Senescence impairs successful reprogramming to pluripotent stem cells

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Declaration of originality

All experiments included in this thesis were performed by me unless otherwise stated in the text.
Abstract

Somatic cells can be reprogrammed into induced pluripotent stem (iPS) cells by overexpression of combinations of transcription factors such as Oct4, Sox2, Klf4 and c-Myc. However, reprogramming is slow and stochastic, suggesting the existence of mechanisms that limit its efficiency. Senescence is an irreversible G1 cell cycle arrest elicited by replicative exhaustion or in response to stresses such as DNA damage, or aberrant expression of oncogenes. The arrest observed during senescence is implemented mainly through activation of p53 and the upregulation of the cyclin-dependent kinase (CDK) inhibitors, p16\textsuperscript{INK4a} and p21\textsuperscript{Cip1}.

In this work the relation between the process of reprogramming to iPS cells and senescence was investigated. The expression of the 4 reprogramming factors from a polycistronic vector in human fibroblasts (IMR90) was shown to induce cell cycle arrest and upregulation of p53, p16\textsuperscript{INK4a} and p21\textsuperscript{Cip1}. Reprogramming-induced senescence (RIS) results from the activation of a DNA damage response, and chromatin remodeling of the \textit{INK4a/ARF} locus, as shown by a decrease in the levels of the H3K27me3 modification following expression of the reprogramming factors. RIS resembles a stress response, which parallels oncogene-induced senescence, however context-dependent differences may also contribute as shown by the fact that ES cells-specific miRNAs can partially bypass RIS. Additionally when expressed individually, each reprogramming factor was shown to have a negative effect over the proliferation of somatic cells.

Since reprogramming initially triggers a stress response with characteristics of senescence it may act as an initial barrier limiting the efficiency of the process. Indeed, ablation of different senescence effectors improved the efficiency of reprogramming, both in mouse and human cells. Additionally, the polycomb protein CBX7 was also shown to increase reprogramming efficiency in a process that may partially dependent on repression of the \textit{INK4a/ARF} locus. The senescence response to expression of reprogramming factors uncovers an important barrier to induced pluripotency but also highlights the importance of tumour suppressor pathways in preventing dedifferentiation during tumorigenesis. Identification of RIS mediators may help to understand this connection and provide safer approaches to increase reprogramming efficiency.
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## Abbreviations

| Ac | acethylated |
|----|-------------|
| AHR | aryl hydrocarbon receptor |
| AID | activation-induced cytidine deaminase |
| AP | alkaline phosphatase |
| ARF | alternative reading frame gene |
| ATM | ataxia telangiectasia mutated |
| ATR | ataxia telangiectasia and Rad3 related |
| ATP | adenosine tri-phosphate |
| BMP | bone morphogenenic protein |
| bp | base pair |
| BrdU | 5-bromo-2-deoxyuridine |
| BSA | bovine serum albumin |
| CBX7 | chromobox protein homolog 7 |
| CDK | cyclin dependent kinase |
| CDKI | cyclin dependent kinase inhibitor |
| CDKN1 | cyclin dependent kinase inhibitors, family 1 |
| CDKN2 | cyclin dependent kinase inhibitors, family 2 |
| cDNA | complementary DNA |
| ChIP | chromatin immunoprecipitation |
| CSC | cancer stem cell |
| C(T) | threshold cycle |
| CXCL | CXC chemokine ligand |
| DAPI | 4,6-diamidino-2-phenylindole |
| dNTP | deoxyribonucleotide triphosphate |
| DMEM | Dulbecco’s modified Eagle’s medium |
| DMSO | dimethyl sulfoxide |
| DNA | deoxyribonucleic acid |
| DNMT | DNA methyltransferase |
| Dox | doycycline |
| DTT | dihiothreitol |
| E.coli | *Escherichia coli* |
| EDTA | ethylene-dinitrili tetraacetic acid |
| EED | embryonic ectoderm development |
| EC | embryonic carcinoma |
EG embryonic germ
EpiS epiblast stem
ES embryonic stem
EZH2 enhancer of zeste homolog 2
FBS foetal bovine serum
FGF fibroblast growth factors
g gram
GFP green fluorescent protein
GLB1 galactosidase, beta 1
GSK3 glycogen synthase kinase 3
H histone
HAT histone acetyltransferase
γH2AX phosphorylated histone 2, variant X
HCA high content analysis
HDAC histone deacetylase
HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hES human embryonic stem
HMT histonemethyltransferase
HP1 heterochromatin protein 1
HPV16 human papilloma virus 16
HRAS Harvey rat sarcoma gene
HSCs hematopoietic stem cells
hTERT human telomerase reverse transcriptase
ICM inner cell mass
IF immunofluorescence
IGF insulin growth factor
IGFBP insulin growth factor binding protein
IL interleukin
INK4 Inhibitor of CDK4
iPS induced pluripotent stem
JMJD3 jumonji domain containing 3
K lysine
Kb kilobase
kDa kiloDalton
KLF4 Krüppel-like factor 4
KSR knockout serum replacement
L  litre
LB  lysogeny broth
LIF leukaemia inhibitor factor
m  mili
M  molar
MAPK mitogen-activated protein kinases
Me3 trimethylated
Mb  mega base pair
MEF mouse embryonic fibroblast
mES mouse embryonic stem
mGS mouse germ stem
mRNA messenger RNA
miRNA microRNAs
MYC myelocytomatosis gene
NEAA non-essential aminoacids
NSC neural stem cell
OCT4 octamer-binding transcription factor 4
OIS Oncogene induce-senescence
OSKM polycistronic cassette containing Oct4, Sox2, Klf4 and c-Myc
PBS phosphate buffered saline
PcG polycomb group
PCNA proliferating cell nuclear antigen
PCR polymerase chain reaction
PEI polyethylenimine
PFA paraformaldehyde
PI propidium iodide
PRC polycomb repressive complex
PRE polycomb response element
p(S/T)Q ATM/ATR phosphorylated serine/threonine
qPCR quantitative PCR
RB retinoblastoma
REX1 RNA exonuclease 1 homolog
RIS reprogramming-induced senescence
RNA ribonucleic acid
ROS reactive oxygen species
rpm rotations per minute
| Abbreviation | Description |
|--------------|-------------|
| SA-β-gal    | senescence-associated β-galactosidase |
| SAHA        | suberoylanilide hydroxamic acid |
| SAHF        | senescence-associated heterochromatic foci |
| SASP        | senescence associated secretory phenotype |
| SCNT        | somatic cell nuclear transfer |
| SDS         | sodium dodecyl sulphate |
| shRNA       | small hairpin RNA |
| SOX2        | SRY (sex determining region Y)-box 2 |
| SSEA        | stage specific embryonic antigen |
| SUZ12       | suppressor of zeste 12 |
| SV40 LT     | simian vacuolating virus 40 large antigen |
| SWI/SNF     | switch/sucrose non-fermenting family of chromatin remodelling ATPases |
| TCDD        | 2,3,7,8-tetrachlorodibenzo-p-dioxin |
| TGF         | tumour growth factor |
| UTR         | untranslated region |
| VPA         | valproic acid |
| WNT         | wingless (Wg) and integration site (INT) gene |
Chapter 1 – Introduction

1.1. The limited lifespan of normal somatic cells

In the beginning of the 20th century when tissue culture techniques were developing, it was believed that primary cells isolated from tissues and organs could be propagated indefinitely if the right culture conditions were used. Alexis Carrel proposed that the longevity of a tissue outside of the organism could exceed greatly its normal duration within the body (Carrel, 1912). Leonard Hayflick and Paul Moorhead, who were working on the isolation of human diploid strains in the 1960s, challenged this idea. They proposed that differentiated normal cells, unlike tumour cells, could only undergo a limited number of cell divisions after which they would enter a state of irreversible growth arrest, which they named cellular senescence (Hayflick, 1965). However this idea was not accepted easily. Some thought that the failure of normal diploid cells to proliferate indefinitely was due to extrinsic causes, including the absence of one or more essential nutrients or growth factors and infection with virus or mycoplasma. Subsequent work showed that senescence is not specific to human fibroblasts; it was described in cultures established from numerous mammalian species and in a variety of cell types other than fibroblasts including keratinocytes (Rheinwald and Green, 1975), vascular smooth muscle cells (Bierman, 1978), lens cells (Tassin et al., 1979) and lymphocytes (Tice et al., 1979).

The work by Hayflick and Moorhead gave birth to the idea that normal somatic cells possessed a “mitotic clock” that dictates their maximum lifespan and highlighted the fact that immortality was a key feature of tumour cells. Nowadays cellular senescence is known to be a fundamental mechanism, implicated in a number of physiological processes and disease states (Campisi et al., 2001).
1.1.1. Main characteristics of cellular senescence

In contrast with quiescence, a state characterized by a growth arrest that can be reversed in the presence of proper physiological signals, the hallmark of cellular senescence is its irreversible nature. Once they reach senescence, growth arrested cells cannot re-enter cell cycle when stimulated, despite remaining metabolically active (reviewed in Campisi and d'Adda di Fagagna, 2007). Although cells reach a state in which they can no longer initiate replication, this happens asynchronously so that at any given moment in active mitotic cultures, the population is a heterogeneous mixture of senescent and non-senescent cells, that are at various stages in their proliferative life (Cristofalo and Sharf, 1973). Early passage cultures consist primarily of cells that exhibit short cell cycle periods. With successive subcultivations, the percentage of cells in the population that exhibit longer generation times and those unable to proliferate, increases. Successful progression through the cell cycle involves the synthesis of new DNA that can be quantified by 5-bromo-2'-deoxyuridine (BrdU) incorporation assays. Senescent cells fail to incorporate BrdU because they are arrested predominantly at the G1 phase of the cell cycle and do not progress to S phase for DNA synthesis.

Together with senescence, apoptosis or programmed cell death protects the organism from the propagation of damaged cells. However while senescence prevents their growth, apoptosis quickly eliminates them. Interestingly, senescent human fibroblasts are resistant to some types of apoptosis such as those caused by growth factor deprivation and oxidative stress (Chen et al., 2000; Hampel et al., 2004). The mechanisms responsible for this resistance are not well understood however they are most probably related to their common regulator p53. Upon stress the decision between life and death, is dependent on p53 that can in some situations preferentially transactivate genes that arrest proliferation, rather than those that induce apoptosis (Das et al., 2007; Jackson and Pereira-Smith, 2006). Indeed,
although p53 has a key role in inducing apoptosis, some p53 target proteins also inhibit apoptosis, including p21$^{CIP1}$, decoy death receptors such as DcR1 and DcR2, the transcription factor SLUG (which represses the expression of PUMA), and several activators of the AKT/PKB (protein kinase B) survival pathways (Janicke et al., 2008). This might explain, to some extent, why senescent cells are so stable in culture, and why they increase with age in vivo.

The senescent state is also characterized by a number of morphological, structural and functional changes. The most obvious morphological changes that accompany the onset of cellular senescence in vitro are an increase in cell size, thin cytoplasm and flattened appearance. Related with this change in morphology, there is a reorganization of the cytoskeleton that seems to be due to down-regulation of actin and upregulation of vimentin (Nishio et al., 2001)(Figure 1.1).

Senescence-associated β-galactosidase (SA-β-gal) activity detectable at pH 6.0 produces a blue perinuclear staining and has been widely used as a senescence marker (Dimri et al., 1995) (Figure 1.1). SA-β-gal activity is due to an increase in $GLB1$ expression, the gene encoding lysosomal β-D-galactosidase. Fibroblasts from patients with autosomal recessive $GM_1$-gangliosidosis, which have defective lysosomal β-galactosidase, do not express SA-β-gal at late passages even though they undergo replicative senescence (Lee et al., 2006a). These results confirmed that (SA-β-gal) activity is a consequence rather than a cause of the senescent state, and is instead related with the increased lysosomal biogenesis that is observed in these cells (Lee et al., 2006a). Indeed, early reports showed that lysosomes increase in number and size in senescent cells (Robbins et al., 1970), therefore it is possible that SA-β-gal activity at a suboptimal pH (lysosomal β-D-galactosidase activity is typically measured at pH 4.5) becomes detectable as result of increased lysosomal content (Kurz et al., 2000). Other results also suggest that an increase in autophagy, that is digestion of the cell’s organelles, functions as an effector mechanism during
cellular senescence (Young et al., 2009) and may be associated with an increase of lysosomal mass and SA β-gal (Gerland et al., 2003). Although widely used as a senescence marker, it is important to note that SA-β-gal activity at pH 6, is not senescence-specific; therefore it must be used in conjunction with other senescence markers (Severino et al., 2000; Yang and Hu, 2005).

Concomitant with the morphologic features described, there is a reorganization of the heterochromatin. Senescence-associated heterochromatic foci (SAHF) have been described in senescent cells undergoing oncogenic stress by expression of an oncogenic version of the proto-oncogene HRAS, and in cells undergoing replicative
exhaustion. SAHF are enriched for markers of heterochromatin, such as heterochromatin protein 1 (HP1) and Lys9 tri-methyl on histone H3 (H3K9me3) and exclude euchromatic markers, such as acetylation of histone 3 on lysine 9 (H3K9Ac) or tri-methylation on lysine 4 (H3K4me3). The reorganization of heterochromatin that gives rise to these foci results in a stable transcriptional repression of E2F target genes (Narita et al., 2003). This stable repression is related to another important feature of senescent cells, its altered gene expression profile.

Several studies have identified senescence-associated gene expression signatures (Mason et al., 2004; Shelton et al., 1999; Trougakos et al., 2006; Yoon et al., 2004). As expected they revealed changes in cell-cycle regulators. Two cell-cycle inhibitors that are often expressed by senescent cells are the cyclin-dependent kinase inhibitors (CDKIs) \textit{CDKN1a} (also termed \textit{Waf1}, encodes p21\textsuperscript{Cip1}) and \textit{INK4a} (also termed \textit{CDKN2a}, encodes p16\textsuperscript{INK4a}). On the other hand, senescent cells repress genes that encode proteins involved in cell-cycle progression; some of them are E2F targets (for example, replication-dependent histones, c-FOS, cyclin A, cyclin B and PCNA) (Pang and Chen, 1994; Seshadri and Campisi, 1990). Interestingly the quest for senescence-specific gene expression signatures also revealed that besides changes in cell-cycle regulators, senescent cells exhibited changes in genes that appeared to be unrelated with growth arrest (Mason et al., 2004; Shelton et al., 1999). These included, the upregulation of multiple secreted factors that are known to alter the tissue microenvironment, and are thought to contribute to age-related pathologies. This senescent-associated secretory phenotype (SASP) can trigger different and, sometimes, opposing effects in the microenvironment and surrounding cells. Work by Judith Campisi’s group suggests that factors secreted by senescent fibroblasts promote cancer progression (Krtolica et al., 2001), however, besides its implication in tumour clearance by the immune system (Xue et al., 2007), several studies suggest that this SASP may also have an important role in establishing and
maintaining the senescent state itself. Some of the factors secreted by senescent cells were shown to have tumour suppressive roles. The plasminogen activator inhibitor (PAI)-1 is necessary and sufficient for the induction of senescence (Kortlever et al., 2006). Insulin-like growth factor-binding protein 7 (IGFBP7) was shown to mediate senescence induced by oncogenic BRAF (Wajapeyee et al., 2008). Equally, pro-inflammatory cytokines and chemokines secreted by senescent cells, such as IL-8, CXCL1, IL-6, and their receptors have been shown to be up-regulated during senescence and their depletion to partially bypass replicative and oncogene-induced senescence (Acosta et al., 2008; Kuilman et al., 2008).

1.1.2. Mechanisms triggering senescence

1.1.2.1. Replicative Senescence

Hayflick and Moorhead observations reveal not only that normal cells had a limited number of cell divisions, but also that there was a high reproducibility concerning the number of population doublings that fibroblast from different embryonic donors underwent (between 40 and 60 population doublings). Additionally they observed that cells frozen at any population doubling level could “remember” how many populations doublings they have been through before being frozen. This implied that cells had some kind of molecular counting mechanism able to register rounds of replication, but the nature of this “replicometer” was unknown (reviewed in Hayflick, 2000). In the early 1970s when the molecular basis of DNA replication was unravelled it became clear that because of the nature of lagging-strand synthesis, DNA polymerases could not completely replicate the 3’ end of linear duplex DNA. Watson described it as the “end-replication problem” (Watson, 1972), however it was Alexey Olovnikov who realized that the repeated shortening of the DNA molecule at each round of DNA replication could explain why normal cells could divide only a limited number of times (Olovnikov, 1973). This idea was later confirmed when the
structure of telomeres was determined. Elizabeth Blackburn described that telomeres of the ciliated protozoan, Tetrahymena thermophila, consisted of a simple sequence of hexameric repeats of the nucleotides TTGGGG (Blackburn and Gall, 1978). The telomeres in human cells also consist of thousands of repeats, but in mammals the sequence is TTAGGG (Moyzis et al., 1988). Once this sequence was known, the length of human telomeres could be measured. The mean telomere length decreased by 2 to 3 kilobase pairs during the entire in vitro lifetime of several strains of cultured normal human diploid fibroblasts (Harley et al., 1990) supporting the idea that telomeric shortening is the replicometer that determines the number of times that a normal cell is able to divide.

However one essential question remained: How do immortal cell lines avoid telomere shortening? The answer to this critical question originated in studies by Greider and Blackburn, who identified telomerase, a ribonucleoprotein enzyme terminal transferase that extends the 3′ end of telomeres and thus elongates them (Greider and Blackburn, 1985). Telomerase was later found to be present in extracts of immortal human cell lines (Counter et al., 1992; Morin, 1989) and in about 90% of all human tumours studied (Chiu and Harley, 1997).

1.1.2.2. Premature senescence and tumour suppression

The replicative exhaustion observed in human cells following extensive passage in culture results from progressive shortening of the chromosomes ends (Bodnar et al., 1998; Harley et al., 1990). The situation in mouse embryonic fibroblasts (MEFs) is different, as they have longer telomeres than human cells (40-60 Kb versus 10 Kb) and in many cases express telomerase (Itahana et al., 2004; Rangarajan and Weinberg, 2003). In MEFs, the main trigger of replicative senescence is the generation of reactive oxygen species (ROS). Consequently,
senescence of MEFs has been compared with a culture shock. In particular, it has been shown that DNA damage caused by oxidative stress is the main cause of senescence onset in murine cells (Parrinello et al., 2003). In this study, it was demonstrated that when MEFs are propagated in 3% oxygen, rather than the commonly used 20% oxygen conditions, they do not suffer senescence. Indeed, throughout the years several studies suggested that senescence can be initiated in human and mouse cells by various cellular stresses. This has been referred to as premature senescence, as opposed to replicative or telomere-dependent senescence (Ben-Porath and Weinberg, 2005). DNA-damaging agents, improper culture conditions and interestingly, activation or overexpression of certain oncogenes can trigger this kind of irreversible growth arrest. In 1997, it was described that activated RAS elicited a proliferative arrest similar to the replicative senescence of human fibroblasts (Serrano et al., 1997). Since this discovery, other oncogenes have been shown to induce a similar response, denominated oncogene-induced senescence (OIS). Importantly, OIS was later proven to take place in vivo (Collado et al., 2005) and pre-malignant lesions were shown to be positive for markers of cellular senescence (Michaloglou et al., 2005). This is in agreement with the fact that cancer is a multistep process that requires both the activation of oncogenes and inactivation of tumour suppressor genes (Hanahan and Weinberg, 2000). As all the evidences are gathered, one idea becomes clear. Regardless of the senescence-initiating signals, cellular senescence in both human and mouse cells can be triggered by an anomalous activation of the DNA-damage pathways that eventually results in p53 activation. This is in agreement with the notion that senescence, as apoptosis, is a mechanism that is induced in situations of stress, and has a role in preventing proliferation of damaged/altered cells (Figure 1.2).
Figure 1.2. Senescence is a tumour suppressive mechanism. (A) Two main mechanisms, senescence and apoptosis, act to prevent the proliferation of altered cells. Senescence can be triggered by several different stimuli, including telomere erosion, oxidative stress and others forms of DNA damage. Activation of oncogenes can also trigger senescence, highlighting the importance of senescence as a tumour suppressor mechanism. (B) Premalignant lesions usually comprise of a heterogeneous population of tumour cells, some undergoing apoptosis (purple) others senescence (blue). These lesions may remain restricted in growth or if cells are able to override tumour suppressive mechanisms, tumour progression to a malignant state will be favoured. (Adapted from (Collado and Serrano, 2006; Gil and Peters, 2006).

1.1.3. Two pathways to senescence

During cell cycle progression, checkpoints assure that cells are “ready” to progress to the next phase and that no defects during DNA replication or chromosome segregation are accumulated. Additionally, two DNA damage checkpoints, during G1/S and G2/M assure that cells that acquired DNA breaks or other forms of DNA damage, do not progress through the cell cycle. Arresting the
cell cycle allows them to repair these defects. If the damage is irreparable due to excessive or continuous DNA damage, cells may enter senescence or undergo apoptosis (reviewed in Malumbres and Barbacid, 2009).

Misregulation of cyclin-dependent kinases (CDKs) whose activity requires binding of regulatory subunits known as cyclins, is at the heart of the cell cycle defects that can lead to unscheduled proliferation. In unstressed cells at G1, the phase preceding the DNA replication phase, mitogenic signals are sensed by D-type cyclins that preferentially bind CDKs 4 and 6. Activation of the CDK4/6-Cyclin D complexes results in partial inactivation of the retinoblastoma protein (RB) and retinoblastoma-like proteins (RBL) 1 and 2 (p107 and p130). This allows expression of E-type cyclins that bind to and activate CDK2, which will further phosphorylate RB proteins thereby inactivating them. Inactivated RB releases E2F transcription factors that will induce transcription of genes necessary for DNA replication and cell cycle progression (reviewed in Weinberg, 1995)(Figure 1.3).

If cells encounter various stresses, such as telomere uncapping or expression of oncogenes, two main pathways are triggered that converge on modulation of CDK activity and are responsible for senescence onset. The p53-p21Cip1 pathway is activated by the DNA damage response mediated by ATM/ATR, and by the ARF protein. ATM/ATR are protein kinases that activate p53 mainly by phosphorylation while ARF activates p53 by inhibiting HMD2 an ubiquitin E3 ligase that targets p53 for degradation. Active p53 induces transcription of genes, such as CDKN1a (p21Cip1) (El-Deiry, et al 1993). The other main pathway is frequently referred as the p16/RB pathway. Both p21Cip1 and p16INK4a are CDK inhibitors that belong to two different families: Cip/Kip or CDKN1 family (p21Cip1, p27Kip1 and p57Kip2) (Gu et al., 1993; Harper et al., 1993; Lee et al., 1995; Matsuoka et al., 1995; Polyak et al., 1994; Toyoshima and Hunter, 1994) and the INK4 or CDKN2 family (p16INK4a, p15INK4b, p18INK4c and p19INK4d) (Hannon and Beach, 1994; Hirai et al., 1995; Serrano
et al., 1993). p16<sup>INK4a</sup> interacts strongly with CDK4 and 6 and inhibits their ability to interact with D-type cyclins that are therefore targeted for degradation. p21<sup>Cip1</sup> inhibits cell cycle progression mainly through the inhibition of CDK2. However, it is known to interact with both CDK4/6-Cyclin D complexes and CDK2-Cyclin E complexes. Inactive CDKs result in unphosphorylated RB proteins that sequester E2F transcription factors, avoiding cell cycle progression (Figure 1.3) (reviewed in Sherr and Roberts, 1999).

**Figure 1.3. Two pathways to senescence.** In unstressed cells and in the presence of mitogenic signals activation of the CDK4/6-Cyclin D complexes results in partial inactivation of RB family proteins. This allows expression of E-type cyclins that bind to and activate CDK2, which will further phosphorylate RB proteins, thus inactivating them. Inactive RB releases E2F transcription factors that activate transcription of genes necessary for entry into S-phase. Senescence-inducing signals usually engage either the p53 or the p16<sup>INK4a</sup>-retinoblastoma protein (RB) tumour suppressor pathways. p53 is negatively regulated by HDM2 (Mdm2 in mice), which facilitates its degradation. HDM2 is negatively regulated by ARF (alternative-reading-frame protein). Active p53 establishes senescence in part by inducing the expression of p21<sup>Cip1</sup>, a cyclin-dependent kinase (CDK) inhibitor that suppresses the phosphorylation and, hence, the inactivation of RB. Engagement of p16-Rb pathway, that is upregulation of p16<sup>INK4a</sup>, also results in inhibition of RB phosphorylation and inactivation, by inhibition CDK4/6. RB halts cell proliferation by inhibiting the activity of E2F, and thereby suppressing expression of genes required for cell-cycle progression.
1.1.3.1. The DNA damage response (DDR)

Several stresses that can lead to senescence converge on a DNA damage response (DDR). Telomeres consist of nucleoprotein complexes at the chromosomes ends, which terminate in a duplex loop that protects the single stranded 3’ DNA overhang. The progressive telomere shortening eventually triggers an alteration in telomere structure, referred to as telomere uncapping, revealing the chromosome end that is recognized as a DNA break (reviewed in Verdun and Karlseder, 2007). This idea is supported by studies that demonstrate that telomere structure, rather than telomere length, is the main determinant of functional telomeres (Karlseder et al., 2002). A DNA damage response also contributes to the onset of oncogene-induced senescence. It has been shown that constitutively active HRAS leads to DNA replication stress that might result from impaired or inappropriately activated origins of replication, a phenomenon known as hyper-replication (Bartkova et al., 2006; Di Micco et al., 2006; Mallette et al., 2007).

Induction of p53 in response to DNA damage is coordinated by the serine/threonine protein kinases ataxia telangiectasia mutated (ATM) and the ataxia telangiectasia and Rad3-related (ATR). ATM plays a crucial part in the response to double strand breaks by activating and executing checkpoint and repair pathways. Other forms of DNA damage, such as replication stress, are mainly regulated by ATR, however these two regulatory kinases share downstream targets such as H2AX (histone 2A variant X) at sites flanking DNA breaks, several DNA damage mediators and p53 itself (Figure 1.4). Accordingly DNA damage markers such as γH2AX (phosphorylated histone 2A variant X), are detected at the chromosomes ends of cells reaching replicative exhaustion and in stalk replication forks in cells suffering from oncogenic stress (Di Micco et al., 2006; Takai et al., 2003).

The mechanisms regulating p53 activity are complex. Amongst them, p53 can be subjected to a range of post-transcriptional changes that include phosphorylation
(Atadja et al., 1995; Webley et al., 2000) and acetylation (Tang et al., 2008). In addition, its levels are tightly controlled by HMD2, (Mdm2 in mice) which targets p53 for degradation. Therefore in unstressed cells p53 has a half-life of a few minutes and remains at low levels and in an inactive form, until some form of stress results in p53 modification and accumulation (reviewed in Vousden and Prives, 2009). Activated p53 can effectively block cell cycle progression by activating the transcription of the cyclin-dependent kinase inhibitor p21Cip1, although several other p53-target genes such as 14-3-3 sigma and GADD45 also contribute to this response (el-Deiry et al., 1993; Vousden and Prives, 2009).

