2

Molecular Mechanisms of Pluripotency in Murine Embryonic Stem Cells

Elisa A. Casanova, Kurt Bürki and Paolo Cinelli
Institute of Laboratory Animal Science, University of Zurich
Switzerland

1. Introduction

Embryonic stem cells (ESCs) are pluripotent cells, which can be isolated from the inner cell mass (ICM) of blastocyst stage embryos. They are defined by two properties: they can indefinitely self-renew *in vitro* and contribute to the formation of all cells of an adult organism, including functional gametes for genome transmission. Due to their pluripotent state ESCs can be used for various applications, like the generation of knockout or transgenic animals, and potentially as a cell source for cell therapy in regenerative medicine. Alternatively self-renewing cells with pluripotent potential can also be generated by specifying germ cells with extrinsic factors (Matsui, 1992) or by reprogramming somatic cells using gene transfection to generate the so called induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka, 2006).

Due to the fundamental characteristics of ESCs, developmental biology, regenerative medicine and cancer biology are more and more interested in understanding the molecular mechanisms controlling stem cells. Even though a lot of efforts have been made in the past years to elucidate the factors that regulate stem cell self-renewal and pluripotency, the precise mechanism of how these processes are regulated remains largely unknown. In this chapter we will give an overview on the known molecular pathways and transcription factors involved in maintaining pluripotency, by especially focusing on the core transcription factors OCT3/4, SOX2, Nanog, and STAT3. Subsequently we will discuss the epigenetics of ESCs and iPSCs and finally conclude this chapter with remarks and discussions.

1.1 Establishing and maintaining ESCs *in vitro*

ESC lines are derived from the preimplantation embryo, precisely from the ICM of the blastocysts. Although the presence of self-renewing, pluripotent, cells is transient *in vivo*, apparently immortal cell lines with these properties can be obtained *in vitro*. Once removed from the blastocyst, the cells of the ICM can be cultured under special conditions, into ESCs, which maintain an undifferentiated status (Evans and Kaufman, 1981; Martin, 1981). Under other conditions, for example by giving the right extrinsic and intrinsic signals, ESCs can differentiate into all the cell types that make up the organism. Pluripotent ESCs are characterized by the expression of specific cell surface glycoproteins such as the stage-specific embryonic antigen 1 (SSEA-1) (Solter and Knowles, 1978) as well as by the presence of transcription factors such as OCT3/4 (Schöler, 1991; Schöler et al., 1989) and Nanog

(Chambers et al., 2003). High expression levels of alkaline phosphatase also characterize ESCs. Furthermore, ESCs exhibit a short G1 phase of the cell cycle (Rohwedel et al., 1996) and a high telomerase activity (Thomson et al., 1998). Other essential properties of ESCs include growth as multicellular colonies, normal and stable karyotypes, and prolonged undifferentiated culture.

Derivation and maintenance of murine ESCs *in vitro* was originally achieved by using mitotically inactivated embryonic fibroblasts (feeders) and/or the cytokine leukaemia inhibitory factor (LIF) in combination with fetal calf serum and/or the growth factor bone morphogenetic protein (BMP) (Smith et al., 1988; Ying et al., 2003). However, the same culture conditions are not sufficient for derivation of ESCs from most of the mouse strains and not at all from the rat. The genetic background strongly affects the efficiency of ESC isolation and almost all lines in use are derived from the strain 129. Even though ESCs were discovered more than 25 years ago only limited number of ESCs of proven ability to colonize the germ-line have been obtained and only a few mouse strains other than 129 (Simpson et al., 1997). Only after adjusting the culture conditions, germ line competent inbred ESCs could be established, e.g. from C57BL6/J (Keskintepe et al., 2007; Ledermann and Bürki, 1991), DBA/1lacJ (Roach et al., 1995), BALB/c (Kawase et al., 1994; Noben-Trauth et al., 1996), and CBA mice (Lodge et al., 2005). Recently it has been shown that extrinsic stimuli are dispensable for derivation and maintenance of the pluripotent state. Culture conditions free from feeders, serum and cytokines were established by using a combination of small-chemical molecules, which inhibit the fibroblast growth factor (FGF)/mitogen-activated protein kinase (MEK)/extracellular signal-related kinase (ERK1/2) and the glycogen synthase kinase 3 (GSK3) (Ying et al., 2008). These culture conditions are known as 3i or 2i and have been applied for derivation of ESCs from non-permissive mouse strains like non-obese diabetic (NOD) mice (Nichols et al., 2009) but also from rat embryos, resulting in the production of the first germ-line competent rat ESCs (Buehr et al., 2008; Li et al., 2008).

The finding that by using the 2i conditions it is nowadays possible to derive ESCs from almost all the mouse strains and importantly also from the rat is surely of high importance in the field of ESC research.

In the following paragraphs we will review the most important molecular and cellular mechanisms that regulate stem cell self-renewal and pluripotency prevalently in mouse ESCs, since they are the most investigated ESCs.

2. Signalling through cytokine receptors: LIF/gp130 pathway

In 1988 Austin Smith and colleagues isolated a soluble glycoprotein that prevents stem cell differentiation and established that ESC self-renewal was dependent on paracrine signals produced from the feeders on which ESCs were cultivated (Smith et al., 1988). The principal factor required for self-renewal was shown to be leukaemia inhibitory factor (LIF) (Gearing et al., 1987; Williams et al., 1988). *Lif* knockout feeders were reported to be unable to support ESCs self-renewal (Stewart et al., 1992), indicating that supply of LIF was a key attribute of feeders. LIF belongs to the family of interleukin (IL)-6-type cytokines and exerts its effects by binding to a two-part receptor complex, which consists of the low-affinity LIF receptor (LIFRβ) and the glycoprotein 130 (gp130). LIF induces heterodimerization of the LIFRβ and gp130 resulting predominantly in the activation of the JAK/STAT signal transduction pathway, which promotes self-renewal in ESCs. Several studies showed that LIFRβ receptor is not sufficient to mediate the signal to maintain ESCs self-renewal, whereas gp130 is (Niwa

et al., 1998; Starr et al., 1997). These results indicate that gp130 is the main component of the activated LIFRβ/gp130 receptor. As we will see in the following chapters activation of LIFRβ/gp130 receptor through the binding of LIF leads also to the activation of the mitogen-activated protein kinase (MAPK) and the phosphatidylinositol-3 phosphate kinase (PI3K) pathways, which together with the JAK/STAT3 pathway are essential for regulating biological responses in ESCs.

In the embryo the epiblast is the transient population of cells from which the foetus is derived. Because of their characteristics, ESCs seem to be the *in vitro* counter part of the epiblast cells *in vivo*. However, in contrast to the LIF dependency of ESCs, early epiblast cells do not require LIF stimulation, since *Lif* -/- embryos develop normally into later stages (Stewart et al., 1992) and embryos carrying mutations on the LIFβR and gp130 receptor develop normally, at least until mid-gestation (Li et al., 1995; Nakashima et al., 1999; Ware et al., 1995). Nevertheless, it has been shown that the embryos do express LIF, LIFβR and the gp130 mRNA indicating a possible function of this pathway also *in vivo*.

Mice can temporary arrest embryogenesis at the blastocyst stage; this phenomenon is called diapause and has evolved in certain mammals to overcome sub-optimal conditions for pregnancy. During diapause the embryos develop to the hatched blastocyst stage but then stop their development remaining unimplanted in the uterus. This situation can persist in mice for weeks, a period during which the epiblast cells have to be maintained pluripotent till the development of the embryo is restored. Interestingly ESCs were first established from diapause embryos (Evans and Kaufman, 1981). It has been shown that development arrested embryos carrying mutation on the LIFβR and the gp130 receptors fail to restore normal embryogenesis (Nichols et al., 2001). These findings highlight the absolute requirement for LIF/gp130 signalling in the epiblast during diapause and give also an explanation why ESCs are LIF dependent. However this is a facultative situation, because the pathway is dispensable for early development without diapause.

2.1 JAK/STAT3 signalling and self-renewal

Binding of the cytokine LIF to the receptor results in conformational changes in the intracellular part of the receptor. Cytosolic tyrosine kinases of the JAK family are then recruited to the receptor. The activated receptor phosphorylates the tyrosine residues in the kinase molecule that become docking sites for the STAT3 transcription factors. When bound to the receptor, STAT3 molecules are phosphorylated on the tyrosine 705 (Tyr705) residues and dimerize with another phosphorylated STAT3. The dimers are then translocated to the nucleus in a regulated manner where they bind to promoter and enhancer regions of their target genes (Fig.1).

Active STAT3 is necessary and sufficient for maintaining pluripotency in ESCs (Cinelli et al., 2008; Matsuda et al., 1999; Niwa et al., 1998). Matsuda and colleagues used a chimeric STAT3-estrogen receptor (STAT3-ER) composed of the entire coding region of STAT3 and the ligand-binding domain of the estrogen receptor. Dimerization of the chimeric STAT3 was activated after treatment with the estrogen derived 4-hydroxy-tamoxifen (4-OHT). ESCs cultivated in presence of 4-OHT were able to self-renew in absence of LIF. In a recent study, by using the same STAT3 inducible system we were able to generate with high efficiency ESCs from the non-permissive FVB/N mouse strain (Cinelli et al., 2008). Wild type FVB/N ESCs derived in presence of LIF were not able to generate chimeras whereas cells derived from transgenic ICMs overexpressing STAT3-ER, in absence of LIF and in presence of 4-OHT, were able to generate germline competent ESCs (Cinelli et al., 2008).

The *Socs* genes (Suppressor of cytokine signalling) are well characterized STAT3 target genes, whose encoded proteins generally act in a negative feedback loop to suppress further STAT3 signalling (O'Sullivan et al., 2007) (Fig 1.). Although overexpression of STAT3 promotes stem cell self-renewal and maintenance of pluripotency in the absence of LIF and in presence of serum (Cinelli et al., 2008; Matsuda et al., 1999), inactivation of STAT3 in LIF-maintained ESCs promotes spontaneous differentiation (Niwa et al., 1998). Even though these lines of evidence establish STAT3 as an essential component of the LIF-dependent self-renewal in ESCs, the downstream target genes of activated STAT3 have remained elusive. In order to isolate these genes, several studies based on chromatin immunoprecipitation (ChIP) analysis or on microarray technology have been performed.

Recently Cartwright et al. indicated a role for the transcription factor c-MYC in self-renewal by functioning as a key target of LIF/STAT3 signalling (Cartwright et al., 2005). Like other genes involved in the maintenance of pluripotency, such as *Nanog* (Chambers et al., 2003), *Klf2*, (Hall et al., 2009), *Pem/Rhox5* (Cinelli et al., 2008; Fan et al., 1999) and *Pramel7* (Cinelli et al., 2008), constitutive expression of c-MYC renders self-renewal independent of LIF (Cartwright et al., 2005). On the other hand expression of a dominant negative form of c-MYC promotes differentiation (Cartwright et al., 2005). c-MYC is a transcription factor that controls many different biological processes, such as cell proliferation, growth, differentiation and apoptosis. Several studies have shown the importance of this gene in embryonic development, since homozygous deletion of *c-Myc* in the mouse results in embryonic lethality before E10.5 of gestation (Davis et al., 1993). Interestingly, overexpression or amplification of the *c-Myc* gene has been detected in numerous solid tumours and blood malignancies (Dang et al., 1999).

Cartwright and colleagues demonstrated by ChIP analysis that the *c-Myc* gene is a direct transcriptional target of the LIF/STAT3 pathway in ESCs and that the sole overexpression of c-MYC is sufficient to maintain self-renewal in absence of LIF (Cartwright et al., 2005). Not surprisingly, c-MYC is one of the four transcription factors found to be able, together with OCT3/4, KLF4, and SOX2, to reprogram somatic cells into undifferentiated, induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka, 2006).

In a recent microarray study we could identify a group of 26 hypothetical STAT3 target genes (Cinelli et al., 2008). In situ hybridization experiments indicated that the expression of four of the up-regulated genes was restricted to the ICM of the blastocysts, pointing to their potential role in maintaining pluripotency in ESCs. Overexpression in ESCs of two of these genes, namely the *Pem/Rhox5* homeobox-containing gene and the *Pramel7* gene, were shown to be sufficient to maintain expression of pluripotency markers in absence of LIF. These results suggest that these two genes could represent possible STAT3 transcriptional target genes, involved in self-renewal and maintenance of pluripotency in ESCs (Cinelli et al., 2008).

The fact that overexpression of the transcription factor Nanog does not increase significantly the level of phosphorylated STAT3, and *vice versa* the overexpression of STAT3 seems not to affect Nanog expression leads to the conclusions that Nanog is not a direct transcriptional target of STAT3, nor does it regulate STAT3 activity (Chambers et al., 2003). Moreover increased STAT3 activity maintains pluripotency even when Nanog expression is reduced (Bourillot et al., 2009), confirming that these two transcription factors are regulated through different signals. Nevertheless, in a recent study it was shown that 55% of the putative STAT3 target genes display binding sites for Nanog, and 41% of the putative Nanog target genes display binding sites for STAT3 (Chen et al., 2008). These results suggest that both

transcription factors co-regulate the expression of a large number of target genes, whose expression is involved in the maintenance of the undifferentiated state in ESCs. Bourillot et al. identified in a microarray study twenty-four STAT3 target genes which showed binding sites for STAT3 and/or Nanog (Bourillot et al., 2009). Knockdown experiments showed increased differentiation in LIF-supportive conditions, confirming that these STAT3 target genes contribute to the maintenance of the undifferentiated state (Bourillot et al., 2009). Among these there were some already identified as STAT3 targets, like *Pim-1* and *Pim-3* kinases (Aksoy et al., 2007) and two genes of the Krüppel-factors family, namely *Klf4* and *Klf5*. The *Klf* genes have been proposed as downstream targets of LIF/STAT3 also by other research groups (Hall et al., 2009; Li et al., 2005; Niwa et al., 2009). Recently Hall et al. showed that OCT3/4 in addition to the LIF/STAT3 signalling activates the Krüppel-factors KLF4 and KLF2 and that their overexpression reduces LIF dependence (Hall et al., 2009). However only KLF2 was able to sustain pluripotency in absence of either LIF or *Stat3*, and was shown to be OCT3/4 induced. KLF4 was shown to be selectively induced by LIF/STAT3 but was not sufficient in absence of the LIF/STAT3 signalling to sustain prolonged ESCs self-renewal. Interestingly, like c-MYC, also KLF4 is one of the four transcription factors that are able to reprogram somatic cells into undifferentiated, self-renewing cells (Takahashi and Yamanaka, 2006).

In the molecular mechanisms involved in the maintenance of pluripotency in ESCs extrinsic stimuli converge with intrinsic circuitries in a synergistic manner propagating the undifferentiated and self-renewing state in ESCs. STAT3 is an important regulator of mouse ESC self-renewal and it is known to inhibit differentiation into both mesoderm and endoderm lineages (Ying et al., 2003) by preventing the activation of lineage-specific differentiation programs. However its mechanisms of action remain to be better elucidated.

2.2 gp130-dependent activation of the MAPK/ERK pathway

Self-renewal and differentiation converge downstream from the LIFβR/gp130 receptor. The binding of the cytokine LIF to the heterodimeric receptor not only activates the JAK/STAT3 pathway but also the mitogen-activated protein kinase (MAPK) pathway that culminates in the activation of the extracellular signal-regulated kinases (ERK1/2). Because it has been shown that ERK regulates early differentiation *in vivo* and *in vitro* (Kunath et al., 2007; Nichols et al., 2009b), the balance between self-renewal and differentiation has to be maintained in order to preserve the undifferentiated state of ESCs.

