

# The role of FGF/Erk signaling in pluripotent cells

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## Summary

Fibroblast growth factor (FGF) signaling controls fundamental processes such as proliferation, differentiation and migration throughout mammalian development. Here we discuss recent discoveries that implicate FGF/Erk signaling in the control of pluripotency and lineage specification in several different stem cell states, including the separation of pluripotent epiblast and primitive endoderm in the blastocyst, the lineage priming of embryonic stem (ES) cells, and in the stabilization of the metastable state of mouse epiblast and human ES cells. Understanding how extrinsic signals such as FGF regulate different stem cell states will be crucial to harvest the clinical promise of induced pluripotent and embryo-derived stem cells.

**Key words:** FGF signaling, Stem cells, Embryonic development

## Introduction

Fibroblast growth factors (FGFs) and their receptor tyrosine kinases control a multitude of developmental processes, including proliferation, survival, migration and differentiation. In this review, we discuss recently emerging evidence that FGF signaling plays an important role in regulating pluripotency and lineage segregation in both the early mouse embryo and in pluripotent mammalian stem cells. Although mouse and human embryonic stem (ES) cells are both derived from the pluripotent inner cell mass (ICM) cells of the pre-implantation blastocyst, they exhibit fundamental differences, whereby mouse ES cells appear to represent a developmentally more 'naïve' pre-implantation state and human ES cells a 'primed' post-implantation state (Nichols and Smith, 2009), similar to mouse epiblast-derived stem cells (EpiSCs) (Brons et al., 2007; Tesar et al., 2007). Intriguing recent findings suggest an important role for FGF/extracellular signal-regulated kinase (Erk) signaling in promoting the transition from a naïve to a primed state and in preventing primed cells from reverting back to a naïve state, in effect stabilizing the primed cell state (Hanna et al., 2009; Li et al., 2009; Greber et al., 2010; Hanna et al., 2010). Modulating FGF/Erk signaling might therefore facilitate the conversion of conventional human ES cells into a more naïve and mouse-like state. The existence of multiple and possibly interconvertible pluripotent stem cell states controlled by the extrinsic signaling environment could have significant implications not only for early developmental biology, but also for the field of reprogramming and induced pluripotency.

## FGF signaling

FGF signaling is activated by a ligand-receptor interaction that results in the autophosphorylation of tyrosine residues in the intracellular region of an FGF receptor (FGFR). The signal is

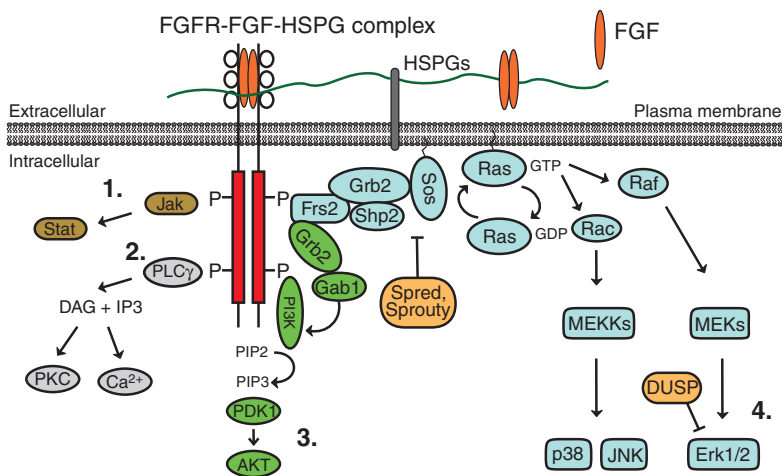
further relayed through four distinct pathways: the Janus kinase/signal transducer and activator of transcription (Jak/Stat), phosphoinositide phospholipase C (PLC $\gamma$ ), phosphatidylinositol 3-kinase (PI3K) and Erk pathways (Dailey et al., 2005) (Fig. 1). The formation of a complex between FGFR, fibroblast growth factor receptor substrate 2 (Frs2 $\alpha$ ), Src homology region 2 domain-containing phosphatase 2 (Shp2; also known as Ptpn11) and growth factor receptor-bound protein 2 (Grb2) facilitates son of sevenless homology (Sos)-Ras-Erk activation, whereas FGFR-Frs2 $\alpha$ -Grb2 complex formation drives PI3K activation via Grb2-associated binding protein 1 (Gab1) (see Fig. 1). FGF signaling is tightly regulated and modulated at multiple levels, both intracellularly and extracellularly. For example, the bioavailability of secreted FGF ligands is regulated by their binding to extracellular heparan sulfate proteoglycans (HSPGs). HSPGs also act as important co-receptors for FGF ligands by facilitating the assembly of activated ligand-receptor complexes (Ornitz and Itoh, 2001). Intracellularly, the FGF signaling cascade is further regulated by negative-feedback mechanisms at multiple levels by dual specificity phosphatases (DUSPs), similar expression to FGF (Sef), Spred and Sprouty proteins (Dailey et al., 2005). Several of these inhibitors are themselves downstream transcriptional targets of the FGF pathway.

## Ligand and receptor expression in the early embryo

Vertebrates have 22 FGF ligands and five receptors (Fgfr1-5), each with specific expression patterns throughout development. Alternative splicing of the receptors provides some degree of ligand specificity (Itoh and Ornitz, 2004). The importance of FGF signaling in the early mouse embryo is well established, as mutations in *Fgf4*, *Fgfr2*, *Frs2a*, *Grb2* and *Erk2* (*Mapk1*) all result in peri-implantation lethality (Feldman et al., 1995; Arman et al., 1998; Cheng et al., 1998; Hadari et al., 2001; Hatano et al., 2003; Saba-El-Leil et al., 2003; Chazaud et al., 2006) (summarized in Table 1). *Fgf4* is the predominantly expressed ligand in the early embryo. It is expressed in the 8- to 16-cell morula but becomes restricted to the epiblast cells of the ICM in the expanded blastocyst, and has been shown to be under the direct regulation of the pluripotency factors Oct4 (Pou5f1) and Sox2 (Yuan et al., 1995). *Fgfr2* appears to be the predominantly expressed receptor in the blastocyst, although it is restricted to the extra-embryonic, trophectoderm (TE) and primitive endoderm (PE) lineages. This differential receptor-ligand expression suggests that epiblast-produced *Fgf4* could be important for the establishment or maintenance of the two extra-embryonic tissues. Although loss of *Fgf5* does not result in any early phenotype, onset of its expression is often used to mark the transition from pre- to post-implantation epiblast (Hebert et al., 1991; Hebert et al., 1994). *Fgf8* and *Fgfr1* are also expressed in the blastocyst, but they appear to function slightly later as loss of their function results in post-implantation epiblast phenotypes with impaired axis formation and mesoderm specification (Deng et al., 1994; Yamaguchi and Rossant, 1995; Meyers et al., 1998).

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**Fig. 1. The FGF-FGFR signaling pathway.** The fibroblast growth factor (FGF)-FGF receptor (FGFR) signaling pathway is regulated at multiple levels. Heparan sulfate proteoglycans (HSPGs) act both as co-receptors and modulators of ligand bioavailability. The four main signaling pathways downstream of receptor activation are the Janus kinase/signal transducer and activator of transcription (Jak/Stat; 1, brown), phosphoinositide phospholipase C (PLC $\gamma$ ; 2, gray), phosphatidylinositol 3-kinase (PI3K; 3, green) and mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/Erk; 4, blue). Dual specificity phosphatases (DUSPs), Sprouty and Sprouty proteins (orange) provide a negative-feedback mechanism to reduce or terminate FGF signaling.

### FGF signaling in embryo-derived stem cells

Four distinct stem cell types can be derived from the early mouse embryo, representing the first cell types of mammalian development: trophectoderm stem (TS) cells from the TE; extra-embryonic endoderm stem (XEN) cells from the PE; ES cells and EpiSCs from the pre- and post-implantation epiblast, respectively (Box 1) (Evans and Kaufman, 1981; Martin, 1981; Tanaka et al., 1998; Kunath et al., 2005; Brons et al., 2007; Tesar et al., 2007). Reflecting the differential FGF ligand-receptor expression in the early embryo, these cell lines show differential dependency on exogenous FGF. TS cells and EpiSCs require exogenous FGF for their derivation and maintenance. Although FGF is routinely

added to XEN cell cultures, it has not formally been tested whether this is crucial for their derivation. By contrast, mouse ES cells are derived and maintained without exogenous FGF. Although *Fgf4* is produced by mouse ES cells and can activate FGF/Erk signaling in an autocrine manner, it is dispensable for the maintenance of the undifferentiated state of mouse ES cells. Indeed, *Fgf4*-null ES cells do not suffer from reduced proliferation, unlike the *Fgf4*-null ICM (Wilder et al., 1997). However, it was recently demonstrated that FGF/Erk signaling is required to instruct ES cells to exit from the self-renewal program and to begin differentiation (Kunath et al., 2007); inhibition of this signaling pathway actually increases the efficiency of ES cell

