Int. J. Dev. Biol. 57: 667-675 (2013)
doi: 10.1387/ijdb.130064dd

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

Signaling pathways dictating pluripotency in embryonic stem cells

DEBASREE DUTTA*

Cancer Research Program, Rajiv Gandhi Centre for Biotechnology, Kerala, India

ABSTRACT Embryonic Stem Cells (ESCs) are derived from the inner cell mass of blastocysts. They have the unique potency to differentiate into diverse lineages. Hence, they are bestowed with the term pluripotency. Several mechanisms have been implicated in maintaining the pluripotency of ESCs. This review will focus on the role of signaling pathways in regulating ESC pluripotency among diverse mammalian species. A novel phylogenetic approach has been designed to understand the structural basis of divergence in the signaling pathways which modulate pluripotency among different species. Detailed insight into different signaling mechanisms indicates inhibition of Extracellular Related Kinase 1/2 (ERK 1/2) signaling as the key component regulating the pluripotency of ESCs. On the basis of recent advances made in this field, it can be hypothesized that expression of the transcription factor KLF4 and inhibition of ERK signaling may promote the establishment and maintenance of true ESCs from different mammalian species.

KEY WORDS: *blastocyst, embryonic stem cell, signaling pathway, ERK, KLF4*

Introduction

The process of embryogenesis is initiated with the fertilization of a sperm with an ovum. The one-celled embryo divides to form the blastocyst (Fig. 1) comprising of inner cell mass (ICM) and trophectoderm. Within the ICM, an epiblast layer of cells form the inner core sandwiched between polar trophblast and hypoblast cells (Fig. 1). This region further develops *in vitro* into ESC. Back in 1981, Evans and Kaufman were the first to derive mouse ESCs (mESCs) from the mouse blastocyst (Evans & Kaufman, 1981). It took more than a decade to establish the first pluripotent human ESCs (hESCs) (Thomson *et al.,* 1998) and primate ESCs (Thomson *et al.,* 1995). ESCs have the inherent capacity to differentiate into any lineage and hence are termed as pluripotent. Also, they have the intrinsic potential to divide or self-renew indefinitely. These two characteristics formed the basis of exploiting ESCs for genetic manipulations (various genetic disorders) or in regenerative medicine and tissue replacement after injury or diseases. So, it is extremely significant to understand the mechanism responsible for the maintenance of pluripotency in ESCs.

Signaling molecules, transcription factors, cell cycle regulators and epigenetic modifications regulates intricate molecular mechanism of maintaining pluripotency of ESCs among wide range of mammalian species. Signaling mechanisms regulating the expression of pluripotency factors POU5F1, NANOG, SOX2 and KLF4 significantly differ in the context of species specification. Both extrinsic and intrinsic factors contribute towards the regulatory mechanisms involved in pluripotency. Extrinsic factors like Leukemia Inhibitory Factor (LIF), Bone morphogenic protein 4 (BMP4) or basic Fibroblast growth factor (bFGF) when supplemented to the ESC culture, induce signals that are transmitted through intracellular components and regulate the expression of pluripotency factors (Pera & Tam, 2010). Intrinsic factors like ERK are present within the cell and in their active form generally induce the differentiation of mESCs. Hence, inhibition of these signaling pathways could maintain the pluripotency of mESCs.

ESCs have been isolated from mice to men. In spite of the importance of farm animals in agricultural and pharmaceutical application, the generation of true ESCs from these animals is still elusive. Animal models mimicking human anatomy are severely required to evaluate the safety and efficacy of cell therapies. But, due to technical problems in culturing and deriving ESCs from these animals and their inability to contribute to germ line, a detailed study on maintaining pluripotency of ESCs has been limited to fewer cases.

This review deals with signaling pathways implicated in maintain-

Abbreviations used in this paper: EpiSC, epiblast stem cell; ERK, extracellular related kinase; ESC, embryonic stem cell.

***Address correspondence to:** Debasree Dutta. Cancer Research Program, Rajiv Gandhi Centre for Biotechnology, Thycaud PO, Poojappura, Thiruvananthapuram, Kerala, India 695014. Tel: 0471-252-9465. Fax: 0471-234-8096. E-mail: debasreedutta@rgcb.res.in

Accepted: 5 June 2013. *Final, author-corrected PDF published online:* 4 November 2013.

ISSN: Online 1696-3547, Print 0214-6282

ing pluripotency in ESCs isolated from different mammalian species. A novel phylogenetic approach has been included to provide a structural basis of the differential role of signaling pathways. The combinatorial role of a signaling pathway and transcription factor has been hypothesized to promote the maintenance and derivation of true ESCs from different mammalian species.

Intrinsic factors in regulation of pluripotency

ERK signaling

ESCs have an intrinsic property to differentiate spontaneously. So, inhibition of differentiation maintains the self-renewal character of mESCs. ERK1/2 signaling autoinductive stimulation by Fibroblast growth factor or FGF4 is implicated in inducing differentiation of mESCs. Suppression of FGF4/ERK signaling pathway promotes the self-renewal of mESCs (Ying *et al.,* 2008). But, inhibition of FGF/ERK signaling resulted in degeneration of mESC culture (Ying *et al.,* 2008). So, to maintain metabolic activity, biosynthetic capacity and overall viability of mESCs, CHIR99021 (CH) is introduced in the culture condition to (Ying *et al.,* 2008) inhibit Glycogen synthase kinase 3 or GSK3 signaling. It could inhibit the differentiation towards neuronal lineage (Ying *et al.,* 2008; Bechard & Dalton, 2009) as well. Hence, dual inhibition of ERK and GSK3 signaling by PD0325901 (PD) and CH respectively restored the pluripotency in mouse as well as rat ESCs in a feeder free condition (Ying *et al.,* 2008; Bechard & Dalton, 2009; Buehr *et al.,* 2008; Li *et al.,* 2008).

Pluripotency in ESCs is maintained by a set of transcription factors. They function through feedback regulatory circuit positively regulating their own genes. These factors activate genes encoding critical components of pluripotency while repress genes important for developmental processes. Maintenance of self-renewal and pluripotency of ES cells require a complex network of transcription factors namely *Pou5f1*, *Sox2* and *Nanog* (Niwa *et al.,* 2000; Masui *et al.,* 2007; Chambers *et al.,* 2007; He *et al.,* 2009). Another factor *Klf4* was earlier found to be dispensable (Nakatake *et al.,* 2006). But, a triple RNAi approach of knocking down *Klf2, Klf4* and *Klf5*, established the role of *Klf* circuitry in maintaining self-renewal state of mESCs (Jiang *et al.,* 2008). ERK1/2 binds at the C-terminal domain of KLF4 and phosphorylates it at Ser123 residue in mESCs (Kim *et al.,* 2012). This phosphorylation downregulate the transcriptional activity of *Klf4* and induces differentiation of mESCs. Thus, inhibition of ERK signaling enhances *Klf4* activity and in turn maintains the undifferentiated state of mESCs (Kim *et al.,* 2012).

On the contrary, downstream to FGF signaling, activation of ERK-signaling is associated with maintenance of pluripotency in hESCs.

PKC signaling

Inhibition of protein kinase C (PKC) isoforms maintain pluripotency in mESCs without the activation of STAT3 or inhibition of ERK/GSK3 signaling pathways (Dutta *et al.,* 2011). Atypical PKC isoform, PKCζ function is involved in the activation of *Nfκb* pathway during mESC differentiation. On inhibition of PKC signaling by the pharmacological drug, Gö6983, downregulation of *Nfκb* target genes were observed (Dutta *et al.,* 2011). Thus, an appropriate activity of PKC-signaling directly balances the self-renewal and differentiation status in mESCs and in rat ESCs as well (Rajendran *et al.,* 2013).

Aurora Kinase A/p53 signaling

Phosphoregulators play a key role in signal transduction. Screening of shRNAs for mESC-associated phosphoregulators revealed an essential role of Aurora kinase A or Aurka in maintaining the self-renewal state of mESCs (Lee *et al.,* 2012). Loss of Aurka is coupled to stimulation in p53 activity and hence induces differentiation of ESCs towards ectoderm and mesoderm lineage (Lee *et al.,* 2012). Thus, Aurka mediated p53 phosphorylation is essential for maintaining self-renewal and pluripotency in mESCs. But, only future studies will establish whether the function of Aurka is conserved among species or not.