Active gp130 receptor can also associate with the protein tyrosine phosphatase SHP-2 (Fukada et al., 1996), which acts as a positive effector of the MAPK signalling cascade. Interaction between active gp130 receptor and SHP-2 phosphatase induces the recruitment of GAB1. The complex formed by the gp130 receptor, SHP-2 and GAB1 proteins, through the activation of further kinases (RAS/RAF and MEK) results in the activation of the ERK1 and ERK2 kinases (Takahashi-Tezuka et al., 1998) (Fig.1).

Burdon et al. confirmed that after stimulation with LIF, ERK1 and ERK2 were activated through phosphorylation of SHP-2 (Burdon et al., 1999). Surprisingly they also showed that suppression of the SHP-2/ERK signalling was not affecting propagation of stem cells, but on the contrary it was enhancing ESCs self-renewal (Burdon et al., 1999). This study indicates that SHP-2/ERK signalling activation is a necessary component of the normal differentiation processes. Differentiation of ESCs into embryoid bodies *in vitro* was associated with an induction of expression of G1 cyclins, a lengthening of the G1 phase and a decrease in the rate of cell division (Savatier et al., 1996). As we mentioned before, ESCs typically exhibit a

short G1 phase of the cell cycle (Rohwedel et al., 1996) and high rate of cell divisions. Entrance into the G1 phase of the cell cycle is a prerogative for cell differentiation. ERK signalling is known to regulate proliferation and survival of somatic cells (Lloyd, 1998), and *in vivo* phosphorylated ERK has been detected from the 2-cell stage till the blastocyst stage (Wang et al., 2004). Incubation of 2-cell stage embryos with an ERK inhibitor results in a developmental arrest at the four-cell stage embryo, however, normal embryo development can be restored once the inhibitor is removed (Maekawa et al., 2007). This confirms that the ERK1/2 pathway is required for progression of early cell division cycles in the preimplantation embryo. In contrast to many mammalian cells, where ERK activity is essential for the cell cycle progression from G0/G1 to S phase (Lewis et al., 1998), during the development from the 2-cell to the 8-cell stage embryo, ERK signalling seems to be essential in the G2/M transition (Maekawa et al., 2007).

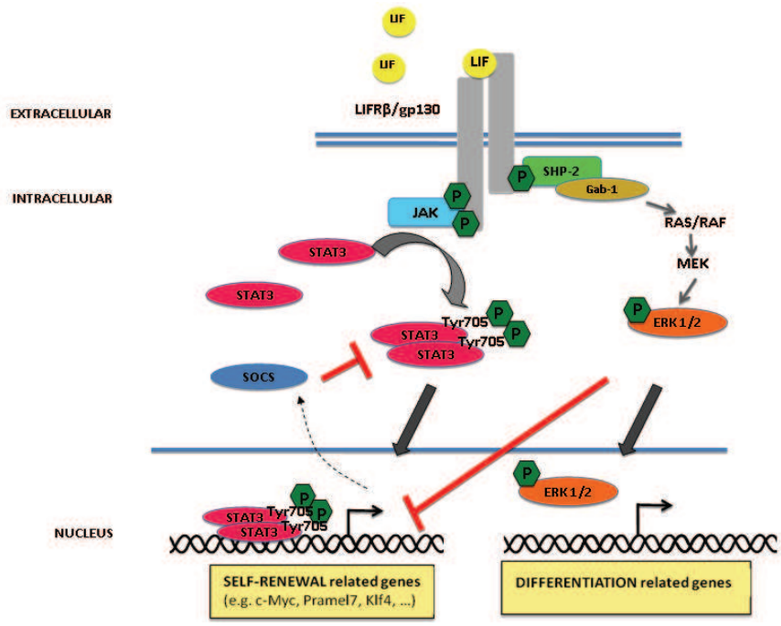


Fig. 1. gp130/LIF dependent STAT3 and MAPK/ERK signalling

Interestingly the ERK pathway may be able to inhibit the JAK/STAT3 pathway at the level of STAT3. It has been shown that ESCs knockout for *Shp2* phosphatase after LIF stimulation showed an increased phosphorylation of STAT3 when compared to the wild type cells (Chan et al., 2003). These data support the evidence that the two pathways seem to converge and thereby determine the choice between self-renewal and differentiation (Fig. 1).

Recently Ying et al. cultivated ESCs in a serum-free medium containing B27- and N2-supplement in presence of selective small-chemical inhibitors of the FGF receptor and the ERK kinase in combination with a GSK3 inhibitor, the so called 3i or 2i conditions (Ying et al., 2008). While the first two inhibitors are involved in selectively blocking differentiation signals induced by the ERK pathway, the third inhibitor is used for blocking the negative regulation on biosynthetic pathways driven by the GSK3 protein (Ying et al., 2008). This work indicates that by inhibiting differentiation-inducing signals from the MAPK pathway by 3i/2i it is possible to maintain self-renewal in absence of LIF/STAT3 stimulation. This was confirmed by the fact that under these conditions neither STAT3 nor SOCS3 activation

was detected. Moreover it was possible to establish *Stat3* null cells, which did not show morphologically differences when compared with the wild type cells. All the different cell lines established under the 3i conditions expressed the typical pluripotency markers like OCT3/4 and Nanog and were able to contribute to chimera formation and germ line transmission (Ying et al., 2008).

2.3 gp130-dependent activation of the PI3K signalling

There are three distinct classes of phosphatidylinositol-3 phosphate kinase (PI3K); members of the class I_A family of PI3Ks are activated via the LIF/gp130 receptor (Fig. 2.). The products of PI3K transmit the signals through downstream effectors including the serine/threonine protein kinase B (PKB, also known as AKT). AKT has been implicated in many cellular processes like regulation of the cell cycle progression, cell death, adhesion, migration, metabolism and tumorigenesis (for review see Brazil et al., 2004).

Initially it was shown that this pathway was implicated in the control of proliferation in ESCs (Jirmanova et al., 2002; Takahashi et al., 2003). Paling et al. demonstrated that LIF induced PI3K signal activation in ESCs is involved not only in the regulation of cell proliferation but also in their self-renewal (Paling et al., 2004). ESCs incubated with LIF and a small chemical PI3K inhibitor showed less alkaline phosphatase activity compared to the untreated control cells, indicating a reduced ability of LIF to promote self-renewal. However these cells did not show altered levels of phosphorylated STAT3 when compared to the control cells. This result led to the conclusion that *Stat3* is not a target of PI3K action. The loss of self-renewal and the consequently differentiation of the cells after inhibition of PI3K was explained by an increase in ERK phosphorylation upon LIF stimulation (Paling et al., 2004). These findings are consistent with other studies were it was reported PI3K playing a role in negatively regulating ERK activity in ESCs (Hallmann et al., 2003). Paling et al. demonstrated that self-renewal was restored after incubation with both ERK and PI3K inhibitor, therefore confirming that the regulation of ERK activity by PI3K signalling contributes to the determination of ESCs self-renewal.

The involvement of PI3K/AKT signalling in the regulation of stem cell systems has been also proposed from studies of conditional *Pten*-deficient mice. PTEN is a lipid phosphatase that antagonizes the physiological and pathological processes of PI3K/AKT pathway (Stiles et al., 2004). It was shown for example that self-renewal of neural stem cells was increased in brain-specific mutant mice lacking *Pten* gene (Groszer et al., 2001). It was therefore interesting to investigate if a constitutive active form of AKT was able to support self-renewal in ESCs. Watanabe and colleagues investigated this hypothesis by generating ESCs expressing a myristoylated, active form of AKT and they demonstrated that constitutive expression of AKT liberates ESCs from LIF-dependence. These cells showed no alteration in the level of phosphorylated STAT3 compared to the control cells, but interestingly showed an increased ERK phosphorylation (Watanabe et al., 2006). These findings are in contrast with the results from Paling et al. that observed an increased ERK phosphorylation upon PI3K inhibition thereby attributing to the PI3K signalling an involvement in controlling ERK phosphorylation and therefore blocking differentiation.

GSK3β (glycogen synthase kinase 3β) is a common target from the PI3K/AKT pathway and the WNT pathway, which phorphorylation leads to its inactivation (Fig. 4.). WNT blocks proteasome-mediated degradation of β-catenin through the inhibition of GSK3β (Moon et al., 2004). Watanabe et al. postulated an involvement of the β-catenin signalling in the AKT-mediated maintenance of the undifferentiated state. Although in the transgenic ESCs

expressing a constitutive active form of AKT, GSK3β phosphorylation was enhanced, β-catenin signalling was not activated (Watanabe et al., 2006). They concluded that AKT-dependent maintenance of the undifferentiatied state in ESCs was independent from the WNT/β-catenin signalling. Long term treatment of ESCs with the PI3K inhibitor LY294002 was shown to inhibit the proliferation and to induce cell cycle arrest at the G1 phase (Jirmanova et al., 2002). It is known that PI3K/AKT controls cell-cycle regulation: AKT promotes the G1 to S phase transition by facilitating the formation of cyclin/CDK complexes (Brazil et al., 2004). ESCs lack cell-cycle inhibitory mechanisms which are acquired only upon differentiation (Burdon et al., 2002). It is therefore possible that AKT-mediated maintenance of self-renewal in ESCs is due to its ability to block the cell-cycle inhibitory mechanisms and consequently block differentiation (Watanabe et al., 2006).

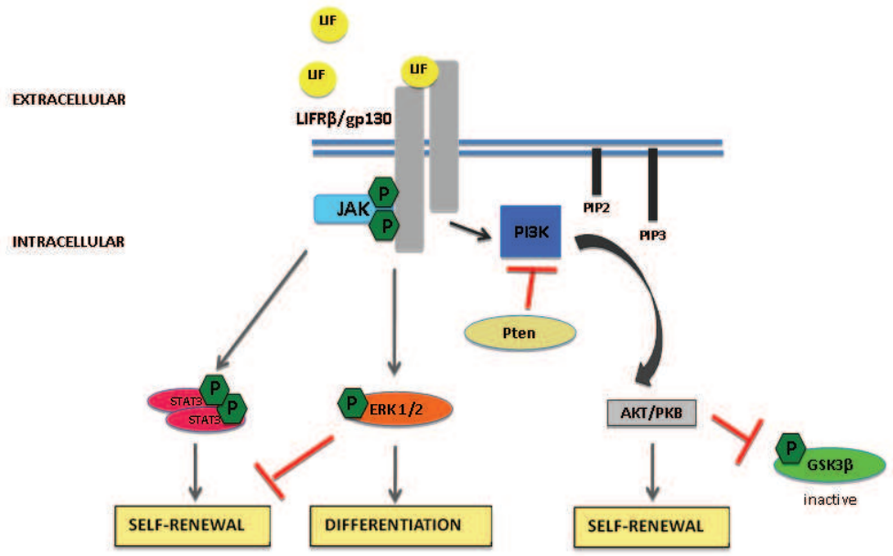


Fig. 2. gp130/LIF dependent PI3K/AKT signalling

As mentioned before GSK3 inhibition together with ERK inhibition promotes self-renewal in absence of LIF and serum. It can be assumed that both the ERK and the PI3K pathways are important for controlling self-renewal and differentiation in ESCs (Fig. 2.).

3. Signalling through the TGF-β pathway

Transforming Growth Factor β (TGF-β) signalling controls diverse sets of cellular processes, including cell proliferation, recognition, differentiation, apoptosis, and specification of developmental fate, during embryogenesis as well as in mature tissues. Moreover it has been implicated in the progression of many cancers, functioning both as an antiproliferative and as a tumor-promoting factor. The TGF-β family members bone morphogenetic proteins (BMPs), Nodal and Activin have been implicated in the development and maintenance of various stem cells, including ESCs.

BMP, Nodal and Activin act through the transmembrane type I and type II serine/threonine kinase receptors, leading to their dimerization. The activated receptor recruits SMAD molecules, which carry then the signal from the cell surface to the nucleus. SMAD molecules can be divided into three classes: the receptor-regulated SMAD (R- SMAD), the Co- SMAD,

and the inhibitory SMAD. The R- SMAD are transcription factors and the group is composed of the SMAD1, -2, -3, -5 and -8. They are activated through direct phosphorylation by the type I receptor and one of their functions is to translocate to the cell nucleus, where they form heteromeric complexes with SMAD4, the only member of the Co-SMAD. Formation of the complex in the nucleus induces association with many DNA binding partners, thereby regulating the transcription of target genes both positively and negatively. The members of the inhibitory SMADs are SMAD6 and SMAD7, they antagonize the TGF-β signalling by binding to the receptor-regulated SMAD and therefore limiting their ability to interact with Co-SMAD and form the transcriptionally active complex (Fig. 3.).
In the following paragraphs we will review the most important functions of the BMP and the Nodal/Activin pathways in regulating pluripotency in ESCs.

3.1 The BMP/SMAD pathway

It has been shown that in serum-free culture, LIF is insufficient to maintain the undifferentiated state of murine ESCs (Ying et al., 2003). On the other hand the overexpression of STAT3 alone is enough to sustain pluripotency in ESCs in a LIF-independent manner, however in presence of serum and feeders (Cinelli et al., 2008; Matsuda et al., 1999). These observations suggest that there must be other factors in the serum or produced by the feeders, which suppress differentiation and concomitantly, efficiently sustain self-renewal in ESCs. One of these signals was shown to be the bone morphogenetic proteins (BMPs). BMPs bind to the Activin receptor-like kinases (ALKs) ALK2, ALK3, and ALK6, and activate the Inhibitor of differentiation (*Id*) genes through the activation of the receptor-regulated SMAD2, SMAD5 and SMAD8 (Fig. 3.) (Ying et al., 2003).

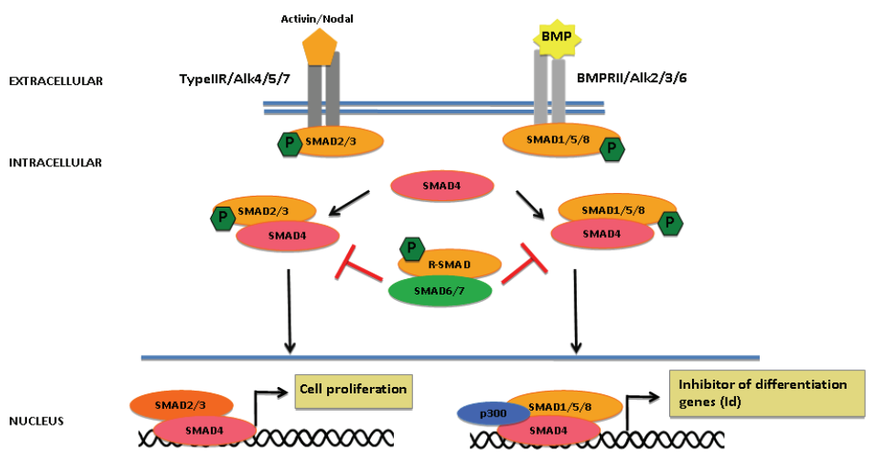


Fig. 3. Signalling through the TGF-β pathway: Nodal, Activin and BMP

In a serum-free culture, ESCs differentiate into neural precursors under the influence of autocrine FGF signal even in presence of LIF (Ying and Smith, 2003) indicating that other signals are required for suppressing neural differentiation (Ying et al., 2003). The combination of LIF and BMP4 or BMP2 was found to be sufficient to maintain the undifferentiated state of ESCs in serum-free medium (Ying et al., 2003). However, withdrawal of LIF and retention of BMP causes differentiation into epithelial-like cells, leading to the conclusion that the self-

renewal response to BMP is dependent on continuous LIF signalling and that the BMP main function is therefore to antagonize the neural differentiation induced by LIF in absence of serum (Ying and Smith, 2003). This was corroborated by the fact that ID overexpression in ESCs enables serum-free self-renewal in the sole presence of LIF. Upon LIF withdrawal, ID overexpressing cells differentiated into non-neuronal precursors, therefore demonstrating that these genes contribute to the ESCs self-renewal by complementing the blockade of other lineages induced by STAT3. Interestingly, overexpression of Nanog rendered BMP or serum requirement dispensable. These findings were partially explained by a constitutively high *Id* expression in these cells, which probably allowed bypassing the BMP signalling thus maintaining ESCs undifferentiated (Ying and Smith, 2003). However these cells were induced to differentiate upon BMP stimulation, indicating that Nanog cannot inhibit in an unlimited manner SMAD-induced differentiation pathways.