**Figure 1.4. The DNA damage response activates p53.** Double strand breaks are recognized by the MRN complex (composed by MRE11, RAD50 and NBS1) and lead to the activation of the kinase ataxia-telangiectasia mutated (ATM) and subsequent amplification of the response by recruitment of other DNA damage signalling proteins. ATM phosphorylates several proteins including MDM2, MDM4, p53 and checkpoint-2 (CHK2), which phosphorylates p53 and other proteins. Single strand breaks become coated with replication protein A (RPA) that attracts the ataxia-telangiectasia and Rad3-related (ATR)- interacting protein (ATRIP) complex which phosphorylates the 9-1-1 complex (constituted by RAD9, RAD1 and HUS1) further activating ATR. Active ATR phosphorylates p53, MDM2, checkpoint-1 (CHK1) and other substrates. The DNA damage response ultimately results in activation of effector molecules such as p53, CDC25 or structural maintenance of chromosomes (SMC1). Activated p53 will induce expression of several genes involved either in cell cycle arrest or apoptosis (Adapted from Campisi and d’Adda di Fagagna, 2007).
1.1.3.2. The INK4b-ARF-INK4a locus

The p53 and RB pathways are regulated by, among other proteins, the products of the INK4b-ARF-INK4a locus. This locus encodes for two CDK inhibitors from the same family, p15\textsuperscript{INK4b} and p16\textsuperscript{INK4a}. Surprisingly, the INK4b-ARF-INK4a locus also encodes for ARF (p19ARF in mice and p14ARF in humans), a tumour suppressor that induces p53. The INK4a and ARF genes each have their own promoters, which produce different transcripts. INK4a consists of exons 1\textalpha, 2 and 3, while ARF is formed by exons 1\textbeta, 2 and 3. Although exons 2 and 3 are common to both transcripts, they are read in different reading frames, therefore there is no homology at their protein sequence (Mao et al., 1995; Quelle et al., 1995) (Figure 1.5).

![Diagram](https://example.com/diagram.png)

**Figure 1.5. The INK4b-ARF-INK4a locus.** This locus in chromosome 9 includes three tumour suppressors genes in close proximity. Both INK4b (green) and INK4a (orange/blue) encode inhibitors of cyclin dependent kinases (p16\textsuperscript{INK4a} and p15\textsuperscript{INK4b}). The two INK4 genes flank the ARF exon 1\textbeta, which is spliced together with the exons 2 an 3 to generate the ARF mRNA (white/red). Exons 2 and 3 are shared between ARF and INK4a (red and blue rectangles) however; ARF is read in a different reading frame originating unrelated proteins. Exons (ex.) are represented by coloured rectangles and arrows designate the gene promoters.
The INK4b/ARF/INK4a locus is one of the most commonly altered regions in human cancers. This region can be subjected to loss of heterozygosity, point mutations or epigenetic alterations, such as promoter hypermethylation, that results in loss of expression (reviewed in Ortega et al., 2002).

Since inactivation of the locus results in the abrogation of three tumour suppressor genes, significant debate has focused on which member of the locus represents the principal tumour suppressor at chromosome 9p21. The individual knockout of each of the three genes in mouse revealed that the three mouse strains are more tumour prone than wild-type mice, however each of them is less tumour prone when comparing with mice that lack both Arf and p16\textsuperscript{Ink4a} (Latres et al., 2000; Sharpless et al., 2004). This implies that there is a synergy between p16\textsuperscript{Ink4a} and Arf in tumour suppression. To further complicate matters, several studies revealed that these proteins have different relevance in senescence control when comparing mouse and human cells. Unlike human cells, which require the inactivation of both the p53 and RB pathways to prevent the onset of senescence, MEFs derived from p53 or Arf null animals maintain their high proliferative potential, do not senesce, and can be propagated indefinitely (Kamijo et al., 1997). On the other hand MEFs derived from Rb or p16\textsuperscript{Ink4a} null mice undergo senescence like wild type MEFs (DePinho, 2000; Krimpenfort et al., 2001; Sharpless et al., 2001; Sherr and DePinho, 2000). In mouse cells, the Arf-p53 pathway seems to be the main pathway controlling senescence. In addition, MEFs from transgenic mice generated by disruption of all Rb family proteins (Rb, p107 and p130) do not senesce (Dannenberg et al., 2000; Sage et al., 2000), suggesting that, in mice, Rb function can be compensated by Rb related proteins. Furthermore, p19\textsuperscript{Arf} has functions that are independent of p53 because when Arf is overexpressed in cells lacking p53, it is still able to induce cell cycle arrest (Weber et al., 2000). In contrast, p14\textsuperscript{ARF} seems to have a less relevant role in human cells. For example while Ink4a and Arf are largely
co-regulated in mouse (Krishnamurthy et al., 2004; Zindy et al., 1997), co-regulation of the two genes in human cells is not well established. In cultured human cells, senescence onset is usually associated with $p16^{\text{INK4a}}$ up-regulation, but not $p14^{\text{ARF}}$ (Huot et al., 2002; Michaloglou et al., 2005; Munro et al., 1999).

1.1.3.3. Epigenetic regulation of the $\text{INK4b-ARF-INK4a}$ locus

Despite of species-specific differences, activation of the $\text{INK4b-ARF-INK4a}$ locus is one of the first events that follow oncogenic activation. Understanding the molecular mechanisms that regulate this locus should help to identify the properties recognized by a cell as malignant as well as the way off recognizing them.

Epigenetic mechanisms, such as the ones establishing chromatin marks present in either the DNA or the associated histones, do not alter the primary DNA sequence but are responsible for changes in gene expression that are heritable through cell division. Methylation of $\text{INK4b-ARF-INK4a}$ promoters has been reported to occur in a variety of tumours. On the other hand methylation of histones proteins at specific residues, which plays a major role in the maintenance of active and silent states of gene expression during development, are also implicated in the regulation of the $\text{INK4b-ARF-INK4a}$ locus.

Polycomb group (PcG) proteins regulate chromatin by directly methylating histones. There are two core PcG protein complexes: polycomb repressive complex 2 (PRC2) and polycomb repressive complex 1 (PRC1). PRC2 establishes the histone code that is subsequently interpreted by PRC1. The core components of the mammalian PRC2 complex are enhancer of zeste homologue 2 (EZH2), embryonic ectoderm development (EED), suppressor of zeste homologue 12 (SUZ12) (Sparmann and van Lohuizen, 2006) (Figure 1.6A). EZH2 is a histone methyltransferase that trimethylates histone H3 at lysine 27 (H3K27me3), an epigenetic mark associated with inactive chromatin. EED and SUZ12 are required for
EZH2 methytransferase activity. Specific DNA sequences firstly identified in *D. melanogaster* and named polycomb repressive elements (PREs), are recognized and bound by PRC2. Repressive histones marks, such as H3K27me3 established by PRC2, are than recognized by the PRC1 (reviewed in Mills, 2010). The latter stabilizes the inactive state by inhibiting transcription initiation (Cao et al., 2002), ATP-dependent chromatin remodelling (Francis et al., 2001) as well as by compacting nucleosome arrays (Francis et al., 2004). There is significant diversity among complexes of the PRC1 family, in part due to the existence of multiple paralogues of each PcG gene. PRC1 homologs of *D. melanogaster* in mammals are expressed differently in distinct tissues or developmental stages. The core of mammalian PRC1 is constituted by: a polycomb component containing a chromo domain (PC)(CBX2, 4, 6, 7 or 8), a polyhomeotic component (PH) (PH 1, 2, or 3) a posterior sex combs element (PSC) (such as BMI or MEL18) and a RING protein (RING1A or B) (Simon and Kingston, 2009)(Figure 1.6A).

PcG proteins such as BMI1 and EZH2 are deregulated during tumorigenesis. BMI1 has been shown to regulate cellular senescence in murine and human cells (Itahana et al., 2003; Jacobs et al., 1999) and is also thought to prevent premature senescence of adult stem cells by repressing the *INK4b-ARF-INK4a* locus (Bruggeman et al., 2005; Molofsky et al., 2005). Other PRC1 member, the polycomb homolog CBX7 was identified in a cDNA screen designed for identification of genes able to bypass senescence in human epithelial cells. Knockdown of CBX7 in MEFs induces senescence whereas its expression extends life span, suggesting that CBX7 has a role in suppressing the *INK4b-ARF-INK4a* locus (Gil et al., 2004).

Oncogene-induced senescence results in reversal of PcG-mediated repression of p16<sub>INK4A</sub>. Senescence induced by oncogenic RAS coincides with the loss of PcG protein binding at the *INK4b-ARF-INK4a* locus, transcriptional repression of EZH2, and coincident transcriptional activation and recruitment of the H3K27me3 histone
demethylase, jumonji domain-containing 3 (JMJD3). There is a loss of the repressive H3K27me3 mark, RNA polymerase II is recruited and p16^{INK4A} expression is induced, culminating in senescence (Agger et al., 2009; Barradas et al., 2009) (Figure 1.6B).

Figure 1.6. Epigenetic activation of the INK4b-ARF-INK4a locus due to oncogenic stress. (A) Composition of PRC2 (left) and PRC1 (right) PcG protein complexes. PRC2 complex contains enhancer of zeste homologue 2 (EZH2), embryonic ectoderm development (EED), suppressor of zeste homologue 12 (SUZ12) and retinoblastoma-binding protein 4 (RBBP4) or RBBP7. PRC1 is constituted by: a polycomb component containing a chromo domain (PC)(CBX2, 4, 6, 7 or 8), a polyhomeotic component (PH) (PH 1, 2, or 3) a posterior sex combs element (PSC) (BMI or MEL18) and a RING protein (RING1A or B) (B) In proliferating cells the INK4b-ARF-INK4a locus (here referred as CDKN2A-CDKN2B) is repressed (OFF) by PCR2 and PRC1 complexes that establish and maintain the repressed state characterized by the acquisition of the silent mark, tri-methylation of lysine 27 in histone 3 (H3K27-Me3). Expression of activated Ras results in recruitment of jumonji domain-containing 3 (JMJD3) leading to demethylation of H3K27-Me3 and consequent eviction of PRC proteins. The inactive mark is substituted by the active mark H3K4-Me3 and RNA polymerase II (RNA Pol II) is recruited resulting in transcriptional activation of the locus (ON). (Adapted from Mills, 2010).
1.2. Developmental potential and cell reprogramming

Mammalian organisms rely on a range of specialized cells to fulfil the diverse functions required for their maintenance and survival. However this extensive array of specialized cells arises from a single cell after fertilisation: the zygote. During development, cells gradually lose developmental potential and became progressively differentiated in order to fulfil their function in somatic tissues. Therefore cells can be classified on the basis of their developmental potential. The zygote is “totipotent” because it retains the potential to give rise to all embryonic and extra-embryonic tissues. Embryonic stem (ES) cells, which are derived from the inner cell mass of the blastocyst, can originate all embryonic but not all extra-embryonic tissues and are therefore classified as “pluripotent”. Further down the line are the “multipotent” adult tissue stem cells, such as hematopoietic stem cells, that can only give rise to cell types within their lineage (Figure 1.7).

1.2.1. Nuclear equivalence and the beginning of cell reprogramming

The notion that differentiation is an unidirectional process that progressively limits the cells developmental potential soon intrigued biologists. In 1892 Weismann suggested that cell specialization was due to the uneven distribution of heritable determinants present in the nucleus. In this way only germ cells would carry a complete set of determinants and could therefore evolve into a whole organism (reviewed in Lensch, 2009). Although several researchers tried to clarify this matter, it was the work by Briggs and King, 60 years later that provided the irrefutable answer. Working with *Xenopus laevis* they established the technique of somatic cell nuclear transfer (SCNT), which consisted of isolating the nucleus of somatic cells
Figure 1.7. Stem-cell hierarchy. The totipotent zygote formed by the fusion of egg and sperm divides to form the inner cell mass (ICM) and the extra-embryonic (EE) tissue of the blastocyst. When isolated from the blastocyst in vitro, the cells of the ICM can be maintained in culture as pluripotent embryonic stem cell (ESC) lines. During development cells become increasingly restricted in their lineage potential and generate tissue-specific, multipotent stem cells. Adult multipotent stem cells are present in different organs and several niches, and are important for tissue maintenance. (Adapted from Eckfeldt et al., 2005.)
from late-stage embryos and transferring them into enucleated oocytes (Briggs and King, 1952). In this way they were able to evaluate the developmental potential of the somatic nucleus and demonstrate that differentiated cells retain the genetic information necessary to generate the whole organism. John Gurdon carried these results further by demonstrating that terminally differentiated cells such as those from the tadpole intestine were likewise capable of being reprogrammed by the cytoplasmatic factors present in the oocyte (Gurdon, 1962). Dolly the sheep was the first mammal to be generated by SCNT using nucleus of a fully specialized mammary gland cell (Wilmut et al., 1997). Importantly, this work not only demonstrated the nuclear equivalence theory, that somatic cells possess the same gene pool as the totipotent zygote, but also implies that differentiation imposes reversible epigenetic, rather than genetic changes. Therefore, the somatic nucleus can be reprogrammed by the cytoplasmatic factors present in the oocyte to establish a different expression programme.

1.2.2. Pluripotency and self-renewal

Many questions remained unanswered following the demonstration of the nuclear equivalence theory. How are the differentiation programs established and maintained? Which factors are able to reprogram the somatic nucleus to reverse differentiation? An important step towards dissecting these mechanisms was the establishment of pluripotent cell lines.

1.2.3.1. Derivation of pluripotent cells lines

Pluripotency describes the functional capacity to generate all cell types by differentiation into any of the three germ layers: endoderm, mesoderm and ectoderm. However, pluripotent cells cannot develop into an adult animal because they lack the potential to contribute to extra-embryonic tissue such as the placenta.
Pluripotency can be found transiently in vivo in the inner cell mass (ICM) of the mammalian blastocyst and in some regions of the epiblast. However, it can also be maintained in vitro under defined conditions that promote growth while inhibiting cellular differentiation. In this way pluripotent cells lines are also characterized by the ability to self-renew: the capacity to divide continually and give rise to identical undifferentiated cells.

Embryonic carcinoma (EC) cells were the first pluripotent cells to be propagated in vitro. These cells are derived from teratocarcinomas, which are malignant germ cell tumours that are comprised of both undifferentiated and differentiated cells belonging to the three germ layers (Kleinsmith and Pierce, 1964; Stevens and Little, 1954).

The derivation of mouse embryonic stem (mES) cells from the inner cell mass (ICM) of mouse blastocysts was first described in 1981 (Evans and Kaufman, 1981; Martin, 1981), while human embryonic stem (hES) cells were derived more than fifteen years later (Shamblott et al., 1998; Thomson et al., 1998). Embryonic stem cells from mouse and human, have both the properties of self-renewal and pluripotency; and can therefore be maintained in culture indefinitely in an undifferentiated state or differentiate in vitro into derivates of the three germ layers and give rise to teratomas when transplanted into nude mice. In the case of mouse ES cells the test of pluripotency can be taken even further. By re-introducing ES cells into developing blastocysts one can verify their contribution to all somatic tissues including the germ line, in a process that originates chimeras (animals that have two different populations of genetically distinct cells).

Besides ES cells, other pluripotent stem cell lines have been successively derived: epiblast-derived stem (EpiS) cells from post-implantation embryos (Brons et al., 2007; Tesar et al., 2007); embryonic germ (EG) cells from primordial germ cells (Matsui et al., 1992; Resnick et al., 1992); and multipotent germ stem (mGS) cells
from explanted neonatal or adult mouse testicular cells (Guan et al., 2006; Kanatsu-Shinohara et al., 2004b; Seandel et al., 2007).

Induced pluripotent stem (iPS) cells constitute another pluripotent cell line that is obtained from somatic cells by expression of defined factors. The assays used to demonstrate pluripotency of ES cells also demonstrate pluripotency of iPS cells. But complete equivalence between iPS cells and their biological counterpart, ES cells, is still a matter of debate.

1.2.3.2. Core transcriptional regulators of pluripotency

The intense study of ES cell biology has uncovered numerous and diverse pluripotency regulators such as transcription factors, signalling transduction molecules, regulatory RNAs and chromatin-modifying enzymes. Three main transcription factors, all highly expressed in the inner cell mass and epiblast of the mouse embryo and in undifferentiated ES cells, have been suggested to be central to the transcriptional regulatory hierarchy that specifies embryonic stem cell identity. These are the POU-family transcription factor Oct4, the homeodomain DNA-binding protein Nanog and the Sox-family transcription factor Sox2. Deletion of any of these genes results in early embryonic lethality due to the inability to maintain pluripotent cells (Avilion et al., 2003; Mitsui et al., 2003; Nichols et al., 1998). While deletion of Oct4 or Sox2 in mouse ES cells lead to conversion to trophectoderm (Masui et al., 2007; Niwa et al., 2000), Nanog deficient mouse embryonic stem cells are still able to self-renew but have an increased propensity to differentiate and cannot give rise to mature germ cells (Chambers et al., 2003).
Co-regulatory and auto-regulatory mechanisms appear to link the three factors in a self-reinforcing circuit. Oct4 and Sox2 can heterodimerise and control expression of a number of genes including Oct4, therefore one of Sox2 functions in maintaining pluripotency seems to be the regulation of Oct4 levels (Masui et al., 2007). Indeed, genome-wide analysis of the occupancy of these three transcription factors reveal that all bind to their own promoters as well as the promoters of the genes encoding the other two factors (Boyer et al., 2005), suggesting that auto-regulatory loops mediate gene expression stability and maintenance of the pluripotent state. In addition, to their own promoters Oct4, Sox2 and Nanog co-occupy hundreds of genes which seem to overlap, suggesting that these three factors act co-ordinately to maintain a pluripotency-associated transcriptional program. Together with ES cell-specific active genes, these regulators also bind genes that are silent in embryonic stem cells and whose expression is associated with lineage commitment and cellular differentiation. Consequently, a key role of these master regulators is probably to counteract differentiation by continuously suppressing expression and activity of lineage specification factors (reviewed in Niwa, 2007) (Figure 1.8).

New regulators of pluripotency (such as Esrrb, Dax1, Sall4, Rex1, Stat3, among others) have been identified by shRNA knockdown screens (Ivanova et al., 2006), proteomics (Wang et al., 2006) and analysis of transcription factor binding sites and gene expression data (Zhou et al., 2007). The extent and complexity of the pluripotency transcriptional network will continue to expand as the understanding of ES cells improves and new tools for their study become available (Orkin et al., 2008).
Figure 1.8. The core embryonic stem cell transcriptional circuit. The homeodomain transcription factors OCT4 (also known as POU5F1) and NANOG, as well as SRY box-containing factor 2 (SOX2), form a transcriptional module that has a central role in maintaining ES cell identity both in mice and humans. The three transcription factors regulate their own and each other’s expression in a highly coordinated manner, involving positive protein–protein and protein–DNA feedback loop interactions. Furthermore, they co-occupy and promote transcription of numerous self-renewal and pluripotency genes and repress the expression of the genes involved in lineage commitment. (Adapted from Macarthur et al., 2009).

1.2.3.3 Epigenetic control of pluripotency

Embryonic stem cells have specific chromatin features that reflect a transcriptionally permissive state. Accordingly, the interaction between chromatin and its structural proteins, such as histone H2B, histone H3 and heterochromatin protein 1 (HP1) is more dynamic in ES cells and therefore their chromatin is more plastic (Meshorer et al., 2006). Additionally, it was shown that chromatin-remodelling proteins are overexpressed in ES cells. These proteins are important to maintain chromatin in an open state and contribute to global transcriptional activity (Efroni et al., 2008).
An important level of gene regulation in ES cells is DNA methylation. Promoters of pluripotency-associated genes are usually hypomethylated in ES cells but hypermethylated and silent in differentiated cells (Farthing et al., 2008). Post-translational modifications of histones can also dictate chromatin structure and transcriptional competence. Tri-methylation of histone H3 on lysine 4 (H3K4me3) is usually associated with transcriptional active chromatin, whereas the tri-methylation of lysine 27 (H3K27me3) with inactive heterochromatin. Interestingly, both modification marks are often found together in promoters of developmental genes constituting domains of “bivalent chromatin” (Azuara et al., 2006; Bernstein et al., 2006). Bivalent marks are thought to allow these genes to remain silent but primed for rapid activation in response to developmental signals. In addition, polycomb-group proteins (PcG) have a central role in regulating pluripotency. Members of the PRC2 or PRC1 bind to the promoters of many developmental genes that are involved in lineage-specification or in later developmental stages, many of them co-bound with Oct4, Sox2 and Nanog (Boyer et al., 2005; Boyer et al., 2006; Jorgensen et al., 2006; Lee et al., 2006b).

1.2.3.4. Extrinsic pluripotency regulators in mouse and human ES cells

Although many of the pluripotency pathways are conserved between mouse and human ES cells, the extrinsic pathways required for pluripotency differ between the two species. Mouse ES cells can be maintained in an undifferentiated state in culture with foetal calf serum supplemented with the leukaemia inhibitory factor (LIF), which activates the STAT3 signalling pathway (Niwa et al., 1998). In addition, signalling through bone morphogenic proteins (BMPs) together with LIF is sufficient for culturing undifferentiated mES cells in serum-free conditions (Ying et al., 2003). BMPs induce the expression of Id (inhibitor of differentiation) proteins through the Smad pathway. However, it is now apparent that self-renewal and pluripotency of
mES cells can be maintained without the addition of extrinsic growth factors, by simply blocking differentiation-inducing signals using inhibitors of the FGF receptors, ERK and GSK3 pathways (Ying et al., 2008).

The delay between the derivation of mouse ES cells in 1981 and of human ES cells in 1998 was primarily due to species-specific ES cell differences. The culture media supplemented with LIF and serum, conditions that allow the derivation of mouse ES cells, resulted only in differentiation of human pluripotent cell lines. It was later shown that hES cells do not require active LIF/STAT3 signalling (Daheron et al., 2004; Humphrey et al., 2004) or BMPs, which in this case promote trophectoderm differentiation (Gerami-Naini et al., 2004; Pera et al., 2004). Instead, FGF and TGFβ/Activin/Nodal signalling are of central importance. Therefore, it now appears largely to be a lucky coincidence that fibroblast feeder layers support both mouse and human ES cells, as the specific factors used to sustain mES cells do not support hES cells.

Basic FGF (bFGF) allows the clonal growth of hES cells on fibroblasts in the presence of a commercially available serum replacement (Amit et al., 2000). The mechanism by which bFGF exert its functions is incompletely known, although one of the effects is suppression of BMP signalling that would otherwise induce differentiation (Xu et al., 2005). Another study demonstrates that FGF is required for maintenance of human-ES-cell-derived fibroblast-like cells (hdFs), which in turn act as a supportive niche for hES cells via IGF-II production. Importantly, the authors show that IGF-II has a direct role in the survival and self-renewal of pluripotent human ES cells. (Bendall et al., 2007). hES cells can also be propagated in feeder-free conditions, conditioned media or serum replacement if a combination of Activin plus FGF2 is used (Beattie et al., 2005; Vallier et al., 2005). It has been argued that human ES cells resemble more closely mEpiS cells than other pluripotent mouse-derived cell types because they also require nodal or activin signals to maintain
pluripotency, whereas mES cells do not (Tesar et al., 2007). In this regard, it has been suggested that the pluripotent state can be classified in two different ways: ICM-like or “naïve” state, and epiblast-like or “primed” state (Hanna et al., 2010; Nichols and Smith, 2009). The first is characteristic of mouse ES cells, and EG cells while the second is typical of the pluripotent cells derived from the mouse epiblast (mEpiS cells) to which hES cells resemble.

Apart from the different extrinsic requirements for maintenance of pluripotency, human and mouse ES cells differ phenotypically. Human ES cells colonies are flatter and display sharper borders when compared with the rounded 3-D shaped mouse ES cells colonies (Pera et al., 2000). In addition, the stage-specific embryonic antigens (SSEA) subtypes and glycolipids present in the cell membrane used as stem cell markers differ between the two species. Mouse ES cells express SSEA-1 (stage-specific embryonic antigen), while human ES cells express other sub-types of stage-specific embryonic antigens (SSEA-3 and SSEA-4) and display other antigens such as the proteoglycans TRA-1–60 and TRA-1–81 (Draper et al., 2002).

1.2.4 Somatic cell reprogramming

The term "cell reprogramming" is used to describe functional and molecular alterations that underlie changes in cell fate. Although most often used in the context of reprogramming adult somatic cells to pluripotent stem cells, reprogramming also includes the conversion of one differentiated cell type into another in a process called “transdifferentiation” or “lineage conversion”. A type of reprogramming that in the “Waddington landscape” can be represented as moving the marble across valleys (Figure 1.9). In the present work, reprogramming refers to the events that lead to a gain in developmental potential, such as induction of pluripotency in somatic cells.
Figure 1.9. Developmental potential illustrated by the Waddington’s epigenetic landscape. Cell populations with different developmental potential are represented as coloured marbles (purple, totipotent; blue, pluripotent; red, multipotent; green, unipotent). Developmental restrictions can be illustrated as marbles rolling down a landscape into one of several valleys. Reprogramming processes are represented as dashed lines. The marble can move uphill (dedifferentiation, gain in developmental potential) or across valleys (transdifferentiation). ICM/ES cells, embryonic stem cells derived from the inner cell mass of the blastocyst; EG cells embryonic germ cells, EC cells embryonic carcinoma cells, mGS cells, multipotent germ cells; iPS cells, induced pluripotent stem cells. (Adapted from Hochedlinger and Plath, 2009).

1.2.4.1 Reversing Differentiation: Why and how?

The derivation of embryonic stem cells ignited an explosion of scientific interest offering unique opportunities for research and disease treatment. The goal is to differentiate these cells in vitro and isolate highly specialised progeny for the purpose of transplantation. However, since tissues or organ transplantation between genetically distinct individuals provoke an immune reaction that can result in graft rejection, the ultimate goal of regenerative medicine is to produce genetically equivalent material, circumventing the risk of rejection or the need for immunosuppressive drugs. In other words the creation of patient-specific pluripotent stem cell lines. In addition, having access to human pluripotent cells bearing mutations found in hereditary diseases would also be an important resource to science and medicine. Three methods have been described that are able to
reprogram the nucleus of a somatic cell to a pluripotent state: somatic cell nuclear transfer (SCNT), cell fusion and transcription factor-induced reprogramming (also referred to as direct cell reprogramming; Figure 1.10).

**Somatic Nuclear Transfer (SCNT)**

As mentioned previously, somatic cell nuclear transfer (SCNT) was developed to assess whether the nuclei of differentiated cells remain equivalent to the nucleus of pluripotent cells and was later used for cloning of several mammalian species (Eggan et al., 2004; Gurdon and Byrne, 2003; Hochedlinger and Jaenisch, 2002; Inoue et al., 2005; Wilmut et al., 1997). However the procedure is technically challenging, inefficient and dependent on the donation of a large number of unfertilized oocytes. Besides, although Byrne and colleagues have successfully derived nuclear-transfer ES cells from primates by improving the SCNT protocol (Byrne et al., 2007), the same was never achieved with human cells. Importantly, even if these technical barriers are one day overcome, this technique faces strong ethical concerns in relation to generation of human embryos exclusively for ES cells production.