Extrinsic factors in regulation of pluripotency

LIF and JAK/STAT3 signaling

Mouse ESCs can be cultured *in vitro* in an undifferentiated state in presence of LIF and serum (Smith *et al.,* 1988). LIF on binding to the receptor activates Janus kinase (JAK) and phosphorylates STAT3 at Tyr705 (Zhong *et al.,* 1994; Niwa *et al.,* 1998). STAT3 essentially maintains the c-MYC level (Cartwright *et al.,* 2005) and induces the KLF4 expression (Hall *et al.,* 2009) in mESCs. Additionally, Stat3 induced Pramel7 activity blocks the phosphorylation of ERK (Casanova *et al.,* 2011). Recently zeta-chain (TCR) associated protein kinase or Zap70, a Syk (spleen tyrosine kinase) family tyrosine kinase, has been found to negatively regulate the JAK/STAT3/c-MYC pathway and thus modulate the self-renewal capacity and differentiation ability of mESCs (Cha *et al.,* 2010). Zap70 interacts with SHP1 phosphatase and inhibits the phosphorylation of JAK, which in turn downregulate the STAT3 dependent c-MYC induction (Cha *et al.,* 2010).

Interestingly, LIF is unable to maintain the undifferentiated state of hESCs. Rather, in pluripotent hESCs, STAT3 do not localize within the nucleus and is incapable to activate its target genes (Dahéron *et al.,* 2004).

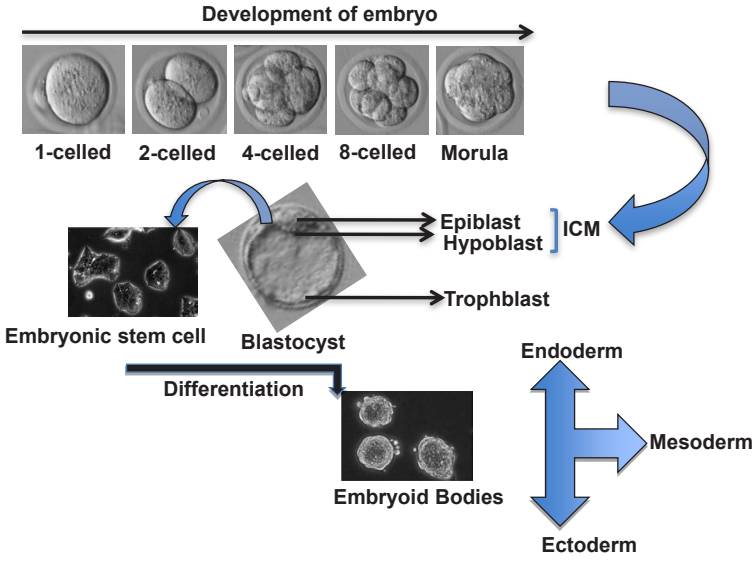

Fig. 1. Derivation and fate of Embryonic stem cells. *Single-celled embryo develops to form blastocyst composed of ICM and trophectoderm. ESCs are derived from the ICM and maintained in an undifferentiated state. Pluripotent ESCs differentiate into different lineages (endoderm, mesoderm and ectoderm) via the formation of embryoid bodies.*

BMP4 signaling

In absence of serum, LIF alone could not maintain the undifferentiated nature of mESCs. But when mESCs are cultured in presence of BMP4 and LIF, they retain the undifferentiated status (Ying *et al.,* 2003). Dimeric BMP4 binds to type II receptor BMPR2 and facilitates the assembly of receptor heteromers (Yu *et al.,* 2005). The constitutively active kinase domains of type II receptors phosphorylate type I receptors, BMPR1 (BMPR1A, earlier known as ALK3 or BMPR1B, earlier known as ALK6) and the type I receptors in turn phosphorylate either of the receptor SMADs (SMAD1or SMAD5 or SMAD8) (Heldin *et al.,* 1997; Kawabata *et al.,* 1998; Derynck & Zhang, 2003). SMAD4 joins as the co-activator to either of the R-SMADs to form the heteromeric complex (Derynck & Zhang, 2003). Phosphorylated receptor-SMAD induces the expression of *Id* genes and represses genes implicated in neuroectoderm like *Neurod*, *Ascl1* (Ying *et al.,* 2003). Downstream to SMAD2, BMP4 signaling modulates the H3K27 demethyase, *Kdm6b* and Dihydropyrimidinase-like 2 or *Dpysl2* in maintaining pluripotency of mESCs (Fei *et al.,* 2010a).

Additionally, BMP4 can steadily attenuate the ERK activity by upregulating ERK-specific dual specificity phosphatase 9 (DUSP9) particularly in mESCs (Li *et al.,* 2012; Qi *et al.,* 2004).

Interestingly, the definite role of BMP4 in regulating pluripotency of hESCs is still unresolved. Earlier, it was demonstrated that BMP4 induces differentiation of hESCs towards extra-embryonic lineages specifically trophblasts and inclusion of Noggin in culture condition prevents the differentiation of hESCs towards the extra-embryonic lineage (Xu *et al.,* 2002). But, in 2011, Bernardo *et al.,* showed that BMP4 is responsible for the induction of differentiation towards mesoderm wherein the cells do express trophblast specific markers but to a lower extent (Bernardo *et al.,* 2011). However, recently, Amita *et al.,* reported the generation of trophoblast specific cells from hESCs in presence of only BMP4 (Amita *et al.,* 2013). The differentiation was induced in a culture condition similar to the one used by Bernardo *et al.,* (2011) although with a reduced level of expression of mesodermal markers. Hence, further validation is required to establish the role of BMP4 signaling in pluripotency of hESCs.

Wnt/β-catenin signaling

The canonical Wnt-pathway has been implicated in pluripotency of mESCs. According to the newly established model of Wnt signaling, Wnt induces the association of the destruction complex composed of DVL1, AXIN, APC, GSK3 and phosphorylated β-catenin with phosphorylated LRP (Clevers & Nusse, 2012). This complex phosphorylates β-catenin but further blocks the ubiquitination by β-TrCP. As a result, newly synthesized β-catenin is accumulated in the cytosol and translocated into the nucleus (Clevers & Nusse, 2012). The β-catenin/CBP complex binds to its target *Tcf* and *Lef* and induces the expression of *Stat3* mRNA (Miyabayashi *et al.,* 2007; Hao *et al.,* 2006). Independent of *Tcf*/*Lef* binding, stabilized β-catenin enhances *Pou5f1* activity for the maintenance of pluripotency in mESCs (Kelly *et al.,* 2011).

In hESCs, it was reported that activating the Wnt/ β-catenin pathway with either WNT3A or a GSK3 inhibitor, BIO ((2'Z,3'E)-6-Bromoindirubin-3'-oxime), maintained the self-renewal of hESCs under feeder-free conditions (Sato *et al.,* 2004). Conversely, there have been reports where WNT3A or GSK3 inhibitors lead to differentiation of hESCs toward primitive streak and definitive endoderm lineages (Bone *et al.,* 2011; Nakanishi *et al.,* 2009). So, the role of Wnt/β-catenin signaling in hESCs is controversial. Recently, Wnt/β-catenin signaling has been found to be inactive in the self-renewal of hESCs (Davidson *et al.,* 2012). During self-renewal in hESCs, POU5F1 reportedly repress β-catenin signaling. So, targeted knockdown of POU5F1 activated β-catenin signaling in hESCs (Davidson *et al.,* 2012). A fluorescent reporter of β-catenin revealed that the enhanced expression of β-catenin is associated with induced differentiation in hESCs (Davidson *et al.,* 2012). Intriguingly, the fate of differentiation of hESCs corresponding to endogenous Wnt signaling is based on the heterogeneous culture of hESCs (Blauwkamp *et al.,* 2012). Human ESCs with a higher expression of Wnt predominantly differentiated towards endodermal and cardiac fates whereas hESCs expressing lower level of Wnt generated cells primarily from neuroectodermal lineage.