These findings clearly indicate that the cooperation between LIF/STAT3 and BMP/SMAD pathway is required for maintaining ESCs in serum-free media. Nevertheless the balance has to be critically regulated, since depletion of the LIF/STAT3 signalling induces BMP-dependent promotion of differentiation and overexpression of SMAD1/4 overrides the effect of LIF and causes non-neural differentiation (Ying and Smith, 2003). Recapitulating, high expression of BMP leads to differentiation into mesoderm and endoderm lineages, whereas neural differentiation is suppressed. On the other side, low level of BMP also induces differentiation into mesoderm. Therefore, support of pluripotency by BMP pathway is highly dose-dependent and needs to be counter-regulated by STAT3 signalling.

However, STAT3 is not the only factor that interacts with BMP, Nanog was also proven to bind to SMAD1 thereby suppressing the formation of the active complex with SMAD4 (Suzuki et al., 2006b). Nanog blocks BMP-induced mesoderm differentiation by binding to SMAD1 and therefore interfering with the recruitment of the co-activator and blocking the transcription of downstream targets responsible for BMP-induced mesodermal specification (Suzuki et al., 2006b). The Nanog promoter contains predicted binding-sites for both STAT3 and Brachyury T, an early mesoderm marker. ESCs transfection with a luciferase construct containing the regulatory sequence, which included both STAT3- and Brachyury T-binding sites, resulted in LIF concentration dependent increase in transcription. The enhancer activity of this region was lost when one or the other binding-site on the Nanog promoter was mutated. Immunoprecipitation assays confirmed then the association of Brachyury T and STAT3, interestingly only after stimulation of the cells with LIF. These results demonstrated that through a cooperative action STAT3 and Brachyury T induced Nanog expression in cells that are initiating to differentiate into mesodermal lineage (Suzuki et al., 2006b).

The p300 protein is an important nuclear factor in the BMP pathway. It interacts with the active R-SMAD and SMAD4 and regulates gene transcription (Fig. 3.). Nanog was found to interfere with the recruitment of the co-activator p300 to the SMAD complex, therefore negatively regulating BMP signalling. Suzuki et al. demonstrated that Nanog inhibits Brachyury T expression by inhibiting the formation of active SMAD/p300 complexes, and therefore maintaining the undifferentiated state of ESCs (Suzuki et al., 2006b). This study, in contrast to the results shown by Ying et al., links BMP signalling more to the differentiation processes than to the maintenance of pluripotency. Ying et al. worked with low concentration of BMP and in presence of LIF; these conditions maintain pluripotency and inhibit mesoderm differentiation (Ying et al., 2003) whereas Suzuki et al. demonstrated with their studies that this mesoderm-inhibition is in part due to a negative effect mediated by Nanog and Brachyury T (Suzuki et al., 2006b).

BMP proteins can bind to the type I ALK3 receptor and to the type II BMPRII receptor (Fig. 3.). Different studies showed that *in vivo* embryos lacking one of these two receptors die because they are unable to undergo gastrulation (Beppu et al., 2000; Mishina et al., 1995), highlighting the essential role of BMP during embryogenesis. The impossibility of generating embryos carrying these mutations leads to the lack of the respective ESCs with the only exception of *Smad4* knockout embryos which die before embryonic day 7.5 and therefore allow the isolation of blastocyst stage embryos and the establishment of viable ESCs (Sirard et al., 1998). SMAD4 as mentioned before is the co-activator molecule, which binds to R-SMADs for forming the active transcriptional complex. These results suggest that there is a SMAD-independent mode of BMP action in the embryos but also in the ESCs. *Alk3* receptor null blastocysts are morphologically identical to the wild type blastocysts; however ICMs of the knockout embryos fail to expand once in culture. By incubating *Alk3* knockout ESCs with an inhibitor for ERK and p38 MAPK pathways Qi and colleagues established and expanded these cells *in vitro* in absence of BMP4. They concluded that BMP4 supports self-renewal by inhibiting the mitogen-activated protein kinase MAPK/ERK pathway (Qi et al., 2004). Anyway the mediators of this inhibition have still to be identified. A more recent study suggests that in the BMP4-induced maintenance of the undifferentiated ESCs status, together with the SMAD proteins, also the PI3K/AKT and the WNT pathway are involved (Lee et al., 2009).
All together these data confirm the complexity of the BMP signalling, where depending on its expression level, presence of active interaction partners in different pathways, and timing of expression during embryogenesis; it strongly influences cell fate decisions both i*n vivo* and *in vitro*.

3.2 The Nodal/Activin pathway

Nodal and Activin ligands bind to the type I and II Activin receptor-like kinases ALKs (ALK4, -5, and -7), which phosphorylate SMAD proteins to regulate gene expression. Nodal/Activin signals are received by the transcription factors SMAD2 and SMAD3, which form complexes with the Co-SMAD4 and are then translocated to the nucleus where they associate with other cofactors to regulate target gene transcription (Fig. 3.). Nodal/Activin signalling is antagonized by Lefty, an extracellular molecule or by the classical TGF-β inhibitor SMAD7. *In vivo* this pathway has been shown to be essential for the induction of mesoderm and endoderm lineages and for the determination of the left-right axis during embryogenesis. *Nodal* knockout show a reduced epiblast cell population that display very low expression of the pluripotency marker OCT3/4 and arrest the development before gastrulation (Robertson et al., 2003).
Nodal expression in mouse ESCs is high, and was found to build an active signalling together with SMAD2. Thus, stimulation of ESCs with Activin or Nodal leads to an increase in SMAD2 phosphorylation and a higher ESCs proliferation, whereas inhibition of SMAD2 activation reduces cell proliferation (Ogawa et al., 2007).
In serum-containing medium, both BMP and Nodal/Activin pathways are autonomously activated in ESCs. Ogawa and colleagues showed that after overexpression of the inhibitors SMAD6 and SMAD7, ESCs proliferation significantly decreased, the effect in SMAD7 transfected ESCs being much more dramatic. It is known that SMAD6 predominantly inhibits BMP-mediated signalling, whereas SMAD7 blocks both BMP and Nodal/Activin pathways. The SMAD7-dependent inhibition of cell proliferation was reversible after excision of the transgene; moreover this was not affecting ESCs pluripotency since injection

of the SMAD7-reverted cells produced live chimeras (Ogawa et al., 2007). In serum-free medium the SMAD7 induced blockade of proliferation is more reduced, leading to the conclusion that soluble TGF-β-related molecules in the serum are also involved in the SMAD7-dependent growth inhibition (Ogawa et al., 2007). In fact, addition of exogenous Activin or Nodal to the serum-free medium caused SMAD2 activation and restored cell proliferation; this was not observed when soluble BMP4 was added to the medium. These results suggested that Nodal and Activin promoted ESCs proliferation through the canonical pathway. Further analyses demonstrated that ESCs autonomously activate Nodal/Activin signalling by producing these ligands in serum-free conditions; in presence of serum the soluble TGF-β-related molecules might increase the endogenous Nodal/Activin activity leading to an enhanced cell proliferation (Ogawa et al., 2007).

Another recent work showed that Nodal signalling acts also through the BMP pathway for regulating self-renewal in ESCs (Galvin et al., 2010). In this study it was observed that treatment of the cells with a chemical SMAD2 inhibitor resulted in an enhanced BMP signalling. The authors found SMAD7 to be the critical component of the Nodal pathway that influences BMP signalling through the regulation of *Id* genes (Galvin et al., 2010).

Although many efforts have been made in order to elucidate the function of Nodal/Activin in mouse ESCs, future work will be needed to determine the molecular mechanisms by which this pathway maintains pluripotency. If the involvement of the Nodal/Activin pathway in maintaining mouse ESCs has still not been completely elucidated, this is not the case for human ESCs (hESCs). In this article we review the molecular mechanisms of pluripotency in ESCs generated from the mouse, being murine ESCs the most investigated cells. Nevertheless it is to mention that the Nodal/Activin pathway plays a fundamental role in the maintenance of pluripotency in hESCs. Like mouse ESCs, hESCs do express all the components of the LIF/STAT3 pathway but in contrast to the mouse cells, cannot be maintained pluripotent in presence of LIF (Humphrey et al., 2004). This indicates that signalling through this pathway is insufficient to prevent differentiation of hESCs and suggests the existence of other pathways involved in the regulation of pluripotency in hESCs. Interestingly, mouse epiblast stem cells derived from the E5.5-E6.5 post-implantation embryos, can also not be maintained undifferentiated in presence of LIF or BMP4. Like hESCs, mouse epiblast stem cells require FGF4 and Nodal/Activin signalling for self-renewal (Brons et al., 2007; Tesar et al., 2007).

In conclusion, even though all the components of the Nodal/Activin pathway are highly expressed in both hESCs and mouse ESCs, the outcome of these signalling is different in the two species. More studies are needed for a better determination of the target genes activated by the Nodal/Activin pathway; nevertheless it is clear that the TGF-β signalling is a critical regulator of stem cell functions.

4. The canonical WNT pathway

The WNT pathway plays crucial roles in controlling genetic programs of embryonic development and adult homeostasis. WNT signals are transduced depending on their functions through different receptors and members: The canonical WNT pathway is known to be involved in transmitting signals for cell fate determination, whereas the non-canonical WNT pathway is involved in controlling cell movements and tissue polarity. In the context of maintaining pluripotency in ESCs, we will focus in the next paragraph only on the canonical pathway.

Canonical WNT signalling starts when the extracellular WNT ligand binds members of the Frizzled and LDL receptor family. The main player of the cascade is the cytoplasmic protein β-catenin. When the WNT ligand activates the pathway, β-catenin translocates to the nucleus where it interacts with other members of the signalling pathway for activating target genes. The tumour suppressor adenomatous polyposis coli (APC) and AXIN are two components of the cytosolic destruction complex responsible for β-catenin degradation in absence of WNT ligand. Casein kinase Iα (CKIα) and glycogen synthase kinase 3β (GSK3β) kinases are also part of the destruction complex and specifically phosphorylate β-catenin (Fig. 4.). This is the signal for ubiquitin ligases, which target β-catenin for proteasomal degradation: Therefore in absence of ligands β-catenin is constantly degraded. On the contrary, in presence of WNT ligands, the membrane receptor LRP5/6 is phosphorylated and bound by Dishevelled (Dsh) and AXIN, which cannot form the destruction complex therefore leading to an accumulation of cytosolic β-catenin. Free β-catenin can enter the nucleus where it binds to the T-cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors (Fig. 4.). The β-catenin-TCF/LEF complex can then activate the transcription of WNT target genes. In absence of β-catenin the TCF/LEF factors are associated with histone deacetylase 1 (HDAC1), forming a complex that promotes gene expression silencing (Kioussi et al., 2002). Once β-catenin is again present it replaces HDAC1 promoting gene expression.

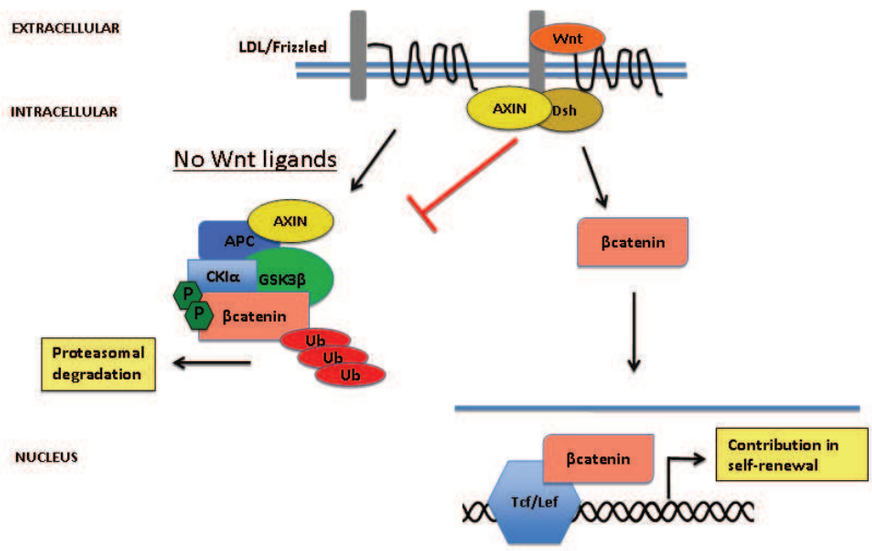


Fig. 4. The canonical WNT pathway

Several publications support the role of the WNT/β-catenin pathway in maintaining pluripotency in ESCs, although the precise mode of action needs still to be clarified. A potential source of WNT ligands is represented by the feeders used for the cultivation of ESCs. A functional screening of different sub-lines of STO feeders, which showed variable ability in maintaining ESCs self-renewal, identified WNT5A and WNT6 ligands to be the determinant factors. Their overexpression in STO feeders is sufficient to maintain ESCs undifferentiated in serum-containing medium and in absence of LIF (Hao et al., 2006). But also WNT3 and WNT3A are able to prevent ESCs differentiation under the same conditions; an effect that can be neutralized by WNT inhibitor. Moreover, ESCs conditionally