**Cell Fusion**

Another method primarily used for the study of the developmental capacity of pluripotent cell lines is cell fusion. Fusion of embryonic carcinoma (EC) cells with somatic cells generates hybrid cells that acquire a stem cell molecular signature and are able to form teratomas in nude mice (Miller and Ruddle, 1976). Similar results can be obtained by fusion with mES cells (Tada et al., 2001) and hES cells (Cowan et al., 2005; Yu et al., 2006). However these hybrid cells are tetraploid, and therefore genetically unstable and still immunologically incompatible. Nevertheless this method
has remained useful as a tool for investigating the mechanisms of cell plasticity and nuclear reprogramming. Importantly, this method has limited applications if DNA replication and cell division are required for complete reprogramming.

Figure 1.10. Three strategies for induction of nuclear reprogramming of somatic cells. Nuclear transfer involves the injection of a somatic nucleus into an enucleated oocyte. From this oocyte a blastocyst is generated from which ES cells can be derived or if development is allowed, give rise to a cloned organism. Cell fusion of somatic cells with ES cells results in the generation of tetraploid multinucleated heterokaryons that quickly acquire features of ES cells. If the fused cells proliferate they will become hybrids that can be euploid (balanced set of chromosomes, 4n) or aneuploid (chromosomes are lost and rearranged) if cells belong to different species. Transcription-factor transduction refers to the generation of induced pluripotent stem (iPS) cells by the ectopic expression of four reprogramming factors (Oct4, Sox2, Klf4 and c-Myc). iPS cells are similar to ES cells in morphology and expression patterns, can self-renew and have pluripotent potential. (Adapted from Yamanaka and Blau, 2010).
Transcription factor-induced reprogramming

The fact that somatic cells can be reprogrammed by nuclear transfer and cell fusion, highlights the presence of factors in the unfertilised oocyte and in the ES cells that are able to induce pluripotency. Based on this, Yamanaka and colleagues selected a group of 24 candidates because they have been implicated in the maintenance of pluripotency (Oct4, Sox2, Nanog) or because they were genes implicated in maintenance and rapid proliferation of mES cells in culture (Stat3, Klf4, β-catenin, E-Ras, c-Myc). To test their ability to induce pluripotency, they expressed these factors by retroviral transduction in mouse embryonic fibroblasts (MEFs) carrying a fusion of the genes encoding β-galactosidase and neomycin resistance expressed from the Fbx15 locus (specifically expressed is ES cells but not required for pluripotency). When MEFs were infected with all 24 factors and cultured on feeders in ES cells media neomycin-resistant, colonies emerged. In a process of step-wise elimination, four transcription factors, Oct4, Sox2, Klf4 and c-Myc, were identified as sufficient to give rise to ES-like colonies that were termed induced pluripotent stem (iPS) cells (Takahashi and Yamanaka, 2006). These first iPS cells were not identical to ES cells since they had different gene expression and DNA methylation patterns, and generated abnormal chimeric embryos. However it was later proven by Yamanaka’s group, as well as by others, that iPS cells capable of germline transmission could be generated (Maherali et al., 2007; Meissner et al., 2007; Okita et al., 2007; Wernig et al., 2007). The reprogramming by defined factors or “direct reprogramming” and its end product, the induced pluripotent stem (iPS) cells soon captivated the attention of the scientific community. Not only would direct reprogramming enable the relatively easy generation of patient-specific pluripotent stem cells, but also it would circumvent the ethical issues associated with the use of human embryos as source for derivation of hES cell lines. In addition, it would be a useful tool to dissect the mechanisms underlying pluripotency.
The generation of iPS cells from human cells was achieved a year later by two research groups (Takahashi et al., 2007; Yu et al., 2007). While Yamanaka’s group used the same combination of 4 factors, Thomson’s group substitute \textit{Klf4} and \textit{c-Myc} with \textit{Nanog} and \textit{Lin-28}. This suggested that the pluripotency network could be induced using different protocols. In the same line cells types other than fibroblasts, were successively reprogrammed to human iPS cells. These included skin keratinocytes (Aasen et al., 2008) neural stem cells (Kim et al., 2008) and umbilical cord blood cells (Giorgetti et al., 2009; Haase et al., 2009). One important achievement was the generation of iPS cells from peripheral blood cells (Loh et al., 2009; Seki et al., 2010; Staerk et al., 2010). This will enable generation of patient-specific iPS cells without the need for invasive skin biopsy, and subsequent time required for fibroblast expansion, bringing iPS cells a step closer to the clinic.

1.2.4.2. Mechanisms underlying reprogramming to iPS cells

Besides their therapeutic implications, iPS cells have been used as tool to understand the pluripotent state and to identify their regulators. However, direct reprogramming is a complex process that remains largely elusive. For example, the individual role of each reprogramming factor is not completely understood. In addition, reprogramming efficiencies are very low varying between 0.01% and 0.5% when using retroviral vectors (reviewed in Amabile and Meissner, 2009). To cap it all, reprogramming is a slow process, taking 2-3 weeks by retroviral delivery, and 10-12 weeks using recombinant proteins. In contrast, somatic nuclear transfer, in which 45-50% of somatic nuclei consistently undergo reprogramming, occurs in approximately 3.5 days (Markoulaki et al., 2008). Fusion of somatic cells with pluripotent cells is the fastest method, with pluripotency markers being detected within 24-48h (Han et al., 2008).
The fact that direct reprogramming to pluripotency is such an inefficient process suggests that several barriers hinder the process; one of the obstacles might involve technical problems such as efficient delivery and optimal stoichiometry of reprogramming factors. This issue was addressed by studies in which "secondary iPS" cells were created. Secondary iPS cells are generated from iPS cell-derived differentiated cells (fibroblast-like cells obtained by in vitro differentiation or in the case of mouse cells by the generation of chimeric mice) that harbour doxycyclin inducible transgenes. (Maherali et al., 2008; Wernig et al., 2008). Despite the fact that viral transgenes are reactivated in most of these iPS cell-derived differentiated cells, and that there is a 50 to 100-fold increase in the reprogramming efficiency, the frequency of reprogramming remains quite low (1% to 3%) (Maherali et al., 2008). This suggests that several biological roadblocks exist and that only a small percentage of cells can slowly overcome them. Two models were proposed to explain these observations.

1.2.4.2.1. Elite versus stochastic model

In the “elite” or deterministic model only some cells in the whole population of somatic cells have the potential to be reprogrammed. This could be due to the existence of somatic stem or progenitor cells in the explanted cell population that would be susceptible to reprogram. In the “stochastic” model all cells in the population are equally amenable to be reprogrammed. However, they have to go through a series of stochastic events to acquire pluripotency (Yamanaka, 2009).

Several studies have proved that a deterministic scenario is unlikely. For example, iPS cells have been derived from different somatic cells types including terminally differentiated somatic cells such as B and T lymphocytes (Hanna et al., 2008). In addition, in one study the monitoring of clonal populations of B cells and monocytes expressing the reprogramming factors, has shown that practically all
somatic cells can give rise to iPS cells, although some may require long times in culture (Hanna et al., 2009).

Importantly, different cell types are reprogrammed with different efficiencies. Somatic stem cells such as haematopoietic stem cells can be reprogrammed quicker and with higher efficiency than cells from them derived such as lymphocytes (10-40% compared with 0.01-1%) (Eminli et al., 2009). Therefore, a combination, of both models most accurately explains the reprogramming kinetics. A series of stochastic events are necessary and sufficient to reprogram any cell type, however some cells, such as adult progenitor and stem cells require fewer of these random events than terminally differentiated cells.

1.2.4.2.2. Sequential events during reprogramming to pluripotency

To understand the impediments and mechanisms of direct reprogramming we have to understand how reprogramming works. Studies in mouse fibroblasts revealed that it begins with the downregulation of somatic markers followed by activation of early pluripotency markers such as SSEA-1 and alkaline phosphatase. A mesenchymal-to-epithelial transition (MET) takes place early during reprogramming of mouse or human fibroblasts to iPS, and TGF-β inhibition promotes this transition (Li et al., 2010; Samavarchi-Tehrani et al., 2010). Endogenous Nanog and Oct4 activation occur later in the process and are usually associated with a fully reprogrammed state (Brambrink et al., 2008; Stadtfeld et al., 2008a) (Figure 1.11.). Underlying this sequence of events, is a genome-wide remodelling of the chromatin to an ES-like state. In differentiated cells many developmental and differentiation-associated genes are active, showing low DNA methylation and enrichment for H3K4me3. During reprogramming chromatin remodelling is necessary for silencing
Figure 1.11. **Landmark events during reprogramming to pluripotency.** When transduced with the reprogramming factors, fibroblasts begin to show alterations in their expression pattern. One of the first events is down-regulation of fibroblast-specific genes. Another early event is the down-regulation of mesenchymal genes, followed by up-regulation of epithelial genes (as a more epithelial nature is essential for successful reprogramming to pluripotency). To acquire a cell cycle profile characteristic of stem cells, down-regulation of tumour suppressor pathways such as the ones involving p16\(^{ink4a}\)/RB and p53/p21\(^{cip1}\) has to take place. Expression of ESC-specific genes such as the one responsible alkaline phosphatase activity, and several membrane markers like SSEA1, are the first to be activated. Pluripotency genes such as Nanog and Oct4, are activated later in the process and are associated with a fully reprogrammed state.

Of lineage-associated genes by establishing repressive epigenetic marks such as H3K27me3, and for some genes the bivalent chromatin structure characteristic of ES cells (Mikkelsen et al., 2008). On the other hand, in differentiated cells early developmental genes are silence by Polycomb-mediated H3K27me3 and all pluripotency-associated genes are hypermethylated. Therefore re-establishment of H3K4me3 active marks and loss of DNA methylation in ES-associated genes has to occur to allow the establishment of an ES-like expression program.

Besides changes in chromatin structure and alteration gene expression, reprogramming involves the acquisition of an ES-like cell cycle regulation and immortality (see section 1.3). Although the timing of these changes is not clear, the fact that partially reprogrammed cells cease proliferation upon doxycyclin redrawal
during secondary iPS reprogramming, suggests that this is a late event (Figure 1.11).

1.2.4.3. Challenges and advances in the generation of iPS cells

Although extremely promising, there are still several obstacles to overcome before direct reprogramming to pluripotency can be applied for clinical use. Several research groups have been working in improving the process, centred in two main objectives: the production of safer iPS cells free of viral integrations, and the improvement of reprogramming efficiency.

1.2.4.3.1. Choice and delivery of reprogramming factors

Although the four factor reprogramming is effective in different cells types and in different species, the endogenous expression of certain reprogramming factors has permitted their exclusion from the four factors cocktail. For example, c-Myc is not necessary to reprogram mouse and human fibroblasts. However, in the absence of c-Myc it takes longer to obtain reprogrammed colonies and the efficiency of the process is much lower (Nakagawa et al., 2008; Wernig et al., 2007). Neural progenitors cells, which express high levels of Sox2 and c-Myc, can be reprogrammed using only Oct4 and Klf4 or Oct4 and c-Myc (Kim et al., 2008). Indeed, Oct4 alone is sufficient to directly reprogram mouse neural stem cells to pluripotency (Kim et al., 2009).

The presence of multiple retroviral integration sites in individual iPS cell clones prohibits their clinical use due to the risk of oncogenic mutations. In addition, while retroviral transgenes are usually silenced by DNA and histone methyltransferases towards the end of the reprogramming process (Lei et al., 1996; Matsui et al., 2010; Stadtfeld et al., 2008a), incomplete silencing can lead to partially reprogrammed cells that fail to activate endogenous expression of pluripotency factors and
depended on the expression of exogenous factors (Mikkelsen et al., 2008; Takahashi and Yamanaka, 2006). Moreover, residual activity or reactivation of viral transgenes in somatic cells derived from iPS cell lines, can lead to defective developmental potential and formation of tumours in chimeric animals (Takahashi and Yamanaka, 2006) (Okita et al., 2007).

Although retroviral vectors are still widely used for factor delivery, if iPS cells are to be use in a therapeutic setting, the creation of integration-free iPS cells will be imperative. Several approaches have been devised to avoid the use of vectors that require integration; unfortunately they are usually associated with low reprogramming efficiencies. The first integration-free iPS cells were generated from mouse hepatocytes using adenoviral vectors (Stadtfeld et al., 2008b), and from MEFs by plasmid transfection (Okita et al., 2008). Importantly transient expression of the four reprogramming factors was sufficient to generate iPS cells supporting the notion that insertional mutagenesis does not play a role in iPS cell generation. Human integration-free iPS cells were later generated by adenoviral infection (Zhou and Freed, 2009), transfection with self-replicating episomal vectors (Yu et al., 2009) or with polycistronic minicircle vectors (Jia et al., 2010).

Due to the low reprogramming efficiency when using non-integrating vectors, some research groups have developed retroviral vectors with loxP sites to allow viral excision from the host genome by transient expression of Cre Recombinase (Kaji et al., 2009; Soldner et al., 2009). This enables the expression of reprogramming factors for longer lengths of time, until reprogramming is complete. In a similar way, two laboratories used piggyBac transposons that can be shuttled in and out of the host genome by transient expression of transposase (Woltjen et al., 2009; Yusa et al., 2009). An alternative for generation of integration-free iPS cells is the delivery of the reprogramming factors as purified proteins (Zhou et al., 2009). Although this is a very attractive approach, the reprogramming efficiency obtained by this method is
particularly low. More recently it was described that iPS could be obtained by administration of synthetic mRNA modified to overcome innate antiviral responses. Importantly the efficiency of this protocol greatly surpasses previously established methods (Warren et al., 2010).

1.2.4.3.2. Increasing reprogramming efficiency: bypassing biological roadblocks

The low efficiency of reprogramming imposes limitations to mechanistic studies and iPS cell potential clinical translation. Therefore great effort has been put into finding candidate genes or molecules that could replace one of the reprogramming factors or interfere with specific pathways or biological processes. Interestingly the quest for enhancing reprogramming has also provided clues on how reprogramming works.

Functional screens will continue to yield new reprogramming factors that can enhance four factors reprogramming or substitute some of the reprogramming factors. One example is the nuclear receptor Nr5a2, which was shown to enhance reprogramming of mouse fibroblasts and to replace Oct4 (Heng et al., 2010). Schöler’s laboratory have devised a functional and quantitative proteomics screen to identify proteins and complexes that contribute to reprogramming, by identifying proteins that are enriched in reprogramming-competent fractions of pluripotent cell extracts. In this way they found that the pluripotent-enriched components of the SWI/SNF complex (or BAF complex) such as Brg1 and BAF155 could significantly increase reprogramming efficiency when used together with the four factors or three factors (excluding c-Myc). A mechanistic insight into BAF complex function revealed that it contributes to a euchromatic chromatin state and enhance binding of reprogramming factors onto promoters of key pluripotency genes, thereby enhancing reprogramming (Singhal et al., 2010).
All these studies, and many other which are not mention here due to space restrictions, have provided useful insights into the mechanisms underlying reprogramming to pluripotency. However the ideal scenario would be to generate iPS cells using fewer factors or without disturbing the host genome. Understanding the events involved in reprogramming will allow us to find which pathways can be modulated using small molecules to increase the efficiency of methods in which integration – free iPS cells are created. In fact, some chemicals have been reported to either enhance reprogramming efficiency or to substitute for some reprogramming factors. Some of these compounds affect chromatin modifications such as the inhibitors of DNA methyltransferases or the histone deacetylase inhibitors while others influence signal transduction pathways.

Proper epigenetic remodelling has proved to be a main roadblock to successful reprogramming. Indeed, during iPS reprogramming a population of cells gets trapped in partially reprogrammed state due to the existence of reprogramming barriers such as epigenetic remodelling. Meissner and colleagues compared the expression patterns of fully reprogrammed and partially reprogrammed cells and discovered that partially reprogrammed cell lines reactivate a distinctive subset of stem-cell-related genes but show incomplete repression of lineage-specifying transcription factors, and DNA hypermethylation at pluripotency-related loci. To overcome the inefficient step of DNA de-methylation, they treated partially reprogrammed cells with 5-aza-cytidine (AZA) and were able to promote their complete reprogramming. Accordingly, addition of this DNMT inhibitor enhances the reprogramming efficiency of mouse fibroblasts by 4-fold (Mikkelsen et al., 2008).

In addition to inhibitors of DNA methylation, several inhibitors of histone post-translational alterations have been identified such as histone deacetylase (HDAC) inhibitors, especially valproic acid (VPA), which have been shown to noticeably increase reprogramming efficiency in MEFs (100-fold for OSK and 50-fold for OSKM)
and in human fibroblasts (20-fold) (Huangfu et al., 2008). Valproic acid is thought to promote a genome wide acetylation that permit somatic cells to adopt a relaxed chromatin structure (Huangfu et al., 2008). In a chemical compound screen, an inhibitor of G9a histone methyltransferase BIX-01294 was found to improve reprogramming of mouse neural progenitor cells transduced with Oct4 and Klf4 (8-fold) (Shi et al., 2008b). BIX-01294 enhances reprogramming of MEFs but only in combination with, a DNA methyltransferase inhibitor (RG108) and a L-calcium channel agonist BayK8644 (Shi et al., 2008a).

Modulation of signalling pathways have also provided ways of improving reprogramming. Inhibition of MEK and GSK3 can bypass the requirement of LIF and BMP signalling in the maintenance of mouse ES cells. This is important to block lineage commitment induced by ERK signalling thereby maintaining the ground state of pluripotency (Burdon et al., 1999; Ying et al., 2008). Inhibition of GSK3, on the other hand, promotes mESCs propagation (Sato et al., 2004). Smith and colleagues used mouse neural stem cells with Oct4 driven GFP and observed that although a considerable percentage of cells acquired stem cell morphology early in the process, only 2% were GFP positive. These pre-iPS colonies presented ES cell-like morphology, showed down-regulation of somatic markers but incomplete activation of pluripotency genes. The use of MEK and GSK3 inhibitors together with LIF (2i/LIF) promoted the transition from pre-iPS cells to fully reprogrammed iPS cells (Silva et al., 2008). Another way to modulate signalling pathways is the addition of recombinant proteins, when Jaenisch and colleagues activated Wnt signaling by addition of recombinant Wnt3a, they observed an improvement in reprogramming to iPS cells. Additionally, they were able to reprogram human cells in the absence of c-Myc or with only two factors, Oct4 and Klf4 (Marson et al., 2008).

Several research groups have performed screenings to find signalling pathways modulators that can replace some of the reprogramming factors. In a high-
content chemical screening to identify small molecules that can replace Sox2 in reprogramming, Eggan and colleagues identified a molecule that functions in reprogramming by inhibiting TGF-β signalling and promotes the complete reprogramming of mouse partially reprogrammed cells by inducing Nanog (Ichida et al., 2009). The same was observed in human cells however in this case in conjunction with inhibition of mitotic activated protein kinase (MAPK) pathway (Lin et al., 2009). Although inhibition of TGF-β signalling improves reprogramming by inducing Nanog in mouse cells, it may also promote the mesenchymal-to-epithelial transition, which is required in the early stages of reprogramming of mouse and human fibroblasts to iPS cells (Li et al., 2010; Samavarchi-Tehrani et al., 2010).

Given the fact that one of the sequential events during reprogramming is the acquisition of immortality, some groups have interfered with senescence and apoptosis pathways to promote reprogramming. Indeed, one of the first studies describing reprogramming of human fibroblasts (usually more difficult to reprogram than mouse cells) required addition of SV40 large T (SV40 LT) and/or the catalytic subunit of telomerase (hTERT) (Park et al., 2008b). In another study siRNAs against p53 and Utf1 was shown to increase reprogramming efficiency of human cells by 100-fold (Zhao et al. 2008). Although there was the notion that interfering with the p53 pathway could improve reprogramming by promoting cell survival and inducing rapid proliferation the exact mechanism by which p53 limits reprogramming was unclear.

Recent work has highlighted the relation between tumour suppressor pathways inactivation and reprogramming to iPS cells, which will be discussed in this work.
1.3. The unlimited lifespan of embryonic stem cells

1.3.1. ES cells do not undergo replicative senescence

One of the more remarkable features of ES cells is that they can self-renew in culture indefinitely, and do not undergo senescence like immortalized cells and transformed cells, yet they are not transformed. For example, hES cells have been shown to continuously grow in culture for two years, which is equivalent to about 500 doubling times (Carpenter et al., 2004; Rosler et al., 2004). Importantly the absence of replicative senescence is not due to acquisition of genotypic abnormalities in culture as hES cells grown continuously in culture show a normal karyotype and have stable genomes (Maitra et al., 2005).

This implies that the pathways responsible for senescence onset are regulated differently in ES cells. Indeed, gene expression profiling identified key differences in cell cycle regulation, regulation of telomeric proteins expression and DNA repair in hES cells (Miura et al., 2004) and in mES cells (Ginis et al., 2004).

1.3.1.1. Embryonic stem cells exhibit telomerase activity

As mentioned before most human cancers contain high levels of telomerase activity underscoring the importance of telomere maintenance in cellular immortality (Blasco, 2005). Introduction of the catalytic sub-unit of telomerase (hTERT) into normal, pre-crisis fibroblasts results in telomerase activity, telomere maintenance, and cell immortalization (Bodnar et al., 1998) but does not by itself result in transformation (Jiang et al., 1999).

In contrast to most somatic cells, stem, germ and tumor cells have high telomerase activity through TERT transcriptional up-regulation (Thomson et al., 1998) and several telomeric proteins such as telomere-associated protein Rif
homologue (RIF) (Brandenberger et al., 2004; Miura et al., 2004). Furthermore, telomerase-deficient mouse ES cells cease to grow after 460–480 divisions, strongly suggesting that telomerase is essential for immortality of ES cells (Niida et al., 1998). Together these observations suggest that telomere maintenance is required for cellular immortality and is likely to play an important role in the immortality of hES cells.

Induced pluripotent stem cells also have telomerase activity (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). Furthermore it has been shown that telomeres are elongated in mouse iPS cells when compared to the parental differentiated cells, and acquire the epigenetic marks of ES cells (such as low density of trimethylated histones H3K9 and H4K20) (Marion et al., 2009b).

Human cells also display telomere elongation after reprogramming (Agarwal et al., 2010). Dyskeratosis congenita (DC), a disorder of telomere maintenance that results in tissue degeneration, is caused by mutations in the dyskerin gene (DKC1). This gene encodes an RNA binding protein whose inactivation destabilizes the levels of telomerase RNA component (TERC) (Mitchell et al., 1999). Surprisingly, fibroblasts from dyskeratosis congenita patients can be reprogrammed to iPS cells, but displayed poor reprogramming efficiency when compared to normal cells (Agarwal et al., 2010). These data show that reprogramming of somatic cells from patients with a genetic impairment in telomere elongation can overcome a critical limitation in telomerase RNA component (TERC) levels to restore telomere maintenance and self-renewal.

### 1.3.1.2. Cell cycle regulation in embryonic stem cells

Although some differences exist in the regulation of the cell cycle between human and mouse ES cells both are characterized by a shortened early G1 phase
(Becker et al., 2006; Savatier et al., 1994). The molecular regulation of the transition through G1 is less well defined in hES and primate ES than it is in mouse ES cells. Mouse ES cells growth in culture is extraordinarily rapid due to a short cell cycle (11–16 hours), owing to a reduction in the duration of G1 phase. While in somatic cells, including adult stem cells, CDK2 activity is periodic and peaks at the G1 to S transition, mouse ES cells have constitutive CDK2 activity independently of the cell cycle phase. Moreover, mouse ES cells express low levels of the D-type cyclins and have almost no detectable CDK4 activity (Savatier et al., 1994; White et al., 2005).

This type of regulation may be linked to cell fate decisions. Mitogen activated protein kinase (MAPK) signalling is important for proliferation of somatic cells, but is also a potent inducer of differentiation. Consequently a short G1 in mouse ES cells allows them to avoid the differentiation-inducing signals mediated by certain mitogenic signalling pathways that are usually active during early G1 in somatic cells (Orford and Scadden, 2008).

A different regulation of cell cycle checkpoints that normally operate in somatic cells allow for a shorter G1 phase in ES cells. Indeed, massive parallel signature sequencing (MPSS) analysis of hES cells showed that several cell cycle regulators such as p53, INK4a (encoding p16\textsuperscript{INK4a}), ARF and CDKN1a (encoding p21\textsuperscript{Cip1}), are not expressed or expressed at very low levels (Brandenberger et al., 2004; Miura et al., 2004). This is in contrast to what has been described in mES cells, which express high levels of p53. However, the p53-mediated response to cell cycle arrest in these cells seems to be inactive. One explanation for this observation is that translocation of p53 to the nucleus in mES cells is insufficient (Aladjem et al., 1998; Ginis et al., 2004; Prost et al., 1998). In addition, expression of MDM proteins, the negative regulators of p53, may also contribute to the inactivation of p53 in mES cells (Ginis et al., 2004). ES cells may have different mechanisms from those present in somatic cells to protect themselves from stress and to maintain a high.
level of genetic stability. mES cells are highly resistant to oxidative stress, and are very efficient in repairing DNA damage induced by radiation (Saretzki et al., 2004). In addition, mES cells are much more proficient at p53-independent apoptosis than differentiated cells (Aladjem et al., 1998; Corbet et al., 1999). Overall, these observations indicate that the absence (hES cells) or the inactivation (mES cells) of the p53 pathway is required for the absence of senescence in ES cells. Further studies have uncovered additional roles for p53 in ES cell biology and new cell cycle regulators, specific of ES cells such as miRNAs, which will be discuss in the following sections.