**Table 1. Expression and reported phenotypes of FGF ligands, receptors and signaling transduction components**

	Early expression pattern	Phenotype	References
<b>Ligands</b>			
Fgf2	Post-implantation	Viable but with cerebral cortex phenotype	Dono et al., 1998
Fgf3	Primitive streak	Viable with tail and inner ear phenotypes	Mansour et al., 1994
Fgf4	Pre-implantation epiblast and in primitive streak	Peri-implantation lethality and reduced blastocyst outgrowth	Feldman et al., 1995
Fgf5	Post-implantation epiblast	Viable with hair phenotype	Hebert et al., 1991; Hebert et al., 1994
Fgf8	Blastocyst and in the primitive streak	Defective gastrulation and absence of mesodermal tissues at E8.5	Meyers et al., 1998
<b>Receptors</b>			
Fgfr1	Pre- and post-implantation epiblast	Impaired gastrulation and mesodermal patterning	Deng et al., 1994; Yamaguchi et al., 1994
Fgfr2	Early morula and extra-embryonic tissues of the blastocyst	Implantation lethality with loss of ICM differentiation and PE formation in vitro	Arman et al., 1998
Fgfr3	Post-implantation	Negative regulator of bone growth	Colvin et al., 1996; Deng et al., 1996
Fgfr4	Post-implantation	Combined loss of Fgfr3/4 causes impaired lung development	Weinstein et al., 1998
<b>Signal transduction</b>			
Frs2 $\alpha$	Ubiquitous	Impaired gastrulation, A-P patterning and TS proliferation	Hadari et al., 2001; Gotoh et al., 2004
Shp2	Ubiquitous	Implantation lethality with TE and mesoderm phenotypes	Saxton et al., 1997; Yang et al., 2006
Grb2	Ubiquitous	Implantation lethality with lack of PE differentiation	Cheng et al., 1998; Chazaud et al., 2006
Erk1	Low or absent expression in pre-implantation embryo	Viable	Pages et al., 1999
Erk2	Early morula and throughout early development	Implantation lethality from TE defects	Hatano et al., 2003; Saba-El-Leil et al., 2003

A-P, anterior-posterior; E, embryonic day; ICM, inner cell mass; PE, primitive endoderm; TE, trophectoderm; TS, trophectoderm stem cell.

### Box 1. Early embryo-derived stem cells

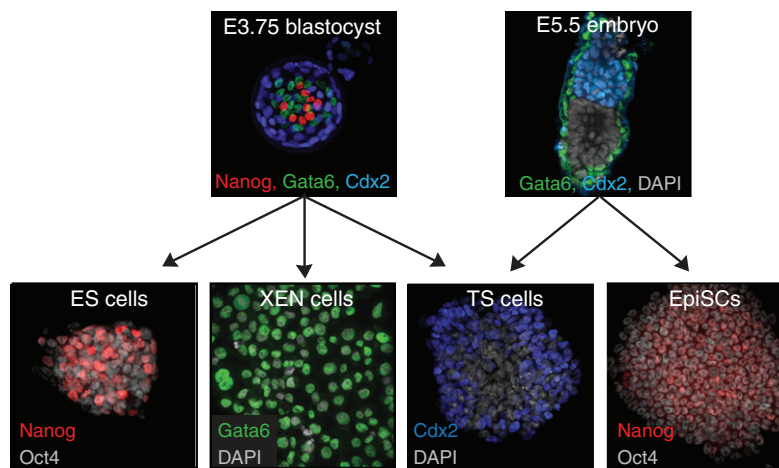
Four stem cell types with distinct dependence upon FGF/Erk signaling can be derived from the pre- and post-implantation mouse embryo.

**Embryonic stem (ES) cells.** ES cells are derived from the Nanog-positive (red in figure) inner cell mass (ICM) population of the blastocyst. Derivation does not depend on FGF but benefits from FGF/Erk signaling inhibition. Autocrine Fgf4/Erk reversibly primes the ES cells for differentiation through downregulation of pluripotency markers, resulting in heterogeneous expression of Nanog within the Oct4-positive cells.

**Extra-embryonic endoderm (XEN) cells.** XEN cells are derived from the Gata6-positive (green) ICM population of the blastocyst, and exogenous FGF is added for their initial derivation but is not necessary for continued derivation. A role of FGF/Erk signaling in established XEN cells has not been reported.

**Trophectoderm stem (TS) cells.** TS cells can be derived from Cdx2-positive (blue) cells from both pre- and post-implantation embryos. Exogenous FGF is required for both their derivation and continued culture. Lack of FGF/Erk signaling results in premature differentiation and apoptosis.

**Epiblast-derived stem cells (EpiSCs).** EpiSCs are derived and cultured with addition of exogenous FGF and activin A, which is similar to human ES cell culture conditions. FGF/Erk signaling is suggested to prevent differentiation and, potentially, to prevent reversion back to a pre-implantation ES cell-like state.



derivation (Kunath et al., 2007; Ying et al., 2008; Nichols et al., 2009a). As we discuss below, this is in stark contrast to human ES cells, which are strictly dependent upon exogenous FGFs for their self-renewal.

### FGF/Erk signaling initiates ES cell differentiation

Until recently, the predominant view of mouse ES cells has been that their pluripotent state must be actively maintained by the addition of extrinsic signaling molecules. The factor that is best known for promoting the self-renewal of ES cells is leukemia inhibitory factor (LIF), which acts through the Jak/Stat3 signaling pathway (Smith et al., 1988; Williams et al., 1988; Ying et al., 2003). ES cells can be maintained in serum-free conditions when LIF is combined with bone morphogenetic protein 4 (Bmp4). Bmp4 induces the inhibitor-of-differentiation (Id) proteins, which are DNA-binding proteins that inhibit the default differentiation of ES cells into neuronal lineages (Tropepe et al., 2001; Ying et al., 2003). However, the view that differentiation is the default pathway for ES cells was challenged when two back-to-back papers showed that autocrine FGF-induced Erk1/2 signaling (Erk1 is also known as Mapk3) occurs in ES cells and is required to initiate their differentiation (Kunath et al., 2007; Stavridis et al., 2007).

Stavridis et al. set out to examine the role of FGF signaling in neural specification using mouse ES cells and the chick embryo (Stavridis et al., 2007). They first showed that FGF signaling activates downstream Erk1/2 and not PI3K or PLC $\gamma$  during neuronal differentiation. Inhibition of either FGFR or Erk1/2 abolished neuronal induction. They could detect induction of the

post-implantation epiblast marker Fgf5 but no reduction in Nanog, and concluded, therefore, that FGF signaling is required for ES cells to differentiate beyond the early epiblast state. Kunath et al. showed that although Fgf4-null ES cells behave normally in the undifferentiated state in the presence of LIF, they are unable to make the transition from pluripotency to neural lineage commitment when LIF is removed (Kunath et al., 2007). The addition of exogenous Fgf4 to Fgf4-null ES cells rescued their differentiation into not only neural, but also mesoderm, lineages. By contrast, the addition of Fgf5 failed to rescue differentiation, suggesting that Fgf4 has a ligand-specific signaling potential. Similar results were observed in Erk2<sup>-/-</sup> mouse ES cells (Kunath et al., 2007). These authors therefore concluded that autocrine Fgf4/Erk signaling is needed for ES cells to exit from self-renewal and initiate differentiation.

Thus, the role of LIF and BMP in promoting ES self-renewal may be to counterbalance the pro-differentiation autocrine Fgf4/Erk signal. Ying et al. hypothesized that the requirement for exogenous factors might be eliminated if autocrine Fgf4/Erk signaling were blocked (Ying et al., 2008). They found that addition of the FGFR inhibitor SU5402 or the Erk signaling inhibitor PD184352 could maintain mouse ES cells in an undifferentiated state in the presence of LIF. These inhibitors could also block differentiation in the absence of LIF, but the cultures progressively degenerated. 6-bromoindirubin-3'-oxime (BIO), a glycogen synthase kinase 3 (GSK3) inhibitor, had previously been shown to promote the maintenance in culture of both mouse and human ES cells (Sato et al., 2004). Using a GSK3 inhibitor (CHIR99021) in combination with FGFR/Erk inhibition, Ying et al. could eliminate the need for

LIF while maintaining the pluripotency and clonogenicity of ES cells (Ying et al., 2008). In vivo, GSK3 is inactivated by the canonical Wnt signaling pathway, resulting in the nuclear accumulation of  $\beta$ -catenin. One of the effects of adding a GSK3 inhibitor would therefore be to mimic canonical Wnt signaling. Inhibition with CHIR99021 did reduce  $\beta$ -catenin phosphorylation and induced a T-cell factor/lymphocyte enhancer factor (TCF/Lef)-responsive promoter, both indicating that the activation of downstream canonical Wnt signaling had occurred. However, addition of recombinant Wnt3 together with FGF/Erk inhibitors failed to fully recapitulate the effect of the GSK3 inhibitor. Furthermore, ES cells that stably express  $\Delta$ NhLef1, a dominant-negative form of lymphoid enhancer binding factor 1 (Lef1; which mediates a transcriptional response to canonical Wnt signaling together with TCF1), which should attenuate canonical Wnt signaling, still responded to GSK3 inhibition. As a result, Ying et al. suggested that GSK3 inhibition might have a Wnt-independent function in promoting the overall viability and proliferation of mouse ES cells. Together, these data show that mouse ES cells can be cultured independently of LIF if the cells are shielded from autocrine FGF/Erk signaling and subjected to GSK3 inhibition.