expressing a constitutive active form of β-catenin were able to maintain the undifferentiated state in a LIF-independent manner (Hao et al., 2006). This study demonstrated that the WNT-mediated maintenance of pluripotency was depending on β-catenin stabilization. Moreover constitutive activation β-catenin induced an upregulation of STAT3 mRNA and protein. In serum-free medium the WNT pathway is not sufficient for sustaining ESCs self-renewal, however if 10 U/ml of LIF are added to the medium, ESCs form compact colonies and show OCT3/4 expression suggesting a synergistic effect of both pathways; the WNT pathway effectively upregulating STAT3 mRNA and the LIF pathway phosphorylating the protein and finally activating STAT3 target gene expression (Hao et al., 2006).
Nevertheless cultivation of ESCs in a feeder- and serum-independent manner with the sole presence of WNT3A conditioned medium is also not sufficient for maintaining ESCs undifferentiated; this is only possible in combination with LIF (10U/ml). The same holds true when ESCs constitutively express an active form of β-catenin (Ogawa et al., 2006).
In *β-catenin* knockout ESCs the expression of the core pluripotency factors Nanog, OCT3/4 and SOX2 is still present indicating that β-catenin regulates the expression of several stemness genes, but is not directly required for maintenance of pluripotency (Anton et al., 2007). Undifferentiated ESCs were shown to accumulate β-catenin in the nucleus (Naito et al., 2006; Takao et al., 2007). It was therefore investigated if the β-catenin-mediated expression of pluripotency factors was controlled through the interaction with TCF/LEF transcription factors. The TCF/β-catenin-mediated gene regulation in ESCs was measured by comparing endogenous TOPFlash activity with a FOPFlash reporter. Interestingly a very minimal TCF/β-catenin activity was detected with this method, indicating that probably β-catenin might interact with factors other than TCF for inducing pluripotency-related gene expression (Anton et al., 2007). Actually other reports demonstrated the interaction of β-catenin with well-known pluripotency factors, such as KLF4, SOX2, and OCT3/4. The expression level of β-catenin was found not to be regulated through the LIF/STAT3 pathway, since LIF depletion for 4 days did not change the mRNA and protein level of β-catenin (Takao et al., 2007). However in the same cells, the amount of β-catenin found exclusively in the nuclei was reduced when compared to the one of ESCs cultivated in presence of LIF. Also by forced expression of an active form of β-catenin, the same authors could cultivate ESCs in absence of LIF. Interestingly, they found Nanog and OCT3/4 to be upregulated in the β-catenin transgenic ESCs compared to the wild type. On the other hand they noticed that after withdrawal of LIF for 3 and 6 days, Nanog and β-catenin expression disappeared at day 3 whereas OCT3/4 expression was decreasing at day 6. These observations suggested the possibility that Nanog could be a potential target of β-catenin (Takao et al., 2007). By luciferase assay *Nanog* promoter activity was examined in ESCs carrying the constitutive active form of β-catenin. In presence of LIF these cells showed higher promoter activity compared to the wild type cells. Furthermore, upon LIF withdrawal the *Nanog* promoter was shown to be still highly active, whereas wild type cells lost its expression and differentiated. Nanog mRNA level correlated with the promoter activity, leading to the hypothesis that *Nanog* promoter contains a β-catenin-responsive element (Takao et al., 2007). Moreover, it was previously shown that the *Nanog* promoter contains OCT3/4-SOX2 binding sites at the promoter region (-322/+50) (Kuroda et al., 2005; Rodda et al., 2005). Mutations in this promoter region in ESCs expressing the active form of β-catenin showed reduced *Nanog* reporter activity in both conditions, with and without LIF, suggesting a reduced β-catenin transcriptional activity. The presence of OCT3/4-SOX2

binding region on the *Nanog* promoter is indispensable for the β-catenin-mediated upregulation of Nanog. These results aroused the question if β-catenin could physically interact with OCT3/4. Pull-down assay confirmed this hypothesis when OCT3/4 was found co-precipitated with β-catenin (Takao et al., 2007). The discrepancy in the results between the Takao and the Ogawa work could be due to the use of different cell lines.
We can conclude that the WNT/β-catenin signalling helps in the maintenance of pluripotency, nevertheless β-catenin alone is not sufficient to maintain the cells in a completely undifferentiated state.

5. Transcription factors that regulate pluripotency in ESCs

A critical role in maintaining ESC identity is played by a set of transcription factors centred on the octamer binding protein 3/4 (OCT3/4) (Nichols et al., 1998; Schöler et al., 1989), the SRY-related HMG-box gene 2 (SOX2) (Yuan et al., 1995), and Nanog (Chambers et al., 2003; Mitsui et al., 2003). Genome-wide studies have highlighted the co-localization of these three transcription factors in ESCs chromatin increasing the complexity of the transcriptional networks that direct ESC identity. The following paragraphs review the current knowledge on the molecular functions and regulation of the transcription factors OCT3/4, SOX2, and Nanog.

5.1 The transcription factor OCT3/4

OCT3/4 is a member of the POU transcription factor family; it recognizes an 8-base pairs DNA sequence found in the promoters and enhancer regions of many ubiquitously expressed and cell-specific genes (Ruvkun and Finney, 1991). The gene encoding OCT3/4 is named *Pou5f1*. OCT3/4 was first identified as an active binding factor in the extract of undifferentiated embryonic stem and embryonal carcinoma cells (Lenardo et al., 1989; Schöler et al., 1989). The presence of OCT3/4 protein in ESCs and embryonal carcinoma cells first suggested an association with the early stage of mouse embryogenesis. During the mouse development, it is first detected in oocytes and its expression declines during the first two embryonic divisions, but it reappears at the 4-8 cell-stage, where it is expressed in all the nuclei. Subsequently it is reduced in the trophectoderm and becomes restricted to the ICM. In the post-implantation embryos, OCT3/4 is localized in the epiblast but disappears as cells undergo differentiation, with expression persisting in the germ cells (Palmieri et al., 1994). The importance of OCT3/4 during early embryogenesis was also highlighted by the fact that embryos lacking the *Pou5f1* gene die after implantation (Nichols et al., 1998) due to the absence of the ICM. Using a conditional expression and repression system in ESCs, the requirement of OCT3/4 in the maintenance of developmental potency was determined (Niwa et al., 2000). A critical amount of OCT3/4 is needed to sustain self-renewal, and up- or downregulation induces differentiation of the ESCs. Overexpression of this factor promotes differentiation into primitive endoderm and mesoderm, whereas repression of it causes loss of pluripotency and differentiation into trophectoderm (Fig. 5.). Effectively, *in vivo* OCT3/4 is abundant in the ICM cells of the blastocyst and down-regulated in the trophectoderm, whereas in the primitive endoderm the level of expression increases (Palmieri et al., 1994). These findings highlight the fundamental function of OCT3/4 in preventing dedifferentiation of epiblast cells or of ESCs into trophectoderm lineage.
Exogenous expression only of OCT3/4 is sufficient to generate pluripotent stem cells from mouse neural stem cells (Kim et al., 2009) and together with three other transcription factors it is essential in the reprogramming of somatic cells into the pluripotent state (Nakagawa et

al., 2008; Takahashi and Yamanaka, 2006). While the other factors involved in reprogramming are replaceable by family members or other factors, without OCT3/4 no reprogramming occurs (Nakagawa et al., 2008). Moreover knockdown experiments in ESCs showed a very dramatic change in gene expression compared to the one caused for example by Nanog or SOX2 knockdown (Ivanova et al., 2006) implying OCT3/4 as a chief selector for ESC fate decision.

LIF/STAT3 signalling does not directly support ESCs renewal by maintaining OCT3/4 expression, but it has been shown that STAT3 and OCT3/4 share some target genes involved in maintaining pluripotency. It is the case for the embryonic ectoderm development (*Eed*) gene, which was shown to be a common downstream target of both transcription factors (Ura et al., 2008). EED is a major component of the Polycomb repressive complex 2 (PRC2), which is involved in the methylation of lysine 27 on the histone H3 (H3K27). As we will describe later epigenetic mechanisms, like dynamical modification of the chromatin, play a fundamental role in maintaining pluripotency and controlling differentiation. EED function is to silence the expression of differentiation-associated genes in self-renewing ESCs and its downregulation induces differentiation (Boyer et al., 2006). Expression of a dominant negative form of STAT3 was shown to induce downregulation of EED, whereas overexpression of STAT3 caused an upregulation. Similar results were found when OCT3/4 was downregulated, suggesting *Eed* to be a common downstream target of both transcription factors (Ura et al., 2008). Further analyses confirmed that STAT3 and OCT3/4 directly bind the promoter region of the *Eed* gene, regulating its expression and thereby inducing silencing of differentiation-associated genes.

Ura et al. found that self-renewal correlates with the presence of high concentration of methylated H3K27 that leads to the transcriptional repression of the gene carrying this signal. Elimination of STAT3 and OCT3/4 causes a reduction of this methylation on the promoter of differentiation marker genes. These results suggested that EED is involved in STAT3- and OCT3/4-dependent gene silencing of differentiation markers and highlight another important function of OCT3/4 transcription factor in maintaining and controlling pluripotency in ESCs.

Of the known OCT3/4 target genes, four have been shown to be essential for the maintenance of pluripotency: *Sox2*, the undifferentiated transcription factor 1 (*Utf1*), *Rex1/Zfp42* and *Nanog*. The regulatory regions of these genes contain an octamer element capable of binding OCT3/4. As we will describe in the following paragraphs, *Sox2* is not only an OCT3/4 target gene but also serves as a cofactor for OCT3/4 (Okumura-Nakanishi et al., 2005). Although OCT3/4 and SOX2 have independent roles in determining other cell types, in pluripotent cells they act synergistically to drive transcription of their target genes. Furthermore, this complex was found to promote transcription of the Nanog homeoprotein (Rodda et al., 2005) (Fig. 5.).

5.2 The transcription factor SOX2

SOX2 is a member of the sex-determining region of the Y chromosome-related (SRY-related) high-mobility group (HMG) box (SOX) family of transcription factors. SOX2 has an expression pattern similar to that of OCT3/4 through the mouse preimplantation development. SOX2 expression is also associated with precursor cells of the developing central nervous system and indeed can be used to isolate these cells. Like OCT3/4, downregulation of SOX2 correlates with a commitment to differentiation and is no longer expressed in cell types with restricted developmental potential. The phenotypic lethal

consequences of the absence of SOX2 expression in the embryo are in contrast to OCT3/4, visible only after implantation (Avilion et al., 2003). This is due to an accumulation of maternal SOX2 in the cytoplasm of the oocytes, which persist in all cells at least until the blastocyst stage, and not like OCT3/4 maternal transcripts, which last only till the 2-cell stage embryo (Palmieri et al., 1994). Thus, mutant embryos lacking the *Sox2* gene die presumably when the maternal SOX2 becomes diluted causing the differentiation of the epiblast cells into trophectoderm or extraembryonic ectoderm (Avilion et al., 2003) (Fig. 5.).

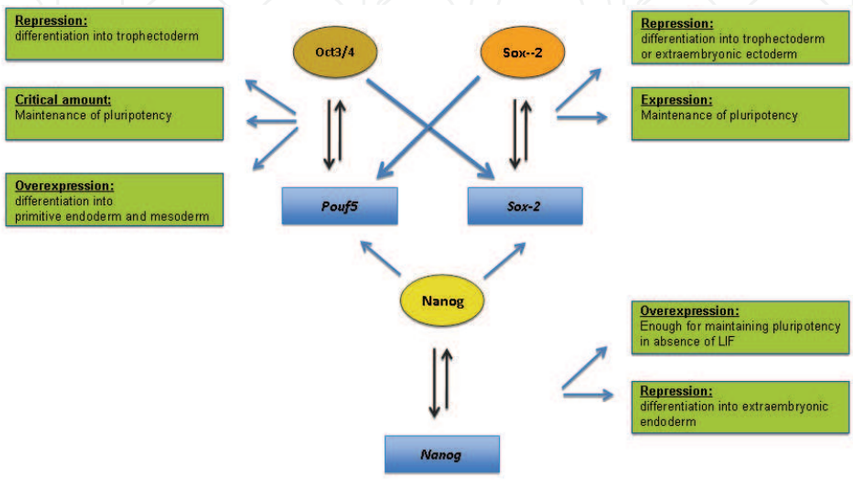


Fig. 5. OCT3/4, SOX2, Nanog core transcription factors: regulatory circuitry

As mentioned before, loss of OCT3/4 causes differentiation of the epiblast cells into trophectoderm derivatives (Niwa et al., 2000). Avilion et al. proposed that the presence of both transcription factors is required for the formation and maintenance of epiblast cells. An upregulation of OCT3/4 and the accompanying downregulation of SOX2 lead to the extraembryonic ectoderm differentiation.

We know that the regulatory regions of the *Sox2* gene contain an octamer element capable of binding OCT3/4 (Tomioka et al., 2002; Yuan et al., 1995). A possible interaction of these two factors was also proposed by the fact that almost all the SOX2-OCT3/4 target genes have both the octamer and sox heptamer elements separated by either 0 or 3 base pairs (Reményi et al., 2003; Williams et al., 2004). The *Pou5f1* gene has different regulatory regions that are important for its expression. The distal enhancer, which contains the conserved region CR4, was shown to be required for ESC-specific OCT3/4 expression (Nordhoff et al., 2001). Two regulatory regions (the SRR1 and SRR2), known to confer ESC-specific expression, were also found in the *Sox2* sequence. Using a ChIP assay Chew et al. first demonstrated that SOX2 and OCT3/4 interact with the enhancers of *Pou5f1* and *Sox2* genes. Moreover OCT3/4 and SOX2 knockdown experiments showed a reduced enhancer activity, confirming that both factors positively control their reciprocal expression (Chew et al., 2005). The same authors hypothesized a transcriptional regulatory network consisting of auto-regulatory and multi-component loops. In an auto-regulatory system the gene product binds to its own regulatory element allowing its continued and stable expression. In a multi-component system, the OCT3/4 factor binds to the *Sox2* regulatory element and *vice versa* generating a bi-stable system with the possibility to switch between the two different states (Chew et al., 2005) (Fig. 5.).

We described before that *Sox2* null embryo die after implantation, because of differentiation of the epiblast cells. The same findings are reproducible also *in vitro*, where ESCs lacking *Sox2* gene differentiate primarily into trophectoderm-like cells. Thus, SOX2 was defined to be indispensable for maintaining ESCs pluripotency. However, the transcription of many OCT3/4-SOX2 target genes was not affected in ESCs null for *Sox2*. These findings suggested that SOX2 regulates the expression of OCT3/4 through the regulation of multiple transcription factors (Masui et al., 2007). So, it seems that the main contribution of SOX2 in ESCs is to maintain OCT3/4 expression. Consistent with this idea is the finding that enforced expression of OCT3/4 can rescue ESCs from differentiation induced by the loss of *Sox2* (Masui et al., 2007). Not to forget is also that SOX2 is one of the four transcription factors, which together are able to induce reprogramming to pluripotency in differentiated cells (Takahashi and Yamanaka, 2006). Furthermore, large-scale ChIP studies have shown the OCT3/4-SOX2 complex closely localized to another important regulator of pluripotency, the Nanog transcription factor.

5.3 The transcription factor Nanog

Nanog is a homeodomain protein, which acts as an intrinsic effector of ESCs self-renewal. Nanog was detected in ESCs, embryonic carcinoma cells, and in the embryonic germ cells. During ESCs differentiation, Nanog mRNA declines markedly, and its expression is retained only in undifferentiated cells. In the mouse embryo its first expression appears in the compacted morula and it is localized to the interior cells, the future ICM. In blastocysts, the expression is confined to the ICM and absent from the trophectoderm. In the later blastocyst, Nanog is further restricted to the epiblast and excluded from the primitive endoderm; it is then down regulated after implantation (Chambers et al., 2003; Mitsui et al., 2003). At day E11.5-E12.5 Nanog expression marks the pluripotent germ cells.

Overexpression of Nanog in ESCs was found to allow proliferation of undifferentiated ESCs in absence of LIF and as well as in the presence of the LIF-antagonist hLIF-05, which blocks the activity of all known LIFR ligands (Chambers et al., 2003). After transfection with Cre-recombinase, in which the *Nanog* expression cassette had been eliminated, these cells reverted to LIF-dependence, demonstrating that this phenotype was directly attributable to Nanog overexpression. Following exposure to a differentiation-promoting agent, like retinoic acid, cells overexpressing Nanog remained, in contrast to the Cre-reverted cells, morphologically undifferentiated and expressed OCT3/4. Cre-reverted cells were injected into mouse blastocysts and contributed to the generation of germline competent chimeras. Even though cells overexpressing Nanog self-renew in a cytokine independent manner, the presence of LIF confers to these cells an enhanced self-renewing capacity (Chambers et al., 2003).

