The brevity of G1 is an intrinsic determinant of naïve pluripotency

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THE BREVITY OF G1 IS AN INTRINSIC DETERMINANT OF NAÏVE PLURIPOTENCY

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RESUME

La brièveté de la phase G1 est une caractéristique fondamentale de l'état naïf de pluripotence

Les cellules souches embryonnaires (cellules ES) sont capables de se multiplier de façon autonome en l'absence de facteurs de croissance et de cytokines, un état appelé "état fondamental de pluripotence". Le cycle cellulaire des cellules ES se caractérise : (*i*) par une expression élevée et uniforme de la cycline E et des complexes Cycline E-CDK2 au cours de la progression dans le cycle cellulaire, contrairement à l'expression périodique atteignant un maximum lors de la transition G1/S observée dans les cellules somatiques, et (*ii*) par une phase G1 très courte (1 heure) dont la traversée ne dépend ni des MAPK ni des points de contrôles régulés par la protéine du rétinoblastome (RB) et p53.

Ces observations soulèvent la question de l'existence d'un lien de cause à effet entre ce phénomène de réplication autonome et la pluripotence. Afin d'y répondre, mon projet de thèse se construit autour de trois axes qui montrent que :

1/ la phase G1 des cellules ES de souris est une phase de sensibilité accrue aux inducteurs de différenciation. Dans ce cas, un raccourcissement de la phase G1 protègerait les cellules ES des signaux déclenchant la différenciation et faciliterait l'autorenouvellement. Le système rapporteur FUCCI ("Fluorescent Ubiquitination-based Cell Cycle Indicator") nous a permis de trier des cellules ES naïves rex1+ de souris en fonction de leur position dans les phases G1, S ou G2 du cycle et d'étudier ensuite la sensibilité de ces populations aux inducteurs de la différenciation dans un test d'autorenouvellement appelé "LIF-rescue assay";

2/ la balance entre autorenouvellement et différenciation est perturbée, (*i*) quand l'expression de la cycline E est altérée, ou (*ii*) quand l'association de la cycline E avec la kinase CDK2 et le centrosome est bloquée. La réduction de l'expression de la cycline E provoque un ralentissement des divisions cellulaires, en particulier un rallongement de la phase G1 et altère l'expression des marqueurs de pluripotence et de différenciation. De la même façon, des cellules ES E1-/-E2-/- ont une faible capacité à s'autorenouveller. D'autre part, l'expression de la Cycline E WT dans les cellules E1-/-E2-/- restaure le phénotype sauvage. De plus, les cellules surexprimant de façon inductible la cycline E WT sont plus résistantes à la différenciation induite par la privation de LIF et cet effet semble dépendant de la fixation à CDK2. Ainsi, la Cycline E participe au contrôle de la pluripotence.

3/ La signalisation par le LIF contrôle la formation et l'activation des complexes Cycline E/CDK2. Dans les cellules ES naïves Rex1+, l'allongement de la durée de la phase G1 induit par la privation de LIF précède, ou est concomitante, à la diminution de

l'expression de marqueurs de pluripotence et à l'activation des marqueurs les plus précoces de la différenciation

Nos résultats suggèrent que l'allongement de G1 résulte en grande partie de l'inhibition réversible des mécanismes de la transition G1/S, et dans une moindre mesure d'un engagement irréversible dans la différenciation. Finalement, nous proposons un modèle dans lequel la signalisation par le LIF régule la transition G1/S et permet le maintien de l'autorenouvellement des cellules ES murines.

Mots clés : Cellules souches embryonnaires, phase G1, pluripotence, Cycline E

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ABSTRACT

The brevity of G1 is an intrinsic determinant of naïve pluripotency

Pluripotency can be captured and propagated *in vitro* from the epiblast of the pre-implantation blastocysts in the form of embryonic stem cells (ESCs). ESCs are capable of unlimited proliferation in an undifferentiated state while maintain the potential to differentiate into cells of all three germ layers in the embryo, including the germline.

Two key features the ES cell mitotic cycle are (i) a vastly elevated and uniform expression of Cyclin E and Cyclin E/CDK2 complexes throughout the cell cycle, as opposed to the oscillatory expression peaking at the G1/S transition described in somatic cells, and (ii) a short G1 phase characterized by the lack of RB- and p53-dependent checkpoints, and reduced dependency on MAPK signalling. During my PhD project, we explored whether and how the regulation of the cell cycle actively sustains self-renewal of mouse ESCs (mESCs). We demonstrated that:

1/ the G1 phase of mESCs is a phase of increased susceptibility to differentiation inducers. Thus shortening of G1 might shield undifferentiated cells from differentiation inducers and help ESCs to self-renew in the pluripotent state. Moreover, CDK2 kinase activity prior to S phase entry is critical for the maintenance of pluripotency.

2/ Knockdown of cyclin E expression results in lowering the proliferation rate, lengthening the G1 phase, and dramatically accelerating the kinetics of differentiation induced with embryoid bodies (EB). Similarly, mESCs harboring a double inactivation of cyclin E1 and E2 (*E1-/-E2-/- null*), display reduced self-renewal abilities in a colony-forming assay. The phenotype of *E1-/-E2-/- null* mESCs could be rescued by overexpressing human cyclin E. Surprisingly, overexpression of a kinase deficient mutant of human cyclin E also rescued the wild-type phenotype. Together, these results suggest that Cyclin E opposes differentiation and supports self-renewal of mESCs by two independent mechanisms, one of which being independent of CDK2 activation.

3/ LIF signalling regulates Cyclin E/CDK2 kinase activity therefore accelerating the G1 to S phase transition.

Finally, we propose a model in which LIF signalling stimulates the G1 to S phase transition to shield mESCs from undesired differentiation signals and help them to self-renew in the pluripotent state.

Key words: embryonic stem cells, G1 phase, pluripotency, Cyclin E

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List of abbreviations

APC	Anaphase Promoting Complex
ATM	Ataxia-Teleangiectasia Mutated
ATR	ATM and Rad3-related
BMP	Bone Morphogenetic Protein
CAK	CDK Activating Kinase
Cdc	Cell division cycle
Cdh1	CDC20 homolog 1
CDK	Cyclin Dependent Kinase
Cdt1	Chromatin licensing and DNA replication factor 1
ChIP	Chromatin ImmunoPrecipation
Cip/Kip	CDK interacting protein/Kinase inhibitory protein
CKI	CDK Inhibitor
CLS	Centrosomal Localization Signal
E	Embryonic day
EB	Embryoid Body
EC	Embryonal Carcinoma
Emi1	Early mitotic inhibitor 1
EpiS	Epiblast Stem
ERK	Extracellular Regulated MAP Kinase
ES	Embryonic Stem
FACS	Fluorescence Activating Cell Sorting
FGF	Fibroblast Growth Factor
FUCCI	Flurorescent Ubiquitination-based Cell Cycle Indicator
GSK3β	Glycogen Synthase Kinase β
hES	human Embryonic Stem

ICM	Inner Cell Mass
INK	Inhibitor of Kinase
IR	Ionizing Radiation
Id	Inhibition of differentiation
Klf	Krüppel-like factor
LIF	Leukemia Inhibitory Factor
mAG	monomeric Azami Green
MAPK	Mitogen Activated Protein Kinase
MCM	Mini Chromosome Maintenance
MEF	Mouse Embryonic Fibroblast
MEK	Mitogen activated protein ERK Kinase
mES	mouse Embryonic Stem
miRNA	microRNA
mKO2	monomeric Kusabira Orange 2
mRNA	messenger RNA
PI	Propidium Iodide
PI3K	Phosphatidyl Inositol 3 Kinase
RA	Retinoic Acid
RISC	RNA- Induced Silencing Complex
Rb	Retinoblastoma protein
TGF	Transforming Growth Factor
siRNA	short interfering RNA
SCF	Skp, Cullin, F box containing complex
Skp2	S phase kinase associated protein 2
STAT3	Signal Transducer and Activator of Transcription 3

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Chapter one:

INTRODUCTION

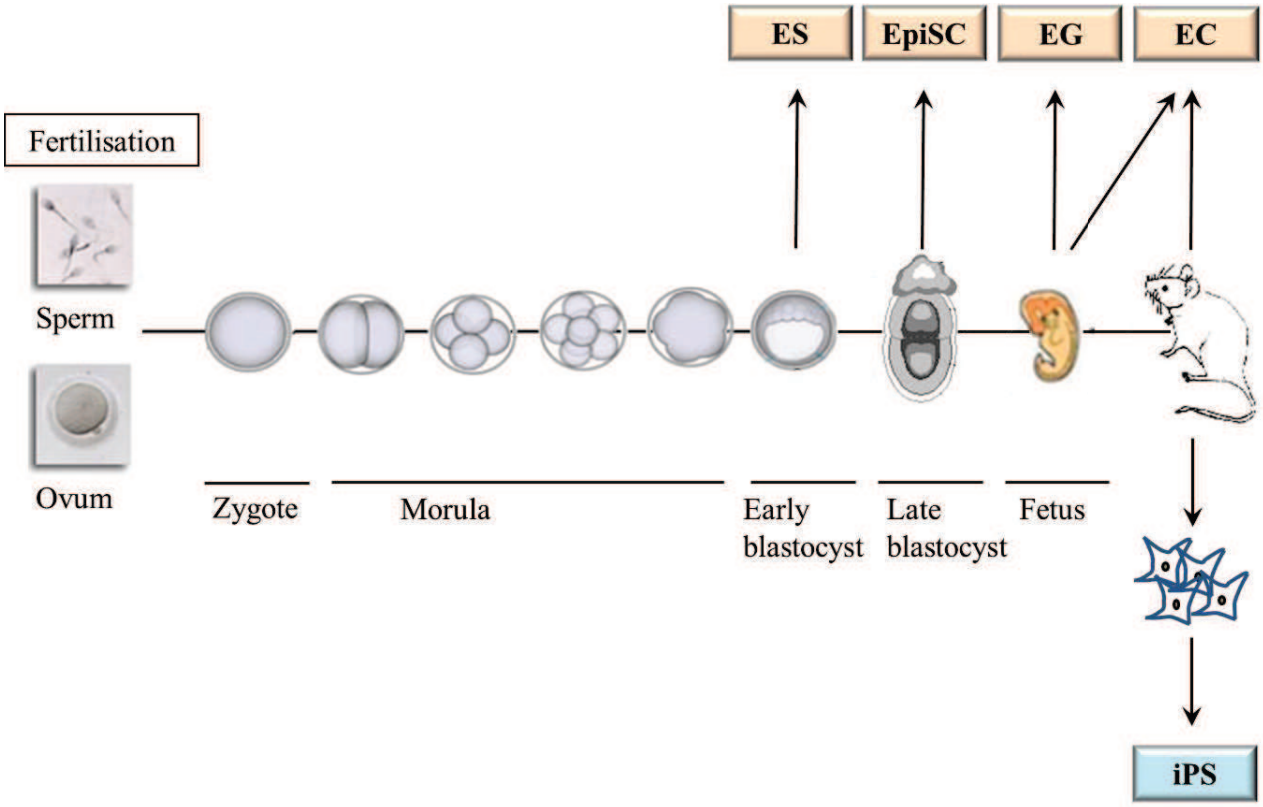

Figure 1: Mouse pluripotent stem cells and their tissue of origin

Embryonal Carcinoma (EC) cells are derived from teratocarcinomas, the Embryonic Stem Cells (ESCs) from the epiblast of early blastocysts, the Epiblast Stem Cells (EpiSCs) from the epiblast of late blastocysts, the induced Pluripotent Stem (iPS) cells from somatic cells forced to reprogrammed, the Embryonic Germ (EG) cells from a specific part of the late embryo called the gonad ridge, the parthenogenetic Embryonic Stem (pES) cells from parthenogenetic blastocysts and the nuclear transferred Embryonic Stem (ntES) cells from blastocysts produced by transfer of a somatic nuclei into an oocyte. See the text for more details.

1 ORIGIN AND PROPERTIES OF EMBRYONIC STEM CELLS

1.1 Definition

Established embryonic stem cell lines are derived from the epiblast of pre- or post-implantation embryos. They are capable of unlimited proliferation in an undifferentiated state while maintain the potential to differentiate into cells of all three germ layers in the embryo, including the germline.

1.2 Derivation

There are several different types of pluripotent stem cells that differ in terms of the sources from whence they are derived. These are the Embryonal Carcinoma (EC) cells, the Embryonic Stem Cells (ESCs), the Epiblast Stem Cells (EpiSCs), the induced Pluripotent Stem (iPS) cells, the Embryonic Germ (EG) cells, the parthenogenetic Embryonic Stem (pES) cells and the nuclear transferred Embryonic Stem (ntES) cells **(Figure 1)**.

Pluripotent stem cell lines were originally isolated from mouse teratocarcinomas which are testicular malignant tumors constituted of a wide type of differentiated cells as well as pluripotent Embryonal Carcinoma cells (EC cells) (Finch and Ephrussi, 1967). EC cells are undifferentiated cells that can be propagated in culture and that retain the capacity at single-cell level to reform multidifferentiated tumors (Kleinsmith and Pierce, 1964, Kahan and Ephrussi, 1970). They typically display high karyotypic instability (Andrews et al., 2005). EC cells are absent from teratomas, the related benign tumors, proving that malignancy is the property of the EC cells (Smith, 2001).

EC cells share common characteristics with ICM cells of the early stage mouse blastocyst. Indeed, embryos prior to gastrulation as well as grafts containing epiblast transplanted into to adult mice can also produce teratocarcinomas (Solter et al., 1970; Stevens, 1970). In a similar manner, it was found that some EC cell lines can participate to embryogenesis when injected into a blastocyst (Brinster, 1974, Papaioannou et al., 1975). Thus EC cells can remain receptive to cues in the embryo microenvironment in an equivalent manner to resident epiblast cells.

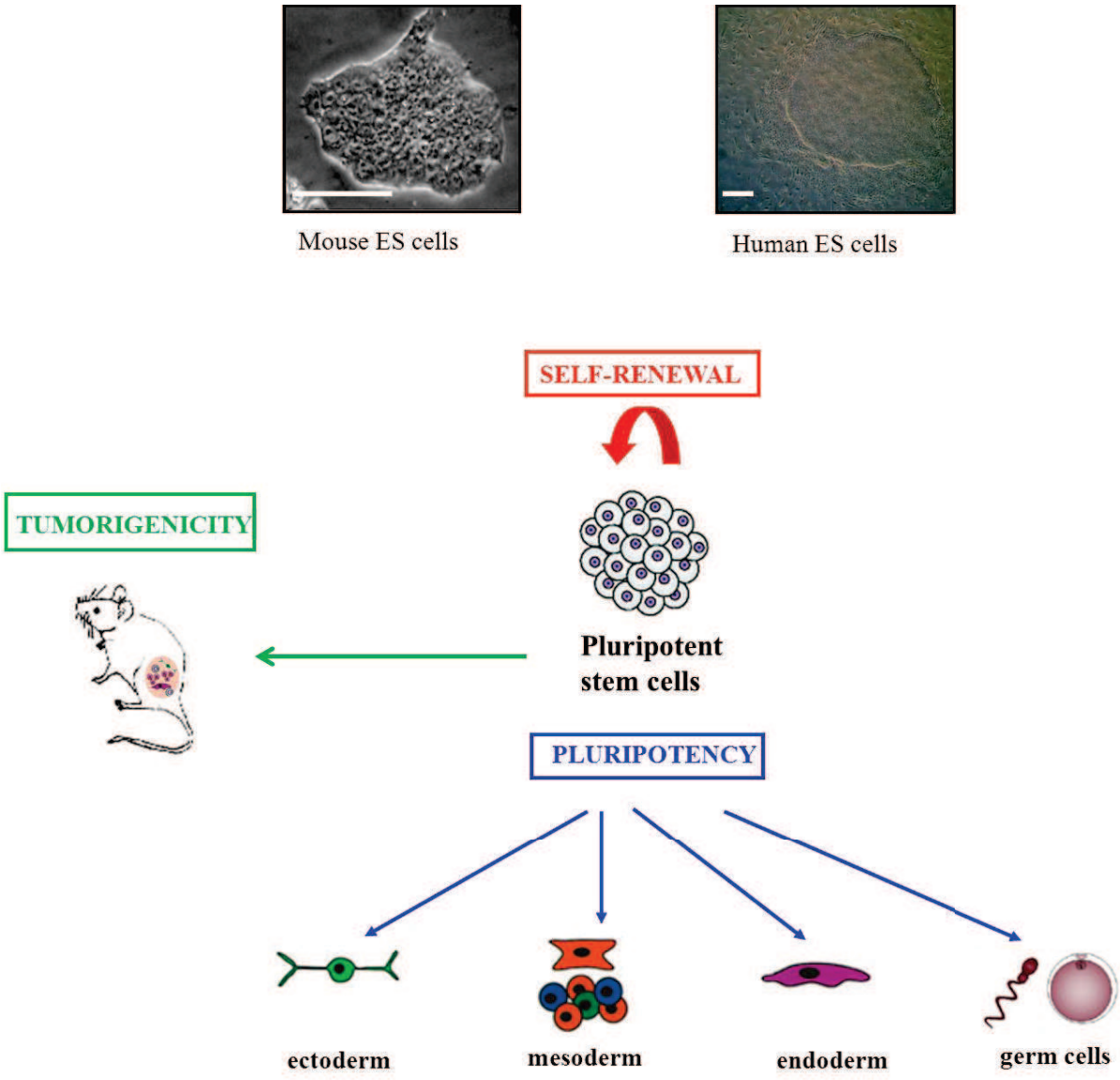

Figure 2: Pluripotent stem cells main properties

Pluripotent stem cells can sustain unlimited proliferative potential in the undifferentiated state while maintaining karyotypic stability. In addition, they display the properties of tumorigenicity and pluripotency, that is to say the capacity to give rise to the three germ layers: ectoderm, mesoderm, endoderm, as well as germ cells.
The scale bar represents 500 µm.

Inspired by studies of teratocarcinomas cells, in 1981, the first ES cell lines were successfully isolated from the early epiblast of the pre-implantation embryo (Evans and Kaufman, 1981; Martin, 1981). This was enabled by the development of appropriate culture conditions involving a mouse embryonic fibroblasts (MEF) feeder layer (Martin and Evans, 1975). This pioneering work culminated in the isolation of pluripotent cell lines from rhesus monkey blastocysts in 1995 and from human blastocysts in 1998 (Thomson et al., 1995; Thomson et al., 1998).

Recently, an additional type of pluripotent cells was derived from the post-implantation epiblast of murine embryos, termed epiblast stem cells (mEpiSCs) (Brons et al., 2007; Tesar et al., 2007). The culture conditions employed were the same as for human blastocysts. mEpiSCs can also be obtained by differentiation of mESCs (Guo et al., 2009). Post-implantation epiblast-derived stem cells represent a unique experimental model for determining whether distinction between mouse and human ESCs reflect species differences or diverse temporal origins.

Despite example from amphibians (Casimir et al. 1988), there has been little evidence to directly support the possibility of cellular dedifferentiation in mammalian cells until recently. Yamanaka and colleagues created induced pluripotent stem cells via a process of direct reprogramming from mouse (Takahashi and Yamanka, 2006) and human (Takahashi et al., 2007) somatic cells. In the initial description, iPS cells were obtained by forced and transient expression of four regulatory transcription factors. This dedifferentiation process of the somatic genome into an embryonic-like pluripotent state remains low and inefficient. In spite of this, it represents a preferred alternative for reprogramming compared to somatic cell nuclear transfer and somatic-cell fusion (Zhao et. al, 2009).

1.3 Properties of ESCs

ES cells are characterized by three cardinal properties: pluripotency, self-renewal and tumorigenicity **(Figure 2)**.

1.3.1 Pluripotency

Totipotent cells have the ability to differentiate into all cell types of an entire organism including the trophoblast lineage. Only the zygote and the blastomeres of the early morula retain this ability (Kelly, 1977).

Pluripotency refers to the capacity of a single cell to generate in a flexible manner all cell lineages of the developing and adult organism excluding the trophoblast lineage. mESCs, as epiblast cells from which they derive, are pluripotent.

mESCs are the ES cell reference in that (*i*) they can be propagated indefinitely *in vitro* while retaining genomic stability and pluripotency, and (*ii*) following injection into host blastocysts and reimplantation of those blastocysts into foster mothers, they contribute to the development of all three germ layers. These reconstituted embryos give rise to chimeric animals harboring ES cell progeny in all organs and tissue types, including the germline (Bradley et al., 1984). Thus ESCs provide a crucial tool for manipulating mouse embryos to study mouse genetics, development and physiology.

In vitro, pluripotent cells are capable of multilineage differentiation in response to particular growth conditions. A standard test to assess pluripotency is the induction of differentiation by culturing cells in suspension without self-renewal signals. Pluripotent cells spontaneously from 3D structures that mimic early embryonic development. These cell aggregates called embryoid bodies (EB) are made up of cells from all three germinal layers: endoderm, ectoderm and mesoderm (Keller, 1995).

When injected into immunosuppressed mice, mESCs formed teratomas, which are benign growths that contain tissues representative of all three germlayers. *In vivo*, the strict proof of pluripotency is the tetraploid complementation which is a technique aggregating a tetraploid eight-cell stage embryo with cells for testing. Resulting embryos are chimera, which originate from cells aggregated with tetraploid embryo. Tetraploid embryos contribute

only to extraembryonic tissues. mESCs exhibit this property. Based on the previous observations mESCs are considered to be the *in vitro* counterparts of the early epiblast.

The primate (human and non-human) ES cells fulfill the criteria of pluripotency (Pera et al., 2000, Wianny et al., 2008). However, it must be stressed that pluripotency in primate ES cells has been evaluated solely on the basis of their ability to differentiate into a large variety of cell types *in vitro*. The demonstration that monkey ES cells, like their murine counterparts, can produce chimaeras *in vivo* is still lacking. This demonstration cannot be done in Humans for ethical reasons (Smith, 2001).

Although mEC cells express some markers common with ICM cells, they have a lower potential for multilineage differentiation. When grown in suspension, mEC cells form EB and when injected into recipient mice, they differentiate forming teratocarcinomas (Evans, 1972). When injected into a mouse blastocyst they can differentiate into several cellular types but cannot participate in the establishment of germ cells (Papaioannou et al., 1975) and they do not contribute to development of a whole embryo after tetraploid complementation (Nagy et., 1993). Human EC cell lines vary in their capacity to differentiate and in their growth requirements in vitro (Andrews, 1998).

mEpiSCs generate derivatives of the three germ layers upon EB-induced differentiation. They can also give rise to differentiated teratomas but and are not able to contribute to blastocyst chimeras. This observation can be attributable to a developmental asynchrony between epiblast stem cells and the pre-implantation embryo context (Brons et al., 2007). mEpiSCs have been propose to represent the *in vitro* counterpart of primed epiblast (Nichols and Smith, 2009).

Pluripotency can be recreated by transcription factor-induced somatic cell reprogramming. iPS cells resemble ESCs in morphology, gene expression profiles and epigenetic status. Remarkably, iPS cells display the ability to form teratomas and to generate germline competent chimeras (Wernig et al., 2007; Okita et al., 2007). More importantly, iPS cells can produce viable mice through tetraploid complementation (Zhao et al., 2009).

1.3.2 Self-renewal

Self-renewal is defined as the potential for unlimited proliferation that enables cell symmetrical division without senescence or differentiation. Moreover, in appropriate culture conditions, pluripotent stem cells can be propagated in the undifferentiated state while maintaining karyotypic stability (Smith, 2001).

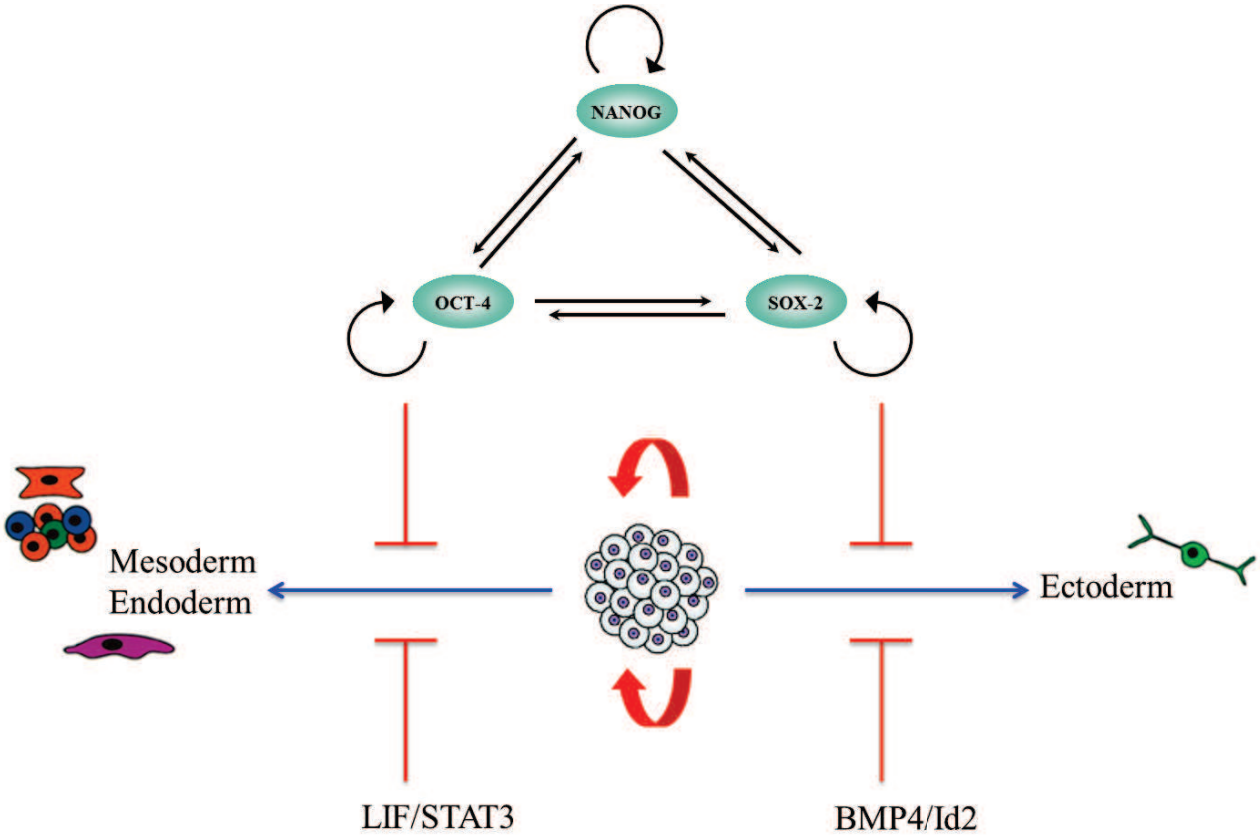

Figure 3: Self-renewal in mouse ES cells

LIF and BMP4 are extrinsic factors maintaining mESCs self-renewal. LIF inhibits differentiation into mesoderm and endoderm via activation of the transcription factor STAT3. BMP4 inhibits differentiation into ectoderm lineage trough activation of the Id2 gene.

Nanog, Oct4 and Sox2 compose the core regulatory network of intrinsic factors. They cooperate with each other and form a positive autoregulatory circuit to regulate ES cell self-renewal and pluripotency in both mouse and human pluripotent stem cells.

1.3.3 Tumorigenicity

Pluripotent embryonic stem cells are derived without transformation or immortalization; they do not undergo either senescence or cell cycle arrest or quiescence. In fact, they can proliferate without apparent limit. They can multiply in the absence of mitogenic signal and are not subject to contact inhibition or anchorage dependence. When injected into immunodeficient mice, teratocarcinomas develop with derivatives of all three germ layers (Burdon et al., 2002). Moreover, ESCs and EC cells display high telomerase activity which maintains telomere length (Stewart et al., 2006). These features are common to transformed cells. What distinguishes ESCs from cancer cell is that the former retain a stable diploid karyotype and a stable phenotype.

1.4 Extrinsic and intrinsic mechanisms sustaining self-renewal and pluripotency

Maintenance of self-renewal and pluripotency relies on stimulation of pluripotent stem cells with growth factors to activate latent signaling pathways, and on the activation of transcription factors and cell cycle regulators. The mechanisms involve the control of proliferation, the inhibition of differentiation, senescence and apoptosis.

1.4.1 Growth factors and signalling pathways

mESCs derivation and propagation is sustained by (i) the cytokine Leukaemia Inhibitory Factor (LIF) provided by either co-culture with a feeder layer of mitotically inactivated mouse fibroblast (Rathjen et al., 1990) or by supplementing the culture medium with LIF (Smith et al., 1988, Williams et al., 1998) and by (ii) foetal calf serum that can be replaced by addition of Bone Morphogenetic Protein (BMP) 4 (Ying, 2003). Inhibition of the LIF pathway results in differentiation into mesoderm and endoderm (Niwa et al., 1998), and inhibition of the BMP4 pathway induces differentiation into ectoderm (**Figure 3**) (Ying, 2003). On the contrary, the majority of EC lines can self-renew in the absence of LIF or of a feeder layer.

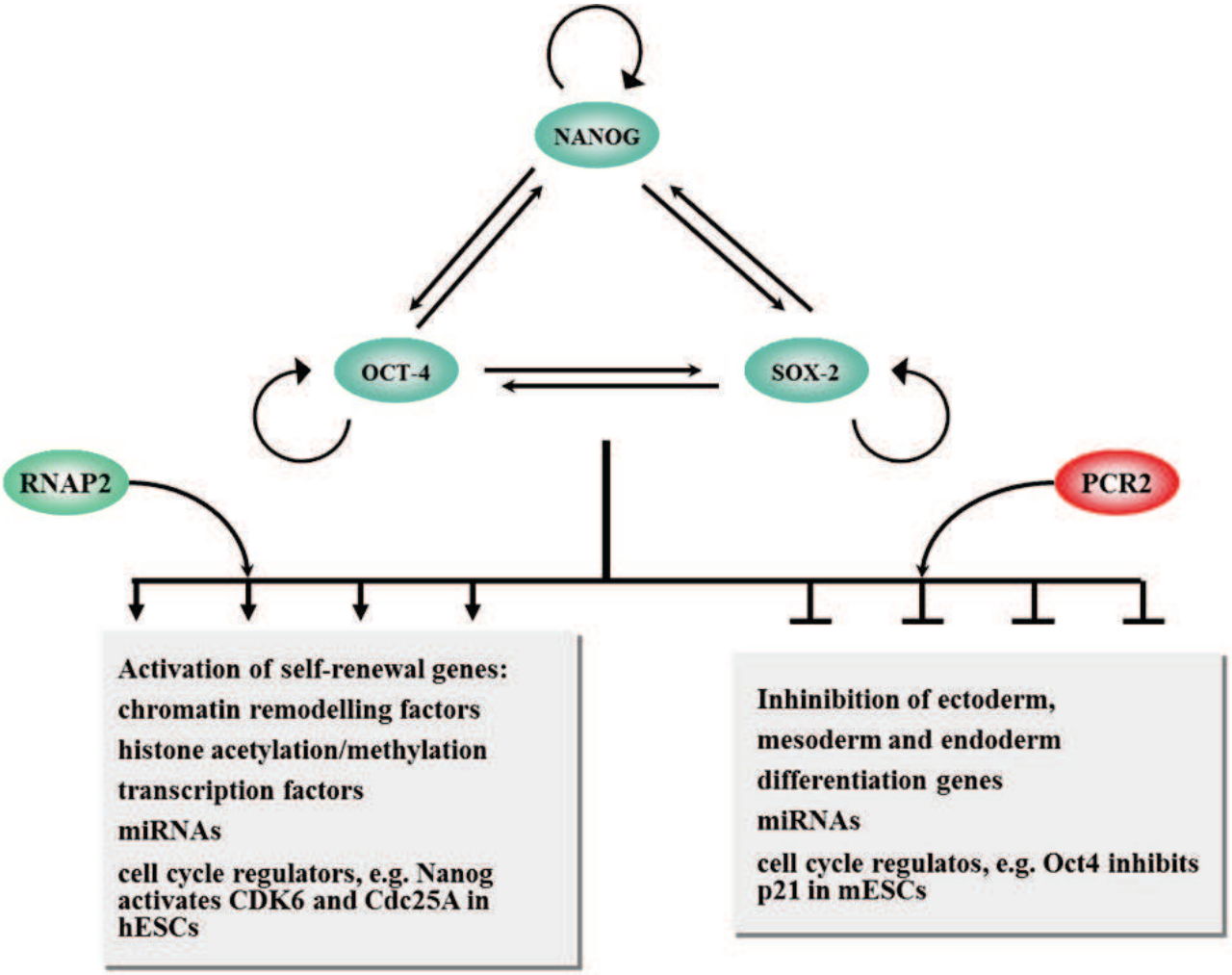

Figure 4: Mechanism of action of the regulatory core

Nanog, Oct4 and Sox2 act cooperatively to sustain self-renewal and pluripotency in ES cells by activating the expression of genes involved in self-renewal and repressing genes initiating differentiation. For more details see section 1.4.2.

PCR2: Polycomb repressive complex 2. RNAP2: RNA polymerase 2

Interestingly, in human and rhesus monkey ES cells, LIF has no effect on sustaining self-renewal (Daheron et al., 2004), and suppression of BMP signaling seems to be critical for maintaining cell pluripotency (James et al., 2005). On the other hand, Nodal or Activin are required for maintenance of an undifferentiated state and pluripotency (James et al., 2005). Propagation of primate ES cells requires culture on MEFs feeder layer or in conditioned medium from MEFs. Addition of FGF2 to the culture medium largely eliminates the need for feeder cells or conditioned medium. Thus FGF2 is a major activator of self-renewal (Xu et al., 2005, Levenstein et al., 2006). Finally, Activin/Nodal- and bFGF-dependent signaling pathways seem to cooperate to sustain self-renewal and maintain pluripotency (Vallier et al., 2005).

mEpiSCs and hESCs share signalling responses that normally function in the late epiblast. Indeed, mEpiSCs derivation and propagation have been achieved by using culture conditions without LIF and including FGF2 and Activin (Brons, 2007; Tesar, 2007) and, like hESCs, they differentiate in the presence of BMP4.

1.4.2 The pluripotency core regulatory network

Three transcription factors, Nanog, Oct4 and its cofactor Sox2, represent the regulatory core of the transcriptional circuitry in pluripotent cells. They act in concert to regulate ES cell self-renewal and pluripotency in both mouse and human pluripotent stem cells.

Gene inactivation experiments clearly indicate that Oct4, Sox2 and Nanog play a determinant role in the specification of mouse pluripotent cells (Nichols et al., 1998, Avilion et al., 2003, Mitsui et al., 2003).

During mES cell differentiation, Oct4, Sox2 and Nanog expression notably declines (Chew 2005, Chambers et al., 2003). A critical amount of Oct4 is crucial for the maintenance of murine and human ES cell self-renewal (Niwa et al., 2000, Hay et al., 2004, Rodriguez 2007). For mESCs as for hESCs in culture, down-regulation of Nanog causes differentiation (Mitsui et al., 2003, Hyslop et al., 2005) whereas its overexpression inhibits differentiation in culture conditions that normally induce differentiation (Chambers et al., 2003, Darr et al., 2006). Surprisingly, it has been demonstrated that *nanog* -/- mESCs, although they are prone to differentiation, can self-renew indefinitely (Chambers et al., 2007). This suggests that Nanog is dispensable for maintaining pluripotency but it is specifically required for its acquisition in the embryo.

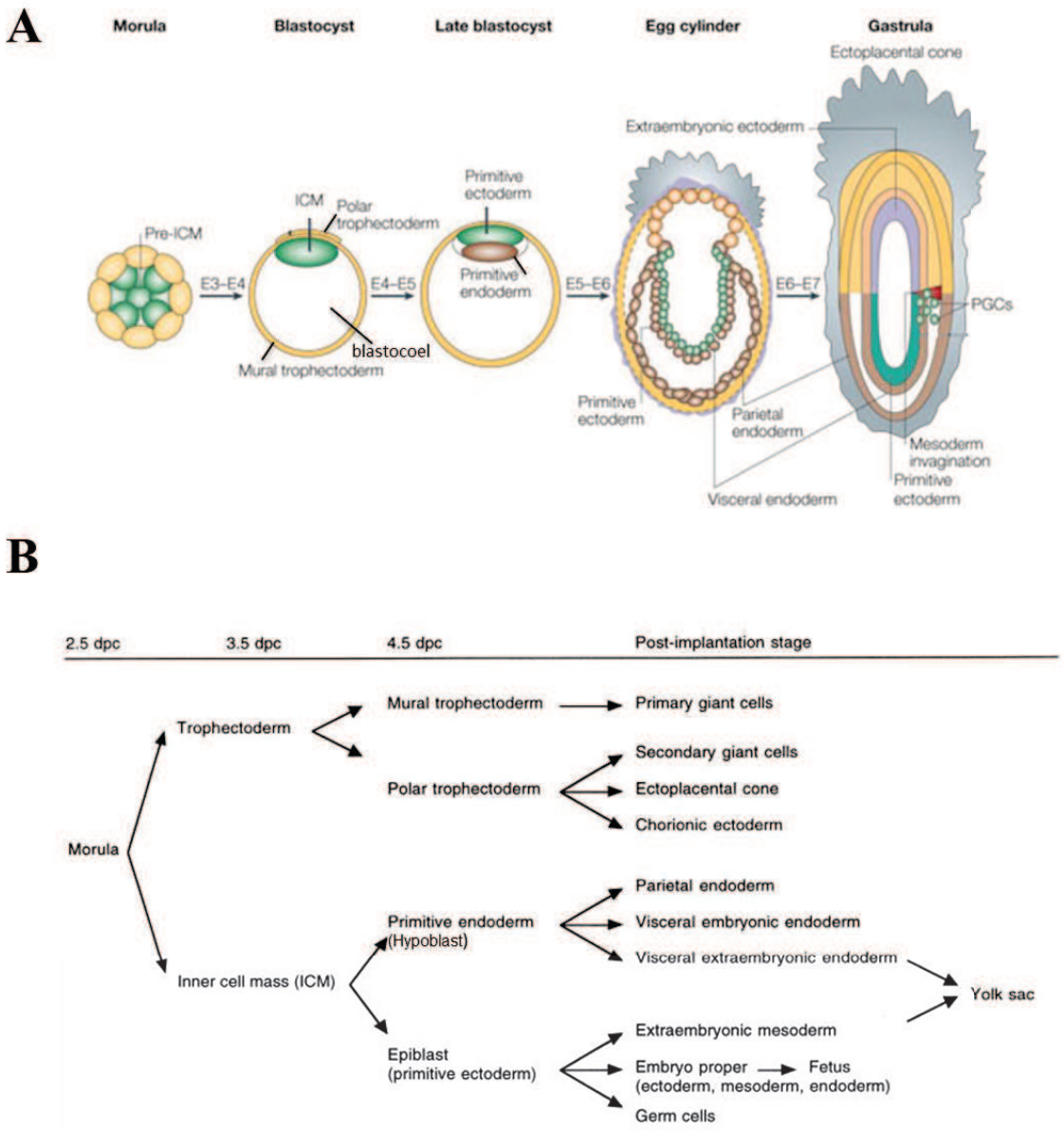

Figure 5: Early developmental stages in the mouse embryo (A) and origin of embryonic lineages (B)

(A) Pluripotent cells of the embryo are tracked in green. Derivatives of the hypoblast are tracked in brown, while lineages deriving from the polar trophectoderm are colored in gray. Finally, trophectoderm and derivatives of the mural trophectoderm are yellow-colored. From left to right:

Morula stage (until E3): after fertilization, cell divisions with no significant growth occur. This results in an embryo containing a core of pre-ICM (inner cell mass).

Early blastocyst (E3-E4): at E3, the morula develops a cavity, the blastocoel, and hence gives rise to the blastocyst. Pre-ICM cells turn into ICM in the inner part. The outer or peripheral layer is called the trophectoderm.

Late blastocyst (E4-E5): as the blastocyst fully expands the ICM further produce the epiblast or primitive endoderm and the hypoblast or primitive endoderm.