The derivation of truly pluripotent ES cells from mammalian species other than mice has been very difficult to achieve. This has been attributed to the inability of most other early embryos to respond to LIF. Ying and colleagues subsequently showed that germline-competent rat ES cells could be successfully generated through the combined use of LIF with FGF/Erk and GSK3 inhibition (Buehr et al., 2008; Li et al., 2008). This approach has also been used to derive germline-competent ES cells from non-permissive mouse strains, such as the non-obese-diabetic (NOD) strain (Hanna et al., 2009; Nichols et al., 2009b). Although LIF itself failed to sustain derived rat ES cells, it did induce Stat3 activation and improved colony formation from single cells when combined with Erk and GSK3 inhibitors (Buehr et al., 2008; Li et al., 2008). This finding suggests that the previous failure to derive ES cells from species other than mice might not solely be attributed to the absence of LIF responsiveness but to differences in endogenous FGF/Erk signaling.

### ES cell cultures contain multiple lineage-primed states

In parallel with these recent findings regarding the important role of FGF/Erk signaling in balancing pluripotency versus differentiation in ES cells, several papers have presented convincing data that pluripotent ES cultures exhibit highly dynamic heterogeneous expression of pluripotency markers, such as Nanog (Chambers et al., 2007; Singh et al., 2007), platelet/endothelial cell adhesion molecule (Pecam1 or CD31) (Furusawa et al., 2004; Furusawa et al., 2006), zinc-finger protein 42 (Zfp42 or Rex1) (Toyooka et al., 2008) and developmental pluripotency-associated 3 (Dppa3 or Stella) (Payer et al., 2006; Hayashi et al., 2008), even though all cells are positive for Oct4 and stage-specific embryonic antigen 1 (SSEA1; Fut4). Furthermore, genes associated with differentiation, such as brachyury (Suzuki et al., 2006), *Gata6* (Singh et al., 2007), *Hex* (*Hhex*) (Canham et al., 2010) and *Sox17* (Niakan et al., 2010), are also expressed in a subset of cells in undifferentiated ES cell cultures.

Nanog is expressed in the mouse blastocyst ICM and in ES and germ cells. The *Nanog* null mutation causes embryonic lethality owing to an inability to form or maintain an epiblast in pre-implantation blastocysts. Conversely, forced expression of Nanog in ES cells maintains cells in an undifferentiated state even in the

absence of LIF (Chambers et al., 2003; Mitsui et al., 2003). Chambers et al. reported that mouse ES cells could be maintained in the absence of Nanog, although they are more prone to differentiation (Chambers et al., 2007). They further showed that Nanog is heterogeneously expressed in normal Oct4<sup>+</sup> ES cell cultures. A knock-in GFP reporter line revealed that ES cells fluctuate between a Nanog-high and Nanog-low state. Nanog-low cells appeared to be poised or 'primed' for differentiation, similar to the *Nanog*-null ES cells. However, Nanog-low cells could still revert back to a Nanog-high state, and Nanog-high cells could regenerate a Nanog-low population.

Heterogeneous and fluctuating expression has also been reported for two additional transcription factors, Rex1 and Stella, which are specifically expressed in pluripotent ICM cells but not in post-implantation epiblast. Toyooka et al. reported that the Rex1-positive population in cultured ES cells is enriched for markers that are expressed in the ICM, such as Nanog, Tbx3, Klf4 and Tcf11, whereas the Rex1-negative population is enriched in post-implantation epiblast markers, such as Fgf5 and brachyury (Toyooka et al., 2008). Hayashi et al. reported that Stella expression undergoes a similar transition and that mouse ES cells cultured on feeder cells maintain more cells in a Stella-GFP-positive state, indicating that the balance between the different populations is influenced by external stimuli. In the case of all three markers, Rex, Stella and Nanog, the positive and negative population of cells, when isolated, can regenerate the heterogeneous expression of the starting population. This suggests that the ES cell population is truly in a metastable state in which a dynamic continuum exists between multiple interconvertible states. It is likely that Nanog-low, Rex1-low, Stella-low expression mark the same population of cells, although this has not been formally proven at the single-cell level. This suggests that ES cultures maintained in LIF undergo a form of lineage priming (Enver et al., 2009), constantly generating a population of cells poised to become post-implantation epiblast (Fig. 2).

The epiblast-primed population might not be the only primed population among ES cells. Singh et al. reported that a subpopulation of ES cells is Nanog-low and positive for Gata6, a PE marker (Singh et al., 2007). When the two populations were sorted by flow cytometry, each population could regenerate the initial heterogeneity. However, when the Nanog-low cells were differentiated, they only induced the expression of extra-embryonic endoderm markers, such as Gata6 and Sparc, whereas Nanog-high cells remained capable of also inducing mesodermal and endodermal transcripts. This suggests that the Nanog-low, Gata6-high cells were primed for extra-embryonic endodermal differentiation. Supporting the existence of a subpopulation that is primed to become PE, Canham et al. identified a subset of ES cells that expresses *Hex* transcripts and is reversibly primed for extra-embryonic endodermal differentiation (Canham et al., 2010). Furthermore, a Sox17-expressing subpopulation has been described in ES cell culture that appears to be irreversibly poised for differentiation to the extra-embryonic endodermal lineage (Niakan et al., 2010) (Fig. 2).

It is still not clear whether all ES cell lines under all culture conditions display the same range of lineage-primed states. Most studies have focused on one particular ES cell line and one lineage marker, making it difficult to compare results from different studies. However, it is clear that ES cultures can undergo fluctuations in gene expression that prime cells for at least two kinds of differentiation: PE and post-implantation epiblast.



## Lineage priming is regulated by exogenous signals

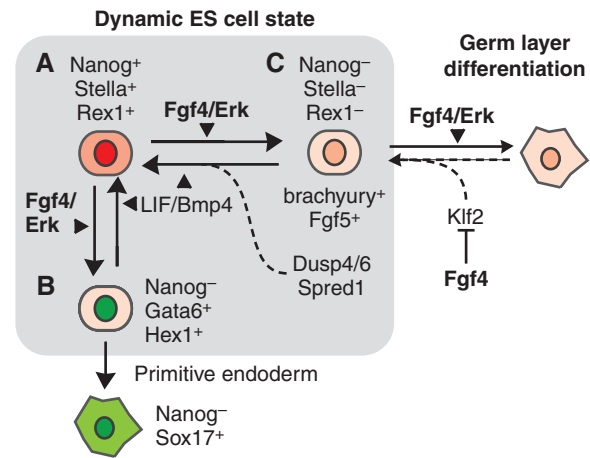
It has been suggested that these fluctuating patterns of gene expression could result from cell-autonomous stochastic variations in the expression of pluripotency-associated genes that are driven by transcriptional noise (Enver et al., 2009; Kalmar et al., 2009). However, external signaling feedback loops could also explain the cyclical fluctuations in the expression of pluripotency genes that have been observed. Niwa et al. recently suggested an elegant model in which heterogeneous expression is maintained by LIF through opposing downstream signaling pathways (Niwa et al., 2009). The authors showed that the LIF-Jak/Stat3 pathway induces Klf4 expression, which preferentially activates Sox2 expression, whereas the LIF-PI3K pathway induces Tbx3, which they suggest activates Nanog expression. They also reported that LIF activates the Erk signaling cascade, which counteracts the PI3K signal and reduces Tbx3 expression, thereby also reducing Nanog levels.

FGF signaling also acts through the Erk pathway and, given that it is required for ES cells to exit from the pluripotent state, it could play a role in modulating the metastable states of these cells. Indeed, it has been shown that manipulating FGF signaling levels in ES cultures can bias the proportions of the different states. Canham et al. showed that the Hex-expressing, PE lineage-primed population can be reduced by blocking Erk signaling or increased by stimulating phosphorylation with sodium vanadate (Canham et al., 2010).

Extracellular HSPGs are key co-activators of FGF receptors. ES cells with targeted mutations in genes involved in the HSPG biosynthesis pathway have been shown to act similarly to *Fgf4*<sup>-/-</sup> ES cells in being unable to exit from the pluripotent state and initiate differentiation (Johnson et al., 2007; Kraushaar et al., 2010; Lanner et al., 2010). *Ndst1/2*<sup>-/-</sup> cells lack the enzymes (N-deacetylase/N-sulfotransferase 1) that sulfate HSPGs, whereas exostoses 1 and 2 (*Ext1/2*)-null cells fail to synthesize proteoglycan chains at all. Such HSPG-deficient ES cells are also enriched for the Nanog<sup>+</sup> Klf4<sup>+</sup> ES population versus the Nanog<sup>-</sup> Fgf5<sup>+</sup> lineage-primed epiblast-like ES cells. Treatment of wild-type cells with sodium chlorate, which blocks sulfation of proteoglycans, had the same effect as inhibition of Erk signaling in terms of enriching for the Nanog<sup>+</sup> population (Lanner et al., 2010). Together, these data implicate the Fgf4-mediated activation of the Erk pathway in triggering the exit of ES cells from a self-renewing into a primed, but reversible, ES cell state. Microarray comparisons of wild-type ES cells with HSPG-deficient, sodium chlorate-treated or with FGFR inhibitor-treated ES cells have revealed the downregulation of *Dusp4* and *Dusp6*, together with the Ras/Erk signaling inhibitor *Spred1* (Lanner et al., 2010), when FGF signaling is inhibited. Active FGF signaling thus both promotes a primed state and induces the expression of inhibitors of FGF signaling to generate a negative-feedback loop that would facilitate reversion back to the original unprimed state. Thus, extracellular signaling feedback controls could drive the heterogeneous and potentially oscillating gene expression patterns that are observed in ES cells. In this way, a complex balance between multiple signaling pathways and their feedback mechanisms may underlie the heterogeneous nature of ES cell cultures (Fig. 2).