A reduction in the level of Nanog causes ESCs to differentiate to extraembryonic endoderm lineages (Chambers et al., 2007; Ivanova et al., 2006) (Fig. 5.). *In vivo* the absence of *Nanog* during embryo development results in early lethality. At developmental day E5.5 the mutant embryos showed disorganized extraembryonic tissues with no discernible epiblast or extraembryonic ectoderm. ICM of *Nanog* null blastocysts failed to proliferate and differentiated into parietal endoderm-like cells, demonstrating that its expression is essential for maintenance of pluripotency of the ICM cells (Mitsui et al., 2003). Interestingly, ESCs upon targeted deletion of the *Nanog* gene can self-renew indefinitely and can contribute to the generation of chimeric animals (Chambers et al., 2007). This indicates that loss of *Nanog* in ESCs does not affect pluripotency once this was established. Nevertheless, even though

Nanog null cells colonize the germ layers of the chimeric mice and they are recruited to the germ line, primordial germ cells lacking *Nanog* fail to mature on reaching the genital ridge (Chambers et al., 2007). These data confirm that Nanog is specifically required for the formation of germ cells.

The fact that STAT3 activation in presence of LIF was not increased in cells overexpressing Nanog (Mitsui et al., 2003) and the fact that these cells did not differentiate in presence of a JAK/STAT3 inhibitor (Chambers et al., 2003), clearly demonstrate the existence of a parallel and LIF/STAT3-independent mechanism sustaining pluripotency in ESCs. *In vivo* these findings are confirmed by the fact that Nanog is absolutely required for epiblast formation, while STAT3 is dispensable. Further analysis showed also that Nanog expression was not altered after incubation of the cells with a MEK inhibitor, indicating that Nanog-dependent maintenance of pluripotency was not due to a repression of the MEK/ERK pathway (Chambers et al., 2003).

Several studies showed that Nanog and OCT3/4 factors overlap substantially in their target genes, causing sometimes, also by cooperation, the activation or suppression of the expression of target genes. Mapping of the binding sites of OCT3/4 and Nanog in the mouse ESC genome showed that a substantial proportion of the genes were occupied by both factors, in some cases Nanog-OCT3/4 were found to co-localize, in other cases they bound indepentently (Loh et al., 2006). Screening for the presence of OCT3/4 and Nanog binding sites after differentiation revealed an enrichment of OCT3/4 or Nanog-bound genes that were induced and repressed upon differentiation. The majority of these genes were repressed and the ones that exhibited the strongest downregulation were bound by both factors (Loh et al., 2006). In the same study it could be shown that the OCT3/4-regulated target genes predominantly repress trophectoderm markers whereas Nanog activates the transcription of *Pou5f1* and *Sox2* evidencing a possible role of Nanog in controlling the levels of both OCT3/4 and SOX2. These factors in turn control the downstream genes involved in the maintenance of pluripotency or inhibition of differentiation (Loh et al., 2006). Interestingly promoter-sequence analyses showed the presence of a Sox-Oct element on the *Nanog* promoter. Two different groups demonstrated that both OCT3/4 and SOX2 can bind to the *Nanog* promoter therefore driving its transcription (Kuroda et al., 2005; Rodda et al., 2005).

ESCs under conventional culture conditions (namely with serum and LIF) are in a dynamic state, that fluctuates between a stable state, in which Nanog expression is high, and an unstable state, where Nanog expression levels are low (Kalmar et al., 2009; Singh et al., 2007). As a consequence ESCs form a heterogeneous population where pluripotent ESCs exhibit a highly variegated gene expression pattern. When induced to differentiate only ESCs with low levels of Nanog expression are able to commit in a stable manner (Chambers et al., 2003). This hypothesis was confirmed by a study where the two different populations were sorted and exposed to differentiation conditions for 3 days (Kalmar et al., 2009). The low-Nanog cells did not change their Nanog expression level, but lost their ability to form colonies and showed neuronal differentiation. On the contrary only a very small part of the high-Nanog cells differentiated and showed reduced Nanog expression. Nevertheless, it seems that both cell types are able to switch from one to the other condition. Moreover the smaller proportion of low-Nanog cells suggests that this could be a transient, short-lived event in the cells. Cultivation of low-Nanog cells population for 24h in conventional culture conditions proved this hypothesis. A subpopulation of cells showed Nanog distribution like the high-Nanog population, confirming that low-Nanog expression is an instable state of the

cells. The same experiment performed only with the high-Nanog cells did not give rise to a subpopulation expressing low level of Nanog, confirming that this is the most stable state. The levels of Nanog expression in an ESC are related to its probability to differentiate or not. In this model the advantage is that in a heterogeneous population, there is always a subpopulation of cells pre-primed for differentiation. Such priming would be an advantage in situations where the cells must be ready within a short period to respond to diverse signalling (Kalmar et al., 2009).

Although there are other transcription factors associated with ESC self-renewal and pluripotency, a large number of studies support the notion that the trio SOX2, OCT3/4 and Nanog are the main regulators that generate and maintain the pluripotent state *in vivo* and *in vitro*. This idea is also corroborated by the fact that all three factors play an essential role in reprogramming somatic cells (Meissner et al., 2007; Okita et al., 2007; Takahashi and Yamanaka, 2006).

6. Regulation of pluripotency by epigenetic mechanisms

In the following paragraph we will discuss some of the emerging evidence of the interaction between the core transcription factors and the epigenetic machinery, which together regulate stem cell pluripotency and differentiation. We will focus on the principal epigenetic modifications and how these influence and regulate the effect of pluripotency factors like Nanog, OCT3/4 and STAT3.

Epigenetic is the study of inherited changes in phenotype or gene expression caused by mechanisms other than changes in the underlying DNA sequence. Non-genetic factors cause cell's genes to behave differently and these changes may remain through cell divisions. The main known epigenetic mechanisms affecting transcription of the genes are: DNA methylation of the CpG islands, modifications of the histones, chromatin remodelling, and small RNA molecules. These modifications induce conformational changes in the chromatin structure: High-condensed chromatin, the so-called heterochromatin, is resistant to transcriptional activation, whereas the more open euchromatin allows transcription factors to bind on the DNA and drive the transcription of genes. Transcription factor regulation of gene expression and chromatin-controlled epigenetic memory systems are closely cooperating in establishing the pluripotent state of ESCs and in maintaining the cell fate decisions throughout development of an organism. ESCs are, in contrast to differentiated cells, highly transcriptional active. This implies that differentiation processes involve downregulation of general transcriptional activity. Therefore differentiation leads to a decrease in the euchromatic nature of the chromosome rendering it more condensed and less accessible for the transcriptional machinery; the resulting heterochromatin leads to a loss of pluripotency.

Histone proteins are subjected to various posttranslational modifications. The N-terminal tails of core histones H2A, H2B; H3 and H4 can be for instance acetylated, methylated, ubiquitinated, and phosphorylated. All these modifications modulate and control the conformation of chromatin, and therefore the transcription of the genes (Margueron et al., 2005). For example two important heterochromatic markers are increased trimethylated lysine 9 on the histone 3 (TriMeK9 H3) and decreased acetylated histone H3 and H4 (AcH3 and AcH4) (Kimura et al., 2004). When these conditions are global, they cause gene repression. These modifications can spread over kilobases of genomic DNA and are transmitted to daughter cells: This is the concept of the epigenetic inheritance (Cavalli and

Paro, 1999). Two other important histone modifications are methylation of lysine 27 on the histone H3 (MeK27 H3), which leads to repression of transcription; and the methylation of lysine 4 on the histone H3 (MeK4 H3) that positively regulates gene transcription by recruiting nucleosome remodelling enzymes and histone acetylases. Methylation of these lysines is catalyzed by thrithorax-and Polycomb-group proteins, which are involved in mitotic inheritance of lineage-specific gene expression programs and play therefore key functions during development (Plath et al., 2003; Ringrose and Paro, 2004).

Bernstein et al. mapped the histone methylation patterns in mouse ESCs and found a novel chromatin modification pattern, the "bivalent domains", which consist of large DNA regions of K27 methylation and smaller regions of K4 methylation that silence developmental-associated genes in ESCs while keeping them in a standby mode for activation. The bivalent histone modification represses gene expression because of the dominant repressive effect of K27 methylation over the K4 methylation. Thus, when ESCs differentiate these genes can rapidly be activated for transcription and the typical bivalent status is lost. Once the cell is committed it shows either K27 or K4 methylation (Bernstein et al., 2006).

The Polycomb protein complexes (PcG) are methyl-transferases responsible for methylation of H3K27. The main function of these complexes is to repress transcription and therefore maintain the cellular identity. They are fundamental during embryonic development and have been associated with many lineage-control gene loci in human and murine ESCs. Recent studies have demonstrated that in ESCs the target genes of PcG highly overlap with those of the core pluripotency markers: Nanog, OCT3/4, and SOX2 (Boyer et al., 2006; Lee et al., 2006). PcG complexes functions as two distinct components, the Polycomb repressive complex 1 (PRC1) and the PRC2 (Francis and Kingston, 2001). Microarray analyses with wild type cells and *Eed* (embryonic ectoderm development) null cells, a component of the PRC2, showed an upregulation of PcG target genes, like for example *Gata3*, *Gata4*, and *Gata6* genes. Moreover most of the PcG target genes were found upregulated upon cell differentiation, highlighting a fundamental role of PcG complex in silencing differentiation-associated genes in ESCs, and therefore contributing to the maintenance of pluripotency (Boyer et al., 2006).

Among the enzymes that modify histones there are also the histone demethylases, which are responsible for removing the methyl groups on the lysine 4 in the histone H3 (Agger et al., 2008). The Jumonji protein family, JARID1 was shown to specifically demethylate the H3K4 lysine, which is usually associated with active transcription (Mikkelsen et al., 2007). The bivalent histone modification theory suggests a mechanism where a cross talk between PcG and demethylases regulates transcriptional and developmental programs. Pasini and colleagues demonstrated that the H3K4 demethylase RBP2 (JARID1A) binds together with the PRC2 complex on PcG target genes effectively regulating their expression. The binding of RBP2 to the target genes is required for maintaining the PRC2-dependent transcriptional silencing (Pasini et al., 2008).

We mentioned before that ESCs show a unique chromatin status that is characterized by hyperdynamic and open chromatin environment. The maintenance of transcriptional permissive chromatin is achieved with general low level of the repressive marks H3K9 methylation and enrichment in active marks like methylation of H3K4 and acetylation of H3 and H4 (Meshorer and Misteli, 2006). These epigenetic processes are required for the maintenance of ESCs pluripotency and plasticity. STAT3 together with OCT3/4 transcription factors can regulate the expression of EED (Ura et al., 2008). However, the

enzymes that catalyze demethylation of the histones also play a fundamental role. OCT3/4 binding sites studies revealed that this factor controls also the architecture of the chromatin by controlling the expression of genes that encode for histone-modifying enzymes (Loh et al., 2006). It was shown with ChIP assay that OCT3/4 clusters within the *Jmjd1a* and *Jmjd2c* histone demethylase genes and after OCT3/4 knockdown their expression was decreased (Loh et al., 2007). Interestingly RNAi-depletion of Nanog had no or little effect on the expression of both JMJD1a and JMJD2c, confirming both genes as OCT3/4 targets. Both JMJD1a and JMJD2c enzymes are involved in the demethylation of the repressive mark H3K9. ESCs differentiated upon knockdown of either JMJD1a or JMJD2c and showed fibroblast-like morphology (Loh et al., 2007). Search for downstream targets identified different genes that were down- or up-regulated depending on which Jmjd was silenced; only few genes were found to overlap between JMJD1a and JMJD2c (Loh et al., 2007). JMJD1a was found to regulate the expression of the *Tcl1* gene, which is known to be required in pluripotent ESCs (Ivanova et al., 2006; Ivanova et al., 2002). Depletion of JMJD1a induced an increase of H3K9Me2 at the *Tcl1* promoter, resulting in its repression and differentiation of the ESCs (Loh et al., 2007). Moreover it is known that *Tcl1* promoter is bound by the OCT3/4 transcription factor (Loh et al., 2006) and it was shown that an increase in H3K9Me2 abolished this binding. Thus, the loss of OCT3/4 binding was probably the cause for downregulation of TCL1 after JMJD1a knockdown. Interestingly overexpression of TCL1 in ESCs rescued the effect of JMJD1a depletion, confirming *Tcl1* as a key effector of JMJD1a. Microarray analyses showed that after JMJD2c knockdown Nanog expression was reduced. With ChIP assay after JMJD2c depletion an enrichment of H3K9Me3 on the *Nanog* promoter region was observed and further analyses demonstrated that JMJD2c directly binds to the *Nanog* promoter, positively regulating its expression (Loh et al., 2007). Like for TCL1 and JMJD1a, overexpression of Nanog in JMJD2c knockdown ESCs was enough for maintaining the undifferentiated state of the cells, demonstrating that Nanog could compensate the loss of JMJD2c. In the end, this study provided the evidence that the histone demethylases JMJD1a and JMJD2c help in the maintenance of pluripotency through the regulation of downstream genes that encode for self-renewal factors.

Histone tails can also be acetylated by acetyl-transferases. The p300 acetyl-transferase catalyzes the acetylation of K9 and K14 of histone H3 which are markers of transcriptionally active chromatin and is required for ESCs to undergo early differentiation (Zhong and Jin, 2009). Interestingly, deletion of p300 did not affect ESCs self-renewal and the expression of Nanog, OCT3/4 and SOX2. However, these cells exhibited an increase in the endoderm marker GATA6 and a reduction of the ectoderm marker FGF5. Embryoid bodies generated from the *p300* null cells had a higher expression of GATA6, resulting in an overgrowth of extra-embryonic endoderm-like cells. Moreover Nanog but not OCT3/4 expression, was much more reduced both at the protein and mRNA levels compared to the wild type embryoid bodies. This suggested that transcription of Nanog during differentiation is dependent on the presence of p300. Overexpression of Nanog in *p300* -/- embryoid bodies could in part rescue the aberrant expression of GATA6, but not for instance the one of FGF5 (Zhong and Jin, 2009). Therefore the unusual phenotype of the knockout embryoid bodies could be caused by an improper regulation of Nanog during differentiation. Reporter activity assay demonstrated that p300 was able to control Nanog expression at the transcriptional level in a dose-dependent manner and independently of the presence or not of LIF in the medium and suggested that p300 might regulate Nanog expression through a regulatory element located at -3.8 to -4.8 kb. Interestingly, previous works showed that

STAT3 and Brachyury T bind to this region of the *Nanog* promoter, regulating early mesoderm differentiation (Suzuki et al., 2006a). By ChIP assays it was confirmed that p300 binds to the same regulatory region and differentiated *p300* knockout ESCs show a significant reduction in H3K9 acetylation at the Nanog/Brachyury T/STAT3 binding site (Zhong and Jin, 2009). In conclusion this study demonstrated that p300 controls transcriptional regulation of Nanog during differentiation through epigenetic modifications of histone acetylation at the Brachyury T/STAT3 binding site on the *Nanog* promoter.
Understanding how epigenetic mechanisms interact with pluripotency factors is of high interest also for the better understanding of reprogramming processes.

