingly, we could observe the following tendency in both experiments: *p53* null > WT > *Ink4a/Arf* null, meaning *p53* null condition as the highest efficient one.

These data suggest that the absence of *Ink4a/Arf* could be crucial for the production of a CM that promotes reprogramming. It is important to note that *p53* null MEFs do not undergo senescence, therefore, these results indicate that WT or *p53* null MEFs respond to damage by producing one or several factors that are absent in the *Ink4a/Arf* null MEFs.

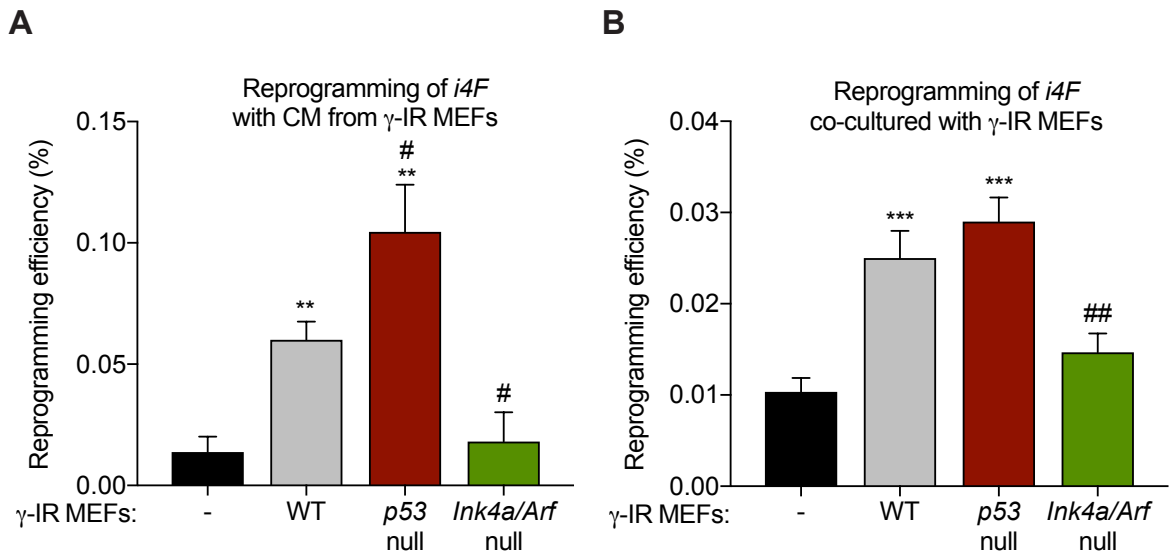

Figure 10. CM and co-culture of senescent cells enhance *in vitro* reprogramming. (A) Reprogramming efficiency of *i4F* MEFs treated with CM from γ-irradiated MEFs of the indicated genotypes was calculated as previous experiments. Initial *i4F* number MEFs was 10^5. **(B)** Reprograming efficiency of *i4F* MEFs co-culture with γ-irradiated MEFs (3:1) of the indicated genotypes was calculated as previously described. Initial *i4F* number MEFs was 10^5. A total of three biological and independent replicates were performed for each experiment (n=3) and graphs represent average ± SD. Statistical significance relative to untreated control was assessed by two tailed unpaired Student's t-test with Welch's correction. ** *P*<0.01, *** *P*<0.001. Comparisons of γ-irradiated *p53* null and *Ink4a/Arf* null CM with γ-irradiated WT CM were also assessed using the same test and are indicated with "#". # *P*<0.05, ## *P*<0.01.

1.3. Identification of pro-reprogramming cytokines in the conditioned media from damaged cells

We first wondered whether the secreted factors present in the CM of damaged WT cells was controlled by a classical SASP mechanism. Rapamycin is known to decrease SASP in senescent cells through the inhibition of mTORC1 (Laberge et al., 2015; Wang et al., 2017). We treated γ-IR WT MEFs with rapamycin for five days after irradiation and CM was collected the following three days in the absence of the drug. We observed that the effect was completely abolished, meaning that secreted SASP factors were involved **(Figure 11)**. Furthermore, we diluted 1:1 γ-IR WT CM with fresh iPS medium to decrease the concentration of SASP factors to determine whether the amount of them was crucial. Indeed, the efficiency was similar to untreated cultures **(Figure 11)**. Therefore, these results suggest that senescent CM contains key factors that facilitate *in vitro* reprogramming in a dose dependent manner.

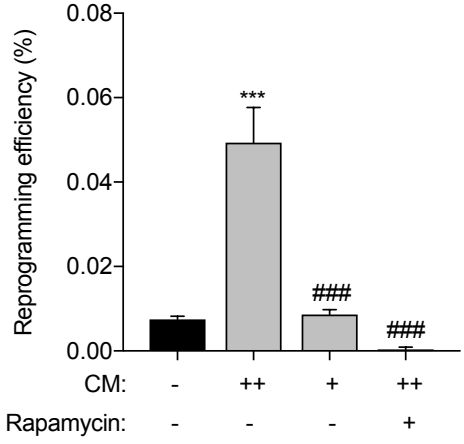

Figure 11. The production of pro-reprogramming factors by damaged cells require active mTOR. Reprogramming efficiency of *i4F* MEFs treated with CM from γ-irradiated WT MEFs as previously done (++), 1:1 CM diluted with fresh medium (+) and CM from γ-irradiated WT MEFs treated with rapamycin. MEFS were cultured with the inhibitor at 12.5 nM for 5 days after irradiation. Then, cells were washed with PBS three times and CM was harvested the following two days. AP staining was performed at day 17 of reprogramming. All values represent the average ± SD of a total of three independent MEF clones for each condition (n=3). Statistical significance to untreated control was assessed using unpaired Student's t-test with Welch's correction. *** P<0.001. Comparisons to usual CM condition (++) were calculated using the same test and are indicated using the symbol "#". ### P<0.001.

To identify the critical pro-reprogramming factors, we analyzed the CMs from WT, *p53* null and *Ink4a/Arf* null γ-IR MEFs from previous experiments in an array with immobilized antibodies for cytokines.

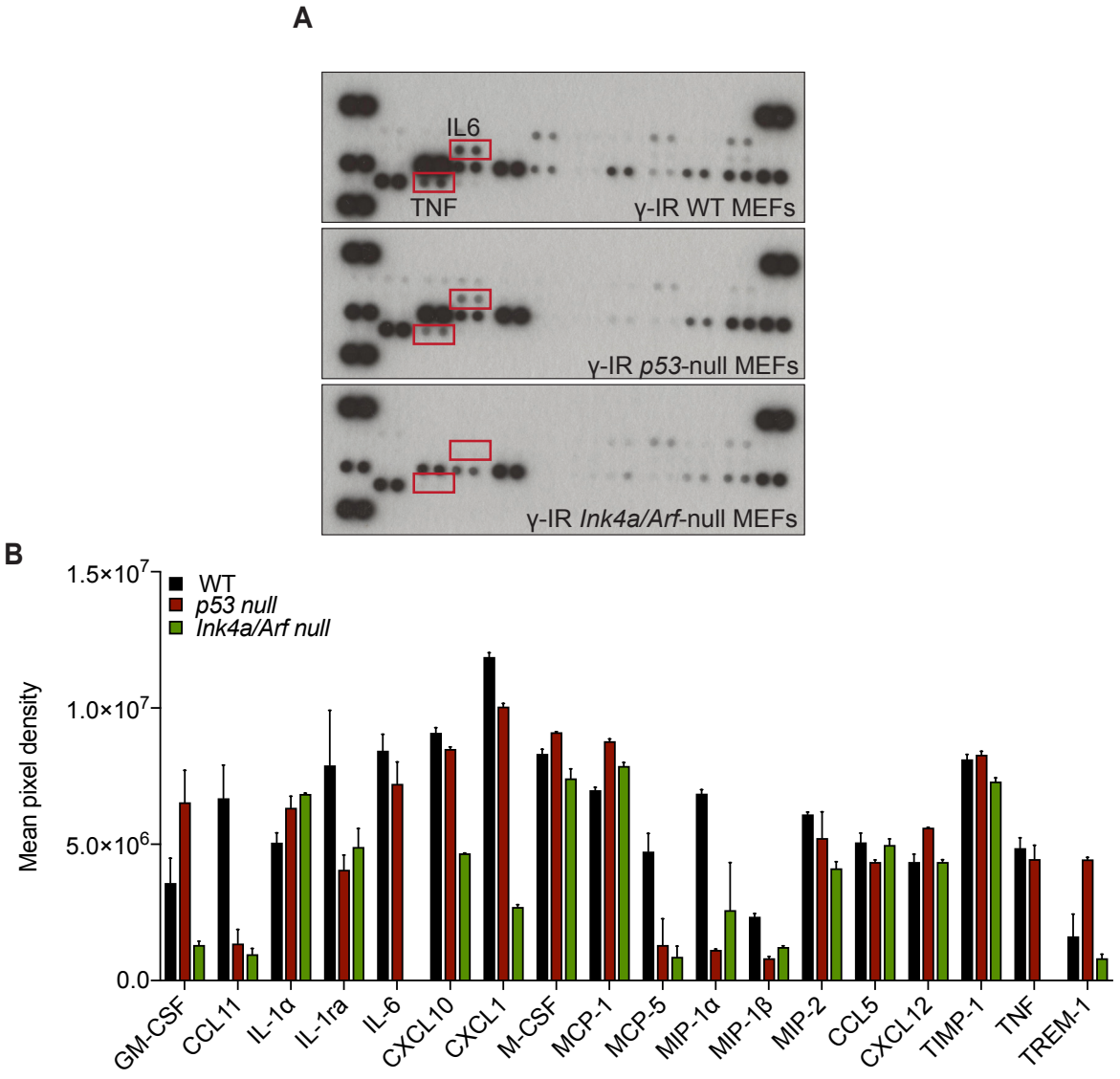

Figure 12. Differential levels of secreted factors in the CMs of irradiated MEFs. (A) Cytokine immunoblot array of the CMs from γ-irradiated MEFs of the three genotypes: WT, *p53* null and *Ink4a/Arf* null 7 days after irradiation. All the cytokines were detected in duplicate. IL6 and TNF are indicated with red rectangles. **(B)** Mean pixel density of the detected cytokines was calculated using Image J software. The data shown is the average ± SD of the duplicate of each molecule.

We searched for proteins that followed the same pattern as the pro-reprogramming activity previously observed in the CMs, that is: high in the CM of irradiated *p53* null and WT MEFs, and low or not present in the CM of irradiated *Ink4a/Arf* null MEFs. The only two detected cytokines that followed this tendency were IL6 and TNF **(Figure 12A and 12B)**.

We also measured the mRNA levels of both cytokines in γ-IR cells at day 3 post-irradiation.

Interestingly, *Il6* and *Tnf* mRNAs were highly expressed in γ-IR *p53* null cells and moderately in WT condition, whereas they were not upregulated in γ-IR *Ink4a/Arf* null cells **(Figure 13A and 13B)**. This differential overexpression correlated well with the increased in *in vitro* reprogramming efficiency of previous experiments **(Figure 10A and 10B)**.

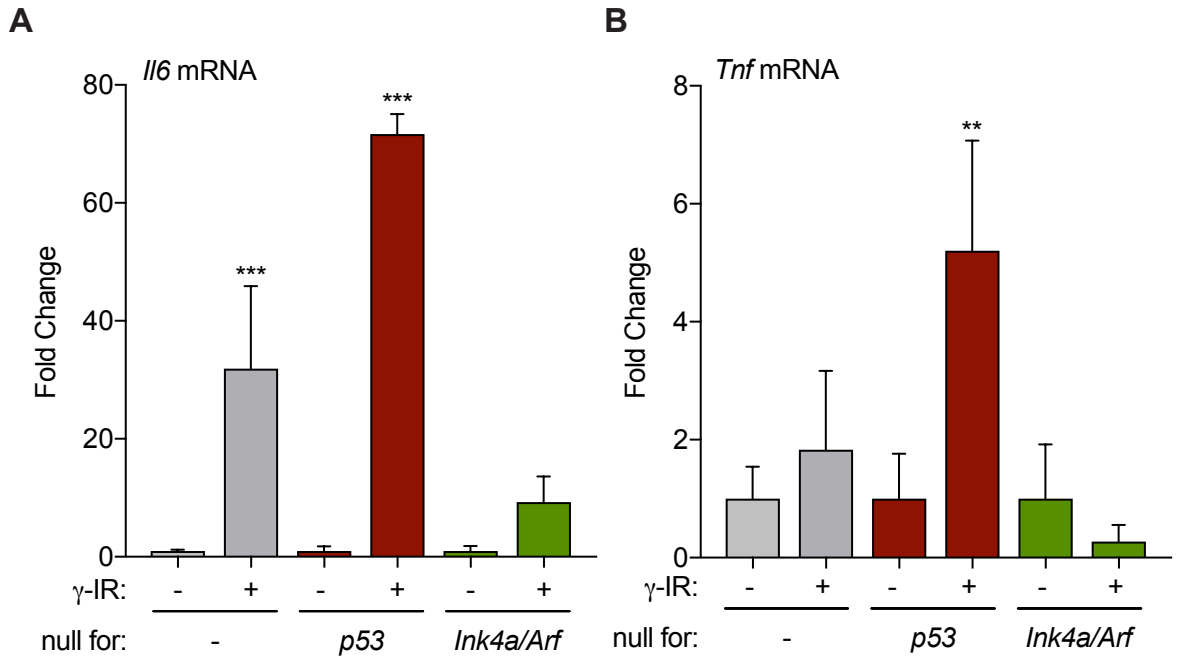

Figure 13. *Il6* and *Tnf* are upregulated after irradiation. (A and B) mRNA levels of *Il6* and *Tnf* in control and γ-irradiated MEFs of the indicated genotypes at day 3 after γ-irradiation. All values represent the average ± SD of a total of three independent MEF clones for each condition (n=3) relative to non-irradiated condition. Statistical significance to untreated or non-irradiated control was assessed using unpaired Student's t-test with Welch's correction. ** $P<0.01$, *** $P<0.001$.

Furthermore, we checked the mRNA levels of other cytokines, which were also present in the CMs, to confirm that only *Il6* and *Tnf* fitted in the model. None of the tested SASP factors followed the expected trend **(Figure 14A)**. Finally, we validated that γ-IR cells were expressing some senescence markers such as *Ink4a* and *Arf* **(Figure 14B)**.

All these results suggest that the cytokines responsible for the beneficial effect on reprogramming were only IL6 and TNF and both of them were overexpressed and secreted after damage in *WT* and *p53* null background. However, *Ink4a/Arf locus* was crucial for their production.