Figure 1.12. The cell cycle of embryonic stem cells. Mouse embryonic stem (ES) cells have a shorter cell cycle when compared to that of most somatic cells (11–16 hours as opposed to 24 hours). An abbreviated G1 phase is responsible for the difference in cell-cycle length. For differentiated cells, the transition through G1 phase requires the mitogen-induced accumulation of cyclin D, resulting in the hyperphosphorylation of the retinoblastoma tumour suppressor protein (RB) by cyclin dependent kinases (CDK4, CDK6 and CDK2). In ES cells CDK2-CyclinE (E/2) is constitutively active throughout the cell cycle, which allows the transition of ES cells from M phase directly to late G1, avoiding the cyclin D-dependent early G1 and shortening the G1 phase. Upon commitment of ES cells, the cell-cycle length is extended as CDK2-cyclinE activity comes under the control of CDK4,6-cyclinD (D/4,6) and phosphorylated RB. + refers to cyclin−CDK activity: +/-, negligible; +, low; ++, intermediate; ++++, high. (Adapted from Orford and Scadden, 2008)
1.4 Aim of this study

Upon expression of the Yamanaka factors, somatic cells undergo a number of changes that lead to acquisition of ES cell-like characteristics and regression to an undifferentiated state. The gain of embryonic stem cell properties during reprogramming to iPS cells involves acquisition of mechanisms to escape replicative senescence. Human embryonic stem cells express low levels of negative regulators of the cell cycle, and display telomerase activity. However, reprogramming efficiency is extremely low and the majority of the population does not acquire these ES cell-like properties. The aim of this study is to characterise the response of human somatic cells to expression of reprogramming factors to understand why only a small subset of the entire population of somatic cells acquire the immortal nature, characteristic of ES cells.
Chapter 2 - Material and Methods

2.1. Cell lines and tissue culture methods

IMR90 human foetal lung fibroblasts and BJ human foreskin fibroblasts were obtained from the American Type Culture Collection (ATCC). MEFs were prepared from 13.5-day-old embryos of CD1 mice. The head and viscera were removed and the remaining tissue was minced and a single cell suspension was obtained by trypsinization (0.1% (v/v)) (Gibco, UK) at 37°C for 15 min. Cells were then cultured until they became confluent and were then frozen in foetal bovine serum (FBS; Sigma) containing 10% dimethyl sulfoxide (DMSO; Sigma). MEFs knockout for p53 and Cdkn1a were a gift from Scott Lowe (Cold Spring Harbor Laboratory - CSHL). MEFs Oct4-GFP were kindly provided by Jose Silva (Wellcome Centre for Stem Cell Research, Cambridge). HEK293T packaging cells were purchased from the American Type Culture Collection (ATCC) and Takara BIO INC. All tissue culture reagents used were purchased from Invitrogen (Invitrogen Ltd, Paisley, UK), unless otherwise stated. IMR90, BJ, MEFs and packaging cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM, Gibco) supplemented with 10% (v/v) FBS and 1% (v/v) antibiotic-antimycotic (Gibco) and grown in humidity at 37°C with 5% CO₂. H7 hES cell line and hiPS cells were maintained in an undifferentiated state in mitotically inactivated MEFs (γ-irradiated; 40Gy) and plated onto 0.1% (w/v) gelatine-coated surfaces (Sigma). hES and iPS cells were maintained in KRS media: Knockout™ Dulbecco’s Modified Eagle Medium (DMEM) medium supplemented with 20% (v/v) Knockout™ Serum Replacement (KSR), 1 mM L-glutamine, non-essential amino acids (NEAA), 0.1 mM β-mercaptoethanol and antibiotics (50 U/ml Penicillin/Streptomycin) supplemented with 4 ng/ml basic fibroblast growth factor (FGF2, PeproTech EC Ltd.).
2.2. Retrovirus production and transduction of target cells

Packaging cells were seeded the previous day to reach a confluence of 70-80% the day of transfection. For each 10 cm plate, 1 ml of serum-free DMEM containing 20µg of expression plasmid and 10µg of helper plasmid was prepared. Ecotropic helper was used for infection of MEFs, amphotropic helper plasmid or a combination of gag-pol and VSV-g plasmids (8µg and 2µg respectively) was used for infection of human fibroblasts. As transfection reagent, 75 µL of linear 25kDa linear polyethylenimine (PEI 1mg/ml (w/v), Polysciences) was used. The mixture was incubated at room temperature for approximately 30 minutes and added drop-wise to each plate. Transfection efficiency was monitored the next day by the presence of GFP (GFP; green fluorescent protein) or Cherry fluorescent protein expressed from a reporter plasmid. The day after transfection, the media of the transduced packaging cells was replaced with fresh media (6ml/plate). In parallel, the target cells, MEFs or IMR90, were plated at a density of 1x10^6 cells per 10cm plate.

After 48hrs, the virus-containing supernatants were collected from packaging cells plates, filtered through 0.45µm pore-sized acetate filters (Anachem) and supplemented with 4µg/ml of polybrene (Sigma). The culture media of the target cells was then removed and replaced by the virus-containing supernatant. Infection took place in 1, 2 or 3 rounds to maximize the efficiency and a period of 2.5-3 hours was left between each round for virus production. Following overnight incubation, the medium was replaced (10ml/plate). The percentage of GFP-positive or Cherry-positive cells was determined by flow cytometry, using as a negative control uninfected cells.

Approximately three days after infection, cells were split and grown for 5-7 days in the presence of puromycin (0.5 µg/ml; Invitrogen) in order to select for transduced cells.
2. 3. Growth assays

2.3.1. Growth curves and colony formation assays

For growth curves 7 \times 10^3 cells were seeded in triplicate in 24-well plates. Cells were fixed on day 0 (day after plating), and after that every two days during a time course of 12 days. For fixation, cells were washed with PBS and fixed with 0.5% glutaraldehyde (w/v) (Sigma-Aldrich), in PBS for 20-30 minutes. All plates were stained simultaneously with 0.2% crystal violet (w/v). Crystal violet was extracted with 10% acetic acid (v/v) and the absorbance at 595 nm (A_{595}) was measured using the Bio-Rad 680XR microplate reader. To determine the growth curves absorbance values at different time points, relative to absorbance of Day 0 were plotted. For colony formation assays, cells were plated at a density of 1 x 10^5 and 5 x 10^4 per 10-cm dish. After approximately 15 days, cells were fixed with glutaraldehyde and stained with crystal violet.

2.3.2 BrdU (5-Bromo-2'-deoxyuridine) incorporation assay

Cells (1-4 x10^3) were plated in 96-well plates in duplicate or triplicates and subsequently incubated with 5-Bromo-2'-deoxyuridine (BrdU, 50 \mu M, Sigma) for 16 hr. After incubation, cells were washed with PBS and fixed for 20 min with 4% (w/v) paraformaldehyde (Sigma). For immunofluorescence staining, cells were first permeabilized for 10 minutes with 0.2% (v/v) Triton X-100 in PBS (Sigma) and incubated for 30 minutes with 1X blocking solution (0.5% (w/v) BSA, 0.2% (w/v) fish skin gelatin (Sigma)). Treatment with DNase I (0.5U/\mu L; Sigma) in presence of 1mM MgCl_2 (Sigma) was performed simultaneously with Alexa Fluor® 488 mouse anti-BrdU antibody (1:200, Invitrogen) incubation for 1h at room temperature. Cells were washed three times with PBS and incubated with DAPI (Invitrogen, 1.5 \mu M). Plates
were examined using In Cell Analyser and High Content Analysis was performed, to discriminate positive BrdU nuclei and total nuclei.

2.3.3. Senescence-associated $\beta$-galactosidase (SA-\$\beta$-gal) assay

Cells were washed once with PBS, fixed with 0.5% glutaraldehyde (w/v), and washed in PBS (pH 6.0) supplemented with 1 mM MgCl$_2$. Cells were stained in X-gal solution (1 mg/ml 5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside [Boehringer], 0.12 mM K$_3$Fe[CN]$_6$, 0.12 mM K$_4$Fe[CN]$_6$, 1 mM MgCl$_2$ in PBS at pH 6.0) overnight at 37°C. After staining, cells were washed with PBS and stored at 4 °C, in the dark. The cell nuclei were stained with 3 µM 4',6-diamidino-2-phenylindole (DAPI) for 30 min at RT. Bright field (BF) and DAPI images were taken using the Olympus CKX41 inverted fluorescence microscope, supplied with a DP20 digital camera. The percentage of SA-\$\beta$-Gal-positive cells was determined upon counting of at least 100 cells.

2.3.4. DNA content analysis

Cells were trypsinized and re-suspended in 200µl of cold PBS. Re-suspended cells were added drop wise to 4 ml of ice cold 70% Ethanol, under vortex for rapid dispersion. Fixation was performed overnight at -20°C. Cells were washed twice in PBS, re-suspended in PI solution (propidium iodide (PI) 40µg/ml, (Sigma); RNAse (100µg/ml (Invitrogen) in PBS) an incubated at 37°C for 30 min. PI stained cells were analysed using the Guava® microcapillary flow cytometry system (Millipore), and the Guava Cell Cycle Software.
2.4. Plasmid amplification and cloning to generate retroviral vectors

2.4.1. Plasmids

OSKM polycistronic cassette was sub-cloned from FUW-TetO-OSKM (Carey et al., 2009) (Addgene) into the EcoRI site in pBABE puro. Full-length cDNAs encoding mouse transcription factors Oct4 and Sox2 and Nanog were PCR-amplified from PMXs plasmids (Takahashi and Yamanaka, 2006) (Addgene) with primers containing BamHI and SalI sites for cloning into pBABE-puro retroviral vector. Retroviral vectors expressing c-Myc and CBX7 (Gil et al., 2004), as well as E6, E7, E6/E7, plasmids; HRAS V12, and plasmid for expression of a shRNA targeting p53 (pRS-shp53) have been described before (Acosta et al., 2008; Brummelkamp et al., 2002). pBABE-KLF4 was kindly provided by Gordon Peters (Cancer Research, UK). The retroviral plasmids used for direct reprogramming of human somatic cells were kindly provided by Ludovic Vallier (MRC Centre for Stem Cell Biology and Regenerative Medicine). pRetroSuper (pRS) plasmids expressing shRNAs against human and mouse Cdkn1a were generated as described in 2.4.4. Plasmids for knockdown of mouse p53 and Ink4a/Arf have been described before (Dickins et al., 2005). Retroviral plasmids for expression of shRNAs in pRetroSuper-Hygromycin (shRNAs) targeting Cdkn1a (p21Cip1), p53 and INK4a were subcloned from pRS-puro plasmids into EcoRI/Xhol restriction sites in pRS-Hygro. MSCV-blast hTR211 and MSCV-blast 302a-d were obtained from Geneservice. For a full list of retroviral vectors, see Table A1 and A2.

2.4.2 Transformation of chemically competent E. coli.

Chemically competent E. coli One Shot® TOP10 or DH5α™ cells (Invitrogen) were thawed on ice and gently mixed with 3 µL ligation product or 10–100 ng plasmid DNA. Following 30 min incubation on ice, cells were heat-shocked for 45
sec at 42 °C and returned to ice for 5 min. After adding 200 µL pre-warmed S.O.C. medium, cells were incubated at 37°C for one hour. The total volume or a dilution of each transformation was spread on a Lysogeny broth (LB) agar plate supplemented with the appropriate antibiotics. The plates were incubated overnight at 37 °C. Single colonies were picked, grown in LB medium containing the appropriate selective antibiotic and analysed by plasmid DNA isolation, polymerase chain reaction (PCR) or sequencing.

2.4.3 Plasmid DNA purification

Recombinant E. coli cultures were grown for 12–16 h at 37 °C, shaking at 150 rpm, in 250 mL LB medium containing the appropriate selective antibiotic. The cells were then pelleted by centrifugation at 6000 × g for 10 min at 4 °C. Large-scale plasmid DNA preparation was carried out using the HiSpeed® Plasmid Purification kit (QIAGEN), based on a modified alkaline lysis procedure. Briefly, bacterial pellet was resuspended in P1 buffer (50 mM Tris-HCl, pH 8.0; 10 mM EDTA; 100 µg mL⁻¹ RNase A). Alkaline lysis buffer P2 (200 mM NaOH, 1% SDS (w/v)) was added to the cells and gently mixed. Buffer P3 (3.0 M KAc, pH 5.5) was added for neutralization. Upon gently mixing, a precipitate formed by proteins, genomic DNA, and detergent is generated. The soluble fraction, containing the plasmid DNA, was applied to an anion-exchange resin under appropriate low-salt and pH conditions. Buffer QC (1.0 M NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol (v/v)) was added to the resin to remove RNA, proteins, and impurities. Plasmid DNA was eluted from the column in a high-salt buffer QF (1.25 M NaCl; 50 mM Tris-HCl, pH 8.5; 15% isopropanol (v/v)) and then desalted by isopropanol precipitation. Plasmid DNA was concentrated and eluted with 500 µL of buffer TE (10 mM Tris-HCl, pH 8.0; 1 mM EDTA). The concentration was determined by measuring the absorbance at 260 nm (A₂₆₀) in a NanoDrop® ND-1000 UV-Vis spectrophotometer.
2.4.4. Generation of shRNA expressing plasmids

Retroviral plasmids for knockdown of human and mouse Cdkn1a were constructed by subcloning oligonucleotides in pRetroSuper (pRS), as previously described (Brummelkamp et al., 2002). Briefly, the BIOPREDsi (www.biopredsi.org/) algorithm was used to computationally predict shRNA target sequences with an optimal knockdown effect for human or mouse Cdkn1a. Three target sequences were used to design and test independent shRNA constructs. For cloning into pRS vectors, the forward and reverse strands of the oligonucleotides were annealed in 100 mM NaCl and 50 mM HEPES, pH 7.4. To achieve proper annealing, the mixture was sequentially incubated in decreasing temperatures (4 min at 90 °C, then for 10 min at 70 °C and slowly cooled to 10 °C or room temperature). The annealed oligos were cloned into BglII and HindIII sites in the pRS vector using T4 DNA ligase (NEB). One Shot® TOP10 competent cells were transformed and plated on LB Amp agar plates. Positive clones were identified by colony PCR, using primers pRS-F (5’–ACCTCCTCGTTGACCC) and pRS-R (5’–TGTGAGGGACAGGGGAG). The PCR products were resolved on a 2.5% (w/v) agarose gel (see Agarose gel electrophoresis) and compared with a negative (empty pRS vector) and positive (previously cloned shRNA) controls. A list of the sense shRNA target sequences is shown in Table A3.

2.4.5. Agarose gel electrophoresis.

For sub-cloning cDNAs, restriction enzyme-digested DNA and PCR products (for cloning primers see Table A4) were analysed by their electrophoretic mobility on an agarose gel. DNA loading buffer Orange G (0.2% (w/v) in 3% glycerol (v/v)) was added to the samples, which were then resolved on a 1–2.5% agarose (w/v) gel with 0.5 µg mL⁻¹ ethidium bromide. Samples were separated at 100 V in TAE buffer (40 mM Tris and 1 mM EDTA (pH 8.0) in 0.1% HAc (v/v)). For DNA visualization, the gel
was exposed to ultraviolet light using the Bio-Rad Gel Doc™ 2000 system. Image analysis was carried out using the Quantity One® (v4.4.1) software (Bio-Rad). The size of the DNA fragments was estimated by using a DNA ladder as a reference.

For extraction of digested DNA or PCR products from agarose gels, the QIAquick Gel Extraction kit (QIAGEN) was used. The DNA fragment was excised from the agarose gel and 3 volumes of buffer QG were added. The gel was dissolved for 10 min at 50 °C and 1 volume of isopropanol added to it next. The sample was then applied to a QIAquick spin column where DNA bound to the silica membrane and contaminants were washed away using buffer PE. DNA was eluted in 30–50 µL buffer EB (10 mM Tris-HCl, pH 8.5). All steps were performed by centrifugation of columns at 8000 × g in a microcentrifuge.

2.4.6. TA cloning

PCR products amplified by Taq polymerase have a single deoxyadenosine (A) at 3’ ends due to Taq’s non-template-dependent terminal transferase activity. This can be used to clone the product into a plasmid vector that has a single overhanging 3’ deoxythymidine (T). For cloning into the pCR 2.1-TOPO® vector (Invitrogen), 2µl of PCR product was mixed with 1µl of Salt Solution, 1µl of pCR 2.1-TOPO® vector and 2µl of water in a final volume of 6µl. The mixture was incubated at room temperature for 10-30 min. Then 2µl of this ligation mixture was transformed into chemically competent E. coli cells as described in 2.4.2.

2.4.7. DNA sequencing.

DNA sequencing was carried out by the MRC Clinical Sciences Centre Genomics Core Laboratory using an automated ABI3730xl DNA analyser (Applied Biosystems). Sequences were viewed using the DNA Strider (v1.4f2) application. A list of sequencing primers is shown in Table A5.
2.5. RNA expression analysis

2.5.1. Total RNA purification

Total RNA was extracted using the RNeasy minikit (QIAGEN) or Trizol reagent if analysis of microRNAs was required. For extraction of RNA using the RNeasy Mini kit (QIAGEN), up to $5 \times 10^6$ cells were harvested and pelleted by centrifugation at 300 $\times$ g for 5 min. The supernatant was aspirated and the cells lysed with guanidine thiocyanate-containing buffer RLT. The lysate was homogenised by centrifuging through a QIAshredder spin column and then 70% ethanol ($v/v$) was added and mixed well by pipetting. The sample was then applied to an RNeasy Mini spin column where total RNA bound to the silica membrane and contaminants were washed away using buffers RW1 and RPE. In order to remove residual DNA, an on-column DNase I digestion was carried out using the RNAse-free DNase set (Qiagen). RNA was eluted in 30–50 $\mu$L RNase-free water. Wash and elution steps were performed by centrifugation at 8000 $\times$ g in a microcentrifuge. The concentration was determined by measuring the absorbance at 260 nm ($A_{260}$) in a NanoDrop® ND-1000 UV-Vis spectrophotometer. The RNA was stored at $-80$ °C for future applications.

2.5.2. Quantitative RT-PCR (RT-qPCR) analysis.

Reverse transcription (RT) and PCR were carried out separately (two-step RT-PCR). In the RT step, 1 $\mu$g RNA was reverse transcribed into cDNA. RNA, diluted in water up to 11 $\mu$L, and oligo (dT)$_{18}$ primer (5 $\mu$M final concentration) were mixed, incubated at 100 °C for 3 min and then chilled on ice for 10 min. First Strand Buffer (FSB), deoxyribonucleotide mix (dNTP; 500 $\mu$M final concentration each), dithiothreitol (DTT; 10 mM final concentration) and 200 U SuperScript™ II reverse
transcriptase (Invitrogen) were added to each reaction. The RT was carried out at 42 °C for 50 min, after which the enzyme was inactivated at 70 °C for 15 min.

RT-qPCR reactions were performed using Opticon 2 (Bio-Rad) using SYBR Green PCR Master Mix (Applied Biosystems) (40 cycles; 95 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s). Primers for RT-qPCR were described before (Barradas et al., 2009; Pereira et al., 2008) or were designed using Primer3 (v0.4.0) software (http://frodo.wi.mit.edu/primer3/) and used at 250 nM final concentration each. To avoid amplification of genomic DNA, the RT-qPCR primers were designed to span an exon-exon junction and controls without reverse transcriptase were included. The TaqMan® Gene Expression Assays (Applied Biosystems) consisted of two unlabelled primers for amplifying the sequence of interest (900 nM final concentration) and a TaqMan® probe, labelled with 6-carboxyfluorescein (6-FAM™), for detecting the sequence of interest (250 nM final concentration). TaqMan PCR was carried out in Opticon 2 (Bio-Rad) using TaqMan® Universal PCR Master Mix (Applied Biosystems, 40 cycles; 95 °C for 15 s, 60 °C for 60 s). Gene expression data was normalised to the ribosomal protein S14 (RPS14) for RT-qPCR and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for TaqMan PCR. The comparative C_T method (ΔΔC_T) was used to quantify differences in the expression level of target genes between different samples. The messenger RNA (mRNA) level of the target gene, normalised to an endogenous reference (housekeeping gene) and relative to a calibrator (empty vector control), was calculated using the following formula: $2^{-\Delta \Delta C_T}$, where $\Delta C_T = C_{T, \text{target}} - C_{T, \text{reference}}$ and $\Delta \Delta C_T = \Delta C_{T, \text{test sample}} - \Delta C_{T, \text{calibrator sample}}$. For a list of RT-qPCR primers and TaqMan® see Tables A6 and A7, respectively.

2.5.3. Purification and analysis of microRNAs expression

For extraction of microRNAs cells were lysed and total RNA was extracted using Trizol reagent (Invitrogen) to avoid the loss of small RNAs. Cell pellets were
first homogenised in 1ml of Trizol, followed by addition of chloroform (200µl). The Phenol-chloroform phase and the aqueous phases were separated by centrifugation (12 000 × g, 4°C, 20 min). Total RNA was precipitated from the aqueous phase using isopropyl alcohol (0.5ml, Sigma) and washed with 75% ethanol (v/v; 1ml). After air-drying, the pellet was dissolved in RNase free water (Sigma). The concentration was determined by measuring the absorbance at 260 nm (A260) in a NanoDrop® ND-1000 UV-Vis spectrophotometer. All RNA samples were stored at – 80°C.

For microRNAs expression analysis, qRT-PCR was performed using individual TaqMan MicroRNA Assays (Applied Biosystems; Table 8). In the reverse transcription (RT) step, cDNA is reverse transcribed from total RNA samples using specific miRNA stem-loop primers from the TaqMan MicroRNA Assays and reagents from the TaqMan® MicroRNA Reverse Transcription Kit. 10µg of total RNA in 5µL, was added to a master mix containing 0.15 µL dNTPs (100 mM), 1µL of reverse transcriptase (50U/µL), 0.19 µL RNase inhibitor (20U/µL), and 1.5 µL 10x reverse transcription buffer in a total of 7µL in RNase free water. Finally, 3µL of microRNAs-specific RT primer was added per reaction. Reverse transcription was performed in a thermal cycler (16 °C for 30 min.; 42 °C for 30 min. and 85°C for 5min). PCR products are amplified from cDNA samples using the TaqMan miRNA-specific forward and reverse PCR primers and TaqMan probe together with the TaqMan® Universal PCR Master Mix. (Applied Biosystems: 40 cycles; 95 °C for 15 s, 60 °C for 60 s). MicroRNAs levels were normalised to the expression of RNU48 and were calculated as described in 2.5.2.

2.6. Immunofluorescence and high content analysis (HCA)

For immunofluorescence, cells were fixed with 4% PFA (w/v) for 30 min, washed with PBS and permeabilised with 0.2% Triton X-100 (v/v) for 10 min. To block unspecific binding of primary antibody, cells were incubated for 30 min in
blocking solution (0.5% BSA (w/v) and 0.2% fish skin gelatin (v/v) in PBS). The cells were then incubated with the primary antibody, diluted in blocking buffer, for 1 h at room temperature. Three washes with blocking buffer for removing unbound primary antibody were performed. The cells were subsequently incubated with the secondary antibody, diluted in blocking buffer, for 1 h at room temperature. Cells were washed with PBS three times (5 min each wash with agitation) before adding 3µM DAPI for 20 min for nuclear staining. Acquisition of Immunofluorescence images was performed using the IN Cell Analyzer 1000 automated high-throughput microscope (GE Healthcare) with 10x or 20x objectives. Image processing was performed using the IN Cell Investigator (v1.7) software (GE Healthcare).

High Content Analysis (HCA) was used for quantification of immunofluorescence images. Two or three (in the case of double staining) fluorescence images corresponding to DAPI and primary antibody/Alexa Fluor® 488-secondary or Alexa Fluor® 594 antibody were acquired for each condition. HCA was performed using the IN Cell Investigator (v1.7) software (GE Healthcare). For the analysis, DAPI staining of the nuclei was used to identify nuclear area and number of cells. The nuclei were defined by using top-hat segmentation, specifying a minimum nucleus area of 100 µm². To define the cell segmentation, a collar segmentation routine was used with a ratio of 1 µm. To determine the cellular expression of the analysed protein, the average intensity of pixels in the reference channel (Alexa Fluor® 488 or 594) within the specified nuclear region (Object Nuclear Intensity) was measured. Each cell was assigned a nuclear intensity value for the specific protein expression and that value was used to set up a threshold filter, which determined high levels (positive) and low levels (negative) expressing cells. The threshold filter used a histogram for data visualisation (Figure 2.1A-B). In order to set the filter cut-off, expression in the control was measured (i.e. p16INK4a expression in wells with empty vector-transduced cells, Figure 2.1A, left panel) to define the negative
population (red population on the histogram). Next, the analysis of the positive control (i.e. \(p16^{INK4a}\) expression in wells with \(H-RAS^{V12}\)-transduced cells, Figure 2.1B) was performed to determine the high-expressing population (green population on the histogram). Once the cut-off was set up, the analysis of the whole experiment was carried out. As a result, the software classified each cell as either positive or negative for the expression of the analysed protein (i.e. \(p16^{INK4a}\) positive (p16) and negative (neg) cells in empty vector vs. \(H-RAS^{V12}\) infected cells, Figure 2.1C) and generated a percentage of both cell populations (positive and negative) per well. The mean of the nuclear intensity was also routinely analysed and equivalent results were obtained. The antibodies used for the analysis were tested with robust controls (shRNAs) to assess their specificity. Where possible, alternative antibodies were used in control experiments to confirm the results obtained. For a list of primary and secondary antibodies used in this study see tables A9 and A10.

2.7. Chromatin immunoprecipitation (ChIP)

ChIP was performed as previously described (Ananthanarayanan et al., 2004). Briefly, proteins and DNA were cross-linked by adding 37% formaldehyde (v/v) drop-wise directly to the cell culture medium, to a final concentration of 1.1%, and incubating for 10 min at RT. The reaction was then terminated by addition of glycine (125 mM final concentration) to the media and incubation for 5 min at RT. Next, the cells were washed twice with 10 mL ice-cold PBS, scraped into 5 mL cold PBS and transferred to a 50 mL Falcon tube. Up to \(4 \times 10^5\) cells were harvested and pelleted by centrifugation at \(400 \times g\) for 5 min at 4 °C. The pellet was carefully resuspended in 1 mL cold PBS and transferred to a 1.5 mL microcentrifuge tube. A centrifugation at \(2000 \times g\) for 5 min at 4 °C followed, after which the supernatant was aspirated and the cell pellet stored at –80 °C.
Figure 2.1. High Content Analysis. IMR90 cells infected with empty vector and RasV12 were seeded in 96-well plates at a density of 4,000 cells per well. The following day, cells were stained by immunofluorescence for p16\textsuperscript{INK4a} and DAPI (nucleus) and subjected to High Content Analysis (HCA). To create a Threshold Filter to distinguish cells expressing high or low levels of p16\textsuperscript{INK4a} we generated a cut-off based on the histograms of p16\textsuperscript{INK4a} nuclear intensity (Nuc Intensity Reference 2) for cells infected with empty vector (A), RasV12 (B). In this example the cut-off was set above a value of nuclear intensity of 235. (C) Representative images showing classification of positive and negative cells in IMR90 transduced with empty vector or RasV12 expressing plasmid.
Chromatin preparation and immunoprecipitation were performed using the EZ-Magna ChIP™ G kit (Millipore), according to manufacturer’s recommendations. These procedures were carried out in the lab of Martin Walsh, Mount Sinai School of Medicine, New York, USA. Succinctly, immunoprecipitation of cross-linked chromatin was conducted with antibodies described in Table 11. After immunoprecipitation, DNA was extracted using the QIAquick PCR purification kit (QIAGEN) and an aliquot was amplified by RT-qPCR using oligonucleotide primers described in Table A12. To confirm target enrichment, each PCR product was evaluated by end-point PCR. The input levels of immunoprecipitated histone H3 were used for normalisation.

2.8. Reprogramming to induced pluripotent stem cells

The reprogramming experiments to compare efficiency of reprogramming in cells with \textit{INK4a}, \textit{Cdkn1a} and \textit{p53} knockdown and empty vectors was carried out in collaboration with Ludovic Vallier, MRC Centre for Stem cell Biology and Regenerative Medicine, Cambridge. The protocol used for direct reprogramming in Ludovic Vallier’s laboratory is similar to the one described before (Park et al., 2008a) which was adapted in our laboratory to compare reprogramming efficiencies.

2.8.1 Transduction of human somatic cells

The plasmids encoding the human reprogramming factors, empty vector control, MSCV-Cbx7 and pRS-shp53 were each transfected together with VSVg helper plasmid into HEK 293T packaging cells expressing gag-pol viral elements (293T GP, Takara Bio Inc). The following day, $1.10^5$ early passage IMR90 cells (p11-p14) were plated per 6-well. Infection was performed as described in 2.2. Two rounds of infection were performed to increase transduction efficiency but avoiding over-stressing the cells. After transduction the media was replaced with fresh media.
2.8.2. Generation and identification of ES-like colonies.