6.1 Induced pluripotent stem cells: an epigenetic revolution

Due to the exceptional characteristics of ESCs, the possibility to generate pluripotent cells from fully differentiated cells by resetting their epigenetic memory has fascinated the scientific world for many years. Originally it was demonstrated that activated enucleated oocytes were able to reprogram differentiated cell nuclei and give rise to viable offspring (Wilmut et al., 1997) or that it was possible to achieve reprogramming of mature cells through fusion with ESCs (Tada et al., 2001). Recently genetic strategies were developed for manipulating and finally re-activating pluripotency mechanisms in terminally differentiated cells (Takahashi and Yamanaka, 2006; Yamanaka 2008). In the following paragraph we will discuss a few examples of epigenetic manipulations, which were shown to help lineage-committed cells to re-activate pluripotency-related genes, giving rise to induced pluripotent stem cells (iPSCs). Yamanaka and colleagues, by transducing four transcription factors (OCT3/4, SOX2, KLF4, and c-MYC) into mouse fibroblasts, could reprogram these cells into iPSCs, which showed an ESC-like transcriptional circuitry and epigenetic landscape. Moreover these cells were demonstrated to be pluripotent being able *in vitro* to differentiate into cell types of the three germ layers and *in vivo* to contribute to germline competent chimeras (Maherali and Hochedlinger, 2008).
Undifferentiated cells usually show transcriptionally highly-active chromatin, which is gradually condensed and therefore less transcriptionally active upon differentiation. It is then easy to understand that reprogramming induces marked epigenetic changes in the differentiated cells making them again competent to respond to pluripotency factors.
We already discussed the importance of the transcription factors OCT3/4, SOX2, KLF4, and c-MYC in the context of ESCs, we therefore refer to the original paper of the Yamanaka laboratory for more information about how these genes were selected for generating iPSCs (Takahashi and Yamanaka, 2006; Yamanaka, 2008). Interestingly, the capacity of reprogramming differentiated cells seems not to be an exclusive characteristic of the four transcription factors (OCT3/4, SOX2, KLF4, and c-MYC). Other studies, in which the same reprogramming technique was used, demonstrated that some of the Yamanaka factors could be replaced (Feng et al., 2009) or used in combination with other factors (Mikkelsen et al., 2008). Importantly only OCT3/4 was found to be indispensable for a proper reprogramming independently from the origin of the parental cells. Moreover by overexpression or knockdown of additional factors, or by using small chemical compounds it was possible to increase the efficiency of the reprogramming process.
As mentioned before DNA methylation plays a fundamental role in regulating gene expression, since highly methylated genes are normally transcriptionally inactive. Thus, the first important change that has to happen in somatic cells undergoing reprogramming is a remodelling of the methylated sites on the chromatin. Moreover for achieving a fully

reprogrammed state iPSCs have to re-activate the endogenous expression of pluripotency-related genes, establish again an open chromatin structure, and re-activate the Polycomb machinery in order to repress lineage specific gene expression.

It has been shown that with the Yamanaka factors, only a small population of the reprogrammed cells showed reactivation of endogenous Nanog expression, contribution to chimera formation, and formation of teratoma. These results demonstrated that most of the infected cells were only partially de-differentiated and their epigenetic landscape was only to some extent changed but did not completely resemble the one of ESCs (Mikkelsen et al., 2008). For example examination of the methylation state of high-CpG-Promoters (HCP), which are usually bivalently marked in ESCs, revealed that partially reprogrammed fibroblasts showed 70% more of HCP with bivalent chromatin structure when compared to the original parental cells. However a comparison with fully reprogrammed iPSCs showed that partially reprogrammed iPSC had 40% less bivalent chromatin structure (Mikkelsen et al., 2008). Further analyses confirmed that in partially reprogrammed cells, the pluripotency-related genes exhibited DNA hypermethylation instead of harbouring the bivalent markers typical for ESC chromatin. The first major reprogramming barrier was shown to be an incorrect induction of DNA methylation changes. Incomplete demethylation of promoters of fundamental pluripotency-related genes such *Nanog* or *Oct3/4* leads to an unsuccessful reprogramming of the cells. Mikkelsen and colleagues demonstrated that treatment of partially reprogrammed iPSCs with a DNA methyltransferase inhibitor (5-aza-cytidine) rapidly and fully reprogrammed these cells into authentic iPSCs (Mikkelsen et al., 2008). Analyses of the methylation state of CpGs near to pluripotency genes such as *Nanog* and *Utf1* confirmed significant demethylation. Identical results were achieved by transient knockdown of DNMT1 in partially reprogrammed cells. Treatment with DNA methyltransferase inhibitors during the reprogramming processes led to an increased efficiency in the generation of ESC-like colonies, however only if the treatment was started 8 days after infection of the cells with the four reprogramming factors (Mikkelsen et al., 2008). Another reason why incomplete reprogramming might occur is the inappropriate activation or failure in the repression of endogenous cell-fate specific transcription factors, which in turn fail to reactivate hypermethylated pluripotency-related genes. Once more these results highlight the importance of a coordinated regulation of transcription factors, which have to be stabilized by correct epigenetic remodelling mechanisms.

Using a chemical approach, aimed at the optimization of the reprogramming efficiency and at the reduction of genetic manipulations due to the integration in the genome of the reprogramming factors, it was demonstrated that by inhibiting G9a histone methyltransferase (BIX-01294) in combination with a L-calcium channel agonist (BayK), the reprogramming process was successfully achieved only by transfecting mouse fibroblast with *Oct3/4* and *Klf4* (Shi et al., 2008). The generated iPSCs were then expanded without the two chemical compounds, demonstrating that once the reprogramming has taken place, they are dispensable. G9a is a histone methyltransferase known to repress gene activity by transferring methyl groups on the histone H3 lysine 9 (H3K9) at target promoters. Bisulphite sequencing analyses of the *Nanog* promoter region confirmed that these iPSCs had demethylated this region allowing the re-activation of Nanog transcription. These data clearly show that inhibition of methyltransferases renders the chromatin structure of lineage-restricted cells more susceptible to the reprogramming, therefore facilitating the re-activation of the transcription of pluripotency factors. This concept was corroborated by another study where it was shown that treatment with histone deacetylase (HDAC)

inhibitors such as valproic acid, after transfection with four or only three reprogramming factors also improved the reprogramming efficiency (Huangfu et al., 2008a). In addition, the combination of HDAC inhibitor and the DNA methyltransferase inhibitor significantly enhanced the kinetics and efficiency of the reprogramming. Furthermore by using HDAC inhibitors it was possible to reprogram human fibroblasts transfecting the cells only with two factors (*Oct3/4* and *Sox2*) instead of four (Huangfu et al., 2008b).

In conclusion, despite having found the essential factors needed for reprogramming differentiated cells, still much has to be clarified before this technique can be used for therapeutical purposes. Reprogramming is a balanced interplay between epigenetic changes and re-activation or suppression of specific gene expression. Controlling these sophisticated processes will surely be the topic for future studies. Nevertheless the combined chemical and genetic approach seems to be nowadays a good combination for driving and facilitating this complicated but powerful process. Moreover a combined approach permits a more precise and time controlled manipulation of the reprogramming processes.

7. Conclusions

We aimed in this chapter at reviewing the current knowledge regarding maintenance of pluripotency in embryonic and induced pluripotent stem cells. Even though in the past years many different key factors could be identified, how they interact between each other and control the pluripotent cell identity is still largely unknown. Understanding these mechanisms is essential because ESCs and iPSCs hold great promise for the therapeutic treatment of human diseases. Successful reprogramming of differentiated cells into iPSCs needs faithful remodelling of epigenetic modifications, because epigenetic aberrations often induce pathological conditions such as cancer. Therefore a thorough understanding of the epigenetic remodelling involved in reprogramming somatic cells is of fundamental interest. The field of stem cell research is one of the great challenges of our times and in the next future extensive studies aimed at the clarification of the correlations between pluripotency and epigenetic will be necessary. The advent of the modern high throughput technologies will open new possibilities in understanding the mechanisms ruling cell fate. All this will lead to the discovery not only of new mechanisms and key players determining pluripotency, but also will allow expanding the knowledge how to drive controlled differentiation of pluripotent cells towards pure populations of precursors and terminally differentiated cells, by identifying the right signals and culture environments.

8. References

Agger, K.; Christensen, J.; Cloos, P. A. C. & Helin, K. (2008). The emerging functions of histone demethylases. *Curr Opin Genet Dev*, 18, 2, 159-168

Aksoy, I.; Sakabedoyan, C.; Bourillot, P.-Y.; Malashicheva, A. B.; Mancip, J.; Knoblauch, K.; Afanassieff, M. & Savatier, P. (2007). Self-renewal of murine embryonic stem cells is supported by the serine/threonine kinases pim-1 and pim-3. *Stem Cells*, 25, 12, 2996-3004

Anton, R.; Kestler, H. A. & Kühl, M. (2007). Beta-catenin signaling contributes to stemness and regulates early differentiation in murine embryonic stem cells. *FEBS Lett*, 581, 27, 5247-5254

Avilion, A. A.; Nicolis, S. K.; Pevny, L. H.; Perez, L.; Vivian, N. & Lovell-Badge, R. (2003). Multipotent cell lineages in early mouse development depend on SOX2 function. *Genes Dev*, 17, 1, 126-140

Beppu, H.; Kawabata, M.; Hamamoto, T.; Chytil, A.; Minowa, O.; Noda, T. & Miyazono, K. (2000). BMP type II receptor is required for gastrulation and early development of mouse embryos. *Dev Biol*, 221, 1, 249-258

Bernstein, B. E.; Mikkelsen, T. S.; Xie, X.; Kamal, M.; Huebert, D. J.; Cuff, J.; Fry, B.; Meissner, A.; Wernig, M.; Plath, K.; Jaenisch, R.; Wagschal, A.; Feil, R.; Schreiber, S. L. & Lander, E. S. (2006). A bivalent chromatin structure marks key developmental genes in embryonic stem cells. *Cell*, 125, 2, 315-326

Bourillot, P.; Aksoy, I.; Schreiber, V.; Wianny, F.; Schulz, H.; Hummel, O.; Hubner, N. & Savatier, P. (2009). Novel STAT3 target genes exert distinct roles in the inhibition of mesoderm and endoderm differentiation in cooperation with Nanog. *Stem Cells*,

Boyer, L. A.; Plath, K.; Zeitlinger, J.; Brambrink, T.; Medeiros, L. A.; Lee, T. I.; Levine, S. S.; Wernig, M.; Tajonar, A.; Ray, M. K.; Bell, G. W.; Otte, A. P.; Vidal, M.; Gifford, D. K.; Young, R. A. & Jaenisch, R. (2006). Polycomb complexes repress developmental regulators in murine embryonic stem cells. *Nature*, 441, 7091, 349-353

Brazil, D. P.; Yang, Z.-Z. & Hemmings, B. A. (2004). Advances in protein kinase B signalling: AKTion on multiple fronts. *Trends Biochem Sci*, 29, 5, 233-242

Brons, I. G. M.; Smithers, L. E.; Trotter, M. W. B.; Rugg-Gunn, P.; Sun, B.; Chuva de Sousa Lopes, S. M.; Howlett, S. K.; Clarkson, A.; Ahrlund-Richter, L.; Pedersen, R. A. & Vallier, L. (2007). Derivation of pluripotent epiblast stem cells from mammalian embryos. *Nature*, 448, 7150, 191-195

Buehr, M.; Meek, S.; Blair, K.; Yang, J.; Ure, J.; Silva, J.; McLay, R.; Hall, J.; Ying, Q.-L. & Smith, A. (2008). Capture of authentic embryonic stem cells from rat blastocysts. *Cell*, 135, 7, 1287-1298

Burdon, T.; Smith, A. & Savatier, P. (2002). Signalling, cell cycle and pluripotency in embryonic stem cells. *Trends Cell Biol*, 12, 9, 432-438

Burdon, T.; Stracey, C.; Chambers, I.; Nichols, J. & Smith, A. (1999). Suppression of SHP-2 and ERK signalling promotes self-renewal of mouse embryonic stem cells. *Dev Biol*, 210, 1, 30-43

Cartwright, P.; McLean, C.; Sheppard, A.; Rivett, D.; Jones, K. & Dalton, S. (2005). LIF/STAT3 controls ES cell self-renewal and pluripotency by a Myc-dependent mechanism. *Development*, 132, 5, 885-896

Cavalli, G. & Paro, R. (1999). Epigenetic inheritance of active chromatin after removal of the main transactivator. *Science*, 286, 5441, 955-958

Chambers, I.; Colby, D.; Robertson, M.; Nichols, J.; Lee, S.; Tweedie, S. & Smith, A. (2003). Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. *Cell*, 113, 5, 643-655

Chambers, I.; Silva, J.; Colby, D.; Nichols, J.; Nijmeijer, B.; Robertson, M.; Vrana, J.; Jones, K.; Grotewold, L. & Smith, A. (2007). Nanog safeguards pluripotency and mediates germline development. *Nature*, 450, 7173, 1230-1234

Chan, R. J.; Johnson, S. A.; Li, Y.; Yoder, M. C. & Feng, G.-S. (2003). A definitive role of Shp-2 tyrosine phosphatase in mediating embryonic stem cell differentiation and hematopoiesis. *Blood*, 102, 6, 2074-2080

Chen, X.; Xu, H.; Yuan, P.; Fang, F.; Huss, M.; Vega, V. B.; Wong, E.; Orlov, Y. L.; Zhang, W.; Jiang, J.; Loh, Y.-H.; Yeo, H. C.; Yeo, Z. X.; Narang, V.; Govindarajan, K. R.; Leong, B.; Shahab, A.; Ruan, Y.; Bourque, G.; Sung, W.-K.; Clarke, N. D.; Wei, C.-L. & Ng, H.-H. (2008). Integration of external signaling pathways with the core transcriptional network in embryonic stem cells. *Cell*, 133, 6, 1106-1117

Chew, J.-L.; Loh, Y.-H.; Zhang, W.; Chen, X.; Tam, W.-L.; Yeap, L.-S.; Li, P.; Ang, Y.-S.; Lim, B.; Robson, P. & Ng, H.-H. (2005). Reciprocal transcriptional regulation of Pou5f1 and Sox2 via the Oct4/Sox2 complex in embryonic stem cells. *Molecular and cellular biology*, 25, 14, 6031-6046

Cinelli, P.; Casanova, E. A.; Uhlig, S.; Lochmatter, P.; Matsuda, T.; Yokota, T.; Rülicke, T.; Ledermann, B. & Bürki, K. (2008). Expression profiling in transgenic FVB/N embryonic stem cells overexpressing STAT3. *BMC Dev Biol*, 8, 57

Dang, C. V.; Resar, L. M.; Emison, E.; Kim, S.; Li, Q.; Prescott, J. E.; Wonsey, D. & Zeller, K. (1999). Function of the c-Myc oncogenic transcription factor. *Exp Cell Res*, 253, 1, 63-77

Davis, A. C.; Wims, M.; Spotts, G. D.; Hann, S. R. & Bradley, A. (1993). A null c-myc mutation causes lethality before 10.5 days of gestation in homozygotes and reduced fertility in heterozygous female mice. *Genes Dev*, 7, 4, 671-682

Evans, M. J. & Kaufman, M. H. (1981). Establishment in culture of pluripotential cells from mouse embryos. *Nature*, 292, 5819, 154-156

Feng, B.; Jiang, J.; Kraus, P.; Ng, J.-H.; Heng, J.-C. D.; Chan, Y.-S.; Yaw, L.-P.; Zhang, W.; Loh, Y.-H.; Han, J.; Vega, V. B.; Cacheux-Rataboul, V.; Lim, B.; Lufkin, T. & Ng, H.-H. (2009). Reprogramming of fibroblasts into induced pluripotent stem cells with orphan nuclear receptor Esrrb. *Nat Cell Biol*, 11, 2, 197-203