This phenomenon further incites a very significant characteristic of ESCs, that is, heterogeneity in culture condition. Basically, this inherent property of ESCs further regulates the response towards different signaling mechanisms and hence will be discussed in a later section.

FGF and Nodal/Activin signaling

FGF signaling is associated with induction of differentiation in mouse and rat ESCs (Ying *et al.,* 2008; Bechard & Dalton, 2009). Activin/Nodal signaling has been reported to promote mESC proliferation (Ogawa *et al.,* 2007) and has been implicated especially in commitment towards mesendoderm (Fei *et al.,* 2010b).

But, FGF signaling could sustain the undifferentiated nature of hESCs. When cultured on feeder layers, hESCs maintain the undifferentiated state only in presence of bFGF (Thomson *et al.,* 1998; Levenstein *et al.,* 2006). Exogenously added bFGF binds to the FGF receptors and activate ERK1/2 signaling responsible for maintaining pluripotency in hESCs (Kang *et al.,* 2005). FGF signaling also inhibits the spontaneous differentiation towards extra-embryonic lineage or neural-induction in hESCs (Greber *et al.,* 2011). Exogenous addition of bFGF induces the expression of insulin growth factor II (IGF-II) from autologously derived hESC fibroblast-like cells (hdFs) (Bendall *et al.,* 2007). The interaction between IGFR1/IGF-II contributes to the maintenance of stemness in hESCs (Bendall *et al.,* 2007).

When cultured on MEFs, addition of bFGF further activates the Nodal/Activin signaling by activating Activin A receptors (Greber *et al.,* 2007). Activin A receptors further phosphorylate SMAD2/3 and form a complex with co-activator SMAD4. The SMAD-complex induces the expression of NANOG in the nucleus and supports the maintenance of pluripotency of hESCs (Vallier *et al.,* 2009a).

PI3 kinase/AKT signaling

Phosphoinositide 3-kinase (PI3K) pathway is important for proliferation, survival, and maintenance of pluripotency in ESC. On activation of PI3K, secondary messengers are produced which transmit their effect through AKT, serine threonine kinase. AKT is implicated in different aspects of cellular metabolism and tumorigenesis. Earlier, a LIF-dependent activation of PI3K pathway was found to be responsible for regulating pluripotency in murine ESCs. On binding of LIF, gp130 is activated. The activated receptor then induces PI3K to phosphorylate PKB/AKT which in turn influences its downstream effectors to maintain the pluripotency in mESCs (Paling *et al.,* 2004). Later, in 2006, the active myristolated form

of AKT was reported to maintain ESC self-renewal independent of LIF or BMP4 or Wnt/β-catenin signaling (Watanabe *et al.,* 2006). Activated PI3K/AKT might inhibit GSK3β signaling and hence could maintain the pluripotency in mouse as well as in primate (monkey) ESCs (Watanabe *et al.,* 2006).

Earlier reports suggested that inhibition of PI3K signaling promotes endodermal and mesodermal differentiation in hESCs (Mclean *et al.,* 2007). But, recently it was demonstrated that in hESCs, PI3/AKT signaling promotes self-renewal by restricting pro-differentiation cues (Singh *et al.,* 2012). In presence of IGF2/ Hergulin and Activin, hESC maintain the self-renewal state. IGF2/ Hergulin is responsible for the activation of PI3K/AKT signaling. Elevated PI3K/AKT activity induces SMAD2 and SMAD3 accumulation and the expression of the target gene NANOG (Singh *et al.,* 2012). Additionally, AKT binding to c-RAF provokes dephosphorylation of ERK. This further inhibits the interaction of ERK with GSK3 resulting in its dephosphorylation. GSK3 signaling in turn inhibits the Wnt/β-catenin pathway (Singh *et al.,* 2012) and thus maintains the undifferentiated state of hESCs. Hence the crosstalk between different signaling pathways could mediate pluripotency in hESCs.

Signaling pathways implicated in pluripotency of ESCs from other animals

Even after 30 years of the first successful derivation of mESC (Evans & Kaufmann, 1981), no convincing pluripotent embryonic stem cells have been reproducibly established from non-rodent or non-primates. Present literature demonstrates the establishment of only ES-like cell lines from these animals as they are deprived of major characters associated with pluripotency or true chimerism (Gandolfi *et al.,* 2012). Different porcine, canine, rabbit, bovine, ovine and cat ES like cells were isolated and cultured. Most of these ES like cells thrive on bFGF signaling for maintenance of pluripotency. Based on the response towards bFGF signaling, they might resemble hESCs rather than mESCs. Signaling pathways implicated in maintaining pluripotency of ES-like cells from diverse mammalian species have been summarized in Table 1. Interestingly, few ES-like cell lines from these domesticated animals require both bFGF and LIF for the maintenance of pluripotency. So, Gandolfi *et. al.* in a recent review concluded that a synergistic action of bFGF and LIF on the expression of NANOG promoted the derivation of ES-like cells (Gandolfi *et al.,* 2012). As robust ESCs from these species are difficult to derive and maintain, so somatic cells from these species have been reprogrammed to induced pluripotent stem cells (iPSCs) with different set of genes (Takahashi *et al.,* 2007). Most frequently but not exclusively, four genes *OCT4*, *SOX2*, *KLF4* and *c-MYC* are expressed in these somatic cells like fibroblast to generate iPSCs. These cells exhibit a pluripotent phenotype resembling ESCs and hence can be used as an alternative to study pluripotency within these species. A recent review by Ezhashi *et al.,* has summarized the characteristics of different set of iPSC lines generated from these animals (Ezhashi *et al.,* 2012).

Structural aspect for divergence

Different signaling mechanisms are implicated in maintaining pluripotency among a wide range of mammalian species. As mentioned earlier, recent evidences indicated that culture of ESCs is a heterogeneous mix of population of cells representing different state and thus fluctuate in the potency to differentiate into different lineages. This heterogeneity is present in both mESCs (Hayashi *et al.,* 2008) and hESCs (Hough *et al.,* 2009). Signaling molecules invoke varied responses from these heterogeneous population of cells in culture condition. But, in a broader sense, hESCs differ from mESCs in various aspects including colony morphology, capacity for colonizing pre-implantation embryo, epigenetic stability among others. Intriguingly, hESCs share common gene expression pattern and signaling responses to stems cells isolated from late epiblast layer of post-implantation mouse embryo, called Epiblast stem cells or EpiSCs (Brons *et al.,* 2007; Tesar *et al.,* 2007). The mEpiSC lines are distinctively different from mESCs both in their epigenetic state as well as signaling responses regulating their differentiation. Mouse EpiSCs maintain the genomic integrity and can be propagated to major somatic cell types. Unlike mESCs