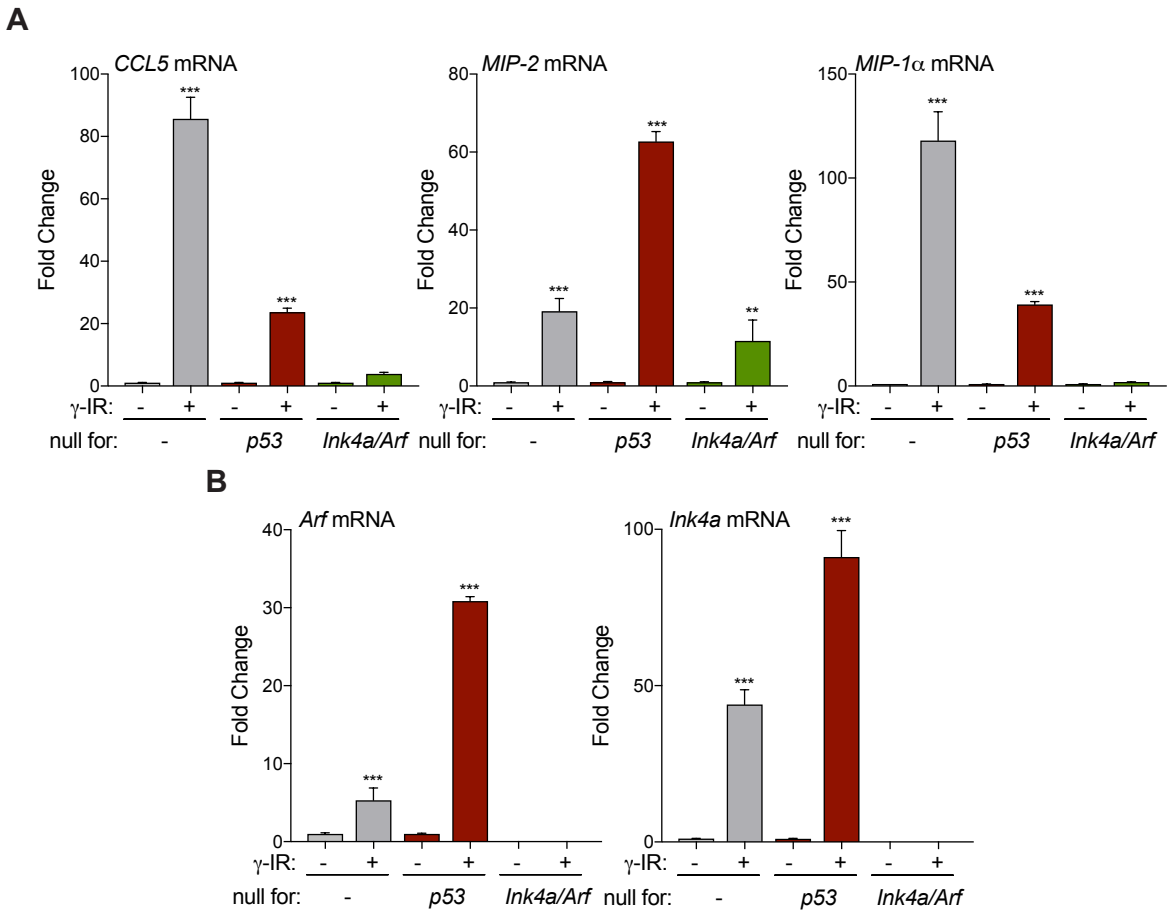

Figure 14. Expression of SASP factors and senescent markers in irradiated MEFs. *CCL5*, *MIP-2* and *MIP-1α* **(A)**, *Ink4a* and *Arf* **(B)** in control and γ-irradiated MEFs of the indicated genotypes at day 3 after γ-irradiation. All values represent the average ± SD of a total of three independent MEF clones for each condition (n=3) relative to non-irradiated condition (-). Statistical significance to untreated or nonirradiated control was assessed using unpaired Student's t-test with Welch's correction. ** $P<0.01$, *** $P<0.001$.

1.4. The *Ink4a* tumor suppressor is required for IL6 and TNF production

The *Ink4a/Arf*, or CDKN2A, *locus* encodes two tumor suppressor proteins, p16INK4A and p19ARF, that positively regulate the Rb and p53 pathways, respectively, and arrest the cell cycle (Chin et al., 1998; Ewen et al., 1993; Narita et al., 2003; Quelle et al., 1995; Serrano et al., 1993). We wondered which of those tumor suppressors was required for triggering the high production and secretion of cytokines. For that, we first downregulated *Ink4a* and *Arf* individually or together using sh*RNAs* in WT MEFs. Then, the cells were irradiated and collected three days later to perform mRNA analyses. We observed that sh*Arf* knockdown MEFs were able to upregulate *Il6* and *Tnf* after damage while sh*Ink4a* and sh*Ink4a/Arf* knockdown MEFs failed **(Figure 15A and 15B)**. These results suggested that the *Ink4a* tumor suppressor was the gene required for the production and secretion of IL6 and TNF.

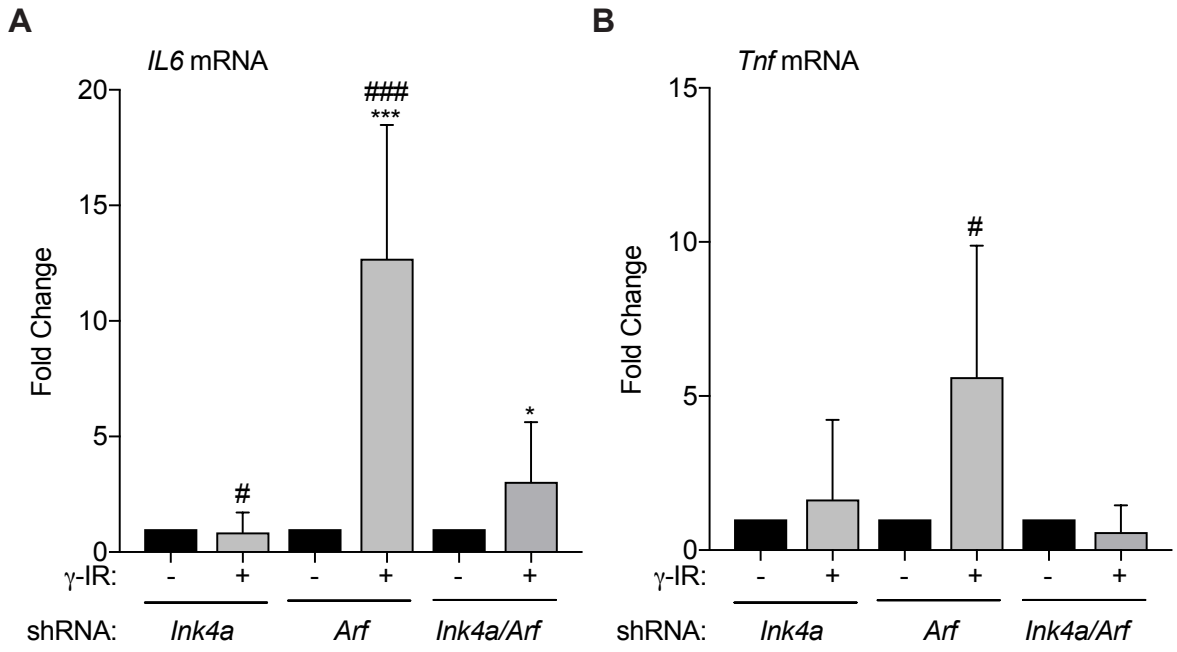

Figure 15. *Ink4a* is essential for cytokine production. (A and B) mRNA levels of *Il6* and *Tnf* in non-irradiated and irradiated MEFs, infected with retroviruses encoding for the indicated shRNAs 3 days post-irradiation. All values represent the average ± SD of a total of three independent MEF clones for each condition (n=3). Statistical significance to nonirradiated control was assessed using two-tailed unpaired Student's t-test with Welch's correction. * $P<0.05$, *** $P<0.001$. Comparisons of γ-irradiated sh*Ink4a* and sh*Arf* to γ-irradiated sh*Ink4a/Arf* were calculated using the same test and are indicated using the symbol "#". # $P<0.05$, ### $P<0.001$.

Therefore, in the absence of *Ink4a*, damaged cells do not secrete IL6 and TNF.

1.5. Senescence and reprogramming co-exist *in vivo*

These results together supported what was observed *in vivo*. The *in vivo* data were obtained by Lluc Mosteiro and Cristina Pantoja in our laboratory (Mosteiro et al., 2016). In summary, the expression of *OSKM in vivo* leads to two concomitant outcomes, senescence and reprogramming, and both co-exist in close proximity within the same tissue. Moreover, both processes are exacerbated in *p53* null *OSKM* mice, together with the production and secretion of cytokines. *Ink4a/Arf* deficiency limits senescence and reprogramming. Finally, IL6 plays a key role on *in vivo* reprogramming and its paracrine action creates a permissive environment for reprogramming.

2. IL6 REQUIREMENT IN REPROGRAMMING

We have demonstrated that senescent cells secrete cytokines that promote reprogramming in a paracrine manner being IL6 and TNF the prime candidates responsible for this effect.

2.1. Study the effect of blocking IL6 during reprogramming

We directly tested the role of these cytokines in reprogramming. First, we added recombinant mouse TNF and anti-TNF antibodies during reprogramming. We observed that *i4F* MEFs were death after 7 days of treatment (data not shown). On the other hand, the group of Helen Blau reported that IL6 could promote reprogramming (Brady et al., 2013) then, we decided to focused only on IL6.

For that, we firstly immunoblocked it in the senescent CM. Fresh CM from γ-IR WT MEFs was harvested, as described in previous experiments, and incubated with anti-IL6 antibodies for 30 minutes. After that, the CM was used for *i4F in vitro* reprogramming. We observed that the beneficial effect of the senescent CM was completely abolished when neutralizing antibodies were added **(Figure 16)**. Surprisingly, reprogramming efficiency of *i4F* MEFs, cultured with the neutralized CM, was even lower than in the untreated cells. This suggested that IL6 was not only coming from the senescent CM but also from cells overexpressing *OSKM*. This was in agreement with the previous data which showed that *OSKM* activation induced strong damage to the cells (Banito et al., 2009) and these damaged cells may be the source of IL6. We hypothesized that both sources of IL6 were neutralized with anti-IL6 antibodies and consequently, reprogramming was completely blocked. Other possibility is that, TNF inhibitory activity would strongly affect reprogramming in the absence of IL6.

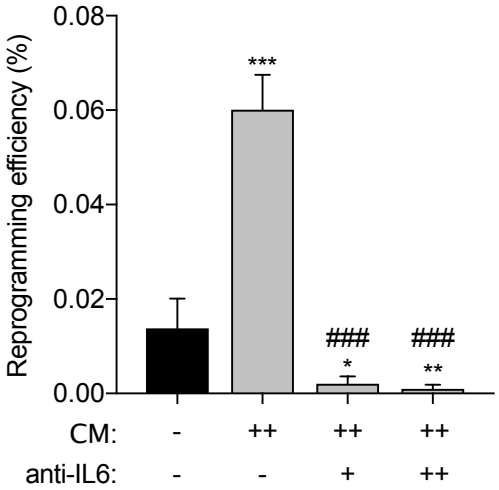

Figure 16. IL6 is the critical factor present in the senescent CM. Reprogramming efficiency of *i4F* MEFs treated with CM from γ-irradiated WT MEFs in the absence (-) or presence of increasing amounts of anti-IL6 in the CM (+: 0.02 mg of antibodies per milliliter of CM, ++: 0.1mg of antibodies per milliliter of CM). AP staining was done at day 17 of reprogramming. All values represent the average ± SD of a total of three independent MEF clones for each condition (n=3). Statistical significance was assessed using unpaired Student's t-test with Welch's correction. Comparisons of each condition to the control without CM are indicated as follows: * $P<0.05$, ** $P<0.01$. Comparisons of each condition to the control without an-IL6 are calculated using the same test and are indicated using the symbol "#". ### $P<0.001$.

To prove that IL6 could be also secreted by *OSKM* overexpressing MEFs and have an impact on *in vitro* reprogramming, anti-IL6 antibodies were added directly into the reprogramming plates, in the absence of any senescent-derived CM or exogenously added IL6. Anti-IL6 treatment during the

whole process of reprogramming decreased significantly the generation of iPS colonies **(Figure 17A)**. Finally, we validated our hypothesis downregulating *Il6* in *i4F* MEFs using sh*RNA*s. First, cells were selected for the sh*RNAs* and then, doxycycline was added to induce *OSKM* expression. *Il6* was downregulated more than 90% before the induction **(Figure 17B)** and importantly, reprogramming efficiency decreased significantly **(Figure 17C)** and was similar to IL6-neutralized reprogramming **(Figure 17A)**.

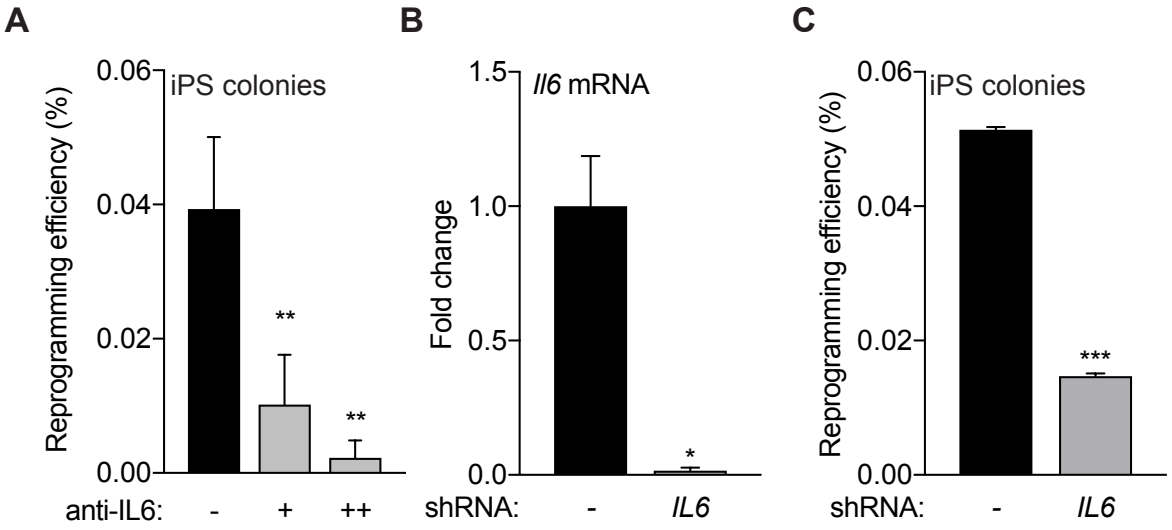

Figure 17. IL6 is produced by the reprogramming culture and it is essential. (A) Reprogramming efficiency of *i4F* MEFs treated with antibodies anti-IL6 at two concentrations, +: 0.02 mg of antibodies per milliliter of iPS medium, ++: 0.1mg of antibodies per milliliter of iPS medium. Quantification of AP^+ colonies was performed at day 12 after *OSKM* induction. **(B)** mRNA levels of *Il6* after sh*RNA* knockdown in *i4F* MEFs. **(C)** Reprogramming efficiency of *i4F* MEFs after knockdown of *Il6* using sh*RNA* at day 14 of reprogramming. All values represent the average ± SD of a total of three independent MEF clones for each condition (n=3). Statistical significance to control condition (-) was assessed using unpaired Student's t-test with Welch's correction. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

These data indicate, first, that IL6 is crucial during *in vitro* reprogramming and that paracrine IL6, coming from surrounding senescent cells or damaged cells, promotes reprograming *in vitro*.