## Fgf4 signaling controls the segregation of epiblast and endoderm lineages in the ICM

The complex involvement of FGF signaling in regulating the pluripotent versus differentiated state in ES cells has some parallels in the mouse embryo itself. As the blastocyst develops, the



**Fig. 2. Dynamic state of mouse ES cells.** Mouse embryonic stem (ES) cells exhibit great heterogeneity, whereby some cells appear to be transiently primed for differentiation. At least three states have been suggested: (A) unprimed cells (Nanog<sup>+</sup> Stella<sup>+</sup> Rex1<sup>+</sup>); (B) cells primed for primitive endoderm (PE) (Gata6<sup>+</sup> Hex1<sup>+</sup>); and (C) cells primed for germ layer differentiation (Fgf5<sup>+</sup> brachyury<sup>+</sup>). Fgf4 signaling has been shown to maintain both primed states. Negative regulators of FGFR signaling, such as *Dusp4/6* and *Spred1*, are positively regulated by FGF signaling and may promote the reversion of primed ES cells to the unprimed state. Leukemia inhibitory factor (LIF) and bone morphogenetic protein (BMP) appear to promote ES self-renewal by counterbalancing the pro-differentiation autocrine Fgf4/Erk signal. The gray box highlights the cell populations that readily revert back to an unprimed state. Dashed lines indicate suggested function.

enclosed ICM cells all express the pluripotency marker Oct4, but as the blastocyst expands ICM cells show mosaic and progressively complementary expression of Nanog versus PE markers, such as Gata6 and *Pdgfra* (Chazaud et al., 2006; Plusa et al., 2008). Using *Pdgfra*<sup>H2B-GFP</sup> reporter mice, which express nuclear histone H2B-GFP, Plusa et al. went further and showed that ICM cells initially express both markers but randomly segregate into the two populations by the expanded blastocyst stage. Over the course of the next day, the *Pdgfra*-expressing cells end up on the blastocoelic surface and form PE, whereas the Nanog-high cells form the epiblast progenitors. The correct location of cells involves both the migration of cells to the appropriate position and the death of cells that are in the wrong place (Plusa et al., 2008).

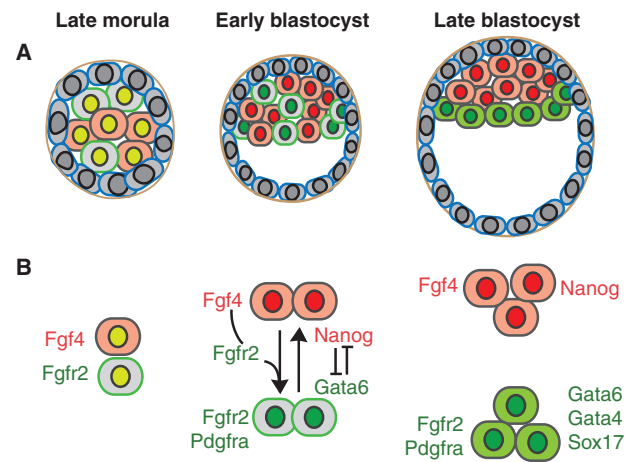
By embryonic day (E) 4.5, the two lineages are irreversibly committed to their future fate, as assessed by chimera analysis (Gardner and Rossant, 1979), but the earlier, apparently stochastic variation in Nanog expression in cells that are all Oct4 positive is very reminiscent of the heterogeneous Nanog expression observed in ES cells. Transcriptional microarray analysis of individual ICM cells has demonstrated that cells can be clustered into two groups that are enriched for many epiblast or PE markers, and not just for Nanog and Gata6 (Kurimoto et al., 2006). A recent study using single-cell quantitative PCR analysis of mouse embryos up to the 64-cell blastocyst stage elegantly provides further support to the mosaic segregation model, as it reported the existence of a temporally progressive inverse correlation between epiblast and PE marker expression (Guo et al., 2010).

Mice carrying mutations in components of the FGF/Erk pathway, including *Fgf4*, *Fgfr2* and *Grb2* (Feldman et al., 1995; Arman et al., 1998; Cheng et al., 1998; Chazaud et al., 2006), all

have peri-implantation lethality and lack PE formation, strongly suggesting a crucial role for this pathway in lineage specification. Pharmacological inhibition of FGF signaling in mouse embryos cultured from the 8-cell to expanded blastocyst stage can completely eliminate the Gata6-positive PE cells (Yamanaka et al., 2010). Conversely, the addition of exogenous Fgf4 to the cultures shifts all ICM cells to adopt the PE fate. The number of ICM cells does not significantly change when embryos are exposed to FGF inhibitors or to exogenous FGF, indicating that there is no selective apoptosis driving this apparent lineage shift. Using the *Pdgfra*<sup>H2B-GFP</sup> reporter mice, we observed the conversion of individual cells from either a GFP-positive or GFP-negative state in response to the inhibition or activation of the FGF/Erk signaling pathway (Yamanaka et al., 2010). Importantly, the fate of the cells could still be modulated after the mutually exclusive Nanog and Gata6 expression had been established at E3.5. The observed plasticity was, however, progressively lost by E4.0 (Fig. 3). Nichols et al. have reported similar results showing that the PE lineage is irreversibly lost when culturing embryos with FGF/Erk inhibition from E2.5-4.5 (Nichols et al., 2009a). Guo et al. also observed the elimination of PE transcripts (*Gata4*, *Sox17*) and the induction of epiblast transcripts (*Nanog*, *Esrrb*) when embryos were cultured in the presence of FGFR inhibitors (Guo et al., 2010).

Fgf4 is suggested to be produced by ICM cells under the direct transcriptional control of Oct4 and Sox2 (Yuan et al., 1995). Interestingly, Guo et al. detected a stronger and earlier inverse correlation between *Fgf4* and *Fgfr2* transcripts in ICM cells than between *Nanog* and *Gata6*, supporting a model in which FGF/Erk signaling variation is upstream of Nanog and Gata6 (Fig. 3). What is still unclear is how the initial variation in the levels of Fgf4 and Fgfr2 expression is established. This initial variation could be stochastic, as suggested for ES cells. However, unlike the ongoing heterogeneity in ES cells, there is only a brief window of time during which expression of lineage-specific genes such as *Nanog* and *Gata6* is still labile and responsive to levels of FGF signaling, before progressive feedback loops lock in cell fate. It is also possible that the initial variation in Fgf4 and Fgfr2 expression among ICM cells could reflect the lineage of these cells during earlier cleavage.

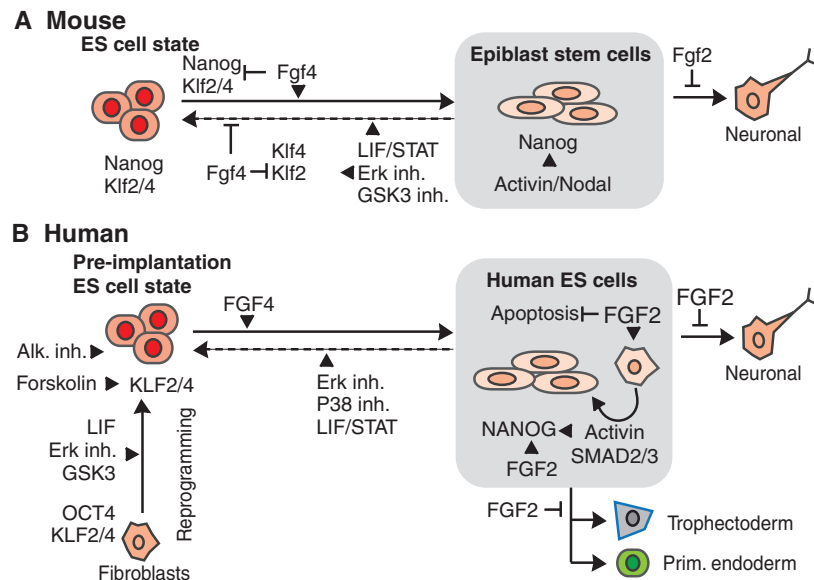
Inside cells are generated through two distinct rounds of asymmetric cell divisions as the embryo divides from the 8- to 16-cell and from the 16- to 32-cell stages (Chisholm and Houlston, 1987; Chazaud et al., 2006). It is thus an attractive model to suggest that these two sets of inside cells could have intrinsic differences that bias them towards either the epiblast or PE fate. Two recent papers attempted to test this by lineage tracing the progeny of the primary and secondary inner cells. Using a transgenic mouse line expressing a membrane-linked GFP, Morris et al. traced cell fate from the 8-cell to the expanded blastocyst stage, when individual cells were scored as being epiblast or PE by morphology (Morris et al., 2010). They reported that primary inner cells are biased towards epiblast, although 50% still give rise to both epiblast and PE, whereas secondary inner cells are heavily biased towards PE. Using a different lineage-tracing method that allowed cell fate to be followed through to the established epiblast and PE lineages post-implantation, Yamanaka et al. could not detect a clear bias towards either lineage (Yamanaka et al., 2010). Although these two papers show apparently conflicting data, in neither case was there an absolute relationship between lineage and fate, indicating that additional mechanisms still influence the final lineage allocation.