TABLE 1

SIGNALING PATHWAYS IN MAINTAINING PLURIPOTENCY OF ESCs AND ES-LIKE CELLS

	Species											
Signaling pathways	**Mouse**	**Rat**	**Rabbit**	**Dog**	**Cat**	**Horse**	**Pig**	**Sheep**	**Bovine**	**Monkey**	**Human**	**References**
Factors intrinsic												
FGF/ERK	D/I	D/I	D/-	ND	ND	ND	ND	ND	ND	ND	D/A	Ying *et al.* 2008; Buehr *et al.* 2008; Li *et al.* 2008; He *et al.* 2009; Greber *et al.* 2011; Honda *et al.* 2009
GSK3	D/I	D/I	ND	ND	ND	ND	ND	ND	ND	ND	D/I	Ying *et al.* 2008; Bechard & Dalton *et al.* 2009; Buehr *et al.* 2008; Singh *et al.* 2012
PKC	D/I	D/I	ND	ND	ND	ND	ND	ND	ND	ND	ND	Dutta *et al.* 2011; Rajendran *et al.* 2013
Arora Kinase A/p53	D/A	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	Lee *et al.* 2012
Factors extrinsic												
LIF and JAK/STAT	D/+	D/-	D/+/-	D/+/-	D/+/-	D/+	D/+/-	D/+/-	D/-	D/-	D/-	Evans & Kaufman, 1981; He *et al.* 2009; Pera & Tam, 2010; Daheron *et al.* 2004; Telugu *et al.* 2011; Behboodi *et al.* 2011; Saito *et al.* 2002; Hayes *et al.* 2008; Maruotti *et al.* 2012; Gandolfi *et al.* 2012; Zhao *et al.* 2011
BMP4/Smad	D/+	D/-	D/-	D/-	ND	ND	ND	ND	ND	D/-	D/-	Ying *et al.* 2003; Tan *et al.* 2011; Kobayashi *et al.* 2008; Ueda *et al.* 2008, Xu *et al.* 2002
Wnt/β-catenin	D/+	ND	D/-	ND	ND	ND	ND	ND	ND	ND	D/-	Miyabayashi *et al.* 2007; Davidson et al. 2012; Hoffmeyer *et al.* 2012; Wang *et al.* 2008
bFGF/Activin/Nodal	D/-	ND	D/+	D/+	D/+	D/-	D/+	D/+	D/+	ND	D/+	Fei *et al.* 2010; Levenstein *et al.* 2006;, Gandolfi *et al.* 2012; Honda *et al.* 2009; Hayes *et al.* 2008; Zhao *et al.* 2011
PI3K/AKT	D/+	ND	ND	ND	ND	ND	ND	ND	ND	D/+	D/+	Paling et al. 2004; Watanabe *et al.* 2006; Singh *et al.* 2012

D- Determined; ND- Not Determined; I- Inhibition; A- Activation; + positively modulate; +/- positively modulate but need other factors; - negatively modulate by inducing differentiation.

but alike hESCs, mEpiSCs are derived and cultured in presence of bFGF/Activin (Brons *et al.,* 2007; Tesar *et al.,* 2007; Grebar *et al.,* 2010). The pluripotency of mEpiSCs is associated with the expression of classical pluripotent markers, *Nanog*, *Pou5f1* and *Sox2* (Brons *et al.,* 2007; Tesar *et al.,* 2007). Hence, a proper characterization of the pluripotent state of mEpiSCs would further enhance our understanding of hESCs. A comparative study of differential characteristics among hESCs vs. mEpiSCs is summarized in Table 2. Mouse EpiSCs culture comprises of both early and late epiblast cells (Han *et al.,* 2010). They express different sets of markers and accordingly, only the minor fraction of early EpiSCs contribute to chimera (Han *et al.,* 2010). This further emphasizes the importance of heterogeneity in culture conditions.

ESCs from species other than mouse and rat might represent the pluripotent state of mEpiSC, as they resemble hESCs. Thus, two distinct phases exist in pluripotency: one that is represented by mESCs is referred as naïve state while the other corresponding to EpiSCs is referred as primed state (Nichols &Smith, 2009, 2011; De Los Angeles *et al.,* 2012). The naïve state refers to those cells that contribute towards the chimeric embryo, maintain X chromosome activation in the female cells and are comparatively refractory to differentiate towards primordial germ cells or PGCs (Nichols & Smith, 2009, 2011). They can be cloned with high efficiency, form dome shaped colonies, are responsive to LIF/STAT3 signaling and destabilized by bFGF and TGFβ/Activin signaling (Nichols & Smith, 2009, 2011). On the contrary, primed pluripotent state refers to those cells that have a limited capacity for colonizing preimplantation embryo; undergone X chromosome inactivation (XiXa) within the female cells and is poised for differentiation to PGCs (Nichols & Smith, 2009, 2011). Their clonal propagation is inefficient, form flattened colonies, are stabilized by bFGF and TGFβ/Activin signaling and are non-responsive to LIF/STAT3 signaling (Nichols & Smith, 2009, 2011). Human ESCs sharing similar characteristics with mEpiSCs, represent the primed state. Even ESCs from Rhesus monkey exist in the primed state of pluripotency as they failed to colonize the pre-implantation blastocyst (Tachibana *et al.,* 2012). So, in effect, the derivation of naïve ESCs from domesticated animals and primates still remain

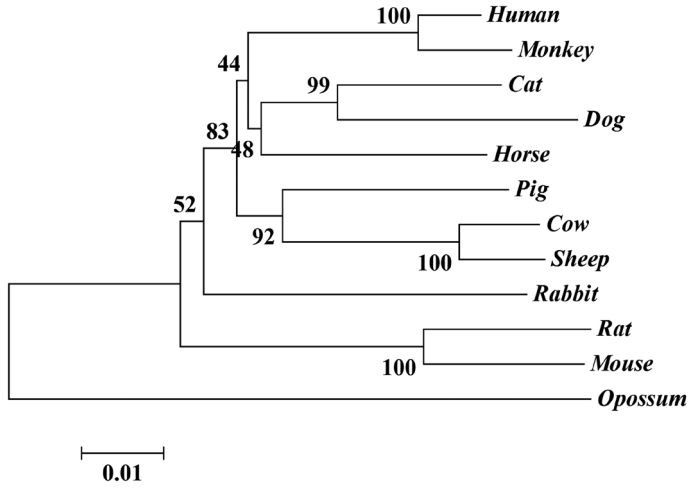

Fig. 2. Phylogenetic tree: point of divergence. *Phylogenetic relationship among mammals based on* Stat3 *gene sequences. The phylogenetic tree is deduced using Mega (Kumar* et al.*, 2004) software after multiple alignments of gene sequences with ClustalW (Thompson* et al.*, 1994). Distances (distance options according to the Jukes-Cantor model) and clustering with the neighbour-joining method was determined by using bootstrap values (Felsenstein, 1985) based on 100 replications. The* Stat3 *sequence from Opossum is used as the outgroup. Numbers at nodes indicate bootstrap values. Bar, 1 substitution per 100 nucleotides.*

elusive. Mouse ESCs are considered to present the naïve state of pluripotency, but while in culture, an intermediate population called intermediate epiblast state cells (IESCs) has been found to exist (Chang & Li, 2013). IESCs are responsive towards both LIF/STAT3 as well as Activin/Smad2/3 signaling (Chang & Li, 2013). So, even naïve mESCs are prone to spontaneous differentiation to an intermediate state corresponding to neither naïve nor primed.

Depending on the differential responses of ESCs or ES-like cells to LIF/STAT3 signaling, a phylogenetic approach has been designed to understand the structural basis of this divergence among different mammalian species. Multigene and multiprotein

TABLE 2

HUMAN ESCs *vs.* MOUSE EpiSCs

	Human ESCs	Mouse EpiSCs	References
Characteristics (differential)			
Source	Peri-implantation embryo	Post-implantation as well as pre-implantation embryo	Thompson *et al.* 1998; Brons *et al.* 2007; Tesar *et al.* 2007
Chimera generation	No	Only with pre-implantation (E5.5) and not from post-implantation (E6.5/E7.5)	Thompson *et al.* 1998; Brons *et al.* 2007; Tesar *et al.* 2007; Najm *et al.* 2011
KLF4	Expressed	Virtually absent or weakly present	Nichols & Smith, 2009; Chan et al. 2009
REX1	Expressed	Absent	Greber *et al.* 2010
FGF5	Absent	Expressed	Greber *et al.* 2010
Signaling pathway			
LIF and JAK/STAT3	Independent	Independent	Dahèron *et al.* 2004; Brons *et al.* 2007; Tesar *et al.* 2007
BMP4	Differentiate towards trophblast or mesoderm	Differentiate towards extraembryonic cells	Xu *et al.* 2002; Blauwkamp *et al.* 2012; Amita *et al.*, 2013; Tesar *et al.* 2007
BMP4 (High Activin & bFGF)	Mesendoderm	Mesendoderm	Vallier *et al.* 2009b
bFGF in pluripotency	Induces NANOG expression	Inhibits differentiation towards neuroectoderm and reversion to mESC by suppressing KLF2	Greber *et al.* 2011; Greber *et al.* 2010
bFGF in differentiation (-Activin & BMP)	Neuroectoderm	Neuroectoderm	Vallier *et al.* 2009b
bFGF/Activin/Nodal	Maintain Pluripotency	Maintain Pluripotency	Thompson *et al.* 1998; Brons *et al.* 2007; Tesar *et al.* 2007
FGF/ERK inhibition	Death or differentiation	Revert to mESCs	Nichols & Smith, 2009; Guo *et al.* 2009; Roode *et al.* 2012