Four days after transduction with reprogramming factors and controls, $1.10^6 \gamma$-irradiated MEFs were plated on 0.1% gelatin (Millipore) coated 10cm dishes. The following day transduced IMR90 were detached using trypsin-EDTA (Invitrogen) and counted. $2.10^5$ cells were plated on feeders in DMEM 10% FBS. At day 7 after transduction cells were washed and the media replaced with hES cell media (KSR hESCs medium: Knock out DMEM + 20% Serum Replacer + FGF2 4ng/ml). Media was changed every day from this point (Timeline, Figure 2.2A). The first hiPSC colonies appeared around day 13 after transduction (Figure 2.2B). Cells were fixed using 4% PFA for 2 min, to avoid inactivation of alkaline phosphatase activity at day 20-24 after transduction and stained with alkaline phosphatase detection kit (Millipore). Briefly, Fast Red Violet solution (0.8g/L), Napthol AS-BI phosphate solution (4mg/mL), and water were mixed in a 2:1:1 proportion and added to each plate. Cells were incubated with staining solution in the dark, for 15min, at room temperature. Plates were scanned and colony quantification was performed using ImageJ software tool to analyse particles.

2.8.3. Colony picking and expansion

hiPS colonies were large enough to be picked around day 18-20 (Figure 2.2B). Colonies were picked under an inverted microscope in sterile conditions using a p10 micropipete. The colony was transferred into a 96-well plate with round bottom with 100uL of hES media (KSR media previously described), and disaggregated into clumps by gently pipeting. The cells were then plated in 24-well 0.1% gelatin coated plates containing feeders. Avoiding breaking the colony into very small pieces is essential, since hiPS and hES cells have very poor survival when plated as single cells. The colonies that survived and grew without undergoing differentiation were mechanically passaged to 12 well plates containing MEFs feeders. Colonies were
repicked if necessary to isolate hiPS cells from differentiated cells or partially reprogrammed cells contaminating the culture. Successfully expanded hiPS cell lines were characterised for the expression of pluripotency markers by immunofluorescence and quantitative q-RT-PCR.

2.8.4. Reprogramming of mouse embryonic fibroblasts

Reprogramming of mouse fibroblasts was performed as described in 2.8.1. and 2.8.2 with some modifications. Three days following transduction with the mouse reprogramming factors (pMXs vectors; (Takahashi and Yamanaka, 2006)) 3.10⁴ MEFs Oct4-GFP were plated on feeders in 6-well plates. Next day fibroblast media was replaced by KSR media supplemented with LIF (1000U/ml, ESGRO, Millipore). GFP-positive colonies were picked and expanded as described in 2.8.3., however instead of mechanical disaggregation, trypsin was used. miPS cells were keep on feeders in KSR media +LIF.
Figure 2.2. Reprogramming to induced pluripotent stem cells. (A) Timeline for generation of induced pluripotent stem cells from human fibroblasts. (B) Bright-field microphotographs acquired with 4x magnification showing morphology of IMR90 cells and hiPS cell colonies.
Chapter 3 – A senescence response is induced by expression of the four reprogramming factors

3.1. Early response to reprogramming factors expression in human fibroblasts

During direct reprogramming to induced pluripotent stem (iPS) cells, expression of a group of transcription factors initiates a sequence of events that ultimately result in gaining of pluripotency and immortality. The study of this sequence of events is not simple. This because the process is very inefficient, resulting in only a small portion of the whole population undergoing reprogramming, but also because it relies on the transduction of four different transcription factors that can give rise to a heterogeneous population, expressing various combinations and levels of each factor.

To study the effect of simultaneous expression of the four reprogramming factors a polycistronic system was used (Carey et al., 2009) coupled with puromycin selection. Carey et al. generated polycistronic viral vectors containing the mouse cDNAs for Oct4, Sox2, Klf4, and c-Myc (OSKM) separated by three different 2A peptides to reprogram human and mouse cells (Carey et al., 2009). These peptides, which are 18 to 22 amino acids long have been used to express multiple proteins from a single transcript, by mediating "ribosomal skipping" (Doronina et al., 2008; Ryan and Drew, 1994; Szymczak et al., 2004). To express the four reprogramming factors in IMR90 fibroblasts, the OSKM reprogramming cassette was transferred to the retroviral vector pBABE, which allows selection of transduced cells based on puromycin resistance (Figure 3.1A). Expression of the four reprogramming factors in IMR90 cells was confirmed by immunofluorescence (Figure 3.1 B).
3.1.1. **OSKM-transduced cells show features of cellular senescence**

IMR90 human fibroblasts were transduced with an empty vector or with the OSKM polycistronic vector. As a control for senescence induction a vector expressing oncogenic RAS (H-\(\text{RAS}^{G12V}\)), which is known to induce senescence in human and mouse cells (Serrano et al., 1997), was used. A short-hairpin against p53 (shp53) was included as a control for bypass of senescence. Three days after transduction, cells were selected by adding puromycin for approximately 7 days (Figure 3.2 A). The early events after the infection of IMR90 human fibroblasts with the OSKM vector were monitored.

Surprisingly, expression of the reprogramming factors caused a decrease in the number and growth of IMR90 cells, as evaluated by colony formation assays, growth curves and a decrease in the percentage of cells incorporating BrdU, at independent time points over the course of 12 days (Figure 3.2 B-D). Growth curves showed that upon expression of the reprogramming factors the observed growth arrest was similar to the strong effect caused by expression of oncogenic RAS (Figure 3.2C). The BrdU incorporation assays revealed that the percentage of cells entering S-phase was lower in OSKM cells when compared with empty vector cells (\(\approx 15\%\) compared to \(\approx 50\%;\) Figure 3.2 D). To confirm that the decrease in cell number upon OSKM expression was due to growth arrest rather than apoptosis, immunofluorescence for cleaved caspase 3, an apoptosis marker was performed. The percentage of cells that scored positive was not significantly different between OSKM and control cells (Figure 3.2 E). To further characterise the growth arrest observed in OSKM-transduced fibroblasts, DNA content analysis was performed. OSKM cells presented a higher percentage of cells arrested in G1 phase and a reduction of S-phase cells (Figure 3.2 F). This is in agreement with the fact that in the experimental conditions used, expression of the four factors induces cells to undergo growth arrest rather than apoptosis.
Figure 3.1. pBABE OSKM vector for expression of the 4 factors in IMR90. (A) The polycistronic cassette encoding the 4 mouse cDNAs was cloned into the retroviral vector pBABE, which contains the gene for puromycin resistance (pBABE-puro OSKM). The four cDNAs are separated by 2A peptides derived from foot-and-mouth disease virus FMDV (“F2A”), insect Thorea asigna virus (TaV, “T2A”) and equine rhinitis A virus (ERAV, “E2A”). (B). Expression of the 4 reprogramming factors (Sox2, Oct4, Klf4 and c-Myc) in IMR90 was confirmed by immunofluorescence staining against the four proteins after selection of transduced cells. Cells transduced with empty vector were used as control. Dapi staining (blue) and Alexa-488 (green) is shown for each sample. Whereas Oct4 and Sox2 are not expressed in these cells; human endogenous c-MYC and KLF4 are detected. (bp) base pairs.
Figure 3.2. Expression of the 4 reprogramming factors induces growth arrest. (A) Timeline of the experiments presented in Fig. 3.2 and 3.3 and 3.4. IMR90 human fibroblasts were infected with a polycistronic vector expressing the 4 factors (OSKM) and selected for 7 days. (B) Crystal violet stained plates of IMR90 cells infected with the indicated vectors. (10^5 cells were seeded per 10cm-dish after selection and fixed 14 days later) (C) Growth curve of IMR90 transduced cells with the indicated vectors. Cells were seeded in triplicate in 24-well plates; one plate was fixed every two days and stained with crystal violet at the end of the time course. The relative cell number corresponds to the absorbance at each time point relative to absorbance at day 0 (day after seeding). (D) Quantification of BrdU incorporation in IMR90 cells transduced with the reprogramming factors and controls (Day 0 is day after seeding). (E) Expression of the 4 reprogramming factors in IMR90 cells do not induce a significant amount of apoptosis as estimated by IF using an antibody recognizing cleaved caspase 3. As a positive control, IMR90 cells were treated with cyclohexamide (100 µg/ml) for 1h, followed by treatment with TNF α (50 ng/ml) for 18 hours. (F) Percentage of cells in different stages of the cell cycle as analyzed by PI staining followed by flow cytometry. Cell cycle profiles for empty vector control and OSKM are shown in Figure A1. Results presented in A-D correspond to one representative experiment (n=3).
OSKM transduced cells displayed a distinct morphology that resembled that of senescent cells, with a large cytoplasm (Figure 3.3A) and a subset of cells were polynucleated. To further prove that the growth arrest had characteristics of senescence, cells were stained for senescence-associated β-galactosidase activity (SA-β-Gal), a widely used marker for identification of senescent cells (Dimri et al., 1995). A higher percentage of cells displayed SA-β-Gal activity when the four reprogramming factors were expressed (around 50% compared with 10% of the cells transduced with empty vector control; Figure 3.3 B). Additionally, the expression of the four factors led to an increase in the percentage of cells showing senescence-associated heterochromatic foci (SAHF; Figure 3.3 C).

Together these results suggest that simultaneous expression of the four reprogramming factors induces senescence in human diploid fibroblasts.

### 3.1.2. Levels of senescence mediators during OSKM-induced senescence

To identify the pathways responsible for senescence induced by expression of the OSKM cassette, the expression of different senescence effectors was analyzed by immunofluorescence. Expression of reprogramming factors activated a DNA damage response as observed by elevated levels of proteins phosphorylated by ATM or ATR (p(S/T)Q) (Figure 3.4A). Interestingly, OSKM expression also caused oxidative stress, as reflected by elevated levels of the oxidized base 8oxoG, a common DNA lesion that results from oxidative stress (Figure 3.4B). Consistently with an activation of a DNA damage response (DDR) in OSKM-transduced cells, an up-regulation of the tumour suppressors p53 and p21Cip1 was observed in these cells (Figure 3.5A-B).
Figure 3.3 OSKM transduced cells present characteristics of senescence. (A) Bright-field pictures acquired with a 10x objective, showing morphology of IMR90 transduced with empty vector control, OSKM, RAS V12 and shp53. Amplification of an area of the picture is shown. (B) Staining for SAβ-Gal activity, showing higher percentage of positive cells in OSKM-transduced cells and in the RASV 12 control. More then 100 cells were counted; error bars correspond to two independent experiments. Pictures were acquired with 20x objective with and without phase contrast, to better identify blue perinuclear staining. (C) Senescence-associated heterochromatic foci (SAHF) are present in cells expressing the OSKM polycistronic vector. Cells were stained with DAPI, and quantification of SAHF was performed using InCell Analyser and the InCell Investigator software. Areas of Dapi stained nuclei with high intensity were identified using a min area of 0.5µm. Nuclei containing more than 5 high intensity areas were scored as positive. Error bars in B and C correspond to two independent experiments.
Figure 3.4 OSKM expression induces a DNA damage response. (A) Expression of the 4 reprogramming factors results in an increase in the cells positive for DNA damage as measured by the presence of proteins containing the phosphorylated residues that are ATM/ATR substrates (p(S/T)Q). Representative images are shown on the right hand side. (B) 8-oxo-Guanine (a common DNA lesion resulting from oxidative stress) levels increase in response to expression of the 4 reprogramming factors. Error bars correspond to duplicates in one representative experiment (n=3).
Additionally up-regulation of \( p16^{\text{INK4a}} \), a feature that is characteristic of senescent cells, was observed (Figure 3.5C). To analyze the contribution of the senescence effectors up-regulated upon OSKM expression, the four reprogramming factors were expressed together with the E6 and/or E7 proteins of HPV16, which inactivate the p53 and the Rb networks, respectively. As shown in Figure 3.5D, expression of either protein partially alleviates the growth arrest induced by the reprogramming factors, while joint expression of E6 and E7 has additive effects. To test specifically for the contribution of \( p16^{\text{INK4a}} \), \( p21^{\text{Cip1}} \), and p53 to the arrest, shRNAs targeting these factors were used. Analysis of the BrdU incorporation of cells infected with the OSKM vector showed that there is a partial rescue when shRNAs against p53, \( p21^{\text{Cip1}} \), and \( p16^{\text{INK4a}} \) are co-transduced, suggesting that they are relevant for the arrest (Figure 3.5E).

To understand the mechanisms underlying reprogramming to pluripotency, Meissner’s group have carried out a comprehensive genomic characterization of cells at various stages of the reprogramming process (Mikkelsen et al., 2008). To do so, they used mouse embryonic fibroblasts (MEFs) isolated from chimeric mice that had been generated from an iPS cell line carrying integrated doxycycline (Dox)-inducible lentiviral vectors with the four reprogramming factors. Treatment of these MEFs with Dox allows expression of the reprogramming factors without the heterogeneity that usually results from multiple transduction with of the 4 factors. To follow the events that occur in the following days they obtained gene expression profiles at days 4, 8, 12 and 16, after Dox administration. On one hand they observed an increase in the proliferative response as noted by the upregulation of genes with functions such as DNA replication (\( \text{Poli}, Rfc4 \) and \( \text{Mcm5} \)) and cell cycle progression (\( \text{Ccnd1} \) and \( \text{Ccnd2} \)). However, a strong increase in the expression levels of the anti-proliferative genes \( \text{Cdkn1a} \) (which encodes \( p21^{\text{Cip1}} \)) and \( \text{Ink4a} \) (which
Figure 3.5. Molecular analysis of senescence induced by expression of reprogramming factors. (A-C) Expression of the 4 reprogramming factors results in an increase in the percentage of cells positive for p53, p21\textsuperscript{Cip1} and p16\textsuperscript{INK4a}. Percentage of positive cells was determined by high content analysis as described in the methods. Error bars correspond to duplicates of one representative experiment (=3). (E, F) The ability of the 4 reprogramming factors to block proliferation of IMR90 fibroblasts was alleviated by co-expression of Human papillomavirus (HPV) E6 and/or E7 or knockdown of p53, p16\textsuperscript{INK4a} or p21\textsuperscript{Cip1} using shRNAs as measured by crystal violet staining (D) or BrdU incorporation (E). For crystal violet staining, cells co-infected with empty vector or OSKM polycistronic vector and vectors containing E6, E7 or both, were selected with puromycin. After selection \(10^5\) cells were plated per 10-cm dish and fixed 12 days later. For BrdU analysis, \(4.10^3\) cells were plated in duplicate per well of a 96-well plate. Next day BrdU was added and incubated for approximately 14 hours before fixing the cells for immunofluorescence analysis. Error bars correspond to duplicates in one representative experiment (n=2).
encodes p16\textsuperscript{INK4a} was also observed (Figure 3.6A). The levels of these negative regulators of the cell cycle remained high during the time course and decline slightly at later times during the 16 days that were necessary to reprogram a small percentage of the population (Mikkelsen et al., 2008)(Figure 3.6A). If an early response to expression of reprogramming factors is up-regulation of senescence effectors, it is likely that intermediate stages during reprogramming still show elevated levels of these regulators. Partially reprogrammed cells are cells that show some of the stem cells features such as alkaline phosphatase activity and expression of some pluripotency markers; but fail to express late pluripotency markers, such as Nanog and endogenous Oct4 (Mikkelsen et al., 2008). Indeed, by re-examination of published data from two studies that characterize the expression of partially reprogrammed cells (Mikkelsen et al., 2008), the expression of p16\textsuperscript{INK4a} and p21\textsuperscript{Cip1} was up-regulated in partially reprogrammed cells derived from MEFs when compared with iPS cells and ES cells (Figure 3.6B and C).

To understand how the levels of these cell cycle regulators change over time following OSKM expression, the protein levels of p53, p21\textsuperscript{Cip1} and p16\textsuperscript{INK4a} were measured over a course of 10 days. The percentage of cells exhibiting high levels of p53 or p21\textsuperscript{Cip1} decreases at later time points in IMR90 cells expressing the reprogramming factors (Figure 3.6A and B), while the percentage of cells with high p16\textsuperscript{INK4a} levels remains higher throughout the time-course (Figure 3.6 C). Whether there is a selection for cells that express lower levels of senescence regulators or the levels tend to decrease with time is still not clear.

Together, these results suggest that there is induction of senescence during the early stages of reprogramming, which will be referred in the following sections as reprogramming-induced senescence (RIS).
Figure 3.6 *Ink4a* and *Cdkn1a* levels during reprogramming of mouse fibroblasts and in partially reprogrammed cells. (A) The graphs presented were generated from data published by (Mikkelsen et al. 2008). These data were retrieved from the microarray data present in supplementary material (S1) of that paper, which refers to gene expression profile generated during doxycycline treatment (days 4, 8, 12, and 16) of MEFs carrying integrated doxycycline-inducible lentiviral vectors expressing the four reprogramming factors. Expression levels of *Ink4a* (encoding p16\(^{INK4a}\)) and *Cdkn1a* (encoding p21\(^{CIP1}\)) upon Dox treatment are relative to the levels of expression in day 0. (B-C) Expression levels of *Ink4a* and *Cdkn1a* are elevated in partially reprogrammed iPS cells when compared with fully reprogrammed iPS cells or ES cells. Partially reprogrammed cells also referred to as Pre-iPSCs, exhibit ES cell morphology, but do not express all pluripotency associated genes, maintain the expression of viral transgenes, retain the epigenetic silencing of the X chromosome, are irresponsible to LIF and are unable to give rise to chimeras. (B) Values were obtained from gene expression profiling data presented in Supplemental Material of (Mikkelsen et al. 2008). Values of expression for *Ink4a* and *Cdkn1a* in partially reprogrammed cell lines (MCV6 and MCV8), iPS cell line (MCV8.1) and ES cells are relative to expression levels in MEFs. Partially reprogrammed cells characteristics are supplied in detail in (Mikkelsen et al. 2008). (C) Values presented for *Ink4a* and *Cdkn1a* correspond to the logarithm (base 2) of the expression in partially reprogrammed (Pre-iPS) and ES and were obtained from Supplemental Data (Table S1) of (Sridharan et al. 2009). The values are normalized to the expression in ES cells.
Figure 3.7. Dynamics of the percentage of cells exhibiting high levels of senescence regulators in OSKM-transduced cells and empty vector control. The protein levels of senescence regulators p53 (a), p21\textsuperscript{Cip1} (B) and p16\textsuperscript{INK4a} (C) were assessed by immunofluorescence in IMR90 cells transduced with the OSKM polycistronic vector or with the empty vector control over the course of 10 days. Graphs correspond to one representative experiment (n=2).
3.3. Epigenetic regulation of the INK4a/ARF locus during RIS

The INK4a/ARF locus is normally subjected to strong epigenetic repression mediated by H3K27 methylation and recruitment of Polycomb-repressive complexes (Gil and Peters, 2006). Activation of the INK4a gene by activated RAS has been shown to occur by reversal of PcG-mediated repression (Barradas et al., 2009). To evaluate the epigenetic status of the INK4a-ARF locus during RIS, chromatin immunoprecipitation (ChIP) assays were performed. IMR90 transduced with OSKM and empty vector control were treated with formaldehyde for cross-linking of DNA and proteins (hES cells were included as a control). Chromatin preparation immunoprecipitation, and quantative PCR was performed by SiDe Li in Martin Walsh’s laboratory (Mount Sinai School of Medicine, New York). The ratio between H3K27 trimethylation (H3K27me3) to total H3 was determined using primer sets described previously (Barradas et al., 2009) (Figure 3.8A). The repressive mark peaked around the INK4a promoter, and a decrease in the levels of the H3K27me3 modification around the INK4a/ARF locus in response to the expression of reprogramming factors was observed (Figure 3.8B). Human ES (hES) cells were included for comparison, showing even higher levels of H3K27me3 than empty vector control IMR90 cells. In parallel, an increase in the H3K4me3 marks around the INK4a/ARF locus was observed, further suggesting that the locus is de-repressed in response to the expression of the reprogramming factors (Figure 3.8C).

Previous work has shown that expression of activated RAS reduces the extent of H3K27me3 at the locus by up-regulating JMJD3 and down-regulating EZH2, leading to a loss of PcG-mediated repression (Barradas et al., 2009). JMJD3 is an H3K27me3 demethylase, whereas EZH2 is a member of the PRC2 complex responsible for the establishment of the H3K27me3 repressive mark. To investigate whether the mechanisms responsible for the chromatin changes observed in the
Figure 3.8. The histone demethylase JMJD3 contributes to the regulation of the INK4a/ARF locus during reprogramming-induced senescence. (A) Schematic figure showing the organization of the INK4a/ARF locus and the location of the primers used. (B, C) Expression of the 4 reprogramming factors results in a loss of H3K27me3 marks (B) and an increase in H3K4me3 marks (C) in the INK4a/ARF locus as analysed by chromatin immunoprecipitation (ChIP). (D) The H3K27me3 histone demethylase JMJD3 is induced in response to the expression of the 4 reprogramming factors. (E) Quantitative RT-PCR showing upregulation of JMJD3 transcript in response to the expression of the 4 reprogramming factors. (F) ChIP analysis showing an enrichment of JMJD3 and RNA pol II and loss of EZH2 at the INK4a promoter in response to expression of the 4 reprogramming factors.
INK4a/ARF locus were similar to those that operate during RAS-induced senescence, the levels of JMJD3 were analysed. An up-regulation of JMJD3 at the mRNA and protein level was observed (Figure 3.8D and E). In agreement with this result, ChIP reflected an increased recruitment of JMJD3 to the INK4a promoter (Figure 3.8F). Interestingly, a decrease in the binding of the H3K27me3 histone methyl transferase EZH2 to the locus was also observed, but only marginal effects on their total levels of expression (Figure 3.8F, data not shown).

Together these results show that the INK4a-ARF locus is activated during reprogramming-induced senescence (RIS). Importantly, chromatin remodelling in this locus is critical, as in hES cells and fully reprogrammed cells, the locus is found in a repressed state.

3.4 Dissecting the role of p53/p21<sup>Cip1</sup> during RIS by individual expression of the four reprogramming factors.

To understand how the p53/p21<sup>Cip1</sup> pathway is engaged during RIS, each of the reprogramming factors was expressed individually in IMR90 fibroblasts. Surprisingly expression of any of them had a negative effect over the growth of IMR90 cells as shown by colony formation assays (Figure 3.9A), growth curves and BrdU incorporation assays (Figure 3.9B). The extent to which the individual factors were able to arrest growth was variable, with Sox2 and Klf4 showing the strongest effect. Interestingly, Nanog, an important stem cell transcription factor that can be omitted from the Yamanaka reprogramming cocktail, had no negative effect over the growth of human fibroblasts (Figure 3.9B, growth curve). Accordingly, expression of the four factors separately also increased the amount of SA-β-galactosidase positive cells, even for Oct4-transduced cells, which do not exhibit a strong growth arrest (Figure 3.9 C). These results suggest that the individual factors are able to induce
senescence, but probably some of them provoke a heterogeneous response. The transcription factor c-Myc for example, clearly induced senescence and p16\textsuperscript{INK4a} up-regulation, but it also gave rise to highly compact proliferative colonies (data not shown; Figure 3.9A).

Next the ability of the individual reprogramming factors to regulate expression of senescence effectors was analysed. Sox2, Klf4, and c-Myc each up-regulated p21\textsuperscript{Cip1} levels. Sox2 expression resulted in a p53-independent up-regulation of p21\textsuperscript{Cip1}, while c-Myc or Klf4 induced both p53 and p21\textsuperscript{Cip1}, although only c-Myc provoked DNA damage (Figure 3.9 D). These results suggest that p21\textsuperscript{Cip1} is activated by redundant signals during reprogramming.

Several microRNAs (miRNAs) have been identified whose expression is linked to pluripotency, such as the miR-290 cluster in mouse cells, (the closest homologues in human cells are the miRNAs that form the miR-371–373 cluster) or the miR-302 cluster that is conserved in mouse and human (Houbaviy et al., 2003; Suh et al., 2004). It has been suggested that these ES cell-specific miRNAs are necessary for the normal proliferation of ES cells, and function by targeting negative cell cycle regulators such as p21\textsuperscript{Cip1}, the RB homolog p130, or LATS2 (Wang et al., 2008).

The levels of miRNAs belonging to the miR-302 and miR-371-3 clusters were measured in IMR90 cells transduced with the OSKM polycistronic vector, and compared with empty vector transduced cells. Human ES cells and three different human iPS cell lines were included (Figure 3.10 A). While expression of miR-302 and miR-372/3 is low or absent in IMR90 fibroblasts, in iPS cells it is reset to levels similar to the ones found in human ES cells. Noticeably, expression of the four reprogramming factors in IMR90 cells did not reset the level of the miR-302 cluster to the ones observed in hES cells, while expression of miR-372 or miR-373 were not
Figure 3.9 Dissecting the role of p53/p21\textsuperscript{Cip1} during RIS. (A) Crystal violet stained plates of IMR90 cells infected with the indicated vectors. \(10^5\) cells were seeded per 10-cm dish after selection and fixed 14 days later. (B) Growth curve of IMR90 transduced cells with the indicated vectors. Cells were seeded in triplicate in 24-well plates; one plate was fixed every two days and stained with crystal violet at the end of the time course. The relative cell number corresponds to the absorbance at each time point relative to absorbance at day 0 (day after seeding). On the right hand side a quantification of BrdU incorporation in IMR90 cells transduced with the reprogramming factors separately and controls is shown. (C) Staining for SA\(\beta\)-Gal activity. More then 100 cells were counted; error bars correspond to two independent experiments. Pictures were acquired with 20x objective. (D) Individual effect of reprogramming factors on the induction of DNA damage (\(\gamma\)H2AX) and the expression of p53 and p21\textsuperscript{Cip1} as measured by immunofluorescence. The expression of c-Myc, and Klf4 induces p53 upregulation. The expression of c-Myc, Klf4 or Sox2 induces the upregulation of p21\textsuperscript{Cip1}. Similar results were obtained in 2 independent experiments. Error bars correspond to two independent experiments.
detected in IMR90 transduced with empty vector or OSKM vector (Figure 3.10 A). This is in agreement with the fact that the miR-302 cluster is regulated by Oct4 and Sox2 (Barroso-del Jesus et al., 2009) however it is possible that time is required to reach expression of adequate levels of these ES cell-specific miRNAs during the reprogramming process or that expression of the cluster is not induced in all cells. These data suggest that uncoupling between these events; expression of the four reprogramming factors and expression of pluripotency-associated miRNAs may have a role in the p21Clp1 induction observed during RIS. To probe this hypothesis, the miR-302 cluster was co-expressed with the four reprogramming factors. Ectopic expression of the miR-302 cluster alleviated the growth arrest induced by OSKM expression in IMR90, as evaluated by BrdU incorporation and colony formation assay (Figure 3.10 B and C). Furthermore, miR-302 prevented the up-regulation of p21Clp1 and p130 observed during RIS (Figure 3.10 C). Therefore, the uncoupling between expression of reprogramming factors and expression of ES cell-specific miRNAs can have a role in the senescence response of somatic cells to OSKM expression.