2.2. Deciphering IL6 role in reprogramming

In 2013, Helen Blau's laboratory demonstrated that IL6 has a transient and early role in heterokaryon reprogramming and is rapidly induced at the earliest phases (Brady et al., 2013). We wondered when IL6 could be crucial on our *in vitro* reprogramming setting and for that, we added anti-IL6 antibodies at different and narrower time windows. We defined three important periods: from day 0 to day 4 which we considered as the early reprogramming phase, from day 4 to 7 as intermediate phase, and from 7 to 12 as late stage. Interestingly, the lowest reprogramming efficiencies were observed when IL6 was blocked during the early phase **(Figure 18)**. However, when anti-IL6 antibodies were added during the intermediate and late stages, the efficiency of reprogramming was not reduced as potently **(Figure 18)**.

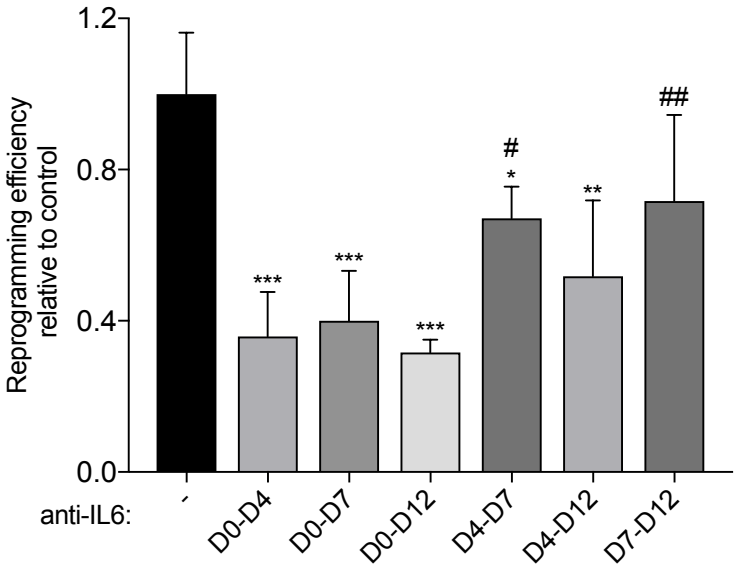

Figure 18. Early role of IL6 in promoting *in vitro* reprogramming. Reprogramming efficiency of *i4F* MEFs treated with antibodies anti-IL6 at 0.1 mg per milliliter of iPS medium. Antibodies were added at specified time windows from day 0 to day 12. Quantification of iPS colonies was done at day 14. All values represent the average ± SD of a total of six independent MEF clones for each condition relative to untreated control (-) (n=6). Statistical significance to control condition (-) was assessed using two-way ANOVA and Bonferroni post-hoc test. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. Comparisons to treated condition from day 0 to day 12 (D0-D12) are calculated using the same test and are indicated using the symbol "#". ## $P<0.01$.

This result was in agreement with Blau's data (Brady et al., 2013) and demonstrated that the main role of IL6 was at the beginning of *in vitro* reprogramming. Late effects cannot be discarded, however, their impact on the process was substantially lower.

2.3. The IL6 and LIF axes are differentially induced in reprogramming

Considering the above results, we decided to evaluate the expression profile of IL6, IL6-type cytokines (mainly LIF), and pluripotency markers during reprogramming. We induced *i4F* MEFs and collected samples at day 1, 3, 6, 8 and 11 of reprogramming and compare the mRNA levels of different genes with MEFs at basal conditions (day 0) and with WT ES cells. Expectedly, pluripotency markers, such as *Nanog*, endogenous *Oct4* (*En-Oct4*) and endogenous Sox2 (*En-Sox2*), were expressed late in reprogramming **(Figure 19A)**. Interestingly, *Il6* was sharply induced from day 3 to day 6 and its receptor (*Il6ra*) did it from day 6 **(Figure 19B)**. This pattern was not followed by *Lif* and *Lifr*. We observed that *Lif* was not induced during reprogramming, probably because it is exogenously added in all our reprogramming experiments, and *Lifr* was upregulated from day 8 **(Figure 19B)**. These data suggests that the first axis that is induced during reprogramming is IL6-IL6R, which could firstly activate the JAK/STAT pathway and, then this is followed by the LIF-LIFR axis which is known to play an important role in the establishment of full pluripotency.

We also observed that the expression levels of *gp130*, *STAT3*, *NKX3-1*, *IL27*, *IL11* and *OSM* increased gradually during reprogramming **(Figure 19C)**. Importantly, the senescent marker *Ink4a* was highly expressed later in reprogramming and this confirmed that *OSKM* overexpression *in vitro* not only induce dedifferentiation but also senescence.

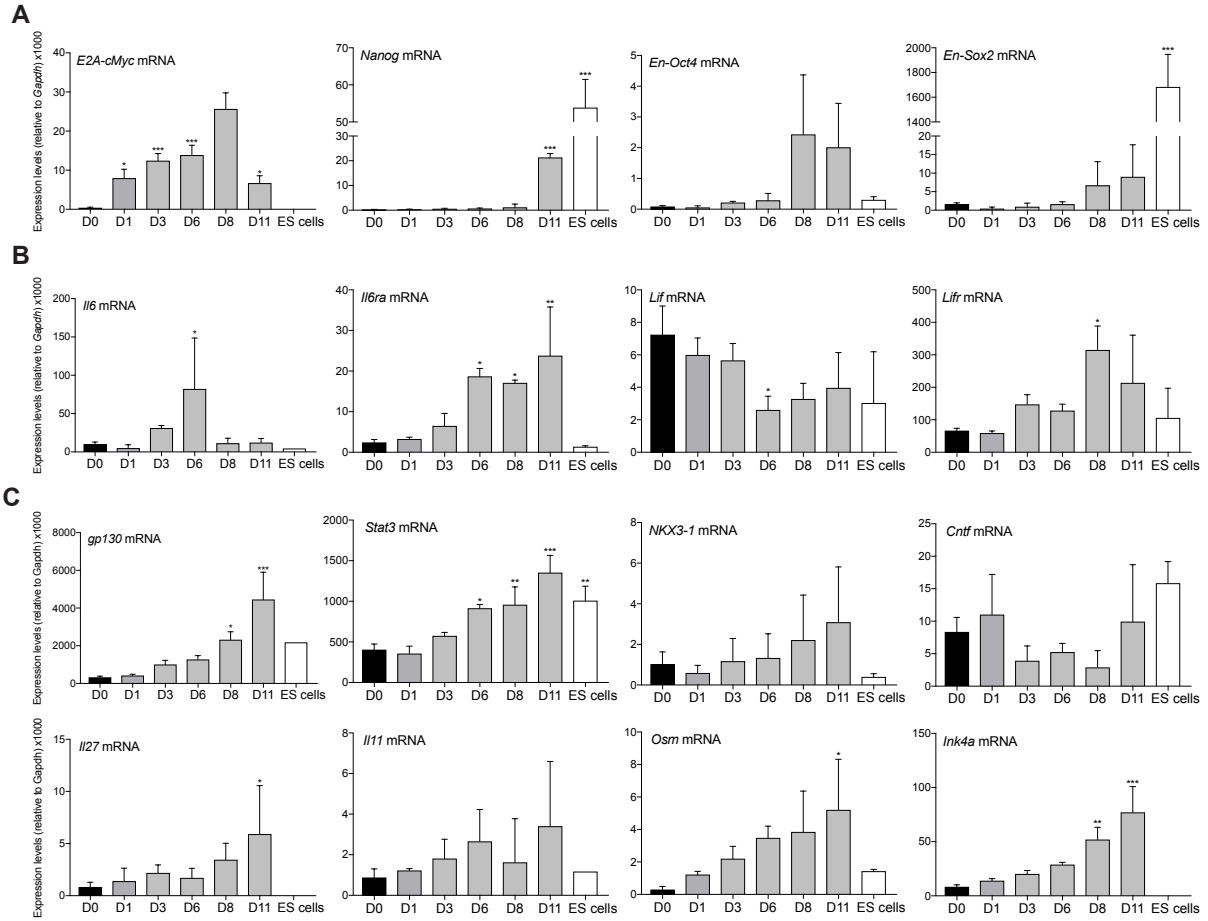

Figure 19. Time course analysis of *i4F* MEFs during *in vitro* reprogramming. mRNA levels of genes at day 1, 3, 6, 8 and 11 after induction with doxycycline. Non-induced MEFs (Day 0, D0) are used to determine the basal expression levels and WT ES cells, the levels in full pluripotency. **(A)** mRNA levels of genes associated to pluripotency: *E2A-cMyc*, *Nanog*, endogenous *Oct4* (En-*Oct4*) and endogenous *Sox2* (En-*Sox2*). **(B)** mRNA levels of IL6 and LIF related genes. **(C)** mRNA analysis of *gp130*, *STAT3*, *NKX3-1*, IL6-related cytokines (*Cntf*, *Il27*, *Il11* and *Osm (Onconstatin M)*) and *Ink4a*. Graphs represent the average ± SD of a total of three independent MEF clones and one WT ES clone (n=3 or 1). Statistical significance to basal levels (D0) was assessed using one-way ANOVA and Bonferroni post-hoc test. * $P<0.05$, ** $P<0.01$, ** $P<0.001$.

2.4. IL6R null MEFs are not reprogrammed *in vitro*

We observed that there was variability in the capacity to immune-block IL6 between the experiments. In some studies, we did not observe a completely reduction of the reprogramming efficiency and we hypothesized that it could be due to incomplete blocking of IL6 and the heterogeneity of MEFs (Singhal et al., 2016). This could obscure or diminish the true activity of IL6 during reprogramming. For that, we decided to study a model deficient for IL6R. Different null models for IL6 and IL6R have been described in the literature (Bernad et al., 1994; Kopf et al., 1994; McFarland-Mancini et al., 2010; Poli et al., 1994; Sommer et al., 2014). We decided to use the IL6R null model because it has been demonstrated that IL6 signaling, both classic signaling and trans-signaling, is completely abrogated in these mice. Moreover in IL6 null mice, compensatory mechanisms, through low affinity binding of cytokines to IL6R (McFarland-Mancini et al., 2010; Sommer et al., 2014), may be activated and then, IL6 function would be masked.

We first generated reprogrammable mice combined with IL6R lox allele and a transgenic

modification where the Cre recombinase was under the control of the mouse *Sox2* promoter, which is active in embryonic epiblast cells. Mice with the *Sox2*-Cre modification effectively deleted exons 4, 5 and 6 and gave rise to *IL6R* null reprogrammable mice. We isolated MEFs from these mice and, as WT control, we used reprogrammable MEFs in combination with the unexcised IL6R lox allele.

Reprogrammable MEFs were induced and iPS colonies were observed at day 7 of reprogramming in WT *i4F* MEFs, whereas they were absent in *IL6R* null *i4F* MEFs **(Figure 20A)**. iPS colonies were quantified at day 11 of reprogramming based on their AP activity and it was confirmed that there were not AP^+ colonies in *IL6R* null *i4F* MEFs **(Figure 20B)**. Atypical colonies were observed in null MEFs **(Figure 20A and 20B)**.These cells did not form dome-shaped and refractile colonies and were formed by small bright individual cells **(Figure 20A)** which will be studied in further detail in the future.

We confirmed that *IL6R* null *i4F* MEFs overexpressed the *OSKM* cassette similarly to WT cells to discard it as a possible reason for unsuccessful reprogramming (*E2A-cMyc* and *Sox2-Klf4*) **(Figure 20C)**. Null cells were unable to upregulate the endogenous pluripotency genes (*En-Oct4*, *En-Sox2* and *Nanog*) **(Figure 20C)**. This data confirmed that *IL6R* null *i4F* MEFs failed to activate the pluripotency circuity and therefore, the transition to iPS cells was impaired. As we observed previously, *Il6ra*, *Il6* and *Lifr* were upregulated during reprogramming of WT cells; however, all these genes remained unchanged in *IL6R* null **(Figure 20C)**. *gp130* and *STAT3* were equally induced in both genotypes indicating that their expression did not depend on IL6 signaling **(Figure 20C)**. Finally, we observed that the downstream mediator of IL6, the transcription factor *NKX3-1*, which is partly responsible for activating endogenous *Oct4* (Mai et al., 2018), was induced during reprogramming of WT *i4F* MEFs as it was reported. However, null *i4F* MEFs were not able to induce its expression and this correlated with the failed upregulation of endogenous *Oct4* **(Figure 20C)**.

This data confirms that IL6 is essential for the transition to iPS cells and it could be due to its involvement in the regulation of the pluripotency circuitry including *Lifr*, *NKX3-1*, *Nanog* and the endogenous reprogramming genes.

All together, we confirm that, on one hand, *Il6* and *Il6r* are upregulated the first seven days of reprogramming and IL6 function is essential in reprogramming, being more important in the early phase. On the other hand, *Lif* and *Lifr* are induced later in reprogramming, from day 8, and this might depend on IL6-IL6R axis, as *IL6R* null cells fail to induce *Lifr*. Moreover, LIF and LIFR have been demonstrated to be essential in the maintenance of ES cells (Yoshida et al., 1994). Based on these observations, we hypothesize that there may be two phases during reprogramming. The first one might depend on the temporally upregulation of IL6-IL6R axis and the second one, on LIF-LIFR axis which is maintained in the full pluripotent state. Moreover, both cytokines might be not interchangeable due to the different expression pattern of their receptors and also because LIF could not overcome the absence of IL6 for successful reprogramming.