studies revealed that phylogenetically, human and monkeys are the nearest relative to the rodents while they are distantly related to the domesticated animals (Hedges, 2002). But, the phylogenetic tree derived from the alignment of *Stat3* gene sequences represents a different picture (Fig. 2). The phylogenetic tree in Fig.2 demonstrates that human and monkeys are closer to the farm animals and not rodents. Even rabbit branches with the cluster composed of these farm animals rather than the rodents. As ES like cells from domesticated animals resemble the hESCs and are responsive to bFGF signaling, this phylogenetic relationship further suggests that they might be distinct from mESCs. Hence, the phylogenetic tree predicts that unless manipulated, ESCs from the domesticated animals and rabbit might not respond to the LIF/STAT3 signaling (Fig. 2). Thus, the structural basis demonstrating a probable picture of deviation in signaling pathways implicated in pluripotency among diverse mammalian species could replicate the experimental findings.

Common pathway in deriving and culturing true ESCs?

So, the next question arises that in spite of spending a considerable effort on deriving and culturing ESCs from livestock for decades, what is the basic hurdle in achieving the promise of true stem cells in agriculture and how can we conquer it? The answer might lie in the fact that there is a basic difference in the development of embryo within different mammalian species (Nichols & Smith, 2009). During early development of embryo, the opportunity for capturing the transient ground state in non-rodents is minimized as compared to rodents (Nichols & Smith, 2009). Additionally, absence of diapause in primates further alleviates ESC derivation (Nichols & Smith, 2009). Despite this hurdle in generating naïve pluripotency, number of successful *in vitro* experiments has been performed to revert primed state into naïve state. Re-expression of the transcription factor KLF4 is essential in the regeneration of naïve mESCs from primed mEpiSCs (Guo *et al.,* 2009) or naïve hESCs from primed hESCs (Hanna *et al.,* 2010). Forskolin or Kellpaullone are used in culture during reversion from primed to naïve state to induce the KLF4 expression (Hanna *et al.,* 2009, 2010). Downstream to JAK/STAT3 signaling, an induced *Klf4* expression along with *Pou5f1* could establish and maintain naïve pluripotency (van Oosten *et al.,* 2012). Additionally, KLF4 regulate the TERT subunit of telomerase, which is required for the maintenance of pluripotency in mESCs (Hoffmeyer *et al.,* 2012). Intriguingly, true porcine ESCs have been derived by the overexpression of KLF4 in the ICM of pig blastocyst (Telugu *et al.,* 2011). These experimental evidences strongly justify the substantial role of *Klf4* in isolating or maintaining true stemness of ESCs. Expression of *Klf4* is further regulated by ERK signaling (Kim *et al.,* 2012). In addition to *Klf4* regulation, inhibition of ERK signaling is associated with the existence of mESCs in ground state of pluripotency (Nichols *et al.,* 2009). In non-permissive NOD mouse strain, inhibition of ERK/GSK3 (2i) signaling enabled the derivation of naïve mESCs (Hanna *et al.,* 2009). Inhibition of ERK signaling is even associated with LIF/STAT3 or BMP4/SMAD signaling or in the biallelic expression of *Nanog* in mESCs (Miyanari & Torres-Padilla, 2012).

On the other hand, inhibition of ERK signaling promotes death or differentiation in hESCs (Nichols & Smith, 2009; Guo *et al.,* 2009). But interestingly, inhibition of ERK signaling has been found to promote establishment of naïve hESCs from primed hESCs or in the generation of naïve hiPSCs. Yamanaka factors, especially KLF4, along with LIF/2i could generate mESC-like hiPSCs (Hanna *et al.,* 2009) while, generation of FGF2-dependent hiPSC is independent of KLF4 expression (Huangfu *et al.,* 2008).

Intriguingly, inhibition of ERK signaling is responsible for the establishment of ground state of pluripotency from the mouse blastocyst. But this role of ERK signaling has been found to be highly species specific. The epiblast/hypoblast differentiation from the common ICM precursor is dependent on FGF/ERK signaling in mouse (Feldman *et al.,* 1995). An inhibition of ERK signaling induces NANOG expression in the epiblast cells and shuts off GATA6 in the hypoblast cells (Feldman *et al.,* 1995). In bovine blastocyst, ERK inhibition could induce NANOG expression without affecting hypoblast formation (Kuijk *et al.,* 2012). But, the segregation of hypoblast from epiblast in human embryo is independent of FGF signaling (Roode *et al.,* 2012). On inhibition of ERK signaling, there is no repression of GATA4 expression. Interestingly, without any deteriorating effect, 2i could still retain the NANOG positive epiblast cells (Roode *et al.,* 2012). This observation significantly indicates that FGF/ERK signaling independent derivation of naïve hESCs might be possible in future.

Conclusion

Defined intrinsic and extrinsic regulations contribute to the maintenance of pluripotency in ESCs from different species. But, neither of them could be universally implicated in maintaining the self-renewal or pluripotent state of ESCs from the entire range of mammalian species. Supported by experimental evidences, this review hypothesized that an induced expression of KLF4 in the ICM of non-permissive strains of non-rodent/primate/human blastocyst followed by an inhibition of ERK signaling might promote naïve state of pluripotency in ESCs. The requirement of a combinatorial action of these two factors is reflected even during the reversion of primed EpiSCs to naïve ESCs. In future, it will be highly significant to understand how ERK signaling and KLF4 overexpression could induce the generation of naïve state of pluripotency in hESCs.

Acknowledgement

This work is partially supported by grant from Department of Biotechnology, India (#BT/PR5754/MED/31/167/2012). I thank Dr. Ananda Mukherjee for his critical comment on the manuscript.

References

AMITA M, ADACHI K, ALEXENKO AP, SINHA S, SCHUST DJ, SCHULZ LC, ROBERTS RM, EZASHI T (2013). Complete and unidirectional conversion of human embryonic stem cells to trophoblast by BMP4. *Proc Natl Acad Sci USA* 110: E1212-1221.

BECHARD M, DALTON S (2009). Subcellular localization of glycogen synthase kinase 3beta controls embryonic stem cell self-renewal. *Mol Cell Biol* 29: 2092-2104.

BEHBOODI E, BONDAREVA A, BEGIN I, RAO K, NEVEU N, PIERSON JT, WYLIE C, PIERO FD, HUANG YJ, ZENG W, TANCO V, BALDASSARRE H, KARATZAS CN, DOBRINSKI I (2011). Establishment of goat embryonic stem cells from *in vivo* produced blastocyst-stage embryos. *Mol Reprod Dev* 78: 202-211.

BENDALL SC, STEWART MH, MENENDEZ P, GEORGE D, VIJAYARAGAVAN K, WERBOWETSKI-OGILVIE T, RAMOS-MEJIA V, ROULEAU A, YANG J, BOSSÉ M, LAJOIE G, BHATIA M (2007). IGF and FGF cooperatively establish the regulatory stem cell niche of pluripotent human cells in vitro. *Nature* 448: 1015-1021.

BERNARDO AS, FAIAL T, GARDNER L, NIAKAN KK, ORTMANN D, SENNER CE, CALLERY EM, TROTTER MW, HEMBERGER M, SMITH JC, BARDWELL L,

MOFFETT A, PEDERSEN RA (2011). BRACHYURY and CDX2 mediate BMP-induced differentiation of human and mouse pluripotent stem cells into embryonic and extraembryonic lineages. *Cell Stem Cell* 9: 144-155.

BONE HK, NELSON AS, GOLDRING CE, TOSH D, WELHAM MJ (2011). A novel chemically directed route for the generation of definitive endoderm from human embryonic stem cells based on inhibition of GSK-3. *J Cell Sci* 124: 1992–2000.