3.5. Discussion and conclusions

3.5.1. The senescence response to expression of reprogramming factors

The mechanisms involved in the reprogramming of somatic cells to a pluripotent-like state remain largely unknown. Upon expression of the reprogramming factors in somatic cells, a sequence of events takes place ultimately originating cells that highly resemble embryonic stem cells. To understand why the reprogramming process is so inefficient we have to unravel the sequential events that result in successful reprogramming and identify the biological barriers. Most protocols used to
Figure 3.10. Expression of ESC-specific miRNAs rescues RIS. (A) Expression of microRNAs of the miR-302 and mir-371-3 cluster analyzed by Taqman in IMR90 cells infected with vector or 4 factors (OSKM), three iPS cells (iPS B6, B7, B8) and H1 hES cells (hES). miR-302a c and d expression is detected at lower levels in OSKM arrested cells when compared to iPS or human ES cells. miR-372 and miR-373 are undetected in human fibroblast as well as in OSKM arrested cells. Values are normalized to IMR90 infected with control vector, in the case of the miR302 cluster, and normalized to hES cells in the case of miR-372 an 373; u.d.l., under the detection limit. (B-C). Expression of the miR-302 cluster (302) alleviates OSKM-induced senescence, as shown by BrdU incorporation (B) and colony formation assays (C). (D and E). IMR90s infected with OSKM vector were subjected to immunofluorescence using antibodies recognizing p21Cip1 and p130 (RBL2, retinoblastoma-like protein 2). Expression of miR-302a-d prevents up-regulation of CDKN1a (encoding for p21Cip1) and RBL2 (encoding for p130), which are both upregulated during reprogramming-induced senescence. (Ctrl: corresponds to miR-Vec-Ctrl, which contains a stuffer DNA derived from the first 211 nt of hTR (Voorhoeve et al., 2006).
reprogram human and mouse cells rely on the fact that high viral titers and high transduction efficiencies result in multiple viral integration events. This is important to assure expression of the four different cDNAs from independent vectors, but also originates heterogeneous populations of cells, composed of cells transduced with different combinations of factors. Heterogeneous populations are difficult to study not only because different phenotypes can be found, but also because over time cells with a growth advantage will be selected. In this work, expression of the four reprogramming vectors from a polycistronic vector enabled the study of the early response of human fibroblasts to the simultaneous expression of the transcription factors known to induce somatic cells to regress to an undifferentiated, pluripotent state. Surprisingly, the first response to expression of reprogramming factors is an anti-proliferative response. Human fibroblasts expressing the OSKM reprogramming cassette show an up-regulation of negative regulators of the cell cycle, such as p53, p21Cip1 and p16INK4a (Figure 3.5 A-C). Accordingly, characterization of OSKM-transduced cells, revealed that they are arrested in the G1 phase of the cell cycle, show lower levels of BrdU incorporation when compared with control cells, and are positive for the senescence associated β-galactosidase activity (SA-β-Gal activity, Figure 3.2 and 3.3). Whereas induction of the p53-p21Cip1 pathway seems to be due to an activation of the DNA damage response, as analysed by up-regulation of phosphorylation of ATM/ATR substrates and existence of the oxidative DNA lesion 8-oxoG, up-regulation of p16INK4a seems to be related to an epigenetic remodelling of the INK4a-ARF locus (Figure 3.8).

The systems for generation of secondary iPS cells are particularly useful to avoid the heterogeneity caused by viral infections from independent plasmids. Somatic cells that give rise to secondary iPS cells express homogeneous quantities of the reprogramming factors, since all cells within the population were derived from a single iPS clone and therefore possess the same combination of reprogramming
factors. In spite of this, the reprogramming efficiency of these cells, although higher than that obtained after primary iPS cell generation, is still extremely low. Meissner’s group took advantage of this system to understand the mechanisms underlying reprogramming. They produced genome-wide gene expression profiles upon induction of the reprogramming factors during reprogramming to secondary iPS cells. Interestingly, they observed an upregulation of antiproliferative genes, such as the CDK inhibitors p21$^{\text{Cip1}}$ and p16$^{\text{Ink4a}}$ (Mikkelsen et al., 2008). The same profiles of gene expression and chromatin status have been performed to compare somatic cells, partially reprogrammed cells, fully reprogrammed cells and ES cells. Mouse partially reprogrammed cells also show increased levels of p21$^{\text{Cip1}}$ and p16$^{\text{Ink4a}}$ reflecting the up-regulation of these genes during reprogramming and suggesting that their inhibition may hinder the process (Mikkelsen et al., 2008) (Sridharan et al., 2009). The work presented in this chapter reveals that human cells respond to expression of the four reprogramming factors by halting proliferation. Importantly, induction of p21$^{\text{Cip1}}$ and p16$^{\text{Ink4a}}$ was also observed when using a different reprogramming system. Work performed by Filipe Pereira, shows that these CDK inhibitors are induced in human B cells during cell fusion mediated reprogramming (Banito et al., 2009). During the course of this work, other groups reported that the expression of the reprogramming factors is sufficient to directly trigger an anti-proliferative response and showed that the expression of the four Yamanaka factors, or combinations of only Oct4, Sox2 and Klf4, in human or mouse fibroblasts can induce p53 and p21$^{\text{Cip1}}$ (Hong et al., 2009; Kawamura et al., 2009).

Therefore, induction of senescence upon reprogramming (reprogramming-induced senescence or RIS) is observed in different cell types, using different reprogramming approaches and it is reflected by elevated levels of senescence effectors such as p21$^{\text{Cip1}}$ and p16$^{\text{Ink4a}}$ in partially reprogrammed cells.
3.5.2. RIS: Stress or context-dependent response?

Although these observations seemed at first surprising, they probably reflect the stress that somatic cells undergo during reprogramming. Indeed, all of the reprogramming factors have been reported to have oncogenic potential. c-Myc and \textit{Klf4} are well-established oncogenes (Dang et al., 2005; Rowland et al., 2005). Oct4 plays a critical role in the genesis of testicular germ cell tumours (Gidekel et al., 2003) and has been shown to cause dysplasia in epithelial tissues (Hochedlinger et al., 2005). SOX2 has been implicated in breast (Chen et al., 2008), esophageal (Bass et al., 2009) and lung cancer (Hussenet and du Manoir, 2010). The fact that the reprogramming factors have oncogenic functions could explain why a senescence response is triggered; and in fact an obvious parallelism can be drawn between oncogene-induced senescence triggered by oncogenic RAS and that induced by expression of the reprogramming factors. Like oncogenic RAS, expression of the OSKM cassette leads to activation of ATM/ATR, which might explain p53 activation, still, the main determinants of p53 activation during reprogramming have not been clearly established. Differences in how p53 is activated during RIS might exist between cells of human and mouse origin, as occurs during oncogene-induced senescence (OIS). For example, a role for p19\textit{Arf} in the activation of p53 during reprogramming in MEFs can be inferred from recent results (Kawamura et al., 2009), but whether p14\textit{ARF} has a similar role in activating p53 during the reprogramming of human cells remains to be determined. Nevertheless it is clear that a DNA damage response (DDR) is mounted coinciding with the expression of the reprogramming factors (Figure 3.4) (Kawamura et al., 2009; Marion et al., 2009a). The triggering factor for this DDR is still unclear, although it has been suggested that the observed pan-nuclear pattern of \textit{γH2AX} staining is compatible with DNA damage induced by aberrant DNA replication.
(Marion et al., 2009a). This would be similar to what occurs during OIS. However, the expression of the four Yamanaka factors in human fibroblasts leads to accumulation of 8-oxoguanine adducts (Figure 3.4B) which are commonly the result of oxidative stress, and c-MYC induces DNA damage in a ROS-dependent (Vafa et al., 2002) rather than DNA replication-dependent manner (Egler et al., 2005). Therefore, the DNA damage response that occurs during reprogramming could be caused not only by aberrant DNA replication but also by the generation of ROS. Accordingly, it was later described that reprogramming is more efficient under low oxygen conditions (Utikal et al., 2009; Yoshida et al., 2009). Similarly is has been reported that vitamin C, a powerful antioxidant is also able to improve reprogramming efficiency (Esteban et al.).

In addition to the activation of p53, the results presented here demonstrate that \( p16^{INK4a} \) is induced early upon the expression of the four Yamanaka factors in human fibroblasts reminiscent of what happens in response to oncogenic RAS and other stress-inducing signals. Although the mechanism behind upregulation of \( p16^{INK4a} \) needs to be explored further, it involves chromatin remodelling, including the loss of H3K27me3 marks around the \( INK4b/ARF/INK4a \) locus. The up-regulation of the histone demethylase JMJD3 observed in reprogramming could be partly responsible for this phenotype, which markedly resembles the one observed during RAS-induced senescence (Agger et al., 2009; Barradas et al., 2009). The \( INK4b/ARF/INK4a \) locus is repressed epigenetically by Polycomb group proteins in both iPS cells and ESCs (Figure 3.8B)(Li et al., 2009). The fact that a derepression of the \( INK4b/ARF/INK4a \) locus is observed when the 4 factors are expressed, suggests that a proper epigenetic reprogramming of this locus could be an important barrier during reprogramming.

The view that somatic cells such as fibroblasts undergo a stress response similar to the one caused by expression of oncogenes is attractive, however, one
should also suppose that the scenario might be more complicated. Some of the reprogramming factors behave in a context-dependent manner. For example, *Klf4* is a context-dependent oncogene, able to induce a growth arrest in normal cells or conversely to bypass senescence induced by *RAS* (Rowland et al., 2005). In the same line it was described that *Sox2* is downregulated in gastric cancer and that it can induce growth arrest and apoptosis when expressed in gastric epithelial cell lines, suggesting that it also has tumour suppressive functions (Otsubo et al., 2008).

The different outcomes of reprogramming factors expression in somatic and ES cells may also result from their ability to regulate distinct target genes when expressed in different contexts. In fact, *Oct4* and *Sox2* genes are known to interact with several partners to activate or repress the transcription of target genes. When this happens, activation of other stem cell-specific genes, including *Nanog*, takes place. However, this is a late event in reprogramming (Silva et al., 2009). Interestingly, *Sox2* is also able to interact with other partners, such as Pax 6 in lens cells (Kamachi et al., 2001) and Brn2 in neural primordium (Tanaka et al., 2004). The availability of interacting partners for the reprogramming factors, and how they influence their ability to transactivate specific genes is a possible explanation for the context-dependent behaviour that these proteins exhibit.

Moreover, the regulation of cell cycle related pathways in ES cells differs significantly from that of differentiated somatic cells. It is most probable that the unique type of cell cycle-regulation of ES cells has a role is the anti-proliferative response observed in somatic cells during reprogramming. Inactivation of negative regulators of the cell cycle enables ES cells to continuously self-renew in culture, and this is achieved in part by the expression of ES cell-specific microRNAs that target *Cdkn1a* and *Rbl2* (Wang et al., 2008). The absence of ESC-specific miRNAs in somatic cells can also contribute to the differential response of reprogramming factors in these cells. This is in agreement with the results presented here that show
how expression of some of these ES-specific microRNAs is able to partially bypass RIS (Figure 3.10). Similarly, it was recently described that Δ40p53, a transactivation-deficient isoform of the tumour suppressor p53, is highly expressed in mouse ES cells and during the early stages of development (Ungewitter and Scrable, 2010). Although the same research group described previously that Δ40p53 impairs the regenerative capacity of adult stem cells (Medrano et al., 2009), this later study reveals that in ES cells the Δ40p53 isoform has a role in maintaining pluripotency and the abbreviated cell cycle that allows for their robust proliferation and growth. Accordingly, Δ40p53 haploinsufficiency results in a rapid downregulation of proliferation and acquisition of a somatic cell cycle. Further analysis demonstrate that Δ40p53 is able to block p53 from binding target promoters, including Nanog and Igf-1R (Ungewitter and Scrable, 2010). The uncoupling between the ES-specific regulation of the pathways that mediate senescence and expression of reprogramming factors during reprogramming, could explain the results presented here. Somatic cells lack the ES cells mechanisms to avoid senescence, therefore while the ES cell-specific transcriptional program correlates with high proliferation in ES cells the outcome in somatic cells may be different. It would be interesting to know whether expression of Δ40p53 is able to bypass RIS and improve reprogramming efficiency.

3.5.3. Different cell fates may limit reprogramming

In conclusion, the results presented here, and those reported by several research groups, reveal an unexpected activation of the anti-proliferative response during reprogramming. The upregulation of senescence mediators due to expression of 4 reprogramming factors (RIS) underlines that senescence is an active barrier that may hinder reprogramming (Kawamura et al., 2009; Marion et al., 2009)(Figure 3.11). Some of the genes activated during reprogramming are common to different
anti-proliferative responses, such as apoptosis, senescence or other forms of cell-cycle arrest raising the question of which anti-proliferative response(s) is triggered during reprogramming. The answer is probably complex. Human fibroblasts expressing reprogramming factors undergo a cell-cycle arrest that presents multiple characteristics of senescence, including p16$^{INK4a}$ up-regulation, an event that is specifically observed during senescence but not in other types of cell-cycle arrest. Although apoptosis was not a significant determinant in the conditions used in this work, it has been described that expression of the reprogramming factors can trigger apoptosis (Figure 3.11); for example, Bax is upregulated in response to the expression of Oct4, Sox2 and Klf4, and expression of its antagonist molecule Bcl2 results in enhanced reprogramming efficiency (Kawamura et al., 2009). Other reports suggest that the expression of the reprogramming factors synergizes with induction of DNA damage to trigger apoptosis, especially in cells that have critically short telomeres or are sensitized by exposure to exogenous DNA-damaging agents, such as ultraviolet or ionizing radiation. In such a scenario, the expression of Bcl2 restores the ability of these cells to be reprogrammed to levels similar to control cells (Marion et al., 2009a). Therefore reprogramming is limited by both anti-proliferative responses, as happens during tumour suppression, in which both senescence and apoptosis are implicated.

The fact that alternative cell fates can be an outcome when reprogramming is initiated might explain the low efficiency of the process. During reprogramming a cell has to “choose” between proliferation, growth arrest, uncontrolled cell growth or apoptosis. Probably only some cells undergo the stochastic sequence of events that allows them to avoid these alternative fates and achieve complete reprogramming to pluripotency. The ones that bypass the initial barrier of senescence and apoptosis still have to overcome additional barriers such as complete epigenetic remodelling and consequent activation of pluripotency genes (Figure 3.11). Acquisition of a stem
cell characteristic cell-cycle regulation, or immortality, seems also to be a late event during reprogramming as partially reprogrammed cells still express high levels of anti-proliferative genes and depend on the expression of exogenous factors for continuous growth (Brambrink et al., 2008; Stadtfeld et al., 2008a).

Figure 3.11. Alternative cell fates limit reprogramming efficiency. During successful reprogramming, the expression of reprogramming factors in somatic cells results in the generation of iPS cells. In some cases, the reprogramming process is not complete and partially reprogrammed iPSCs that have undergone incomplete chromatin remodelling are obtained. Alternatively, the expression of the reprogramming factors can cause senescence (RIS), apoptosis, or contribute to the oncogenic transformation of the resulting cells. ESC, embryonic stem cell; iPSCs, induced pluripotent stem cells; KLF4, Kruppel-like factor 4; OCT4, octamer 4; RIS, reprogramming-induced senescence; SOX2, SRY-box 2. Adapted from (Banito and Gil, 2010).
Chapter 4 – Modulating senescence to improve reprogramming efficiency

4.1 Inhibition of senescence mediators improves reprogramming efficiency.

The results presented in the previous chapter reveal a link between senescence and the reprogramming process. If senescence limits reprogramming, inhibiting this response should increase reprogramming efficiency. To test this idea, the expression of \textit{INK4a}, \textit{CDKN1a}, or \textit{p53} was knocked down using shRNAs in BJ and IMR90 human fibroblasts (Figure 4.1A). Reprogramming experiments were carried out by Tamir Rashid and Imbisaat Geti in Ludovic Vallier’s laboratory in the MRC Centre for Stem Cell Biology and Regenerative Medicine, Cambridge. Human fibroblasts transduced with empty vector or shRNAs targeting the three senescence effectors, were infected with retroviruses expressing the human reprogramming factors and cultured in the appropriate conditions to promote the appearance of human iPS (hiPS) cell colonies. After 21 d, colonies were analyzed for expression of \textit{NANOG} and \textit{TRA-1-60} by immunofluorescence. Double-positive colonies were counted as fully reprogrammed iPS cell colonies, whereas morphologically distinct colonies negative for both markers were scored as partially reprogrammed (Figure 4.1 B-D). The number of fully reprogrammed colonies was higher in cells expressing shRNAs targeting the expression of senescence effectors. (Figure 4.1 C). Similar experiments were performed with IMR90 cells using morphological criteria to determine hiPS cell colonies and partially reprogrammed colonies (Figure 4.2 B). In these experiments, IMR90 cells proved more difficult to reprogram than BJ cells and could only be reprogrammed when senescence mediators were knockdown (Figure 4.2B).
Figure 4.1. Inhibition of senescence improves reprogramming efficiency of BJ human fibroblasts. (A) BJ human fibroblasts were infected with shRNAs targeting senescence effectors. Quantitative RT–PCR showing the levels of the transcripts for p53, CDKN1A (encoding for p21<sup>Cip1</sup>), and INK4a (encoding for p16<sup>INK4a</sup>). BJ fibroblasts were transduced with retrovirus expressing OCT-4, SOX2, KLF4, and c-MYC and grown in culture conditions compatible with pluripotent stem cell growth. (B) Bright-field microphotographs showing distinct morphology of fully reprogrammed and partially reprogrammed iPS cells. (C) and (D). Colonies were analyzed by immunofluorescence for NANOG and TRA-1-60. The number of positive colonies is shown as the mean ± SD of three independent experiments.
Figure 4.2. Inhibition of senescence improves reprogramming efficiency of IMR90 human fibroblasts. (A) IMR90 human fibroblasts were infected with shRNAs targeting senescence effectors. Quantitative RT–PCR showing the levels of the transcripts for p53, CDKN1A (encoding for p21<sup>Cip1</sup>), and INK4a (encoding for p16<sup>INK4a</sup>). IMR90 fibroblasts were transduced with retrovirus expressing OCT-4, SOX2, KLF4, and c-MYC and grown in culture conditions compatible with pluripotent stem cell growth. (B) and (C) Colonies were counted based on morphologic criteria to distinguish fully and partially reprogrammed cells. The number of positive colonies is shown as the mean ± SD of three independent experiments. Bright-field microphotographs showing distinct morphology of fully reprogrammed and partially reprogrammed iPS cells is shown on the right hand side.
In addition to an increase in the number of fully reprogrammed cells, knockdown of senescence effectors resulted in an increased number of partially reprogrammed iPS cell colonies in both BJ and IMR-90 cells, suggesting that senescence limits the initial stages of reprogramming to pluripotency (Figure 4.1D and 4.2C).

The hiPS cell colonies generated from IMR90 or BJ fibroblasts with reduced levels of senescent factors were individually picked and expanded. All the hiPS cell lines analyzed (n=8) expressed pluripotency markers including OCT4, SOX2, NANOG, and TRA-1-60. Representative images of hiPS derived from BJ transduced with empty vector, shRNA against INK4a and p53 are shown in Figure 4.3. Additionally, hiPS generated in this experiment expressed other pluripotency markers such as DNMT3b, REX1, CRIPTO and hTERT (Figure 4.3B). The hiPS lines (n = 8) could be differentiated in vitro into extraembryonic tissues and into derivatives of the three germ layers ectoderm, mesoderm, and endoderm. Representative images are shown in Figure 4.4.

Similar experiments were performed in mouse fibroblasts. MEFs expressing shRNA against Ink4a/Arf, Cdkn1a or p53 were generated (Figure 4.5A) and subsequently reprogrammed in Ludovic Vallier's laboratory. These experiments also revealed increased reprogramming efficiency upon depletion of senescence effectors as analysed by quantification of alkaline phosphatase positive colonies (Figure 4.5B). Reprogramming of MEFs knockout for Cdkn1a or p53 also revealed a higher number of alkaline phosphatase colonies when compared with wild type MEFs (Figure 4.5C).

Taken together, these data demonstrate that inhibition of senescence improves the efficiency of reprogramming of somatic cells, supporting the idea that senescence imposes a barrier to successful reprogramming to pluripotency.
Figure 4.3. hiPS cell lines generated upon knockdown of p53 or p16\textsuperscript{INK4a} express pluripotency markers (A) hiPS cell lines generated from BJs transduced with vector control and shRNAs against p53 or p16 express pluripotency markers. (B) Q-RT-PCR for pluripotency markers showing expression in IPS and hES cells. Expression of DNMT3B, Rex1, and Cripto is relative to levels in BJ fibroblasts. Expression of hTERT is relative to levels in hES (H1) cells.
Figure 4.4. hiPS cells generated upon knockdown of p53 or p16\textsuperscript{INK4a} can differentiate into extra-embryonic tissues and into derivatives of the three germ layers. (A) hiPS cell lines generated from BJ cells transduced with vector control and shRNAs against p53 or INK4a were cultured in growth conditions to promote differentiation. Representative images of immunofluorescence for extra-embryonic markers (CDX2 and GATA6), mesendoderm (BRACHYURY and SOX17) and neuroendoderm (SOX2, PAX6 and SOX1) are shown. Protocols for differentiation are described in (Vallier et al., 2009).
Figure 4.5. Enhanced efficiency of reprogramming in MEFs knockout or knockdown for senescence effectors. (A) MEFs were infected with the indicated vectors and selected. Q-RT-PCR showing the levels of the transcripts for Tp53, Cdkn1a (encoding for p21Cip1) and Ink4a (encoding for p16Ink4a) in the cells used for the reprogramming experiments showed in (B). (B). MEFs expressing shRNAs targeting Ink4a/Arf, Cdkn1a or p53 were transduced with retrovirus expressing Oct-4, Sox2, Klf4 and c-Myc and grown in culture conditions compatible with pluripotent stem cells growth. The number of alkaline phosphatase (AP) positive colonies was quantified. (C) MEFs knockout for p53 or Cdkn1a (p53−/−, p21−/−) and wild type MEFs (WT) were transduced with retrovirus expressing Oct-4, Sox2, Klf4 and c-Myc, and and grown in culture conditions compatible with pluripotent stem cells growth. The number of alkaline phosphatase (AP) positive colonies was quantified. The number of positive colonies is shown as the mean ± SD of three representative experiments.
4.2 CBX7 expression improves reprogramming efficiency

To further study the link between senescence and reprogramming the reprogramming protocol was set up in the laboratory. The Polycomb group protein CBX7 is able to bypass replicative senescence by repressing the INK4a/ARF locus (Gil et al., 2004). Interestingly, CBX7 expression was found to be upregulated in miPS cells and mES cells when compared with differentiated cells (Mikkelsen et al., 2008). To test the ability of CBX7 to increase reprogramming efficiency, early passage IMR90 cells were transduced with the four factors (from independent plasmids), either with empty vector, a vector expressing mouse CBX7 or shp53 as a positive control for improvement of reprogramming efficiency. Infection conditions were maximized to assure high infection rates (above 75%). IMR90 infected with empty vector only, were kept in parallel throughout the experiment as a negative control for reprogramming. Transduced IMR90 cells were plated on feeders five days following transduction, and maintained in conditions that support hES cells growth for approximately fifteen days. ES-like colonies appeared approximately 13 days post-infection (after 8 days of changing to ES-cell growing conditions), but additional colonies, with varied morphology appeared at different stages previously. Addition of CBX7 to the reprogramming cocktail resulted in an increase in the number of alkaline phosphatase positive colonies (Figure 4.6 A and B). Reprogramming efficiency, expressed as number of AP-positive colonies per 100 cells transduced, was similar to the ones reported in the literature (0.01%-0.1% (Stadtfeld and Hochedlinger, 2010)(Figure 4.6C), although this depends on several factors such as transduction efficiency and type and passage number of somatic cells to reprogram. In parallel to plating cells on feeders to promote generation of ES-cells like colonies, cells were plated five days after transduction for immunofluorescence analysis. The percentage of cells with high levels of p16INK4a was higher in cells infected with the 4 factors,
however in cells infected with CBX7, the amount of cells showing high expression of p16\textsuperscript{INK4a} was lower (Figure 4.6D). Immunofluorescence for p53 protein was also performed. The percentage of cells showing high p53 levels was greater in cells set to reprogram in the presence of empty vector or CBX7 expression plasmid. However, addition of an shRNA targeting p53 clearly avoided its up-regulation during reprogramming (Figure 4.6E).

To assure that successful reprogramming was achieved in these experiments, colonies were picked and expanded for characterization. Two hiPS cells lines were successfully isolated. Their morphology was strikingly similar to the one of hES cells (Figure 4.7). During picking and expansion, some cell lines fail to attach or grow in undifferentiated state, since hES cells and iPS cells cannot survive as single cells and tend to differentiate when grown in low density conditions. Additionally, the carry over of partially reprogrammed cells, represent a problem, since these cells tend to grow faster and take over the cell culture (Figure 4.7 bottom panel, pre-iPS). The two hiPS isolated were positive for pluripotency markers, such as OCT4, TRA-1-60 and TRA-1-81, as determined by immunofluorescence (Figure 4.8A). IMR90 fibroblasts and hES cells were included as controls. Quantitative PCR for endogenous expression of the pluripotency factors OCT4 and SOX2 (Figure 4.8B) revealed that these genes were expressed at levels comparable with those of hES cells. Similar results were obtained for NANOG, DNMT3B, REX1 and hTERT (Figure 4.8B). The partially reprogrammed cell line shown in Figure 4.7 (pre-iPS) was also included, and as expected it shows incomplete activation of pluripotency markers (Figure 4.8B). Finally hiPS cells were analyzed for expression of exogenous factors. Partially reprogrammed cells are characterized by dependence on exogenous factors expression. The mRNA levels of exogenous factors were much lower in hiPS cells lines when compared with partially reprogrammed cells, suggesting that the transgenes are silenced in these cells (Figure 4.8).
Figure 4.6. Cbx7 enhances reprogramming efficiency of human fibroblasts. (A) IMR90 fibroblasts passage 12, were transduced with the 4 factors, together with empty vector control, a vector expressing mCbx7 and a vector expressing a small hairpin targeting p53. Five days after transduction 2. 10^5 cells were plated on feeders. From day 7, cells were maintained in media that supports growth of hES cells, and fixed on day 20-24. Alkaline phosphatase (AP) staining was performed to visualized ES-like colonies. Cells infected only with empty vector were kept in the same conditions throughout the experiment and used as a negative control for AP staining. (B) AP positive colonies were counted using Image J software. Error bars correspond to the standard deviation from three independent experiments. (C) Reprogramming efficiency is presented as number of AP positive colonies per 100 cells infected. The total number of AP positive colonies in each plate was counted. The efficiency of retroviral transduction was measured in parallel using a red fluorescent protein (cherry) retroviral plasmid, followed by FACS analysis on day 5. (D) and (E) Immunofluorescence analysis of p16^{INK4a} (D) and p53 (E) proteins. Cells were plated in duplicate in 96-well plates, five days after transduction, and correspond to the same cells plated for the reprogramming experiment presented in (A). ★★ p<0.005 (two-tailed t-test).
Figure 4.7. Morphology of hiPS cell lines generated in this study. Bright-field microphotographs acquired at three different magnification (4x, 10x and 20x) showing morphology of IMR90 (passage 15), two iPS cells lines (picked and expanded from a 4F+Vector plate), hES cells H7 and one pre-iPS cell line. The morphology of iPS cells is strikingly similar to the morphology of hES cells, which have large nuclei and a low cytoplasm to nucleus ratio. Pre-iPS, or partially reprogrammed cell lines clearly differ in morphology when compared to hES cells.
Figure 4.8. iPS cell lines generated in 4.6 express pluripotency markers. (A) Expression of the pluripotency markers OCT4, TRA-1-60 and TRA-1-81 was analyzed by immunofluorescence in the two iPS cell lines successfully picked and expanded together with controls (IMR90 cells and hES H7 cells). Pictures were acquired with 10x magnification. (B) qPCR for pluripotency markers showing expression in iPS, partially reprogrammed cells (pre-iPS) and hES cells. Expression is relative to levels in IMR90 fibroblasts.
Figure 4.9. hiPS cell lines have silenced the expression of exogenous reprogramming factors. The exogenous expression of reprogramming factors was measured by Q-RT-PCR in the partially reprogrammed cell line (pre-iPS).