**Fig. 3. Fgf4-Fgfr2 signaling drives stable segregation of epiblast and primitive endoderm.** (A) The inner cell mass (ICM) of the early blastocyst consists of a heterogeneous population of epiblast (Epi, red) and primitive endodermal (PE, green) progenitors that express Nanog or Gata6, respectively, in a mutually exclusive manner. (B) Nanog and Gata6 inhibit each other and thereby segregate the two lineages. Titrated Fgf4/Erk signaling drives this lineage segregation, as addition of either exogenous Fgf4 or FGF/Erk inhibitor reverts all cells to PE or Epi, respectively. Importantly, the fate of the cells can still be modulated after the mutually exclusive Nanog and Gata6 expression has been established. This plasticity is however progressively lost by embryonic day 4.0 (E4.0). Supporting a lineage-instructive role of Fgf4, it has been shown that segregation of Fgf4 and Fgfr2 precedes Nanog and Gata6 expression. ICM cells are color coded to match cells in A,B. Trophoctoderm is in gray.

### FGF signaling in EpiSCs: stabilizing a metastable state?

The derivation of a new pluripotent stem cell type (the EpiSC) directly from the early post-implantation epiblast (Brons et al., 2007; Tesar et al., 2007) has shed new light on the evolving roles of FGF signaling in the transition from one pluripotent state to another. EpiSCs express the pluripotency markers Oct4 and Sox2 and can differentiate into derivatives of all germ layers in vitro. However, they cannot contribute to normal development in chimeras and they differ transcriptionally and epigenetically from ES cells. Importantly, they express markers of the post-implantation epiblast, such as *Fgf5*, and female cells are X-inactivated, unlike ES cells. Maintenance of these cells in the undifferentiated state in culture requires exogenous FGF and activin A, clearly distinguishing them from mouse ES cells. In the context of EpiSCs, FGF signaling has been demonstrated to act by inhibiting both forward differentiation to the neural lineage and reversion to a pre-implantation mouse ES cell-like state (Greber et al., 2010) (Fig. 3). Both these findings are surprising as neuronal differentiation is generally thought to be promoted by FGF signaling, and EpiSCs were proposed to have irreversibly lost the potential to revert back to an ES cell-like state. Results from Stavridis et al. support a neuronal suppression function of FGF in EpiSCs (Stavridis et al., 2010). They have shown that although FGF signaling is crucial for making the transition from ES cell to a post-implantation epiblast state, the subsequent transition to neuroectoderm is inhibited by FGF signaling.



**Fig. 4. FGF/Erk signaling stabilizes the metastable state of EpiSCs and hES cells.** (A) FGF/Erk signaling has a dual function in mouse EpiSCs: preventing neuronal differentiation and blocking reversion back to a pre-implantation ES cell-like state (Greber et al., 2010). Klf4 overexpression facilitates EpiSCs to revert back to a pre-implantation ES cell-like state (Guo et al., 2009). Inhibition of FGF/Erk signaling (Erk inh.) also facilitates such reversion by relieving FGF/Erk repression of endogenous Klf2 in EpiSCs (Greber et al., 2010). (B) FGF/Erk signaling in human ES (hES) cells maintains the pluripotent state and blocks neuronal, trophectoderm and primitive endoderm differentiation. FGF regulates NANOG expression both directly and indirectly via activin/NODAL induction in hES-derived feeder cells. It is unclear whether FGF/Erk signaling prevents reversion into a pre-implantation ES cell-like state, but combined ERK1/2 and P38 inhibition facilitates this conversion (Xu et al., 2010). A similar pre-implantation ES cell-like state can be achieved through genetic reprogramming by KLF2/4 overexpression in the presence of LIF, ERK1/2 and GSK3 $\beta$  (Li et al., 2009; Hanna et al., 2010). This state is further stabilized by activin/NODAL signaling inhibition (Alk. inh.) or addition of Forskolin. Forskolin induces cAMP signaling and induces endogenous KLF2/4 expression, thereby stabilizing the pre-implantation state following removal of reprogramming transgenes. The gray shading highlights the common mouse EpiSC and hES cell state.

This dual effect ensures that the EpiSC state is more stable than that of the epiblast-like cells seen in normal metastable ES cultures. However, it is possible to convert EpiSCs back to an ES-like state. Guo et al. and Hall et al. reported that this reversion from EpiSCs to the ES-like state does not occur spontaneously but depends upon the forced expression of Klf2 or Klf4 (Guo et al., 2009; Hall et al., 2009). However, using the distal *Oct4* promoter that is preferentially expressed in ES cells and not in post-implantation epiblast cells to drive GFP, Bao et al. reported a modest but spontaneous reversion of EpiSCs back to an ES cell-like state following their extended culture in media containing serum and LIF (Bao et al., 2009). These cells showed several hallmarks of true conversion, including X reactivation and the potential to contribute to somatic tissues and germ cells in chimeras. Greber et al. showed that this spontaneous conversion is greatly enhanced by combined FGF/Erk and GSK3 $\beta$  inhibition in LIF-containing media (Greber et al., 2010). Mechanistically, they showed that the switch in culture conditions induces Klf2 but not Klf4. Since Klf2 and Klf4 appear to have overlapping functions, they suggest that FGF suppression of Klf2 normally prevents EpiSCs from spontaneously reverting to an ES cell-like state, which is in line with the data presented by Guo et al. (Guo et al., 2009) (Fig. 3). Hanna et al. also reported that EpiSCs can be converted to an ES cell phenotype by application of Erk and GSK3 inhibition, and demonstrated that this reverted state is stable in permissive mouse strains, such as 129, but not in non-permissive strains, such as NOD (Hanna et al., 2009). Removal of the inhibitors leads to a conversion back to an EpiSC state for NOD lines but not for 129 lines. Thus, the two pluripotent states – ES-like and EpiSC-like – may have an intrinsic potential for interconversion, but the

relative stability of the two states may vary according to genetic background. In all cases, stable interconversion is still a relatively rare event, which suggests that there is still more to discover about the control of the different stem cell states. However, the modulation of FGF signaling is a key element in all cases.

### Human ES cells: the enigmatic role of FGF signaling

Although human and mouse ES cells are derived from the ICM of the blastocyst and rely on similar transcriptional regulators, such as Nanog, *Oct4* and Sox2, they require completely different culture conditions for their self-renewal in vitro. Indeed, although human ES (hES) cells show increased STAT3 phosphorylation in response to exogenous LIF, LIF addition does not seem to enhance self-renewal in culture (Thomson et al., 1998; Reubinoff et al., 2000; Sumi et al., 2004). Numerous protocols for hES cell culture have been reported, utilizing a variety of growth factors, such as FGF2, activin A, transforming growth factor  $\beta$ 1 (TGF $\beta$ 1) and Wnt. The common denominator in all these protocols is the use of exogenous FGF2. Indeed, the combination of FGF and activin signaling, which maintains mouse EpiSC self-renewal, is the most common and effective culture condition for the maintenance of hES cells as well. Comparison of gene expression profiles among hES cells, mouse ES cells and mouse EpiSCs showed that hES cells were more similar to mouse EpiSCs than to mouse ES cells (Brons et al., 2007; Tesar et al., 2007), which has led to the suggestion that the pluripotent cells captured from human blastocysts are actually more equivalent to the post-implantation-derived EpiSC than they are to the mouse ES cell (Brons et al., 2007; Tesar et al., 2007; Rossant, 2008).



The mode of action of FGF signaling in promoting hES self-renewal seems to be different from that by which FGF signaling blocks both differentiation and reversion to an ES-like state in mouse EpiSCs (Greber et al., 2007; Greber et al., 2010) (Fig. 4). In contrast to mouse ES cells, hES cells and EpiSCs are reported to exhibit a high degree of spontaneous differentiation into both extra-embryonic lineages (Xu et al., 2002; Brons et al., 2007; Tesar et al., 2007). It has been suggested that FGF is crucial for blocking such extra-embryonic differentiation. Inhibition of the FGF receptors or of downstream Erk signaling in hES cells induces the TE markers CDX1/2 and gonadotrophin- $\alpha/\beta$  and the PE marker SOX7 (D'Amour et al., 2005; Li et al., 2007). Furthermore, activated HRas robustly induces the extra-embryonic differentiation of ES cells (Lu et al., 2008). FGF has also been shown to cooperate with activin signaling through SMAD2/3 to activate NANOG expression in hES cells (Vallier et al., 2005; Li et al., 2007; Greber et al., 2007).

Some of the activity of FGF may be indirect via its action on the supporting feeder cells in hES cultures. Examining the transcriptional response of feeder cells to FGF2 identified the induction of TGF $\beta$ 1 and activin A in these cells, which can subsequently promote the undifferentiated state of hES cells (Greber et al., 2007). hES cells can be cultured in the absence of feeders or of feeder-conditioned media, but in these conditions the ES cells may generate their own 'niche' cells, which are flattened cells that respond to exogenous FGF2 in a similar manner to feeder cells (Greber et al., 2007; Bendall et al., 2007). Bendall et al. reported that the predominant FGF receptor, FGFR1, is expressed exclusively in these endogenous feeder-like cells and that they secrete TGF $\beta$ 1 and insulin-like growth factor 2 (IGF2) in response to exogenous FGF2 (Bendall et al., 2007). They then showed that the function of exogenous FGF in promoting ES self-renewal could be replaced by addition of IGF2 alone, and suggested that the role of FGF is in fact an indirect one, via its effect on the niche in which hES cells grow. However, this extreme model has been challenged in subsequent publications. Wang et al. reported that exogenous IGF2 alone was insufficient to maintain undifferentiated growth in defined (TeSR1) culture conditions (Wang et al., 2009) and suggested that FGF has a direct role in blocking caspase-activated apoptosis through anoikis in ES cells. An anti-apoptotic action of FGF is also supported by data from Eiselleova et al., who were able to detect all four FGF receptors and weak FGF2 binding to undifferentiated hES colonies (Eiselleova et al., 2009). However, after a few days of differentiation, they did detect increased binding of FGF2 to peripheral OCT4-negative cells, as shown by Bendall et al. (Bendall et al., 2007). It seems likely that FGF2 acts both directly and indirectly on hES cells to maintain the undifferentiated state, promoting expression of pluripotency markers, such as OCT4 and the DNA methyltransferase DNMT3B, while reducing expression of TE markers and preventing cell death and apoptosis.