Egg cylinder (E5-E6): implantation occurs at E6. The primitive endoderm transforms into a cup-shaped structure and gives rise to the parietal and visceral endoderm lineages.

Gastrula (E6-E7): the embryo starts the gastrulation i.e. the formation of the three germ layers and of the primordial germ cells (PGCs).

Adapted from Boiani and Scholer, 2005

All three transcription factors form a positive autoregulatory circuit in which each member activates its own expression as well as the expression of the other two members of the group. They act cooperatively by activating a set of genes involved in self-renewal maintenance and by silencing another set of genes responsible for initiating differentiation (**Figure 4**) (Lee et al., 2006, Boyer et al., 2006). Their expression constitutes a hallmark characteristic for pluripotency.

It has been proposed that unique intrinsic determinants impose cell identity and extrinsic signals suppress activation of differentiation pathways (Chambers and Smith, 2004).

1.5 The ground and the primed pluripotency states

Pluripotency is generated naturally in the epiblast during mammalian embryogenesis and this state can be captured and stabilized in culture.

1.5.1 Insights into early embryogenesis in the mouse

The following paragraph describes very succinctly the first stages of mouse embryo development so as to have a better understanding of when, where and how pluripotency is attained in the embryo.

The fertilized mammalian embryo undergoes several rounds of cell divisions. By embryonic day 3 (E 3.0), the morula develops a cavity called the blastocoel (**Figure 5**). At this stage the embryo becomes the blastocyst, the first structure in which cell specialization occurs. Two distinct lineages emerge: an outside epithelium, the trophoblast and an inner core of cells, the inner cell mas (ICM). Between E 3.5 and E4.5, the ICM segregates into the pluripotent epiblast and a second extraembryonic lineage, the hypoblast (Gardner, 1983). At 4.5 days also called the late blastocyst stage, the newly formed epiblast is a cluster of 10 to 20 unspecialized cells located between the trophoblast and the hypoblast. It generates the whole embryo and when injected into another blastocyst it contributes to all lineages. Shortly after implantation i.e about E6.0, the blastocyst develops further into the egg cylinder. The epiblast transforms into a cup-shaped structure and becomes primed for lineage specification and commitment in response to stimuli from adjacent tissues. At E6.5, The embryo stars gastrulation.

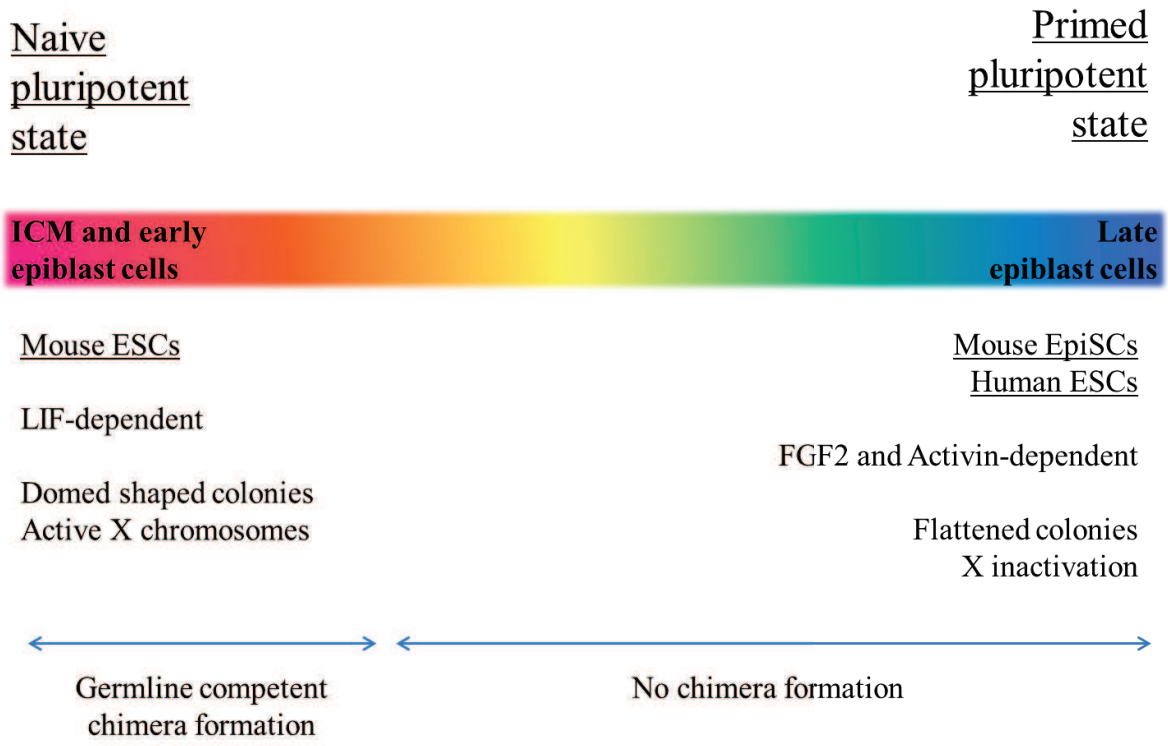

Figure 6: Different flavors of pluripotency

Gradient showing cell populations with different characteristics and developmental potentials. Pluripotent stem cells are classified into two distinct states: a primitive, naive LIF-dependent state represented by mouse ESCs analogous to IMC and early epiblast cells, and a primed FGF2/Activin-dependent state observed in mouse EpiSCs and in human ESCs analogous to late epiblast cells. Recent findings demonstrate that naive and primed pluripotent states are interconvertible.

In the embryo epiblast, pluripotent cells persist only transiently before inevitably undergoing differentiation or cell death. Establishing a pluripotent stem cell culture therefore involves capture of this transient phase of pluripotency (Smith, 2001).

1.5.2 Capturing pluripotency in vitro

Functionally and developmentally distinct pluripotent embryonic stem cell lines can be derived from pre- or post-implantation embryos under different growth factor conditions (**Figure 6**).

Oct4 and its partner Sox2 are expressed ubiquitously in morula and are present throughout the ICM until after segregation of the hypoblast (Avilion et al., 2003, Chazaud et al., 2006, Palmieri et al., 1994). Expression of these factors is therefore too wide to define the epiblast. In contrast, Nanog is exclusively expressed in the nascent epiblast (Silva et al., 2009). Nanog expression at E4.5 demarcates and specifies the embryo founder compartment. It has been proposed that the epiblast constitutes the ground state, meaning a fully unrestricted population the harbours the requisite developmental potency and flexibility to produce all embryonic lineages (Nichols and Smith 2009).

The naïve early epiblast pluripotency may be captured *in vitro* under appropriate culture conditions in the form of mESCs. When injected into another blastocyst, mESCs retain the capacity to colonize an embryo and to contribute to all lineages, including the germline, as epiblast cells do. mESCs express gene markers characteristic of the ICM and the early epiblast, such as Rex1 and Stella. They also share epigenetic features such as the presence of two active X chromosome in female cells. In culture, mESCs form dome-shaped colonies with packed round-cell morphology and display a high single cell cloning efficiency of more than 90%.

Pluripotency can also be captured and propagated *in vitro* from the epiblast of post-implantation embryos in the form of epiblast stem cells. These mEpiSCs express the core pluripotency factors and also markers of the late epiblast but no early ICM marker. mEpiSCs differ to mESCs in global gene expression profile and growth factor dependency. They can

give rise to differentiated teratomas but cannot contribute to chimera formation. Moreover they have inactivation of the X chromosome in XX cells (Brons 2007; Tesar 2007; Nichols and Smith 2009). This phenomenon is also observed in late epiblast where one of the X chromosomes will be randomly silenced (Guo et al., 2009, Lengner et al., 2010). In culture, they form flattened colonies and they cannot be propagated efficiently from single cells.

For all these reasons, mESCS and mEpiSCs are believe to represent two different stages of pluripotency, (i) a naive state illustrated by mESCs that are assumed to be analogous to ICM and early epiblast and (ii) a primed state closer to commitment into differentiation illustrated by mEpiSCs that are the equivalent of late epiblast of the embryo (Nichols and Smith, 2009).

Although not identical, human ESCs share several features with primed mEpiSCs and are distinct from naive mESCs. hESCs and mEpiSCs are comparable in their colony morphology, low clonogenicity, dependence on FGF2 and Activin signalling pathways (Brons et al., 2007, Xu et al., 2008), inactivation of the X chromosome in female cell lines (Silva et al., 2008). These similar characteristics suggest that the blastocyst stage at which hESCs are derived would be analogous to that of mouse post-implantation blastocyst from which mEpiSCs are derived. These observations lead to the conclusion that primate ESCs are more similar to mEpiSCs than to LIF dependent mESCs.

One may ask whether the failure to produce true primate ES cells reflect an evolutionary divergence of development in which the state of naïve pluripotency is rapidly transited so that the opportunity to capture true pluripotency in primates is reduced, or whether current culture conditions are unsuitable to capture this state *in vitro*. It should be taken into account a unique property of rodents that is their potential for embryonic diapause. This corresponds to a stage of arrested embryonic development due to a delayed implantation (Lopes et al., 2004). LIF signalling is essential for epiblast formation *in vivo* when diapause is observed (Nichols et al., 2001). This phenomenon may increase the likelihood to capture the true pluripotent state *in vitro*. Notice that for mammals that do not undergo diapause, true ESCs have not been derived.

1.5.3 Reversion from the primed to the naïve state of pluripotency

As previously described, mEpiSCs and hESCs have distinct properties and have less developmental potential than naïve mESCs.

FGF2-dependent mEpiSCs were converted into LIF-dependent cells by transiently overexpressing the pluripotency factors Klf2, Klf4 and Nanog and inhibiting MEK kinases and GSK3b. The final population was able to contribute to blastocyst chimeras and even colonize the germline (Hall et al., 2009).

Hanna et al (2010) were able to rewire the identity of hESCs into a more immature state. An already established hESC line was reverted to a naïve pluripotent state by transient expression of Oct4 and Klf4 or Klf4 and Klf2 in medium containing LIF and inhibitors of GSK3b and ERK1/2 pathways (Hanna et al., 2010). Addition of Forskolin, a molecule that was previously used for the propagation of human embryonic germ cells, was necessary for stabilization of the phenotype. After 8 to 12 days of culture on feeders, colonies with naïve mESCs-like morphology appeared. Their clonogenicity was dramatically increased to more than 85%. Cells were positive for pluripotency markers. After *in vitro* differentiation markers of all three lineages were expressed and they were able to form teratomas. These naïve hESCs shared defining features with mESCs such as dependence on the same signalling pathways, state of the X chromosome activation and similar global gene expression profile. A cross-species hierarchical clustering showed that ICM-derived mESCs and naïve hESCs formed a distinct group apart from conventional hESCs and mEpiSCs. Nevertheless after 15-20 passages, naïve hESCs stopped proliferating and begin to differentiate. According to the authors, this may be due to a toxic effect of Forskolin or its inability to fully substitute for ectopic gene expression. These findings support the notion that distinct state of pluripotency can be established and maintained by specific culture conditions. Indeed stabilization of the naïve pluripotency may require for instance inhibition of MEK and GSK3b.

1.5.4 Heterogeneous populations within undifferentiated ES cell cultures

Based on the expression of the ICM specific markers, Stella and Rex1, two teams identified subpopulations of self-renewing mES cells. The cell culture contains ICM-like populations, which are Stella+ and Rex1+/Oct4+ cells. Stella- Rex1-/Oct4+ cells are assumed to be equivalent to late epiblast. These subsets can convert into each other spontaneously. They show different potency *in vitro* and *in vivo*. For instance, cells negative for Stella or Rex1 differentiate more efficiently. mES cells grown in conventional culture conditions would represent intermediate states between ICM like cells and EpiSCs. This dynamic

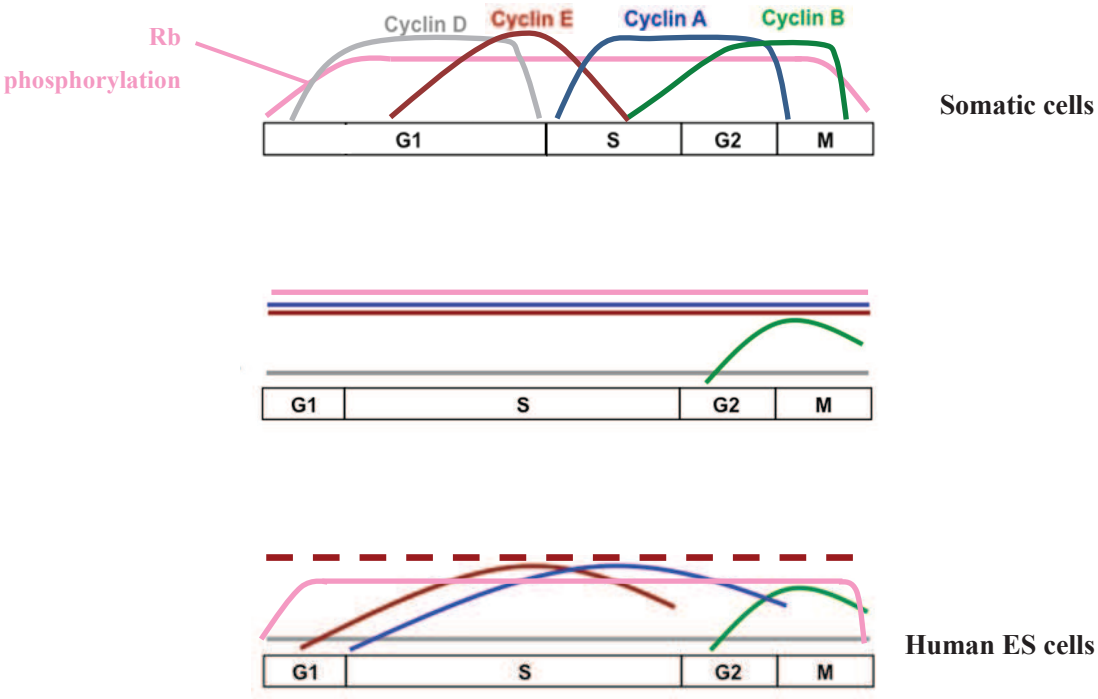

Figure 7: Cyclins expression and Rb phosphorylation status in ES and somatic cell cycles

In somatic cells, Cyclins concentrations and Rb phosphorylation levels vary in a cyclical fashion during the cell cycle.

In mouse ES cells, both Cyclin E- and Cyclin A-CDK2 complexes are present and active throughout the cell cycle suggesting that ES cells are constitutively primed for DNA replication. Moreover hypophosphorylated Rb is undetectable.

In rhesus monkey ES cells Cyclin E level does not fluctuate. By contrast, Cyclin A level seems to fluctuate. Rb is predominantly in a hyperphosphorylated form.

In human ES cells, Cyclin E expression may be robust and non-oscillatory (Filipczyk et al., 2007) or cell cycle dependent (Neganova et al., 2009; Ghule et al., 2007). Conversely, reports agree on a periodic expression of Cyclin A. Likely, Rb undergoes a G1-specific dephosphorylation event.

Adapted from Conklin and Sage, 2009.

equilibrium reflects cellular plasticity and metastability (Hayashi et al., 2008, Toyooka et al., 2008).

Retroviral marking of single human ESCs shows that culture of these cells contains subpopulations with distinct functional properties and different biological potential (Stewart 2010). Subsets of pluripotent cells can be distinguished by distinct pattern of cell-surface marker expression, gene expression and growth and differentiation properties. Each subset yield different efficiency in clonogenicity, embryoid body formation and teratoma formation experiments. Apparently these different states of pluripotency are interconvertible under certain conditions.

The diversity of pluripotent murine and human stem cells offers a unique opportunity to explore the regulation of the cell cycle in established ES cell lines, during early embryogenesis and reprogramming.

2 THE UNIQUE CELLE CYCLE STRUCTURE OF PLURIPOTENT STEM CELLS

2.1 General overview of the somatic cell cycle

It is useful to remind the established principles underpinning growth control in mammalian cells of somatic origin to better be aware of how unique pluripotent cell cycle features are.

Proliferating somatic cells exhibit a canonical cell cycle in which the G1 phase (8-12 hours) is longer than the S phase. The transition from one cell cycle to the next is controlled by Cyclin-Dependent Kinases (CDKs), the activity of which is regulated by activators (Cyclins) and inhibitors (Cip/Kip and INK4). Proper progression through the cell cycle is monitored by checkpoints. The G1, the S, the DNA damage and the spindle assembly checkpoints ensure correct DNA replication, chromatin packaging, DNA integrity and attachment of chromosomes, respectively. Activation of theses checkpoints induces cell cycle arrest to allow the cell to repair the defect (Bartek et al., 2004).

Precise temporal regulatory decisions during the gap phases (G1 and G2) prepare the cell for the event of DNA replication (S phase) and for entering mitosis (M phase), in the

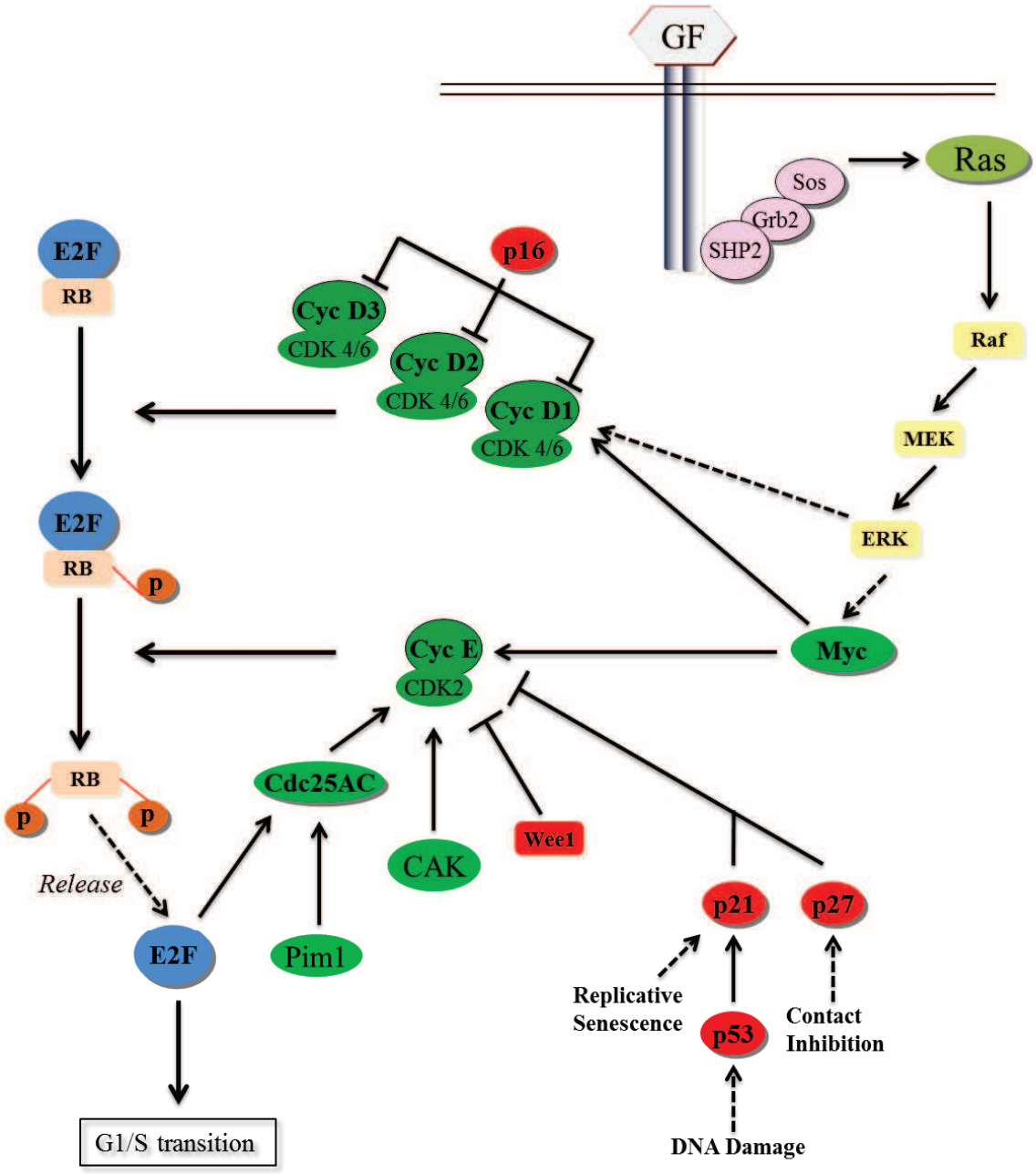

Figure 8: The G1 to S phase transition in somatic cells

Growth factors activate the Ras/ERK signaling pathway that induces Cyclin D production. In early G1 phase, Rb is partially phosphorylated by Cyclin D/CDK complexes. Partial phosphorylation of Rb leads to partial release of E2Fs transcription factors, which activate Cyclin E transcription. Likewise, Myc also activates Cyclin E. Upon Pim1 binding, Cdc25A activates Cyclin E/CDK2 complexes as well as CAK contrarily of Wee1. Next, Cyclin E/CDK2 totally releases E2Fs and facilitates entry into the S phase. Additionally, members of the family Cip/Kip (p21, p27, p57) and INK4 (p15, p16, p18, p19) inhibit CDKs kinase activities. p21 transcription is either activated by p53 following DNA damage or as a consequence of replicative senescence. p27 is activated in response to contact inhibition.

correct order. Cyclin/CDK kinases become activated at precise points of the cell cycle in somatic cells (**Figure 7**). The Cyclin D/CDK4-6 complexes are formed during the G2 phase that precedes mitosis. They are maintained through the subsequent G1 phase and their levels drop abruptly when cells enter the S phase. The Cyclin E/CDK2 complexes are present during the late G1 phase and until the beginning of the S phase. Then CDK2 forms new complexes with Cyclin A which are progressively replaced by Cyclin B1/CDK1 complexes during the transit through the G2 phase. The level of those latter complexes collapses shortly before re-entry into G1 (Ekholm and Reed, 2000). The cell cycle regulation of D-type (D1, D2 and D3), E-type (E1 and E2), A-type (A1 and A2) and B1 Cyclins plays a key role in this process.

Growth factor-induced activation of tyrosine receptors initiates a kinase cascade involving the sequential activation of Ras, Raf, MEK and ERK kinases which induces the activity of transcription factors that will finally regulate Cyclin D expression (**Figure 8**) (Lavoie et al., 1996). Cyclin D binds and activates CDK4 or CDK6 kinases. These complexes initiate the phosphorylation of the retinoblastoma protein (Rb), the partial release of its binding partner E2F and ultimately the G1 progression. Indeed, hypophosphorylated Rb (G1-specific) inhibits the expression of genes required for S phase entry, such as Cyclin E and Cdc25A, by sequestering the E2F family transcription factors (Dyson, 1998). Next, Cyclin E regulates CDK2 to complete Rb phosphorylation consolidating a positive feedback loop that confers irreversibility to the G1/S transition (Geng et al., 1996). Freed E2F activate expression of their targets, which include key components of the machinery needed for DNA replication and S phase progression. In conclusion, Cyclin D acts as a transducer of extracellular mitogenic signals in order to trigger the transition from the G1 to the S phase of the cell cycle.

Notice that in the absence of mitogenic signalling, cells exit the cell cycle at some point during G1 and enter into a quiescent state. This point is known as the restriction (R) point and beyond it cells become irreversibly committed to complete the cell cycle and require no further mitogenic cues.

2.2 ESCs exhibit an unusual cell cycle distribution

In mouse ESCs

Mouse ESCs have an unusual rapid cell cycle with truncated gap phases. The mean generation time is 11 hours. Its most striking feature is a very short G1 phase of approximately 1.5 hours which accounts for 15% of the total cell cycle duration. The proportion of time devoted to the S phase is ~60% that is about 6 to 7 hours (Savatier et al., 1994, Stead et al., 2002). At the molecular level, this is underpinned by a high and constant level of Cyclin E/CDK2- and Cyclin A/CDK2-associated kinases activities, in addition with low levels of the Cip/Kip family members of CDK inhibitors throughout the cell cycle (Savatier et al., 1996; Stead et al., 2002). Besides, Rb is exclusively in its hyperphosphorylated form (specific to the S and G2/M phases in the somatic cell cycle), regardless of cell cycle position. This observation suggests mESCs cells are constitutively primed for DNA replication (Savatier et al., 1994, Stead et al., 2002).

It is worth to note that the cell cycle time of epiblast cells at E6 is 9 hours. When differentiation is induced, the length of the G1 phase increases substantially, thus changing significantly the cell cycle profile.

In embryonal carcinoma cells

A low proportion of cells in G1 was reported in murine embryonal carcinoma cells, the malignant stem cells of teratocarcinomas (Mummery et al., 1987b). Upon synchronization, EC cells that were expose to retinoic acid, a very powerful inducer of differentiation, were more vulnerable to differentiation signal while progressing through G1 phase and, by contrast, became more resistant when being in S phase. This result indicates that G1 phase corresponds to a window of increased sensitivity to differentiation inducers. Thus the physiological reason of the unusually short G1 phase i.e constitutive transit through G1 may be to constraint the temporal opportunity for differentiation so to sustain the undifferentiated state.

The differentiation process of human and murine EC cells is accompanied by a decrease in growth rate. Accordingly to Mummery et al (1984,1987a), the modification of human EC cells cell cycle kinetics is due to an increase in the duration of both G1 and S phases, whereas for murine EC cells it is mainly attributable to an increase in the duration of the S phase only (Mummery et al., 1984, Mummery et al., 1987a). Note that the methods used in these studies do not allow to distinguish accurately the cell cycle phases, so the precise duration of each phase is difficult to evaluate.

In rhesus monkey ESCs

Rhesus ESCS (rESCs) share several cell cycle characteristics with mESCs. By time-lapse video imaging, the cell cycle length was calculated. It varies from 12 to 20 hours with a median value of 15 hours. Hence within a population of undifferentiated rESCs, there is a high heterogeneity in terms of growth rate. Using flow cytometry and quantitative analyses of cell cycle kinetics, Fluckiger et al (2006) showed that proliferating rESCs have a cell cycle phase distribution as follows: G1: 16± 3%, S: 56± 3 %, G2/M: 28± 5%. rESCs exhibit an extremely rapid transit through G1 phase (less than 2 hours). As for their mESCs, one molecular mechanism that supports this cell cycle feature is high and cell cycle independent levels of Cyclin E and Cyclin A, as demonstrated by in situ immunofluorescence analysis (Fluckiger et al., 2006).

After induction of differentiation, cell cycle distribution changes remarkably. There is a large increase in the proportion of G1 cells concomitant with a decrease in the proportion of S cells: G1: 45± 2%, S: 29± 6%, G2/M: 26± 5%. Also, Cyclin E and Cyclin A levels decrease dramatically.

In human ESCs

Human ES cells rapidly proliferate with a doubling time of 15-16 hours. 65% of asynchronous growing cells are in S phase. Immunoflurorescence on mitotically synchronized cells showed that S phase duration is 8 hours, G2 phase lasts 4 hours and M phase is about 1 hour. Lastly, hESCs display a G1 phase of only 2.5-3 hours which is significantly shorter when compared with somatic cells (Becker et al., 2006).

In another report, hESCs were sorted based on their level of GCTM2, a stem cell surface marker. Cells strongly positive for GCTM2 ($GCTM^{HIGH}$) are Oct4 positive whereas 85% of cells weakly positive ($GCTM^{LOW}$) and 5% of cells negative ($GCTM^{NEG}$) are Oct4 positive. The three cell populations have significantly different G1 and S fractions. $GCTM^{HIGH}$ have the highest proportion of S-phase cells (52±4%) and the lowest proportion of cells in G1 (24±3). The $GCTM^{LOW}$ population had a significantly lower proportion of cells in S phase (35±3%), while the percentage of cells in G1 was larger (45±4%). $GCTM^{NEG}$ cells had the lowest S-phase fraction (17±3%) and the highest proportion of cells in G1 (59±12%) (Filipczyk et al., 2007).

These features are supported partially by absent or barely detectable mRNA and protein levels of Cip/Kip and INK4 inhibitors (Becker et al. 2006; Miura et al., 2004, Neganova et al., 2009; Sengupta et al., 2009, Zhang et al., 2009).

Menchon et al (2011) evaluated the cell cycle structure of two hESC and two hiPSC lines. They observed a high resemblance between the cell cycle profiles of undifferentiated (G1 phase: 17-33%; S phase: 54-72%) and of differentiated hESCs and hiPSCs (G1 phase: 87-93%; S phase: 2-8%). They concluded there is a conserved cell cycle structure between pluripotent stem cell of embryonal origin and from de-differentiation process (Menchon et al., 2011).

Pluripotent stem cells share several fundamental properties of the cell cycle. Rapid division of ESCs is associated with an unusual cell cycle structure with a short G1 phase. This is one key difference between somatic and pluripotent stem cells. Nevertheless, murine ESC cell cycle profile, while similar, is not equal to that of primate ESCs. This may be due to hESCs sub optimal culture conditions which can affect cell cycle progression. But the most plausible hypothesis is that murine and human ESCs represent two stages of development. Thereby the differences observed may reflect the properties of two developmentally distinct pluripotent populations. It is consistent with the hypothesis that hESCs are more similar to mEpiSCs than to mESCs (Brons, 2007; Tesar, 2007). In the following pages, we will investigate the molecular mechanisms underlying the rapid proliferation rate of ES cells.

2.3 The Ras/ERK→Cyclin D/CDK→RB-E2F dependent pathway

In somatic cells, in response to mitogenic signals, the Ras/Extracellular signal-regulated protein kinase (ERK) pathway activates the expression of Cyclin D1 which will at last ensure the passage from G1 to S phase.

In mouse and rhesus monkey ESCs

Murine ES cells display a short G1 phase during which hypophosphorylated Rb is undetectable (Savatier et al., 1994). It is thus likely that Rb proteins are rephosphorylated immediately after mitosis and consequently held in a biochemically inactive state throughout the cell cycle. As a result E2F target genes would be transcribed independently of cell cycle progression. This is consistent with these reports (Stead et al., 2002, White et al., 2005 and Jirmanova et al., 2002).

Several lines of evidence support the notion that murine ESCs do not employ Ras/ERK signalling to regulate the expression of G1 Cyclins, nor rely on D-type Cyclins to regulate the phosphorylation of the retinoblastoma protein:

1/ Inactivating the ERK signaling does not impair proliferation. For instance, specific restriction of ERK activity by a pharmacological inhibition of MEK, the upstream activator of ERK, does not alter the cell cycle kinetics (Jirmanova et al., 2002). On the contrary, suppression of ERK activity enhances self-renewal, suggesting that Ras/ERK signaling has a prodifferentiative effect on mESCs (Burdon et al., 1999).

2/ The basal expression of Cyclin D1 protein depends on PI3K signaling, and not on the Ras/ERK pathways, and it seems to be uncoupled from mitogenic stimulation (Jirmanova et al., 2002). It is also noticeable that serum starvation has no impact on mESCs proliferation but eventually contributes to apoptosis (Schratt et al., 2001).

3/ Cyclin D1 and Cyclin D3 are present in low amounts in mES cells, whereas Cyclin D2 is absent. CDK4-associated kinase activity is virtually absent. Cyclin D3/CDK6 exhibit high Rb kinase activity but in some cell types it has been implicated in controlling differentiation independently of its role in cell cycle regulation (Faast et al., 2004, Grossel and Hinds, 2006).

4/ Mouse ES are refractory to the growth inhibitory activity of $p16^{INK4}$ (Savatier et al., 1996). $p16^{INK4}$ is a specific inhibitor of CDK4 and CDK6 which acts by preventing the association of D Cyclins with the CDKs (Sherr and Roberts, 1999). Cyclin D3/CDK6 complexes evade inhibition by $p16^{INK4}$ (Faast et al., 2004).

5/ The inactivating disruptions of the retinoblastoma gene family members do not seem to compromise ES cell proliferation, but do reduce differentiation in experimental teratocarcinomas (Dannenberg et al., 2000, Sage et al., 2000).

6/ mESCs share striking similarities with mouse embryonic fibroblasts deficient for the three Rb family members. Both cellular types fail to arrest in G1 at confluence and following DNA damage (Neganova and Lako, 2008). They both display comparable cell cycle profiles with up to 65% in S phase and they both escape replicative senescence and are immortal (Dannenberg et al., 2000, Sage et al., 2000).

It has thus been proposed that mESCs lack the so called restriction point that is to say that their cell cycle control is mitogen-independent.

In monkey rhesus ESCs, immunoblot analysis showed a predominance of the hyperphosphorylated form of RB in undifferentiated cells which reflects the cell cycle distribution of rESCs in which S and G2/M phases represent almost 85% of the total cell cycle. On the contrary the hypophosphorylated form is detected in differentiated cells (Fluckiger et al., 2006). Rhesus ESCs where deprived of serum for 24 hours i.e a duration sufficient for every cell to pass at least once through any serum checkpoint. No alteration of

the cell cycle structure was detected. Neither the blockage of the MEK activity resulted in any significant alteration of the cell cycle structure. This suggests that as mESCs, rESCs do not require persistent mitogenic stimulation and functional activation of MEK signaling to progress through G1/S transition.

In human ES cells

Apparently human ES cells, unlike murine ES cells, possess elevated expression of Cyclin D2 and CDK4 mRNA (Becker et al., 2006). Cyclin D2 protein is remarkably expressed compared to Cyclin D1 and its levels increase during G1 and S phases. In differentiated cells, roles are reversed suggesting a particular function of Cyclin D2 linked to pluripotency (Becker et al., 2009). Cyclin D1, CDK1, CDK2 and CDK4 protein expression are constant throughout the cell cycle. Moreover, immunoprecipitation studies coupled with kinase assays showed that D-type Cyclins could associate with CDK4 or CDK6 and form active complexes (Neganova et al., 2009). Nevertheless, accordingly to Filipczyk report, Oct4+ hESCs are negative for all Cyclin D proteins and unresponsive to CDK4 inhibitor treatment. Furthermore, both phosphorylated and unphosphorylated forms of Rb are present in hESCs (Neganova et al., 2009, Filipczik et al., 2007). Filipczik et al demonstrated that Rb undergoes a G1-specific dephosphorylation event. Within the Oct4+ population, immunofluorescence analysis using specific antibodies detected 20% of cells expressing the hypophosphorylated form and 99% the hyperphosphorylated one. Hypophosphorylated form of Rb never coincides with Cyclin A expression (which oscillates in hESCs as discussed below). These properties can be interpreted as the capacity to respond to growth factor signaling suggesting that there may be a potential operative regulatory control of the G1/S transition in human ES cells.

The previous hypothesis is in conflict with the observations of another group that suggests that short term self-renewal can be carried out autonomously i.e hESCs are primed for at least two rounds of cell proliferation without any requirement for external growth factors. This observation would be consistent with the lack of a functional E2F/pRb mediated transition in late G1 phase (Becker et al., 2009).

An alternative restriction point is required for self-renewal of human ES cells

Since Rb/E2F dependent growth control seems to be inactivated in hESCs, Becker and colleagues (2009) proposed that self-renewal is regulated by Cyclin/CDK dependent phosphorylation of $p220^{NPAT}$. In somatic cells, Cyclin E/CDK2 kinase activity is required for

activation of the regulatory pathway $p220^{NPAT}$/HiNF-P/Histone genes that controls the onset of the S phase. It consists in the Cyclin E/CDK2 dependent phosphorylation of $p220^{NPAT}$, a histone gene regulator factor, which associates with the transcription factor HiNF-P to induce the histone H4 expression at the G1/S transition. It ensures correct packaging of newly replicated DNA. This process is facilitated by targeting $p220^{NPAT}$ to specific nuclear organelles (Ghule et al., 2007). In hESCs, depletion of either Cyclin D2 or $p220^{NPAT}$ blocks cell cycle progression in early G1 with a concomitant reduction in the number of organelles containing phosphorylated $p220^{NPAT}$ and a decrease in histone H4 gene expression. Taken together, these data strongly suggest that in hESCs the Cyclin D2/CDK4 dependent induction of $p220^{NPAT}$/HiNF-P/Histone genes pathway is required for cell cycle progression beyond the G1/S transition (Becker et al., 2009).

Within committed (Oct4-negative) hESCs, 65% expressed the hypophosphorylated form of Rb and only 12% the hyperphosphorylated one. As previously discussed, $GCTM^{NEG}$ cell fraction displays a G1 elongation, almost no expression of Oct4 when compared to GCTM2+ cells and important levels of active Rb protein. Only in the $GCTM^{NEG}$ subpopulation, CDK4 inhibition delays G1 suggesting that commitment changes the cell cycle control parameters (Filipczik et al., 2007). Indeed, undifferentiated hESCs which are refractory to external growth factor deprivation at least in short term cultures (Becker et al., 2009) become dependent on the Rb/E2F switch only during lineage programming.

2.4 The role of Cyclin E and Cyclin A in self-renewal and pluripotency

2.4.1 Permanent vs. periodic expressions

In mouse ESCs

In mouse ES cells, both Cyclin E and Cyclin A expressions are vastly elevated and non-phasic. Cyclin E- and Cyclin A-CDK2 complexes are present and active throughout the cell cycle suggesting that ES cells are constitutively primed for DNA replication (Jirmanova et al., 2002). Actually, cell division is driven by unusually high Cyclin E- and Cyclin A-CDK2 activity all over cell cycle progression (Stead et al., 2002). When differentiation is induced, the observed lengthening of the G1 is associated with the establishment of cell cycle-dependent CDK2 activity and activation of a functional Rb-E2F pathway. This is correlated with cell

cycle-regulated synthesis of Cyclin E and A and an increase in p21/p27 inhibitors (White et al., 2005).

In rhesus monkey ESCs

Undifferentiated rESCs express high level of Cyclin E and Cyclin A that dramatically decrease upon differentiation induced by low density culture in feeder-free conditions for 5 days (Fluckiger et al., 2006). In situ immunofluorescence revealed that among the Oct4+ population, 97% of the cells expressed Cyclin E. This indicates that Cyclin E level does not fluctuate during cell cycle progression as for murine ES cells. By contrast, the Cyclin A level seems to fluctuate in rhesus ESCs as 78% of cells expressed Cyclin A in an asynchronously growing cell population.

In humans ESCs

There are some discrepancies between several reports that attempt to describe the mRNA levels, the protein expression and the kinase activity of cell cycle regulators.

Accordingly to Filipczyk et al (2007), like mESCs, Oct4+ hESCs show robust and non-oscillatory expression of Cyclin E by immunofluorescence analysis (Filipczyk et al., 2007). Conversely, Neganova et al (2009) and Ghule (2007) et al reported a cell cycle dependent expression of Cyclin E by the means of immunoblot analysis in synchronously growing hESCs. Cyclin E protein level increase during G1 and is further upregulated during progression through S (Neganova et al., 2009, Ghule et al., 2007). Furthermore, Neganova et al (2009) presented some evidence indicating that all CDK activities display modest periodicity in hESCs. This divergence could be explained by different analysis method sensitivity, intrinsic variability between the hESCs lines, or low quality of the cell culture which leads to "contamination" by differentiated cells.

Reports agree on a periodic expression of Cyclin A. Indeed, Cyclin A protein levels are upregulated in the late G1/S through G2/M (Ghule et al., 2007, Neganova et al., 2009). Flow cytometry assay associated with propidium iodide staining showed, in $GCTM^{HIGH}$ and $GCTM^{NEG}$ hESCs fractions, that Cyclin A protein expression raised in S and G2 phases followed by downregulation in M and absence in G1 phases.

Besides active complexes between Cyclin E or Cyclin A and CDK2 were detected and the CDK2-associated kinase activity was higher during S phase (Neganova et al., 2009). In this work it is proposed that, unlike mESCs, most of the cell cycle regulators display a cell

cycle dependent expression. This encompasses Cyclin E, Cyclin A, Cyclin B1, CDK2-, CDK4-, and CDK6-associated kinase activities. These findings differ from Ghule et al (2007), who showed that only Cyclins A and E are cell cycle regulated. Finally, all these data is consistent with the fact that the absence of cell cycle regulation of Rb can be partially accounted by an elevated CDK2-associated kinase activity.