Francis, N. J. & Kingston, R. E. (2001). Mechanisms of transcriptional memory. *Nat Rev Mol Cell Biol*, 2, 6, 409-421

Fukada, T.; Hibi, M.; Yamanaka, Y.; Takahashi-Tezuka, M.; Fujitani, Y.; Yamaguchi, T.; Nakajima, K. & Hirano, T. (1996). Two signals are necessary for cell proliferation induced by a cytokine receptor gp130: involvement of STAT3 in anti-apoptosis. *Immunity*, 5, 5, 449-460

Galvin, K. E.; Travis, E. D.; Yee, D.; Magnuson, T. & Vivian, J. L. (2010). Nodal signaling regulates the bone morphogenic protein pluripotency pathway in mouse embryonic stem cells. *J Biol Chem*, 285, 26, 19747-19756

Gearing, D. P.; Gough, N. M.; King, J. A.; Hilton, D. J.; Nicola, N. A.; Simpson, R. J.; Nice, E. C.; Kelso, A. & Metcalf, D. (1987). Molecular cloning and expression of cDNA encoding a murine myeloid leukaemia inhibitory factor (LIF). *EMBO J*, 6, 13, 3995-4002

Groszer, M.; Erickson, R.; Scripture-Adams, D. D.; Lesche, R.; Trumpp, A.; Zack, J. A.; Kornblum, H. I.; Liu, X. & Wu, H. (2001). Negative regulation of neural stem/progenitor cell proliferation by the Pten tumor suppressor gene in vivo. *Science*, 294, 5549, 2186-2189

Hall, J.; Guo, G.; Wray, J.; Eyres, I.; Nichols, J.; Grotewold, L.; Morfopoulou, S.; Humphreys, P.; Mansfield, W.; Walker, R.; Tomlinson, S. & Smith, A. (2009). Oct4 and LIF/Stat3 Additively Induce Krüppel Factors to Sustain Embryonic Stem Cell Self-Renewal. *Cell stem cell*, 5, 6, 597-609

Hallmann, D.; Trümper, K.; Trusheim, H.; Ueki, K.; Kahn, C. R.; Cantley, L. C.; Fruman, D. A. & Hörsch, D. (2003). Altered signaling and cell cycle regulation in embryonal stem cells with a disruption of the gene for phosphoinositide 3-kinase regulatory subunit p85alpha. *J Biol Chem*, 278, 7, 5099-5108

Hao, J.; Li, T.-G.; Qi, X.; Zhao, D.-F. & Zhao, G.-Q. (2006). WNT/beta-catenin pathway up-regulates Stat3 and converges on LIF to prevent differentiation of mouse embryonic stem cells. *Dev Biol*, 290, 1, 81-91

Huangfu, D.; Maehr, R.; Guo, W.; Eijkelenboom, A.; Snitow, M.; Chen, A. & Melton, D. (2008a). Induction of pluripotent stem cells by defined factors is greatly improved by small-molecule compounds. *Nat Biotechnol*,

Huangfu, D.; Osafune, K.; Maehr, R.; Guo, W.; Eijkelenboom, A.; Chen, S.; Muhlestein, W. & Melton, D. (2008b). Induction of pluripotent stem cells from primary human fibroblasts with only Oct4 and Sox2. *Nat Biotechnol*,

Humphrey, R. K.; Beattie, G. M.; Lopez, A. D.; Bucay, N.; King, C. C.; Firpo, M. T.; Rose-John, S. & Hayek, A. (2004). Maintenance of pluripotency in human embryonic stem cells is STAT3 independent. *Stem Cells*, 22, 4, 522-530

Ivanova, N.; Dobrin, R.; Lu, R.; Kotenko, I.; Levorse, J.; DeCoste, C.; Schafer, X.; Lun, Y. & Lemischka, I. R. (2006). Dissecting self-renewal in stem cells with RNA interference. *Nature*, 442, 7102, 533-538

Ivanova, N. B.; Dimos, J. T.; Schaniel, C.; Hackney, J. A.; Moore, K. A. & Lemischka, I. R. (2002). A stem cell molecular signature. *Science*, 298, 5593, 601-604

Kalmar, T.; Lim, C.; Hayward, P.; Muñoz-Descalzo, S.; Nichols, J.; Garcia-Ojalvo, J. & Martinez Arias, A. (2009). Regulated fluctuations in nanog expression mediate cell fate decisions in embryonic stem cells. *PLoS Biol*, 7, 7, e1000149

Kawase, E.; Suemori, H.; Takahashi, N.; Okazaki, K.; Hashimoto, K. & Nakatsuji, N. (1994). Strain difference in establishment of mouse embryonic stem (ES) cell lines. *Int J Dev Biol*, 38, 2, 385-390

Kim, J. B.; Sebastiano, V.; Wu, G.; Araúzo-Bravo, M. J.; Sasse, P.; Gentile, L.; Ko, K.; Ruau, D.; Ehrich, M.; van den Boom, D.; Meyer, J.; Hübner, K.; Bernemann, C.; Ortmeier, C.; Zenke, M.; Fleischmann, B. K.; Zaehres, H. & Schöler, H. R. (2009). Oct4-induced pluripotency in adult neural stem cells. *Cell*, 136, 3, 411-419

Kimura, H.; Tada, M.; Nakatsuji, N. & Tada, T. (2004). Histone code modifications on pluripotential nuclei of reprogrammed somatic cells. *Mol Cell Biol*, 24, 13, 5710-5720

Kioussi, C.; Briata, P.; Baek, S. H.; Rose, D. W.; Hamblet, N. S.; Herman, T.; Ohgi, K. A.; Lin, C.; Gleiberman, A.; Wang, J.; Brault, V.; Ruiz-Lozano, P.; Nguyen, H. D.; Kemler, R.; Glass, C. K.; Wynshaw-Boris, A. & Rosenfeld, M. G. (2002). Identification of a Wnt/Dvl/beta-Catenin --> Pitx2 pathway mediating cell-type-specific proliferation during development. *Cell*, 111, 5, 673-685

Kuroda, T.; Tada, M.; Kubota, H.; Kimura, H.; Hatano, S.-y.; Suemori, H.; Nakatsuji, N. & Tada, T. (2005). Octamer and Sox elements are required for transcriptional cis regulation of Nanog gene expression. *Mol Cell Biol*, 25, 6, 2475-2485

Lee, M. Y.; Lim, H. W.; Lee, S. H. & Han, H. J. (2009). Smad, PI3K/Akt, and Wnt-dependent signaling pathways are involved in BMP-4-induced ESC self-renewal. *Stem Cells*, 27, 8, 1858-1868

Lee, T. I.; Jenner, R. G.; Boyer, L. A.; Guenther, M. G.; Levine, S. S.; Kumar, R. M.; Chevalier, B.; Johnstone, S. E.; Cole, M. F.; Isono, K.-i.; Koseki, H.; Fuchikami, T.; Abe, K.;

Murray, H. L.; Zucker, J. P.; Yuan, B.; Bell, G. W.; Herbolsheimer, E.; Hannett, N. M.; Sun, K.; Odom, D. T.; Otte, A. P.; Volkert, T. L.; Bartel, D. P.; Melton, D. A.; Gifford, D. K.; Jaenisch, R. & Young, R. A. (2006). Control of developmental regulators by Polycomb in human embryonic stem cells. *Cell*, 125, 2, 301-313

Lenardo, M. J.; Staudt, L.; Robbins, P.; Kuang, A.; Mulligan, R. C. & Baltimore, D. (1989). Repression of the IgH enhancer in teratocarcinoma cells associated with a novel octamer factor. *Science*, 243, 4890, 544-546

Lewis, T. S.; Shapiro, P. S. & Ahn, N. G. (1998). Signal transduction through MAP kinase cascades. *Adv Cancer Res*, 74, 49-139

Li, M.; Sendtner, M. & Smith, A. (1995). Essential function of LIF receptor in motor neurons. *Nature*, 378, 6558, 724-727

Li, P.; Tong, C.; Mehrian-Shai, R.; Jia, L.; Wu, N.; Yan, Y.; Maxson, R. E.; Schulze, E. N.; Song, H.; Hsieh, C.-L.; Pera, M. F. & Ying, Q.-L. (2008). Germline competent embryonic stem cells derived from rat blastocysts. *Cell*, 135, 7, 1299-1310

Lloyd, A. C. (1998). Ras versus cyclin-dependent kinase inhibitors. *Curr Opin Genet Dev*, 8, 1, 43-48

Lodge, P.; McWhir, J.; Gallagher, E. & Sang, H. (2005). Increased gp130 signaling in combination with inhibition of the MEK/ERK pathway facilitates embryonic stem cell isolation from normally refractory murine CBA blastocysts. *Cloning Stem Cells*, 7, 1, 2-7

Loh, Y.-H.; Wu, Q.; Chew, J.-L.; Vega, V. B.; Zhang, W.; Chen, X.; Bourque, G.; George, J.; Leong, B.; Liu, J.; Wong, K.-Y.; Sung, K. W.; Lee, C. W. H.; Zhao, X.-D.; Chiu, K.-P.; Lipovich, L.; Kuznetsov, V. A.; Robson, P.; Stanton, L. W.; Wei, C.-L.; Ruan, Y.; Lim, B. & Ng, H.-H. (2006). The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. *Nat Genet*, 38, 4, 431-440

Loh, Y.-H.; Zhang, W.; Chen, X.; George, J. & Ng, H.-H. (2007). Jmjd1a and Jmjd2c histone H3 Lys 9 demethylases regulate self-renewal in embryonic stem cells. *Genes Dev*, 21, 20, 2545-2557

Maekawa, M.; Yamamoto, T.; Kohno, M.; Takeichi, M. & Nishida, E. (2007). Requirement for ERK MAP kinase in mouse preimplantation development. *Development*, 134, 15, 2751-2759

Maherali, N. & Hochedlinger, K. (2008). Guidelines and techniques for the generation of induced pluripotent stem cells. *Cell stem cell*, 3, 6, 595-605

Margueron, R.; Trojer, P. & Reinberg, D. (2005). The key to development: interpreting the histone code? *Curr Opin Genet Dev*, 15, 2, 163-176

Martin, G. R. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci USA*, 78, 12, 7634-7638

Masui, S.; Nakatake, Y.; Toyooka, Y.; Shimosato, D.; Yagi, R.; Takahashi, K.; Okochi, H.; Okuda, A.; Matoba, R.; Sharov, A. A.; Ko, M. S. H. & Niwa, H. (2007). Pluripotency governed by Sox2 via regulation of Oct3/4 expression in mouse embryonic stem cells. *Nat Cell Biol*, 9, 6, 625-635

Matsuda, T.; Nakamura, T.; Nakao, K.; Arai, T.; Katsuki, M.; Heike, T. & Yokota, T. (1999). STAT3 activation is sufficient to maintain an undifferentiated state of mouse embryonic stem cells. *EMBO J*, 18, 15, 4261-4269

Matsui, Y. (1992). [Regulation of murine primordial germ cell (PGC) growth by peptide growth factors]. *Tanpakushitsu Kakusan Koso*, 37, 15, 2935-2946

Meissner, A.; Wernig, M. & Jaenisch, R. (2007). Direct reprogramming of genetically unmodified fibroblasts into pluripotent stem cells. *Nat Biotechnol*, 25, 10, 1177-1181

Meshorer, E. & Misteli, T. (2006). Chromatin in pluripotent embryonic stem cells and differentiation. *Nat Rev Mol Cell Biol*, 7, 7, 540-546

Mikkelsen, T. S.; Hanna, J.; Zhang, X.; Ku, M.; Wernig, M.; Schorderet, P.; Bernstein, B. E.; Jaenisch, R.; Lander, E. S. & Meissner, A. (2008). Dissecting direct reprogramming through integrative genomic analysis. *Nature*, 454, 7200, 49-55

Mikkelsen, T. S.; Ku, M.; Jaffe, D. B.; Issac, B.; Lieberman, E.; Giannoukos, G.; Alvarez, P.; Brockman, W.; Kim, T.-K.; Koche, R. P.; Lee, W.; Mendenhall, E.; O'Donovan, A.; Presser, A.; Russ, C.; Xie, X.; Meissner, A.; Wernig, M.; Jaenisch, R.; Nusbaum, C.; Lander, E. S. & Bernstein, B. E. (2007). Genome-wide maps of chromatin state in pluripotent and lineage-committed cells. *Nature*, 448, 7153, 553-560

Mishina, Y.; Suzuki, A.; Ueno, N. & Behringer, R. R. (1995). Bmpr encodes a type I bone morphogenetic protein receptor that is essential for gastrulation during mouse embryogenesis. *Genes Dev*, 9, 24, 3027-3037

Mitsui, K.; Tokuzawa, Y.; Itoh, H.; Segawa, K.; Murakami, M.; Takahashi, K.; Maruyama, M.; Maeda, M. & Yamanaka, S. (2003). The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. *Cell*, 113, 5, 631-642

Moon, R. T.; Kohn, A. D.; De Ferrari, G. V. & Kaykas, A. (2004). WNT and beta-catenin signalling: diseases and therapies. *Nat Rev Genet*, 5, 9, 691-701

Naito, A. T.; Shiojima, I.; Akazawa, H.; Hidaka, K.; Morisaki, T.; Kikuchi, A. & Komuro, I. (2006). Developmental stage-specific biphasic roles of Wnt/beta-catenin signaling in cardiomyogenesis and hematopoiesis. *Proc Natl Acad Sci USA*, 103, 52, 19812-19817

Nakagawa, M.; Koyanagi, M.; Tanabe, K.; Takahashi, K.; Ichisaka, T.; Aoi, T.; Okita, K.; Mochiduki, Y.; Takizawa, N. & Yamanaka, S. (2008). Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. *Nat Biotechnol*, 26, 1, 101-106

Nakashima, K.; Yanagisawa, M.; Arakawa, H. & Taga, T. (1999). Astrocyte differentiation mediated by LIF in cooperation with BMP2. *FEBS Lett*, 457, 1, 43-46

Nichols, J.; Chambers, I.; Taga, T. & Smith, A. (2001). Physiological rationale for responsiveness of mouse embryonic stem cells to gp130 cytokines. *Development (Cambridge, England)*, 128, 12, 2333-2339

Nichols, J.; Jones, K.; Phillips, J.; Newland, S.; Roode, M.; Mansfield, W.; Smith, A. & Cooke, A. (2009). Validated germline-competent embryonic stem cell lines from nonobese diabetic mice. *Nat Med*,

Nichols, J.; Zevnik, B.; Anastassiadis, K.; Niwa, H.; Klewe-Nebenius, D.; Chambers, I.; Schöler, H. & Smith, A. (1998). Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell*, 95, 3, 379-391

Niwa, H.; Burdon, T.; Chambers, I. & Smith, A. (1998). Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3. *Genes Dev*, 12, 13, 2048-2060

Niwa, H.; Miyazaki, J. & Smith, A. G. (2000). Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nat Genet*, 24, 4, 372-376

Noben-Trauth, N.; Köhler, G.; Bürki, K. & Ledermann, B. (1996). Efficient targeting of the IL-4 gene in a BALB/c embryonic stem cell line. *Transgenic Res*, 5, 6, 487-491

Nordhoff, V.; Hübner, K.; Bauer, A.; Orlova, I.; Malapetsa, A. & Schöler, H. R. (2001). Comparative analysis of human, bovine, and murine Oct-4 upstream promoter sequences. *Mamm Genome*, 12, 4, 309-317

O'Sullivan, L. A.; Liongue, C.; Lewis, R. S.; Stephenson, S. E. M. & Ward, A. C. (2007). Cytokine receptor signaling through the Jak-Stat-Socs pathway in disease. *Mol Immunol*, 44, 10, 2497-2506