Given this complex interplay, it is not clear how to relate the effects of FGF signaling in hES cells with its known roles in modulating the metastable pluripotent states in mouse ES cells, EpiSCs and embryos. If hES cells are really like mouse EpiSCs, it might be possible to drive them towards a state more similar to the mouse ES cell by blocking FGF/Erk signaling and GSK3, as observed in the mouse. However, it has been clearly shown that blocking Erk signaling alone causes hES cells to lose their self-renewal capacity (Li et al., 2007). No successful direct derivation of hES cells through FGF/Erk signaling inhibition has been reported so far. However, human induced pluripotent stem (iPS)

cells with morphological and transcriptional characteristics more similar to those of mouse ES cells have been established through genetic reprogramming in the presence of Erk and GSK3 $\beta$  inhibition (Li et al., 2009; Hanna et al., 2010). Li et al. reported that supplementing the inhibitor cocktail with activin A signaling inhibitor is crucial for preventing differentiation. Hanna et al. showed that induced pre-implantation hES cells require at least the transient expression of *OCT4* and *KLF4* and degenerate upon removal of the transgenes unless the inhibition cocktail was supplemented with Forskolin (Fig. 4). This suggests that a pre-implantation ES cell state can also be stabilized in human cultures. It has also recently been reported that it is possible to achieve conversion of hES cells to a mouse-like ES cell phenotype through the combined use of LIF with Erk and p38 (Mapk14) inhibitors (Xu et al., 2010). It will be interesting to find out whether fluctuations and interconversions between these different pluripotent states occur as in the mouse, and whether they can be modulated by the same or different external signaling pathways.

### Conclusions

FGF signaling clearly plays multiple and sometimes contrasting roles in the early embryo and pluripotent stem cells. In one setting, FGF/Erk maintains pluripotency and in another it drives lineage differentiation. It is still unclear how such diversity is generated from seemingly identical FGF signaling responses. As ligands and receptors show restricted expression patterns throughout early development, it is possible that different ligand-receptor combinations could facilitate different signaling outputs. Much of the recent work on FGF signaling in pluripotent stem cells has focused on the FGF/Erk signaling axis, whereas the potential roles of FGF-activated PI3K, PLC $\gamma$  and Jak/Stat signaling have been largely overlooked. Niwa et al. have shown that parallel LIF signaling through Jak/Stat and PI3K pathways maintains the pluripotent ES cell state, whereas Erk signaling appears to destabilize it (Niwa et al., 2009). This finding suggests that FGF-activated pathways might also exhibit such differential and opposing functions. Eiselleova et al. also showed that the FGF signal is transduced via PI3K-Akt, as well as through the Erk pathway in hES cells (Eiselleova et al., 2009). This raises the possibility of differential functions of FGF in hES cells that are dependent on the particular downstream signaling pathway activated. Fine-tuning the balance between multiple and opposing signals downstream of FGF receptors could therefore generate contrasting functional outcomes, either maintaining pluripotency or instructing lineage differentiation. This fine-tuning might be further influenced by additional extracellular signals, such as LIF, BMP and Wnt, with downstream signaling responses that may interconnect with the FGF pathways. Recent data using global phosphoproteomics found that several pluripotency-associated proteins exhibit regulated phosphorylation in hES cells, suggesting that there might be direct post-transcriptional regulation of the pluripotent state (Brill et al., 2009; Van Hoof et al., 2009). The role of FGF and other signaling pathways in activating downstream pathways in ES cells at the post-transcriptional and transcriptional levels remains to be elucidated. A fuller understanding of the extrinsic and intrinsic pathways that regulate interchangeable and metastable pluripotent stem cell states will have a significant impact on the field of reprogramming and iPS cells.

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**Competing interests statement**

The authors declare no competing financial interests.