2.4.2 Cyclin A is essential in mESCs whereas Cyclin E is dispensable

Mammalian cells express two A-type Cyclins (A1 is testis specific whereas A2 is ubiquitously expressed in all proliferating cells) and two E-type Cyclins (E1 and E2 are coexpressed in all proliferating cells). Male mice lacking Cyclin A1 are sterile (van der Meer et al., 2004) whereas Cyclin A2 *null* embryos die shortly after implantation (Murphy et al., 1997). This reflects the necessity for Cyclin A2 in early embryo development for cell proliferation. Cyclin E1 or E2 knockout mouse embryos develop relatively normally whereas double knockout display profound abnormalities in the placenta. Nevertheless the tetraploid complementation method rescues the embryonal lethality. Thus Cyclin E1 and E2 are largely dispensable for mouse embryogenesis (Geng et al., 2003).

Kalaszczynska et al (2009) investigated the requirements for Cyclin A in cell proliferation using as model conditional knockout mice that lack both A-type Cyclins. Cyclin A *null* fibroblasts proliferate relatively normally and are able to re-enter the cell cycle from quiescence. The phosphorylation of Cyclin E and Cyclin A substrates remained unperturbed. The expression of cell cycle regulators was unchanged except for Cyclin E. Its pattern of expression resembles the combined patterns of Cyclin E and A in wild type fibroblasts. It is likely that Cyclin E is responsible for the normal proliferation of Cyclin A *null* fibroblasts. Shortly, Cyclin E *null* mouse fibroblasts proliferate normally during continuous growth. They display normal Cyclin A2 expression and Cyclin A2-associated kinase activity (Geng et al., 2003, Parisi et al., 2003).

Fibroblasts *null* for both E-type Cyclins and both A-type Cyclins display no CDK1 or CDK2 catalytic activity, are not able to replicate DNA and arrest in G2/M transition. Thus, in mouse fibroblasts either Cyclin E or A must be present to allow DNA synthesis and progression from G2 to M. This tells that they have the same set of targets, involved in centrosome duplication, DNA replication licensing, transcription and cell cycle progression Kalaszczynska et al., 2009).

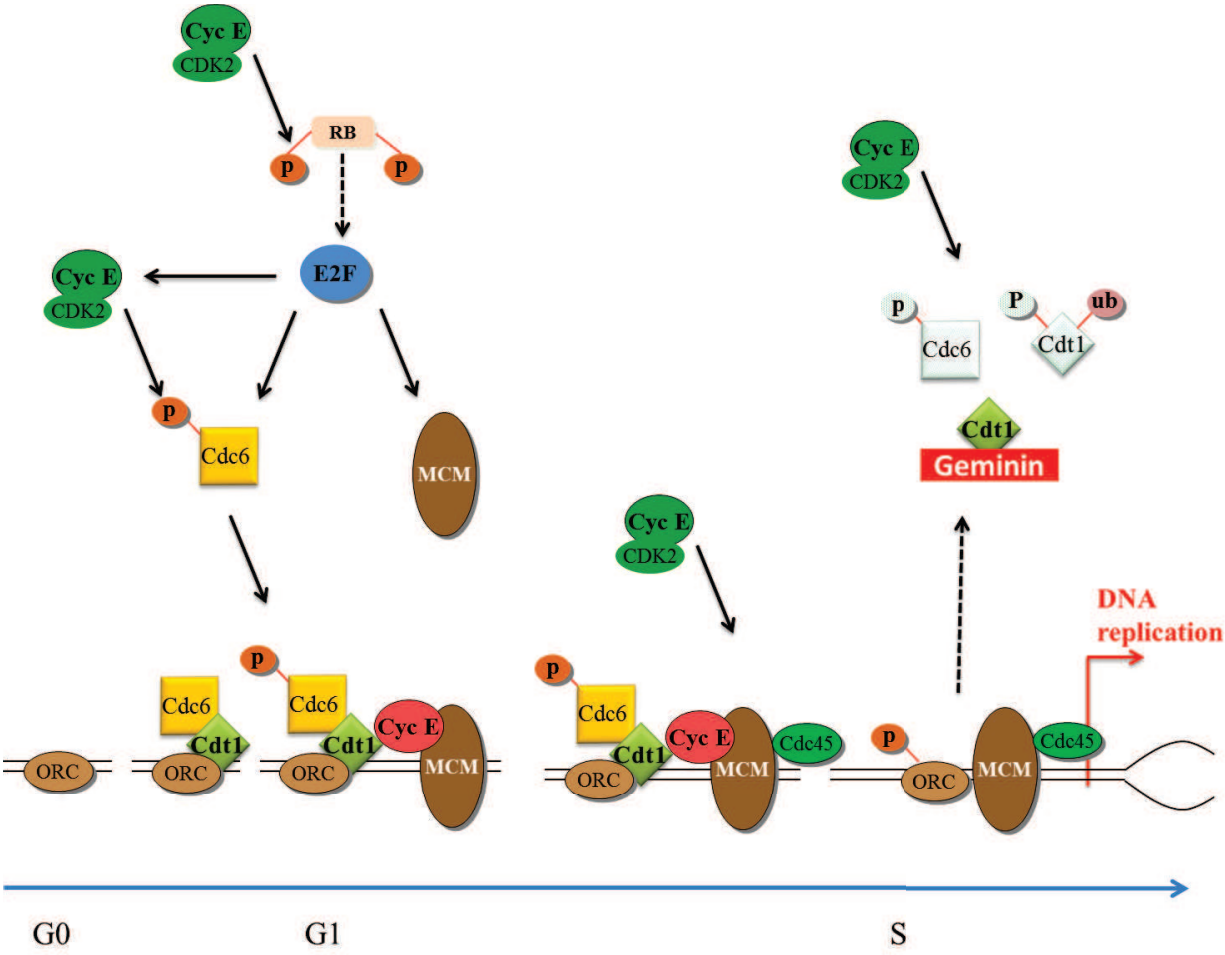

Figure 9: Assembly of prereplication complexes during cell cycle reentry of somatic cells

The pre-replication complex forms at the origin of replication and is made up of the following factors: the origin recognition complex (ORC), the regulatory proteins Cdc6 and Cdt1, and the Minichromosome Maintenance proteins (MCMs), the putative helicase complex. Once sequentially assembled, this complex of proteins indicates that the replication origin is ready for activation and DNA replication can begin. First, Cdc6 and Cdt1 leave the ORCs, and then MCM unwinds the double stranded DNA allowing the pre-initiation complex to bind. Regulation of replication is important to prevent the DNA from being replicated more than once during each cell cycle. For instance, Cdt1 is inactivated during S, G2 and early M phases by at least two pathways: (1) Geminin binds to Cdt1 and blocks interaction with MCM, (2) Cyclin E/CDK2 phosphorylates Cdt1 and targets it to ubiquitin-mediated degradation.

During G0 to S progression, in a CDK2 independent fashion, Cyclin E is necessary for MCM loading into the pre-replication complexes hence allowing S phase entry (function depicted on pink). Cyclin E/CDK2 kinase activity contributes to the assembly of DNA pre-replicative complexes by releasing E2Fs from Rb and by stabilizing other components (function depicted on green).

Adapted from Geng et al., 2007.

In mESCs, total deletion of Cyclin A is incompatible with proliferation. Besides in wild type mESCs Cyclin A/CDK2 complexes are by far more abundant than Cyclin E/CDK2 complexes. This may confer to Cyclin A a fundamental role in cell cycle machinery making it essential for mESCs cell cycle progression and in all likelihood for all pluripotent stem cells (Kalaszczynska et al., 2009). It still has to be investigated whether its function is CDK-dependent or if CDK activity per se is required to maintain ESC identity.

On the other hand, works on mouse fibroblasts deficient in both E-type Cyclins reveal interesting data. Cyclin E *null* fibroblasts are unable to reenter the cell cycle from quiescent state because they fail to incorporate the MCM helicase into the DNA pre replication complexes during G0 to S progression (Geng et al., 2003). The authors ectopically express in Cyclin E *null* fibroblasts a kinase deficient (KD) mutant of Cyclin E1 that retains the ability to interact with CDK2 but the complexes are unable to phosphorylate their substrates. Interestingly, the KD mutant corrects the phenotype by partially restoring MCM loading and consequently allowing S phase entry. In conclusion, Cyclin E acts locally at DNA replication origins by mediating the loading of MCM helicase and thus the assembly of functional pre-replication complexes in cells progressing from G0 to S phase (**Figure 9**). This is a unique, non-redundant and kinase-independent function of Cyclin E (Geng et al., 2007). It should be recalled that Cyclin E/CDK2 kinase activity also contributes to the assembly of pre replicative complexes by phosphorylating RB, leading to activation of E2F transcription factors and to induction of their target genes such as MCM (Arata et al., 2000). Cyclin E/CDK2 also stabilize these complexes by phosphorylating other components (Mailand and Diffley, 2005). These kinase related functions can be compensated by other functionally redundant Cyclins since Cyclin E *null* cells are capable for continuous cycling.

2.4.3 CDK2 makes a connection between G1 length and early commitment

The robust expression of Cyclin E/CDK2 complexes observed in ESCs may result, either in active proliferation by shortening the whole cell cycle duration, or in sustaining self-renewal by shortening the duration of G1 thus minimizing the risks of spontaneous commitment to differentiation, or in both. Previous reports suggested that the rapid G1 phase progression of ESCs might be underpinned by high and precocious Cdk2 activity (Stead

2002) and that Cdk2 activity might be crucial for both cell-cycle regulation and cell-fate decisions in human ESCs.

Synchronized mouse ESCs treated with a specific pharmacological inhibitor of CDK2 displayed a two hour prolongation of the G1 phase, from 3 to 5 hours, without G1 cell cycle arrest. Next, a prolonged treatment was shown to increase from 20% to 50% the proportion of cells in G1. Thus prolonged downregulation of CDK2 establishes a somatic cell-like cell cycle. It also responsible for changes in morphology and in gene expression indicating differentiation. All these observations were confirmed by siRNA knockdown of CDK2 (Koledova et al., 2010). This observation corroborates the role of high and persistent CDK2 activity in driving mouse ES cell cycle (Stead et al., 2002).

Human ESCs were treated with a specific CDK2 inhibitor, Roscovitine, led to visible smaller colonies and changes on the cell cycle structure of $GCTM^{HIGH}$ cells (G1: 34±2; G2/M; 8±3% compared with the DMSO control G1: 19±3%; G2/M: 18±4%). Intriguingly, there were no significant differences in the proportions of GCTM2 positive cells in Roscovitine (52±3%) or DMSO (46±3%) treated cultures. CDK2 inhibition results in G1 prolongation, accumulation of cells with hypophosphorylated Rb and loss of Oct4+ cells. Thus lengthening of G1 seems to be linked with an increased spontaneous differentiation of hESCs (Filipczyk et al., 2007).

Another study supports the previous one. In hESCs, CDK2 expression was transiently downregulated by RNA interference. 48 hours post-transfection, CDK2 expression was down regulated by 90% generating changes in colony morphology; 97% of the cells were arrested in G1, pluripotency markers protein expression was decreased while the expression of markers of differentiation was initiated. By day 4, 50% of normal expression of CDK2 was achieved, Nanog and Cip/Kip inhibitors expression was increased and normal cell cycle was resumed. By day 6, colonies had a normal morphology. This data suggests that in hESCs changes in cell cycle structure and lineage commitment were reversible after CDK2 transient knockdown (Neganova et al., 2009).

Koledava's and Neganova's studies on mESCs and on hESCs diverge in effects of Cdk2 down-regulation: while mESCs slowdown their G1 progression, hESCs arrest in G1. This might be explained by the technique used to achieve CDK2 inhibition on which depends the extend of the loss of expression.

Another group also established a link between the mode of proliferation and the induction of differentiation. Human ES cells early lineage commitment modifies their cell cycle kinetics as they acquire a lengthened G1 phase (from 3 to ~10 hours) within 72 hours

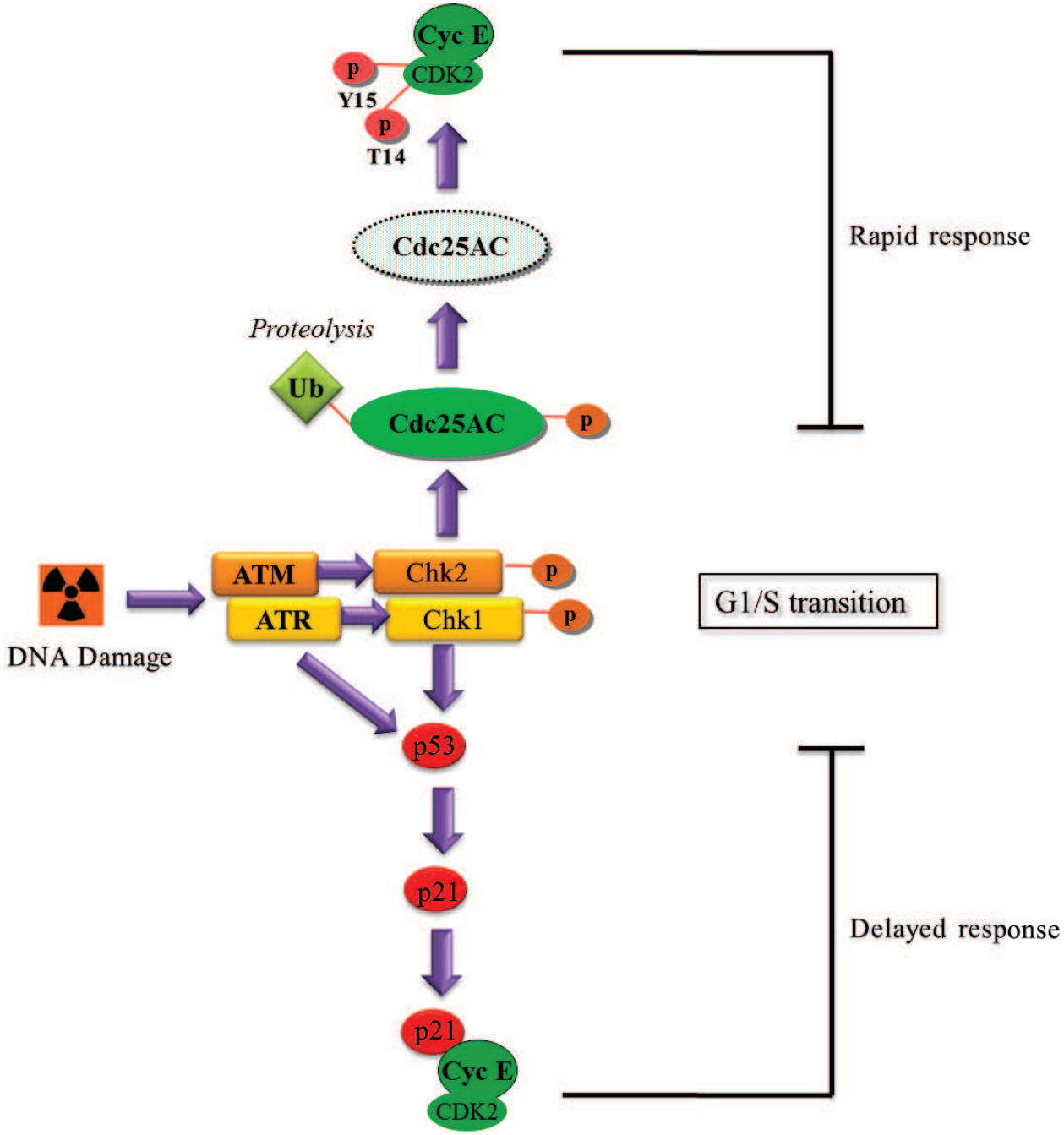

Figure 10: DNA damage response pathways at the G1/S transition in somatic cells

DNA damage triggers a rapid cascade of phosphorylation events involving the ATM/ATR and Chk1/Chk2 and eventually Cdc25A and p53. In the rapid response, the Cdc25A phosphatase is targeted for rapid ubiquitin/proteasome-mediated degradation. Hence CDK2 kinase is "locked" in an inactive form with inhibitory phosphorylations on threonine 14 (T14) and tyrosine 15 (Y15). In the delayed response, p53 is stabilized, accumulates in the nucleus and activates the transcription of a number of genes including the p21 CDK inhibitor. When accumulated to a threshold level, p21 binds and inhibits Cyclin E/CDK2 complexes and thereby secures the maintenance of the G1 arrest. Cyclin E/CDK2 integrates checkpoint pathways inducing both rapid and delayed G1 arrest upon DNA damage.

after induction of differentiation (culture on plastic coverslip, with serum and without FGF2). The process is reflected by loss of pluripotency factors (Becker et al., 2009).

CDK2 expression can be inhibited by the cell cycle arrest inducers p15, p16 and p21 among others. Recently, Ruiz et al (2011) showed, by the means of an inducible overexpressing system, that induction of p15 or p16 is followed as early as 24 hours later by a significant increase of the proportion of cells in G1. Moreover p21 overexpression leads to a permanent arrest in G1 and to a massive cell differentiation.

The previous observations are compelling arguments to address a potential role for the cell cycle machinery in the maintenance pluripotency.

2.5 Response to DNA damage in ES cells

Cell cycle checkpoints or safeguards for genomic integrity (Pardee, 1974) are activated in somatic cells after DNA damage during the G1/S, G2/M transitions and S phase, and converge to Cyclin/CDK inactivation. First, DNA lesion sensors ATM/ATR phosphorylate downstream targets, Chk1 and Chk2, which subsequently stabilize and activate the tumor suppressor p53 or degrade Cdc25A (**Figure 10**) (Bartek and Lukas, 2001). The former is a transcription factor that activates the expression of the CDK inhibitor p21. The latter is a phosphatase that removes inhibitory phosphates from CDK2. In somatic cells, DNA damage results in rapid decrease of the S phase fraction, due to the activation of the p53-p21 axis and the Chk1/Chk2 kinase dependent degradation of Cdc25A that finally leads to CDK2 inactivation. These two mechanisms prevent G1 cells with DNA damage from entering S phase so to permit DNA repair or apoptosis (Di Leonardo et al., 1994, Mailand et al., 2000). Replication errors are the most common source of double strand breaks (DSB) in proliferating cells. DSB can also be induced by ionizing irradiation (IR) or ultraviolet (UV) light.

The mutations rate in mouse ESCs is 100-fold lower that in embryonic or adult somatic cells (Cervantes et al., 2002). Besides the efficiency of repair of UV-induced DNA damage is decreased when mouse embryonal carcinoma cells are induced to differentiate (Rasko et al., 1993). Also, human pre-implantation embryos express genes that function in DNA repair pathways (Wells et al., 2005). These suggest that pluripotent stem cells have a remarkable DNA maintenance response. One may ask how extremely rapid proliferation is compatible with DNA stability and genomic integrity. Several studies agree that pluripotent

stem cells avoid DNA damaging agents deleterious effects by readily undergoing high levels of apoptosis. For instance, it has been shown that highly proliferating cells of the gastrulation embryo undergo important ATM- and p53- dependent apoptosis in response to very low IR (Heyer et al., 2000). Does the surveillance mechanism preventing propagation of DNA defects relies on the G1 checkpoint in all pluripotent cells? There are conflicting data regarding p53 subcellular localization, transactivation activity and apoptosis triggering capacity upon DNA damage. Additionally, some authors claim that mouse and human ESCs undergo important differentiation upon environmental insults while others affirm that there is no alteration in pluripotency markers expression.

2.5.1 Lack of DNA damage checkpoint in G1 in mouse and in rhesus ES cells

Aladjem et al. reported that mouse ES cells do not activate p53-dependent DNA damage responses. Indeed, even if mESCs synthetize important quantities of p53, they do not undergo cell cycle arrest at G1 checkpoint in response to DNA damage, nucleotide depletion or overexpression of this tumor suppressor. p53 appears to be nonfunctional because it translocates inefficiently to the nucleus (Aladjem et al.,1998, Hong and Stambrook, 2004, Chuykin et al., 2008). This correlates with an undetectable expression of p53-target p21 gene (Stead et al., 2002, Chuykin et al., 2008). Also Chk2 has an altered intracellular localization making it unavailable to phosphorylate Cdc25A (Hong and Stambrook, 2004). After IR, a large number of cells are arrested in G2, consistent with an active G2 checkpoint (Hirao et al., 2000), and a majority of cells are in S phase, consistent with an S phase arrest (Hong and Stambrook, 2004).

Although mESCs do not undergo G1 checkpoint in response to DNA damage, they still have functional mechanism for detection of DNA defects. Indeed, mESCs exposure to IR induces accumulation of activated ATM (Chuykin et al., 2008). Another report demonstrated that the two pathways controlling the G1 checkpoint and converging to CDK2 inhibition are activated but do not fulfill their final goal. By using synchronized mESCs and mouse fibroblast in mitosis and irradiated in G1, it was demonstrated that p53 is activated and that p21 is accumulated in both cells types, without changes in the expression of mESCs pluripotency markers. Following introduction of DSB, Cdc25A is degraded but this is not mediated by the kinases Chk1 and Chk2 because they are sequestered in the centrosomes. One could expect that p21 synthesis and Cdc25A degradation would inhibit the kinase activity of

CDK2. But on the contrary, Koledova et al (2010) showed that the high CDK2 activity is unresponsive to DNA damage. They hypothesized that CDK2 is refractory to p21 because level of expression is insufficient and it avoids inhibitory phosphorylations by localizing in the cytoplasm and in the centrosomes. Actually, centrosomes have been proposed to shelter the proteins they harbor and to constitute cell cycle control centers in somatic cells (Doxsey et al., 2005). The conclusion of this study is that CDK2 constitutes the driving force of the rapid escape of mESCs from G1 after DNA damage which is the cause for G1 checkpoint deficiency (Koledova et al., 2010).

In a similar manner, human embryonal carcinoma cells lack DNA damage-induced G1 cell cycle arrest even though p53 is highly expressed (Wang et al., 2009). Instead undifferentiated hEC cells exhibit S and G2 phase delay while their differentiated counterparts display G2 arrest in response to IR. Before or after retinoic acid-induced differentiation, DNA damage activated the ATM-Chk1/Chk2-p53 pathway. Only in undifferentiated hEC cells, Cyclin E/CDK2 complexes were expressed at high level and remain high after DNA damage. Only in differentiated cells hEC cells, G2/M complexes Cyclin B/ CDK1 were up-regulated following DNA damage treatment. The authors of this study uncovered new evidence for the biological importance of S and G2 cell cycle checkpoints, in the context of an absent G1 checkpoint. Since after irradiation undifferentiated hEC cells have better survival rate, more efficient DNA repair and exhibit an enhanced delay in S phase. S phase arrest may serve as a protective mechanism that gives extra time to repair DNA lesions before they are transmitted to the daughter cells (Wang et al., 2009).

For rhesus monkey ESCs, after IR the percentage of cells in the G1 phase decrease from 22% to 7% whereas for cells in G2 phase an increase from 36% to 70% was observed. This result suggests that rESCs do not have a functional DNA damage response pathway for growth arrest in G1. By contrast, they exhibit growth arrest in the G2 phase. This observation strongly suggests that rESCs, like their murine counterparts, lack a DNA damage checkpoint in G1 (Fluckiger et al, 2006).

2.5.2 Do human ES cells lack the DNA damage checkpoint in G1 phase?

First of all notice that mESCs undergo low spontaneous apoptosis and differentiation (Corbet et al., 1999), in contrast to hESCs that often display high rates of spontaneous apoptosis (30%, Dravid et al., 2005) and differentiation (40%, Ezashi et al., 2005) in routine culture. May a differential regulation of the G1 checkpoint contribute to these differences species-related? There are many discrepancies between findings that we will summarize.

In response to exposure to IR, the cell cycle distribution of pluripotent human ES cells was investigated by flow cytometric analysis of DNA content and immunostaining with the mitosis specific marker, phospho-H3. It was shown that irradiation resulted in a temporary arrest of the cell cycle at the G2 phase, but not at G1. hESCs resumed cycling by 16 hours following irradiation but with prominent mitotic spindle defects. By 48 hours, the cell cycle distribution closely resembled to non-irradiated hES cells. Hence, hESCs display a lack of G1/S cell cycle arrest and temporary arrest cell cycle progression in the G2 phase. Further, the authors found that irradiated hESCs activate and properly localize the checkpoint signaling proteins ATM, Chk2, and p53, resulting in a temporary G2 arrest-ATM dependent before the cells resume mitosis. A weakly increase in p21 protein expression following induction of DSB is observed but the level is still insufficient to inhibit CDK2 activity. Importantly, pluripotency factors protein levels do not change over the 24 hours period after irradiation, suggesting that hESCs remain pluripotent (Momcilovic et al., 2009).

All of these findings were later extended to hiPSCs with mitotic cells starting to reappear 24 hours after irradiation (Momcilovic et al., 2010).

Another report showed that in hESCs, IR results in rapid and robust p21 transcriptional activation via p53 which may induce cell cycle arrest. Simultaneously, HiNF-P and $p220^{NPAT}$ are down-regulated which may decrease histone H4 gene expression. Thus hESCs may have a functional checkpoint that monitors genomic integrity and prevents the cell cycle dependent expression of histone H4 genes in response to DNA damage (Becker 2007). One weakness of this study is that p21 protein levels were not investigated. It may be possible to have a mRNA induction without any protein expression as shown for p21 in the same cell line in Filion et al work (Filion et al., 2009).

Different studies show that hESCs undergo p53-p21 dependent (Neganova et al., 2011) or independent (Barta et al., 2010) G1 checkpoint. The first research group showed that in hESCs downregulation of CDK2 kinase activity leads to blockage of DNA replication, activation of ATM, Chk1, Chk2 and p53, degradation of Cdc25A and up regulation of p21,

thus triggering the activation of the G1 checkpoint. Also, downregulation of CDK2 kinase activity induces DNA damage and increases apoptosis. In addition, depletion of CDK2 activates and at the same time inhibits different pools of genes involved in apoptosis. The authors suggest that CDK2 plays a role in the execution of apoptosis and in DNA repair (Neganova et al., 2011).

The second research group was able to delay hESCs in G1 phase and activate the classical molecules involved in the G1 checkpoint activation under low doses of UV light in a dose dependent manner and without inducing massive apoptosis. Upon treatment of synchronized hESCs, p53 accumulated in the nucleus and likely activated p21 transcription. In spite of this, p21 protein levels were similar to that in non-irradiated cells. In parallel, the kinases Chk1 and Chk2 localized in the nucleus and mediated Cdc25A degradation. Besides, pharmacological inhibition of Chk1 and Chk2 abrogated UV-induced G1 arrest. Cells that fail to progress to the S phase showed reduced CDK2 activity compared to control cells. Finally, radiation stress provoked loss of CDK-activating Cdc25A, thereby low CDK2 activity, and execution of G1 checkpoint without any contribution of the p53-p21 axis (Barta et al., 2010).

Furthermore, hESCs repair several types of DNA damage more efficiently than differentiated cells and together with hiPSCs have higher expression of DNA damage signaling and repair genes (Maynard et al., 2008). This is consistent with the existence of enhanced surveillance pathways ensuring the high capacity for genomic stability in pluripotent stem cells.

2.5.3 DNA damage induces an alternative pathway: apoptosis

According to Corbet et al (1999), apoptotic response to UV radiation is p53-dependent because it is reduced to near background levels in *p53 null* mESCs. However, p53 *null* mESCs still exhibited low but significant levels of apoptosis in response to UV indicating that p53 independent apoptotic pathway also exists in these cells (Corbet et al., 1999). In addition, although IR does not induce apoptosis in mESCs, it significantly reduces their clonogenic potential except for p53 *null* mESCs (Corbet et al., 1999). Contrary to the previous report, another study showed that mouse ESCs exposed to DNA damaging agents went through apoptosis in a p53-independent manner. Indeed p53-/- mESCs have similar kinetics of apoptosis as wild-type cells (Aladjem et al., 1998). Furthermore, mouse EC cells undergo p53

dependent apoptosis and to a lesser extend p53 independent apoptosis (Lutzker and Levine 1996).

In human ESCS cultures, massive cell death occurs within hours after exposure (Momcilovic et al., 2009). Human iPSCs exhibit profound sensitivity to DNA damaging agents even more than hESCs cultured by the same group. This is attributed to different culturing methods (Momcilovic et al., 2010). According Qin et al (2007), UV irradiation of hESCs induces p53 induced apoptosis through a mitochondrial pathway (Qin et al., 2007).

2.5.4 DNA damage induces an alternative pathway: differentiation

Cell cycle arrest for DNA repair and apoptosis may not be sufficient after certain types of DNA damage. Therefore an alternative way to maintain genetic stability is inducing differentiation into cell types that are sensitive to the tumour suppressor p53.

It has been suggested that p53 prompt mESCs to commitment but this function is opposed by high levels of expression of a truncated p53 transactivation deficient isoform known to maintain p53 in an inactive state. When overexpressed, the isoform Δ40p53 protect mESCs from cell death without altering their cell cycle structure. By contrast, in mESCs deficient in Δ40p53 cell cycle progression is deeply affected, with the cells acquiring a somatic cell cycle profile. Thus Δ40p53, by blocking p53, enhances survival and promotes a highly proliferative state (Ungewitter and Scrable, 2010).

p53 may be a regulator of pluripotency because it responds to DNA damage signals by inducing differentiation. As a matter of fact, Lin et al demonstrated in mESCs that after UV irradiation p53 binds to the Nanog promoter, recruits co-repressors and suppresses Nanog expression, thus causing lineage commitment. After induction of differentiation, the observed down-regulation of Nanog expression correlates with p53 stabilization and transcriptional activity (Lin et al., 2005). In a similar manner, in human ESCs UV irradiation-induced p53 accumulation suppresses Nanog and Oct4 expression through direct binding to their promoters (Qin et al., 2007). Interestingly, p53 is unable to activate the transcription of its canonical target genes even if it binds to their promoter regions. This failure may be explain by a default in recruiting the cofactors required for this process. However, after UV treatment p21 protein levels increase by 2 fold. Reducing p53 expression by RNA interference reduces both spontaneous and DNA damage-induced apoptosis as well as spontaneous differentiation and increased single cell survival and proliferation rates when compared to control cells. The

authors propose a model where p53 accumulation and induction of apoptosis is caused by stress inducing culture conditions In conclusion, a novel role of p53 in promoting spontaneous differentiation, in addition to survival, has been revealed.

In support of this finding, another study showed that activation of p53 with a small molecule activator, Nutlin, induces rapid differentiation of hESCs by promoting accumulation of cell in G1 phase in a p21 dependent manner (Maimets et al., 2008).

Lastly, upon LIF starvation and retinoic acid treatment on mESCs, p53 proteins levels were reduced but by contrast the transactivational activity of p53 was increased. Similar findings have been reported for EC cells (Lutzker and Levine 1996, Oren 1982). Upon differentiation, a functional p53-mediated DNA damage response pathway was restored (Aladjem et al., 1998, Lin et al., 2005). Interestingly, human EC cells display a higher survival rate after IR when compared to their differentiated counterparts (Wang el al., 2009).

2.5.5 Biological meaning of DNA damage response in pluripotent stem cells

We observed pluripotent stem cells have set different levels of control such as DNA damage detection, DNA repair, cell cycle arrest, apoptosis and differentiation that operate together to protect genome stability.

The biological meaning of eliminating damaged cells would be to avoid transmission of damaged genomes in early embryogenesis which finally could compromise multiple cell lineages. Indeed, at this developmental stage, rapid cell divisions would be incompatible with DNA repair mechanisms. On the other hand, since G1 phase may be considered a time of increased sensitivity to differentiation cues in pluripotent EC cells (Mummery et al., 2007), loss of G1/S cell cycle arrest may protect pluripotent cells from differentiation. The physiological explanation for G2 arrest may be that the predominant mechanism for repairing DSB in mouse and human ESCs is error-free homologous recombination repair, and it critically depends on the presence of the homologous sequence on the sister chromatin which is only present during G2. Under this context, cells that are in G1 at the time when DNA damage is inflicted may undergo differentiation or apoptosis, and those cells that are in G2 phase of the cell cycle would arrest and attempt repair of the damage.

The divergences between mouse and human ESCs data and between research group observations are difficult to reconcile. A plausible explanation can be experimentally addressed by the use of different cell lines, use of different DNA damaging agents inducing a

variety of lesions of several magnitudes, use of different experimental regimes (synchronized cells, cells in exponential growth phase, growth factors, oxygen level, type of passaging, presence of MEF layer). Assou et al (2007) found lack of consistency in pluripotent stem cells expression profiles which may reflect natural variability in the cell types present in culture. It is hence reasonable to consider ES cell culture being inherently heterogeneous, with one cell type lacking canonical p53 stress response but being interconvertible with a second type able to undergo p53 activation. Additionally, differences observed can be afforded by technical or statistical bias (Assou et al., 2007). Finally, one may speculate that species-specific regulations reflect the differences between the developmental stages of the embryo from which ESCs are derived (Brons et al., 2007; Tesar et al., 2007).

2.5.6 A particular situation: the telomeres

Uncapped telomeres eventually are recognized as DNA damage and trigger apoptosis (Karlseder et al., 1999). In human fibroblasts, erosion of chromosome overhangs is detected by ATM and activates the p53-p21 axis (Herbig, 2004). The telomerase holoenzyme, that encompasses the Telomerase Reverse Transcriptase (TERT) unit and the telomerase RNA template (TR), maintains the structure of chromosome ends. In mouse somatic cells, deletion of either mTR or mTERT results in progressive loss of telomeres, genomic instability, aneuploidy and eventual reduced growth rate (Niida et al., 1998, Liu et al., 2000). Studies in human tumors and differentiated somatic cells have revealed roles of the telomerase weakly related to stabilization of telomeres length. Among extratelomeric roles, there are antiapoptotic functions (Cao et al., 2002), modulation of growth-controlling genes and acceleration of cell proliferation (Smith et al., 2003, Geserick et al., 2006). Cancer cells, germline cells and ES cells display abundant telomerase expression. Upon differentiation of ESCs, EC cells or some immortalized cells telomerase expression declines (Yang et al., 2008).

Overexpression of mTERT in mouse ES cells and in their differentiated progeny, promotes cell proliferation, improves resistance to apoptosis and oxidative stress. Thus telomerase can be considered as a “survival enzyme”. Besides mTERT overexpression enhances differentiation towards the hematopoietic lineage with no karyotypic instability (Armstrong et al., 2005).

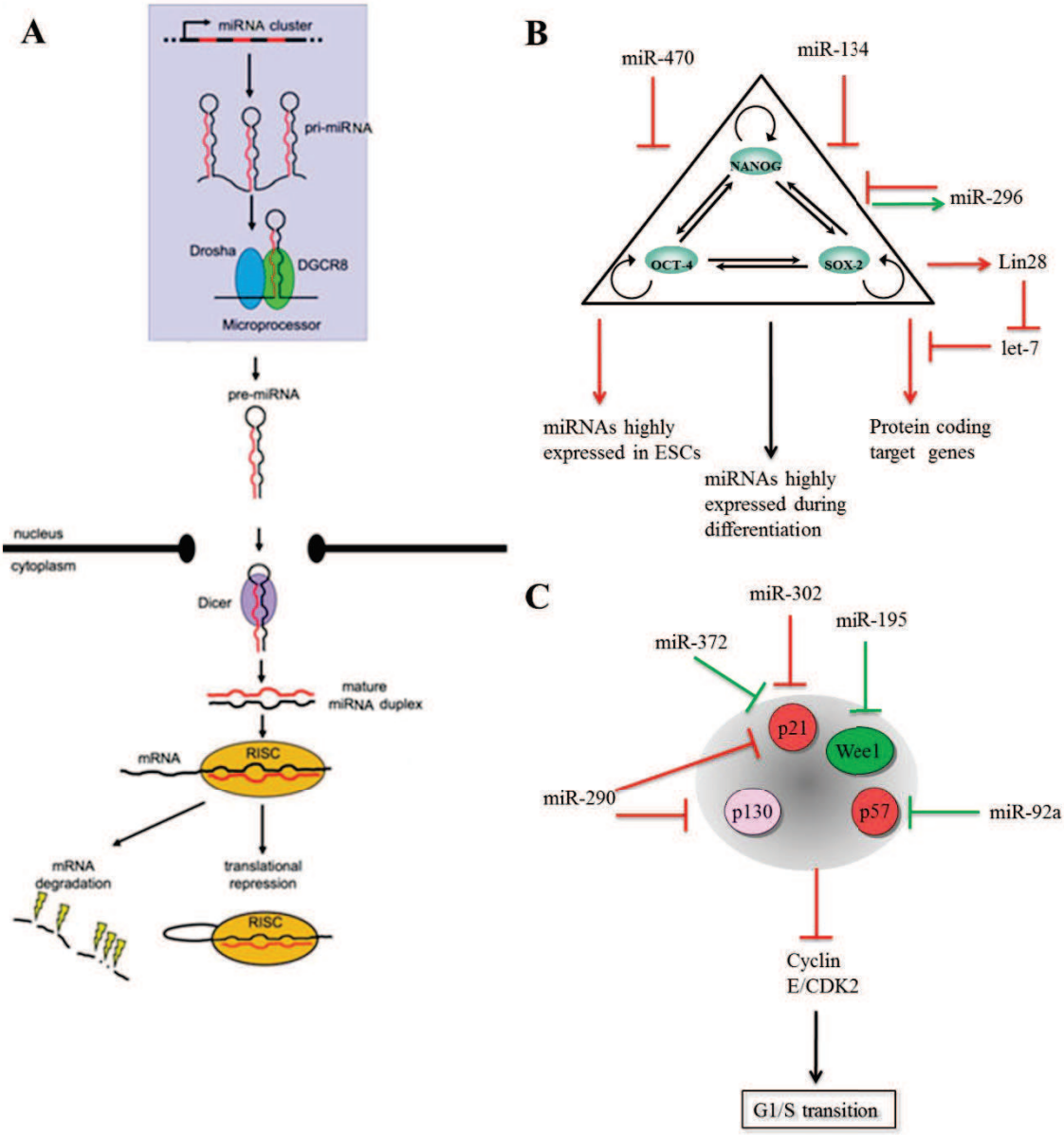

Figure 11: miRNAs regulate stem cell fate

(A) Biogenesis of miRNAs

Most miRNAs promoters characterized to date are typical of RNA polymerase II transcribed genes and give rise to a capped, spliced and polyadenylated primary precursor (pri-miRNA), formed by one or several concatenated hairpin structures consisting of a stem and a terminal loop. The pri-miRNA is recognized and cleaved by a protein complex constituted by Drosha and DGCR8 to release an intermediate stem–loop structure (pre-miRNA) that will be transported to the cytoplasm. There the loop is further processed by the Dicer complex to release a mature mi-RNA duplex, which is finally incorporated into the RISC. The downstream regulatory effects are the degradation of the mRNA or its translational repression. From Tiscornia et al., 2010.

(B) Interplay between miRNAs and the core regulatory network

Only the regulation in mouse ESCs is represented here. The core of transcription factors controlling pluripotency shapes the miRNA signature of ESCs: some miRNAs are highly expressed in ESCs (e.g. miR-302 and miR-290/371 clusters) while others are activated during differentiation (in ESCs their expression is inhibited by Polycomb proteins). Concomitantly, miRNAs can repress the expression of the pluripotency transcription factors, ultimately establishing a network that tightly controls the undifferentiated pluripotent state.

(C) miRNAs regulate the G1 to S transition

Regulations in human ESCS are depicted in green, those in mouse ESCs are in red. Negative regulators of Cyclin E/CDK2 are represented inside the gray circle. By controlling cell cycle regulators expression, Embryonic Stem cell-Cell Cycle regulating miRNAs may activate Cyclin E/CDK2 complexes hence promoting S phase entry. In hESCs, the miR-302 cluster has been reported to have a positive effect on the G1 to S phase transition. The underlying mechanism still has to be clarified.