BLAUWKAMP TA, NIGAM S, ARDEHALI R, WEISSMAN IL, NUSSE R (2012). Endogenous Wnt signalling in human embryonic stem cells generates an equilibrium of distinct lineage-specified progenitors. *Nat Commun* 3: 1070.

BRONS IG, SMITHERS LE, TROTTER MW, RUGG-GUNN P, SUN B, CHUVA DE SOUSA LOPES SM, HOWLETT SK, CLARKSON A, AHRLUND-RICHTER L, PEDERSEN RA, VALLIER L (2007). Derivation of pluripotent epiblast stem cells from mammalian embryos. *Nature* 448: 191-195.

BUEHR M, MEEK S, BLAIR K, YANG J, URE J, SILVA J, MCLAY R, HALL J, YING QL, SMITH A (2008). Capture of authentic embryonic stem cells from rat blastocysts. *Cell* 135: 1287-1298.

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: 885-896.

CASANOVA EA, SHAKHOVA O, PATEL SS, ASNER IN, PELCZAR P, WEBER FA, GRAF U, SOMMER L, BÜRKI K, CINELLI P (2011). Pramel7 mediates LIF/STAT3-dependent self-renewal in embryonic stem cells. *Stem Cells* 29: 474-485.

CHA Y, MOON BH, LEE MO, AHN HJ, LEE HJ, LEE KA, FORNACE AJ JR, KIM KS, CHA HJ, PARK KS (2010). Zap70 functions to maintain stemness of mouse embryonic stem cells by negatively regulating Jak1/Stat3/c-Myc signaling. *Stem Cells* 28: 1476-1486.

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: 1230-1234.

CHAN KK, ZHANG J, CHIA NY, CHAN YS, SIM HS, TAN KS, OH SK, NG HH, CHOO AB (2009). KLF4 and PBX1 directly regulate NANOG expression in human embryonic stem cells. *Stem Cells* 27: 2114-2125.

CHANG KH, LI M (2013). Clonal isolation of an intermediate pluripotent stem cell state. *Stem Cells* 31: 918-927.

CLEVERS H, NUSSE R (2012). Wnt/β-catenin signaling and disease. *Cell* 149: 1192-1205.

DAHÉRON L, OPITZ SL, ZAEHRES H, LENSCH MW, ANDREWS PW, ITSKOVITZ-ELDOR J, DALEY GQ (2004). LIF/STAT3 signaling fails to maintain self-renewal of human embryonic stem cells. *Stem Cells* 22: 770-778.

DAVIDSON KC, ADAMS AM, GOODSON JM, MCDONALD CE, POTTER JC, BERNDT JD, BIECHELE TL, TAYLOR RJ, MOON RT (2012). Wnt/β-catenin signaling promotes differentiation, not self-renewal, of human embryonic stem cells and is repressed by Oct4. *Proc Natl Acad Sci USA* 109: 4485-4490.

DE LOS ANGELES A, LOH YH, TESAR PJ, DALEY GQ (2012). Accessing naïve human pluripotency. *Curr Opin Genet Dev* 22: 272-282.

DERYNCK R, ZHANG YE (2003). Smad-dependent and Smad-independent pathways in TGF-beta family signalling. *Nature* 425: 577-584.

DUTTA D, RAY S, HOME P, LARSON M, WOLFE MW, PAUL S (2011). Self-renewal versus lineage commitment of embryonic stem cells: protein kinase C signaling shifts the balance. *Stem Cells* 29: 618-628.

EVANS MJ, KAUFMAN MH (1981). Establishment in culture of pluripotential cells from mouse embryos. *Nature* 292: 154–156.

EZASHI T, TELUGU BP, ROBERTS RM (2012). Induced pluripotent stem cells from pigs and other ungulate species: An alternative to embryonic stem cells? *Reprod Domest Anim* 47: 92-97.

FEI T, XIA K, LI Z, ZHOU B, ZHU S, CHEN H, ZHANG J, CHEN Z, XIAO H, HAN JD, CHEN YG (2010a). Genome-wide mapping of SMAD target genes reveals the role of BMP signaling in embryonic stem cell fate determination. *Genome Res* 20: 36-44.

FEI T, ZHU S, XIA K, ZHANG J, LI Z, HAN JD, CHEN YG (2010b). Smad2 mediates Activin/Nodal signaling in mesendoderm differentiation of mouse embryonic stem cells. *Cell Res* 20: 1306-1318.

FELDMAN B, POUEYMIROU W, PAPAIOANNOU VE, DECHIARA TM, GOLDFARB M (1995). Requirement of FGF-4 for postimplantation mouse development. *Science* 267: 246-249.

FELSENSTEIN J (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39: 783–791.

GANDOLFI F, PENNAROSSA G, MAFFEI S, BREVINI T (2012). Why is it so difficult to derive pluripotent stem cells in domestic ungulates? *Reprod Domest Anim* 47: 11-17.

GREBER B, COULON P, ZHANG M, MORITZ S, FRANK S, MÜLLER-MOLINA AJ, ARAÚZO-BRAVO MJ, HAN DW, PAPE HC, SCHÖLER HR (2011). FGF signalling inhibits neural induction in human embryonic stem cells. *EMBO J* 30: 4874-4884.

GREBER B, LEHRACH H, ADJAYE J (2007). Fibroblast growth factor 2 modulates transforming growth factor beta signaling in mouse embryonic fibroblasts and human ESCs (hESCs) to support hESC self-renewal. *Stem Cells* 25: 455-464.

GREBER B, WU G, BERNEMANN C, JOO JY, HAN DW, KO K, TAPIA N, SABOUR D, STERNECKERT J, TESAR P, SCHÖLER HR (2010). Conserved and divergent roles of FGF signaling in mouse epiblast stem cells and human embryonic stem cells. *Cell Stem Cell* 6: 215-226.

GUO G, YANG J, NICHOLS J, HALL JS, EYRES I, MANSFIELD W, SMITH A (2009). Klf4 reverts developmentally programmed restriction of ground state pluripotency. *Development* 136: 1063-1069.

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: 597-609.

HAN DW, TAPIA N, JOO JY, GREBER B, ARAUZO-BRAVO MJ, BERNEMANN C, KO KINARM, WU GUANGMING, STEHLING M, DO JT, SCHÖLER HR (2010). Epiblast stem cell subpopulations represent mouse embryos of distinct pregastrulation stages. *Cell* 143: 617–627.

HANNA J, CHENG AW, SAHA K, KIM J, LENGNER CJ, SOLDNER F, CASSADY JP, MUFFAT J, CAREY BW, JAENISCH R (2010). Human embryonic stem cells with biological and epigenetic characteristics similar to those of mouse ESCs. *Proc Natl Acad Sci USA* 107: 9222-9227.

HANNA J, MARKOULAKI S, MITALIPOVA M, CHENG AW, CASSADY JP, STAERK J, CAREY BW, LENGNER CJ, FOREMAN R, LOVE J, GAO Q, KIM J, JAENISCH R (2009). Metastable pluripotent states in NOD-mouse-derived ESCs. *Cell Stem Cell* 4: 513-524.

HAO J, LI TG, QI X, ZHAO DF, ZHAO GQ (2006). WNT/beta-catenin pathway up-regulates Stat3 and converges on LIF to prevent differentiation of mouse embryonic stem cells. *Dev Biol* 290: 81-91.

HAYASHI K, LOPES SM, TANG F, SURANI MA (2008). Dynamic equilibrium and heterogeneity of mouse pluripotent stem cells with distinct functional and epigenetic states. *Cell Stem Cell* 3: 391-401.

HAYES B, FAGERLIE SR, RAMAKRISHNAN A, BARAN S, HARKEY M, GRAF L, BAR M, BENDORAITE A, TEWARI M, TOROK-STORB B (2008). Derivation, characterization, and *in vitro* differentiation of canine embryonic stem cells. *Stem Cells* 26: 465-473.

HE S, NAKADA D, MORRISON SJ (2009). Mechanisms of stem cell self-renewal. *Ann Rev Cell Dev Biol* 25: 377-406.