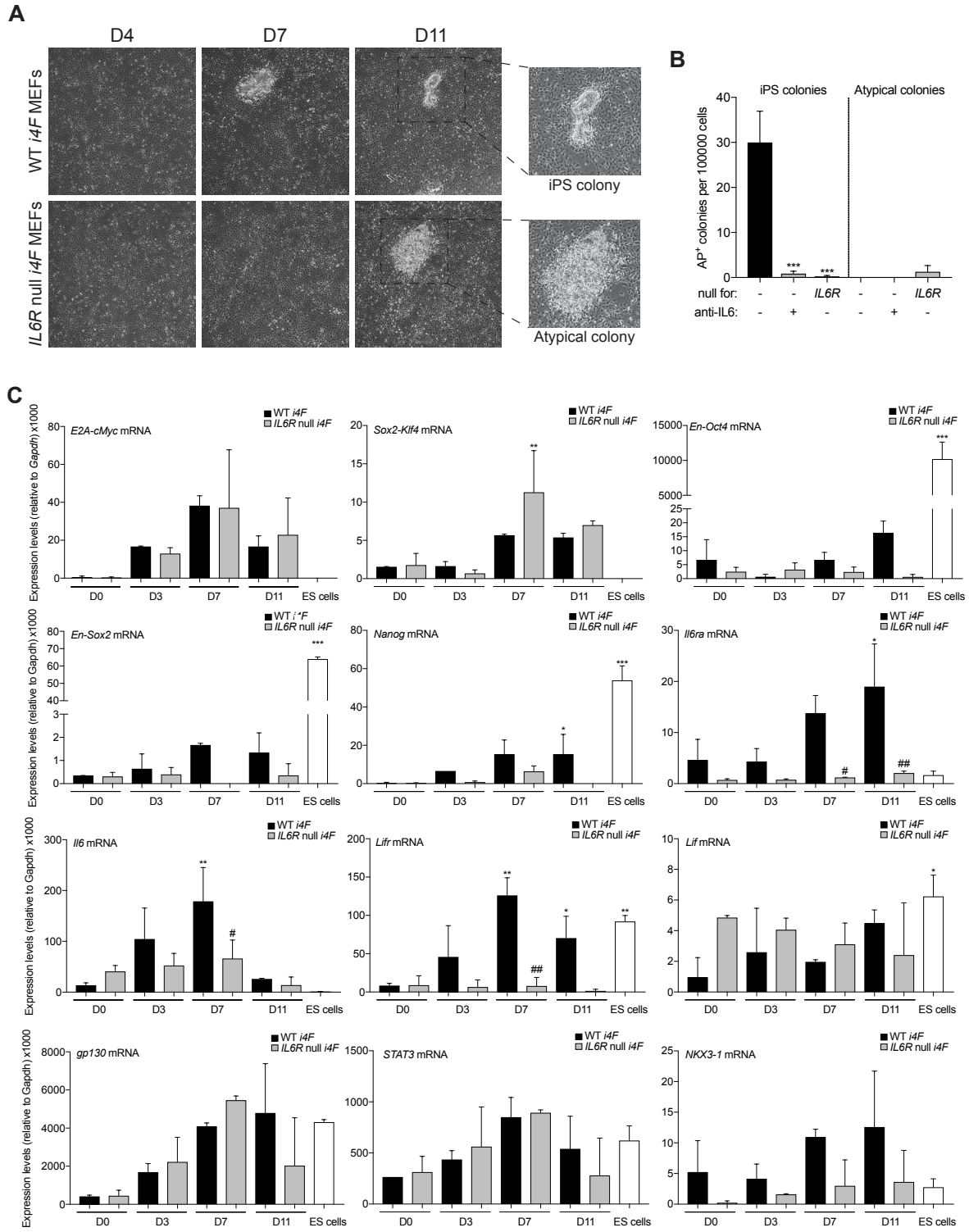

Figure 20. *IL6R* null *i4F* MEFs are not reprogrammed *in vitro*. (A) Representative bright field pictures of WT *i4F* and *IL6R* null *i4F* MEFs at day 4, 7 and 11 of reprogramming. The pictures on the right showed the area depicted by a dashed line at higher magnification. **(B)** On the left, quantification of the total AP⁺ colonies (iPS colonies) and on the right, the quantification of atypical colonies per 100000 plated cells in all the conditions at day 11 of reprogramming. **(C)** mRNA levels of *E2A-cMyc*, *Sox2-Klf4*, *En-Oct4*, *En-Sox2*, *Nanog*, *Il6ra*, *Il6*, *Lifr*, *Lif*, *gp130, STAT3* and *NKX3-1* during reprogramming (day 0, 3, 7 and 11) of WT *i4F* and *IL6R* null *i4F* MEFs. WT ES cells were used as control of pluripotency. In (B) and (C) the values represent the average ± SD of a total of four independent MEF clones or one clone of WT ES cells (n=4 or 1). Statistical significance to WT *i4F* untreated condition (B) or WT *i4F* MEFs at day 0 (C) was assessed using one-way ANOVA and Bonferroni post-hoc test. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. In (C), comparisons between WT and null *i4F* MEFs at the same day were calculated using the same test and are indicated using the symbol "#". # $P<0.05$, ## $P<0.01$.

2.5. Analysis of the interplay between the IL6 and LIF pathways

To address our two-phases hypothesis, we first wondered whether the activation of LIF-LIFR axis from the beginning would make reprogramming independent of IL6.

2.5.1. Reprogramming of *i4F* neonatal astrocytes

We looked for primary cells with natural constitutive expression of *Lifr*. Astrocytes are specialized glial cells that have been shown to express *Lifr, gp130* and *Osmr* (Alfonsi et al., 2008; Hsu et al., 2015). Therefore, these cells could be a model to challenge our hypothesis. We isolated primary astrocytes from 1 day old (P1) neonatal reprogrammable mice and compared the expression profile of *Lif*, *Lifr*, *Il6* and *Il6ra* genes relative to MEFs in basal conditions. As expected, *Lifr* expression was higher in astrocytes than in MEFs together with *Il6ra* as well as some specific markers for astrocytes (*Aldh1l1*, *Aqp4* and *Gfap)* **(Figure 21A)**. Once this was validated, reprogramming was induced in *i4F* astrocytes together with the anti-IL6 neutralizing treatment as it was done previously. Flatter iPS-like colonies appeared at day 20 of induction in both conditions, untreated and anti-IL6 treated **(Figure 21B)**. The flatter morphology of the colonies and the timing resembled the previously published astrocyte reprogramming (Nakajima-Koyama et al., 2015). Besides, the colonies were positive for AP activity **(Figure 21C)**. Interestingly, there were not differences in reprogramming efficiency **(Figure 21D)**. Therefore, these data suggested that reprogramming of astrocytes was independent of IL6-IL6R axis and could rely on LIF-LIFR one.

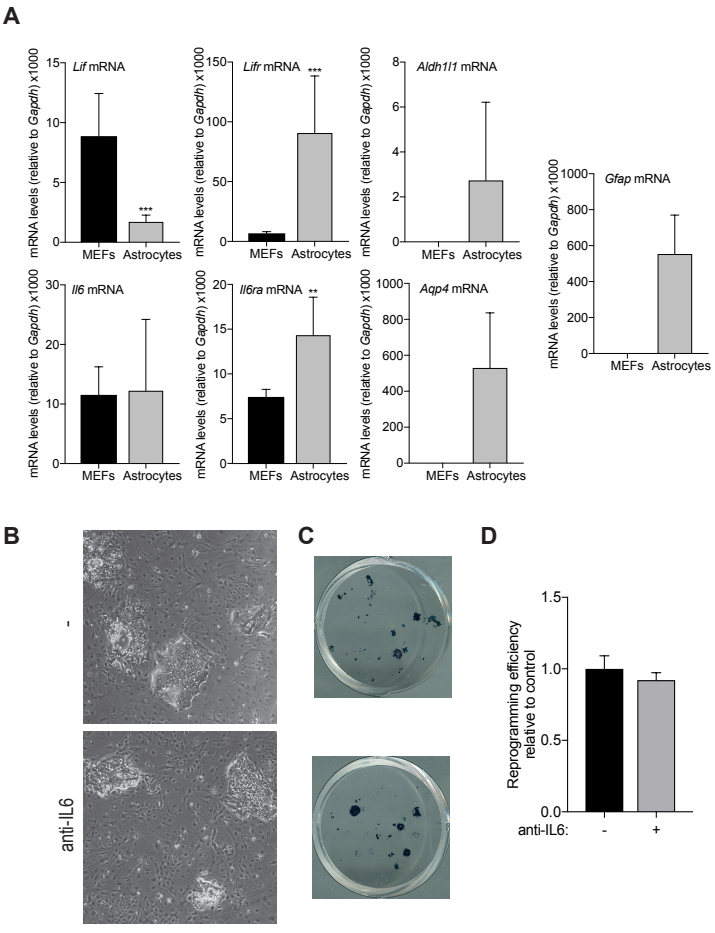

Figure 21. Reprogramming of astrocytes is independent of IL6-IL6R axis. (A) mRNA levels of *Lif*, *Lifr*, *Il6*, *Il6ra*, *Aldh1l1*, *Aqp4* and *Gfap* in MEFs and astrocytes in basal conditions. **(B)** Representative bright field pictures of untreated (-) and treated *i4F* astrocytes with anti-IL6 antibodies at 20 day of *in vitro* reprogramming. **(C)** Plates stained for AP activity at day 20. Picture at the top correspond to untreated astrocytes and at the bottom anti-IL6 treated cells. **(D)** Reprogramming efficiency of *i4F* astrocytes treated with antibodies anti-IL6 at 0.1 mg/ml in iPS medium. In (A), graphs represent the average ± SD of a total of three independent MEF clones and five astrocyte clones (n=3 or 5). In (D), values represent the average ± SD of a total of three independent astrocyte culture for each condition (n=3). Statistical significance to MEFs (A) or untreated control (D) was assessed using unpaired Student's t-test with Welch's correction. ** P<0.01, *** P<0.001.

We also performed a time course mRNA analysis of astrocytes during reprogramming. We verified that the *OSKM* cassette was induced after doxycycline treatment (*E2A-cMyc* expression) and that endogenous genes, like *Oct4* (*En-Oct4*), were expressed at the end of the process **(Figure 22A and 22B)**. *Nanog* was also upregulated by day 11 **(Figure 22B)** and this demonstrated that successful reprogramming was achieved and colonies could be true iPS cells (isolation and characterization of individual clones have not been done yet). Interestingly, *Il6* was sharply induced at day 1 and *Il6ra* was not **(Figure 22C)** in contrast to the reprogramming of fibroblast **(Figure 19B)**.

The above data support our idea that reprogramming depends firstly on IL6 and secondly on LIF signaling. However, when LIF-LIFR signaling is active from the beginning, as it is the case in astrocytes, reprogramming was independent of IL6.

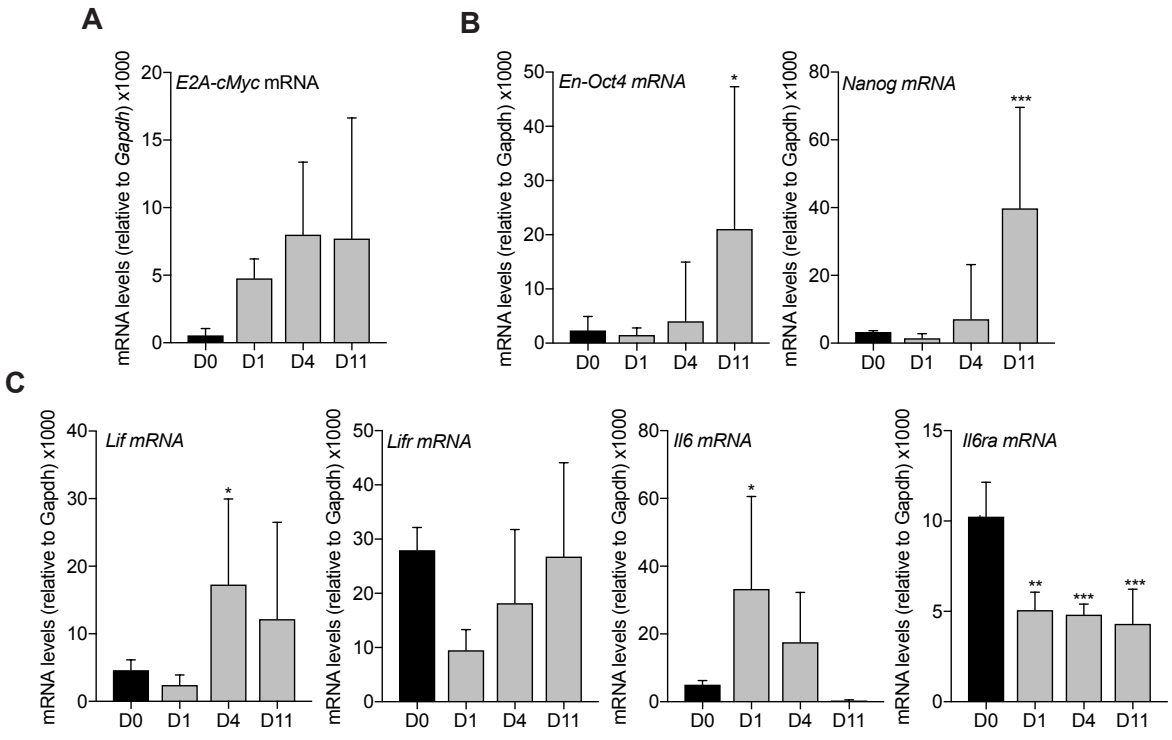

Figure 22. mRNA expression profile of astrocytes during *in vitro* reprogramming. (A, B and C) mRNA levels of *E2A-cMyc* (A), *En-Oct4*, *Nanog* (B), *Lif*, *Lifr*, *IL6* and *Il6r* (C) at day 0, 1, 4 and 11 of reprogramming. All values represent the average ± SD of a total of six independent astrocyte clones for each condition (n=6). Statistical significance to basal condition (day 0) was assessed using one-way ANOVA and Bonferroni post-hoc test. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

2.5.2. Overexpression of *Lifr* in MEFs and its consequences on *in vitro* reprogramming

To confirm these encouraging results, we decided to test which consequences may have the overespression of *Lifr* in our initial fibroblast model where *Lifr* mRNA level was low until day 8. To overexpress exogenous mouse *Lifr* in MEFs, we used a lentiviral vector encoding for *Lifr* ORF (NM_013584.2) under the control of a doxycycline-responsive promoter (Tet-On). First, reprogrammable MEFs were transduced and selected for *Lifr* with blasticidin and, then, were reprogrammed in the presence of doxycycline, LIF and antibodies anti-IL6, as it was done in previous experiments. The transcriptional activator (rtTA), in the presence of doxycycline, is able to activate not only the polycistronic cassette *OSKM* but also *Lifr*. We first measured mRNA

levels of *Lifr* 5 days after doxycycline treatment to confirm that the resistant cells were indeed overexpressing it **(Figure 23A)**. Interestingly, iPS colonies were firstly observed in *Lifr i4F* MEFs, at day 6, in contrast to WT *i4F* MEFs (usually appearing from day 10) and AP^{+} colonies were quantified at day 10. Furthermore, *Lifr i4F* MEFs were efficiently reprogrammed even in the presence of anti-IL6 antibodies **(Figure 23B)**.

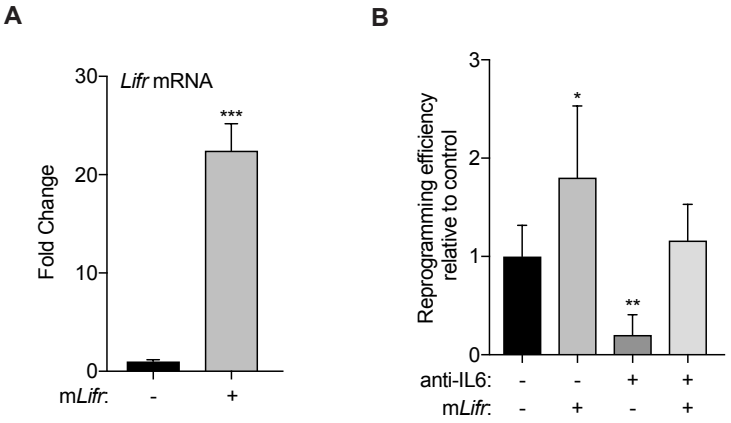

Figure 23. High *Lifr* levels makes reprogramming of murine fibroblasts independent on IL6. (A) mRNA levels of mouse *Lifr* 5 days after induction with doxycycline relative to control MEFs (-). **(B)** Reprogramming efficiency, relative to standard condition, of WT *i4F* MEFs overexpressing *Lifr* in combination with anti-IL6 antibodies treatment. Values represent the average ± SD of a total of three independent MEF clones for each condition (n=3). Statistical significance to control condition (-) was assessed using unpaired Student's t-test with Welch's correction in (A) and one-way ANOVA and Bonferroni post-hoc test in (B). * P<0.05, ** P<0.01, *** P<0.001.