Stably hTERT-overexpressing human ES cells can form teratomas as control cells whereas differentiation into embryoid bodies is quite refractory. This cells show lower spontaneous differentiation in culture and higher colony-forming ability when dissociated into single cells. Cell cycle analysis shows an increase of S phase length at the expense of the G1 phase, an increase in Cyclin D1 and Cdc6 levels and an enhancement of E2F-dependent transcription. By the contrary, transient downregulation of the hTERT induces loss of typical colony morphology, a decrease in proliferation, a reduction of Oct4, Nanog, Cyclin D1 and Cdc6 expression, an increase in the expression of lineage commitment markers and in apoptosis rate. Moreover the authors demonstrated that changes in cell cycle and proliferation precede differentiation induced by loss of hTERT. It is yet unclear how telomerase participates in transcriptional regulation (Armstrong et al., 2005).

Altogether these results indicate that telomerase plays a key role in the maintenance of pluripotency, control of cell cycle dynamics and *in vitro* differentiation capacities of mESCs and hESCs.

2.6 Pluripotency depends on miRNA control

A new class of regulators, microRNAs (miRNAs), has been shown to control self-renewal, differentiation, G1 progression and G1 to S phase transition in mouse and human embryonic stem cells.

miRNAs are ~22 nt small noncoding RNAs that repress gene expression at the post-transcriptional level via base pairing to complementary sites located in the target messenger RNAs. Like transcription factors, a single miRNA can regulate the expression of numerous genes. Besides, miRNAs are highly evolutionary conserved (Bartel, 2004). Their biogenesis begins with the transcription of long and hairpin-shaped transcripts (pri-miRNA) that are cleaved by a protein complex constituted by Drosha and DGCR8 to produce ~60-70 nt long precursors (**Figure 11 A**) (pre- miRNA). Notably, many pri-miRNAs accumulate without being efficiently processed, until specific developmental or environmental cues arise (Thomson et al., 2006). pri-miRNAs are exported to the cytoplasm where they associate with a complex containing the ribonuclease Dicer. This nuclease further cleaves pre-miRNAs generating mature miRNAs, which nested within the protein complex constitute the miRNA-Induced Silencing Complex (RISC). This in turn recognizes target mRNAs to finally modify the output of many protein coding genes.

2.6.1 Hinder miRNA biogenesis affects both proliferation and differentiation

In mouse, ablation of two essential components of miRNA biogenesis machinery, Dicer and DGCR8, results in similar embryonal phenotypes, namely lethality by at early stages of development (Bernstein el al., 2003). DGCR8 depleted mESCs have a similar morphology to wild type control but display an altered cell cycle profile, with a slight increase of cells in G1. What is more DGCR8 *null* mESCs cannot efficiently silence the ESC program even under stringent differentiation conditions (Wang et al., 2007). Likewise, Dicer *null* mESCs experience a complete proliferation block but continued culture eventually gives rise to clones that proliferate at rates comparable with that of DGCR8 *null* mESCs (Murchison et al., 2005) *In vitro* differentiation by embryoid bodies formation does not abolish Oct4 expression nor activate differentiation markers. Moreover*, in vivo* they do not contribute to chimera formation (Kanellopoulou et al., 2005). Both cell lines no longer fulfill the criteria that define stem cells, nevertheless they maintain the expression of pluripotency markers. Dicer- and Drosha-*null* human ESCs have similar phonotypes to what has been described in mouse. Particularly, they are characterized by G1/S and G2/M transition delays (Qi et al., 2009). Clearly, miRNAs are central players in the regulation of pluripotent stem cell functions, especially in the G1 to S phase progression and in the proper ESC differentiation.

2.6.2 ESCs are characterized by a defined miRNA signature

Two studies identified unique stem cell specific miRNAs, highly expressed in ESCs and drastically down regulated following differentiation. Murine and human miRNA families have high homology with similar genomic organizations, expression patterns and common sets of target genes. For instance, murine miR-290 cluster is homologous to human miR-307 and murine and human miR-302 clusters are homologous too (Houbaviy et al. 2003; Suh et al., 2004). These miRNAs are collectively called Embryonic Stem cell-Cell Cycle regulating (ESCC) miRNAs.

Interestingly, many of the ESC-specific miRNAs are co-transcribed as polycistronic transcripts, suggesting common upstream regulation (**Figure 11 B**). Actually in murine ES cells, the core pluripotency factors co-occupied the promoters for 55 distinct miRNA transcription units that are divided into two groups: one group of miRNAs that is preferentially expressed in pluripotent cells (ESCC miRNAs), and a second, Polycomb-

occupied group that is silenced in ES cells and is poised to contribute to cell fate-decisions during differentiation (Boyer et al. 2005; Marson et al., 2008). Transcription factor occupancy of the promoters of miRNAs is conserved in human ESCs suggesting a common gene regulation. For instance, the expression of miR-302 is dependent on Oct4 and Sox2 in mouse and human ESCs (Marson et al., 2008; Card et al., 2008). Conversely, miR-134, miR-296 and miR-470, upregulated on retinoic acid-induced differentiation of mouse ESCs, have been shown to inhibit Nanog, Oct4 and Sox2 (Tay et al., 2008). Likewise, miR-145 represses the pluripotency machinery of hESCs (Xu et al., 2009).

Transfection of let-7 miRNA into DGCR8-/- mESCs rescued the differentiation phenotype, allowing cells to shut down the pluripotency program more efficiently. Mature let-7 family members are essentially absent in ESCs and accumulate only upon differentiation, eventually being broadly expressed in differentiated tissues. It has been shown that let-7 directly inhibits downstream targets of the pluripotency factors (Melton et al., 2010). Interestingly, in somatic cells let-7 directly targets genes whose products promote G1/S or G2/M transitions, including CDK6, Cdc25 and Cyclin D2 (Johnson et al., 2007). Besides, let-7 miRNAs are negatively regulated by Lin 28, an RNA-binding protein which promoter is occupied by Oct4, Sox2 and Nanog (Viswanathan et al., 2008). In conclusion, miRNAs are connected to the basic molecular circuit of pluripotency.

Interestingly, ESCC miRNAs do not rescue the capacity to silence the self-renewal program under differentiation conditions of miRNA-deficient ESCs. In addition, it has been established that silencing of pluripotency markers requires de novo methylation (Feldman et al., 2006). It has been shown that members of the miR-290 cluster repress p130, an Rb related protein that in turns inhibits *de novo* methyltransferases, hence preventing epigenetic silencing of mouse pluripotency factors explaining the observed phenotype (Sinkkonen et al., 2008). This shows that the miRNA ESC component can interact with the DNA methylation machinery. Furthermore, that the set of lineage-specific miRNAs directly downstream of the core pluripotency factors could help poise ES cells for rapid and efficient differentiation.

2.6.3 ESC-specific miRNAs modulate the G1 to S phase transition

Although there is no consensus about the expression of many cell cycle regulators throughout ESCs cell cycle, there is a general agreement on the unique role of miRNAs in ESCs cell cycle progression.

A screening strategy of individual miRNAs introduced in the miRNA-deficient mESCs background showed that 14 miRNAs can fully restore the short G1 phase (Wang et al., 2008). These rescuing miRNAs belong mainly to the miR-290, miR-302 and miR-17-92 clusters. Members of these three clusters were identified as direct or predicted suppressors of Cyclin E/CDK2 upstream inhibitors (Sinkkonen et al., 2008; Wang et al. 2008). For instance normal cell growth can be partially restored by introducing mature miRNAs miR-195 and miR-372 (**Figure 11 C**). They regulate respectively Wee1, a negative regulator of the G2/M kinase Cyclin B/CDK, and p21, a negative regulator of the G1/S kinase Cyclin E/CDK2 (Qi et al., 2009).

It has been reported that the miR-302 cluster promotes cell cycle progression in human ESCs by targeting Cyclin D1, although the mechanism is yet unclear. Besides, miR-302 is predicted to target many others cell cycle regulators (such as Rb, p130, and E2F1) and it is positively regulated by Oct4/Sox2. This data provides evidence for a link between the core pluripotency network and the cell cycle regulation (Card et al., 2008). Also, miR-92a has been identified as a regulator of the G1/S transition in human ESCs by repressing p57 (a member of the Cip/Kip family of inhibitors of CDKs) (Sengupta et al., 2009). Similarly, the miR-302b member, by regulating Cyclin D2 expression, turned out to be necessary to maintain self-renewal and inhibit neuronal differentiation of human EC cells (Lee et al., 2008).

The features that distinguish pluripotent from differentiated cells are clearly the result of complex regulatory interactions between transcription factors, cell cycle regulators, chromatin remodeling proteins, signaling molecules and miRNA.

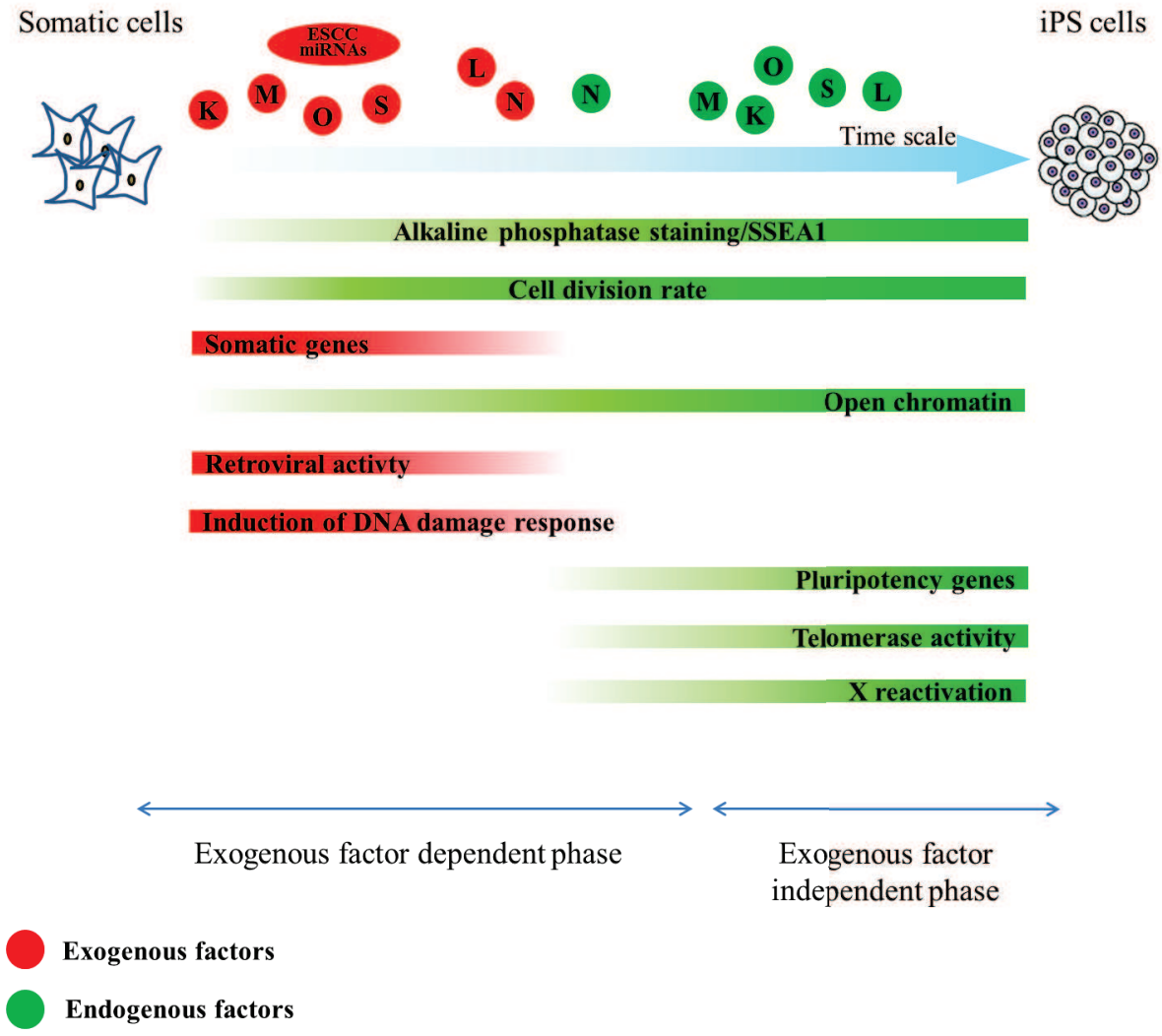

Figure 12: Transcription factor-induced reprogramming is a stepwise progress

Induction of cellular proliferation via c-Myc or ESCC miRNAs, alkaline phosphatase activation and SSEA1 expression are early events. Ectopic expression of the transcription factors cocktails triggers a DNA damage response and either a cell cycle arrest or apoptosis. In partially reprogrammed cells, somatic specific genes are efficiently silenced, endogenous transcription factors are activated, transgenes are efficiently silenced, telomerase activity is increased and Nanog is required to acquire pluripotency. Modulation of the expression of proteins controlling the G1 to S phase transition enhances reprogramming kinetics and efficiency.

O: Oct4, S: Sox2, K: Klf4, M: c-Myc, L: Lin28, N: Nanog

3 THE CELL CYCLE DURING THE ACQUISITION OF PLURIPOTENCY

3.1 During reprogramming

Pluripotent cells can be created outside the embryo by reprogramming somatic cells either by fusion with pre-existing pluripotent cells (Miller and Ruddle, 1976) or by transfection with regulatory transcription factors (Takahashi and Yamanaka, 2006). It consists to converted somatic cells to a pluripotent state molecularly and functionally similar to that of ESCs. It is based on the remodeling of epigenetic marks and so the rewiring of transcriptional regulatory networks of specialized cells to an undifferentiated, pluripotent state *in vitro*.

3.1.1 How to induce pluripotency?

Ectopic gene expression reprograms cell fate…

Revolutionary studies in the stem cell field have shown that it is possible to reprogram pluripotency in somatic cells by overexpression of two cocktails of transcription factors, namely Oct4, Sox2, Klf4, c-Myc in mouse fibroblasts (**Figure 12**) (Takahashi et al., 2006, 2007) and Oct4, Nanog, Sox2, Lin28 in human fibroblasts (Yu et al., 2007). Oct4, Sox2 and Nanog constitute the core of pluripotency whereas Klf4, Lin28and c-Myc have oncogenic properties. Klf4 belongs to the Krüppel-like family of transcription factors, extensively studied for their roles in cell proliferation, differentiation and survival, especially in the context of cancer. Its ability to inhibit p53 (Rowland and Peeper, 2006), a suppressor of Nanog expression, may be important in the reprogramming process by preventing cell cycle exit and apoptosis. Lin28 is an mRNA-binding protein that acts as a transcriptional enhancer and stabilizer. c-Myc belongs to a family of proto-oncogenes transcription factors known to regulate cell growth, cell proliferation, DNA replication, inhibition of cellular differentiation and metastasis (Welstead et al., 2008). The induction of its target genes with role in proliferation such as p21, Cyclin D and E2F2 may enable cells to cycle.

It is important to note that c-Myc is dispensable for the generation of iPSCs, though the kinetics and efficiency of reprogramming are impaired (Nakagawa et al., 2008; Wernig et al., 2008). Interestingly, as Sox2, Klf4, Nanog and Lin28, c-Myc can be replaced with similar efficiencies. Differences in their expressions in different cell types could explain why exogenous contribution is dispensable for iPSCs formation in some cell types. For instance, neural progenitors do not require ectopic c-Myc for reprogramming because they express high endogenous levels (Han et al., 2008).

Over the past few years new procedures for somatic reprogramming have been described including virus-based inducible approaches (Brambrink et al., 2008), transposon PiggyBac technology (Kaji et al., 2009), protein delivery (Zhou et al., 2009) or modified mRNA administration (Warren et al., 2010) of various combinations of transcription factors or small molecules. Nonetheless, reprogramming is a very inefficient process with 0.01-0.1% success rate (Okita et al., 2007) suggesting that there are unknown limiting steps necessary for the generation of iPSCs. For instance, senescence is one of the barriers that impair the process of dedifferentiation. Also, the ectopic expression of Oct4, Sox2, Klf4 and c-Myc triggers a DNA damage response and either a cell cycle arrest or apoptosis by up regulating the expression of p53 and p21 (Banito et al., 2009).

Some cells types such as keratinocytes are more amenable to reprogramming. Differences in reprogrammability may be explained by differences in the cell cycle status, endogenous expression of the reprogramming factors or viral infectivity for a given cell type (Maherali et al., 2008).

Epigenetic events have also been implicated in the reprogramming process. For example, it has been shown that valproic acid, which acts primarily as a histone deacetylase inhibitor, can enhance the efficiency of reprogramming (Huangfu et al., 2008), and that DNA methylation impedes iPSC derivation (Mikkelsen et al., 2008). Also telomeres lengthen significantly in iPS cells in comparison to the parental source, and progressively shorten after differentiation back into fibroblast-like cells, concomitantly with telomerase activation and down-regulation, respectively (Yehezkel et al., 2011).

The question of when and how all these reprogramming factors participate has begun to be elucidated.

... via a gradual process

Factor induced reprogramming progress in a stepwise fashion where individual reprogramming factors play distinct, stage-specific roles.

Firstly, alkaline phosphatase (AP) and stage-specific embryonic antigen 1 (SSEA1) expression are activated. In partially reprogrammed cells, somatic specific genes are efficiently silenced while the embryonic pluripotency regulators are not fully induced. Klf4 and c-Myc have a major contribution to this first steps. In addition of maintaining cells in a proliferative state, c-Myc performs a role in silencing genes associated with differentiation by broadly modifying the epigenetic signature of a cell (Brambrink et al., 2008; Stadtfeld et al., 2008; Knoepfler, 2008; Sridharan et al., 2009). Similarly, Klf4 facilitates chromatin remodeling towards a more dynamic state (Mak et al., 2010). According to Sridharan et al, c-Myc would enable cells to become more receptive to further effects of other transcription factors. Myc participates at multiple stages of iPSCs formation, since it is likely that cells need to be in a proliferative state throughout the reprogramming process.

Secondly, reactivation of endogenous Oct4, Sox2, Nanog and telomerase occur, accompanied with efficient transgene silencing, marking fully reprogrammed cells that can contributes to adult chimeras and give germline transmission (Stadfeld et al., 2008; Brambrink et al., 2008). Nanog is essentially dispensable during the first stage but becomes essential for dedifferentiated intermediates to transit to ground state pluripotency probably by facilitating cooperative binding of the core pluripotency factors to their cognate ES cell targets (Silva et al., 2009).

It is a gradual process that seems to be dependent on cellular proliferation and cell cycle progression. The observed low efficiency supports a model in which all cells are not equally responsive to the different factors and suggests that additional stochastic events are needed.

3.1.2 How the cell cycle structure and its control impacts on the establishment of the pluripotent state?

Established iPSC lines have a cell cycling time and a G1 length indistinguishable from that of hESCs indicating that reprogramming of somatic cells re-establishes the pluripotent cell cycle and more importantly restores the abbreviated G1 phase (Ghule et al., 2011). In the process of erasing the somatic program and establishing the ESC-like phenotype, when is the cell cycle structure modified and what are the implications?

The transcription factor-induced reprogramming can be used as a model to address whether the acquisition of stem cell properties is linked to cell cycle regulation. It has been demonstrated that the signature cell cycle structure of pluripotent cells is acquired as an early

event (Ruiz et al., 2011; Smith et al., 2010). A time-lapse imaging approach enabled to visualize the first events in the path to iPSC state. The first noticeable changes are a faster proliferation and a decrease in cell size only 24 hours after the induction of the reprogramming factors (Smith et al., 2010). Another study supports this evidence. Ruiz et al showed that cell cycle distribution of emerging Oct4 positive/ Nanog negative iPS colonies is similar to that observed in established iPSCs. Besides induction of cell proliferation by modulating the expression of proteins involved in the control of the G1 to S phase transition, such as Rb, E-type and D-type Cyclins and CDK inhibitors, enhances reprogramming efficiency which perfectly correlates with a higher rate of cells entering the S phase. This result is due to a burst in the number of cells amenable to being reprogrammed. Conversely, cell cycle arrest successfully blocks the process of reprogramming and it is sufficient to drive human ES cells toward irreversible differentiation. Altogether, these data highlights the dependency of ESC identity on the atypical mode of cell cycle regulation (Ruiz et al., 2011).

Another group investigated the cell cycle dynamics of human neonatal fibroblasts 48 hours after viral mediated overexpression of Oct4 or Sox2 or Nanog. Precociously and using just one of the reprogramming factors, an increase in the proportion of cells in the S phase was observed going from 1% to 19%. This shows that changes in cell cycle are likely to happen very early during the reprogramming process even before the cells start to re-express their own pluripotency markers (Lako et al., 2009).

Furthermore, inactivation of p53 or CDK inhibitors is able to enhance the reprogramming ability of this currently rather inefficient process (Hong et al., 2009; Li et al., 2009; Utikal et al. 2009, Hanna et al., 2009). It is therefore likely that interference with cell cycle inhibitors or key tumor suppressors that govern the G1 to S transition may dramatically increase the potential of any somatic cell to form an iPS clone. Hanna et al proposed that reprogramming is a stochastic event where all cells have the potential to generate iPSCs albeit with distinct numbers of cell divisions until giving rise to an iPSC. There are two modes of accelerating this process based on cell division rate dependency: (1) inhibition of p53 or p21 entails cells to divide more rapidly, and so increases the number of daughter cells from partially reprogrammed cells, resulting in an increase of the probability of the stochastic event to occur. It is also possible that nuclear changes during cell division may facilitate the acquisition of epigenetic marks that activate the core pluripotency network. (2) Nanog overexpression enhances the reprogramming efficiency without changing the cell division rate. This model supports the hypothesis that promoting the cell cycle improves reprogramming efficiency (Hanna et al., 2009; Ruiz et al 2010).

In the context of cell cycle control, if we assume that one of key change that must occur is the shortening of G1 phase to a length more appropriate to pluripotent cells, the role for c-Myc oncogene may be a requirement for the parental and partially reprogrammed cells to be cycling when exposed to reprogramming factors. It has been shown that c-Myc signals through all three D-type Cyclins to transmit oncogenic information in MEF (Bouchard et al., 2001, Yu et al., 2005). Furthermore, c-Myc transcriptional network do not overlap with those of Oct4, Sox2 and Klf4and it is already engaged at the intermediary step of reprogramming (Sridharan et al., 2009). This is consistent with the idea that c-Myc targets are preferentially involved in the regulation of cellular proliferation pathways as distinct from the endogenous pluripotency network.

Similarly, when human ESCs or iPSCs are reverted to a more pluripotent state similar to the naïve pluripotent mESCs state, the average doubling time was slightly decreased by ~20% (Hanna et al., 2010).

3.1.3 ESCC miRNA link proliferation and efficient reprogramming

Mouse ESCC miRNAs were found to enhance the efficiency of Klf4, Oct4- and Sox2-induced reprogramming to an extend similar to c-Myc. The mechanism of how ESCC miRNAs can replace the proto-oncogene is still unclear. It seems likely that the main contribution is to promote cell cycle progression (Judson et al., 2009). Additionally, suppression of the let-7 family of miRNAs modestly enhances reprogramming of mouse fibroblasts to iPSCs (Melton et al., 2010). The RNA-binding protein Lin 28 is an inhibitor of let-7 expression (Viswanathan et al., 2008). Taken together, these results indicate that Lin28-mediated inhibition of let-7 expression promotes cell de-differentiation through a predominantly cell division rate-dependent mechanism (Hanna et al., 2009).

Manipulation of miRNAs levels is a plausible route for generating iPSCs as demonstrated by Lin and colleagues in 2010. By inducible expression of the four members of the miR-302 miRNA family to levels closely similar to that found in human ESCs, human hair follicle cells were fully reprogrammed. Ectopic miR-302 miRNAs silenced multiple epigenetic regulators thus achieving global demethylation of the genome. Subsequently, Oct4, Sox2 and Nanog expression were activated which in turns further stimulates miR-302 expression. The positive feedback between epigenetic, transcriptional and post-transcriptional events stabilizes the pluripotent phenotype. Work of Subramanyam et al (2011) confirm that multiple targets of miR-302 and miR-372 promote dedifferentiation of human fibroblasts to

iPSCs by acting on multiple downstream pathways such as cell cycle regulation, epigenetic regulation, cell signaling and epithelial-mesenchymal transition.

These data provides some evidence on the importance of the cell cycle machinery in establishing and maintaining ESC identity. c-Myc is not the only component of the reprogramming transcription factor network that plays a role in cell cycle control. For instance, Sox2 and Oct4 control the cell cycle miRNAs (see section 2.6.2), and Nanog regulates G1 progression at the transcriptional level (see section 3.1.4).

3.1.4 Interplay between the reprogramming core factors and the cell cycle components

Combined overexpression of Oct4, Sox2, Lin28 and Nanog is able to reprogram somatic cells to a pluripotent phenotype (Yu et al., 2007), a processes during which the long G1 phase becomes an abbreviated pluripotent G1 phase. This means that during transcription factor-mediated reprogramming changes in expression or post-transcriptional modifications of cell cycle regulators have to occur.

Two research groups demonstrated that Nanog overexpression in mouse ESCs confers pluripotency independently of LIF signaling (Chambers et al., 2003; Mitsui et al., 2003). On the other hand, it has been shown in human ES cells that Nanog overexpression enhances G1 to S phase transition. This is achieved by two downstream cell cycle effectors of Nanog, CDK6 and CDC25A. First, the authors demonstrated that Nanog overexpression enhances proliferation by accelerating S phase entry without interfering with the ability of multi-lineage differentiation. In addition, Nanog down regulation halts cell cycle progression in G1 phase but also in S phase and in the G2 to M transition. Immunoblot for key cell cycle components of the G1 to S phase transition combined with ChIP analysis revealed that Nanog directly binds the regulatory regions of CDK6 and CDC25A and regulates their expression. Other experiment showed that overexpression of either CDK6 or CDC25A resulted in a faster S phase than in control cells, and that it rescued the delay in cell cycle entry observed in hESCs with Nanog knockdown. CDK6 knockdown resulted in retention of cells in G1 phase and CDC25A knockdown provokes retention of cells at the G1 phase and in G2/M transition. Cdc25A phosphatase could act via enhancing the activity of any CDK. These results suggest Nanog control of G1/S transition depends on CDK6 and CDC25A, and that other effectors mediate its effect on S phase progression and G2 to S transition (Zhang et al., 2008).

Likewise, it has been shown that Oct4 promotes G1 to S phase transition. Inducible downregulation of Oct4 expression in mouse ESCs resulted in doubling the cell division time, arrest in G1 phase and up regulation of p21 expression. Moreover, it was shown in the same study that Oct4 directly represses p21 promoter. Thus, the authors proposed that Oct4 stimulates S phase entry likely by inhibiting p21 expression (Lee et al., 2010). Another recent work on human ESCs suggests that E2F may act as a cofactor for Oct4 at the promoters of Oct4 target genes (Chavez et al., 2009).

Interestingly, Singh et al (2007) showed evidence for Nanog and Gata6 heterogeneous and mutually exclusive expression in mouse ESC culture. $Nanog^{HIGH}$ population expresses genes involved in cell cycle regulation, whereas $Nanog^{LOW}$ / $Gata6^{HIGH}$ population has an increased expression of cell cycle inhibitory genes (Singh et al., 2007).

Lin 28 has been shown to play a positive role in the regulation of Cyclin A, Cyclin B and CDK4 at the translational level in mouse ESCs. Actually, repression of Lin28 results in a decrease of cell proliferation while its overexpression has the opposite effect. Lin 28 associates specifically with ribonucleoprotein particles containing mRNAs for Cyclin A, Cyclin B and CDK4. Finally, changes in Lin 28 expression lead to corresponding changes in the level of these proteins (Xu et al., 2009). This is a hint on how Lin 28 enhances the reprogramming efficiency (Yu et al., 2007) by accelerating cell division (Hanna et al., 2009).

Finally, global gene analysis experiments have shown that Oct4 regulates CDK4 transcription (Boyer et al., 2005; Greco et al., 2007), that D-type Cyclins and CDK6 promoters are bound by SOX2 and that CDK1 is a target of Nanog and Sox2 (Boyer et al., 2005).

Altogether, generation of pluripotent cells provides a picture of how the fundamentals of pluripotency are linked with cell division cycles by describing the role of master transcription factors play in regulating the ES cell cycle positively. In conclusion, there is an intimate link between mechanisms of pluripotency and cell cycle regulation.

3.2 During embryogenesis

For general insights into embryogenesis please refer to section 1.5.1. Here, we will focus on the cell cycle features of mouse cells during early embryogenesis.

A remarkable feature: rapid division rates

Post-fertilization divisions of the mammalian embryo generate the first extra embryonic tissue, the trophoblast that will give rise to the placenta and to the chorion and do not produce the embryo proper. Early cell divisions are not unusually fast but instead have well defined G1 and G2 phases. Moreover, at this stage of development inhibition of DNA replication triggers cell cycle arrest. The fertilized egg reaches the 2 cell stage at 1.5 day post-coitum (O'Farrell et al., 2004). In human embryo the cycle time for cells in the cleavage stage is approximately 36 hours (Odorico et al., 2001). The epiblast cells of the postimplantation E5.5 to E6.0 mouse blastocyst have a mean cycle time of 11.5 hours. But a day later when gastrulation begins, at E6.5 to E7.0, epiblast cells have a mean cell cycle time of only 4.4 hours. Mouse gastrulation occurs at the egg cylinder stage (E6.5) and it corresponds to the formation of the embryonic germ layers in conjunction with the primitive streak formation. The latter is the structure that establishes the bilateral symmetry and that initiate the germ layers formation. At the onset of gastrulation, the epiblast cells have short cell cycle time of 7-7.5 hours (Lawson et al., 1991) and a G1 phase that lasts 1.5-2 hours. These durations shorten in cells of the primitive streak. These cells are characterized by a cell cycle time of 3-3.5 hours with a further shortening of all three phases of the cell cycle G1(<30 min), S (2-2.75 hours) and G2 (<20 min). Cells of the primitive streak are believed to have virtually eliminated G1 (MacAuley et al., 1993).

The events surrounding mouse gastrulation display some unique properties namely the rapid speed, an extremely short G1 phase, no cell growth and lack of checkpoints (Snow, 1977; MacAuley et al., 1993). Upon DNA damage cells rather undergo a p53 and ATM dependent apoptotic response (Heyer et al., 2000). Rapid cell division is achieved by structuring the cell cycle so that it lacks gap phases and consists purely of alternating rounds of DNA replication (S phase) and chromosome segregation (M phase). Although the proliferative burst associated with this phase of development has been well documented, the molecular mechanisms underpinning this sudden change remains largely unknown. Necessarily, they imply highly dynamic regulation of cell cycle parameters.

As gastrulation and embryonic development progresses, commitment towards specific cell lineages is accompanied by a deceleration of cellular expansion (Snow, 1977), so that terminal differentiation is coupled to withdrawal from the cell cycle. Equally important in the execution of developmental programs is the arrest of growth once the program is complete (Parker et al., 1995).

Finally, rapid proliferation rates in early embryo are encountered in lower vertebrates such as Xenopus (Murray and Kirschner, 1989) and in the fruit fly Drosophila (Edgar and

Lehner, 1996). Early divisions consist of alternating rounds of DNA replication and mitosis lacking intervening G1 and G2 gap phases. Thus, rapid proliferation is an intrinsic property of pluripotent stem cells easily explained by the need of sufficient cell number to initiate gastrulation.

Another remarkable feature: unusual cell cycle molecular mechanisms

Epiblast cells of preimplantation and early postimplantation embryos have unusual mechanisms of CDK regulation, namely low Cyclin D1 and Cyclin D3 expression as well as low CDK4 related kinase activity. Consistently, preimplantation development is independent of exogenous growth factors (Biggers et al. 1997).

Gene knock-out experiments in mice give quite unexpected results. Homozygous knockout mice for D-type Cyclins, CDK2, CDK4 and CDK6 are viable at least until midgestation indicating that those cell cycle regulators are not essential for proliferation of most cells types during early embryo development. Interestingly, while Cyclin E has been shown to be critically required for the development of drosophila (Knoblich et al., 1994) and Caenorhabditis elegans (Fay and Han, 2000), this is clearly not the case in mouse embryos. Cyclin E has been shown to de dispensable for embryonic development, but critical to extraembryonic development (Geng et al., 2003). This suggests an evolutionary acquired redundancy between cell cycle proteins. Intriguingly, other reports indicate that mice lacking other components of the cell cycle machinery, namely Rb (Wu et al., 2003), as well as the E2F partner DP1 (Kohn et al., 2003), also die due to placental abnormalities, revealing the specific requirement for these proteins in extraembryonal development. Studies of genetic mouse models have highlighted the extraordinary plasticity of the embryonic cell cycle with an important functional redundancy and compensation of its components (Artus et al., 2006). In addition, although genes taking part in the Rb pathway are expressed during early mouse development, they are dispensable for this period of development (Ciemerych and Sicinski, 2005). Rb proteins are hyperphosphorylated throughout preimplantation development (Xie et al., 2005).

It is not until gastrulation that D-type Cyclins and CDK4 activity are detected (Wianny et al., 1998). At the onset of gastrulation, cell cycle of embryonic cells becomes dependent of the activation of the E2F-pRb pathway, Cyclin E/CDK2 activity is down regulated and G1/S regulation is incorporated (White et al., 2005). This correlates with lineage specific commitment. The modulation of the cell cycle progression is achieved by both time and tissue specific expression of cell cycle regulatory proteins as well as the integration of external cues

generated by cell cell contact and signaling pathways (Artus and Cohen-Tannoudji, 2008). For instance, the ability of p21 to function as an inducible cell cycle inhibitor suggests that it may also function to mediate the cell cycle duration during development (Parker et al., 1995). Nevertheless, possible contributions of cell cycle regulators to developmental programs have still to be explored.

Small RNAs are likely to play a pivotal role in mammalian embryogenesis. Interestingly, maternally contributed miRNAs are key factors in the maintenance self-renewal renewal and pluripotency during the pre and postimplantation stages of development. miRNAs are non-essential during preimplantation embryonic development nevertheless they function throughout postimplantation development (Suh and Blelloch, 2011). Suh and Blelloch speculate that the suppression of miRNA function enables the massive epigenomic reprogramming that occurs after fertilization in preparation for new gene expression.

Numerous data have shown that ES and EpiS cells are strikingly similar to their inner cell mass counterparts, thus they are considered to be a useful *in vitro* tool to understand cell cycle mechanisms in early development and how they change during differentiation. A legitimate question is at what extend observations made in pluripotent stem cells *in vitro* correspond to the *in vivo* situation since they are not subject to the same selective pressure in each environment. The native environment of pre implantation embryos is hypoxic. But long term culture of mouse ESCs under low oxygen culture inhibits self-renewal and lead to differentiation (Jeong et al., 2007). By the contrary, human ESCs highly proliferate in the pluripotent state under hypoxia (Forristal et al., 2010).

3.3 Does a unique cell cycle structure actively establishes pluripotency, sustains self-renewal and contributes to inhibit differentiation?

Epiblast cells as well as pluripotent stem cells derived from the embryo or by molecular reprogramming *ex vivo* are believe to exist in two different stages of pluripotency, naive or primed, that reflect different origins and developmental potentials. Although these states are molecularly and phenotypically similar, they are not identical. As we have discussed, pluripotent stem cells exhibit a very unusual cell cycle structure, characterized by a short G1 phase and a high proportion of cells in S phase. This is associated with a unique mechanism of cell cycle regulation, underpinned by lack of retinoblastoma control, lack of G1

checkpoint at least for naïve cells, as well as non-oscillatory and robust activity of Cyclin E/CDK2-associated kinase activity throughout the cell cycle. Modification of the cell cycle parameters results in either apoptosis or differentiation in cell culture or disruption of proper embryogenesis. Moreover, on path towards pluripotency one of the first steps is to increase cell divisions rate. Thereby, cell cycle is expected to play a major role in establishing and maintaining pluripotency.

Unlike somatic cells, neither CDK4 nor CDK6 kinase related activities are implicated in the regulation of the Rb phosphorylation status in human or murine pluripotent stem cells. Elevated Cyclin E-Cyclin A/CDK2 activity is likely to be implicated in persistent Rb phosphorylation independently of Cyclin D/CDKs and in sustaining the accelerated G1/S transition.

To address the question of whether unique cell cycle features are needed to achieve pluripotency and define cell fate, we engineered ES cells that can be situated cells in a specific cell cycle compartment and we made use of gain and loss of function technologies. We focused on studying the cell cycle structure, and more specifically the G1 length, link to the state of pluripotency, which depends on distinct intrinsic controls and on particular extracellular signal requirements. We may wonder if modifying the rate of G1 to S phase transition in ES cells directly results in the modification of pluripotency. If that is the case, which would be the molecular pathways involved? Presumably, Cyclin E, by driving the G1/S transition, may contribute to the regulation of the balance between self-renewal and differentiation. Nevertheless, pluripotency can be acquired without Cyclin E and even without CDK2. This suggests that Cyclin E contribution to maintenance of pluripotency may be CDK2 independent. We propose evidences directly linking G1 length to maintenance of the naïve or primed state of pluripotency.

4. THE FUCCI SYSTEM

4.1 Getting into the basics of the Fucci system

4.1.1 The initial system established in somatic cells

Miyawaki's group developed a surprisingly precise visual indicator of cell cycle status in living cells based on the expression of an orange-emitting fluorescent protein (mKO2) fused to human Cdt1 licensing factor during the G1 phase, and a green-emitting fluorescent protein (mAG) fused to human Geminin (Gem) licensing factor during the S, G2 and M phases (**Figure 13 A**). mKO2:hCdt1 is abruptly degraded at the G1/S transition by SCF^{Skp2}-dependent destruction of Cdt1. Similarly, mAG:hGem is abruptly degraded during late mitosis by APC^{Cdh1}-dependent destruction of Geminin (Sakaue-Sawano el al., 2007). In somatic cells, because the SCF^{Skp2} complex is a direct substrate of the APC^{Cdh1} complex but also functions as a feedback inhibitor of APC^{Cdh1} (Vodermaier, 2004; Benmaamar and Pagano, 2005), these two E3 ligase activities oscillate reciprocally during the cell cycle. The APC^{Cdh1} complex is activated at the metaphase/anaphase transition and G1 phases, while the SCF^{Skp2} complex is active in the S, G2 and early M phases.

This system was named Fluorescent Ubiquitination-based Cell Cycle Indicator (Fucci). Truncated forms of human Cdt1 and of human Geminin were used. They display a nuclear subcellular localization. Cdt1 (amino acids 30 to 120) accumulates during G1 phase in the nuclei but it is degraded during S, G2 and M phases by ubiquitin mediated proteolysis. Inversely, Gem (amino acids 1 to 110) is present only during S, G2 and M phases. In Hela cells and mouse mammary epithelial cells, it was observed that, since green fluorescence associated to mAG:hGem disappeared rapidly in late M phase and orange fluorescence associated to mKO2:hCdt1 became detectable in early G1 phase, a small gap without any fluorescent signal was detected in daughter cells. By contrast, during orange to green conversion, orange and green fluorescence always overlapped to yield a yellow nucleus.

Finally, the Fucci system allows to follow cell cycle progression with high spatiotemporal resolution in a multicellular context during various biological events. It is a powerful technique designed to track and to observe cellular processes directly in cultured cells and within a living animal e.g. investigate tumor development *in vivo*, study embryonic development.