**References**

- Arman, E., Haffner-Krausz, R., Chen, Y., Heath, J. K. and Lonai, P. (1998). Targeted disruption of fibroblast growth factor (FGF) receptor 2 suggests a role for FGF signaling in pregastrulation mammalian development. *Proc. Natl. Acad. Sci. USA* **95**, 5082-5087.
- Bao, S., Tang, F., Li, X., Hayashi, K., Gillich, A., Lao, K. and Surani, M. A. (2009). Epigenetic reversion of post-implantation epiblast to pluripotent embryonic stem cells. *Nature* **461**, 1292-1295.
- Bendall, S. C., Stewart, M. H., Menendez, P., George, D., Vijayaragavan, K., Werbowetski-Ogilvie, T., Ramos-Mejia, V., Rouleau, A., Yang, J., Bosse, M. et al. (2007). IGF and FGF cooperatively establish the regulatory stem cell niche of pluripotent human cells in vitro. *Nature* **448**, 1015-1021.
- Brill, L. M., Xiong, W., Lee, K. B., Ficarro, S. B., Crain, A., Xu, Y., Terskikh, A., Snyder, E. Y. and Ding, S. (2009). Phosphoproteomic analysis of human embryonic stem cells. *Cell Stem Cell* **5**, 204-213.
- Brons, I. G., Smithers, L. E., Trotter, M. W., Rugg-Gunn, P., Sun, B., Chuva de Sousa Lopes, S. M., Howlett, S. K., Clarkson, A., Ahrlund-Richter, L., Pedersen, R. A. et al. (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, Q. L. and Smith, A. (2008). Capture of authentic embryonic stem cells from rat blastocysts. *Cell* **135**, 1287-1298.
- Canham, M. A., Sharov, A. A., Ko, M. S. and Brickman, J. M. (2010). Functional heterogeneity of embryonic stem cells revealed through translational amplification of an early endodermal transcript. *PLoS Biol.* **8**, e1000379.
- Chambers, I., Colby, D., Robertson, M., Nichols, J., Lee, S., Tweedie, S. and Smith, A. (2003). Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. *Cell* **113**, 643-655.
- Chambers, I., Silva, J., Colby, D., Nichols, J., Nijmeijer, B., Robertson, M., Vrana, J., Jones, K., Grotewold, L. and Smith, A. (2007). Nanog safeguards pluripotency and mediates germline development. *Nature* **450**, 1230-1234.
- Chazaud, C., Yamanaka, Y., Pawson, T. and Rossant, J. (2006). Early lineage segregation between epiblast and primitive endoderm in mouse blastocysts through the Grb2-MAPK pathway. *Dev. Cell* **10**, 615-624.
- Cheng, A. M., Saxton, T. M., Sakai, R., Kulkarni, S., Mbamalu, G., Vogel, W., Tortorice, C. G., Cardiff, R. D., Cross, J. C., Muller, W. J. et al. (1998). Mammalian Grb2 regulates multiple steps in embryonic development and malignant transformation. *Cell* **95**, 793-803.
- Chisholm, J. C. and Houliston, E. (1987). Cytokeratin filament assembly in the preimplantation mouse embryo. *Development* **101**, 565-582.
- Colvin, J. S., Bohne, B. A., Harding, G. W., McEwen, D. G. and Ornitz, D. M. (1996). Skeletal overgrowth and deafness in mice lacking fibroblast growth factor receptor 3. *Nat. Genet.* **12**, 390-397.
- Dailey, L., Ambrosetti, D., Mansukhani, A. and Basilico, C. (2005). Mechanisms underlying differential responses to FGF signaling. *Cytokine Growth Factor Rev.* **16**, 233-247.
- D'Amour, K. A., Agulnick, A. D., Eliazar, S., Kelly, O. G., Kroon, E. and Baetge, E. E. (2005). Efficient differentiation of human embryonic stem cells to definitive endoderm. *Nat. Biotechnol.* **23**, 1534-1541.
- Deng, C. X., Wynshaw-Boris, A., Shen, M. M., Daugherty, C., Ornitz, D. M. and Leder, P. (1994). Murine FGFR-1 is required for early postimplantation growth and axial organization. *Genes Dev.* **8**, 3045-3057.
- Deng, C., Wynshaw-Boris, A., Zhou, F., Kuo, A. and Leder, P. (1996). Fibroblast growth factor receptor 3 is a negative regulator of bone growth. *Cell* **84**, 911-921.
- Dono, R., Texido, G., Dussel, R., Ehmke, H. and Zeller, R. (1998). Impaired cerebral cortex development and blood pressure regulation in FGF-2-deficient mice. *EMBO J.* **17**, 4213-4225.
- Eiselleova, L., Matulka, K., Kriz, V., Kunova, M., Schmidtova, Z., Neradil, J., Tichy, B., Dvorakova, D., Pospisilova, S., Hampl, A. et al. (2009). A complex role for FGF-2 in self-renewal, survival, and adhesion of human embryonic stem cells. *Stem Cells* **27**, 1847-1857.
- Enver, T., Pera, M., Peterson, C. and Andrews, P. W. (2009). Stem cell states, fates, and the rules of attraction. *Cell Stem Cell* **4**, 387-397.
- Evans, M. J. and Kaufman, M. H. (1981). Establishment in culture of pluripotential cells from mouse embryos. *Nature* **292**, 154-156.
- Feldman, B., Poueymirou, W., Papaioannou, V. E., DeChiara, T. M. and Goldfarb, M. (1995). Requirement of FGF-4 for postimplantation mouse development. *Science* **267**, 246-249.
- Furusawa, T., Ohkoshi, K., Honda, C., Takahashi, S. and Tokunaga, T. (2004). Embryonic stem cells expressing both platelet endothelial cell adhesion molecule-1 and stage-specific embryonic antigen-1 differentiate predominantly into epiblast cells in a chimeric embryo. *Biol. Reprod.* **70**, 1452-1457.
- Furusawa, T., Ikeda, M., Inoue, F., Ohkoshi, K., Hamano, T. and Tokunaga, T. (2006). Gene expression profiling of mouse embryonic stem cell subpopulations. *Biol. Reprod.* **75**, 555-561.
- Gardner, R. L. and Rossant, J. (1979). Investigation of the fate of 4-5 day post-coitum mouse inner cell mass cells by blastocyst injection. *J. Embryol. Exp. Morphol.* **52**, 141-152.
- Gotoh, N., Laks, S., Nakashima, M., Lax, I. and Schlessinger, J. (2004). FRS2 family docking proteins with overlapping roles in activation of MAP kinase have distinct spatial-temporal patterns of expression of their transcripts. *FEBS Lett.* **564**, 14-18.
- Greber, B., Lehrach, H. and 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, J. Y., Han, D. W., Ko, K., Tapia, N., Sabour, D., Sterneckert, J., Tesar, P. et al. (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, J. S., Eyres, I., Mansfield, W. and Smith, A. (2009). Klf4 reverts developmentally programmed restriction of ground state pluripotency. *Development* **136**, 1063-1069.
- Guo, G., Huss, M., Tong, G. Q., Wang, C., Li Sun, L., Clarke, N. D. and Robson, P. (2010). Resolution of cell fate decisions revealed by single-cell gene expression analysis from zygote to blastocyst. *Dev. Cell* **18**, 675-685.
- Hadari, Y. R., Gotoh, N., Kouhara, H., Lax, I. and Schlessinger, J. (2001). Critical role for the docking-protein FRS2 alpha in FGF receptor-mediated signal transduction pathways. *Proc. Natl. Acad. Sci. USA* **98**, 8578-8583.
- Hall, J., Guo, G., Wray, J., Eyres, I., Nichols, J., Grotewold, L., Morfopoulou, S., Humphreys, P., Mansfield, W., Walker, R. et al. (2009). Oct4 and LIF/Stat3 additively induce Kruppel factors to sustain embryonic stem cell self-renewal. *Cell Stem Cell* **5**, 597-609.
- Hanna, J., Markoulaki, S., Mitalipova, M., Cheng, A. W., Cassidy, J. P., Staerk, J., Carey, B. W., Lengner, C. J., Foreman, R., Love, J. et al. (2009). Metastable pluripotent states in NOD-mouse-derived ESCs. *Cell Stem Cell* **4**, 513-524.
- Hanna, J., Cheng, A. W., Saha, K., Kim, J., Lengner, C. J., Soldner, F., Cassidy, J. P., Muffat, J., Carey, B. W. and 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.
- Hatano, N., Mori, Y., Oh-hora, M., Kosugi, A., Fujikawa, T., Nakai, N., Niwa, H., Miyazaki, J., Hamaoka, T. and Ogata, M. (2003). Essential role for ERK2 mitogen-activated protein kinase in placental development. *Genes Cells* **8**, 847-856.
- Hayashi, K., Lopes, S. M., Tang, F. and Surani, M. A. (2008). Dynamic equilibrium and heterogeneity of mouse pluripotent stem cells with distinct functional and epigenetic states. *Cell Stem Cell* **3**, 391-401.
- Hebert, J. M., Boyle, M. and Martin, G. R. (1991). mRNA localization studies suggest that murine FGF-5 plays a role in gastrulation. *Development* **112**, 407-415.
- Hebert, J. M., Rosenquist, T., Gotz, J. and Martin, G. R. (1994). FGF5 as a regulator of the hair growth cycle: evidence from targeted and spontaneous mutations. *Cell* **78**, 1017-1025.
- Itoh, N. and Ornitz, D. M. (2004). Evolution of the Fgf and Fgfr gene families. *Trends Genet.* **20**, 563-569.
- Johnson, C. E., Crawford, B. E., Stavridis, M., Ten Dam, G., Wat, A. L., Rushton, G., Ward, C. M., Wilson, V., van Kuppevelt, T. H., Esko, J. D. et al. (2007). Essential alterations of heparan sulfate during the differentiation of embryonic stem cells to Sox1-enhanced green fluorescent protein-expressing neural progenitor cells. *Stem Cells* **8**, 1913-1923.
- Kalmar, T., Lim, C., Hayward, P., Munoz-Descalzo, S., Nichols, J., Garcia-Ojalvo, J. and Martinez Arias, A. (2009). Regulated fluctuations in nanog expression mediate cell fate decisions in embryonic stem cells. *PLoS Biol.* **7**, e1000149.
- Kraushaar, D. C., Yamaguchi, Y. and Wang, L. (2010). Heparan sulfate is required for embryonic stem cells to exit from self-renewal. *J. Biol. Chem.* **285**, 5907-5916.
- Kunath, T., Arnaud, D., Uy, G. D., Okamoto, I., Chureau, C., Yamanaka, Y., Heard, E., Gardner, R. L., Avner, P. and Rossant, J. (2005). Imprinted X-inactivation in extra-embryonic endoderm cell lines from mouse blastocysts. *Development* **132**, 1649-1661.
- Kunath, T., Saba-El-Leil, M. K., Almousaillekh, M., Wray, J., Meloche, S. and Smith, A. (2007). FGF stimulation of the Erk1/2 signalling cascade triggers transition of pluripotent embryonic stem cells from self-renewal to lineage commitment. *Development* **134**, 2895-2902.
- Kurimoto, K., Yabuta, Y., Ohinata, Y., Ono, Y., Uno, K. D., Yamada, R. G., Ueda, H. R. and Saitou, M. (2006). An improved single-cell cDNA amplification method for efficient high-density oligonucleotide microarray analysis. *Nucleic Acids Res.* **34**, e42.
- Lanner, F., Lee, K. L., Sohl, M., Holmbo, K., Yang, H., Wilbertz, J., Poellinger, L., Rossant, J. and Farnebo, F. (2010). Heparan sulfation-dependent fibroblast growth factor signaling maintains embryonic stem cells primed for differentiation in a heterogeneous state. *Stem Cells* **28**, 191-200.