HEDGES SB (2002). The origin and evolution of model organisms. *Nat Rev Genet* 3: 838-849.

HOFFMEYER K, RAGGIOLI A, RUDLOFF S, ANTON R, HIERHOLZER A, DEL VALLE I, HEIN K, VOGT R, KEMLER R (2012). Wnt/β-catenin signaling regulates telomerase in stem cells and cancer cells. *Science* 336: 1549-1554.

HELDIN CH, MIYAZONO K, TEN DIJKE P (1997). TGF-beta signalling from cell membrane to nucleus through SMAD proteins. *Nature* 390: 465-471.

HONDA A, HIROSE M, OGURA A (2009). Basic FGF and Activin/Nodal but not LIF signaling sustain undifferentiated status of rabbit embryonic stem cells. *Exp Cell Res* 315: 2033-2042.

HOUGH SR, LASLETT AL, GRIMMOND SB, KOLLE G, PERA MF (2009). A continuum of cell states spans pluripotency and lineage commitment in human embryonic stem cells. *PLoS One* 4: e7708.

HUANGFU D, OSAFUNE K, MAEHR R, GUO W, EIJKELENBOOM A, CHEN S, MUHLESTEIN W, MELTON DA (2008). Induction of pluripotent stem cells from primary human fibroblasts with only Oct4 and Sox2. *Nat Biotechnol* 26: 1269-1275.

JIANG J, CHAN YS, LOH YH, CAI J, TONG GQ, LIM CA, ROBSON P, ZHONG S, NG HH (2008). A core Klf circuitry regulates self-renewal of embryonic stem cells. *Nat Cell Biol* 10: 353-360.

KANG HB, KIM JS, KWON HJ, NAM KH, YOUN HS, SOK DE, LEE Y (2005). Basic fibroblast growth factor activates ERK and induces c-fos in human embryonic stem cell line MizhES1. *Stem Cells Dev* 14: 395-401.

KAWABATA M, IMAMURA T, MIYAZONO K (1998). Signal transduction by bone morphogenetic proteins. *Cytokine Growth Factor Rev* 9: 49-61.

KELLY KF, NG DY, JAYAKUMARAN G, WOOD GA, KOIDE H, DOBLE BW (2011). β-catenin enhances Oct-4 activity and reinforces pluripotency through a TCF-independent mechanism. *Cell Stem Cell* 8: 214-227.

KIM MO, KIM SH, CHO YY, NADAS J, JEONG CH, YAO K, KIM DJ, YU DH, KEUM YS, LEE KY, HUANG Z, BODE AM, DONG Z (2012). ERK1 and ERK2 regulate embryonic stem cell self-renewal through phosphorylation of Klf4. *Nat Struct Mol Biol* 19: 283-290.

KOBAYASHI M, TAKADA T, TAKAHASHI K, NODA Y, TORII R (2008). BMP4 induces primitive endoderm but not trophectoderm in monkey embryonic stem cells. *Cloning Stem Cells* 10: 495-502.

KUIJK EW, VAN TOL LT, VAN DE VELDE H, WUBBOLTS R, WELLING M, GEIJSEN N, ROELEN BA (2012). The roles of FGF and MAPK signaling in the segregation of the epiblast and hypoblast lineages in bovine and human embryos. *Development* 139: 871-882.

KUMAR S, TAMURA K, NEI M (2004). Mega3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* 5: 150–163.

LEE DF, SU J, ANG YS, CARVAJAL-VERGARA X, MULERO-NAVARRO S, PEREIRA CF, GINGOLD J, WANG HL, ZHAO R, SEVILLA A, DARR H, WILLIAMSON AJ, CHANG B, NIU X, AGUILO F, FLORES ER, SHER YP, HUNG MC, WHETTON AD, GELB BD, MOORE KA, SNOECK HW, MA'AYAN A, SCHANIEL C, LEMISCHKA IR (2012). Regulation of Embryonic and Induced Pluripotency by Aurora Kinase-p53 Signaling. *Cell Stem Cell* 11: 179-194.

LEVENSTEIN ME, LUDWIG TE, XU RH, LIANAS RA, VANDENHEUVEL-KRAMER K, MANNING D, THOMSON JA (2006). Basic fibroblast growth factor support of human embryonic stem cell self-renewal. *Stem Cells* 24: 568-574.

LI P, TONG C, MEHRIAN-SHAI R, JIA L, WU N, YAN Y, MAXSON RE, SCHULZE EN, SONG H, HSIEH CL, PERA MF, YING QL (2008). Germline competent embryonic stem cells derived from rat blastocysts. *Cell* 135: 1299-1310.

LI Z, FEI T, ZHANG J, ZHU G, WANG L, LU D, CHI X, TENG Y, HOU N, YANG X, ZHANG H, HAN JD, CHEN YG (2012). BMP4 Signaling Acts via dual-specificity phosphatase 9 to control ERK activity in mouse embryonic stem cells. *Cell Stem Cell* 10: 171-182.

MASUI S, NAKATAKE Y, TOYOOKA Y, SHIMOSATO D, YAGI R, TAKAHASHI K, OKOCHI H, OKUDA A, MATOBA R, SHAROV AA, KO MS, NIWA H (2007). Pluripotency governed by Sox2 via regulation of Oct3/4 expression in mouse embryonic stem cells. *Nat Cell Biol* 9: 625-635.

MCLEAN AB, D'AMOUR KA, JONES KL, KRISHNAMOORTHY M, KULIK MJ, REYNOLDS DM, SHEPPARD AM, LIU H, XU Y, BAETGE EE, DALTON S (2007). Activin a efficiently specifies definitive endoderm from human embryonic stem cells only when phosphatidylinositol 3-kinase signaling is suppressed. *Stem Cells* 25: 29-38.

MARUOTTI J, MUÑOZ M, DEGRELLE SA, GÓMEZ E, LOUET C, DÍEZ C, DE LONGCHAMP PH, BROCHARD V, HUE I, CAAMAÑO JN, JOUNEAU A (2012). Efficient derivation of bovine embryonic stem cells needs more than active core pluripotency factors. *Mol Reprod Dev* 79: 461-477.

MIYABAYASHI T, TEO JL, YAMAMOTO M, MCMILLAN M, NGUYEN C, KAHN M (2007). Wnt/beta-catenin/CBP signaling maintains long-term murine embryonic stem cell pluripotency. *Proc Natl Acad Sci USA* 104: 5668-5673.

MIYANARI Y, TORRES-PADILLA ME (2012). Control of ground-state pluripotency by allelic regulation of Nanog. *Nature* 483: 470-473.

NAJM FJ, CHENOWETH JG, ANDERSON PD, NADEAU JH, REDLINE RW, MCKAY RD, TESAR PJ (2011). Isolation of epiblast stem cells from preimplantation mouse embryos. *Cell Stem Cell* 8: 318-325.

NAKANISHI M, KURISAKI A, HAYASHI Y, WARASHINA M, ISHIURA S, KUSUDA-FURUE M, ASASHIMA M (2009). Directed induction of anterior and posterior primitive streak by Wnt from embryonic stem cells cultured in a chemically defined serum-free medium. *FASEB J* 23: 114–122.

NAKATAKE Y, FUKUI N, IWAMATSU Y, MASUI S, TAKAHASHI K, YAGI R, YAGI K, MIYAZAKI J, MATOBA R, KO MS, NIWA H (2006). Klf4 cooperates with Oct3/4 and Sox2 to activate the Lefty1 core promoter in embryonic stem cells. *Mol Cell Biol* 26: 7772–7782.

NICHOLS J, SILVA J, ROODE M, SMITH A (2009). Suppression of Erk signalling promotes ground state pluripotency in the mouse embryo. *Development* 136: 3215-3222.

NICHOLS J, SMITH A (2009). Naive and primed pluripotent states. *Cell Stem Cell* 4: 487-492.

NICHOLS J, SMITH A (2011). The origin and identity of embryonic stem cells. *Development* 138: 3-8.

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: 2048-2060.