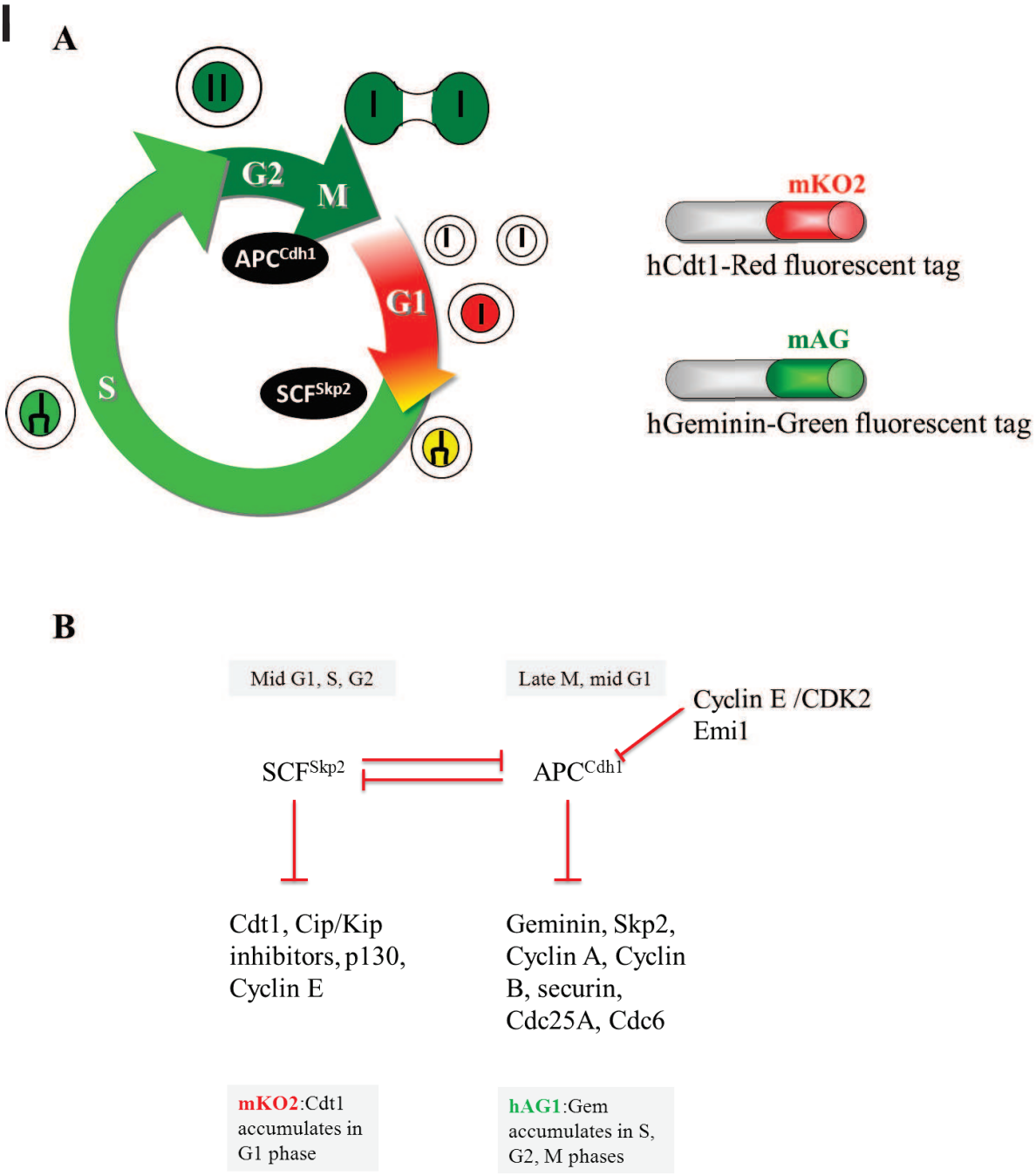

Figure 13: The Fluorescent Ubiquitination-based Cell Cycle Indicator

(A) Based on the mutually exclusive expression of a red (mKO2:hCdt1) and a green (mAG:hGem) fluorescent probes, the Fucci system enables to track live cells and to follow their cell cycle progression. mKO2:hCdt1 is degraded at the G1/S transition by SCF^{Skp2} and mAG:hGem is degraded during late mitosis by APC^{Cdh1}. Thereby, cells in early G1 are white-colored, those in late G1 are red, those transiting from G1 to S are yellow, those in S phase are light green and finally cells in G2 are dark green. Cell DNA content is also represented.

(B) The APC^{Cdh1} / SCF^{Skp2} switch orchestrates the coordination of events required for cell cycle progression by mediating the destruction of key regulatory proteins such as Cyclins and their inhibitors. It is thought that SCF^{Skp2} ubiquitin ligase controls the G1 to S transition, whereas APC^{Cdh1} ubiquitin ligase controls the exit from mitosis. A subset of cell cycle regulatory substrates for each E3 ligase is listed. Note that by targeting the SCF-component Skp2 for destruction, APC^{Cdh1} not only regulates exit from mitosis, but also controls the duration of G1.

4.1.2 Molecular mechanism underlying the APC^{Cdh1} / SCF^{Skp2} switch in somatic cells

By targeting specific substrates for proteasome degradation, the two ubiquitin ligase complexes SCF^{Skp2} and APC^{Cdh1} also drive cell cycle progression. Importantly, the irreversible nature of proteolysis is complement to the intrinsically reversible regulation through phosphorylation and other post-translational modifications. Cdh1and Skp2 are co-activators of APC and SCF, respectively, and therefore contribute to the regulation of protein degradation, by providing substrate specificity in a cell cycle regulated manner (**Figure 13 B**). APC^{Cdh1} activity is responsible for the degradation of Geminin, Skp2, Cyclin A, Cyclin B, the anaphase inhibitor securin and the CDK2 activating phosphatase Cdc25A. Thereby APC^{Cdh1} is essential for proper chromosome segregation, mitotic exit and regulates the timing for S phase entry (Li and Zhang, 2009). CDK-dependent phosphorylation of Cdh1 inhibits APC^{Cdh1} activity (Vodermaier, 2004). Among SCF^{Skp2} targets there are Cdt1, Cip/Kip inhibitors, the Rb related protein p130 and Cyclin E. Thus SCF^{Skp2} contributes to cell cycle re-entry (Benmaamar and Pagano, 2005).

Two direct substrates of the APC^{Cdh1} and SCF^{Skp2} complexes, Geminin and Cdt1, are involved in ‘‘licensing’’ of replication origins (Nishitani et al., 2000). This carefully regulated process ensures that replication occurs only once in a cell cycle. Geminin inhibits pre-replication complex assembly between S phase and the metaphase/anaphase transition by preventing Cdt1 from recruiting MCM proteins to chromatin (**Figure 9**) (McGarry and Kirschner, 1998).

Since, ES cells have a completely different expression pattern of Cyclins /CDKs compared to that in somatic cells, one could expect also a different regulation of the APC^{Cdh1} / SCF^{Skp2} switch.

4.2 A distinct mode of regulation of the APC^{Cdh1} / SCF^{Skp2}switch in pluripotent stem cells

In mouse ES cells, Geminin was shown to act as a prodifferentiation factor, additionally to inhibiting DNA replication origins licensing. Geminin is expressed in the nucleus at a constant level in mESCs progressively acquiring a neural fate. Its overexpression promotes neural gene expression. It regulates neural commitment by maintaining the

chromatin of neural genes in a state of high acetylation and accessibility. *In vitro,* it regulates histone acetylation. Surprisingly, Geminin knockdown did not result in genome overreplication, neither affected self-renewal nor altered proliferation or cell viability (Yellajoshyula et al., 2011). Conversely, another report concluded that Geminin inhibits neural differentiation in mouse EC cells (Seo et al., 2005).

On the other hand, another recent report claims that silencing of Geminin in mouse ESCs and EC cells causes loss of stem cell identity and promotes differentiation towards trophoblast lineage mimicking depletion of Oct4 (Yang et al., 2011). In contrast to Yellajoshyula et al (2011) report, here an increase of the DNA content was observed following downregulation of Geminin. Moreover, the authors demonstrated that Geminin sustains Oct4, Sox2 and Nanog expression by antagonizing the chromatin-remodeling protein Brg1. This suggests that Geminin may link cell cycle control and pluripotency. Furthermore they showed that Geminin is expressed throughout the cell cycle, particularly during G1 phase in contrast to somatic cells. They argued that APC^{Cdh1} activity is very low in G1 in mouse pluripotent cells because of the high expression of its inhibitor Emi1. This hypothesis would be consistent with the fact that its targets e.g. Cyclin A and B do not oscillate during the cell cycle. However, it is well known that Cyclin B is the only cell cycle regulator that has a periodic expression in pluripotent stem cells (Stead et al., 2002). Finally, they speculated that Geminin might only be absent for a very short window during late telophase, or in very early G1, to allow pre replication complexes to assemble (Yang et al., 2011).

Fujii-Yamamoto et al (2005) conducted some experiments on synchronized cells that revealed that Geminin is highly expressed throughout the cell cycle of mouse ESCs in the cytosolic comportment only. This study also showed that Cyclin E1, Cyclin A2 and Cyclin B1 protein levels and their associated kinase activities are prominently detected at all the cell cycle stages and did not show striking fluctuation. These findings differ with the cell cycle dependent expression of Cyclin B in pluripotent stem cells described by Stead et al (2002). According to this subcellular localization, Geminin would not probably fulfill its function in preventing overreplication of DNA.

In undifferentiated human ES cells a study showed that Skp2, a regulatory subunit of the SCF ubiquitin ligase complex, is highly expressed all over the cell cycle but decline rapidly following induction of differentiation (Bar-On et al., 2010). Inversely, APC^{Cdh1} that targets Skp2 for degradation is present but inactive in hESCs and becomes active in the differentiate cell state. Two hypotheses may explain the inactive state of APC^{Cdh1}: (1) high levels of its specific inhibitor Emi1, which decline upon differentiation, and (2) Cdh1 fail to

activate the ubiquitin ligase complex possibly by Cyclin/CDK inhibitory phosphorylations. Thereby APC^{Cdh1} can become active only after induction of differentiation which is consistent with the down regulation of the levels of its targets Cyclin A and Cyclin B. All of this convey to constitutive expression of Geminin and a lack of Cdt1.

Clearly the previous studies show inconsistent data on Geminin effects on self-renewal, genomic integrity and pattern of expression. In spite of this, the important conclusion may be that for all cell cycle phases APC^{Cdh1} activity is low in pluripotent stem cells, thereby Geminin is protected from degradation while SCF^{Skp2} is active thus leading to Cdt1 degradation.

The findings on these key regulatory proteins raise the questions of how the Fucci system based on the expression of mKO2:Cdt1 and hmAG1:Gem fusion proteins still works in ES cells? More specifically, why overexpressing the truncated form of Geminin (1/110) does not induce neural fate acquisition? Why the red and green fluorescence detected are rather mutually exclusive and nuclear?

Chapter two:

RESULTS

The brevity of the G1 phase is an intrinsic determinant of naive pluripotency

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INTRODUCTION

The proliferation of mammalian cells is controlled largely during the G1 phase of their growth cycles. The decision to initiate a new round of DNA synthesis is dependent upon phosphorylation and functional inactivation of the retinoblastoma protein (pRB). This phosphorylation is driven largely by components of the cell cycle apparatus, specifically cyclins and cyclin-dependent kinases (CDKs). Of prime importance are complexes of D-type cyclins (cyclin D1, D2 and D3) and CDK4 or CDK6. Their accumulation is exquisitely regulated by extracellular signals transduced *via* the SOS-RAS-MEKK-MAPK pathway (Weinberg, 1995; Lukas, 1996; Bartek, 1997). Phosphorylation of pRB by cyclin D-CDK4/6 results in the activation of E2F family transcription factors, which then act in synergy with Myc to transcriptionally activate cyclin E and Cdc25A, resulting in the accumulation of active cyclin E-CDK2 complexes (Bartek, 2001). Cyclin E is a rate-limiting activator of CDK2 (Resnitzky, 1994; Hwang, 2005) and cyclin E-CDK2 kinase, the driving force of the G1-to-S phase transition (Lukas, 1997). After S-phase entry, cyclin E-CDK2 complexes are degraded by phosphorylation and ubiquitination-mediated proteolysis (Welcker, 2003). The cyclin E-CDK2 kinase can be temporarily inhibited in response to DNA damage, *via* activation of p53 and subsequent activation of the CDK2 inhibitor p21 (Bartek, 2001).

Embryonic stem cells (ESC) are derived from the early epiblast of the pre-implantation blastocyst. ESCs are rapidly proliferating mammalian cells, which exhibit an unusual cell cycle structure characterized by a G1 phase of less than two hours (Savatier, 1994). In the case of mouse ESCs (mESCs), the brevity of the G1 phase is associated with a unique mechanism of cell cycle regulation, underpinned by lack of MAPK, cyclin D and pRB control (Savatier, 1996; Jirmanova, 2002; White, 2005), lack of functional p53-p21 pathway in response to DNA damage (Aladjem, 1998), active transcription of E2F target genes, and

robust activity of cyclin E-CDK2 complexes throughout the cell cycle (Stead, 2002; White, 2005). Hence, the mESC mitotic cycle appears to be constitutively primed for DNA replication. As mESCs differentiate, their cell cycle structure changes dramatically so as to incorporate a longer G1 phase, and their mechanism of cell cycle regulation changes to that typically seen in other mammalian cells including the acquisition of p53- and RB-dependent checkpoints.

One of the most remarkable feature of the mESC mitotic cycle is the continuous - *i.e.* non oscillatory - expression of cyclin E and cyclin E-associated kinase activity throughout the cell cycle, as opposed to the G1-specific expression as observed in somatic cells (Stead, 2002). Studies in the rhesus monkey and human indicate that primate ESCs share many of the mESC cell-cycle characteristics. In particular, we showed that rhesus ESCs do not arrest in G1 after DNA damage, and express cyclin E uniformly throughout the cell cycle (Fluckiger, 2006). Similar observations were made with human (Filipczyk, 2007; Momcilovic, 2009) and rabbit ESCs (unpublished data). Hence, cell cycle-independent expression of cyclin E seems to be consubstantial with pluripotency. The reason why pluripotent stem cells express cyclin E and cyclin E:CDK2 complexes uniformly throughout the cell cycle is presently unknown. It might simply reflect the fundamental requirement of rapid proliferation of epiblast cells to initiate gastrulation in the developing embryo. It is possible, however, that it might be involved in actively sustaining the pluripotent state. This hypothesis is supported by the observation that roscovitin, a pharmacological inhibitor of CDK2, induces differentiation of human ESCs (Filipczyk, 2007). Moreover, the murine teratocarcinoma-derived Embryonal Carcinoma (EC) stem cells are more susceptible to differentiation when they are in G1 phase as compared to the other phases of the cell cycle (Mummery, 1987). Whether the same holds true for ESCs remains to be determined. Of equal importance in exploring the functional relationship between cell cycle and pluripotency is the identification of the pathways that

regulate cyclin E expression and cyclin E:CDK2 kinase activity in ESCs.

Making use of the Fucci reporter system, we examined the cell-cycle dependency of differentiation commitment, and we explored the role of LIF signalling in the regulation of the G1-to-S phase transition. We also examined the role of cyclin E and cyclin E-CDK2 kinase in mESCs self-renewal.

MATERIAL AND METHODS

Plasmid construction and shRNA design

The pFucci G1 orange and pFucci S/G2/M green plasmids (MBL International Corporation, ref AM-VS0605) were digested with XbaI and BamHI. The resulting 1045 bp and 1106 bp fragments, containing mKO2:Cdt1(30:120) and hmAG1:Geminin(1:110) coding sequences, were sub-cloned between the HindIII and BamHI sites in *pGAE-CAG-eGFP-WPRE* (Wianny, 2008) to generate *pGAE-mKO2:Cdt1* and *pGAE-mAG:Geminin*, respectively.

Lentiviral expression vectors of rat and human cyclin E were generated using the MultiSite Gateway® Technology (Invitrogen, http://tools.invitrogen.com/content/sfs/manuals/multisite_gateway_man.pdf). Myc-tagged rat cyclin E cDNAs (wild-type and S180D mutant) were amplified by PCR from $pINDCycE^{WT}$ and $pINDCycE^{S180D}$, respectively (Matsumoto, 2004), using primers that contain *att* recombination sites [AttB1-K-ratCycE-S: 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGCCACCATGGAGCAAAAGCTCATTCTGAA-3'; AttB2-ratCycE-AS: 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCTCATTCTGTCTCCTGCTCACTGCT-3'. Human cyclin E cDNAs (wild-type and KD-E mutant) were amplified by PCR from $pCS2cycE1^{WT}$ and $pCS2cycE1^{KD}$, respectively (Geng, 2007),

using the following primers [AttB1-K-hCycE-S: 5'-GGGGACAAGTTTGTACAAA AAAGCAGGCTTCGCCACCATGCCGAGGGAGCGCAGGGAGCGG-3'; AttB2-hCycE-AS: 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCTCACGCCATTTCCGGCCCGCT GCT-3'. PCR products were subsequently cloned into *pDONR™221* using Gateway BP clonase enzyme mix. In parallel, the CAG promoter (CMV early enhancer/chicken β-actin) from *pGAE-CAG-eGFP-WPRE* (Wianny, 2008) was cloned into *pDONRP4-P1R*. The resulting two entry vectors were then recombined into *2K7neo* lentivector (Suter, 2006) using Gateway LR plus clonase enzyme mix to generate *p2K7neo-ratCycE*^{WT}, *p2K7neo-ratCycE*^{S180D}, *p2K7neo-hCycE*^{WT}, and *p2K7neo-hCycE*^{KD}.

Expression vectors of rat and human cyclin E suitable for conditional expression induced by doxycycline and dexamethasone (Anastassiadis, 2002) were generated. Myc-tagged rat cyclin E cDNAs (both wild-type and S180D mutant) were amplified by PCR from *pINDCycE*^{WT} and *pINDCycE*^{S180D}, respectively (Matsumoto, 2004), using primers containing SalI and NotI sites (underlined) (Myc-cycE-SalI-F: 5'-ACGCGTCGACACCATG GAGCAAAAGCTCATTTC-3'; Myc-cycE-NotI-R: 5'-AAGGAAAAAAGCGGCCGCTCAT TCTGTCTCCTGCTCAC-3'). Human cyclin E cDNAs (wild-type and KD-E mutant) were amplified by PCR from *pCS2cycE1*^{WT} and *pCS2cycE1*^{KD}, respectively (Geng, 2007), using the following primers [hCycE1SalIF: 5'-ACGCGTCGACACCATGCCGAGGGAGCG CAGGGAC-3'; hCycE1Not1R: 5'-AAGGAAAAAAGCGGCCGCTCACGCCATTTCCGGC CCGC-3']. The PCR products were subsequently subcloned between the SalI and NotI sites in *pSport1-SV40-PolyA* to generate *pSport1-cDNA* plasmids. In parallel, *pTet/CMV-INS2-Hygro* was digested with SalI and SwaI. The resulting 5,046 bp *Tet/CMV-INS2-Hygro* fragment was subcloned into each *pSport1-cDNA* plasmids by red/ET recombination to generate *pSport1-tet/CMV-INS2-cDNA-Hygro* vectors.

shRNA sequences were designed using the si*DESIGN*®Center application of Dharmacon (http://www.dharmacon.com). shRNA sequences were cloned into *pLenti6/BLOCK-iT-PGKneo*^{r}. The sequences of selected shRNAs and the interference resulting from transfection of pENTRY-shRNA vectors in CGR8 cells are given in **Table I.**

ESC culture, differentiation, and electroporation.

All ES cell lines were routinely cultured in Glasgow's Modified Eagle's Medium (GMEM) supplemented with 10% fetal calf serum (PerbioScience CRC0406) and 1000 U/ml of LIF. To induce differentiation, cells were allowed to aggregate in hanging drops in ES cell medium without LIF (100 cells/drop). After three days, embryoid bodies were collected and further grown in suspension for 1 to 7 days in non-adherent Petri dishes. ES cells were electroporated with linearized plasmids at 260 V and 500 µF in a 0.4 cm cuvette. Cells were plated at 5.10^{6} cells per 10 cm dish, cultured in the presence of 1 µg/ml puromycine or 100 µg/ml hygromycine B for 7 days.

LIF rescue assay

ES cells were plated at 10^{3} cells per gelatin-coated 100 mm tissue culture dish in complete ES cell medium. Cells were exposed to medium without LIF for 12 to 48 hrs and were subsequently cultured in normal ES cell medium with 1000 U/ml of LIF for 6 days. To detect cells expressing alkaline phosphatase, dishes were fixed in methanol for 15 min, and then stained for 15 min with a solution containing 1 mg/ml Fast Red TR salt™ (Sigma) dissolved in 0.1 M Tris, pH 9.2, containing 200 mg/ml naphtol AS-MX phosphate. The percentage of undifferentiated, mixed and differentiated colonies were counted in triplicates for each treatment.

Production of lentiviral vectors and infection of ESCs

For production of SIV-derived lentivectors, 293T cells were transfected with a mixture of DNAs containing 7.5 µg of the *pGRev* plasmid encoding the vesicular stomatitis virus glycoprotein (VSV-G) envelope, 4 µg of the *pSIV3*$^{+}$ plasmid encoding the gag, pol, tat and rev proteins, and 11.5 µg of the *p2K7neo* plasmid encoding the cDNA of interest (or *pLenti6/BLOCK-iT-PGKneo*^{r} encoding shRNA) using the calcium phosphate precipitation technique. The following day, cells were refed with 5 ml of DMEM and further cultured for 24 hours. The supernatant was then collected, cleared by centrifugation (3000 RPM, 15 minutes) and passed through a 0.8µm filter.

For infection, CGR8 cells were plated at a density of 10^4 cells in 24-well plates in 1 ml of medium composed of 100 µl of ES cell medium and 900 µl of culture supernatant from virus-producer cells. After 48 hours, ES cells were trypsinized, replated at 10^4 cells per gelatin-coated 10 mm tissue culture dish and further cultivated in complete ES cell medium. ESCs infected with pLenti6/BLOCK-iT-PGKneo^{r} were cultivated in medium supplemented with 250 µg/ml of G418 to kill non infected cells.

Semi-quantitative and real-time PCR analysis. Total RNA was extracted using RNAeasy kits with on-column DNAse digestion (Qiagen, http://www.qiagen.com). Reverse transcription was performed using M-MLV Reverse Transcriptase (Promega Corp., Madison, WI, http://www.promega.com) and Random Primers (MWG-Biotech AG, Ebersberg, Germany, http://www.mwg-biotech.com), according to the manufacturer's recommendations. PCR reactions were performed with a Perkin-Elmer thermal cycler, operating on a regimen of 94°C for 30 sec, 55 to 62°C (according to primers) for 30 sec, and 72°C for 60 sec. Quantitative PCR was performed with Step One Plus thermal cycler (Applied Biosystem), operating on a regimen of 94°C for 45 sec, 55 for 45 sec and 72°C for 45 sec, during 40

cycles with Quantitec SYBR Green (Qiagen). In all cases target genes were normalised to *β-actin*. Primers, annealing temperatures and number of cycles are provided in **Table II**.

Flow cytometry and fluorescent microscopy

ESCs were analysed using a FACS LSR II (Beckton-Dickinson) equipped with 355, 488, and 561nM lasers, or sorted on an Aria cell sorter (Beckton-Dickinson) equipped with 405, 488, and 633nM lasers. Data were recorded and analysed using DiVa software.

Western blotting.

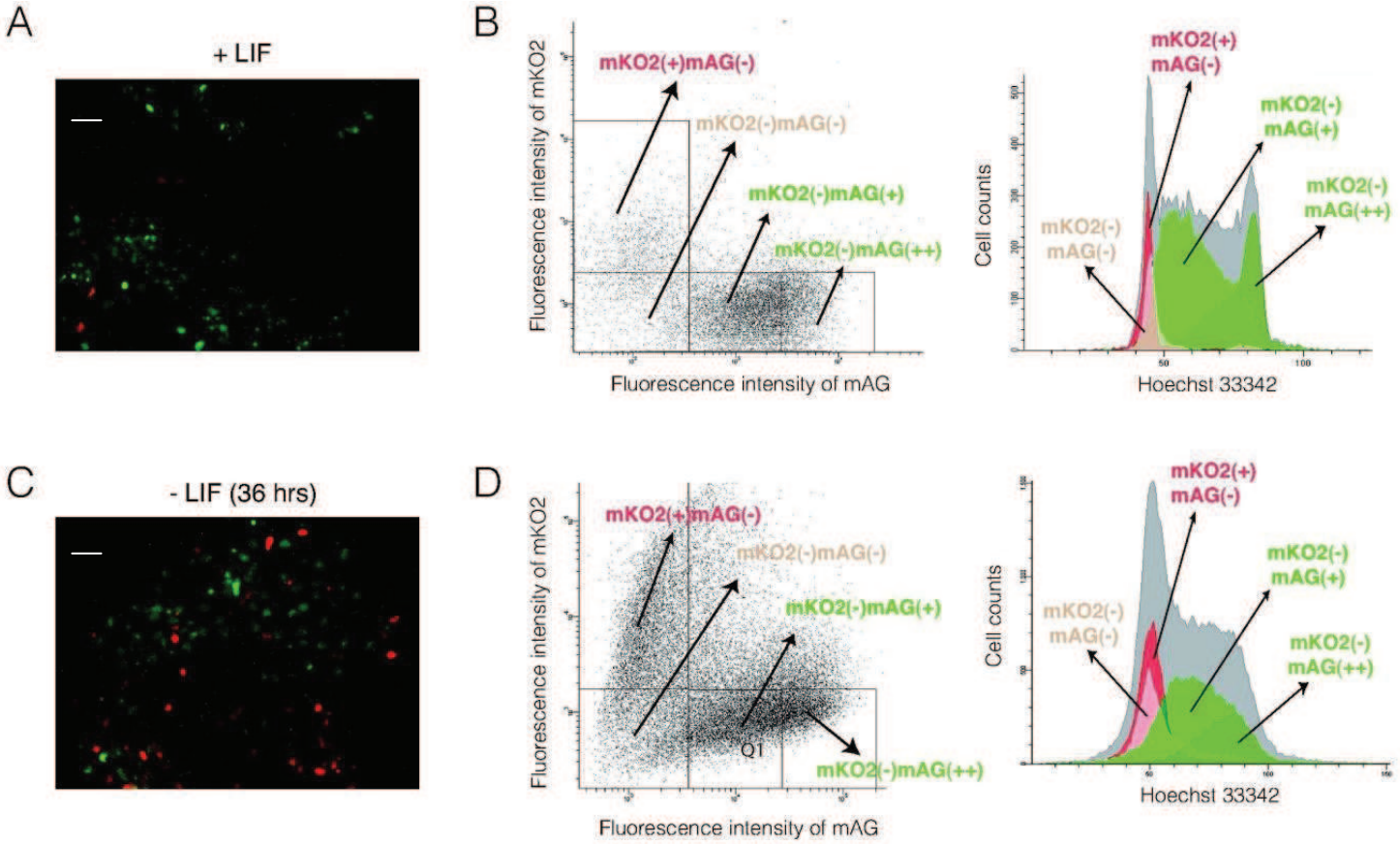
ES cells were washed and scraped in ice-cold PBS, centrifuged and frozen at -80°C. Cell pellets were lysed in 20 mM Hepes pH 7.4, 150 mM NaCl, 50 mM NaF, 1% Triton X-100, 0.5% NP40, 1 mM dithiothreitol, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride and cocktail of protease inhibitor (Roche Diagnostic) for 1 hr at 4°C. Protein lysates were cleared by centrifugation (14,000 RPM for 20 min). For immunoprecipitation, lysates (500 µg of proteins) were mixed with 1-5 µg antibodies [Santa Cruz : anti-CDK2 (sc-163), Upstate Biotechnology : anti-cyclin E (#06-459)] coupled to 25 µl of protein A- or protein G-Sepharose and incubated for 4-6 hours. Immunoprecipitates or whole cell lysates (30 µg) were electrophoresed on SDS-polyacrylamide gel and electroblotted onto nitrocellulose. After overnight treatment with blocking buffer (50 mM Tris-Hcl pH 7.6, 150 mM NaCl, 0.1% tween, 5% dry milk), the membranes were probed with specific monoclonal or polyclonal antibodies [Santa Cruz : anti-cyclin E (sc-481), anti-phospho Cdk1/CDK2 (Tyr15) (sc-7989), anti-CDK2 (sc-6248), Cell Signaling Technology : anti-phospho CDK2 (Thr160) (#2561)]. Blots were incubated with horseradish peroxidase-coupled anti-mouse or anti-rabbit and developed using enzymatic chemiluminescence reagents (ECL, Amersham, Buckinghamshire, U. K., http://www.amersham.com).

Kinase assay. Immune complexes bound to protein A-sepharose beads were washed twice in lysis buffer, then twice in kinase buffer (50 mM Hepes-NaOH, 10 mM $MgCl_2$, 1 mM NaF, 1 mM Na_2VO_4), then resuspended in 50 µl kinase buffer containing 200 µg/ml histone H1, 20 µM ATP, 1 µCi g^{32}P-ATP and incubated for 20 min at 30°C. Phosphorylated histone H1 was then resolved on a 7.5% SDS polyacrylamide gel. After electrophoresis, the gel was fixed in 40% methanol + 7.5% acetic acid (30 min), washed twice in 5% methanol + 7.5% acetic acid for 1 hour, dried and autoradiographed.

RESULTS

Generation of ESCs expressing the Fucci reporter

The Fluorescent Ubiquitination cell-cycle indicator (Fucci) reporter system makes use of two distinct fluorescence-emitting proteins to distinguish cells in the G1 phase from cells in the other phases of the cell cycle (Sakaue-Sawano, 2008). It thus allows direct visualization of cell cycle progression as well as FACS-sorting. We engineered an ESC line stably expressing the Fucci reporters. To this aim, mouse CGR8 ESC were dually transduced with two lentiviral vectors expressing the orange-emitting Cdt1:mKO2 and the green-emitting Geminin:mAG fusion proteins, both driven by the robust CAG promoter. One clone, hereafter called ESC-Fucci, which expressed both fluorescent proteins at high levels was chosen for subsequent studies (**Figure 1A**). Flow cytometry analysis of orange and green fluorescence, associated with DNA staining with Hoechst, showed that (*i*) 10.4% of ESC-Fucci cells have a 2n DNA content and lack fluorescence above background [mKO2(-)mAG(-)], which most probably corresponds to cells in early G1 (**Figure 1B**), (*ii*) 9.4% have a 2n DNA content and exhibit an orange fluorescence [mKO2(+)mAG(-)], which corresponds to cells progressing toward the


A
+ LIF
B
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mKO2(-)mAG(+)
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Fluorescence intensity of mKO2
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mKO2(+)
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Cell counts
Hoechst 33342
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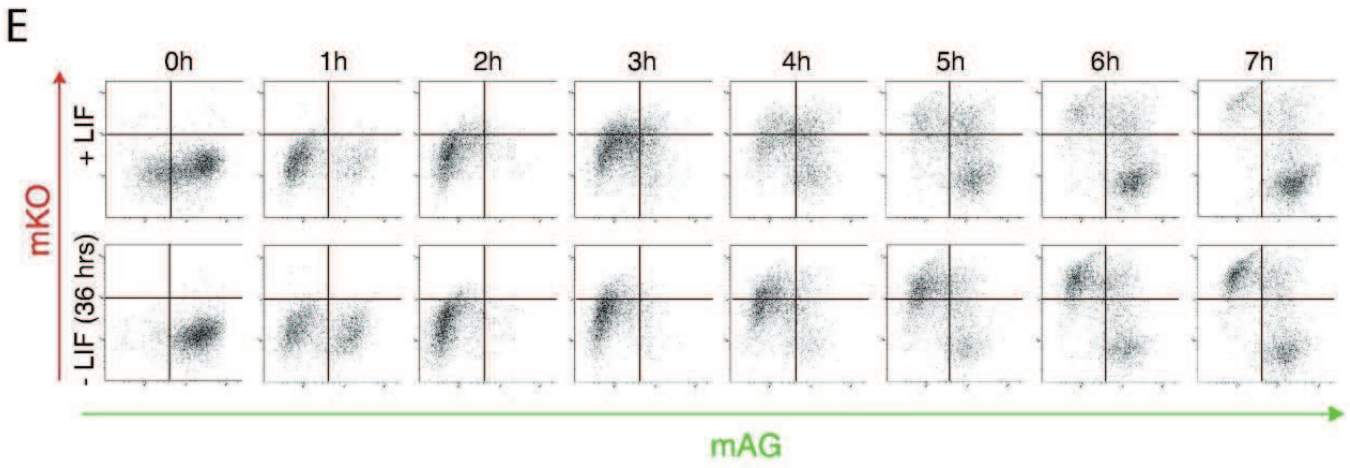
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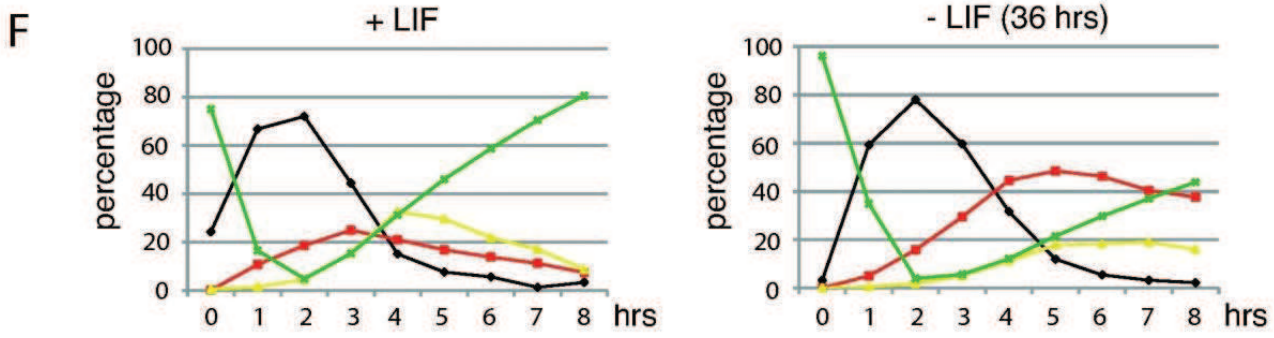
F
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- LIF (36 hrs)
percentage
100
80
60
40
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0
0 1 2 3 4 5 6 7 8
hrs

G1/S transition, and (*iii*) 74.8% have a > 2n DNA content and express the green fluorescence [mKO2(-)mAG(+)]. mAG-positive cells that exhibit a low green fluorescence seem to be mainly in S-phase [hereafter called [mKO2(-)mAG(+)] cells], whereas those with a strong green fuorescence are mainly in G2 phase [hereafter called [mKO2(-)mAG(++)] cells]. Deprivation of LIF for 36 hours dramatically increased the percentage of [mKO2(+)mAG(-)] cells, which reflects the lengthening of G1 that accompanies differentiation (Savatier, 1994) (**Figure 1C,D**). To confirm that the shifts from [mKO2(-)mAG(-)] to [mKO2(+)mAG(-)] then from [mKO2(+)mAG(-)] to [mKO2(-)mAG(+)] reflect the transitions from early to late G1 phase, then from late G1 to S phase, ESC-Fucci were cultured in colcemid for 5 hrs, then synchronized by mitotic shake-off (**Figure 1E**). Between 1 and 8 hours after release from the mitotic block, four successive waves of cells were observed, (*i*) a first wave of [mKO2(-)mAG(-)] cells (early G1), (*ii*) a second wave of [mKO2(+)mAG(-)] cells (late G1), (*iii*) a third wave of [mKO2(+)mAG(+)] cells (G1/S transition), and (*iv*) a fourth wave of [mKO2(-)mAG(+)] cells (S and G2 phases), consistent with the rapid progression from the G1 to the S phase that characterizes ESCs (Savatier, 1994). In a parallel experiment, ESC-Fucci were deprived of LIF for 36 hours prior to synchronization. The first wave of [mKO2(-)mAG(-)] cells peaked at 2 hours, as previously observed with LIF-stimulated cells. In contrast, the waves of [mKO2(+)mAG(-)], [mKO2(+)mAG(+)], and [mKO2(-)mAG(+)] cells were delayed, indicating that withdrawal of LIF lengthens the G1 phase and delays entry into S phase.

Figure 1: Characterization of mESC-Fucci. (A,C) Typical fluorescence image of mESC expressing mKO2-hCdt1 and mAG-hGem in th presence of LIF (**A**), or after LIF withdrawal for 36 hours (**C**). The scale bar represents 30μM. **(B,D)** Dot plots of mESC-Fucci (+LIF) (**B**) and LIF-deprived mESCs (-LIF 36hr) (**D**) showing red [mKO2(+)mAG(-)], yellow [mKO2(+)mAG(+)], and green [mKO2(-)mAG(+)] fluorescence, or no fluorescence [mKO2(-)mAG(-)]. The histograms show the position of each cell type in the cell cycle after staining with Hoechst33342 followed by FACS analysis. (**E,F**) Synchronization of mESC-Fucci in the presence of LIF, or after LIF withdrawal for 36 hours, using colcemid block. **(E)** Dot plots of [mKO2(-)mAG(-)], [mKO2(+)mAG(-)], [mKO2(+)mAG(+)], and [mKO2(-)mAG(+)] cells following release from the mitotic block at the times indicated. **(F)** Percentages of each cell phenotype according to time after release from the mitotic block.

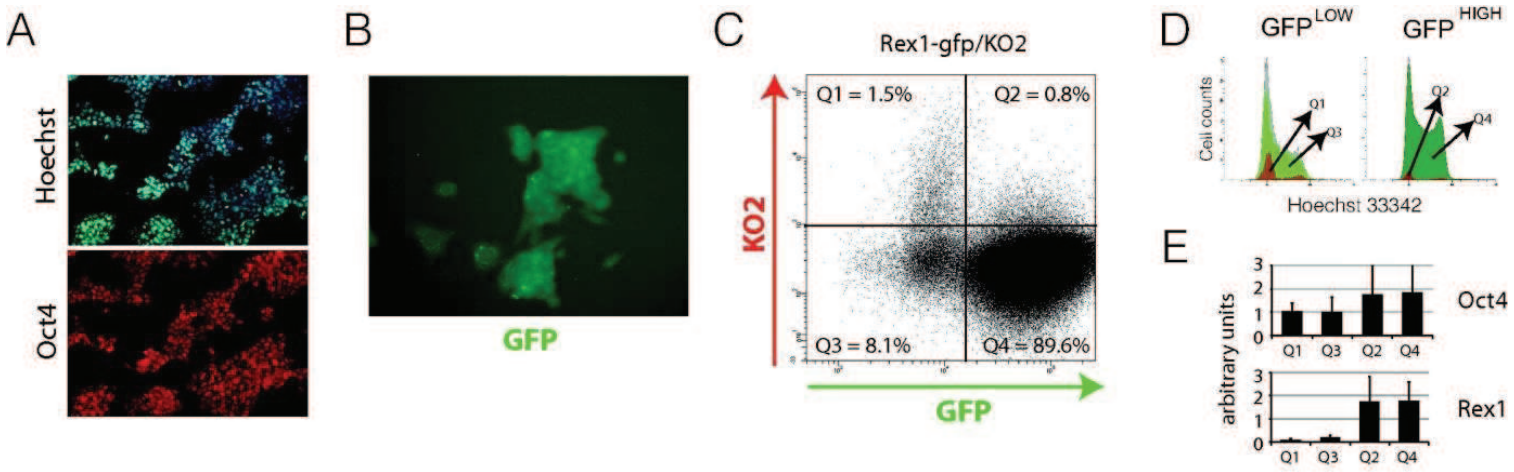
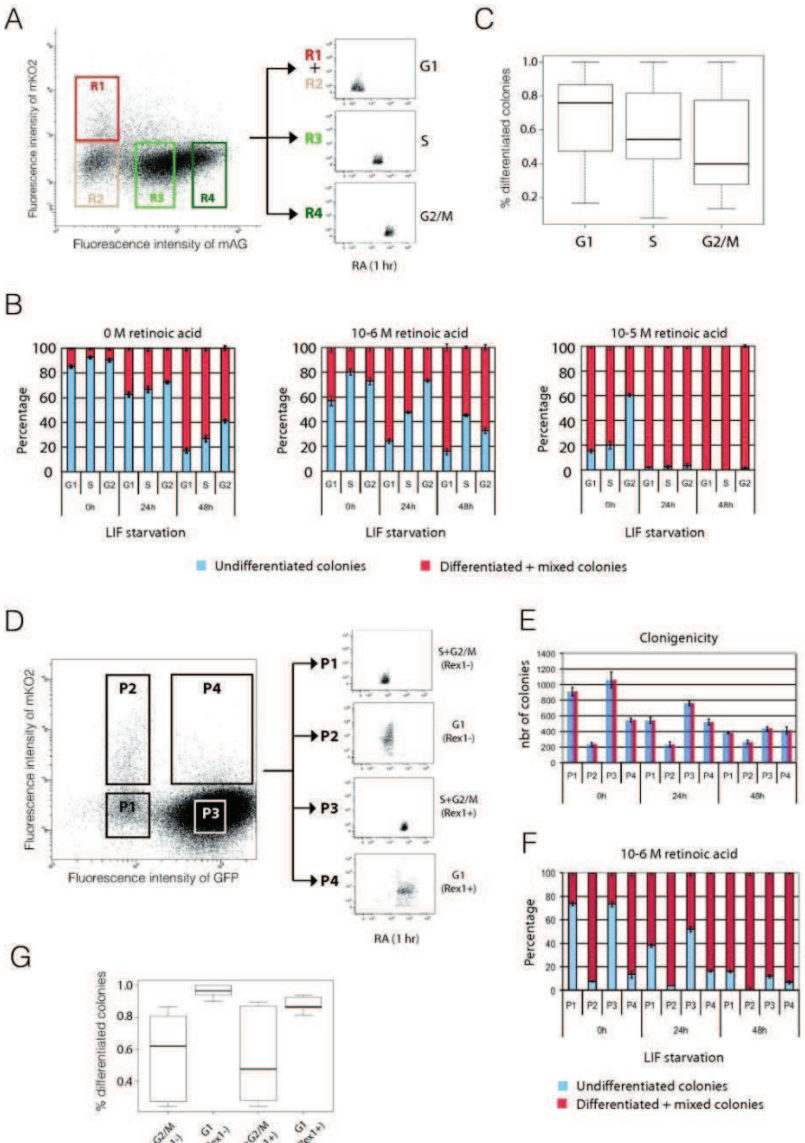


Figure 2: Characterization of *Rex1*$^{GFP\text{-}bla}$*/Oct4*$^{CFP\text{-}puro}$*/mKO2* mESCs. (A) Immunostaining of Oct4 expression in *Rex1*$^{GFP\text{-}bla}$*/Oct4*$^{CFP\text{-}puro}$*/mKO2* mESCs after puromycine selection. **(B)** Typical fluorescence image of *Rex1*$^{GFP\text{-}bla}$*/Oct4*$^{CFP\text{-}puro}$*/mKO2* mESCs in th presence of LIF. **(C)** Dot plot of *Rex1*$^{GFP\text{-}bla}$*/Oct4*$^{CFP\text{-}puro}$*/mKO2* mESCs showing GFP fluorescence associated with Rex1 expression, and red fluorescence associated with mKO2 expression, analyzed by flow cytometry. **(D)** Histograms showing the position of the Q1 [GFP^{low}/mKO2(+)], Q2 [GFP^{high}/mKO2(+)], Q3 [GFP^{low}/mKO2(-)], and Q4 [GFP^{High}/mKO2(-)] fractions in the cell cycle after staining with Hoechst33342. **(E)** Histograms showing the expression levels of Oct4 and Rex1 in each fraction.