These results suggest that high levels of *Lifr* not only overcome the absence of IL6 but also accelerate the kinetics of reprogramming of murine fibroblasts.

2.6. Study the replacement of reprogramming factors by IL6

In 2013, Blau's laboratory demonstrated that IL6 can functionally replace *cMyc* (Brady et al., 2013). However, they did not tested the ability of IL6 to replace any other reprogramming factor. To investigate this, MEFs were transduced with all the possible combinations of *OSKM* transcription factors (*OSKM*, *OSK*, *OKM*, *SKM*, *KM*, *SK*, *SM*, *OS*, *OK* and *OM*) and treated with recombinant mouse IL6 (rmIL6). *OSKM* and *OSK* conditions were able to induce the formation of iPS colonies and it was slightly enhanced by the presence of IL6 as it was expected **(Figure 24A)**. Surprisingly, we could observe iPS-like colonies in *OKM*, *SKM*, *KM* and *SM* IL6-treated conditions **(Figure 24A and 24B)**. Ten colonies of each condition were isolated to have individual clones however, only *OKM* plus IL6 derived iPS-like colonies survived and were expanded, even in the absence of the cytokine. These results suggested that IL6 may replace *Sox2* during reprogramming of MEFs.

PIM1 has been reported to be a downstream mediator of IL6 and its inhibition reduced *in vitro* reprogramming (Brady et al., 2013). We wondered whether *PIM1* overexpression, mimicking IL6 activation, may also replace *Sox2* in reprogramming. MEFs were transduced with all the possible combinations, as in the previous experiment, together with a vector encoding for human *PIM1* (h*PIM1*). MEFs overexpressed more than 50 times h*PIM1* at the end of reprogramming **(Figure 24D)**. Expectedly, iPS colonies appeared in *OSKM* and *OSK* conditions **(Figure 24C)** and h*PIM1* overexpression slightly enhanced the efficiency only in *OSK* suggesting that could activate

endogenous *cMyc*, as IL6 did. Interestingly, iPS colonies were observed in *OKM* plus h*PIM1*, as we observed with IL6 treatment, whereas no colonies were observed only with *OKM* **(Figure 24B and 24C)**.

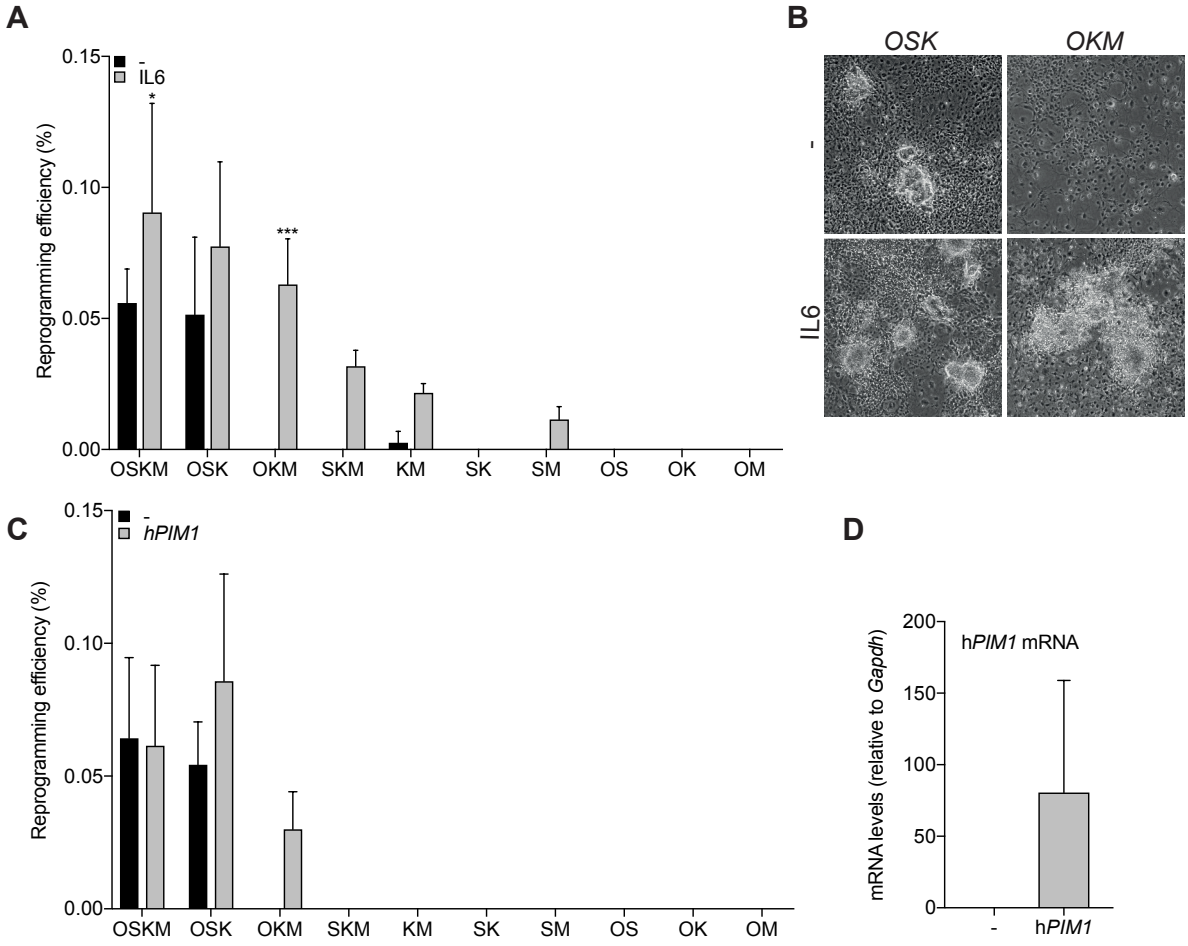

Figure 24. *Sox2* is replaced by IL6 and *PIM1* during *OKM in vitro* reprogramming. **(A)** Reprogramming efficiency of MEFs transduced with specific combinations of reprogramming factors *OSKM* and treated with recombinant mouse IL6 at 100 ng/ml. AP staining was performed at day 14 of reprogramming. **(B)** Representative bright field pictures of untreated (-) and treated (IL6) MEFs transduced with *OSK* or *OKM* retroviruses. **(C)** Reprogramming efficiency of MEFs transduced with human *PIM1* and specific combinations of reprogramming factors *OSKM*. AP staining was done at day 14 of reprogramming. **(D)** mRNA levels of human *PIM1* after reprogramming. In (A), (C) and (D) the values represent the average ± SD of a total of three independent MEF clones for each condition (n=3). In (A) and (C), statistical significance to untreated MEFs of each condition (-) was assessed using using two-way ANOVA and Bonferroni post-hoc test. In (D), statistical significance to control MEFs (-) was assessed using unpaired Student's t-test with Welch's correction *** $P<0.001$.

These data suggest a new function of IL6, namely, to replace *Sox2* in *OKM in vitro* reprogramming of MEFs and this happens through PIM1 kinase. Nevertheless, a deeper analysis is required to understand the mechanisms involved.

2.7. The intermediate reprogramming states are affected by IL6

Reprogramming is a low and inefficient process where many cells fail to reprogram and intermediate populations are generated. Some of them are trapped and cannot continue the transition while other intermediate populations become iPS cells with high efficiency (more than 90% of efficiency). Many groups previously characterized these intermediate stages of

reprogramming using surface makers, mass cytometry and RNA-seq (Brambrink et al., 2008; Lujan et al., 2015; O'Malley et al., 2013; Polo et al., 2012; Schwarz et al., 2018; Stadtfeld et al., 2008). Taking into account that reprogramming was impaired when IL6 was neutralized, we examined whether the formation of some intermediates stages might be affected.

A well-studied transition during *in vitro* reprogramming is the switch from Thy1^{+} to SSEA^{+} cells. Thy1 is a surface marker highly expressed in fibroblasts (Rege and Hagood, 2006) whereas SSEA1 is another marker highly expressed in ES cells (Cui et al., 2004). Downregulation of Thy1 is an early event starting at day 1-2 of reprogramming (Polo et al., 2012; Stadtfeld et al., 2008). A subset of Thy1^{+}cells remain and are refractory to reprogramming. Thy1^{-} cells initiate SSEA1 expression at day 3 at low levels and increase gradually throughout reprogramming (Polo et al., 2012; Stadtfeld et al., 2008). We analyzed at day 3, 7 and 11 of reprogramming the expression of Thy1 and SSEA1 in *i4F* MEFs treated with anti-IL6. MEFs in basal conditions presented two populations (Thy1^{+} and Thy1^{-}) while SSEA1 was completely absent as it has been published (Stadtfeld et al., 2008) **(Figure 25A)**. FACS analysis of the reprogramming samples revealed that by day 7, cells activated SSEA1 expression and SSEA1^{low} cells were detected in untreated and anti-IL6 treated condition at similar levels (~0.50%) **(Figure 25B)**. However, differences in SSEA1 expression were observed by day 11. In control conditions (-), the percentage of SSEA1^{high} cells increased up to ~15% of the parental population (live cells) compare to only ~5% in anti-IL6 treated samples **(Figure 25B and 25D)**.

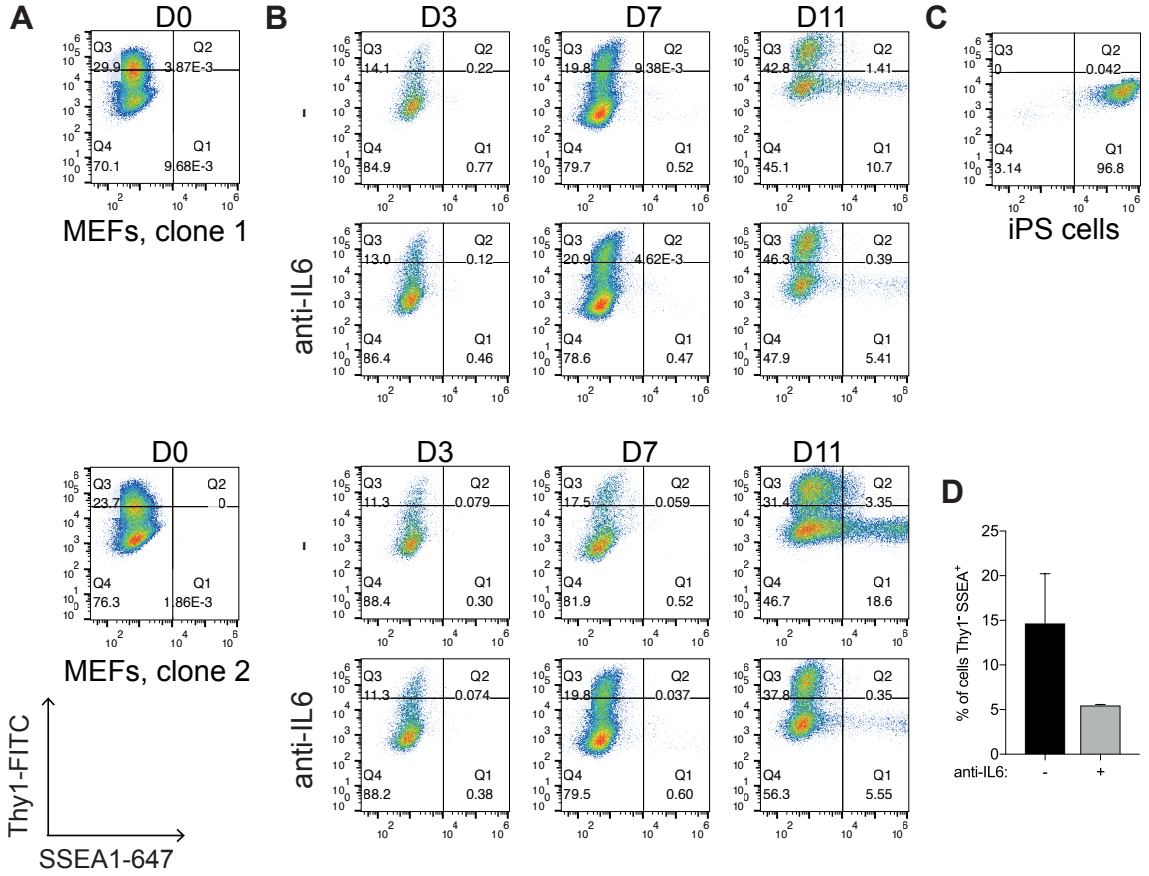

Figure 25. Thy1 and SSEA1 transition is affected during anti-IL6 treated reprogramming. (A, B and C) FACS plots showing Thy1 and SSEA1 expression in two independent *i4F* MEF clones (A), WT iPS cells (C) and reprogramming samples at day 3, 7 and 11 in untreated (-) and anti-IL6 treated conditions (B). **(D)** Percentage of Thy1^{-} SSEA1^{+} cells at day 11 of reprogramming. In (D), the values represent the average ± SD of a total of three independent MEF clones (n=3). Statistical significance to untreated MEFs (-) was assessed using two-tailed unpaired Student's t-test with Welch's correction.

Regarding Thy1 expression, we could not conclude how it was downregulated because we did not sort the cells to start with a homogeneous Thy1^{+} population. The percentage of Thy1^{+} increased at the end of reprogramming process however, this could be due to proliferation of failed states **(Figure 25B)**. Therefore, this experiment suggests that SSEA^{high} expression may be impaired in anti-IL6 treated *i4F* MEFs.

On the other hand, platelet derived growth factor receptor β (PDGFRβ) has been reported to be rapidly downregulated in reprogramming and its expression is completely lost in iPS cells (Schwarz et al., 2018). We decided to investigate whether this early event was affected by IL6. We performed FACS analysis at different time points of reprogramming analyzing PDGFRβ and SSEA1 markers. MEFs displayed a homogeneous PDGFRβ^{high} expression while it was not present in iPS cells **(Figure 26A and 26C)**. We observed that by day 3, ~60% of the live cells were PDGFRβ^{-} and by day 11 ~70% in both conditions without clear differences. The highest downregulation (from 90% to 40% of PDGFRβ^{+} cells) was before day 3 of reprogramming **(Figure 26B)**. Therefore, it could be interesting to analyze also earlier time points to evaluate possible differences in PDGFRβ lost. Regarding SSEA1 expression, differences between untreated and treated conditions were observed similarly to the previous FACS panel analysis **(Figure 26B and 26D)**.