NIWA H, MIYAZAKI J, SMITH AG (2000). Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nat Genet* 24: 372-376.

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: 55–65.

PALING NR, WHEADON H, BONE HK, WELHAM MJ (2004). Regulation of embryonic stem cell self-renewal by phosphoinositide 3-kinase-dependent signaling. *J Biol Chem* 279: 48063-48070.

PERA MF, TAM PP (2010). Extrinsic regulation of pluripotent stem cells. *Nature* 465: 713-720.

QI X, LI TG, HAO J, HU J, WANG J, SIMMONS H, MIURA S, MISHINA Y, ZHAO GQ (2004). BMP4 supports self-renewal of embryonic stem cells by inhibiting mitogen-activated protein kinase pathways. *Proc Natl Acad Sci USA* 101: 6027-6032.

RAJENDRAN G, DUTTA D, HONG J, PAULA, SAHA B, MAHATO B, RAY S, HOME P, GANGULY A, WEISS ML, PAUL S (2013). Inhibition of protein kinase C signaling maintains rat embryonic stem cell pluripotency. *J Biol Chem* 288: 24351-24362.

ROODE M, BLAIR K, SNELL P, ELDER K, MARCHANT S, SMITH A, NICHOLS J (2012). Human hypoblast formation is not dependent on FGF signalling. *Dev Biol* 361: 358-363.

SAITO S, UGAI H, SAWAI K, YAMAMOTO Y, MINAMIHASHI A, KUROSAKA K, KOBAYASHI Y, MURATA T, OBATA Y, YOKOYAMA K (2002). Isolation of embryonic stem-like cells from equine blastocysts and their differentiation in vitro. *FEBS Lett* 531: 389-396.

SATO N, MEIJER L, SKALTSOUNIS L, GREENGARD P, BRIVANLOU AH (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: 55–63.

SINGH AM, REYNOLDS D, CLIFF T, OHTSUKA S, MATTHEYSES AL, SUN Y, MENENDEZ L, KULIK M, DALTON S (2012). Signaling network crosstalk in human pluripotent cells: a Smad2/3-regulated switch that controls the balance between self-renewal and differentiation. *Cell Stem Cell* 10: 312-326.

SMITH AG, HEATH JK, DONALDSON DD, WONG GG, MOREAU J, STAHL M, ROGERS D (1988). Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. *Nature* 336: 688-690.

TACHIBANA M, SPARMAN M, RAMSEY C, MA H, LEE HS, PENEDO MC, MITALIPOV S (2012). Generation of chimeric rhesus monkeys. *Cell* 148: 285-295.

TAKAHASHI K, TANABE K, OHNUKI M, NARITA M, ICHISAKA T, TOMODA K, YAMANAKA S (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131: 861-872.

TAN T, TANG X, ZHANG J, NIU Y, CHEN H, LI B, WEI Q, JI W (2011). Generation of trophoblast stem cells from rabbit embryonic stem cells with BMP4. *PLoS One* 6: e17124.

TELUGU BP, EZASHI T, SINHA S, ALEXENKO AP, SPATE L, PRATHER RS, ROBERTS RM (2011). Leukemia inhibitory factor (LIF)-dependent, pluripotent stem cells established from inner cell mass of porcine embryos. *J Biol Chem* 286: 28948-28953.

TESAR PJ, CHENOWETH JG, BROOK FA, DAVIES TJ, EVANS EP, MACK DL, GARDNER RL, MCKAY RD (2007). New cell lines from mouse epiblast share defining features with human embryonic stem cells. *Nature* 448: 196-199.

THOMPSON JD, HIGGINS DG, GIBSON TJ (1994). Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Res* 22: 4673–4680.

THOMSON JA, ITSKOVITZ-ELDOR J, SHAPIRO SS, WAKNITZ MA, SWIERGIEL JJ, MARSHALL VS, JONES JM (1998). Embryonic stem cell lines derived from human blastocysts. *Science* 282: 1145–1147.

THOMSON JA, KALISHMAN J, GOLOS TG, DURNING M, HARRIS CP, BECKER RA, HEARN JP (1995). Isolation of a primate embryonic stem cell line. *Proc Nat Acad Sci USA* 92: 7844–7848.

UEDA S, KAWAMATA M, TERATANI T, SHIMIZU T, TAMAI Y, OGAWA H, HAYASHI K, TSUDA H, OCHIYA T (2008). Establishment of rat embryonic stem cells and making of chimera rats. *PLoS One* 3: e2800.

VALLIER L, MENDJAN S, BROWN S, CHNG Z, TEO A, SMITHERS LE, TROTTER MW, CHO CH, MARTINEZ A, RUGG-GUNN P, BRONS G, PEDERSEN RA (2009a). Activin/Nodal signalling maintains pluripotency by controlling Nanog expression. *Development* 136: 1339-1349.

VALLIER L, TOUBOUL T, CHNG Z, BRIMPARI M, HANNAN N, MILLAN E, SMITHERS LE, TROTTER M, RUGG-GUNN P, WEBER A, PEDERSEN RA (2009b). Early cell fate decisions of human embryonic stem cells and mouse epiblast stem cells are controlled by the same signalling pathways. *PLoS One* 4: e6082.

VAN OOSTEN AL, COSTA Y, SMITH A, SILVA JC (2012). JAK/STAT3 signalling is sufficient and dominant over antagonistic cues for the establishment of naive pluripotency. *Nat Commun* 3: 817.

WANG S, SHEN Y, YUAN X, CHEN K, GUO X, CHEN Y, NIU Y, LI J, XU RH, YAN X, ZHOU Q, JI W (2008). Dissecting signaling pathways that govern self-renewal of rabbit embryonic stem cells. *J Biol Chem* 283: 35929-35940.

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: 2697-2707.

XU RH, CHEN X, LI DS, LI R, ADDICKS GC, GLENNON C, ZWAKA TP, THOMSON JA (2002). BMP4 initiates human embryonic stem cell differentiation to trophoblast. *Nat Biotechnol* 20: 1261-1264.

YING QL, 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: 281-292.

YING QL, 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: 519-523.

YU PB, BEPPU H, KAWAI N, LI E, BLOCH KD (2005). Bone morphogenetic protein (BMP) type II receptor deletion reveals BMP ligand-specific gain of signaling in pulmonary artery smooth muscle cells. *J Biol Chem* 280: 24443-24450

ZHAO Y, LIN J, WANG L, CHEN B, ZHOU C, CHEN T, GUO M, HE S, ZHANG N, LIU C, LIU M, HUANG J (2011). Derivation and characterization of ovine embryonic stem-like cell lines in semi-defined medium without feeder cells. *J Exp Zool A Ecol Genet Physiol* 315: 639-648.

ZHONG Z, WEN Z, DARNELL JE JR (1994). Stat3: a STAT family member activated by tyrosine phosphorylation in response to epidermal growth factor and interleukin-6. *Science* 264: 95-98.

Further Related Reading, published previously in the *Int. J. Dev. Biol.*

Signaling pathways during maintenance and definitive endoderm differentiation of embryonic stem cells.
Lina Sui, Luc Bouwens and Josué K. Mfopou.
Int. J. Dev. Biol. (2013) 57: 1-12

Rediscovering pluripotency: from teratocarcinomas to embryonic stem cells.
Ivana Barbaric and Neil J. Harrison.
Int. J. Dev. Biol. (2012) 56: 197-206

Reprogramming cell fate to pluripotency: the decision-making signalling pathways.
Daniela Sanges and Maria-Pia Cosma.
Int. J. Dev. Biol. (2010) 54: 1575-1587

Porcine induced pluripotent stem cells analogous to naïve and primed embryonic stem cells of the mouse.
Bhanu Prakash V.L. Telugu, Toshihiko Ezashi and R. Michael Roberts.
Int. J. Dev. Biol. (2010) 54: 1703-1711

Natural and artificial routes to pluripotency.
Winfried H. Krueger, Lindsey C. Swanson, Borko Tanasijevic and Theodore P. Rasmussen.
Int. J. Dev. Biol. (2010) 54: 1545-1564

5 yr ISI Impact Factor (2011) = 2.959