The G1 phase duration varies according to the pluripotency state.

Mouse ESCs display a mosaic expression of transcription factor Zfp42 (Rex1). Only $Rex1^+$ cells can efficiently colonize the blastocyst and contribute to embryo development, a characteristic feature of ESCs in the naïve state of pluripotency (Toyooka, 2008). We therefore asked if $Rex1^+$ and $Rex1^-$ ESCs differ in their cell-cycle distribution. To address this issue, we made use of the *Rex1*$^{GFP\text{-}bla}$/*Oct4*$^{CFP\text{-}puro}$ ESC line, which carries a knock-in of the Green Fluorescent Protein (GFP) and the *bla*^{r} selectable genes into *Rex1*, and a knock-in of the Cyan Fluorescent Protein (CFP) and the *puro*^{r} selectable genes into *Oct4*. *Rex1*$^{GFP\text{-}bla}$/*Oct4*$^{CFP\text{-}puro}$ ESCs were further engineered by lentiviral vector infection to express the orange-emitting Cdt1:mKO2 fusion protein. The resulting cell line, *Rex1*$^{GFP\text{-}bla}$/*Oct4*$^{CFP\text{-}puro}$/*mKO2,* was continuously cultivated in puromycin to eliminate the $Oct4^-$ spontaneously-differentiating cells. This was monitored by the disappearance of CFP(-) cells (**Figure 2A**). In the absence of blasticidin selection, 90% of the cells exhibited a strong GFP fluorescence (GFP^{high}), and the remaining 10% exhibited a weak GFP fluorescence (GFP^{low}) (**Figure 2B,C**). Real-time PCR analysis showed that the GFP^{low} and GFP^{high} phenotypes correspond to $Rex1^-$ and $Rex1^+$ cells, respectively (**Figure 2E**). Then, we measured the percentages of cells expressing mKO2 in the two cell populations. mKO2(+) cells represented 1.5 ± 0.1% of the GFP^{low} and 0.8 ± 0.05% of the GFP^{high} cell populations (**Figure 2B,C**). Moreover, the mean intensity of mKO2-associated fluorescence was dramatically higher in $Rex1^-$ cells than in $Rex1^+$ cells. Together, these observations indicate that a larger fraction of the $Rex1^-$ ESC population is in G1 phase compared to the $Rex1^+$ population.


A
Fluorescence intensity of mKO2
Fluorescence intensity of mAG
R1
R2
R3
R4
G1
S
G2/M
RA (1 hr)
C
% differentiated colonies
G1
S
G2/M
B
0 M retinoic acid
10-6 M retinoic acid
10-5 M retinoic acid
Percentage
LIF starvation
0h
24h
48h
Undifferentiated colonies
Differentiated + mixed colonies
D
P1
P2
P3
P4
Fluorescence intensity of GFP
S+G2/M (Rex1-)
G1 (Rex1-)
S+G2/M (Rex1+)
G1 (Rex1+)
RA (1 hr)
E
Clonigenicity
nbr of colonies
F
10-6 M retinoic acid
Percentage
LIF starvation
Undifferentiated colonies
Differentiated + mixed colonies
G
% differentiated colonies

G1 is a phase of increased susceptibility to retinoic acid-induced differentiation.

We made use of the ESC-Fucci to ask whether the susceptibility of ESCs to retinoic acid (RA)-induced differentiation varies according to their position in the cell cycle. ESC-Fucci were FACS-sorted into three distinct cell populations, corresponding to G1 [[P1: mKO2(-)mAG(-)] + [P2: mKO2(+)mAG(-)]], S [P3: mKO2(-)mAG(+)], and G2 [P4: mKO2(-) mAG(++)], respectively. Because of the relative scarcity of G1 cells, P1 and P2 cells were pooled into a single G1 population (**Figure 3A**). FACS-sorted cells were treated with 10^{-6} and 10^{-5} M RA (or with DMSO alone) for 1 hour, and subsequently analysed in a LIF rescue assay (Aksoy, 2007). This assay measures the capacity of ESCs to withstand transient LIF starvation and, subsequently, to form alcaline phosphatase-positive (AP^+) undifferentiated colonies when plated at clonal density. mESC-Fucci were cultivated for 0, 24, and 48 hours in the absence of LIF, and subsequently re-stimulated with LIF for the 5 to 7 days. The percentages of undifferentiated (AP^+), mixed (AP^+/AP^-), and differentiated (AP^-) colonies was calculated (**Figure 3B,C**). The percentage of "mixed + differentiated" colonies was significantly increased when cells were treated with 10^{-6} M RA during the G1 phase, as compared to cells treated during the other phases of the cell cycle. At 10^{-5} M, RA dramatically

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Figure 3: Cell-cycle dependency to RA-induced differentiation. (A) FACS-sorting of mESC-Fucci in three distinct fractions, R1+R2 [mKO2(-)mAG(-) + mKO2(+)mAG(-)], R3 [mKO2(-)mAG(+)], and R4 [mKO2(-)mAG(++)], corresponding to cells in G1, S, and G2/M phases, respectively. **(B)** Histogram showing the percentages of undifferentiated (blue), mixed and differentiated (red) colonies observed in a LIF-rescue assay after treatment with 0, 10^{-6} and 10^{-5} M retinoic acid for 1 hour, followed by plating on gelatin-coated dishes at a density of 100 cells/cm^2. The duration of LIF starvation varies from 0 to 48 hours, as indicated. **(C)** The box-and-whiskers plot displays the median (line inside the box), the first and the third quartile (lower and upper edges of the box); the whiskers indicate the lowest and the highest values within 1.5 times the interquartile range. As boxplots limits overlap and to confirm the tendency observed, we performed a general linear model to quantify the effects of the factors time of LIF starvation, retinoic acid dose and cell cycle phase on the percentage of differentiated colonies. **(D)** FACS-sorting of *Rex1*$^{GFP\text{-}bla}$*/Oct4*$^{CFP\text{-}puro}$*/mKO2* mESCs in four distinct fractions, P1 [GFP^{low}/mKO2(-)], Q2 [GFP^{low}/mKO2(+)], Q3 [GFP^{high}/mKO2(-)], and Q4 [GFP^{High}/mKO2(+)], corresponding to $Rex1^-$/S-G2-M, $Rex1^-$/G1, $Rex1^+$/S-G2-M, and $Rex1^+$/G1 cells, respectively. **(E)** Histogram showing the clonigenicity of each fraction, after plating at a density of 100 cells/cm^2. **(F)** Histogram showing the percentages of undifferentiated (blue), mixed and differentiated (red) colonies observed in a LIF-rescue assay after treatment 10^{-6} M retinoic acid for 1 hour, followed by plating on gelatin-coated dishes at a density of 100 cells/cm^2. The duration of LIF starvation varies from 0 to 48 hours, as indicated. **(G)** Box-and-whiskers plot displaying the median, the first and the third quartile, and the lowest and the highest values within 1.5 times the interquartile range. Significant differences were confirmed by statistical analysis with a general linear model. (**B,E,F**) Means and standard errors were calculated from three replicates.

increased the percentage of "mixed + differentiated" colonies upon treatment in any phase of the cell cycle. Again, the effect was more pronounced when cells were treated during the G1 phase. Of note, even without RA treatment, the G1 phase cells tended to give more "mixed + differentiated" colonies, as compared to the S and G2/M cells, which suggests that cells have an increased sensitivity to differentiation when they are transiting through the G1 phase prior to FACS-sorting and re-plating at low density.

Since the $Rex1^-$ cells appeared to have a significantly longer G1 phase as compared to the $Rex1^+$ cells, the increased sensitivity of G1 phase cells to RA observed in ESC-Fucci might result from a sub-population effect rather than from a cell-cycle effect. To eliminate the confounding effect of cell population heterogeneity, we sought to eliminate the $Rex1^-$ cells from the population prior to the RA assay. To this aim, the *Rex1*$^{GFP\text{-}bla}$*/Oct4*$^{CFP\text{-}puro}$*/mKO2* were FACS-sorted into four distinct cell populations, corresponding to S/G2/M $Rex1^-$ cells [P1: mKO2(-)GFP^{low}], G1 $Rex1^-$ cells [P2: mKO2(+)GFP^{low}], G1 $Rex1^+$ cells [P3: mKO2(-)GFP^{high}], and S/G2/M $Rex1^+$ cells [P4: mKO2(+)GFP^{high}], respectively (**Figure 3D**). FACS-sorted cells were treated either with 10^{-6} M RA for 1 hour, and subsequently analysed in the LIF rescue assay. The percentage of "mixed + differentiated" colonies was significantly increased when GFP^{high} cells were treated with 10^{-6} M RA during the G1 phase, as compared to same cells treated during the other phases of the cell cycle (**Figure 3E,G**). Strikingly, the clonigenicity of G1 phase GFP^{high} cells was significantly reduced ($p < 0.001$) as compared to GFP^{high} cells in the other phases of the cell cycle (**Figure 3F**). The same held true for GFP^{low} cells ($Rex1^-$), which exhibited both an increased sensitivity to 10^{-6} M RA and a reduced clonigenicity when they were in G1 phase, as compared to the other phases of the cell cycle. Collectively, these data indicate that the $Rex1^+$ pluripotent stem cells become more susceptible to commitment into differentiation when transiting through the G1 phase.

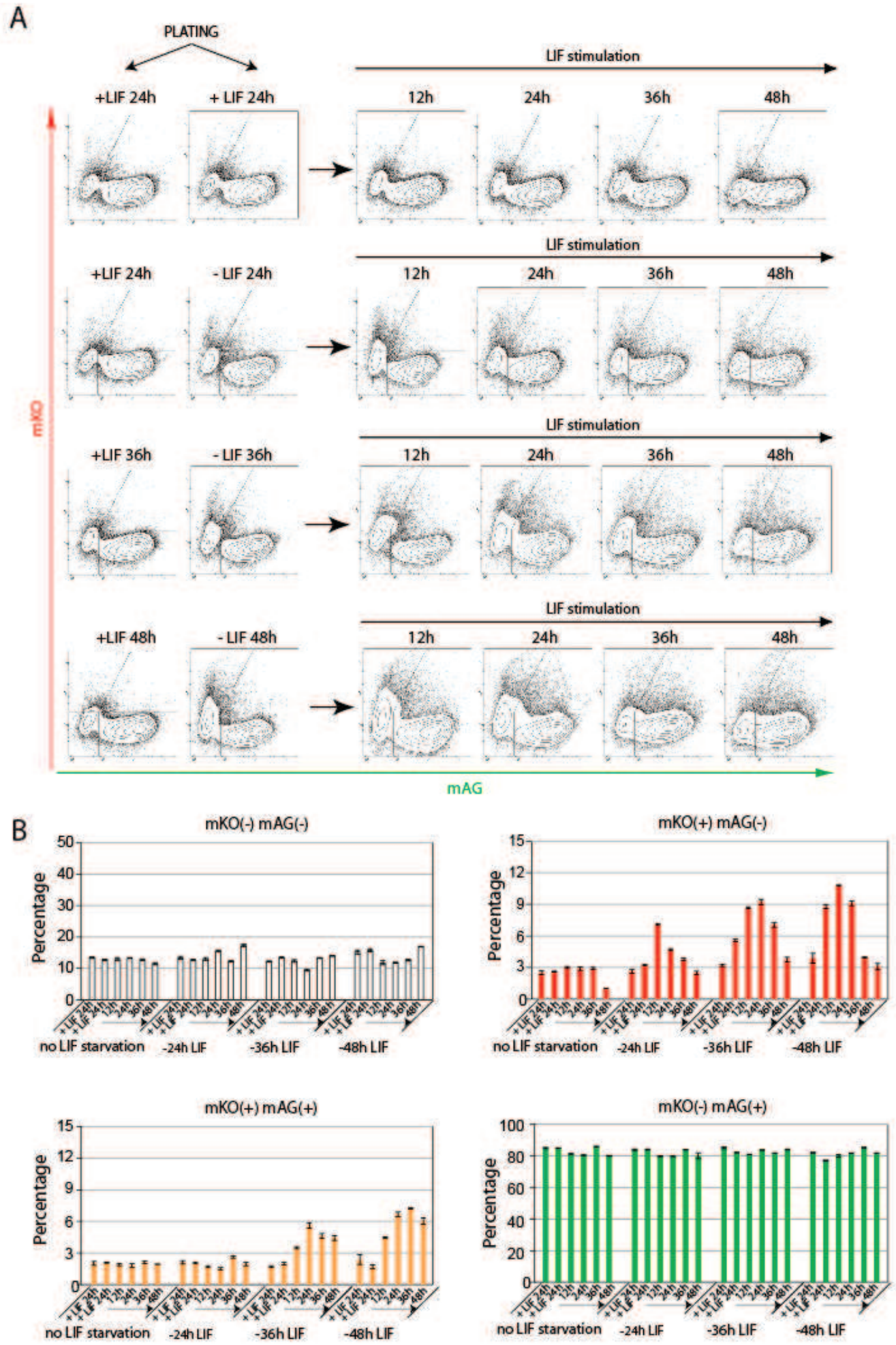
A
PLATING
LIF stimulation
+LIF 24h
+ LIF 24h
12h
24h
36h
48h
+LIF 24h
- LIF 24h
+LIF 36h
- LIF 36h
+LIF 48h
- LIF 48h
mKO
mAG
B
mKO(-) mAG(-)
mKO(+) mAG(-)
mKO(+) mAG(+)
mKO(-) mAG(+)
Percentage
no LIF starvation
-24h LIF
-36h LIF
-48h LIF

FIGURE 4

LIF stimulation accelerates the G1-to-S phase transition

Differentiation of ESCs induced by LIF withdrawal delays the G1/S transition (Savatier, 1994; Savatier, 1996). However, it is not clear if the lengthening of the G1 phase takes place before, during, or after the appearance of the first lineage markers. To address this question, ESC-Fucci were LIF-deprived for 0, 24, 36, and 48 hours, and restimulated with LIF for 12, 24, 36, and 48 hrs. We found that the percentage of G1 phase ESCs ([mKO2(+)mAG(-)] phenotype) increased 1.23-, 1.74-, and 2.27-fold upon LIF deprivation for 24, 36, and 48 hours, respectively (**Figure 4A,B**). After stimulation with LIF for 12 hours, these percentages further increased 2.2-, 1.56-, and 1.23-fold (upon LIF starvation for 24, 36, and 48 hours, respectively), then progressively returned to their original levels after 48 hours of stimulation. The 12 hour delay in G1 phase shortening induced by the addition of LIF might reflect the cell cycle duration, as each cell had to pass through the G1 phase to undergo cell cycle acceleration. Of note, after LIF starvation for 36, and 48 hours, followed by the restimulation for 48 hours, some cells continued to accumulate mKO2 protein and went off-scale on the dot plots (**Figure 4A**, bottom right panels). These are most likely cells which were irreversibly committed to differentiation, therefore, failed to re-contract the G1 phase upon LIF stimulation. It is also worthy of note that the percentages of ESCs with a [mKO2(-)mAG(-)] phenotype (early G1 phase cells) varied to a far lesser extent during LIF withdrawal and stimulation, indicating that the kinetics of transit through the late G1 phase is the most sensitive to LIF. The transient increase of the G1 phase duration was paralleled by an alteration in gene expression patterns, namely a decreased expression of pluripotency markers (*Oct4*, *Nanog*, and *Rex1*), and an increased expression of lineage-specific markers

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Figure 4: Role of LIF signaling in the regulation of G1 phase duration. (A) Contour plots of mESC-Fucci showing fluorescence levels associated with mAG and mKO2, after withdrawal of LIF for 0, 24, 36, and 48 hours (h), followed by re-stimulation for 12, 24, 36, and 48 hours. **(B)** Histograms showing the percentages of mKO(-)mAG(-) (white), mKO(+)mAG(-) (red), mKO(+)mAG(+) (yellow), and mKO(-)mAG(+) (green) cells in each experimental conditions defined in A.

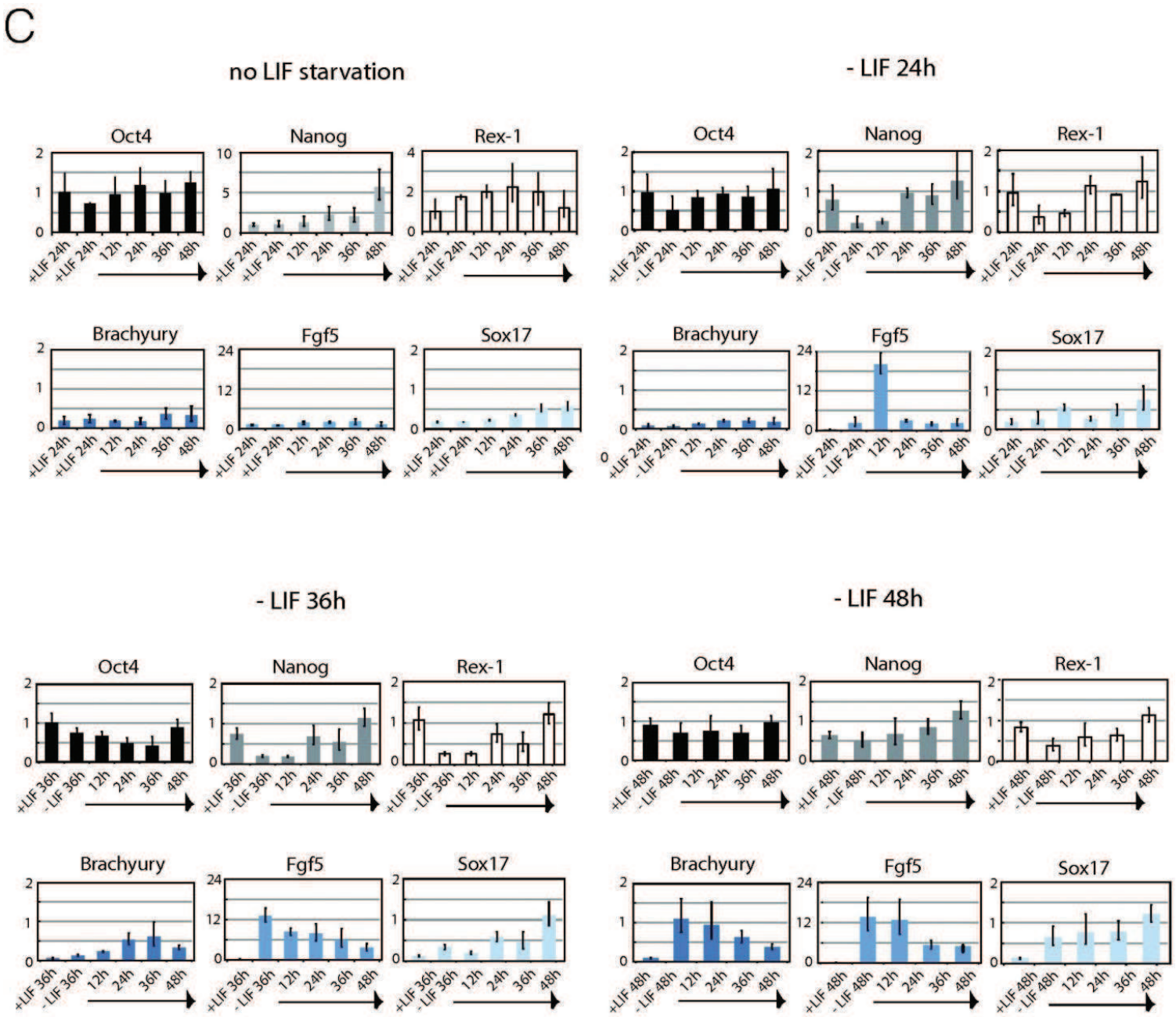


Figure 4: Role of LIF signaling in the regulation of G1 phase duration (C) Histograms showing the expression levels of *Oct4*, *Nanog*, *Rex1*, *Brachyury*, *Fgf5*, and *Sox17*, in each experimental conditions defined in (**A**). **(B,C)** Means and standard errors were calculated from three replicates.

(*Brachyury*, *Fgf5*, and *sox17*). All returned to original levels after stimulation with LIF for 48 hours, except for *Sox17* expression, which continued to rise (**Figure 4C**). Thus, variations of the G1 phase duration induced by transient withdrawal of LIF occur concomitantly with changes in gene expression levels.

To exclude the possibility that the lengthening of the G1 phase induced by transient LIF starvation might actually result from an increase in the percentage of $Rex1^-$ cells in the population, we made use of the $Rex1^{GFP\text{-}bla}/Oct4^{CFP\text{-}puro}/mKO2$ ESC line to study the cell-cycle response of $Rex1^+$ mESCs to LIF stimulation. $Rex1^{GFP\text{-}bla}/Oct4^{CFP\text{-}puro}/mKO2$ cells were LIF-deprived for 0, and 24 hours, then restimulated with LIF for 0, 12, 36, and 48 hours (**Figure 5A**). LIF starvation resulted in a 4.4-fold increase in the percentage of $Rex1^-$ cells, which returned to normal after stimulation for 48 hours (**Figure 5B**). Consistent with the data obtained with ESC-Fucci, we found that the percentage of $Rex1^+$ ESCs with a ([mKO2(+)] phenotype increased 2.8-fold upon LIF deprivation for 24 hrs, then returned to its original level after 24 hrs of re-stimulation with LIF (**Figure 5C**). By contrast, the percentage of $Rex1^-$ ESCs expressing a [mKO2(+)] phenotype only increased 1.3-fold after LIF-deprivation. These data indicate that stimulation of LIF signaling results in a shortening of the G1 phase, relative to the other phases of the cell cycle, in the $Rex1^+$ ESC population. So as to match the observed changes in the G1 phase duration with alterations in gene expression patterns in the $Rex1^+$ cell population, the GFP^{high} population (both mKO2-positive and negative cells) were FACS-sorted, and the expression of pluripotency and lineage-specific markers subsequently analysed by quantitative RT-PCR (**Figure 5D**). Only *Nanog* and *Fgf5* displayed significant changes of their expression levels among the six genes tested. In contrast, *Oct4*, *Rex1*, *Brachyury* (*T*) and *Sox17* levels remained constant. To conclude, our results indicate that G1 phase lengthening is an early response to LIF withdrawal that takes place before, or concomitantly with, a transient activation of early lineage-specific markers.

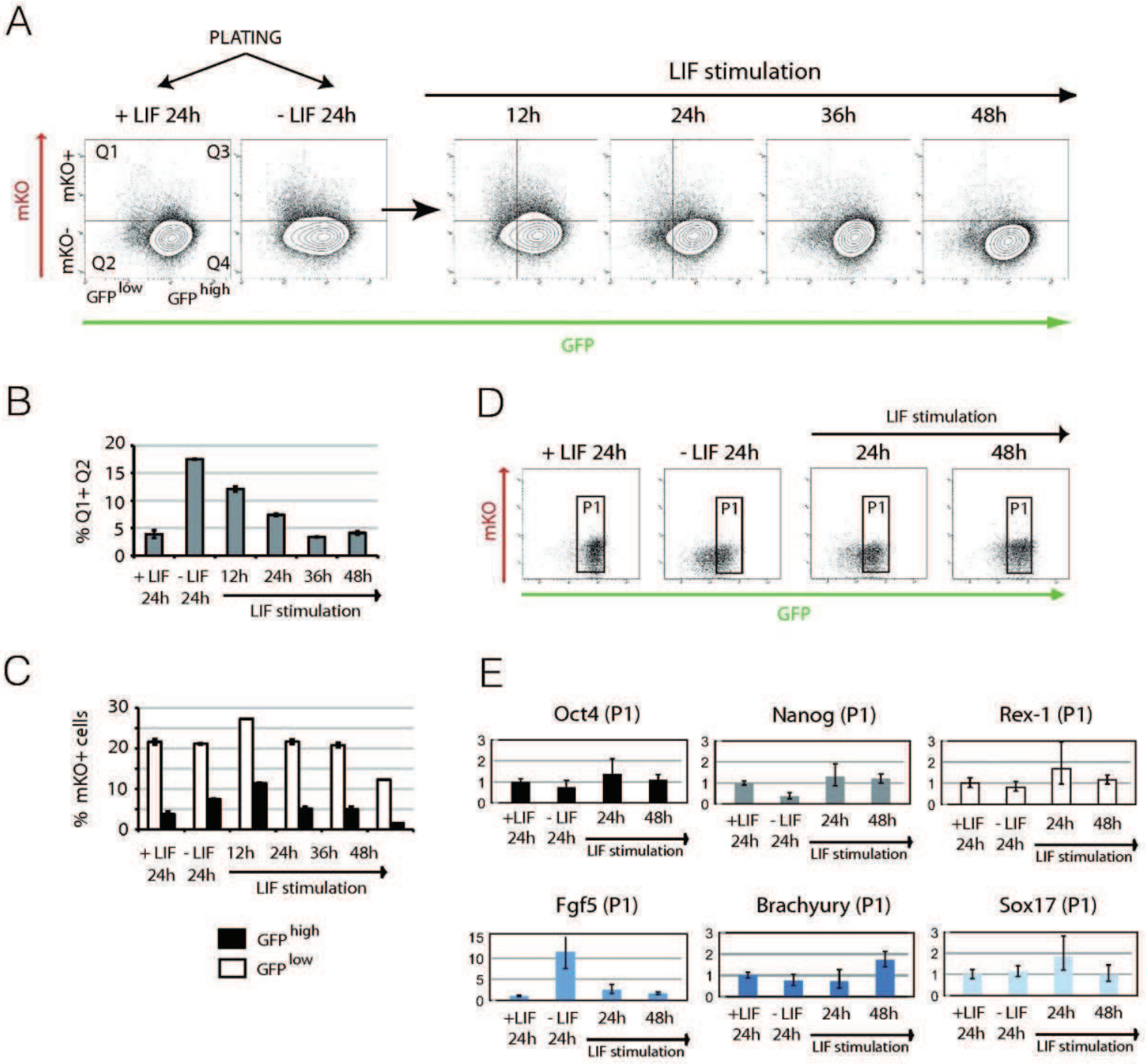


Figure 5: Role of LIF signaling in the regulation of G1 phase duration in Rex1^{+} mESCs. (A) Dot plots of *Rex1*$^{GFP\text{-}bla}$/*Oct4*$^{CFP\text{-}puro}$/*mKO2* mESCs showing fluorescence levels associated with GFP (Rex1 expression) and mKO2, after withdrawal of LIF for 24 hours, followed by re-stimulation for 12, 24, 36, and 48 hours. **(B)** Histogram showing the percentage of GFP^{low} (Rex1^{-}) cells [GFP^{low}/mKO2(-)] + [GFP^{low}/mKO2(+)] (→ [(Q1+Q2)/(Q1+Q2+Q3+Q4)]. **(C)** Histograms showing the percentages of [GFP^{low}/mKO2(+)] [Rex1^{-} in G1 phase, Q1/(Q1+Q2)] and [GFP^{high}/mKO2(+)] [Rex1^{+} in G1 phase, Q3/(Q3+Q4)]. **(D)** FACS-sorting of the GFP^{high} cells from the *Rex1*$^{GFP\text{-}bla}$/*Oct4*$^{CFP\text{-}puro}$/*mKO2* mESCs treated as indicated. **(E)** Histograms showing the expression levels of *Oct4*, *Nanog*, *Rex1*, *Brachyury*, *Fgf5*, and *Sox17*, in the P1 (GFP^{high}) fractions. **(B,C,E)** Means and standard errors were calculated from three replicates.

LIF stimulation enhances the formation of active cyclin E1:Cdk2 complexes

Cyclin E:Cdk2 complexes are known to play a key role in the transition from the G1 to the S phase of the cell cycle in mammalian cells (Bartek, 2001; Moroy, 2004). In pluripotent stem cells, cyclin E:Cdk2 complexes are present and active throughout the cell cycle, as opposed to the G1-specific expression observed in somatic cells (Stead, 2002). It has been proposed that continuous expression of active cyclin E:Cdk2 complexes results in autonomous replication and enhanced self-renewal (Stead, 2002; Burdon, 2002). Since we found that LIF influenced the duration of the G1 phase, we asked if LIF could also regulate the formation of active cyclin E:Cdk2 complexes in ESCs. To examine the role of LIF in regulating the accumulation of cyclin:Cdk protein complexes and the regulation of their kinase activity, ESCs were LIF-deprived for 24 hours and restimulated with 10.000 U/ml of LIF for 1 to 8 hours (**Figure 6**). The activity of cyclin E-CDK2 complexes was down-regulated following LIF starvation (2.2 ± 0.6-fold), and were reinduced by LIF stimulation (2.5 ± 0.6-fold). The accumulation of cyclin E:Cdk2 complexes was also increased by LIF stimulation. By contrast, levels of cyclin E and Cdk2 subunits remained unchanged. At least two phosphorylations regulate the activity of the Cdk2 subunit independently from cyclin E binding, a Cdc25A-dependent activating dephosphorylation of phosphorylated-Tyr15 (Blomberg, 1999; Sandhu, 2000), and a Cdk-activating kinase (CAK)-dependent activating phosphorylation of Thr160 (Chiariello, 2000; Lents, 2002). Stimulation with LIF for 1 hour decreased the level phosphorylated-Tyr15-CDK2, indicating that ES cells expressed a larger fraction of active CDK2. In contrast, the level of phosphorylated-Thr160 was not influenced. Collectively, these data indicate that LIF signaling regulates the activity of cyclin E:Cdk2 complexes but at least two independent mechanisms, (*i*) the stimulation of the activating dephosphorylation of Cdk2, and (*ii*) the accumulation of active cyclin E:Cdk2 complexes.

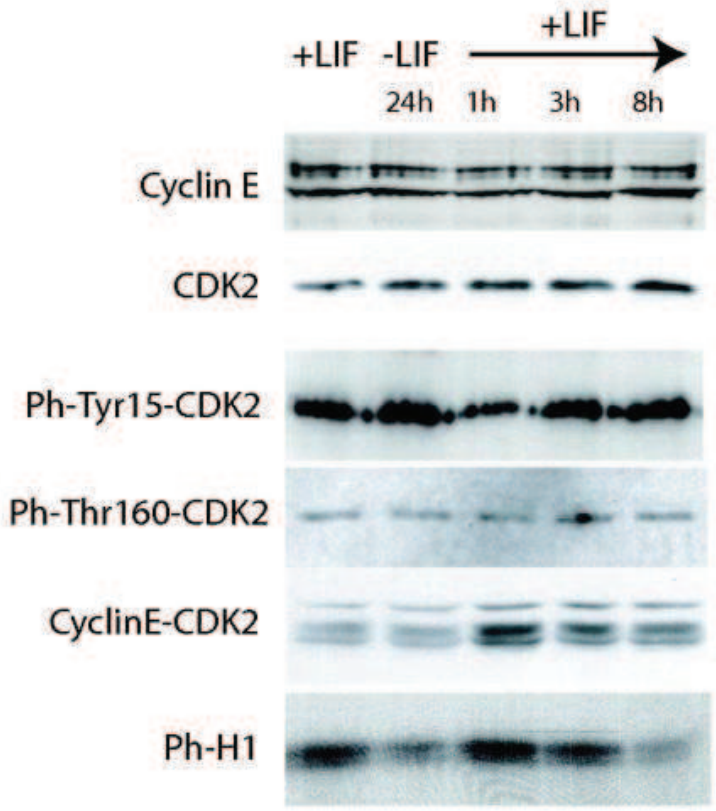


Figure 6: Regulation of cyclin E:Cdk2 kinase by LIF. ES cells (+ LIF) were plated at a density of 2 x 10^6 cells per 10 cm dish, LIF-starved for 24 hours (- LIF), then restimulated with LIF for the time indicated. Cyclin E, Cdk2, Ph-Tyr15-Cdk2, and Ph-Thr160-Cdk2 levels are measured in whole cell lysates. E-CDK2 indicates immunoprecipitation of Cdk2 followed by immunoblotting and detection of cyclin E; Ph-H1 : cyclin E-dependent kinase activity measured on histone H1.

Down-regulation of cyclin E1 expression induces premature expression of endoderm lineage-specific markers.

Cyclin E1, the main regulatory subunit of CDK2, is a rate-limiting activator of the G1-to-S phase transition in mammalian cells. Suppression of cyclin E expression results in slowing down transit through the G1 phase, and ultimately G1 arrest at the restriction point (Bartek, 2001; Moroy, 2004). We thus asked if lowering the level of cyclin E1 expression in mESCs delayed the G1-to-S phase transition, lengthened the G1 phase, and subsequently weakened self-renewal. To this aim, cyclin E1 expression was knocked-down in ESC-Fucci, by infection with an interfering lentiviral vector expressing cyclin E1 shRNAs. Forty-eight hours after infection, the ESC-Fucci were dissociated, replated at clonal density, and further grown for 7 days in LIF-supportive conditions, in the presence of G418 to kill the non-infected cells. The resulting colonies were stained for AP activity and the percentages of undifferentiated, mixed, and differentiated colonies calculated. No significance difference in those percentages was observed between mESCs infected with the cyclin E1-interfering vector and control cells infected with a lentivector expressing a scrambled shRNA sequence (**Figure 7A**). However, both the number and the size of colonies were reduced in ESC-ShCylE1 compared to control (**Figure 7B,C**). Moreover, the FACS analysis showed a significant increase in the percentage of [mKO2(+)mAG(-)] cells in the population of ESC-Fucci infected with the cyclin E1-interfering vector, indicating a lengthening of the G1 phase (**Figure 7D**). One clone exhibiting strong interference, hereafter named ESC-ShCylE1, was selected to make EBs and analyse the expression of pluripotency and lineage-specific markers by real-time PCR (**Figure 7E**). ESC-ShCylE1-derived EBs exhibited premature down-regulation of pluripotency markers (*Oct4*, *Nanog*), premature up-regulation of endoderm-specific markers (*GATA-4*, *GATA-6*, *Sox17*), and loss of ectoderm-specific markers (*Fgf5*, *nestin*), as compared to control cells. These data indicate that decreased cyclin E expression provokes premature

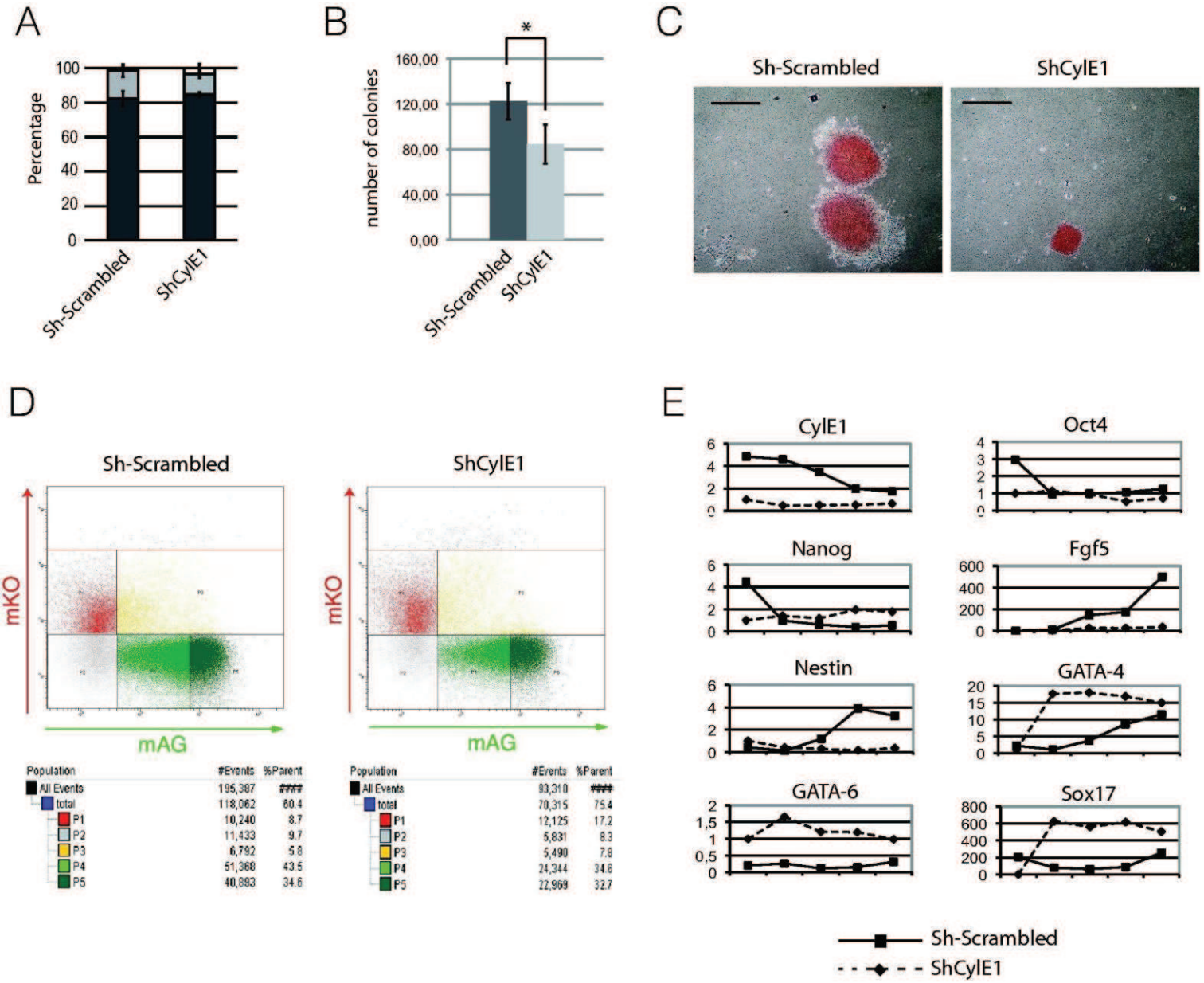


Figure 7: Knockdown of cyclin E1 expression. (A) Histogram showing the percentages of undifferentiated (blue), mixed (red) and differentiated (green) colonies observed in a clonal assays after knockdown of cyclin E1 expression by means of lentiviral infection with the *pLenti6/BLOCK-iT-PGKneo*^{R} interfering vectors. Means and standard errors were calculated from three replicates using ANOVA test. **(B)** Histogram of the total number of colonies obtained after knockdown of cyclin E1 expression. Means and standard errors were calculated from three replicates (*, $p < 0.05$ calculated with student's t test). **(C)** Typical AP+ colonies observed after infection of ESC with interfering lentiviral vectors. The scale bar represents 500 µM. **(D)** Dot plots of mESC-Fucci after infection with cyclin E1-interfering and control lentivectors. **(E)** Real-time PCR analysis of *CylE1*, *Oct4*, *Nanog*, *GATA-4, GATA-6, Sox17, Fgf5*, and *Nestin* expression during differentiation induced by EB formation (day 1 to day 6).

differentiation into endodermal cells, at the expense of the ectodermal lineage.