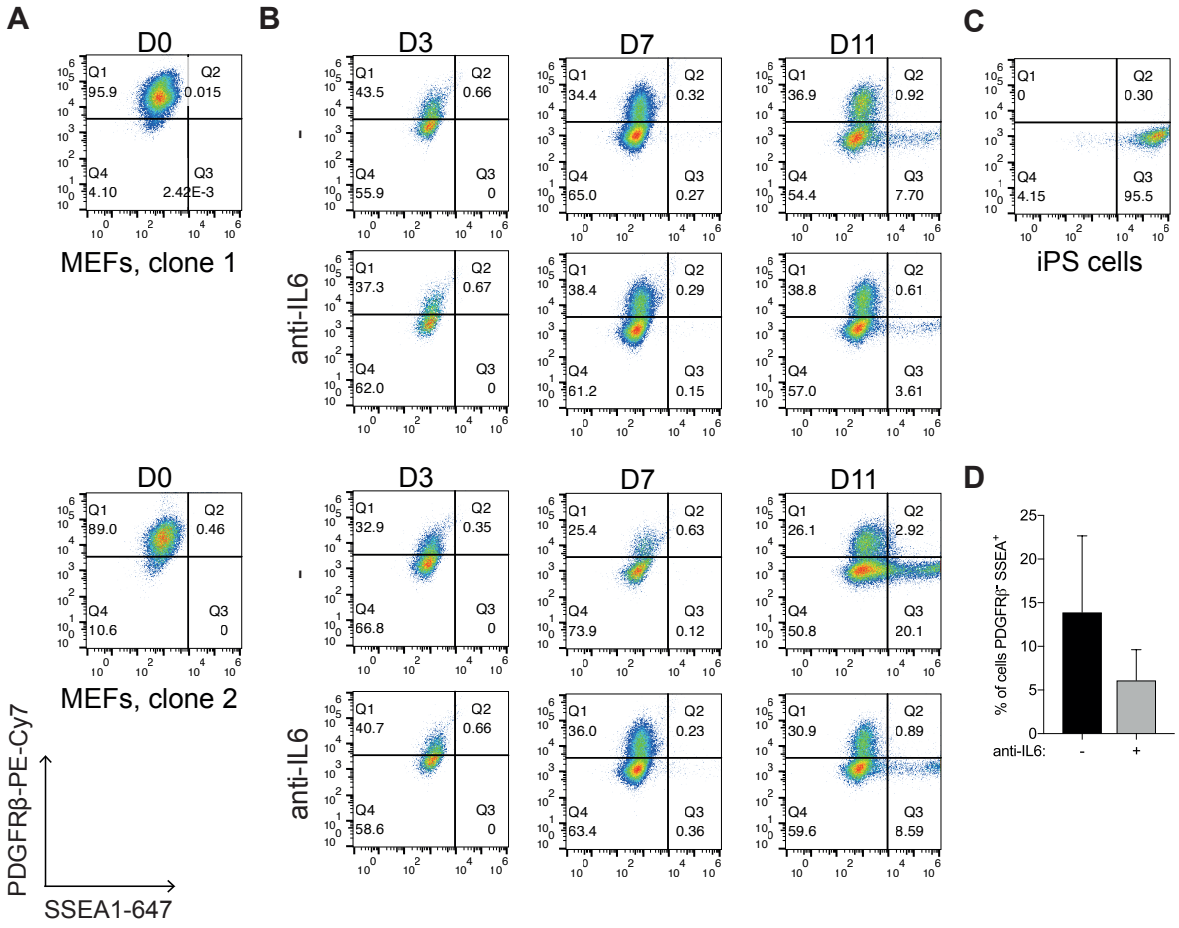

Figure 26. PDGFRβ and SSEA1 transition is affected during anti-IL6 treated reprogramming. (A, B and C) FACS plots showing PDGFRβ and SSEA1 expression in two independent *i4F* MEF clones (A), WT iPS cells (C) and reprogramming samples at day 3, 7 and 11 in untreated (-) and anti-IL6 treated conditions (B). **(D)** Percentage of PDGFRβ^{-} SSEA1^{+} cells at day 11 of reprogramming. In (D), the values represent the average ± SD of a total of three independent MEF clones (n=3). Statistical significance to untreated MEFs (-) was assessed using using two-tailed unpaired Student's t-test with Welch's correction.

Based on these two FACS panels, we conclude that IL6 deficiency could also impact on the expression of SSEA1. We hypothesize that it could be a consequence of the non-upregulation of *Lifr* during reprogramming **(Figure 20C)** which would block the activation of the pluripotent network (SSEA1 by FACS, *Nanog* by mRNA) and then, formation of iPS colonies was impaired **(Figure 16, 17 and 18)**.

2.8. Explore downstream mechanisms of IL6

Once we have evaluated the possible functions of IL6 in reprogramming, we explored the molecular mechanism and signaling pathways downstream IL6. When IL6 binds to the soluble or membrane-bound IL6R and then, to the homodimer of gp130, three intracellular signalling pathways are triggered: JAK/STAT3, PI3K/AKT and MAPK/ERK (Section 1.3. of the introduction).

We first assessed how STAT3 was activated and phosphorylated after IL6 treatment. WT MEFs were treated with rmIL6 and samples were harvested for mRNA and protein analysis. STAT3 can be phosphorylated at the residues tyrosine 705 (Tyr705) or serine 727 (Ser727). The first post-translational modification is associated to pluripotency and maintenance of mouse ES cells and is driven by JAK proteins. The second one is associated to neural differentiation of ES cells and is regulated directly by of MAPK proteins (Huang et al., 2013). We evaluated the phosphorylation at Tyr705 as we were interested in pluripotency. STAT3 was rapidly phosphorylated 30min after adding IL6 **(Figure 27A)** however, from 1 hour to 4 hours, the levels decreased as SOCS proteins may be activated and inhibited the pathway. Interestingly, STAT3 was reactivated and rephosphorylated 6 hours after IL6 treatment which could be due to new mRNA *Il6* synthesis and secretion **(Figure 27A and 27B)**. These preliminary results were in agreement with a previous study that concluded that STAT3 was phosphorylated after IL6 stimulation in a biphasic pattern and the second wave of activation required new synthesis of IL6 as well as the association of IL6R to EGFR (Wang et al., 2013).

Furthermore, *Lifr* mRNA was upregulated two days after rmIL6 treatment and suggested that IL6 could activate downstream mediators that may regulate *Lifr* expression. Therefore, *Lifr* may not be induced in the absence of IL6 and this is what we have observed in reprogramming of *IL6R* null *i4F* MEFs **(Figure 20C)**. Therefore, all together reinforced our idea of two phases of reprogramming, the first one dependent on IL6 and another one on LIF.

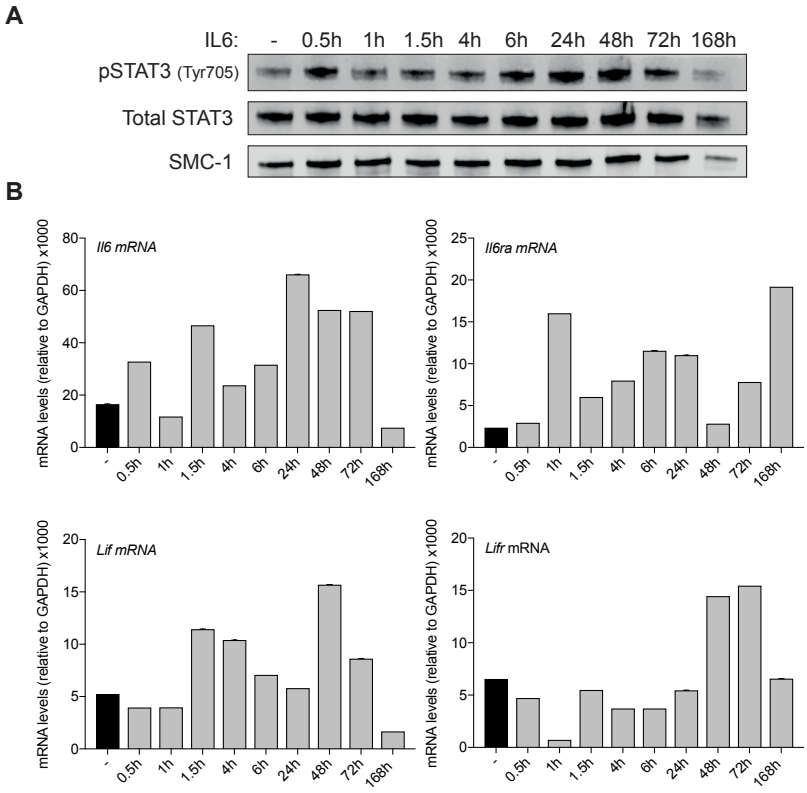

Figure 27. IL6 treatment induces STAT3 activation and *Lifr* mRNA expression in MEFs. **(A)** Immunoblot analyses of the indicated proteins (pSTAT3^{Tyr705}, total STAT3 and SMC-1) of MEFs extracts, 0.5 h to 168 h after rmIL6 treatment at 100 ng/ml. **(B)** mRNA levels of *Il6*, *Il6ra*, *Lif* and *Lifr* in MEFs after rmIL6 treatment at different time points. The values represent the average ± SD of three technical replicates of one independent MEF clone (n=1). Statistical significance was not evaluated.

Next, we wondered whether STAT3 could be activated by *OSKM* overexpression and also whether it may be affected by blocking downstream mediators of IL6 like JAK and PIM proteins. *i4F* MEFs were induced with doxycycline (Dox) and treated with rmIL6 alone or in combination with specific inhibitors of JAK (JAKi) and PIM1 (PIMi) for three days. *OSKM* induction for three days led to activation of STAT3 **(Figure 28)** however, when cells were treated with JAKi or PIMi, the activation was abolished. JAKi was able to completely block STAT3 phosphorylation while PIMi did it to a lesser extent, probably due to its lower level in the signaling cascade **(Figure 28)**. Surprisingly, when IL6 was added, the levels of phosphorylated STAT3 were rescued only in PIMi condition. This suggested that PIM kinases were not involved in the activation of STAT3, which is consistent with the concept that PIM1 is downstream of STAT3.

We compared STAT3 activation pattern with NFκB activation which has been associated with IL6 and PIM kinases (Nihira et al., 2010). IL6 activated NFκB inducing its phosphorylation while *OSKM* overexpression by itself failed. Both inhibitors, JAKi and PIMi, block the induction oh phospho-NFκB however, when cells were treated with JAKi together with IL6, the activation was rescued. This actually confirmed that NFκB was regulated only by IL6-PIM kinases axis independently of *OSKM* expression and STAT3.

These preliminary results indicated that IL6 regulated STAT3 through JAK proteins, as it has been reported, and NFκB through PIM kinases independently of STAT3.

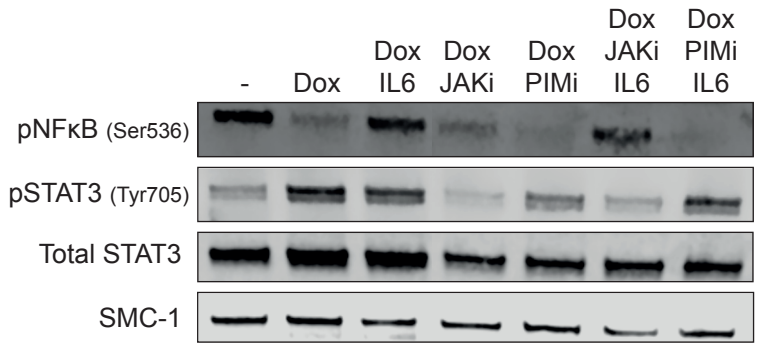

Figure 28. IL6 regulates STAT3 and NFκB activation through JAK or PIM proteins respectively. Immunoblot analyses of $pNF\kappa B^{Ser536}$, $pSTAT3^{Tyr705}$, total STAT3 and SMC-1 proteins in induced *i4F* MEFS treated with different combinations of rmIL6 at 100 ng/ml, JAK inhibitor (JAKi) at 1 μM and PIM1 inhibitor (PIMi) at 100 nM.

Altogether, the second part of the thesis demonstrates that IL6 cytokine plays an important role on *in vitro* reprogramming. Its absence completely blocks the dedifferentiation of fibroblasts as well as the acquisition of pluripotency markers such as *Nanog*, SSEA1 and most importantly *Lifr*. In line with this results, we observed that $Lifr^{high}$ cells were efficiently reprogrammed when IL6 was blocked. Finally, we propose a reprogramming model divided in two phases: the first one depend on the rapidly upregulation of IL6-IL6R axis that, through still unknown mechanism could activate LIFR axis to proceed to the second phase. Moreover, IL6 is able to replace *Sox2* transcription factor in the reprogramming cocktail and we believe this is through PIM1 kinase. All this knowledge will be important not only to understand reprogramming and find mechanisms to improve it but also, to manipulate this molecules *in vivo* and increase plasticity within the tissues to improve the regenerative capacity or their response to different damages.

Discussion

1. *INK4A* DEPENDENT SASP FACTORS PROMOTE REPROGRAMMING

Embryonic stem (ES) cells are pluripotent cells that can be propagated *in vitro* from the inner cell mass of the early blastocyst. They have the capacity of self-renewal and can generate all the tissues of the adult organism. Because of this, they constitute the ideal cell source for cell therapies through a process involving, first, differentiation into the therapeutic somatic cells and then, transplantation. To circumvent the ethical issues raised by the use of human embryos, many studies have focused on obtaining pluripotent cells *in vitro* without using embryos. This was achieved in 2006 by the seminal work of Takahashi and Yamanaka demonstrating that mouse fibroblasts could be converted into pluripotent stem cells, termed induced pluripotent stem cells or iPS cells, by the forced expression of four transcription factors, *Oct4*, *Sox2*, *Klf4* and *cMyc* (*OSKM*), which are important in ES cells (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). Common features are shared by iPS and ES cells in terms of their capacity to differentiate into the three germ layers, gene expression profile, DNA methylation or cell surface markers, among others (Polo et al., 2012; Takahashi and Yamanaka, 2006). Therefore, this technique overcame the concerns about the use of human embryos and offered enormous clinical potential. The efficiency of the standard protocol remains very low, usually around 0.1% of cells form iPS colonies, and many studies have refined the protocol by using different sets of factors or small molecules to improve the efficiency and the kinetics (Federation et al., 2014; Ichida et al., 2009; Li et al., 2012; Maherali and Hochedlinger, 2009; Masuda et al., 2013; Zhang et al., 2014).

1.1. Senescent cells improve the formation of iPS cells through pro-reprogramming factors present in the SASP

In order to address how the efficiency of inducing pluripotency could be increased, we explored the paracrine activity of senescent cells and their influence on reprogramming. Senescence is a cellular response to damage characterized by the secretion of a plethora of pro-inflammatory factors collectively termed senescence-associated secretory phenotype (SASP) (Coppé et al., 2014; Lopes-Paciencia et al., 2019). However, senescent phenotype has been reported to be heterogeneous and dynamic. Comparing three different senescence-inducing stimuli (oncogene-induced senescence (OIS), replicative senescence and ionizing radiation-induced senescence), only 55 genes were commonly upregulated (Hernandez-Segura et al., 2017). We generated conditioned medium (CM) derived from cells rendered senescent with the three previous inducers, and these CMs were tested for their effect on reprogramming. Interestingly, only γ-IR CM increased reprogramming efficiency **(Figure 7)**. SASP from OIS has been shown to propagate senescence to surrounding cells through mainly TGF-β family ligands and IL1-α (Acosta et al., 2013). If this phenomena were also induced in our *i4F* cells treated with the OIS CM, we hypothesize that cells would be permanently arrested and it could explain why we did not observe beneficial effect on reprogramming using this CM.