Together these results show that a protocol for hiPS cell generation and comparison of reprogramming efficiencies was successfully developed in the laboratory. Importantly, CBX7 was found to enhance reprogramming efficiency, further highlighting the importance of bypassing the senescence roadblock to achieve successful reprogramming to pluripotency.
In addition to human fibroblasts, mouse embryonic fibroblasts were also reprogrammed. Reprogramming of mouse fibroblasts has several advantages when compared with reprogramming of human cells. Reprogramming efficiencies are higher for mouse than for human cells (around 0.1% versus 0.01%), ES-like colonies emerge sooner and the use of reporter genes can facilitate the identification of fully reprogrammed colonies (reviewed in Stadtfield and Hochedlinger, 2010). Additionally, MEFs are easier to transduced with retroviral vectors (transduction efficiency is usually above 90%).

MEFs Oct4-GFP were transduced with the four reprogramming factors (4F) or with Oct4, Sox2 and Klf4 (3F). After 4 days, the cells were plated on feeders and grown in conditions that support mES cells growth. ES-like colonies emerged early in the process (10 days after transduction) in 4F-transduced MEFs but not all exhibited GFP expression. Fourteen days following transduction several alkaline phosphatase colonies exhibiting ES-like morphology were detected using 4F reprogramming. MEFs transduced with 3F gave rise to ES-like colonies later (16 days after transduction), but most colonies exhibited ES-like morphology and expressed GFP (Figure 4.10.A). Fifteen GFP-positive colonies were picked and expanded from 3F-transduced MEFs. After expansion these cells retained alkaline phosphatase activity, (Figure 4.10.B), GFP-expression and stained positive for Nanog (Figure 4.10.C), suggesting that they were fully reprogrammed miPS cells. However further characterization, such as expression of other pluripotency markers and silencing of exogenous factors need to be performed in the future.

As discussed previously, reprogramming of mouse cells has advantages over reprogramming of human cells, which makes it a good system for routine assessment of elements modulating reprogramming efficiencies. It will be interesting to test the ability of CBX7 to improve reprogramming of mouse cells both with four or three reprogramming factors.
Figure 4.10. Reprogramming of MEFs Oct4-GFP. (A) MEFs Oct4-GFP were infected with retroviral vectors encoding the mouse reprogramming factors (pMXs vectors from (Takahashi and Yamanaka, 2006)) and grown in conditions that support mES cells growth. Plates were fixed and stained for alkaline phosphatase activity at day 14 and 20 post-infection. After 14 days numerous colonies were identified in cells infected with the 4F whereas with colonies appeared later in cells transduced with 3F (around day 16). On the right hand side bright field picture with 20x magnification of a colony generated with 3F is shown. The colony is GFP-positive showing reactivation of the Oct4 locus. (B and C) Three miPS cell lines were successfully picked and expanded from colonies generated with 3F. The three miPS cell lines are AP positive (B), Oct4-positive and express Nanog (C). mES cells were included as control.
4.3. Discussion and conclusions

4.3.1. Senescence represents a roadblock for successful reprogramming

In the previous chapter a senescence response to the expression of the four reprogramming factors was characterized. A corollary to the fact that senescence and apoptosis are triggered during reprogramming is that the inhibition of those responses could increase the efficiency of pluripotency induction. The results presented in this chapter demonstrate that inhibition of senescence mediators, p53, INK4a and CDKN1a improves reprogramming efficiency of human and mouse fibroblasts. Indeed, Park and colleagues were among the first groups to describe the reprogramming of human adult somatic cells to ESC-like pluripotent cells by using the four Yamanaka factors in combination with the SV40 large T antigen (SV40 LT) and/or hTERT (Park et al., 2008b). Interestingly, these two factors are involved in senescence control. Later, Zhao and colleagues reported that knocking down p53 and Utf1 could greatly increase reprogramming efficiency, but the mechanism behind it was still unclear (Zhao et al., 2008). At the same time that the work present here was published, several groups showed that knocking down p53 or its transcriptional target CDKN1a in human or mouse cells can significantly increase the efficiency of reprogramming (Hong et al., 2009; Kawamura et al., 2009; Marion et al., 2009a; Utikal et al., 2009). The expression of MDM2 or a dominant-negative mutant of p53 also results in enhanced reprogramming, whereas the activation of p53 through different strategies reduced the reprogramming efficiency (Kawamura et al., 2009; Marion et al., 2009a; Sarig et al., 2010), emphasizing the importance of controlling p53 activity to modulate reprogramming.

The low efficiency of reprogramming has been attributed to different biological roadblocks. The transition to a pluripotent state is accompanied by genome-wide remodelling of chromatin modifications such as DNA and histone methylation.
patterns from a somatic to a pluripotent state (Maherali et al., 2007; Mikkelsen et al., 2008). The promoters of pluripotency genes such as Oct4 and Nanog are silenced in somatic cells by DNA methylation (Gidekel and Bergman, 2002), therefore one of the reprogramming roadblocks is the proper demethylation of key pluripotency genes. Different reports have characterized cells that failed to fully reprogram (partially reprogrammed or Pre-iPS cells) and suggest that they are trapped in a late step of reprogramming. Inhibition of DNA methylation, knockdown of lineage-specific genes, or treatment with MEK and GSK3 inhibitors (Mikkelsen et al., 2008; Silva et al., 2008) can either convert some of these pre-iPS to iPS or increase the proportion of fully reprogrammed iPS versus pre-iPS (Figure 4.10). This work demonstrates that inhibiting or alleviating senescence leads to an increase in the number of fully and partially reprogrammed colonies (Figure 4.1 and 4.2). By inhibiting senescence mediators, the number of cells able to surpass the early barrier imposed by RIS is higher, resulting in higher numbers of both pre-iPS and fully reprogrammed iPS (Figure 4.10, right panel). A combination of both strategies could be used to enhance reprogramming efficiency synergistically. These results highlight an interesting link between reprogramming and tumour suppressors and emphasise how stressful the process of reprogramming must be for the cell. Additionally, senescence and reprogramming are deeply intertwined processes: a direct comparison of the ability of young and old cells to be reprogrammed shows that the closer cells are to the onset of senescence and therefore the higher the levels of INK4a and CDKN1a they express, the more difficult it is to reprogram them. Fibroblasts from older mice, which express high levels of the Ink4b/Arf/Ink4a locus products, are less efficient in generating iPS cells (Li et al., 2009), further linking ageing with decreased reprogramming efficiency. Similar experiments performed in late generation Terc−/− MEFs, which have critically short telomeres, emphasize the difficulty of reprogramming cells that are already aged or stressed (Marion et al., 2009b).
Therefore senescence seems to limit reprogramming in two slightly different ways, by functioning as an anti-proliferative/stress response as well as by limiting the number of cells in a population that are refractory to be reprogrammed due to pre-existent high levels of senescence regulators.

Figure 4.11. Senescence as an early barrier during reprogramming. Partially reprogrammed (Pre-iPS) cells are trapped in a late state in the reprogramming process due to incomplete epigenetic resetting. Strategies including inhibition of DNA methylation, knockdown of lineage specific genes (Mikkelsen et al., 2008) or treatment with 2 inhibitors (Silva et al., 2008) can convert Pre-iPS cells to fully reprogrammed iPS cells or increase the proportion of fully vs. partially reprogrammed cells (middle panel). Knocking down critical senescence effectors results in increased numbers of both partially and fully reprogrammed cells, presumably by reducing the proportion of cells that succumb to the first barrier (right panel).
Although the efficiency of reprogramming can be improved by interfering with crucial anti-proliferative genes, the consequence of dismantling cell-intrinsic tumour suppressive mechanisms is too detrimental to consider, as it would affect the safety of the resulting iPS cells. Indeed, iPS cells derived from p53−/− MEFs have increased chromosomal instability (Marion et al., 2009a). Additionally, single-cell imaging during reprogramming of mouse fibroblasts in the presence of a hairpin against p53 revealed that although p53 inhibition expand the global population responsive to reprogramming, there is a higher number of aberrantly reprogrammed cells compared with control cells (Smith et al., 2010). It is obvious that reprogramming methods that include inactivation or deletion of p53 are too unsafe, as, even cells that have been heavily burdened with DNA damage are still able to acquire pluripotent properties. However this approach may help to establish useful cellular models for a variety of diseases in which the target somatic cells are particularly difficult to reprogram.

Sensible alternatives may also exist to benefit from disabling senescence to enhance reprogramming efficiency without compromising the integrity and safety of the resulting iPS cells. This may be achieved by identifying the mediators of senescence induction during reprogramming, as well as by having a complete understanding of ES cell-specific mechanisms of cell-cycle regulation. For example, Rem2 GTPase, a suppressor of the p53 pathway implicated in maintenance of hES self-renewal and pluripotency, has been shown to improve reprogramming efficiency (Edel et al., 2010). Rem2 enhances the process by accelerating the cell cycle and protects cells from apoptosis by suppressing p53 and regulating cyclin D1 expression and localization. In this way the authors suggest that imposing cell-cycle features that are specific of hES cells might safely improve reprogramming (Edel et al., 2010). Although this is an interesting idea, the oncogenic potential of Rem2 needs to be further studied.
A possible strategy is to transiently inhibit senescence, by using either small interfering RNAs or chemical compounds. In this regard, two additional studies have provided safer methodologies for improving reprogramming by modulating senescence pathways. Reprogramming cells in low oxygen conditions (Utikal et al., 2009; Yoshida et al., 2009) or in the presence of antioxidants such as vitamin C (Esteban et al., 2010) has been shown to enhance the generation of iPS cells. Consistent with the results presented in this work, Pei's group report that one of the important metabolic activities that occur during the early stages of the reprogramming process is a significant increase in the level of reactive oxygen species (ROS). Addition of the natural compound vitamin C, results in an increase in cell proliferation and extension of cell lifespan, suggesting that vitamin C is able to overcome the senescence roadblock during the reprogramming process. Furthermore, vitamin C alleviates senescence by reducing but not abolishing p53 and p21 expression levels (Esteban et al., 2010). Therefore this study provides a good example of how senescence can be modulated to improve reprogramming efficiency in a safer way. Understanding of the senescence roadblock during reprogramming will hopefully provide similar additional strategies.

4.3.2. The role of the Polycomb protein CBX7 in reprogramming

CBX7, a polycomb group protein that is part of the PRC1 complex, is involved in suppression of the INK4a/ARF locus (Bernard et al., 2005; Gil et al., 2004) is also able to enhance the generation of partially and fully reprogrammed colonies. Analysis of p16\textsuperscript{INK4a} after transduction with the 4 reprogramming factors together with CBX7 showed that the percentage of cells with high p16\textsuperscript{INK4a} levels was lower when compared with reprogramming in the presence of empty vector control.
These results suggest that CBX7 can improve reprogramming by avoiding p16INK4a up-regulation and repressing the \textit{INK4a/ARF} locus. However it is possible that the ability of CBX7 to enhance reprogramming is due to additional factors. Indeed, it has been described that Polycomb group proteins have a critical role in the chromatin-remodelling events required for the direct conversion of differentiated cells toward pluripotency (Pereira et al., 2010). In ES cells, pluripotency is maintained by expression of ES cell-specific factors but also by suppression of developmental pathways by recruitment of repressive chromatin remodelling complexes (Boyer et al., 2006). Pereira et al. showed that members of the PRC2 and PRC1 complex have a role, not only in the maintenance of the pluripotent state, but also in the reprogramming abilities of ES cells during cell fusion reprogramming. Interestingly, the inability of PRC2 mutant ES cells to reprogram is dominant suggesting that mutant ES cells may express factors that block the efficient reprogramming of differentiated cells. As stated before, the \textit{INK4a/ARF/INK4a} locus is repressed epigenetically by Polycomb group proteins in both iPS cells and ESCs (Figure 3.8B) (Li et al., 2009). However the authors show that human \textit{INK4a} levels are not changed when comparing fusion of wild type or PRC2-deficient ESCs. Although this suggests that senescence induction is unlikely to be the cause of PRC2-\textit{null} reprogramming defects, it is important to note that in this reprogramming system the ability to induce a pluripotency transcriptional program is dissociated from the acquisition of the type of cell cycle regulation characteristic of ES cells. Therefore, although senescence regulators are induced in this scenario, their down-regulation is not fundamental in these settings because interspecies heterokaryons cease proliferation and cannot divide further (Bhutani et al., 2010). It will be interesting to understand what is the exact role of CBX7 in improving reprogramming. The fact that there is an increase in both partially and fully reprogrammed colonies suggests that CBX7 is acting in part by alleviating senescence and increasing the number of cells.
that bypass the initial senescence barrier. However, to understand whether CBX7 improves reprogramming by repressing the INK4a/ARF locus, an interesting experiment would be to compare the ability of CBX7 to enhance the reprogramming efficiency using MEFs wt and MEFs that are knockout for Inka4/Arf.

4.3.3. Comparing reprogramming efficiencies

Identification of RIS mediators will be one of the focuses of future research (see chapter 5, future work). Therefore, establishing an in house reprogramming protocol was also crucial for future work taking place in the laboratory. Genes that may have a role in mediating RIS will be tested for the ability to modulate reprogramming efficiency. Alkaline phosphatase staining can be used to score total, partially and fully reprogrammed colonies. Since RIS imposes an early barrier to reprogramming, the main purpose will be comparing total number of alkaline positive colonies. However, characterisation of fully reprogrammed cells was also important to assure that proper reprogramming was achieved in these experiments. There is a considerable degree of heterogeneity in the colonies arising during reprogramming with a high proportion being partially reprogrammed or simply transformed colonies. In the case of mouse cells drug selection cassettes linked to the pluripotency genes Oct4 and Nanog, which are activated in fully reprogrammed colonies have been used. However this approach requires genetic engineering of mice, so it cannot be applied in the case of human cells. Importantly, several studies report the isolation of high quality iPS cells without drug selection, but by simply using morphological criteria (Blelloch et al., 2007; Meissner et al., 2007). This is more easily applicable to hiPS cells, which display a distinct morphology (Figure 4.7). Indeed, several studies that report generation of hiPS cells rely on morphologic criteria (Takahashi et al., 2007; Yu et al., 2007). Live cell imaging of human fibroblasts during reprogramming
revealed that, similar to mouse fibroblasts, it begins with down-regulation of fibroblasts markers, such as CD13. Early pluripotency markers such as SSEA4 and TRA-1-60 appear later, however not all of these cells are set to reach a fully reprogrammed state (Chan et al., 2009). Additionally, Chan et al. report that pluripotency markers such as Nanog may be present in partially reprogrammed cells and that the fully reprogrammed state in hiPS is associated with additional activation of the pluripotency markers REX1 and DNMT3B (Chan et al., 2009). Interestingly the two hiPS cells lines generated in the lab are positive for those markers, whereas the partially reprogrammed cell line express lower levels of NANOG when compared with hES and hiPS and no REX1 or DNMT3B (Figure 4.8B). These results suggests that successful reprogramming to iPS cells was achieved and that the protocol used is suitable to compare reprogramming efficiencies. However if the quantification of fully reprogrammed versus partially reprogrammed colonies is required, double staining with pluripotency markers and/or morphology features will also be used in addition to quantification of alkaline positive colonies.
Chapter 5 – Discussion and future work

The generation of induced pluripotent stem cells, not only opened a vast range of possibilities for stem cell therapy but also provided a new tool to investigate pluripotency and to study specific diseases. iPS cells can be produced from adult cells from any individual, thus they could solve the ethical issues associated with ES-cell use and circumvent the need for immunologically matched cell donors for transplantation. Still, despite their potential, it is not known whether iPS cells will be an effective treatment for human diseases. In fact, it is important to bear in mind that even embryo-derived ES cells have only been tested in a few settings, and that their effectiveness and safety are not well established. Importantly, if iPS cells are to be used in a clinical setting, several issues have to be addressed. Previous work has paved the way to develop methods for the generation of integration-free iPS cells, but probably the most worrying topic is the relationship between reprogramming and tumorigenesis. The idea that inhibiting tumour suppressive pathways enhances reprogramming highlights this concern. In this work a link between these senescence effectors and the expression of the 4 reprogramming factors is demonstrated. Similarly, several research groups have described how inhibition of the two main senescence mediators pathways, p53-p21\(^{\text{Cip1}}\) and p16\(^{\text{INK4a-RB}}\), improves reprogramming efficiency.

5.1. The tumour suppressor p53 limits de-differentiation

The p53 tumour suppressor was first linked to reprogramming when mouse germ cells deficient for p53, were shown to be able to give rise to ES-like cells (Kanatsu-Shinohara et al., 2004a). Later, another research group reported that knocking down p53 and Utf1 could greatly increase reprogramming efficiency, but
the mechanism behind it was still unclear (Zhao et al., 2008). In this work the
activation of p53, and its downstream target p21\(^{Cip1}\) in response to expression of
reprogramming factors is described. Two research groups have reported similar
observations upon expression of the OSKM, OSK or SO (Hong et al., 2009)
(Kawamura et al., 2009).

The link between reprogramming and p53 is somehow intuitive. Indeed,
reprogramming highlights the ability of somatic cells to revert their fate towards a
state of pluripotency, a de-differentiation process that resembles tumorigenesis.
Sarig et al. have also reported that a mutant form of p53 could increase
reprogramming efficiency to higher levels than the ones obtained with p53 knockout
MEFs (Sarig et al., 2010). This further supports the link between tumorigenesis and
the de-differentiation of somatic cells into an embryonic state.

Undoubtedly, loss of p53 plays an important role in gain of pluripotency, but to
completely understand why p53 is induced during reprogramming a better
comprehension of its role in stem cell biology is necessary. Several studies have
implicated p53 in regulating pluripotency, for example p53 has been shown to
directly suppress the expression of Nanog (Lin et al., 2005). Similarly, p53 activation
has been related with differentiation; soon after differentiation the phosphorylation of
p53 in specific residues is increased (Lin et al., 2005). This suggests that p53 may
also have a role in preventing dedifferentiation, an idea supported by the fact that
germs cells can be reprogrammed to pluripotent stem cells when p53 is absent
(Kanatsu-Shinohara et al., 2004a). On the other hand it is intriguing that DNA
damage in ES cells does not lead to a p53-dependent cell cycle arrest or apoptosis
like in somatic cells (Aladjem et al., 1998). In fact it has been shown that although
present at high levels in mouse ES cells the p53 protein is in the cytoplasm and
translocated inefficiently to the nucleus upon stress conditions (Aladjem et al., 1998)
whereas in human ES cells is not present at all, or at very low levels. It has also
been shown that p53 is important for self-renewal of mouse ES cells because ES cells knockout for p53 have reduced self-renewing capability. Hu et al. showed that p53 up-regulates LIF expression and in this way self-renewal of cells with impaired p53 is avoided (Hu et al., 2007). Recently, it was reported that Δ40p53, an isoform of p53 which is specifically expressed is ES cells, is essential to maintain pluripotency. The authors suggest that this isoform regulates pluripotency by regulating the full-length p53 and promoting IGF signalling (Ungewitter and Scorable, 2010).

In light of the role of p53 in inhibiting dedifferentiation, the fact that p53 is activated during reprogramming and that its inactivation leads to enhanced reprogramming efficiency is logical. However, the several layers of p53 regulation make difficult to understand its role in stem cell biology. As these mechanisms become clearer, the easier will be to understand p53 dynamics during reprogramming.

5.2. Reprogramming the INK4a/ARF locus

In addition to p53, inhibition of the p16\(^{\text{INK4a}}\) and p19\(^{\text{Arf}}\) genes has been also shown to improve reprogramming efficiency. Li et al showed that the Ink4a/Arf locus is silenced during iPS reprogramming. They argue that this silencing occurs early in reprogramming, implying a direct effect of the reprogramming factors on this locus. There is, however, no consensus about the timing of downregulation of Ink4a/Arf expression during reprogramming (Li et al., 2009; Utikal et al., 2009). The fact that the Polycomb protein CBX7 improves reprogramming supports the view that repression of the INK4a/ARF locus is important for cells to bypass an early barrier during the process. In the reprogramming experiments performed in the laboratory, the percentage of cells positive for p16\(^{\text{INK4a}}\) was higher than in control cells 10 days after infection. These results imply that epigenetic remodeling of the locus is not one of the first events during reprogramming of human cells. This is also supported by
the fact that partially reprogrammed cells express high levels of *INK4a* when compared to fully reprogrammed cells (Mikkelsen et al., 2008; Sridharan et al., 2009).

During reprogramming the epigenetic memory of differentiated cells is reset. However differentiated cells can accumulate abnormal epigenetic changes that can contribute to pathological conditions. The *INK4a/ARF* locus is frequently aberrantly silenced in cancer. A recent study showed how aberrant methylation of the *INK4a* gene in immortalized human fibroblasts could be reverted during reprogramming to iPS cells. These results suggest that the *INK4a* locus is not aberrantly silenced during reprogramming and that it can even be reverted to a “normal” state in iPS cells. (Ron-Bigger et al., 2010). The authors also suggest that the *INK4a* gene in hES cells although expressed at low levels is not completely repressed and in this way they differ from the results observed in mES cells (Li et al., 2009). Further work will clarify how the *INK4a/ARF* locus is regulated during reprogramming and in pluripotent human ES cells.

### 5.3. Is cell division required for reprogramming?

The results presented in this work as well as the work performed by several groups clearly demonstrate a link between activation of tumour suppressor pathways and reprogramming. By inhibiting these pathways we prevent senescence, apoptosis and cell cycle arrest, thereby increasing reprogramming efficiency. Interestingly, work by Jaenish and colleagues suggest that almost all cells are capable of being reprogrammed, even without inhibiting p53 or immortalizing the cells (Hanna et al., 2009). When p53 was absent the process was accelerated due to an increase in the proliferation rate. Therefore the authors claim that p53 inhibition influences reprogramming kinetics rather than the overall efficiency of reprogramming (Hanna et al., 2009). It is still to determine whether this is cell-type specific, as in this case a
homogeneous population of B cells obtained from secondary iPS cell-generated mice, was used. It has been described that contrary to what happens with B cells, there is a refractory population of fibroblasts that cannot give rise to iPS cells even after prolonged time in culture (Stadtfeld and Hochedlinger, 2010). Utikal et al. have also addressed this issue by comparing the reprogramming efficiency of wild-type MEFs growing in 15% foetal bovine serum, with that of p53 knockout MEFs in 0.5% serum. Although the proliferation rate was lower in the MEFs deficient for p53 growing in low serum conditions, the reprogramming efficiency was still higher when compared to wild-type MEFs (Utikal et al., 2009). Additionally, live imaging studies of fibroblasts during reprogramming, revealed that after expression of reprogramming factors in MEFs, several distinct cell types based on broad morphological and proliferative characteristics, arise. As expected, most cells failed to initiate reprogramming and generally resembled the initial somatic fibroblast population, which the authors classified as exhibiting arrested/apoptotic or slow-dividing phenotypes. However a subpopulation of cells (about 1% of the total population) characterized by high rates of proliferation and small size, appears early in the process. Tracing back iPS colonies to their source, showed that they arise from this sub-population (Smith et al., 2010). These results are in agreement with the ones presented in this work that suggest that an initial senescence barrier limits reprogramming efficiency. Whether cells maintained in culture for long periods of time are able to bypass RIS and reprogram is still unclear. However since, bypassing RIS could involve spontaneous inactivation of tumour suppressors this should be avoided.

Apart from the senescence barrier, only a subset of cells from the initial population of fibroblasts is able to down-regulate differentiation markers and activate pluripotency genes. This implies that some cells are refractory to reprogramming. One of the limiting factors to achieve successful reprogramming is the demethylation
of pluripotent loci. This can be achieved by passive demethylation, due to inhibition of the DNA methyltransferase, DNMT1 during DNA replication, or by an active mechanism that is independent of DNA replication (Hanna et al., 2010). A recent study provided evidence suggesting that an active demethylation mechanism independent of DNA replication and cell division takes place during cell fusion mediated reprogramming. The authors show that reprogramming towards pluripotency in single heterokaryons is efficiently initiated without cell division or DNA replication and depends on activation-induced cytidine deaminase (AID), required for promoter demethylation and induction of OCT4 and NANOG gene expression (Bhutani et al., 2010). This is a very interesting study that enables the characterization of early events in reprogramming to pluripotency. However it is important to point out that it is still not known whether demethylation of the OCT4 gene detected in heterokaryons after cell fusion actually reflects the events that lead to fully reprogrammed iPS cells. Indeed, the levels of OCT4 observed after cell fusion are much lower (100-fold) when compared with ES cells and its promoter is partially demethylated (Bhutani et al., 2010). This is reminiscent of partially reprogrammed cells that can express low levels of OCT4 without achieving proper promoter demethylation (Mikkelsen et al., 2008).

Maybe the question lies on whether we can consider that a cell that cannot divide but express pluripotency markers is a fully reprogrammed cell. In other words, we need to understand whether proliferation and pluripotency are dissociable identities or if the in vitro pluripotent state of ES cells implies rapid proliferation and immortality. Early studies performed in embryonic carcinoma (EC) cells led to the idea that the length of G1 phase is directly linked to the responsiveness of pluripotent stem cells to differentiation signals (Jonk et al., 1992; Mummery et al., 1987). In this way, the short G1 characteristic of ES cells, would limit the time that cells are exposed to differentiation cues. Indeed, there is evidence that inhibition of
G1 progression compromises pluripotency (Filipczyk et al., 2007). Additionally, it has been proposed that mitosis is essential for changes in global transcriptional regulation systems, as it involves displacement of transcription factors from condensed chromatin (Egli et al., 2008). If this is the case, inhibition of senescence mediators would increase reprogramming efficiency by limiting the cells that are trapped in a senescent state and by increasing the proliferation rate and facilitating the changes required to achieve pluripotency. Whether replication is necessary for direct reprogramming is not completely clear, and may be context-dependent, however understanding the unique mode of cell cycle regulation of ES cells and its implication in pluripotency will certainly continue to be a focus of research.