Enforced expression of cyclin E1 harnesses self-renewal.

As knockdown of cyclin E1 expression induced premature differentiation, we asked if overexpression of cyclin E1 resulted in a reinforcement of self-renewal and delayed differentiation. ESCs were co-electroporated with *irTetR-VP16-GBD*, a plasmid expressing a dexamethasone (Dex)-dependent irtTA and the $puro^R$ selectable gene, and *pSport1-tetCMVcylE*^{WT}*pA-INS*, a plasmid expressing a rat wild-type (WT) cyclin E cDNA under the control of the doxycycline (Dox) promoter and the $hygro^R$ selectable gene. *pSport1-tetCMVcylE*^{S180D}*pA-INS*, a plasmid expressing a mutant form carrying the S180D mutation that inactivates cyclin E function was electroporated in parallel. Stably transfected cells were selected with hygromycin and puromycin, and dually resistant clones were analysed by real-time PCR to measure Dox+Dex-induced expression of WT or S180D cyclin E1. Two clones, rCylE1^{WT}-cl3 and rCylE1^{S180D}-cl8, which showed a 1.6- and 3.5-fold increase in cyclin E1 RNA level after Dox+Dex treatment (**Figure 8A**), were analysed in a LIF-rescue assay (**Figure 8B**). rCylE1^{WT}-cl3 cells treated with Dox+Dex produced a significantly higher percentage of undifferentiated colonies, as compared to untreated cells, in standard culture conditions (86.1 ± 0.01% for treated *versus* 78.6 ± 1.5% for untreated cells), or after LIF-deprivation for 12 hrs (84.6 ± 1% for treated *versus* 68.1 ± 0.9% for untreated cells), 24 hrs (75.0 ± 1% for treated *versus* 60.2 ± 1.2% for untreated cells), 36 hrs (42.0 ± 1.2% for treated *versus* 34.7 ± 1.1% for untreated cells), and 48 hours (19.3 ± 1% for treated *versus* 6.9 ± 0.5% for untreated cells). In contrast, Dox+Dex-treated and untreated rCylE1^{S180D}-cl8 cells showed no significant differences in the yield of undifferentiated colonies at all-time points analysed. It can be concluded that ESCs overexpressing cyclin E show a higher resistance to LIF deprivation.

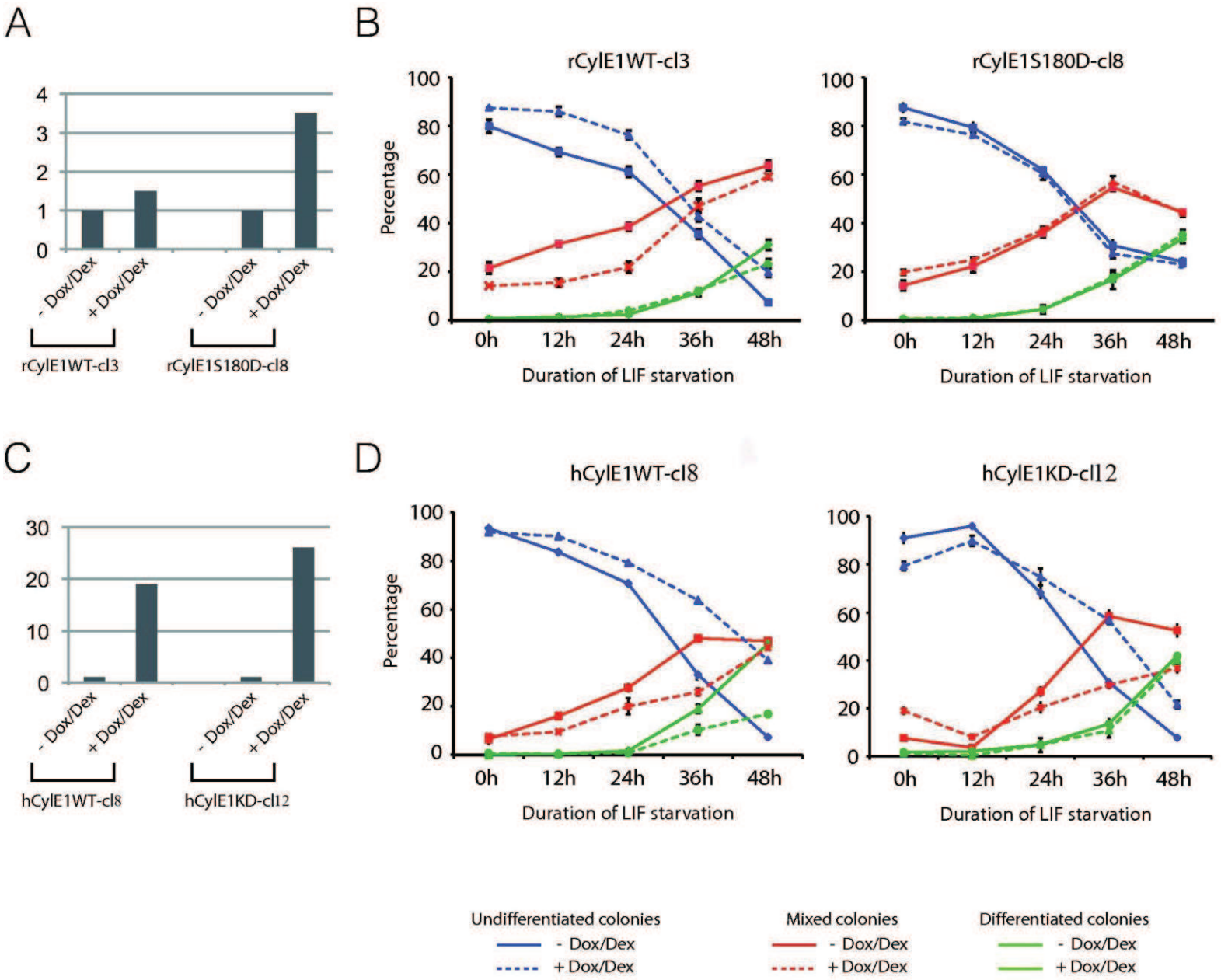


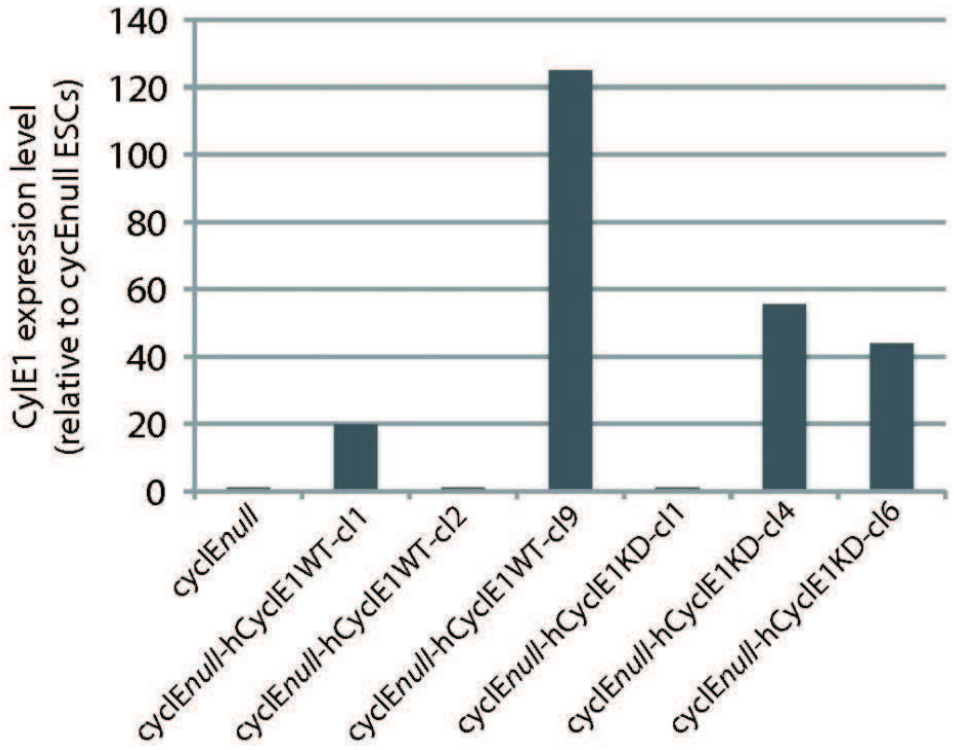
Figure 8: Enforced expression of cyclin E1 harnesses self-renewal. (A) Real-time PCR analysis of rat cyclin E1 expression in ESC-*irTetR-VP16-GBD* electroporated with *pSport1-tetCMVcylE*^{WT}*pA-INS*, or *pSport1-tetCMVcylE*^{S180D}*pA-INS*, before and after treatment with Dox/Dex for 48 hours. **(B)** LIF-rescue assay with rCylE1^{WT}-cl3 and rCylE1^{S180D}-cl8 ESCs. **(C)** Real-time PCR analysis of human cyclin E1 in ESC-*irTetR-VP16-GBD* electroporated with *pSport1-tetCMVhcylE*^{WT}*pA-INS*, or *pSport1-tetCMVhcylE*^{KD}*pA-INS*, before and after treatment with Dox/Dex for 48 hours. **(D)** LIF-rescue assay with hCylE1^{WT}-cl8 and hCylE1^{KD}-cl12 ESCs. **(B,D)** Means and standard errors were calculated from three replicates.

To assess whether stimulation of ESC self-renewal by cyclin E1 is CDK2-dependent, both human WT cyclin E1 and a kinase-deficient (KD-E) mutant were overexpressed with the Dox/Dex-inducible expression system. The KD-E mutant was previously shown to be unable to direct phosphorylation of histone H1, albeit retained the ability to interact with CDK2 (Geng, 2007). Two clones, hCylE1^{WT}-cl8 and hCylE1^{KD}-cl12, which showed a 19- and 26-fold increase in cyclin E1 RNA level, respectively (**Figure 8C**), were analysed in a LIF-rescue assay. As compared to untreated cells, hCylE1^{WT}-cl8 cells treated with Dox+Dex produced a significantly higher percentage of undifferentiated colonies, when LIF-deprived for 12 hours (90.1 ± 0.3% for treated *versus* 83.5± 0.5% for untreated cells), 24 hours (79.1 ± 3.3% for treated *versus* 70.6 ± 2.07% for untreated cells), or for 36 hours (63.7 ± 2.8% for treated *versus* 33± 1.8% for untreated cells) and for 48 hours of LIF deprivation (38.9± 2.8% for treated *versus* 7.3± 0.1% for untreated cells) (**Figure 8D**). Dox+Dex-treated and untreated hCylE1^{KD}-cl12 cells also showed significant differences in the yield of undifferentiated colonies at 24h, 36h and 48h LIF deprivation time points analysed. These data indicate that activation of CDK2 is not required for overexpressed cyclin E1 to reinforce self-renewal.

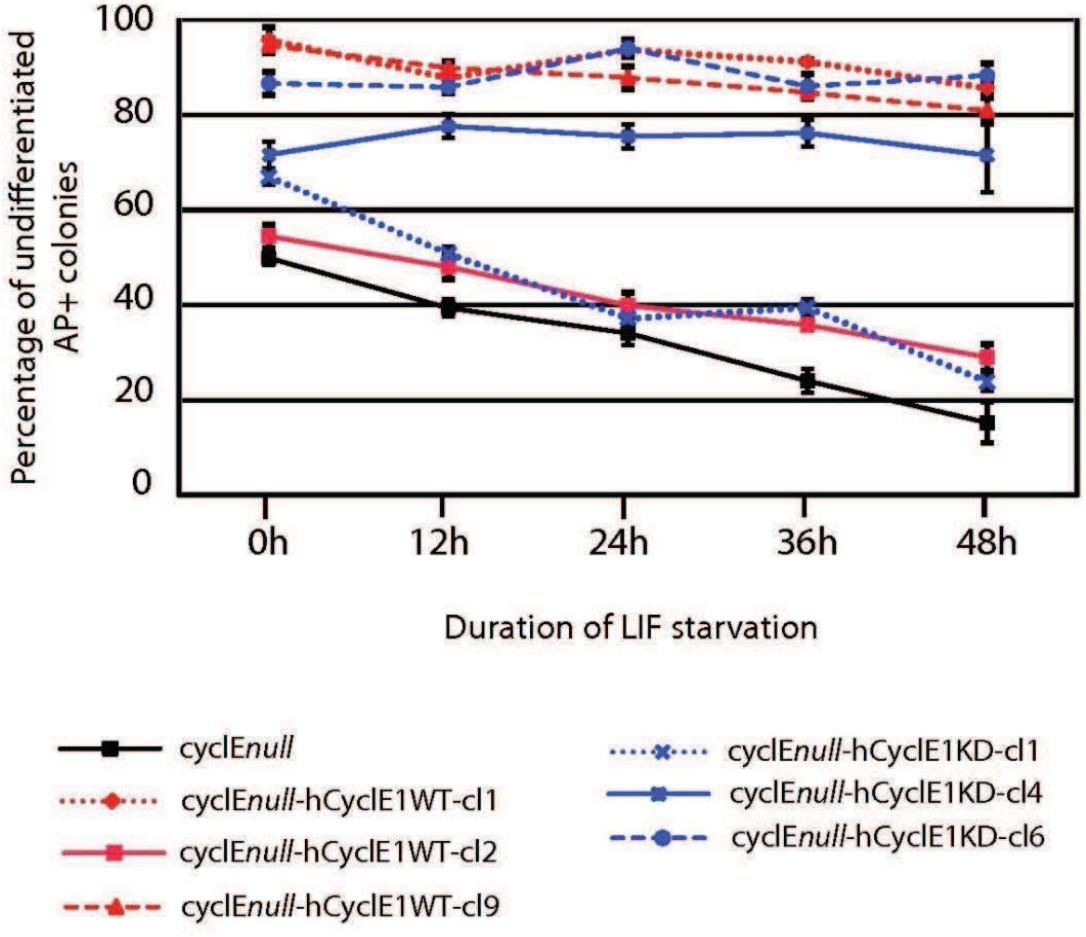
Enforced expression of KD-E blocks differentiation of cyclin E-*null* ESCs.

Since the KD-E mutant is able to rescue the growth deficit of cyclin E-*null* fibroblasts (Geng, 2007), we asked if it is also able to sustain self-renewal in cyclin E-*null* ESCs. To this goal, cyclin E-*null* ESCs were transduced with lentiviral vectors expressing WT cyclin E1 and the KD-E mutant. We verified that the levels of the ectopically expressed cyclin E1 matched the levels of endogenous mouse cyclin E1 in wild-type cells (**Figure 9A**). Consistent with our previous findings, introduction of WT cyclin E resulted in a dramatic increase in their self-renewal capacities measured in the LIF-rescue assay (**Figure 9B**). Introduction of the KD-E

A


CylE1 expression level
(relative to cycEnull ESCs)
140
120
100
80
60
40
20
0
cyclEnull
cyclEnull-hCyclE1WT-cl1
cyclEnull-hCyclE1WT-cl2
cyclEnull-hCyclE1WT-cl9
cyclEnull-hCyclE1KD-cl1
cyclEnull-hCyclE1KD-cl4
cyclEnull-hCyclE1KD-cl6

B


Percentage of undifferentiated
AP+ colonies
100
80
60
40
20
0
0h
12h
24h
36h
48h
Duration of LIF starvation
cyclEnull
cyclEnull-hCyclE1WT-cl1
cyclEnull-hCyclE1WT-cl2
cyclEnull-hCyclE1WT-cl9
cyclEnull-hCyclE1KD-cl1
cyclEnull-hCyclE1KD-cl4
cyclEnull-hCyclE1KD-cl6

mutant resulted in a comparable effect on self-renewal capacities (compare clones CycE*null*-hCylE1^{KD}-4 and CycE*null*-hCylE1^{KD}-6 with clones CycE*null*-hCylE1^{WT}-1 and CycE*null*-hCylE1^{WT}-9). Clones CycE*null*-hCylE1^{KD}-1 and CycE*null*-hCylE1^{WT}-2, which both expressed a cyclin E1 transgene at a low level, exhibited a reduced effect on self-renewal capacities after introduction into cyclin E-*null* ESCs. Surprisingly, 48 hours after withdrawal of LIF, both CycE*null*-hCylE1^{KD} and both CycE*null*-hCylE1^{WT} clones still formed as many undifferentiated colonies as in the continuous presence of LIF, whereas control cells formed three times less. Together, these observations strongly suggest that forced expression of WT cyclin E1, or KD-E, results in differentiation blockade.

Figure 9: Enforced expression of KD-E blocks differentiation of cyclin E-*null* ESCs. (A) Real-time PCR analysis of human cyclin E1 expression in ESCs infected with lentiviral vectors expressing WT cyclin E1 and the KD-E mutant, and subsequently cloned. Cyclin E1 expression levels in individual clones are normalized to cyclin E1 level measured in CycE*null* cells. **(B)** LIF-rescue assay with CycE*null*-hCylE1^{WT}-1, CycE*null*-hCylE1^{WT}-2, CycE*null*-hCylE1^{WT}-9, CycE*null*-hCylE1^{KD}-1, CycE*null*-hCylE1^{KD}-4, and CycE*null*-hCylE1^{KD}-6. Means and standard errors were calculated from three replicates.

Supplementary Table I : Sequence of shRNA and expression levels of cyclin E after transfection of pENTRY-shRNA plasmids measured by real-time PCR.

Gene Symbol	shRNA Sequence	N°	Efficiency % WT expression RT-PCR
CycE (3')	AAAGACAGTGGTGTGGAAGCA	1	86
	AAGATGCCTTGGATGGAAGTG	2	44
	CCGGTGTTGTAGGTTGCTGTT	3	62
	AAGTGCTGCTATGTCTATCAA	4	76
Scra	AAGGATAGCGGTAGGAGACTA	Sc	100
CycE (CDS)	GCAGATCGCAGAGCTTCTAGA	1	34
	GGAGGATCATGTTAAACAAAG	2	
Scra	GTCTTCATACATCCGTCTAGT	Sc	100

Supplementary Table II: Oligonucleotide sequences.

Gene	5' Primer	3' reverse
mAct	TTCTTTGCAGCTCCTTCGTTGCC	TTTGCACATGCCGGAGCCGTTG
mOct4	ATGCAAATCGGAGACCCTGGTGC	AGCCCAAGCTGATTGGCGATGTG
mNanog	AAGCCATGCGCATTTTAGCACCC	AAGGAACCTGGCTTTGCCCTGAC
mRex1	TGTGTGCAGAGTGTGGCAAAGC	TGGGTGCGCAAGTTGAAATCCAG
mKlf4	GAAGACCAGGATTCCCTTGA	CCAAGCACCATCATTTAGGC
mFgf5	AGCGCGACGTTTTCTTCGTCTTC	ATGGAAACCGATGCCCACTCTGC
mBra	TGAAGGCGCCTGTGTCTTTCAGC	TGGCTGGTGATCATGCGTTGC
mSox17	TTGGAAGGCGTTGACCTTGGCAG	TTCATGCGCTTCACCTGCTTGC
mCycE	TGGAGTTGATGCAGAAGGTC	ATG GCT TTC TTT GCT TGG GC
hCycE	AGCACTTCAGGGGCGTCGC	CTGGGGAGAGGAGAAGCCC

Chapter three:

COMPLEMENTARY RESULTS AND DISCUSSION

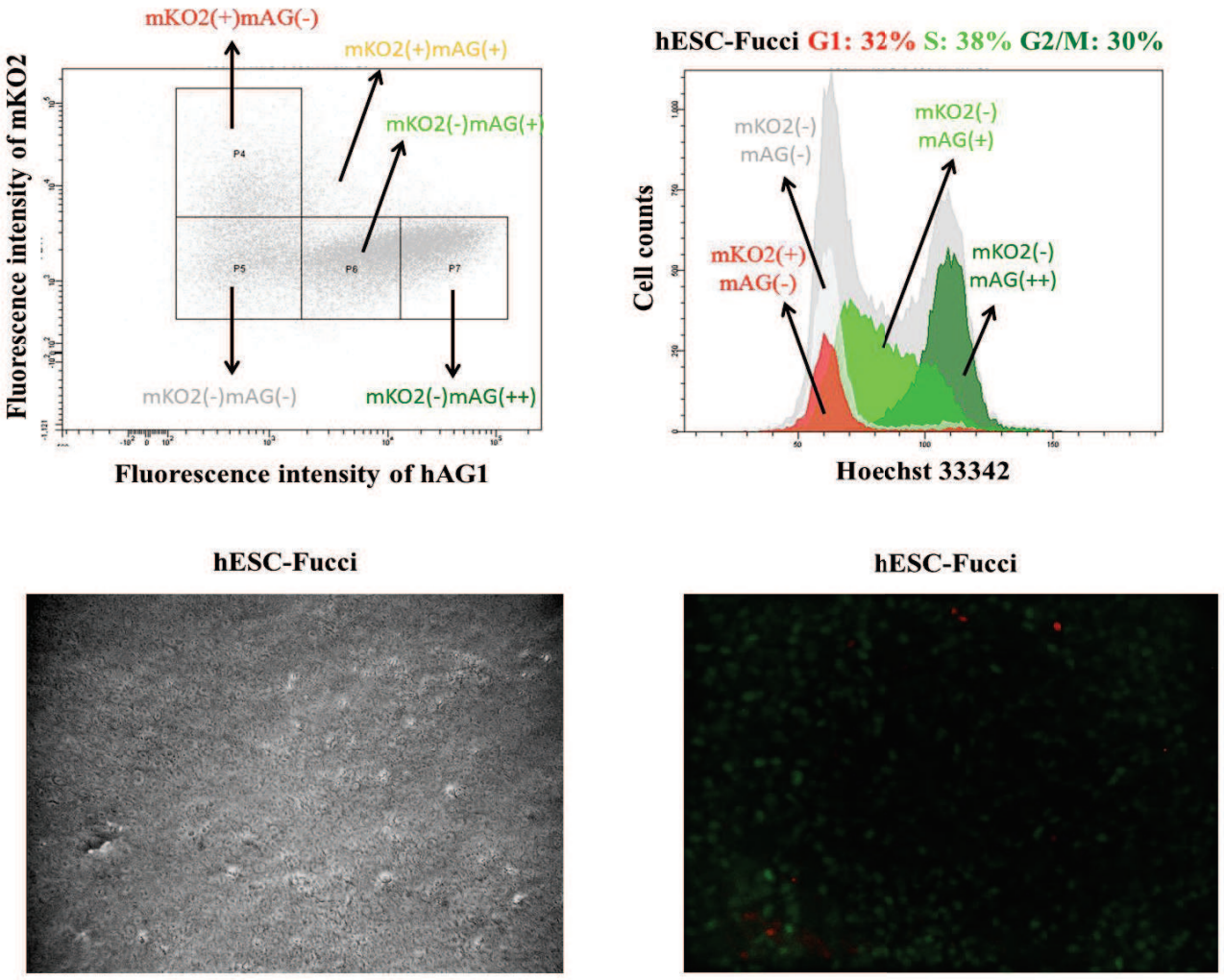


Figure 14: The Fluorescent Ubiquitination-based Cell Cycle Indicator

The human ESC-Fucci cell line uses the same Fucci principle as for mouse ESC-Fucci. Hoechst 33342 staining combined with FACS analysis validated the color-DNA content combinations. At the top left, dot plot of hESC-Fucci on normal culture conditions. At the top right, cell cycle profile showing red [mKO2(+)mAG(-)], yellow [mKO2(+)mAG(+)],light green [mKO2(-)mAG(+)], dark green [mKO2(-)mAG(++)] fluorescence, or no fluorescence [mKO2(-)mAG(-)]. At the bottom, phase contrast and fluorescence microscopy images showing the same colony of undifferentiated hESC-Fucci.

I Validating the Fucci system in ESCs

Mouse ES cell lines stably expressing the cell cycle probes mKO2:hCdt1 and mAG:hGem were engineered thereby generating mESC-Fucci. Flow cytometry analysis combined with Hoechst staining revealed that cells displaying specific combinations of orange and green fluorescence are associated with distinct DNA content and cell size. This result was further confirmed by flow cytometry analysis of synchronized cells. Consequently, this reporter system allows to distinguish live cells in early G1 (no fluorescence) and late G1 phase (orange fluorescence) from those in S (low green fluorescence) and G2 phases (high green fluorescence). Furthermore, time lapse video microscopy confirmed the gradual and orderly succession of events, namely orange fluorescence followed by yellow and then green conversion in a single cell, followed by the emergence of two green daughter cells that initially do not display any fluorescence. In summary, this exceptional tool facilitates cell cycle studies of live cells.

We also engineered a human ES cell line stably expressing the cell cycle probes mKO2:hCdt1 and mAG:hGem. The characteristic pattern of red, orange and green fluorescences was observed (**Figure 14**).

Given the atypical mode of regulation of the APC^{Cdh1} / SCF^{Skp2}switch in pluripotent stem cells described in the section, one could expect the Fucci reporters not to work in ES cells as in somatic cells. However, this is clearly not the case. Even if it seems difficult to reconcile recent data about Cdt1 and Geminin expression in ES cells and what is observed in the ESC-Fucci system, we may propose two tentative explanations:

1/ The first one consists in pointing out that the studies presented in the chapter of Introduction section 4.2 lack of consistency and are contradictory between them and with previously established data. Thus their conclusions should be taken with prudence. Moreover, since it is a recent topic of research in pluripotent stem cells field, we should await for new studies to be performed to confirm the underlying mechanisms.

Let's take a look on what happens in a scenario where APC^{Cdh1} is not active hypothetically by a lack of function of its regulators Cdh1. It has been shown that Cdh1 *null* mice died around E9.5 due to placental defects. Cdh1 deficient MEFs could be propagated for 6 passages only in culture, indicating that Cdh1 is not absolutely required for cell division.

Nevertheless, in its absence MEFs cycle slowly and have some difficulties in completing cell division, causing genome instability manifested as binucleation. Unexpectedly, the levels of Cyclin B1 went down in the absence of Cdh1, whereas there was little change in Cyclin A levels. This correlates with the ES cell phenotype (Li et al., 2008). Downregulation of APC^{Cdh1} during mitotic exit and G1 results in the unscheduled accumulation of target proteins including Cyclins A and B, Geminin and Cdc6. This has the clear potential to promote (1) premature entry into S phase (Bashir et al., 2004), (2) aberrant DNA replication namely overreplication (Vaziri et al., 2003), abnormal loading of replication origins (McGarry & Kirschner, 1998) and (3) cytokinesis defect in mouse and human cells (Engelbert et al., 2008, Garcia-Higuera et al., 2008). In addition, in Cdh1 depleted human cells, activation of p53/p21 and p16 expression occurs, suggesting a DNA damage response and premature senescence (Engelbert et al., 2008; Li et al., 2008). In conclusion, downregulation of APC^{Cdh1} is very likely to be incompatible with non-cancerous cell biology.

2/ The second explanation is that the Fucci system relies on the expression of truncated forms of Cdt1 and Geminin that may contain the appropriate elements subjected to cell cycle dependent degradation. Indeed, the truncated form of Geminin (1-110) contains the destruction box recognized by APC^{Cdh1}. One can speculate that some other parts of the protein contain elements that allow Geminin to escape from APC^{Cdh1}-dependent degradation in pluripotent stem cells. Since SCF^{Skp2} does not recognize a specific sequence of amino acids on the primary structure of the protein but rather recognizes its substrates trough phosphorylation of consensus sequences, it is hard to verify that the truncated form of Cdt1 stills contains that specific region.

2 IMPACT OF CELL CYCLE STRUCTURE ON PLURIPOTENCY

This section aims to clarify whether the molecular regulation of cell division in self-renewing stem cells is coupled to the maintenance of pluripotency, *i.e* whether self-renewal in the undifferentiated state is related to their unusual mode of cell cycle regulation.

2.1 Pluripotent mitotic cell cycle appears to be constitutively primed for DNA replication

2.1.1 Distinctive properties of the cell cycle machinery are a fundamental characteristic of undifferentiated pluripotent stem cells

Pluripotent stem cells, including mouse and human embryonic stem cells, mouse epiblast stem cells, mouse and human induced pluripotent stem cells, and embryonal carcinoma cells, share unique conserved features. They divide rapidly with most of the cell cycle time devoted to the S phase. The very short G1 phase is devoid of a restriction point and a G1 checkpoint. Instead they rely on S and G2/M checkpoints or undergo apoptosis to maintain genomic stability. In the case of mouse ES cells, it has been shown that constitutive replication is sustained by the constitutive activity of Cyclin E/CDK2 and Cyclin A/Cdk2 kinase complexes. This elicits constitutive hyperphosphorylation of Rb, which allows constitutive expression of E2F target genes and rapid progression towards and through S phase.

The expression of Rb in an inactive state and of components of the Ras/ERK pathway may be explained by a need for commitment. That is that their presence would render cells poised to implement G1 regulation immediately on withdrawal of the self-renewal stimulus (Burdon et al., 2002). Strikingly, Rb triple knockout (TKO) mouse embryonic fibroblasts are completely insensitive to senescence inducing signals, display a strong increase of their proliferation rate and have chromatin in an open configuration, just as pluripotent stem cells. Although TKO MEFs retained anchorage dependence, they lack proper G1 control and possess some characteristics of transformed cells (Dannenberg et al., 2000; Sage et al., 2000). In mouse ES cells, concomitant ablation of all three pocket proteins does not affect their

growth characteristics. However, it strongly impairs their differentiation capacity (Dannenberg et al., 2000). This suggests that Rb family members are required for differentiation perhaps by controlling chromatin remodeling (Macaluso et al., 2006).

Cell cycle regulators can influence stem cell identity. For instance, in human ESCs and iPSCs, the expression of p27 was low in undifferentiated conditions and increases during differentiation suggesting that low levels of p27 are important for maintaining hESC self-renewal and pluripotency. p27 has to be maintain at a given level of expression because its overexpression leaded to significant cell cycle arrest in G1, and its knockdown induced a slight decrease of G1 phase length associated with an up-regulation of Brachyury and Twist gene expression. Brachyury is an early indicator of mesoderm formation and Twist is a marker of the epithelial to mesenchymal transition Gene expression and ChIP analysis revealed that p27 regulates negatively and directly Twist (Menchon et al., 2010). This data uncovers a novel role for cell cycle components as regulators of pluripotency, in addition to their function as CDK inhibitors, which consists in binding chromatin to regulate gene expression.

2.1.2 Upon differentiation the cell cycle structure and its regulation are remodeled

Flow cytometry analysis on mESC-Fucci cells in an undifferentiated state or coaxed to commitment revealed how the cell cycle distribution is coordinated with differentiation. Self-renewing mESC cell cycle distribution shows a low proportion of cells in G1phase (20%) and a high proportion of cells in S, and G2/ M phases. After differentiation, the proportion of cells in G1 increases dramatically at the expense of S, G2 and M cells.

White et al (2005) demonstrated that as pluripotent stem cells differentiate into embryoid bodies the rate of cell divisions decreases accompanied by a substantially lengthening of the G1 phase. At the molecular level, this is associated with the establishment of a periodic expression of Cyclin E1 and A2 due to a transcriptional regulatory mechanism. This entails the switch from constitutive to cell cycle regulated CDK2 activity. Without cell cycle dependent pRB kinase activities, the pocket proteins pRb present in the cell become hypophosphorylated in an oscillatory fashion thus activating a functional Rb-E2F pathway. Hence, E2F target genes such as cyclin E1 and A2 are transcribed dependently of the cell cycle position. Moreover the observed up- regulation of CDK inhibitors such as p21 and p27

is likely to be important for the collapse of CDK2 activity. In summary, changes in cell cycle dynamics, notably the acquisition of a full G1 phase, during differentiation correspond to changes in CDK activity and in status of the pRB-E2F pathway. An open question is which is the initiating step that triggers the transition from constitutive to periodic regulation.

The observations made on murine and human ESCs are consistent with the fact that in *Drosophila* and *Xenopus* embryos it has been shown that a decrease in Cyclin E/CDK2activity is required for induction of differentiation (Knoblich et al., 1994; Li et al., 1999).

2.2 First hints on a relationship between G1 phase length and pluripotency

The possible link was first addressed over 20 years ago by Christine Mummery in embryonal carcinoma cells. She demonstrated that following exposure of synchronized EC cells in G1 to retinoic acid, cells lost their anchorage independent growth capacity and the cell cycle duration was increased. These early studies in EC cells revealed that their sensitivity to a differentiation inducer depends on their cell cycle position indeed cells preferentially initiate differentiation while traversing the G1 phase (Mummery et al., 1987). The underlying principle is G1 phase constitutes a window of time during which differentiation cues can accumulate. When a certain threshold level is attained, a differentiation pathway is triggered. The model predicts that a longer G1 phase, that is an extended "window of opportunity", would make cells more sensitive to differentiation signals and that cell cycle remodelling would be a prerequisite for efficient differentiation.

2.3 Is there a relationship between G1 phase length and pluripotency in mouse ES cells?

2.3.1 Inhibition of CDK2 activity impairs self-renewal

There is some evidence in the literature that downregulation of CDK2 results in accumulation or arrest of cells in G1 and is sufficient to induce differentiation of mouse and human ESCs (Filipczyk et al., 2007; Koledova et al., 2009; see section 2.4.3 for more details). This differentiation, however, does not necessarily reflect increased propensity to differentiate

in G1. It may also be caused by the arrest itself irrespective of phase, or even by direct effect of CDK2 on pluripotency of hESCs. Similar concerns apply to studies which showed that chemical arrest of the cell cycle in G2 using Nocodazole also promotes the differentiation of hESCs (Kallas et al., 2011). Thus, a causal relation between the G1 phase and differentiation of ESCs has not yet been fully established, and it remains unclear whether ESCs are more sensitive to differentiate in their G1 phase versus other phases. Furthermore, it can be hypothesized that prolongation of the G1 phase may be sensed and interpreted as stress, leading to differentiation. Indeed, in mouse ES cells UV irradiation induces differentiation as an alternative to apoptosis and senescence. The previous observations are compelling arguments to address a potential role for the cell cycle machinery in the maintenance pluripotency.

2.3.2 G1 phase of ESCs is a period of increased susceptibility to differentiation inducers

We studied the susceptibility of mESC-Fucci to retinoic acid (RA), a powerful inducer of differentiation, in a LIF rescue assay. This cellular model allows to sort cells accordingly to their position in the cell cycle with high accuracy. The LIF rescue assay aims to evaluate the capacity of ESCs to resist transient loss of self-renewal inducing signals, therefore to remain pluripotent in sub optimal culture conditions. Our results clearly showed that cells in G1 phase are more prone to differentiate and display reduced clonigenic capacities when compared with cells in the other phases of the cell cycle, no matter what their state of pluripotency is. Notably, cells transiting through the G1 phase have an intrinsic sensitivity to differentiation.

2.3.3 Loss of expression of Cyclin E lengthens G1 phase and perturbs self-renewal

We showed by FACS analysis that the outcome of a reduced Cyclin E expression in mESCs-Fucci is a longer G1 phase which corresponds to a delayed G1 to S phase transition. This was accompanied by a low clonigenicity and smaller colonies in the colony-forming assay. Surprisingly, no alteration in the self-renewal capacities evaluated in a LIF rescue assay was noticed. The size of the colonies at the end of the experiment could explain why we were unable to detect any changes. Indeed, the colonies were small which lead us to consider the

possibility that by letting them grow a few more days the balance would shift for certain towards pluripotency or differentiation. Next, a 90% loss of expression was achieved by RNA interference and resulted in a dramatic reduction of growth rate. Another mean of assessing mESC self-renewal is by analyzing the kinetics of self-renewal and lineage specific markers during differentiation induced by embryoid body formation. This experimental protocol revealed that a 10% residual expression of Cyclin E1 results in disrupted differentiation capacities.

Moreover $E1^{-/-}$ $E2^{-/-}$ mESCs displayed reduced self-renewal capacities compared to wild type mESCs. Interestingly, ectopic expression of Cyclin E rescued the wild type phenotype.

2.3.4 Gain of expression of Cyclin E improves self-renewal

It has been shown that ectopic expression of wild type Cyclin E accelerates S phase entry (Matsumoto and Maller, 2004). We demonstrated that mESCs overexpressing Cyclin E display a decreased rate of spontaneous differentiation and an enhanced resistance to LIF starvation in the colony-forming assay. In addition, analysis of pluripotency and lineage specific markers during EB formation revealed that overexpression of wild type Cyclin E resulted in delayed extinction of self-renewal markers and delayed activation of lineage specific markers (data not shown)We have not been able yet to show that enforced expression of Cyclin E shortens G1 in mESCs. Nevertheless, these data suggest that forced expression of Cyclin E protect mESCs from differentiation inducers by shortening G1 phase.

To conclude the G1phase is plausibly a time interval of acute susceptibility to differentiation inducers. Thus, the longer the G1 phase is, the more likely ESCs spontaneously commit to differentiation.

The observation that commitment to differentiation is cell cycle dependent has been extended to cancer cells, somatic cell lines and adult stem cells. We will focus in the next section in the link between cell cycle length and the differentiation process established in corticogenesis.

2.4 There is a relationship between G1 phase length and pluripotency during neurogenesis

The following section aims to provide further evidence of a connection between cell cycle regulation and cell fate decisions in stem cells.

With the onset and progression of neurogenesis, mammalian neural precursors start to switch from divisions that generate additional stem cells (expansion) to divisions that generate committed progenitors (differentiation). On top of this parameter, the rate of division of neural precursors concomitantly decreases. Ultimately, these two parameters constitute the control mechanisms of neural production. There is evidence that shows that a number of cellular components capable of influencing cell fate, proliferating-promoting signals and environmental cues exert their influence through G1 cell cycle components (Cremisi et al., 2003). Likewise, recent data suggest that cell cycle effectors determine the mode of division of progenitors and thereby influence cell fate determination.

The lengthening of the G1 phase of neuroepithelial cells is a cause of neurogenesis (Calegari and Huttner, 2003). Artificial lengthening of the cell cycle, by inhibition of CDK1 and CDK2 complexes, can causally contribute to neural progenitors switching from proliferative to neuron-generating divisions and may have important implications for the expansion of somatic stem cells in general. The authors proposed the following model: the longer the stem cell stays in G1, the more likely they are to be subjected/sensitive to differentiation signals (**Figure 15 A**). Additionally, another report demonstrated that regulation of the length of G1 phase, via p27 and Cyclin E expression, can impact on cell fate of primate cortical precursors *ex vivo*. It also provides evidence that a short G1 phase is associated with a higher frequency of cell cycle reentry when compared to a longer G1 phase and that short G1 might shield precursors from signals that induce differentiation (Lukaszewicz et al., 2005).