This result, together with previous data, suggests that senescence has a dual role in reprogramming. On one hand, the expression of the cell cycle regulators *p53*, *p16INK4A*, *p19ARF*

and *p21CIP1* prevents the proliferation of the cells and their ablation significantly improve the efficiency of reprogramming (Kawamura et al., 2009; Li et al., 2009; Marión et al., 2009; Utikal et al., 2009). Moreover, *OSKM* expression, *in vitro* and *in vivo*, induces senescence by increased levels of *p16INK4A*, *p53*, SAβGAL activity and DNA damage (Banito et al., 2009; Chiche et al., 2017; Mosteiro et al., 2016) and this could explain why reprogramming is highly inefficient. Therefore, senescence is a cell-intrinsic barrier for *in vitro* reprogramming. On the other hand, senescent cells secrete multiple cytokines and growth factors which have been shown to recruit and activate the immune system as well as modulate tumor development (Faget et al., 2019). Our work has revealed a new beneficial function for SASP since the CM from senescent cells improves the formation of iPS cells **(Figure 7, 8 and 9)**. Therefore, senescence can be considered as a cell-extrinsic promoter of dedifferentiation.

1.2. Ink4a/Arf and *p53* tumor suppressors regulate the induction of cytokines IL6 and TNF, and this correlates with reprogramming

Our data demonstrate that the tumor suppressors *Ink4a/Arf* and *p53* are involved in the production of pro-reprogramming factors. We observed that the SASP from damaged *p53* null cells was more abundant compared to WT cells **(Figure 12)**. This is in agreement with a previous report which demonstrated that loss of *p53* exacerbated the paracrine activity of the cells after irradiation (Coppé et al., 2008). Regarding *Ink4a/Arf*, we observed that this *locus* was essential for the production of SASP **(Figure 12)** and its deletion completely eliminated the effect of the CM on reprogramming **(Figure 10)**. Indeed, these differences in SASP correlated with the effect of the senescent CMs derived from cells of the three genotypes (WT, *p53* null and *Ink4a/Arf* null), that is: the highest induction of SASP, had the highest beneficial impact on reprogramming. We next identified two cytokines, IL6 and TNF, as the candidate factors highly secreted after damage in WT and *p53* null cells, but absent in *Ink4a/Arf* null cells **(Figure 12 and 13)**. Importantly, these results were recapitulated during *in vivo* reprogramming. *i4F* mice deficient for *p53* present more dysplastic foci as well as higher incidence of teratoma concomitant with higher levels of damage (γH2AX), inflammation (pSTAT3, pNFκB and macrophage infiltration) and, importantly, senescence (SASP factors including IL6, p21 and SAβGAL). This phenotype was absent in reprogrammable mice deficient for *Ink4a/Arf* (Mosteiro et al., 2016). All these evidences indicate that activation of *OSKM, in vitro* and *in vivo,* induces at the same time damage and senescence in some cells, and dedifferentiation in other cells, and both processes appeare associated.

1.3. Ink4a is required for the induction of IL6 and TNF after damage

Ink4a and *Arf* are two tumor suppressors encoded by the *locus* CDKN2A and both regulate cell cycle arrest activating Rb and p53 pathway respectively. We next addressed the individual contribution of these two tumor suppressors in the secretion of IL6 and TNF. *In vitro*, we found that MEFs only deficient for *Arf*, induced the expression of the cytokines similarly to WT MEFs, whereas *Ink4a* only null cells did not **(Figure 15)**. Importantly, this is in agreement with *in vivo*

data showing that *Arf* has a minimal role in *OSKM*-induced senescence and IL6 secretion (Mosteiro et al., 2018). Therefore, *Ink4a* plays a dual role in reprogramming: it acts as a cell-intrinsic barrier to reprogramming by inducing cell cycle arrest in damaged cells, and, at the same time it acts as cell-extrinsic promoter of reprogramming by promoting senescence and the secretion of pro-reprogramming cytokines.

2. IL6 REQUIREMENT IN REPROGRAMMING

We identified IL6 and TNF as candidate factors that could enhance *in vitro* reprogramming. The objective of the second part of this thesis was to evaluate deeply their impact on reprogramming.

2.1. IL6 is the pro-reprogramming factor essential for the acquisition of pluripotency

TNF is a pleiotropic cytokine which has multiple roles in infections, immunity and inducing apoptosis (Chau et al., 2004). We could not evaluate the effect of this factor on reprogramming because cells died during TNF treatment and iPS colonies were not formed. This could be explained by the combined effect of the pro-apoptotic BCL proteins induced after damage and the intracellular pro-apoptotic signaling of TNF treatment (Chau et al., 2004). *OSKM* expression induces damage, senescence and then, the expression of pro-apoptotic factors which could accelerate TNF function in apoptosis. Moreover, it has been reported recently that TNF reduces reprogramming efficiency by two fold (Mahmoudi et al., 2018). Therefore, we conclude that TNF is not the pro-reprogramming candidate we were searching for. Indeed, TNF appears as an anti-reprogramming factor whose negative impact in the SASP is surpassed by the actions of pro-reprogramming factors.

Consequently, we decided to focus on IL6. Helen Blau's laboratory demonstrated that IL6 is induced early during heterokaryon reprogramming. Indeed, IL6 is able to replace *cMyc* from Yamanaka's cocktail treating the cells only for three days and, in combination with *OSKM* increases the efficiency of reprogramming (Brady et al., 2013). First, we demonstrated that incubating the γ-IR CM with neutralizing antibodies anti-IL6 eliminated the beneficial effect of the SASP on reprogramming **(Figure 16)**. Then, we observed that *OSKM* successful reprogramming was blocked in the presence of antibodies anti-IL6 or when IL6 was downregulated in *i4F* MEFs **(Figure 16 and 17)**. Even more, this was confirmed by using a genetic model where *IL6R* is deleted, lacking both classic signaling and trans-signaling driven by IL6 **(Figure 20)**. These results suggest two different and not exclusive interpretations. On one hand, IL6 is essential for reprogramming and in its absence the process of dedifferentiation would be impaired. On the other hand in the absence of functional IL6, TNF inhibitory activity could affect reprogramming negatively and then the process would be blocked.

Recently, Helen Blau's group also observed that partially knockdown of *IL6R* is sufficiency to block reprogramming (Mai et al., 2018). All these results together also suggest that IL6 signaling is not only critical in reprogramming but also that it could be secreted by damaged cells, which are overexpressing *OSKM,* and would influence the cells acquiring pluripotency features in a paracrine manner. It is published that *OSKM* activation *in vitro* induces senescence (Banito et al., 2009) and we hypothesize that this damaged cells could be the responsible for secreting IL6 which affects to the acquisition of pluripotency acting in a paracrine manner **(Figure 29)**.

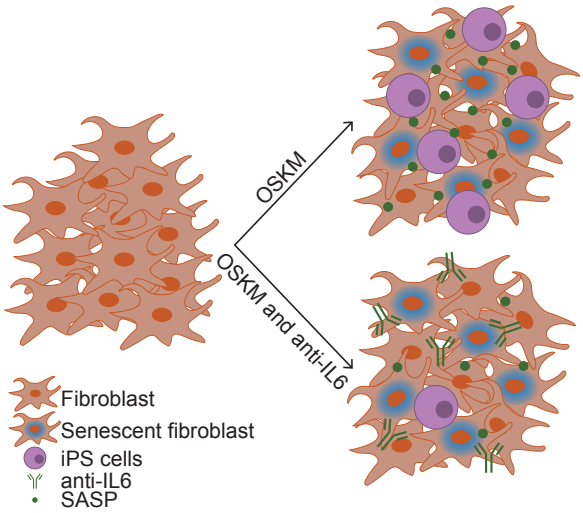

Figure 29. Representation of the connection between senescence, reprogramming and IL6. *OSKM* expression has two mutually exclusive outcomes: in some cells it induces dedifferentiation to form induced pluripotent stem (iPS) cells, in other cells it induces senescence that triggers the secretion of soluble factors which are part of the senescence-associated secretory phenotype (SASP) and promote reprogramming in a paracrine manner. When neutralizing antibodies against IL6 (anti-IL6) are added, reprogramming decreased.

Importantly, these results were recapitulated *in vivo*. Our group has demonstrated that WT and *p53* null *i4F* mice treated with anti-IL6 antibodies have lower levels of senescence, SASP factors and reprogramming (Chiche et al., 2017; Mosteiro et al., 2016). Moreover, *Ink4a/Arf* null *i4F* mice treated with recombinant IL6, have higher degree of dysplasia in the pancreas (Mosteiro et al., 2018). These evidences demonstrate that IL6 is the critical cytokine linking senescence and reprogramming *in vitro* and *in vivo* and, its action is dominant over TNF.

At present, we are studying the consequences of deleting *IL6R* either in all tissues or only in myeloid cells including monocytes, macrophages and granulocytes. Previous work from our laboratory suggests that the immune system could also have an additional role on *in vivo* reprogramming and furthermore, macrophages are recruited efficiently in the process (Mosteiro et al., 2016). Interestingly, there are some reports connecting IL6 and macrophages. Activation of STAT3 signaling in macrophages forced their polarization into M2 macrophages which plays important roles in tissue repair (Yin et al., 2018). Based on this, we hypothesize that after *OSKM* expression followed by STAT3 activation, macrophages might polarize to M2 contributing to the repair of the tissue after damage. This could have important consequences in protocols of regeneration, where not only *OSKM* would induce dedifferentiation and plasticity in the tissue but also would generate an immune environment which favors repair.

2.2. IL6 and LIF axes are differentially induced in reprogramming

Standard protocols of reprogramming are based on the addiction of exogenous recombinant LIF, but not IL6. This could be explained by how stem cells are maintained and the important role of LIF during embryo development. ES cells were firstly derived by culturing the inner cell mass on top of non-proliferative fibroblasts which were found to secrete LIF into the medium (Smith et al., 1988). ES cells can be maintained in a self-renewal state in the presence of LIF, CNTF, CT-1 or OSM since all of them signal through LIFR-gp130 (Conover et al., 1993; Pennica et al., 1995; Rose et al., 1994). In contrast, ES cells cannot be cultured in the presence of IL6 because they do not express IL6R, but can be maintained with IL6-sIL6R through trans-signaling that is only dependent on gp130 (Yoshida et al., 1994).

We evaluated the expression profile of IL6-IL6R and LIF-LIFR during reprogramming. We observed two different waves of induction. The first starts from day 3 to day 6, where *Il6* is sharply induced together with *Il6ra*. The second one expands from day 8 until the end of the process where *Lifr* is highly upregulated **(Figure 19)**. Interestingly, this pattern was lost in *IL6R* null MEFs where *Il6* and *Il6r* were undetectable and *Lifr* remained unchanged upon induction of *OSKM* **(Figure 20C)**. Moreover, pluripotency genes, like *Nanog* and endogenous *Sox2*, were not induced in null cells in contrast to WT ones **(Figure 19 and 20C)**. *NKX3-1* has been described as a downstream mediator of the IL6-STAT3 network which activates endogenous *Oct4* during reprogramming (Mai et al., 2018). In line with this study, we observed that WT *i4F* cells induced the expression of NKX3-1 as well as endogenous *Oct4* however, null cells failed.

Furthermore, IL6 deficiency also affects to the acquisition of SSEA1 marker **(Figure 25 and 26)**. SSEA-1 is a well-known carbohydrate antigenic epitope on the cell surface of preimplantation embryos at 8-cell stage, teratocarcinomas and undifferentiated cells including ES and iPS cells, but not in differentiated cells (Cui et al., 2004). It is synthesized by *Fut9* and null mice are normal and viable, suggesting that SSEA1 is not essential for embryonic development (Kudo et al., 2004). It is involved in cell-cell interactions however, little is known about its regulation. The acquisition of this marker in reprogramming has been well studied and it is used to track pluripotency in reprogramming (Polo et al., 2012; Stadtfeld et al., 2008). When neutralizing anti-IL6 antibodies were added during reprogramming, the percentage of $Thy1^{-}$ $SSEA1^{+}$ cells at the end of the process is 50% lower than in untreated MEFs. In summary, we conclude that in the absence of IL6 signaling, the early pluripotent network is silenced and this eventually blocks the late pluripotent machinery including cell surface markers.

2.3. The expression of *Lifr* is a key event of IL6-IL6R in reprogramming

Since the pluripotency network requires LIF-LIFR signaling, we hypothesized that the failure to express *Lifr* could be the key defect present in cells lacking IL6 signaling that could explain their impaired reprogramming. To address this hypothesis, we generated cells that constitutively express *Lifr*. We examined two cellular models: primary neonatal astrocytes and *Lifr*-transduced MEFs. Astrocytes are a type of glia cells in the central nervous system that are differentiated from neural stem cells. LIFR has been shown to be implicated in their development and regulates the expression of some astrocytes markers like *Gfap* (Alfonsi et al., 2008). Astrocytes have been previously reprogrammed *in vitro* (Nakajima-Koyama et al., 2015). Interestingly, we have observed that astrocyte reprogramming is independent of IL6 signaling **(Figure 21)**. Moreover, *i4F* astrocytes upregulated pluripotency genes like *Nanog* and endogenous *Oct4* at the end of reprogramming meaning that cells acquired full pluripotency **(Figure 22)**. This result was recapitulated in MEFs overexpressing *Lifr*. Interestingly, the forced expression of the receptor in WT MEFs not only increased slightly the efficiency of reprograming in standard conditions but also accelerates the acquisition of pluripotency. iPS colonies usually appear around day 10-12 after *OSKM* induction however, when it is combined with *Lifr,* the colonies are formed around day 6. Moreover, *Lifr*-transduced MEFs were successfully reprogrammed in the absence of IL6.

Therefore, these results demonstrate that LIF-LIFR signaling is required for reprogramming and their expression could depend on IL6.

We conclude that the key role of IL6-IL6R signaling during the early phase of reprogramming is the transcriptional activation of *Lifr*, presumably through STAT3. Upon expression of *Lifr*, cells become sensitive to LIF and LIF-LIFR drives the transition to pluripotency. On the light of these results, we are planning to overexpress *Lifr* in *IL6R* null *i4F* MEFs. We expect to rescue the unsuccessful formation of iPS cells and we will study the expression of the failed upregulated genes (*Nanog*, endogenous *Oct4* and *NKX3-1*) as well as the transitions from Thy1 and PDGFRβ positive to SSEA1 positive cells.

2.4. IL6 replaces *Sox2* in *OKM* reprogramming through the PIM1 kinase

Helen Blau's laboratory demonstrated that IL6 is able to replace *cMyc* from the reprogramming cocktail and they have also shown that an IL6-activated transcription factor, NKX3-1, can replace *Oct4* (Brady et al., 2013; Mai et al., 2018). However, *cMyc* is dispensable for direct reprogramming of fibroblasts, although the efficiency is lower (Nakagawa et al., 2008; Wernig et al., 2008), and they did not demonstrate that exogenous IL6 can replace any of the core reprogramming factors. Given these precedents, we decided to investigate if IL6 could replace any of the Yamanaka factors.