Ogawa, K.; Nishinakamura, R.; Iwamatsu, Y.; Shimosato, D. & Niwa, H. (2006). Synergistic action of Wnt and LIF in maintaining pluripotency of mouse ES cells. *Biochem Biophys Res Commun*, 343, 1, 159-166

Ogawa, K.; Saito, A.; Matsui, H.; Suzuki, H.; Ohtsuka, S.; Shimosato, D.; Morishita, Y.; Watabe, T.; Niwa, H. & Miyazono, K. (2007). Activin-Nodal signaling is involved in propagation of mouse embryonic stem cells. *J Cell Sci*, 120, Pt 1, 55-65

Okita, K.; Ichisaka, T. & Yamanaka, S. (2007). Generation of germline-competent induced pluripotent stem cells. *Nature*, 448, 7151, 313-317

Okumura-Nakanishi, S.; Saito, M.; Niwa, H. & Ishikawa, F. (2005). Oct-3/4 and Sox2 regulate Oct-3/4 gene in embryonic stem cells. *J Biol Chem*, 280, 7, 5307-5317

Paling, N. R. D.; Wheadon, H.; Bone, H. K. & Welham, M. J. (2004). Regulation of embryonic stem cell self-renewal by phosphoinositide 3-kinase-dependent signaling. *J Biol Chem*, 279, 46, 48063-48070

Palmieri, S. L.; Peter, W.; Hess, H. & Schöler, H. R. (1994). Oct-4 transcription factor is differentially expressed in the mouse embryo during establishment of the first two extraembryonic cell lineages involved in implantation. *Dev Biol*, 166, 1, 259-267

Pasini, D.; Hansen, K. H.; Christensen, J.; Agger, K.; Cloos, P. A. C. & Helin, K. (2008). Coordinated regulation of transcriptional repression by the RBP2 H3K4 demethylase and Polycomb-Repressive Complex 2. *Genes Dev*, 22, 10, 1345-1355

Plath, K.; Fang, J.; Mlynarczyk-Evans, S. K.; Cao, R.; Worringer, K. A.; Wang, H.; de la Cruz, C. C.; Otte, A. P.; Panning, B. & Zhang, Y. (2003). Role of histone H3 lysine 27 methylation in X inactivation. *Science*, 300, 5616, 131-135

Qi, X.; Li, T.-G.; Hao, J.; Hu, J.; Wang, J.; Simmons, H.; Miura, S.; Mishina, Y. & Zhao, G.-Q. (2004). BMP4 supports self-renewal of embryonic stem cells by inhibiting mitogen-activated protein kinase pathways. *Proc Natl Acad Sci USA*, 101, 16, 6027-6032

Reményi, A.; Lins, K.; Nissen, L. J.; Reinbold, R.; Schöler, H. R. & Wilmanns, M. (2003). Crystal structure of a POU/HMG/DNA ternary complex suggests differential assembly of Oct4 and Sox2 on two enhancers. *Genes Dev*, 17, 16, 2048-2059

Ringrose, L. & Paro, R. (2004). Epigenetic regulation of cellular memory by the Polycomb and Trithorax group proteins. *Annu Rev Genet*, 38, 413-443

Roach, M. L.; Stock, J. L.; Byrum, R.; Koller, B. H. & McNeish, J. D. (1995). A new embryonic stem cell line from DBA/1lacJ mice allows genetic modification in a murine model of human inflammation. *Exp Cell Res*, 221, 2, 520-525

Robertson, E. J.; Norris, D. P.; Brennan, J. & Bikoff, E. K. (2003). Control of early anterior-posterior patterning in the mouse embryo by TGF-beta signalling. *Philos Trans R Soc Lond, B, Biol Sci*, 358, 1436, 1351-1357; discussion 1357

Rodda, D. J.; Chew, J.-L.; Lim, L.-H.; Loh, Y.-H.; Wang, B.; Ng, H.-H. & Robson, P. (2005). Transcriptional regulation of nanog by OCT4 and SOX2. *J Biol Chem*, 280, 26, 24731-24737

Rohwedel, J.; Sehlmeyer, U.; Shan, J.; Meister, A. & Wobus, A. M. (1996). Primordial germ cell-derived mouse embryonic germ (EG) cells in vitro resemble undifferentiated stem cells with respect to differentiation capacity and cell cycle distribution. *Cell Biol Int*, 20, 8, 579-587

Ruvkun, G. & Finney, M. (1991). Regulation of transcription and cell identity by POU domain proteins. *Cell*, 64, 3, 475-478

Sato, N.; Meijer, L.; Skaltsounis, L.; Greengard, P. & Brivanlou, A. H. (2004). Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. *Nat Med*, 10, 1, 55-63

Savatier, P.; Lapillonne, H.; van Grunsven, L. A.; Rudkin, B. B. & Samarut, J. (1996). Withdrawal of differentiation inhibitory activity/leukemia inhibitory factor up-regulates D-type cyclins and cyclin-dependent kinase inhibitors in mouse embryonic stem cells. *Oncogene*, 12, 2, 309-322

Schöler, H. R. (1991). Octamania: the POU factors in murine development. *Trends Genet*, 7, 10, 323-329

Schöler, H. R.; Balling, R.; Hatzopoulos, A. K.; Suzuki, N. & Gruss, P. (1989). Octamer binding proteins confer transcriptional activity in early mouse embryogenesis. *EMBO J*, 8, 9, 2551-2557

Shi, Y.; Desponts, C.; Do, J. T.; Hahm, H. S.; Schöler, H. R. & Ding, S. (2008). Induction of pluripotent stem cells from mouse embryonic fibroblasts by Oct4 and Klf4 with small-molecule compounds. *Cell stem cell*, 3, 5, 568-574

Simpson, E. M.; Linder, C. C.; Sargent, E. E.; Davisson, M. T.; Mobraaten, L. E. & Sharp, J. J. (1997). Genetic variation among 129 substrains and its importance for targeted mutagenesis in mice. *Nat Genet*, 16, 1, 19-27

Singh, A. M.; Hamazaki, T.; Hankowski, K. E. & Terada, N. (2007). A heterogeneous expression pattern for Nanog in embryonic stem cells. *Stem Cells*, 25, 10, 2534-2542

Sirard, C.; de la Pompa, J. L.; Elia, A.; Itie, A.; Mirtsos, C.; Cheung, A.; Hahn, S.; Wakeham, A.; Schwartz, L.; Kern, S. E.; Rossant, J. & Mak, T. W. (1998). The tumor suppressor gene Smad4/Dpc4 is required for gastrulation and later for anterior development of the mouse embryo. *Genes Dev*, 12, 1, 107-119

Smith, A. G.; Heath, J. K.; Donaldson, D. D.; Wong, G. G.; Moreau, J.; Stahl, M. & Rogers, D. (1988). Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. *Nature*, 336, 6200, 688-690

Solter, D. & Knowles, B. B. (1978). Monoclonal antibody defining a stage-specific mouse embryonic antigen (SSEA-1). *Proc Natl Acad Sci USA*, 75, 11, 5565-5569

Starr, R.; Novak, U.; Willson, T. A.; Inglese, M.; Murphy, V.; Alexander, W. S.; Metcalf, D.; Nicola, N. A.; Hilton, D. J. & Ernst, M. (1997). Distinct roles for leukemia inhibitory factor receptor alpha-chain and gp130 in cell type-specific signal transduction. *J Biol Chem*, 272, 32, 19982-19986

Stewart, C. L.; Kaspar, P.; Brunet, L. J.; Bhatt, H.; Gadi, I.; Köntgen, F. & Abbondanzo, S. J. (1992). Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor. *Nature*, 359, 6390, 76-79

Stiles, B.; Groszer, M.; Wang, S.; Jiao, J. & Wu, H. (2004). PTENless means more. *Dev Biol*, 273, 2, 175-184

Suzuki, A.; Raya, A.; Kawakami, Y.; Morita, M.; Matsui, T.; Nakashima, K.; Gage, F. H.; Rodríguez-Esteban, C. & Belmonte, J. C. I. (2006a). Maintenance of embryonic stem cell pluripotency by Nanog-mediated reversal of mesoderm specification. *Nature clinical practice Cardiovascular medicine*, 3 Suppl 1, S114-122

Suzuki, A.; Raya, A.; Kawakami, Y.; Morita, M.; Matsui, T.; Nakashima, K.; Gage, F. H.; Rodríguez-Esteban, C. & Izpisúa Belmonte, J. C. (2006b). Nanog binds to Smad1 and blocks bone morphogenetic protein-induced differentiation of embryonic stem cells. *Proc Natl Acad Sci USA*, 103, 27, 10294-10299

Tada, M.; Takahama, Y.; Abe, K.; Nakatsuji, N. & Tada, T. (2001). Nuclear reprogramming of somatic cells by in vitro hybridization with ES cells. *Curr Biol*, 11, 19, 1553-1558

Takahashi, K. & Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell*, 126, 4, 663-676

Takahashi-Tezuka, M.; Yoshida, Y.; Fukada, T.; Ohtani, T.; Yamanaka, Y.; Nishida, K.; Nakajima, K.; Hibi, M. & Hirano, T. (1998). Gab1 acts as an adapter molecule linking the cytokine receptor gp130 to ERK mitogen-activated protein kinase. *Molecular and cellular biology*, 18, 7, 4109-4117

Takao, Y.; Yokota, T. & Koide, H. (2007). Beta-catenin up-regulates Nanog expression through interaction with Oct-3/4 in embryonic stem cells. *Biochem Biophys Res Commun*, 353, 3, 699-705

Tesar, P. J.; Chenoweth, J. G.; Brook, F. A.; Davies, T. J.; Evans, E. P.; Mack, D. L.; Gardner, R. L. & McKay, R. D. G. (2007). New cell lines from mouse epiblast share defining features with human embryonic stem cells. *Nature*, 448, 7150, 196-199

Thomson, J. A.; Itskovitz-Eldor, J.; Shapiro, S. S.; Waknitz, M. A.; Swiergiel, J. J.; Marshall, V. S. & Jones, J. M. (1998). Embryonic stem cell lines derived from human blastocysts. *Science*, 282, 5391, 1145-1147

Tomioka, M.; Nishimoto, M.; Miyagi, S.; Katayanagi, T.; Fukui, N.; Niwa, H.; Muramatsu, M. & Okuda, A. (2002). Identification of Sox-2 regulatory region which is under the control of Oct-3/4-Sox-2 complex. *Nucleic Acids Res*, 30, 14, 3202-3213

Ura, H.; Usuda, M.; Kinoshita, K.; Sun, C.; Mori, K.; Akagi, T.; Matsuda, T.; Koide, H. & Yokota, T. (2008). STAT3 and Oct-3/4 control histone modification through induction of Eed in embryonic stem cells. *J Biol Chem*,

Wang, Y.; Wang, F.; Sun, T.; Trostinskaia, A.; Wygle, D.; Puscheck, E. & Rappolee, D. A. (2004). Entire mitogen activated protein kinase (MAPK) pathway is present in preimplantation mouse embryos. *Dev Dyn*, 231, 1, 72-87

Ware, C. B.; Horowitz, M. C.; Renshaw, B. R.; Hunt, J. S.; Liggitt, D.; Koblar, S. A.; Gliniak, B. C.; McKenna, H. J.; Papayannopoulou, T. & Thoma, B. (1995). Targeted disruption of the low-affinity leukemia inhibitory factor receptor gene causes placental, skeletal, neural and metabolic defects and results in perinatal death. *Development (Cambridge, England)*, 121, 5, 1283-1299

Watanabe, S.; Umehara, H.; Murayama, K.; Okabe, M.; Kimura, T. & Nakano, T. (2006). Activation of Akt signaling is sufficient to maintain pluripotency in mouse and primate embryonic stem cells. *Oncogene*, 25, 19, 2697-2707

Williams, D. C.; Cai, M. & Clore, G. M. (2004). Molecular basis for synergistic transcriptional activation by Oct1 and Sox2 revealed from the solution structure of the 42-kDa

Oct1.Sox2.Hoxb1-DNA ternary transcription factor complex. *J Biol Chem*, 279, 2, 1449-1457

Williams, R. L.; Hilton, D. J.; Pease, S.; Willson, T. A.; Stewart, C. L.; Gearing, D. P.; Wagner, E. F.; Metcalf, D.; Nicola, N. A. & Gough, N. M. (1988). Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. *Nature*, 336, 6200, 684-687

Wilmut, I.; Schnieke, A. E.; McWhir, J.; Kind, A. J. & Campbell, K. H. (1997). Viable offspring derived from fetal and adult mammalian cells. *Nature*, 385, 6619, 810-813

Yamanaka, S. (2008). Induction of pluripotent stem cells from mouse fibroblasts by four transcription factors. *Cell Prolif*, 41 Suppl 1, 51-56

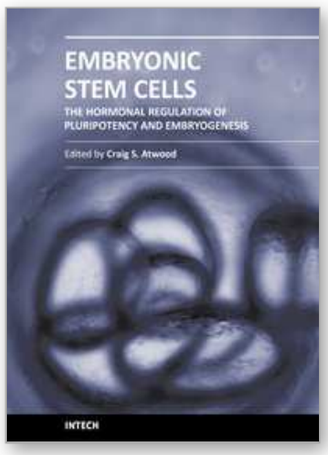
Ying, Q.-L. & Smith, A. G. (2003). Defined conditions for neural commitment and differentiation. *Meth Enzymol*, 365, 327-341

Ying, Q.-L.; Wray, J.; Nichols, J.; Batlle-Morera, L.; Doble, B.; Woodgett, J.; Cohen, P. & Smith, A. (2008). The ground state of embryonic stem cell self-renewal. *Nature*, 453, 7194, 519-523

Ying, Q. L.; Nichols, J.; Chambers, I. & Smith, A. (2003). BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. *Cell*, 115, 3, 281-292

Yuan, H.; Corbi, N.; Basilico, C. & Dailey, L. (1995). Developmental-specific activity of the FGF-4 enhancer requires the synergistic action of Sox2 and Oct-3. *Genes Dev*, 9, 21, 2635-2645

Zhong, X. & Jin, Y. (2009). Critical roles of coactivator p300 in mouse embryonic stem cell differentiation and Nanog expression. *J Biol Chem*, 284, 14, 9168-9175



Embryonic Stem Cells: The Hormonal Regulation of Pluripotency and Embryogenesis

Edited by Prof. Craig Atwood

ISBN 978-953-307-196-1
Hard cover, 672 pages
Publisher InTech
Published online 26, April, 2011
Published in print edition April, 2011

Pluripotency is a prerequisite for the subsequent coordinated differentiation of embryonic stem cells into all tissues of the body. This book describes recent advances in our understanding of pluripotency and the hormonal regulation of embryonic stem cell differentiation into tissue types derived from the ectoderm, mesoderm and endoderm.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Elisa A. Casanova, Kurt Bürki and Paolo Cinelli (2011). Molecular Mechanisms of Pluripotency in Murine Embryonic Stem Cells, Embryonic Stem Cells: The Hormonal Regulation of Pluripotency and Embryogenesis, Prof. Craig Atwood (Ed.), ISBN: 978-953-307-196-1, InTech, Available from: http://www.intechopen.com/books/embryonic-stem-cells-the-hormonal-regulation-of-pluripotency-and-embryogenesis/molecular-mechanisms-of-pluripotency-in-murine-embryonic-stem-cells

INTECH
open science | open minds

InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

IntechOpen