Conversely, shortening G1 duration of cortical precursors promotes their self-renewal (Pilaz et al., 2009). In this study, a 25% reduction of G1 duration was achieved by imposed expression of Cyclin D1 and Cyclin E1, two factors shown to be rate limiting for G1/S transition. This caused a biased decision between proliferative versus differentiative division

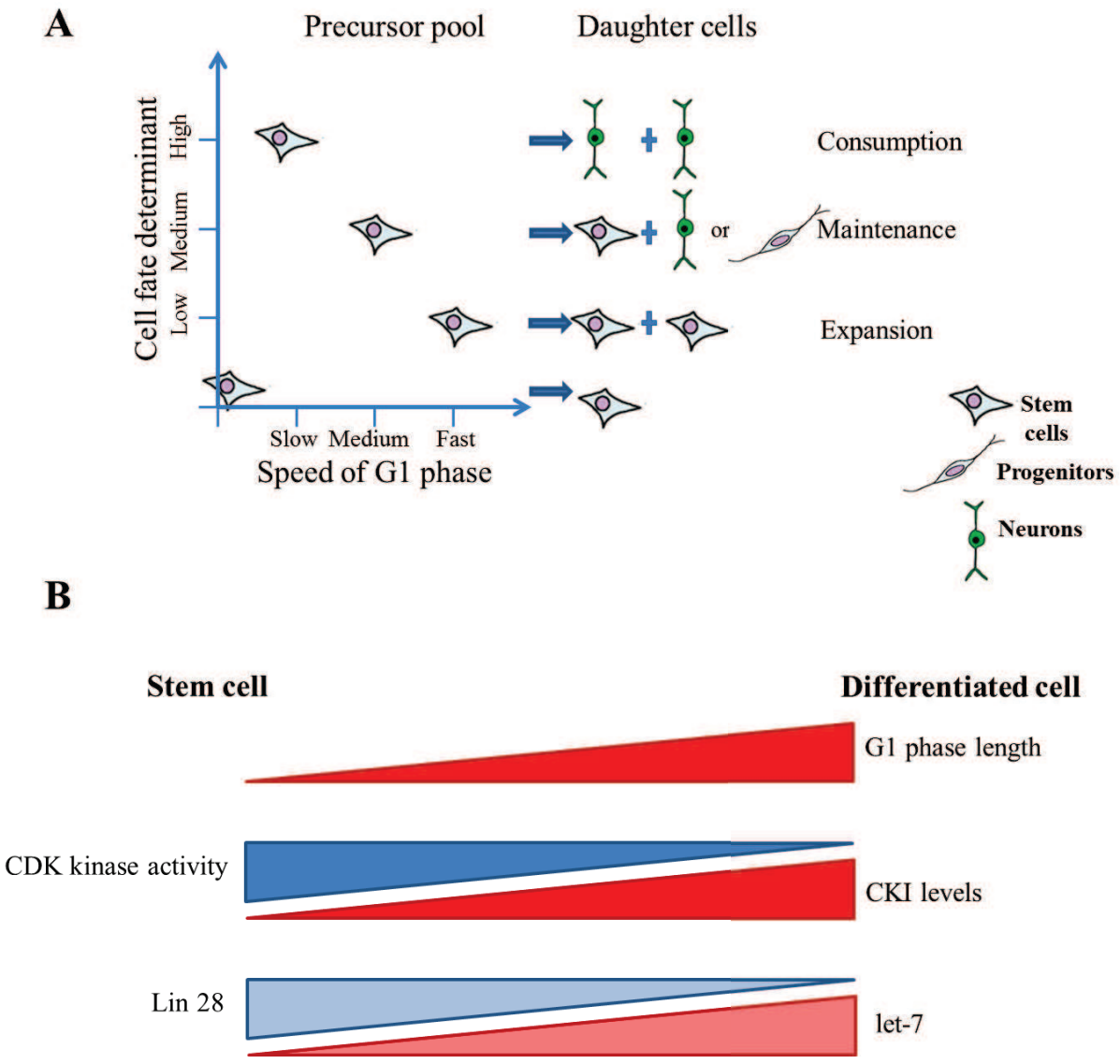


Figure 15: The cell cycle length hypothesis in neurogenesis

(A) The speed of the G1 phase influences the final effect of a cell fate determinant. Thus, this effect will be high, medium or low depending on whether the speed of G1 is slow, medium or fast, respectively, and eventually will trigger consumption, maintenance or expansion of the precursor pool. During quiescence, G1 does not progress (speed=0) and cell fate determinants do not act, which allows the precursor pool to be maintained.

From Salomoni and Calegari , 2010.

(B) Cell cycle regulators (e.g. CDKs, CKIs) influence G1 phase duration, and thereby cell cycle progression, thus modulating cell commitment. Coupled inverse regulation of Lin28 and let-7 illustrates how opposed signals modify the balance self-renewal/differentiation. Indeed the expression of the let-7 miRNAs is tightly regulated in a development- and cell-specific manner by various mechanisms such as blockade by Lin-28, an RNA editing protein that helps to promote pluripotency (Kawahara et al., 2011).

at the expense of neurogenesis. Consistent with this study, overexpression of Cyclin D1/CDK4 in neural stem cells has been shown to shorten G1 phase by 30%, prevent their differentiation and promote expansion of progenitors (Lange et al., 2009).

In a similar manner, hematopoietic cell number is maintained by a delicate balance between cell proliferation, differentiation and death driven by cyclins, CDKs, and CDK inhibitors. The decision to proliferate or remain in a quiescent state is central to the maintenance of the hematopoietic system, and is a decision largely executed by cell cycle regulation. Indeed, prevention of continuous division by halting cells at the G0/G1 phase prevents exhaustion of progenitor populations. Shortening G1 duration would promote precursors expansion. By contrast, leaving quiescence and entering early G1 (or increasing its length) would favor stem cell differentiation (Orford and Scadden, 2008). This is consistent with the cell cycle regulators being involved almost exclusively in regulation of G1 progression and G1/S transition in this cellular compartment. These observations are in agreement with the hypothesis that G1 length is crucial to influence cell fate.

In summary, cell cycle regulators influence cell cycle kinetics by modifying rates of cell cycle progression and cell cycle reentry of the precursor pool, thus modulating its size and eventually neural production. It has been argued that the G1 phase duration is an integral part of the cellular mechanism regulating the mode of division. A prolonged G1 phase facilitates the integration of signals that influence cell fate and ultimately is coupled to lineage specification. In other words, G1 lengthening is both necessary and sufficient to cause to cause the switch from expansion to differentiation. Altogether, this suggests that cell cycle regulation and stem cell potency are highly coordinated (**Figure 15 B**).

Interestingly, in mouse cerebral cortex, cell cycle regulators serve diverse functions independently of their role in cell proliferation. They can participate in cell migration, axonal elongation synaptic maturation, to name a few (Frank and Tsai, 2009).

Pluripotent embryonic stem cells, in contrast to the progenitors, do not exit the cell cycle during their first differentiation steps, while in the contrary terminally differentiated cells are maintained in cell cycle arrest (Skapek et al., 2006). Nevertheless, these mechanisms can be reversed to make phenotypically mature cells divide beyond their normal capacity and gain pluripotent features. This is what reprogramming consists in.

3 LIF MODULATES DURATION OF THE G1 PHASE

3.1 LIF regulates G1 to S phase transition

Since LIF is a key regulator of pluripotency, we asked of it also participates in the regulation of the pluripotent cellc cycle. To this aim, we examined the cell cycle response of mESCs to transient LIF starvation, followed by restimulation. This experimental paradigm aimed to uncouple the short term effects on the cell cycle from the long term effects on differentiation. Indeed, differentiation of ESCs induced by LIF withdrawal delays the G1 to S phase transition (Savatier et al., 1994, 1996). It was therefore necessary to analyse the effects of LIF starvation before the irreversible commitment to differentiation occurred.

mESCs-Fucci were LIF deprived and subsequently restimulated for different period of times. Examination of the duration of each phase of the cell cycle revealed that the kinetics of transit through the late G1 phase is the most sensitive to LIF starvation/restimulation. We demonstrated that LIF withdrawal induces the lengthening of the G1 phase, and that subsequent stimulation restore the original duration. These changes in the duration of the late G1 phase are tightly associated with variations in the expression levels of pluripotency and lineage-specific markers. Crucially, these variations in markers levels are transient, which indicates that LIF starvation/restimulation does not induce irreversible commitment into differentiation.

Even in the more drastic condition (*i.e.* 48 hours of LIF starvation), the pluripotency and lineage-specific markers returned to original levels after stimulation with LIF for 48 hours, suggesting no loss of pluripotency. However, this has to be further confirmed. This finding is in conflict with previous data suggesting that upon 36 hours of LIF withdrawal mESCs are irreversibly commitment to differentiation into various lineages and to apoptosis (Boeuf et al., 2001). In addition, mES cells grown without LIF for 48 hours would have lost their pluripotency, as suggested by their inability to colonize embryos (Burdon et al., 1999). These differences may be explained by the initial state of pluripotency which correlates with the quality of culture conditions and the genomic background of the cell lines.

Here, we showed that LIF signaling accelerates the transit through the G1 phase. It represents an argument in favor of the hypothesis that a short G1 phase shields ESCs from differentiation inducers and promotes self-renewal in the pluripotent state. Reducing the duration of G1 could be one mechanism by which LIF signaling inhibits ESC differentiation.

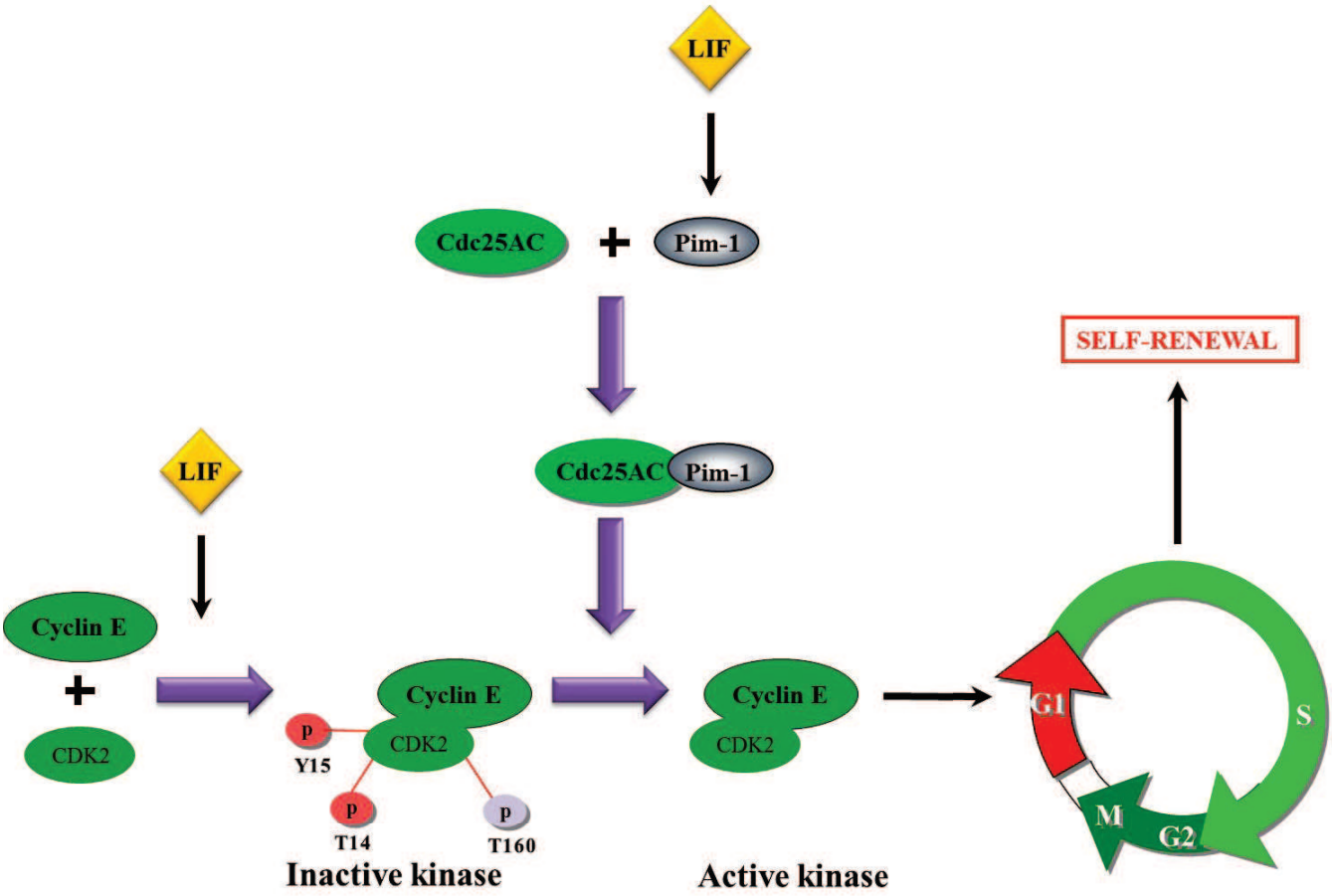


Figure 16: Schematic diagram showing the regulation of G1/S transition by LIF in mouse ESCs

LIF signaling regulates the activity of Cyclin E/CDK2 complexes by at least two independent mechanisms:

the stimulation of the activating dephosphorylation of CDK2: LIF stimulation increases Pim1 levels as well as Pim1/Cdc25A complex formation. Activated Cdc25A dephosphorylates CDK2 on the inhibitory Tyr15. Levels of CDK2 activating phosphorylation on Thr160 remain unchanged.

the accumulation of active Cyclin E:CDK2 complexes.

Thereby, LIF influences the duration of the G1 phase.

3.2 LIF regulates Cyclin E/CDK2 complex activity in mESCs

mESCs were LIF-deprived for 24 hours and subsequently re-stimulated for 1 to 8 hrs. After LIF re-stimulation, Cyclin E and Cdk2 subunits levels showed no significant alteration, while both the amount of Cyclin E/CDK2 complexes and their kinase activity transiently increased. These observations demonstrate the capacity of LIF to regulate the activity of the S-phase-promoting Cyclin E/CDK2 kinase.

The mechanisms of this regulation are still not clear. Both published and unpublished data of the laboratory indicate that LIF stimulation enhances the transcription of the LIF/STAT3 target gene *Pim-1* (Aksoy et al., 2007; Bourillot et al., 2009). The serine/threonine kinase Pim-1 phosphorylates, thereby activates the phosphatase Cdc25A (Mochizuki et al. 1999; Blomberg and Hoffmann, 1999; Bartek and Lukas., 2001). This in turn stimulates the activating dephosphorylation of Tyr15 on the CDK2 subunit. Dephosphorylation of Tyr15-Cdk2 is a prerequisite to the activation of the Cyclin E/CDK2 kinase. Together, these results suggest that LIF regulates Cyclin E/CDK2 complexes by activating the Pim1-Cdc25A-CDK2 regulatory pathway (unpublished data) (**Figure 16**).

In addition, we examined the effects of its overexpression in the context of conditional expression induced by doxycycline and dexamethasone in a LIF rescue assay. The experimental scheme is represented in **figure 17 A**. A 2.6 fold increase in Pim1 mRNA was observed after Dox+Dex treatment. Pim1 overexpressing mESCs display a higher capacity to self-renew as well as a higher resistance to LIF starvation (**Figure 17 B** and Aksoy et al., 2007). Further experiments have to be performed to confirm the role of Pim1 in the LIF dependent regulation of Cyclin E/CDK2 complexes.

4 REGULATION OF CYCLIN E FUNTIONS

4.1 Transcriptional regulation

In somatic cells, Cyclin E expression is induced or repressed by proteins of the E2F-transcription factor family (Moroy and Geisen, 2004), and by multiple signaling pathways such as Hedgehog (Duman-Scheel et al., 2002) and TGF-beta (Geng et al., 1996). Mitogens such as cMyc also activate Cyclin E expression (Perez-Roger et al., 1997).

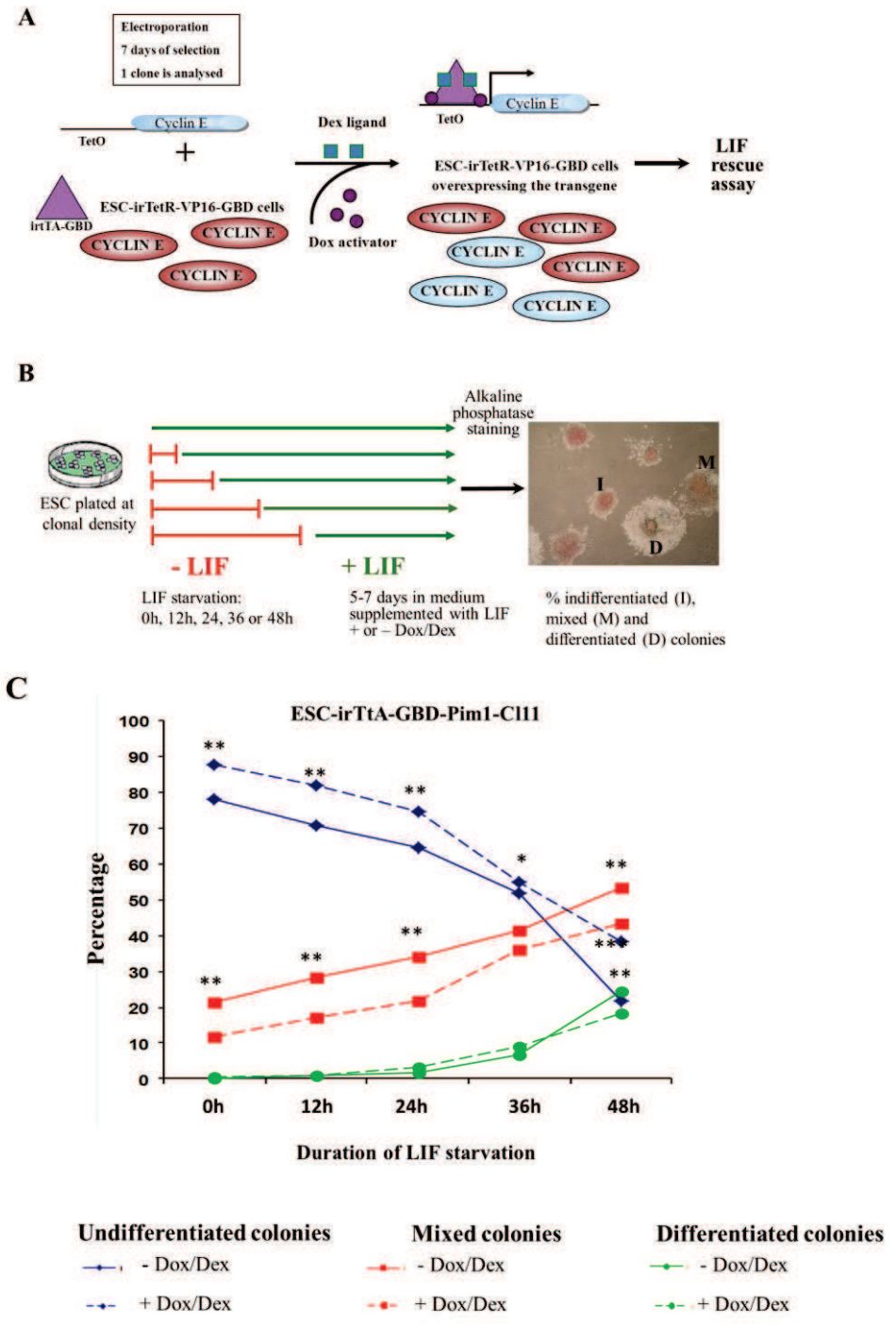


Figure 17: Pim1 enhances the resistance of mouse ESC to LIF starvation

(A) Inducible expression system based on dexamethasone/doxycycline (Dox/Dex) treatment resulting in stable and dose dependent expression of the transgene. **(B)** Analysis is performed by a LIF rescue assay. **(C)** Pim1 overexpression results in a reinforcement of self-renewal and delayed differentiation. Pim1 is likely to be a rate-limiting factor for self-renewal (*, $p < .05$; **, $p < .01$; ***, $p < .001$, calculated with the Student t test).

Note that Cyclin E2 is not expressed in pluripotent stem cells while Cyclin E1 is expressed robustly throughout the cell cycle (Stead et al., 2002; Filipzik et al., 2007). E2F factors have been implicated in the activation of Cyclin E transcription (White et al., 2005). It remains to determine if the expression of CylE1, the gene encoding Cyclin E, is regulated by Oct4, Sox2, Nanog, or Klf4 among other transcription factors that sustain the pluripotent self-renewing state. This question could be addressed, by conditionally overexpressing or down regulating pluripotency genes. This would further point to an important role of Cyclin E in the regulation of pluripotency.

4.2 By activating CDK2

In somatic cells, CDK activity is a major factor that influences the rate of cell cycle progression and the length of cell cycle phases (Bremner et al., 1995; Resnitzky and Redd, 1995). In ESCs, CDK2 plays a role in sustaining the pluripotent undifferentiated state. However, it was shown that CDK2 is a non-essential protein as CDK2 knockout embryos develop normally and Cdk2 deficient mice are defective only in meiosis (Ortega et al., 2003). We aimed to elucidate if the Cyclin E effects we observed in mESCs function through CDK2 kinase interaction or activation. For this purpose two mutant forms were overexpressed. The S180D mutant was presented by Matsumoto and Maller (2004) as unable to bind Cdk2 and thereby lacking associated kinase activity. According to their work, overexpression of this mutant form accelerates entry into S phase. The Kinase Deficient mutant (KD-E) has two amino acid residues, that make direct contact with the CDK2 activation segment, mutated thus preventing CDK2 activation upon Cyclin E binding (Geng et al., 2007). The KD-E mutant is able to rescue the growth deficit of cyclin E-*null* fibroblasts (Geng et al., 2007).

In the context of the doxycycline/dexamethasone regulated overexpression system, we studied the self-renewal capacity of the engineered cells in a LIF rescue assay to determine if the finely tuned balance between self-renewal and differentiation was perturbed. Conforming to Matsumoto and Maller (2004) data, we could have expected a positive effect of the S180D mutant on maintaining self-renewal. Nevertheless in none of our experiments the overexpression of the S180D mutant has any effect. We propose that this mutant form of Cyclin E has an inactive function because its conformation is completely disrupted.

Surprisingly, mESCs overexpressing the KD-E mutant resulted in a reinforcement of self-renewal. Furthermore, in cyclin E-*null* background, overexpression of KD-E rescued the

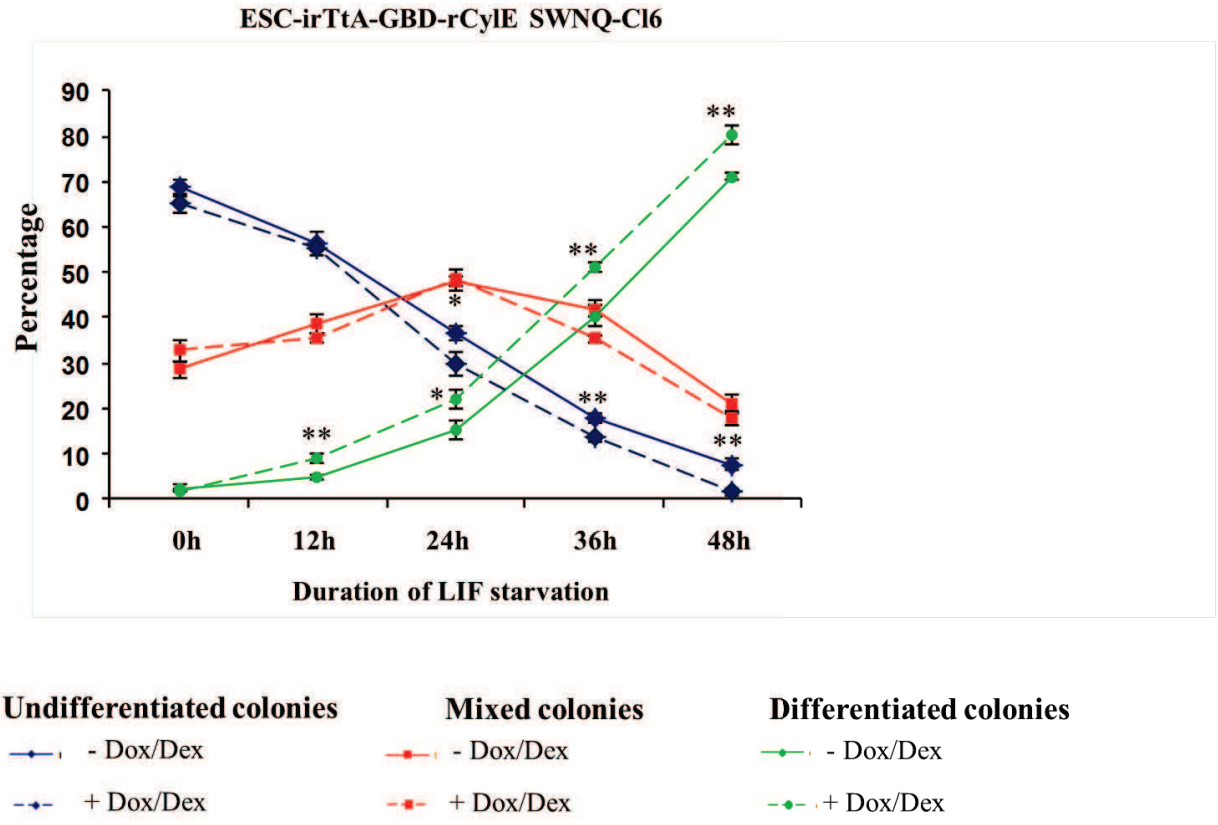


Figure 18: Cyclin E enhances the resistance of mouse ESC to LIF starvation via its centrosomal localization

The mouse cell line ESC-irTtA-GBD-rCylE SWNQ-Cl6 was analyzed in a LIF rescue assay with or without Dox/Dex treatment. Overexpression of the mutant lacking the centrosome binding site impairs self-renewal capacities (*, $p < .05$; **, $p < .01$; ***, $p < .001$, calculated with the Student t test).

wild-type phenotype from $E1^{-/-}$ $E2^{-/-}$ mESCs. These results suggest that Cyclin E is able to support self-renewal of mESCs independently from CDK2 activation. It has been shown that Cyclin D also conveys CDK independent functions (Fu et al., 2004).

4.3 By binding to the centrosome

The centrosome is an organelle that serves as the main microtubule organizing center. Its replication occurs during the S phase, and similarly to DNA replication, it is a semi-conservative processes regulated by CDK2 (Meraldi et al., 1999). It is a well-known regulator of cell cycle progression. Moreover, abnormal amplification of centrosomes leads to cell death, genomic instability or G1 arrest (Bourke et al., 2007, Mikule et al., 2007). This mechanism may act as an additional checkpoint.

Several studies have proposed a role for centrosomes in the regulation of G1/S and G2/M transitions and DNA damage checkpoint signaling because multiple proteins involved in cell cycle control and DNA damage response are found to localize to centrosomes (Doxsey et al., 2005).

In section 2.5.1, we discussed that in mouse ES cells CDK2 related activity is unmodified after changing levels of regulators Cdc25A and p21. Immunolocalization studies have implicated its cellular localization. Indeed, in mouse ESCs, CDK2 predominantly localizes to centrosomes whereas Cdc25A localizes to cytoplasm and nuclei and was not detected at centrosomes. Thus centrosomal CDK2 may be sheltered from its regulators (Koledova et al., 2010). Moreover, centrosomes may serve as sites for sequestration of checkpoint components Chk1 and Chk2 (Hong and Stambrook, 2004; Koledova et al., 2010). Divoky research group suggested a novel role for centrosomes in the coordination of cell fate decision and of cell cycle regulatory events by acting as scaffolds that promote interactions between various regulatory components and that prevent reaction that may limit self-renewal.

In 2004, Matsumoto and Maller identified a centrosomal localization signal (CLS) on Cyclin E that is essential for both centrosomal targeting and for promoting DNA synthesis in a CDK2 independent fashion somatic cells. Interestingly, they demonstrated that Cyclin E localizes to the centrosome from G1 through S phase and that preventing endogenous Cyclin A or Cyclin E localization to the centrosome inhibits DNA synthesis.

Inducible overexpression of the CLS deficient mutant resulted in no positive effect on self-renewal capacities evaluated in a LIF rescue assay (**Figure 18**). Briefly, ESCs were co-

electroporated with irTetR-VP16-GBD, a plasmid expressing a dexamethasone (Dex)-dependent rtTA and the puroR selectable gene, and pSport1-tetCMVcylESWNQpA-INS, a plasmid expressing the CLS deficient mutant cDNA under the control of the doxycycline (Dox) promoter and the hygroR selectable gene. A clone showing 1.9 fold-induction after Dox+Dex treatment was analyzed in a LIF-rescue assay. ESC-irTtA-GBD-cylE SWNQ-Cl6 cells treated with Dox+Dex produced a significantly higher percentage of differentiated colonies, as compared to untreated cells after LIF-deprivation for 12 hrs (9.0 ± 1.1% for treated versus 5 ± 0.6% for untreated cells), 24 hrs (22.0 ± 1.9% for treated versus 15.2 ± 2.1% for untreated cells), 36 hrs (51.0 ± 1% for treated versus 40.4 ± 2.2% for untreated cells), and 48 hours (80.3 ±2. 1% for treated versus 71.0 ± 0.7% for untreated cells). This is in agreement with the CLS mutant not being able to permit S phase entry when overexpressed.

5 CELL CYCLE CHARACTERISTICS DEPEND ON THE STATE OF PLURIPOTENCY

5.1 G1 phase length varies according to the state of pluripotency

Naïve (ICM-like) and primed (epiblast-like) pluripotent stem cells have differential Rex1 expression that correlates with distinct developmental potential (Toyooka et al., 2008) and cell cycle distributions (our data). They can convert into each other under normal culture conditions. Whereas Oct4 is continuously expressed in the ICM and in the epiblast at the pre- and post-implantation embryo, Rex1 is commonly used as a landmark of naïve pluripotency and is strongly expressed in the ICM but down regulated in the epiblast (Rogers et al., 1991; Pelton et al., 2002). $Rex1^{+}/Oct4^{+}$ are assumed to be analogs to ICM stage and $Rex1^{-}/Oct3/4^{+}$ cells are estimated to be equivalent to epiblast. The later do not contribute to chimera formation, express at a lesser extent Nanog and at a higher extent Fgf5, a marker of primitive ectoderm in early development (Toyooka et al., 2008).

We engineered the mES cell line $Rex1^{GFP\text{-}bla}/Oct4^{CFP\text{-}puro}/mKO2$, a tool that offers a unique opportunity to examine the changes in cell cycle regulation during early commitment. Within the Oct4 positive population, a 1:9 ratio of GFP^{high}-Rex^{+} associated cells against GFP^{low}-$Rex1^{-}$ associated cells was observed as described by Toyooka and collaborators

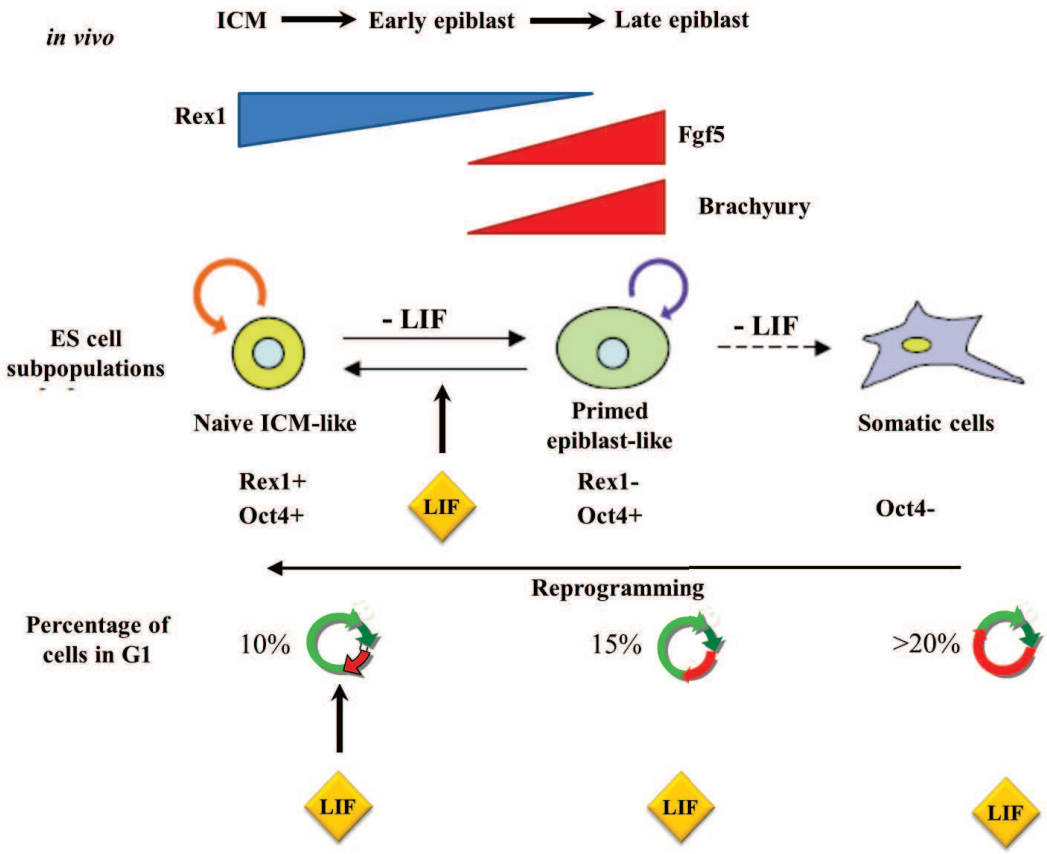


Figure 19: G1 length and regulation by LIF varies according to the pluripotency state

The mouse ES cell line Rex1$^{GFP\text{-}bla}$/Oct4$^{CFP\text{-}puro}$/mKO2 was used to examine the changes in cell cycle regulation during early commitment achieved by LIF deprivation. Rex1^{+} and Rex1^{-} ESCs differ in their cell-cycle distribution. Stimulation of LIF signaling results in a shortening of the G1 phase, relative to the other phases of the cell cycle, in the Rex1^{+} ESC population exclusively. For Rex1^{-} LIF does not regulate G1 length but their commitment to differentiation.

Adapted from Toyooka et al., 2008

(Toyooka et al., 2008).We showed that, within the Rex1$^-$ ESC population, a larger fraction of cells is transiting in G1 phase compared to the Rex1$^+$ population. This means that a lengthening of G1 occurs when cells convert to the Rex1$^-$ phenotype. Both Rex1$^+$ and Rex1$^-$, when transiting through G1 phase, were more prone to commitment into differentiation and exhibited a reduced clonigenicity.

In an experiment of LIF starvation/restimulation, we showed that within the Oct4$^+$ population, LIF promotes transition through G1 in the Rex1$^+$ fraction only (**Figure 19**). By contrast, cells in the Rex1$^-$ do not respond to LIF treatment. LIF does not regulate their cell cycle structure but only their commitment to differentiation. Gene expression analysis of the whole Rex1$^+$ subpopulation revealed variations in Nanog and Fgf5 levels only. The increase in Nanog expression during LIF restimulation may be explained by its property of establishing rather that maintaining pluripotency (Chambers et al., 2007). It would act as a molecular switch to turn on the naive pluripotent program which is consistent with the decrease in the percentage of Rex1$^-$ cells. The increase of Fgf5 expression following LIF starvation can be interpreted as a marker of early commitment, which is reversible.

EpiSCs, which are derived from late epiblast, have low expression level of Rex1 and a higher expression level of genes that can be detected in post-implantation embryos, such as Fgf5, compared with ES cells derived from the ICM. EpiSCs and the Rex1$^-$ fraction of ESCs have similar characteristics but the crucial difference is that Rex1$^-$ cells can keep their status only very transiently and revert to Rex1$^+$ within a short period. Environmental changes (e.g. culture medium supplemented with LIF and BMP) are sufficient to interconvert EpiSCs into ESC-like cells at very low frequency (Bao et al. 2009). A higher efficiency of conversion is achieved by overexpressing Klf4 (Guo et al., 2009) or the activated LIF transducer STAT3 (Yang et al., 2010). We already mentioned that the transition from Rex1$^-$ to Rex1$^+$ ESCs is accompanied by a further shortening of the G1 phase. It would be interesting to determine if the conversion from EpiSCs to naïve ESCs is also paralleled by a shortening of the G1 phase.

5.2 Naive pluripotent stem cells are able to achieve autonomous replication

In contrast to somatic cells, which depend on mitogenic signaling to proceed through the R point, ESCs do not require exogenous growth factors to enter the cell division cycle, are not subject to contact inhibition, and do not enter quiescence upon growth factor withdrawal (Burdon et al., 2002; Fluckiger et al., 2006; Becker et al., 2009). All these features imply that they are able of autonomous replication when the specific requirements for self-renewal are fulfilled. It has been recently shown that the inhibition of differentiation pathways, using compounds that inhibit MAPK and GSK3 in serum-free conditions, enhances reprogramming and promotes maintenance of the naive pluripotent phenotype (Nichols et al., 2009b).

The pluripotent state captured for human ESCs so far resembles to that of mouse EpiSCs. It can be reverted to a naïve pluripotent state mouse ESC-like. This is accompanied by acquisition of colony morphology, gene expression and signaling dependence more similar to mESCs and by a reduction of doubling time (Hanna et al., 2010). In a similar manner, using a reprogramming approach, Buecker et al. succeeded in establishing LIF responsive hES cell lines which display hallmark characteristics of mESCs, including the dome-shaped tightly packed mESC morphology, the high proliferation rate and the higher clonigenicity. The average population doubling time went from 36 hours to 22 hours (Buecker et al., 2010). We speculate that this reduction results from cell-cycle acceleration, including shortening of the G1 phase, and reduced apoptosis.

Nevertheless, it seems that rapid cell proliferation is not always linked with pluripotent identity. Indeed, Ohtsuka and Dalton claim that some human ES cell lines have a cycling time of around 32 to 38 hours (Ohtsuka and Dalton 2008; Dalton 2009). Cell division time of epiblast cells in the early human embryo remains unknown. There is another example that illustrates that rapid cell divisions are not necessary synonym for pluripotent state. Partially reprogrammed cells have established a short G1 phase but are not fully pluripotent (Chan et al., 2009)

Chapter four:

GENERAL CONCLUSION

Self-renewing pluripotent stem cells display unique transcriptional and post transcriptional regulation networks, a poised epigenetic state that maintains chromatin in a form ready for rapid cell fate decisions and a cell cycle characterized by an extremely short G1 phase without robust checkpoint controls. Irrespective of any species differences, cell cycle regulation in ES cells is fundamentally different from that of other somatic cell types, and an understanding of the processes that maintain the unique features of pluripotent stem cell cycle regulation appear critical to understanding pluripotency.

We have proposed strong evidence indicating that self-renewal and pluripotency of embryonic stem cells are related to their unusual mode of cell cycle regulation and specifically to their abrogated G1 phase. Accordingly, commitment of pluripotent stem cells to differentiation is cell cycle dependent. Moreover, we showed that LIF sustains self-renewal and shields mouse ESCs from differentiation, at least in part, by regulating the G1 length. Thus, the G1 duration defines an intrinsic characteristic of the state of pluripotency. We have proved that Cyclin E, usually considered as positive regulator of proliferation, plays also a role in maintaining self-renewal. Cyclin E expression opposes differentiation in an apparently CDK2 independent fashion. Alternatively, Cyclin E may facilitate G1 transit and/or enhances self-renewal of pluripotent stem cells via its binding of to the centrosome. Further investigation to clarify this issue is needed.

Understanding the molecular mechanisms underpinning pluripotency will undoubtedly facilitate the use of pluripotent stem cells as a possible source for cell replacement therapy to treat diseases or syndromes like Parkinsonism, spinal cord injury, diabetes and heart failure.

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