5.4. Pluripotency, reprogramming and transformation

Beyond the technological implications, the results presented in this work also emphasize the relationship between reprogramming and tumorigenesis, and illuminate interesting biological processes. Most tumours have defects in the p53 and p16\(^{INK4a}\)/RB pathways, which were shown to enhance and accelerate reprogramming. It is thus tempting to speculate that this ability is necessary for tumour initiation or maintenance. The initial idea that cancer arises from de-differentiation of fully committed specialized cells has been replaced in favour of the cancer stem cell hypothesis (Reya et al., 2001). The belief is that in many cancer types, tumour growth and propagation is sustained by the so-called cancer stem cells (CSCs), which represent a subpopulation of tumour cells with the ability to self-renew and maintain tumour growth (Clarke and Becker, 2006). CSCs were first identified in leukaemias (Lapidot et al., 1994) and later shown to be crucial for certain solid tumours (Gupta et al., 2009), although their relevance for other tumours, such as melanoma, remains unclear (Quintana et al., 2008). The fact that inhibiting
tumour suppressor pathways enhances reprogramming to a more undifferentiated state suggests that alterations in the p53 or the p16^{INK4a}/RB pathways have an impact on tumorigenesis, not only by affecting proliferation, but also by contributing to reprogramming somatic cells to a more dedifferentiated state. Related mechanisms may exist, as shown by the fact that p53 loss increases the pool of CSCs by promoting the symmetrical cell divisions of cancer stem cells (Cicalese et al., 2009). Interestingly, deletion of the three RB family proteins triggers the reprogramming of MEFs to generate CSC-like cells, and cells that resemble CSCs can even be generated from MEFs that only lack RB1 if they are forced to grow beyond contact inhibition (Liu et al., 2009). Another study reported how simultaneous inhibition of RB and p53 pathways (by inhibiting Arf) in mice leads to extensive loss of differentiation of post-mitotic myocytes (Pajcini et al., 2010). These results implicate the Ink4a/Arf locus in the lack of regenerative potential in mammals, as the authors emphasise, but also highlight how inactivation of tumour suppressor pathways during tumorigenesis might lead to a more undifferentiated state.

Two studies, published during the course of this work further underscore the link between pluripotency and tumorigenesis. Aggressive, poorly differentiated tumours were shown to express high levels of ES cell-associated factors (Ben-Porath et al, 2008). Additionally, the expression of some oncogenes such as c-Myc in epithelial tumours is sufficient to reactivate an ES cell-like transcriptional signature (Wong et al., 2008). However, in a very recent study, different regulatory modules based on gene expression in ES cells, were defined: a core pluripotency module, a Myc module and a PRC module. The authors argue that the ES cell-like module identified before (Ben-Porath et al, 2008) (Wong et al., 2008) is composed mainly of Myc genes rather than core pluripotency module genes (Kim et al., 2010). Although some issues still need clarification, these observations suggest that reprogramming to a more dedifferentiated state occurs during tumour progression and might be
favoured by alterations in crucial tumour suppressors. If the acquisition of stem-cell-like properties goes hand-in-hand with tumorigenesis, it is reasonable to think that the mechanism triggered by expression of the reprogramming factors, which ultimately leads to reprogramming, elicits the tumour suppressor pathways that protect cells against uncontrolled growth. In fact, the cellular response to the expression of the reprogramming factors or stem-cell-specific genes mimics the senescence response observed during OIS, emphasizing the parallels between RIS and OIS (Fig 5.1).

Figure 5.1. Parallels between reprogramming and oncogenic transformation. (A) Aberrant oncogene expression triggers senescence (OIS) in primary cells, which limits oncogenic transformation. The expression of the reprogramming factors also triggers senescence (RIS), limiting the efficiency of reprogramming. (B) As a consequence, when senescence is disabled, cells are more susceptible to either oncogenic transformation or reprogramming. OIS, oncogene-induced senescence; RIS, reprogramming-induced senescence.
The fact that there is an intricate relationship between pluripotency and tumorigenesis suggests that some of the events that lead to reprogramming to iPS cells may overlap with the ones involved in tumorigenesis. ES cells are derived from the cells of the inner cell mass of the embryo, which are forced to grow in culture. Thus, they could represent a cell type that does not exist in nature; indeed, they share characteristics with tumour cells. ES cells, like tumour cells, grow independently of the presence of mitogenic stimuli, have low levels of proteins that are part of tumour suppressor pathways frequently inactivated in cancer and express telomerase. Crucially, one of the widely used pluripotency test, the ability to cause teratomas when cells are injected into nude mice, also reflects tumorigenic potential. Unfortunately this unwanted similarity might be somehow unavoidable. In fact, embryo-derived hES cells share with iPS cells one of the main challenges that hinder their use in a therapeutical setting. The ability to properly differentiate them and more importantly the stability of the differentiated state will dictate the safety of using hES or iPS cells for therapy as the presence of undifferentiated cells will give rise to tumours. Thus, one ongoing challenge is to learn how to achieve accurate differentiation of ES and iPS cells into specific cell types. Nevertheless, if senescence pathways that guard the genome against oncogenic mutations also guard cells against de-differentiation we have to be absolutely sure that they are intact and operating in iPS cells.

5.5. Future work: Identification of RIS mediators

5.5.1. Genetic screen for bypass of RIS

As stated before, identification of mediators of senescence induction during reprogramming might provide a safer and more efficient way of improving
reprogramming than inhibiting key tumour suppressor pathways. To set up a screen assay for bypass of RIS an inducible system for expression of the four reprogramming factors was generated. The OSKM polycistronic cassette was cloned into a retroviral vector containing a tetracyclin responsive element (pBABE TRE puromycin). For inducible expression, IMR90 cells were transduced with the retroviral vectors containing the OSKM cassette, HRAS\textsuperscript{V12} or Cherry fluorescent protein under the control of the TRE, together with a vector constitutively expressing the M2rtTA transactivator with hygromycin resistance. The inducibility and the leakiness of the system were first assessed by analysing the expression of the Cherry fluorescent protein (Figure 5.2A). Cherry expression was detected one day after treatment with doxycyclin (4 \mu g/ml) and continued to increase until day 5 post-treatment. In contrast, Cherry was not detected in the absence of doxycyclin or in the control cells (Figure 5.2A). OSKM expression led to a decrease in the percentage of cells incorporating BrdU over a time course of five days (from 50% to 10%). Similar results were observed with the RASV12 control. Furthermore, inhibition of cell growth by induction of OSKM expression was assessed by crystal violet staining of low-density plates (Figure 5.2C).

To identify genes involved in RIS a genetic screen using an shRNA library is going to be performed. IMR90 containing inducible OSKM will be transduced with a genome-wide shRNA library and maintained in parallel in the presence and absence of doxycyclin. The frequency of the shRNAs in both settings will be determined by massive parallel sequencing using next generation sequencing. Hopefully, this screening will allow identification of genes, whose loss of function leads to bypass of OSKM-induced senescence. Therefore, enrichment of shRNAs will uncovered genes that may be involved in the establishment and maintenance of the growth arrest induced by the expression of the reprogramming factors.
Figure 5.2. OSKM-inducible system for identification of RIS mediators. (A) Cells were transduced with a vector containing the Cherry fluorescent protein under the control of a TET responsive element (Cherry$^{\text{TRE}}$), and a vector constitutively expressing the M2rtTA transactivator. Selection with puromycin and hygromycin over approximately 8 days allowed isolation of double transduced cells. In the absence of doxycyclin there is no cherry expression cells whereas upon doxycyclin addition (Dox) there is an induction of the fluorescent protein. Microphotographs were acquired with 10x magnification on day 5; a DAPI/Cherry merge is shown. (B) The same system was used for inducible expression of OSKM and HRAS$^{V12}$ (OSKM$^{\text{TRE}}$ and RAS$^{\text{TRE}}$ respectively). BrdU incorporation assays show how proliferation is arrested when these cells are grown in presence of doxycyclin. (C) Crystal violet stained plates showing difference in growth in the presence of Dox in OSKM$^{\text{TRE}}$ and RAS$^{\text{TRE}}$ when compared with control cells (EV$^{\text{TRE}}$). 10$^5$ cells were plated in duplicate per 10-cm dish. Next day 4$\mu$g/ml of doxycyclin was added to one set of plates. Plates were fixed and stained with crystal violet 14 days later.
In particular it will be interesting to identify mediators that are specific to the arrest induced by the reprogramming factors. By comparing shRNAs able to bypass RIS with the ones able to bypass oncogene induced-senescence, it will be possible to avoid inhibiting genes whose activity is required for protecting cells against uncontrolled growth and transformation. Modulating the activity of these candidate genes or the pathways in which they are involved may constitute potential strategies to improve reprogramming efficiency. Additionally, it will be interesting to unravel which pathways are activated and may contribute to RIS, to further understand the link between gain of pluripotency and tumorigenesis.

### 5.5.2. SILAC analysis for identification of proteins enriched during RIS

A complementary strategy to understand which genes and pathways may regulate the senescence induced during reprogramming is to generate gene or protein expression profiles. Stable isotope labelling with amino acids in cell culture (SILAC) is a simple and efficient approach for labelling proteins for mass spectrometry-based quantitative proteomics. Early passage IMR90 cells were grown for two passages in SILAC media containing 'light' (L-Arginine and L-Lysine) or 'heavy' (H-Arginine and H-Lysine) amino acids, to allow incorporation into newly synthesized proteins. Each population was transduced with the OSKM retroviral vector and empty control vector and selected with puromycin. Seven days after transduction, cells were harvested and mixed. Mass spectrometry and peptide quantification analysis was performed by Bram Snijders in the Biomolecular Mass Spectrometry and Proteomics Laboratory (MRC Clinical Science Centre).

Functional annotation using the DAVID bioinformatics resources (database for annotation, visualization and integrated discovery) revealed that the list of proteins with L/H rations lower than 0.5 (2 times down-regulation) were enriched for protein
involved in cell cycle progression (Benjamini, 1.5x10^{-22}), mitosis (1.3x10^{-20}) and DNA replication (9.7x10^{-19}). Therefore these preliminary data reflect the arrest in cell cycle described before and validate the assay. Proteins such as Cyclin B1, DNA replication licensing factors (MCM2-7), BUB1B and the Antigen of KI-67 are among the list of down-regulated proteins. Another example is topoisomerase II alpha (TOP2A), which has also been identified as down regulated in RAS-induced senescence (Mason et al., 2004). Similarly, proteins that are up-regulated in OSKM-transduced cells, are related to signal peptide or secreted proteins (1.1x10^{-6}) and lysosome (4.7x10^{-4}). Interestingly beta galactosidase was one of the proteins enriched in the OSKM sample, as well as other lysosome-related proteins. Some of the proteins detected and over-represented in OSKM cells have been implicated before in replicative and oncogene-induced senescence: tissue inhibitor of metalloproteinase 1 and 3 (TIMP1, TIMP3), creatine kinase type-B (CKBB) and insulin growth factor binding proteins 6 and 7 (IGFBP6 and 7) (Chang et al., 2002; Shelton et al., 1999; Trougakos et al., 2006; Wajapeyee et al., 2008).

The similarly between the protein or genes expression profiles between RIS and in OIS reflects the parallelisms between these two processes, as discussed before. However, the aim will be also the identification of pathways which specifically operate during RIS, and which could be safely modulated to improve reprogramming. For this purpose it will be interesting to compare these protein profiles with the ones obtained by SILAC analysis of senescence induced by activated RAS (Juan Carlos Acosta, unpublished data) to identify RIS-specific modulators.

Inhibition of the aryl hydrocarbon receptor (AHR) has recently been described to promote of ex vivo expansion of hematopoietic stem cells (HSCs) (Boitano et al., 2010). Interestingly, AHR was found 4-fold overrepresented in OSKM cells. AHR is
normally in the cytoplasm in an inactive form. Upon ligand binding to chemicals such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), its dissociation from chaperones results in AHR translocation to the nucleus where it function as a transcription factor (Puga et al., 2002). Besides inhibition of AHR being implicated in HSCs expansion, it has been shown that knock out mice for AHR exhibit defects in hematopoiesis (Singh et al., 2010). Consequently, Singh et al. suggest that AHR has a role in maintaining proper quiescence of hematopoietic stem cells thereby restricting their excessive or unnecessary proliferation (Singh et al., 2010). Indeed, several studies suggest a role for the AHR in cell cycle control, although the precise mechanism remains ill defined. Some observations suggested that in the absence of an exogenous ligand, the AHR promotes cell cycle progression (reviewed in Marlowe and Puga, 2005). In contrast, early studies have demonstrated that treatment with AHR ligands, such as TCDD, inhibits cell proliferation. It was described that AHR binds to and synergizes with RB to repress E2F-dependent transcription therefore inducing cell cycle arrest (Puga et al., 2000). Additionally, it has been suggested that AHR silencing may be associated with cancer progression and it acts as a tumour suppressor in hepatocellular carcinoma (Fan et al., 2010). It would be interesting to determine whether inhibiting AHR has an effect in reprogramming efficiency or whether AHR has a role in RIS. The fact that this receptor is silenced in a subset of tumours and that its function is necessary to maintain normal quiescence of HSCs suggest that its inhibition might be unsafe. However several AHR antagonist have been described (Boitano et al., 2010; Prud'homme et al., 2010) that would allow transient inactivation of the receptor without permanently compromising its activity.

Although a total of 5710 proteins have been detected, the sensitivity of this technique is limited. However, a total of 225 proteins were found to be more than 2 fold up-regulated while 242 proteins were 2-fold down-regulated. Before trying to understand their link with RIS, additional work is being performed to validate these
results. Reverse labelling will be performed and the mass spectrometry and peptide quantification analysis will be repeated. Additionally, analysis of the protein profiles in low serum conditions could enrich for proteins specific of the OSKM-induced arrest and discard the ones over-represented as a consequence of the difference in proliferation rates in the two populations.

5.6. Concluding remark

The study of reprogramming to pluripotency has yielded some mechanistic clues about how this process works, which have been used to improve the technology. Unexpectedly, the study of the reprogramming process is also revealing new information on, for example, the links between pluripotency and transformation. Perhaps the study of reprogramming, which has mostly been seen as a markedly artificial process with promising applications will also increase our understanding of other crucial aspects of basic biology (Ramalho-Santos, 2009).

The fact that the same alterations that drive oncogenic progression can influence the reprogramming of somatic cells to iPS cells supports the view that tumour suppressor pathways involved in protecting cells against uncontrolled cell growth, can also have a role in protecting cells against dedifferentiation. In this regard inactivation of these pathways could be involved in the creation of CSCs, described to support the progression of specific cancer types. Furthermore, it suggests that derivation of safe iPS cells could present enormous challenges. Hopefully, the high pace and quality of cancer and stem cell biology research will soon provide answers for all these questions.
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## Appendix

### Table A1. Retroviral constructs.

| Vector  | cDNA       | Antibiotic resistance |
|---------|------------|-----------------------|
| pBABE   | Empty Vect. | puromycin             |
| pBABE   | OSKM       | puromycin             |
| pBABE   | Sox2       | puromycin             |
| pBABE   | Oct4       | puromycin             |
| pBABE   | Klf4       | puromycin             |
| pBABE   | c-Myc      | puromycin             |
| pBABE   | Nanog      | puromycin             |
| pLPC    | Cherry     | puromycin             |
| pBABE   | GFP        | puromycin             |
| pBABE   | RAS V12    | puromycin             |
| MSCV    | Cbx7       | puromycin             |
| LXSN    | Empty Vect. | neomycin             |
| LXSN    | E6         | neomycin             |
| LXSN    | E7         | neomycin             |
| LXSN    | E6/E7      | neomycin             |
| pRS     | Empty Vect. | puromycin             |
| pRS     | sh-hp53    | puromycin             |
| pRS     | sh-hp16    | puromycin             |
| pRS     | sh-hp21    | puromycin             |
| MLP     | sh-mp53    | puromycin             |
| MLP     | sh-mp16/Arf | puromycin         |
| pRS     | sh-mp21    | puromycin             |
| pRS     | sh-hp53    | hygromycin            |
| pRS     | sh-hp16    | hygromycin            |
| pRS     | sh-hp21    | hygromycin            |
| MSCV    | hTR211     | blasticidin           |
| MSCV    | 302a-d     | blasticidin           |
| MSCV M2rtTA | M2rtTA      | hygromycin            |
| pBABE TRE OSKM | OSKM     | puromycin             |
Table A2. Plasmids acquired from Addgene

| Vector | cDNA       | Antibiotic resistance | Addgene ref. |
|--------|------------|------------------------|--------------|
| pMXs   | Oct4       | -                      | 13366        |
| pMXs   | Sox2       | -                      | 13367        |
| pMXs   | Klf4       | -                      | 13370        |
| pMXs   | Myc T58A   | -                      | 13372        |
| pMXs   | Nanog      | -                      | 13354        |
| FUW    | Tet-OSKM   | zeocin                 | 20321        |
| FUW    | M2rtTA     | zeocin                 | 20342        |

Table A3. shRNA target sequences.

| Gene      | Target sequence                      |
|-----------|--------------------------------------|
| INK4a     | GAGGAGGTGCGGCGGCGCTGC                |
| CDKN1a    | TCCCAATGCTGAATATACA                  |
| hp53      | GTAGATTACCACTGGAGTC                  |
| Cdkn1a    | CTCCAGTCTCCAAACTTTAAA                |
| mp53      | CACTACAAGTACATGTGTA                  |
| Ink4a/Arf | CCGCTGGGTGCTTTGTGT                   |
### Table A4. Primers for cloning

| Primer      | Sequence (5' to 3')                                               |
|-------------|-------------------------------------------------------------------|
| Sox2 BamHI  | GGGATCCATGTATAACATGATGGAGACGG                                     |
| Sox2 Sal I  | GGTCGACTCACATGTGCGACAGGG                                          |
| Nanog BamHI | GGAGATCCATGAGTGTGGGTCTTCCTGG                                      |
| Nanog Sal I | GGTCGACTCATATTTCACCTGGTGAGTC                                      |
| Oct4 Bgl II | GGAGATCTATGGCTGGACACCTGGGACACCTGGCTTCAG                           |
| Oct4 Sal I  | GGTCGACTCATTTTGAAATGCATGGGAGAGCC                                  |
| M2rtTA BamHI| GGGATCCATGTCTAGACTGGACAAGAG                                       |
| M2rtTA Sal I| GGTCGACTTACCCGGGAGCAGCATGTCAA                                     |

### Table A5. Sequencing primers.

| Primer      | Sequence (5' to 3')                                               |
|-------------|-------------------------------------------------------------------|
| M13 F (-20) | GTAAACGAGCAGCCAG                                                  |
| M13 R       | CAGGAAACAGCTATGAC                                                 |
| pBABE F     | CTTTATCCAGCCCTCAC                                                 |
| pBABE R     | ACCCTAACTGACACACATTC                                              |
| pMSCV F     | CCGCTTGAACCTTCTCGTACC                                             |
| pMSCV R     | GAGACGTGCTACTTCCATTTGTCC                                         |
| pRS F       | GCTGAGCTCATCAACCCGCT                                              |
| pRS R       | TGCGGAGGAGGAGGAG                                                  |
| TOPO II F   | CGAGCTCGGATCCACTAGTAA                                             |
| TOPO II R   | CGGCCAGTGAAATGTGAATACAG                                           |
Table A6. RT-qPCR primers.

| Target gene | Sequence (5' to3') |
|-------------|--------------------|
| **RPS14**   | TCACCCGCCCTACACATCAAAACT  
            | CTGCAGTGCTGTCAGAGG     |
| **P53**     | CCGCAGTCAGATCTCTAGCG   
            | AATCATCATCTGGTGACGGCCT |
| **INK4**    | CGGTCGGAGGCCGATCCAG    
            | GCACGGGTGGAGCAGACGTGCTT |
| **CDKN1a**  | GCTTTCGGAGTGTTGGAGAATCT |
| **Rps14**   | CACGACTCTCTCCCTCACCTA  
            | AATCATCCAGTGCAGCTT |
| **p53**     | AACTGCAGAGGCAGCAGTCTT  
            | GTGTGCATGACGTGCAATC |
| **Ink4a/Arf** | GCAATTCGACTCTGCAACGTGAG |
| **Cdkn1a**  | CTCGATGTCGGCAGACGTCTT  
            | CCATGAGCAGCATCAGCTT |
| **Endogenous OCT4** | CAGGTTTTTGCTCTACCTAGCT |
| **Exogenous OCT4** | CTCGATGGTCTCCCTCACCTA |
| **Endogenous SOX2** | ATGTCCCAGCACAGCTAGCT |
| **Exogenous SOX2** | CAGGATGACACTGCCAGCCTA |
| **Exogenous-KLF4** | TCCTGCTTCTACAAATGGGACT |
| **Exogenous-MYC** | GCATAGTGTGGATCCAGCCTT |
| **NANOG**   | GCTGAAATAGCCGATCATGG   
            | GTAAGAGCTTGTGGCCAGAAA |
| **DNMT3B**  | AGGTTCGAGACGTGCTT      
            | GCCAGCATCATCAGAACC |
| **hTERT**   | CTGACAGATGACGTGCCTC    
            | GCCTAGCGAAATGACTGCA |
| **REX1**    | CAGCATCTAAACAGCTTCGAGAAT |
### Table A7. TaqMan® Gene Expression Assays (Applied Biosystems)

| Target gene | Catalogue n. |
|--------------|--------------|
| GAPDH        | 4333764F     |
| JMJD3        | Hs.00389749-m1 |

### Table A8. TaqMan® microRNA Assays (Applied Biosystems)

| Target miRNA | Catalogue no. | Mature microRNA sequence/target sequence |
|--------------|---------------|-----------------------------------------|
| RNU48        |               | GAUGACCCCAGGUAAACUGAGUGUGUCGCUG         |
|              |               | AUGCCAUCCGCACGCAGCCGACUGACC             |
| hsa-miR-302a | 529           | UAAGUGCUUCAAUGUUUUGGUGA                 |
| hsa-miR-302b | 531           | UAAGUGCUUCAAUGUUUAGUAG                  |
| hsa-miR-302c | 533           | UAAGUGCUUCAAUGUUUCAGUGG                |
| hsa-miR-302d | 535           | UAAGUCUUCAAUGUUUGAGUGU                 |
| has-miR-372  | 560           | AAAGUGCUUGCAUUUGACGGU                 |
| has-miR-373  | 561           | GAAGUGCUUCAUUGGGGUGU                 |
### Table A9. Primary antibodies.

| Target                  | Clone | Company        | Catalogue no. | Source |
|-------------------------|-------|----------------|----------------|--------|
| 8-Oxoguanine            | 483.15| Millipore      | MAB3560        | mouse  |
| γH2AX                   | JBW301| Millipore      | 05-636         | mouse  |
| BrdU                    | –     | Invitrogen     | A21303         | mouse  |
| H3K27me3                | poly  | Millipore      | 07-449         | rabbit |
| p16**INK4**a            | JC-8  | CRUK           | –              | mouse  |
| p21**Cip1**             | CP74  | Sigma          | P 1484         | mouse  |
| p21**Cip1**             | poly  | Abcam          | ab18209        | rabbit |
| p53                     | DO-1  | Santa Cruz     | sc-126         | mouse  |
| P-(Ser/Thr)Q            | poly  | Cell Signaling | 2851           | rabbit |
| Cleaved caspase3        | 5A1E  | Cell Signaling | 9664           | rabbit |
| JMJD3                   | poly  | Aviva Systems Biology | ARP40102 | rabbit |
| c-MYC                   | poly  | Santa Cruz     | sc-764         | rabbit |
| Nanog                   | poly  | Abcam          | ab80892        | rabbit |
| Oct4                    | C-10  | Santa Cruz     | sc-5279        | mouse  |
| Klf4                    | poly  | Abcam          | ab34814        | rabbit |
| Sox2                    | poly  | Abcam          | ab15830        | rabbit |
| Gata 6                  |       | Abcam          | ab22600        |        |
| Sox1                    | poly  | R&D Systems    | AF3369         | goat   |
| Brachury                | poly  | R&D Systems    | AF2085         | goat   |
| Sox17                   | poly  | R&D Systems    | AF1924         | goat   |
| Cdx2                    | CDX2-88 | BioGenex      | MU392A-UC     | mouse  |
| Pax6                    | poly  | Covance        | PRB-278P       | rabbit |
| TRA-1-60                | -     | Santa Cruz     | sc-21705       | mouse  |
| TRA-1-81                | -     | BD Pharmingen  | 560072         | mouse  |

### Table A10. Secondary antibodies.

| Target   | Label   | Company      | Catalogue no. | Source |
|----------|---------|--------------|---------------|--------|
| mouse    | Alexa Fluor® 488 | Invitrogen | A11029        | goat   |
| mouse    | Alexa Fluor® 594 | Invitrogen | A11032        | goat   |
| rabbit   | Alexa Fluor® 488 | Invitrogen | A11034        | goat   |
| rabbit   | Alexa Fluor® 594 | Invitrogen | A11037        | goat   |
**Table A11. Antibodies for ChIP.**

| Target       | Clone | Company     | Catalogue no. | Source |
|--------------|-------|-------------|---------------|--------|
| EZH2/KMT6    | poly  | Abcam       | ab3748        | rabbit |
| H3K27me3     | poly  | Diagenode   | CS-069-100    | rabbit |
| H3K4me3      | poly  | Diagenode   | pAb-003-050   | rabbit |
| Histone H3   | poly  | Abcam       | ab1791        | rabbit |
| RNA Pol II   | 4H8   | Abcam       | ab5408        | mouse  |

**Table A12. Primers for ChIP.**

| Position | Sequence                                                                 | Location                                                                 |
|----------|--------------------------------------------------------------------------|--------------------------------------------------------------------------|
| PS2      | F-GTGGGTCGCCAGTCTGCAGTTA R-CCTGTTGCAACCAGAGTGGAG                         | p14<sup>ARF</sup>, exon 1                                               |
| PS3      | F-GGAGCGATGATCGTCCGTTATC R-TGAAATCCTCCAGTTCCAAGGCTATGCC                  | 4.5 kb downstream of p14<sup>ARF</sup> start site                        |
| PS4      | F-GCACTTGCCCTCCAGGTATA R-TGATAGTTCAAGGCTATGCC                             | 15 kb downstream of p14<sup>ARF</sup> start site                        |
| PS5      | F-CTCAAAAGGGGATAATTCAGAGACG R-AAAGCTTAAAGAAGCTCCAGACAC                   | 1 kb upstream of p16<sup>INK4a</sup> promoter                           |
| PS6      | F-ACCCCGATTCAGTTTGCCAG R-AAAAAGGAAATCCGGCCCCG                             | 0.2 kb upstream of p16<sup>INK4a</sup> promoter                          |
| PS8      | F-GCCAGGGAGAGGAGATGAGAGAG R-CCTGAGTTGACATCCAGTCATTGG                     | 0.2 kb downstream of p16<sup>INK4a</sup>, exon 1                         |
| PS11     | F-TAGGAGGAGGCTTCCAGTACATAC R-TGAGTTGAGTCCAGAGA                            | 0.8 kb downstream of p16<sup>INK4a</sup>, exon 3                         |
| CCND1    | F-CACCCAGCTACAGGGGAGTTTG R-TTCCACTTCGCACAGAGGAG                          | 0.1 kb downstream of CCND1 promoter                                      |
Figure A1. Cell cycle profiles. For cell cycle analysis, cells were fixed and stained with propidium iodide as described in 2.3.4. Cells were analysed using Guava® microcapillary flow cytometry system (Millipore), and the Guava Cell Cycle Software. Cell cycle profiles for empty vector control (pBABE) and OSKM are presented.
Publications

Parts of the work presented here have been published as follow:

Banito, A., and Gil, J. (2010). Induced pluripotent stem cells and senescence: learning the biology to improve the technology. EMBO Rep 11, 353-359.

Banito, A., Rashid, S.T., Acosta, J.C., Li, S., Pereira, C.F., Geti, I., Pinho, S., Silva, J.C., Azuara, V., Walsh, M., et al. (2009). Senescence impairs successful reprogramming to pluripotent stem cells. Genes Dev 23, 2134-2139.