- Li, J., Wang, G., Wang, C., Zhao, Y., Zhang, H., Tan, Z., Song, Z., Ding, M. and Deng, H. (2007). MEK/ERK signaling contributes to the maintenance of human embryonic stem cell self-renewal. *Differentiation* **75**, 299-307.
- Li, P., Tong, C., Mehrian-Shai, R., Jia, L., Wu, N., Yan, Y., Maxson, R. E., Schulze, E. N., Song, H., Hsieh, C. L. et al. (2008). Germline competent embryonic stem cells derived from rat blastocysts. *Cell* **135**, 1299-1310.
- Li, W., Wei, W., Zhu, S., Zhu, J., Shi, Y., Lin, T., Hao, E., Hayek, A., Deng, H. and Ding, S. (2009). Generation of rat and human induced pluripotent stem cells by combining genetic reprogramming and chemical inhibitors. *Cell Stem Cell* **4**, 16-19.
- Lu, C. W., Yabuuchi, A., Chen, L., Viswanathan, S., Kim, K. and Daley, G. Q. (2008). Ras-MAPK signaling promotes trophoblast formation from embryonic stem cells and mouse embryos. *Nat. Genet.* **40**, 921-926.
- Mansour, S. L. (1994). Targeted disruption of int-2 (fgf-3) causes developmental defects in the tail and inner ear. *Mol. Reprod. Dev.* **39**, 62-68.
- 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**, 7634-7638.
- Meyers, E. N., Lewandoski, M. and Martin, G. R. (1998). An Fgf8 mutant allelic series generated by Cre- and Flp-mediated recombination. *Nat. Genet.* **18**, 136-141.
- Mitsui, K., Tokuzawa, Y., Itoh, H., Segawa, K., Murakami, M., Takahashi, K., Maruyama, M., Maeda, M. and Yamanaka, S. (2003). The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. *Cell* **113**, 631-642.
- Morris, S. A., Teo, R. T., Li, H., Robson, P., Glover, D. M. and Zernicka-Goetz, M. (2010). Origin and formation of the first two distinct cell types of the inner cell mass in the mouse embryo. *Proc. Natl. Acad. Sci. USA* **107**, 6364-6369.
- Niakan, K. K., Ji, H., Maehr, R., Vokes, S. A., Rodolfa, K. T., Sherwood, R. I., Yamaki, M., Dimos, J. T., Chen, A. E., Melton, D. A. et al. (2010). Sox17 promotes differentiation in mouse embryonic stem cells by directly regulating extraembryonic gene expression and indirectly antagonizing self-renewal. *Genes Dev.* **24**, 312-326.
- Nichols, J. and Smith, A. (2009). Naïve and primed pluripotent states. *Cell Stem Cell* **4**, 487-492.
- Nichols, J., Silva, J., Roode, M. and Smith, A. (2009a). Suppression of Erk signalling promotes ground state pluripotency in the mouse embryo. *Development* **136**, 3215-3222.
- Nichols, J., Jones, K., Phillips, J. M., Newland, S. A., Roode, M., Mansfield, W., Smith, A. and Cooke, A. (2009b). Validated germline-competent embryonic stem cell lines from nonobese diabetic mice. *Nat. Med.* **15**, 814-818.
- Niwa, H., Ogawa, K., Shimosato, D. and Adachi, K. (2009). A parallel circuit of LIF signalling pathways maintains pluripotency of mouse ES cells. *Nature* **460**, 118-122.
- Ornitz, D. M. and Itoh, N. (2001). Fibroblast growth factors. *Genome Biol.* **2**, REVIEWS3005.
- Pages, G., Guerin, S., Grall, D., Bonino, F., Smith, A., Anjuere, F., Auberger, P. and Pouyssegur, J. (1999). Defective thymocyte maturation in p44 MAP kinase (Erk 1) knockout mice. *Science* **286**, 1374-1377.
- Payer, B., Chua de Sousa Lopes, S. M., Barton, S. C., Lee, C., Saitou, M. and Surani, M. A. (2006). Generation of stella-GFP transgenic mice: a novel tool to study germ cell development. *Genesis* **44**, 75-83.
- Plusa, B., Piliszek, A., Frankenberg, S., Artus, J. and Hadjantonakis, A. K. (2008). Distinct sequential cell behaviours direct primitive endoderm formation in the mouse blastocyst. *Development* **135**, 3081-3091.
- Reubinoff, B. E., Pera, M. F., Fong, C. Y., Trounson, A. and Bongso, A. (2000). Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. *Nat. Biotechnol.* **18**, 399-404.
- Rossant, J. (2008). Stem cells and early lineage development. *Cell* **132**, 527-531.
- Saba-El-Leil, M. K., Vella, F. D., Vernay, B., Voisin, L., Chen, L., Labrecque, N., Ang, S. L. and Meloche, S. (2003). An essential function of the mitogen-activated protein kinase Erk2 in mouse trophoblast development. *EMBO Rep.* **4**, 964-968.
- Sato, N., Meijer, L., Skaltsounis, L., Greengard, P. and 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**, 55-63.
- Saxton, T. M., Henkemeyer, M., Gasca, S., Shen, R., Rossi, D. J., Shalaby, F., Feng, G. S. and Pawson, T. (1997). Abnormal mesoderm patterning in mouse embryos mutant for the SH2 tyrosine phosphatase Shp-2. *EMBO J.* **16**, 2352-2364.
- Singh, A. M., Hamazaki, T., Hankowski, K. E. and Terada, N. (2007). A heterogeneous expression pattern for Nanog in embryonic stem cells. *Stem Cells* **25**, 2534-2542.
- Smith, A. G., Heath, J. K., Donaldson, D. D., Wong, G. G., Moreau, J., Stahl, M. and Rogers, D. (1988). Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. *Nature* **336**, 688-690.
- Stavridis, M. P., Lunn, J. S., Collins, B. J. and Storey, K. G. (2007). A discrete period of FGF-induced Erk1/2 signalling is required for vertebrate neural specification. *Development* **134**, 2889-2894.
- Stavridis, M. P., Collins, B. J. and Storey, K. G. (2010). Retinoic acid orchestrates fibroblast growth factor signalling to drive embryonic stem cell differentiation. *Development* **137**, 881-890.
- Sumi, T., Fujimoto, Y., Nakatsuji, N. and Suemori, H. (2004). STAT3 is dispensable for maintenance of self-renewal in nonhuman primate embryonic stem cells. *Stem Cells* **22**, 861-872.
- Suzuki, A., Raya, A., Kawakami, Y., Morita, M., Matsui, T., Nakashima, K., Gage, F. H., Rodriguez-Esteban, C. and Izpisua Belmonte, J. C. (2006). Maintenance of embryonic stem cell pluripotency by Nanog-mediated reversal of mesoderm specification. *Nat. Clin. Pract. Cardiovasc. Med.* **3 Suppl.** **1**, S114-S122.
- Tanaka, S., Kunath, T., Hadjantonakis, A. K., Nagy, A. and Rossant, J. (1998). Promotion of trophoblast stem cell proliferation by FGF4. *Science* **282**, 2072-2075.
- Tesar, P. J., Chenoweth, J. G., Brook, F. A., Davies, T. J., Evans, E. P., Mack, D. L., Gardner, R. L. and McKay, R. D. (2007). New cell lines from mouse epiblast share defining features with human embryonic stem cells. *Nature* **448**, 196-199.
- Thomson, J. A., Itskovitz-Eldor, J., Shapiro, S. S., Waknitz, M. A., Swiergiel, J. J., Marshall, V. S. and Jones, J. M. (1998). Embryonic stem cell lines derived from human blastocysts. *Science* **282**, 1145-1147.
- Toyooka, Y., Shimosato, D., Murakami, K., Takahashi, K. and Niwa, H. (2008). Identification and characterization of subpopulations in undifferentiated ES cell culture. *Development* **135**, 909-918.
- Tropepe, V., Hitoshi, S., Sirard, C., Mak, T. W., Rossant, J. and van der Kooy, D. (2001). Direct neural fate specification from embryonic stem cells: a primitive mammalian neural stem cell stage acquired through a default mechanism. *Neuron* **30**, 65-78.
- Vallier, L., Alexander, M. and Pedersen, R. A. (2005). Activin/Nodal and FGF pathways cooperate to maintain pluripotency of human embryonic stem cells. *J. Cell Sci.* **118**, 4495-4509.
- Van Hoof, D., Munoz, J., Braam, S. R., Pinkse, M. W., Linding, R., Heck, A. J., Mummery, C. L. and Krijgsveld, J. (2009). Phosphorylation dynamics during early differentiation of human embryonic stem cells. *Cell Stem Cell* **5**, 214-226.
- Wang, X., Lin, G., Martins-Taylor, K., Zeng, H. and Xu, R. H. (2009). Inhibition of caspase-mediated anoikis is critical for basic fibroblast growth factor-sustained culture of human pluripotent stem cells. *J. Biol. Chem.* **284**, 34054-34064.
- Weinstein, M., Xu, X., Ohyama, K. and Deng, C. X. (1998). FGFR-3 and FGFR-4 function cooperatively to direct alveogenesis in the murine lung. *Development* **125**, 3615-3623.
- Wilder, P. J., Kelly, D., Brigman, K., Peterson, C. L., Nowling, T., Gao, Q. S., McComb, R. D., Capocchi, M. R. and Rizzino, A. (1997). Inactivation of the FGF-4 gene in embryonic stem cells alters the growth and/or the survival of their early differentiated progeny. *Dev. Biol.* **192**, 614-629.
- 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. and Gough, N. M. (1988). Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. *Nature* **336**, 684-687.
- Xu, R. H., Chen, X., Li, D. S., Li, R., Addicks, G. C., Glennon, C., Zwaka, T. P. and Thomson, J. A. (2002). BMP4 initiates human embryonic stem cell differentiation to trophoblast. *Nat. Biotechnol.* **20**, 1261-1264.
- Xu, Y., Zhu, X., Hahm, H. S., Wei, W., Hao, E., Hayek, A. and Ding, S. (2010). Revealing a core signaling regulatory mechanism for pluripotent stem cell survival and self-renewal by small molecules. *Proc. Natl. Acad. Sci. USA* **107**, 8129-8134.
- Yamaguchi, T. P. and Rossant, J. (1995). Fibroblast growth factors in mammalian development. *Curr. Opin. Genet. Dev.* **5**, 485-491.
- Yamaguchi, T. P., Harpal, K., Henkemeyer, M. and Rossant, J. (1994). fgfr-1 is required for embryonic growth and mesodermal patterning during mouse gastrulation. *Genes Dev.* **8**, 3032-3044.
- Yamanaka, Y., Lanner, F. and Rossant, J. (2010). FGF signal-dependent segregation of primitive endoderm and epiblast in the mouse blastocyst. *Development* **137**, 715-724.
- Yang, W., Klamn, L. D., Chen, B., Araki, T., Harada, H., Thomas, S. M., George, E. L. and Neel, B. G. (2006). An Shp2/SFK/Ras/Erk signaling pathway controls trophoblast stem cell survival. *Dev. Cell* **10**, 317-327.
- Ying, Q. L., Nichols, J., Chambers, I. and 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, Q. L., Wray, J., Nichols, J., Battle-Morera, L., Doble, B., Woodgett, J., Cohen, P. and Smith, A. (2008). The ground state of embryonic stem cell self-renewal. *Nature* **453**, 519-523.
- Yuan, H., Corbi, N., Basilico, C. and Dailey, L. (1995). Developmental-specific activity of the FGF-4 enhancer requires the synergistic action of Sox2 and Oct-3. *Genes Dev.* **9**, 2635-2645.