For this, MEFs were reprogrammed with all the possible combinations of the Yamanaka factors in the presence or absence of exogenous IL6. Interestingly, expandable iPS-like colonies were only observed in the combination with *OKM*, suggesting that IL6 can replace *Sox2*. Moreover, when the combinations of Yamanaka factors were combined with overexpression of PIM1 (a kinase activated by IL6-IL6R-STAT3), iPS colonies were again observed only in the combination with *OKM*. This supports the idea that IL6 can replace *Sox2* through PIM1 kinase. *PIM1* is a target gene of STAT3 that contributes to IL6 intracellular signaling (Brady et al., 2013). In pluripotency, PIM1 plays an important role in the maintenance of ES cells. Knockdown of *PIM1* in pluripotent stem cells favours their differentiation, whereas its overexpression protects the self-renewal capacity of the cells and inhibits apoptosis (Aksoy et al., 2007). *In vivo*, we have demonstrated that the activation of *OSKM* combined with an inhibitor for PIM1 strongly reduced reprograming (Mosteiro et al., 2016). However, the connection between PIM1 and *Sox2* has not been addressed yet. One hypothesis could be that PIM1 induces the phosphorylation and activation of NFκB (Nihira et al., 2010) which has been reported to be important for the expression of *Sox2* in breast cancer stem cells (Vazquez-Santillan et al., 2016). In the immediate future, we are planning to study *OKM*-derived iPS cells as well as their expression profile during reprogramming to decipher the mechanisms involved.

3. PROPOSED REPROGRAMMING MODEL REGARDING IL6 AND LIF

Together, these results indicate that IL6 is produced, probably by damaged cells, during *in vitro* reprogramming and its role is essential for the formation of iPS colonies. IL6 induction and its secretion are tightly regulated processes by the tumor suppressors *Ink4a/Arf* and *p53*. p53 is a negative regulator of SASP therefore, its absence is associated to an exacerbated secretion of factors, including IL6, which creates a favouring environment for *in vivo* and *in vitro* reprogramming. On the other hand, *Ink4a/Arf locus*, through *Ink4a*, positively modulates SASP and its absence is a cell-extrinsic barrier for reprogramming.

Importantly, *Il6* and *Il6r* are induced early in reprogramming in contrast to *Lifr,* which is expressed later but maintained in the final pluripotent state, iPS cells, and also ES cells **(Figure 30)**. Furthermore, the expression of *Lifr* depends on the previous signaling of IL6 since *IL6R* null cells do not upregulate it during reprogramming. We hypothesize that IL6 and LIF drive two phases of reprogramming independently but connected.

Firstly, IL6 is induced and secreted early in reprogramming and acts in a paracrine and autocrine manner. It binds to IL6R-gp130 of *OSKM*-expressing cells to induce the phosphorylation of JAK and STAT3. Then, four downstream mediators are activated: IL6R, NKX3-1, PIM1 and LIFR **(Figure 30)**. In tumoral cells, IL6/JAK/STAT3 has been shown to form a positive feed-forward loop in which STAT3 induces both IL6 and IL6R (Chang et al., 2013; Rokavec et al., 2015). Also, NKX3-1 is induced and triggers the expression of the endogenous *Oct4* and activates one of the core transcription factors for pluripotency (Mai et al., 2018). On the other hand, PIM1 is activated, as it is described (Brady et al., 2013). Moreover, our results demonstrate that it replaces *Sox2* from the reprogramming cocktail and it could activate the expression of the endogenous gene or mimic the activity of the reprogramming transcription factor. Finally, IL6/JAK/STAT3 would also activate *Lifr* presumably through STAT3. In this regard, it is worth mentioning that there are STAT3 binding sites in the *Lifr* promoter based on the Gene Transcription Regulation Database (Yevshin et al., 2017). It is published that *cMyc* negatively regulates miR125, which binds directly to the 3'UTR region of the *Lifr* mRNA and inhibits its expression in brain tumors (Salm et al., 2015). Moreover, IL6 and STAT3 are known to positively regulates *cMyc* (Brady et al., 2013; Cartwright et al., 2005). Therefore, as a possible mechanism, IL6 dependent phosphorylation of STAT3 could activate *Lifr* transcription directly and also through the release of miR125 inhibition by *cMyc*. Finally, the full establishment of LIF-LIFR axis would drive the pluripotency circuitry

This study has elucidated the molecular basis of *in vitro* cellular reprogramming that will be translated to an *in vivo* context. Our aim is to manipulate and increase the plasticity in those tissues that could affect tissue repair processes as well as aging.

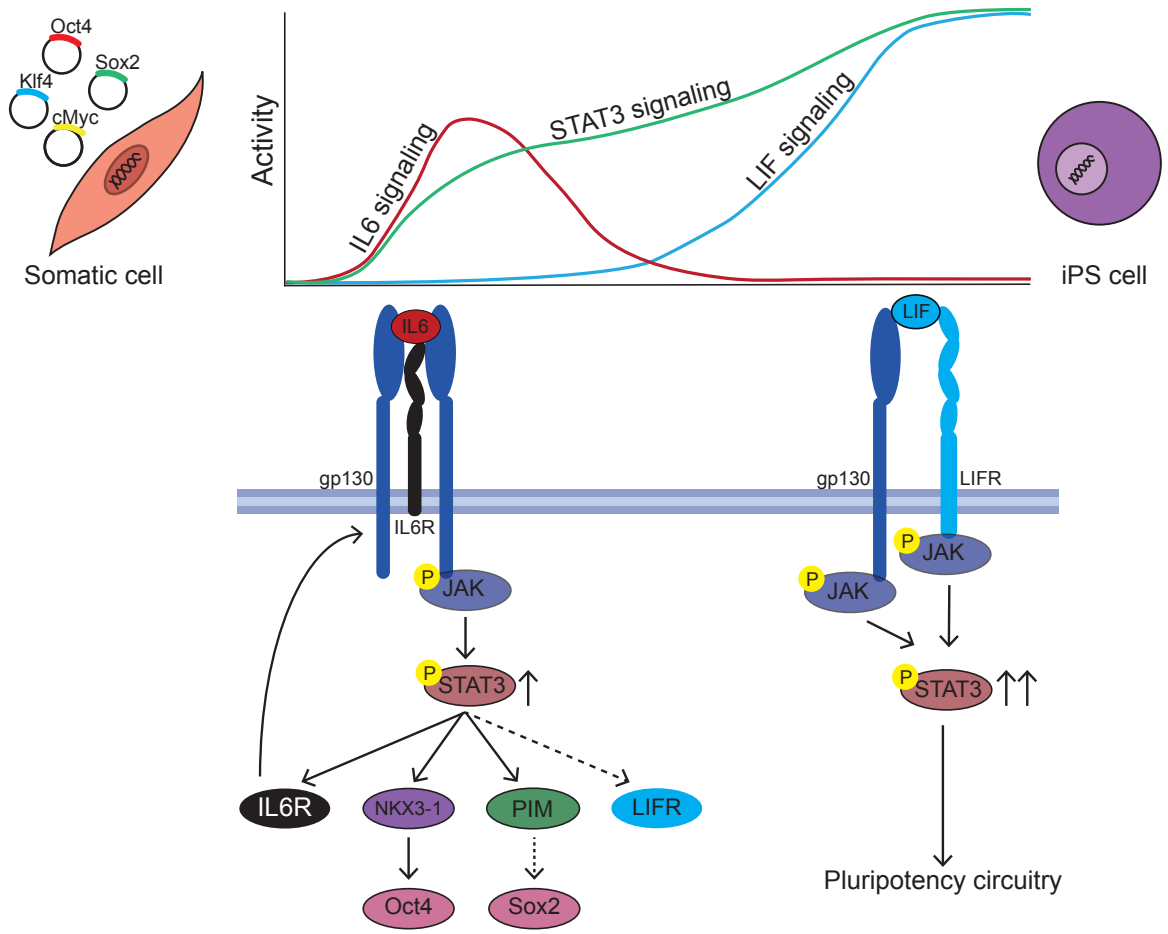

Figure 30: Proposed model of the interplay between IL6 and LIF during *in vitro* reprogramming. In the early phases of *OSKM* induced reprogramming of fibroblasts, IL6 is produced and binds to IL6R-gp130. The, JAK/STAT3 pathway is activated and induces the downstream mediators: IL6R which binds to the membrane, *NKX3-1* which activates *Oct4*; PIM1 which may activate *Sox2* and LIFR. This LIFR binds to the membrane and form a complex with LIF and gp130 to activate the late pluripotency circuitry which maintains the pluripotent state. Dashed lines mean unknown mechanism.

Conclusions

1. Paracrine SASP factors promote *in vitro* reprogramming

1.1. The conditioned media (CMs) derived from replicative senescence and oncogene-induced senescence do not favor reprogramming, while γ-irradiation induced CM promotes the formation of iPS cells.

1.2. *Ink4a, Arf* and *p53* regulate the production of pro-reprogramming factors upon damage: the *Ink4a/Arf locus* is a positive modulator, while *p53* is a negative regulator.

1.3. The levels of two secreted factors, IL6 and TNF, from γ-IR senescent cells correlate with the enhancement in reprogramming. Both are expressed and secreted after damage in WT and *p53* null MEFs in contrast to *Ink4a/Arf* null cells.

1.4. *Ink4a*, but not *Arf*, is necessary for the expression of *Il6* and *Tnf*.

1.5. Senescence cells promote *in vitro* reprogramming through the secretion of paracrine SASP factors and this is regulated by *Ink4a*.

2. In vitro reprogramming requires IL6 for the activation of the pluripotent network

2.1. IL6, coming from the exogenous CM or from *OSKM*-damaged cells, is crucial for *in vitro* reprogramming and its action is dominant over the negative impact of TNF.

2.2. IL6 and IL6R are induced rapidly in reprogramming while LIF and LIFR are upregulated later. Moreover, this expression profile correlates with the early role of IL6 during the process.

2.3. In the absence of IL6 signaling during the early stages of reprogramming, *i4F* MEFs do not induce key pluripotency genes, such as *Lifr*, *Nanog*, *NKX3-1*, *Oct4* or *Sox2*, as well as surface markers (SSEA1) impairing the acquisition of the pluripotent state. However, when LIF-LIFR axis is highly expressed from the beginning, reprogramming is independent of IL6.

2.4. IL6 replaces *Sox2* in *OKM* reprogramming through the *PIM1* kinase.

2.5. Reprogramming depends on the activation of the JAK/STAT3 pathway which is firstly induced by IL6-IL6R axis and later by LIF-LIFR, which is maintained to regulate the pluripotent state.

Conclusiones

1. Los factores paracrinos del SASP favorecen la reprogramación *in vitro*

1.1. El medio condicionado de células senescentes, inducidas por replicación o por expresión de oncogenes, no favorece la reprogramación. Sin embargo, el medio condicionado derivado de células irradiadas senescentes aumenta la reprogramación promoviendo la formación de células iPS.

1.2. *Ink4a, Arf* y *p53* regulan la producción de los factores que favorecen la reprogramación tras un daño: el *locus Ink4a/Arf* lo modula positivamente, mientras que *p53* lo inhibe.

1.3. Los niveles de IL6 y TNF secretados por las células senescentes irradiadas correlaciona con el aumento en la reprogramación. Ambos factores se expresan y se secretan tras el daño en fibroblastos WT y deficientes para *p53*. Sin embargo, MEFs deficientes para el *locus Ink4a/Arf* no expresan dichos factores.

1.4. *Ink4a*, y no *Arf*, es necesario para la expresión de *Il6* y *Tnf*.

1.5. Las células senescentes favorecen la reprogramación celular mediante la secreción de factores (SASP) que actúan de manera paracrina, y esto es regulado por el gen *Ink4a*.

2. La reprogramación *in vitro* requiere la citoquina IL6 para la activación de la pluripotencia

2.1. La citoquina IL6, del medio condicionado senescente o de las células dañadas que expresan *OSKM*, es crucial para la reprogramación celular y su efecto es dominante respecto al impacto negativo de TNF.

2.2. IL6 y IL6R se inducen rápidamente en la reprogramación mientras que LIF y LIFR se inducen en etapas tardías. Además, este perfil de expresión correlaciona con la función de IL6 en las primeras etapas del proceso.

2.3. Cuando la señalización inducida por IL6 no está activa en las primeras etapas de la reprogramación, los MEFS reprogramables no activan genes importantes de pluripotencia como *Lifr*, *Nanog*, *NKX3-1*, *Oct4* o *Sox2*, así como marcadores de membrana (SSEA1) y por lo tanto, la adquisición del estado pluripotente queda bloqueado. Sin embargo, cuando el eje LIF-LIFR está activo desde el inicio, la reprogramación es independiente de IL6.

2.4. IL6 reemplaza a *Sox2* en la reprogramación inducida por los factores de transcripción *OKM* a través de la quinasa *PIM1*.

2.5. La reprogramación celular depende de la activación de la ruta JAK/STAT3 que es inducida en primer lugar por el eje IL6-IL6R y después por LIF-LIFR, que se mantiene para regular la pluripotencia.

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Annex

Published and in preparation articles directly related to this PhD thesis:

- Lluc Mosteiro, Cristina Pantoja[#], **Noelia Alcazar**[#], Dafni Chondronasiou, Pablo J. Fernández-Marcos, Miguel Rovira, Maribel Muñoz-Martin, Carmen Blanco-Aparicio, Joaquin Pastor, Gonzalo Gómez, Alba de Martino, Maria Abad and Manuel Serrano. (2016) Tissue damage and senescence provide critical signals for cellular reprogramming *in vivo*. *Science 354*, 6315.
(#: These authors contributed equally to this work)

- **Noelia Alcazar**, Manuel Serrano. Requirement of IL6 in reprogramming of somatic cells into embryonic pluripotency. (*In preparation*)

Other published articles

- Adelaida R. Palla, Daniela Piazzolla, **Noelia Alcazar**, Marta Cañamero, Osvaldo Graña, Gonzalo Gómez-López, Orlando Dominguez, Marta Dueñas, Jesús M. Paramio and Manuel Serrano. (2015) The pluripotency factor NANOG promotes the formation of squamous cell carcinomas. *Scientific Reports* 5, 10205.

- Cian J. Lynch, Raquel Bernad, Ana Martínez-Val, Marta N. Shahbazi, Sandrina Nóbrega-Pereira, Isabel Calvo, Carmen Blanco-Aparicio, Carolina Tarantino, Elena Garreta, Laia Richart-Ginés, **Noelia Alcazar**, Osvaldo Graña-Castro, Gonzalo Gómez-López, Irene Aksoy, Maribel Muñoz-Martin, Sonia Martinez, Sagrario Ortega, Susana Prieto, Elisabeth Simbloeck, Alain Camasses, Camille Stephan-Otto Attolini, Agustin F. Fernandez, Marta I. Sierra, Mario F. Fraga, Joaquin Pastor, Daniel Fisher, Nuria Montserrat, Pierre Savatier, Javier Muñoz, Magdalena Zernicka-Goetz and Manuel Serrano. Global hyperactivation of enhancers stabilizes human and mouse naïve pluripotency. *Nature Cell Biology* (in press).