Signaling pathways during maintenance and definitive endoderm differentiation of embryonic stem cells

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ABSTRACT Embryonic stem cells (ESCs) have the potential to be used as unlimited resources for tissue replacement therapy, thereby compensating for organ donor shortage. To reach this goal, the molecular principles governing early differentiation events in the developing embryo need to be addressed, understood and properly implemented in vitro. Studies carried out in several vertebrate models have established that Nodal/Activin A, BMP, WNT and FGF signaling pathways regulate early embryo development and that these pathways are similarly used during germ layer formation by cultured ESCs. However, differences have also been identified in the way these pathways function or interact in mouse vs. human ESCs, making it sometimes difficult to extrapolate findings from one system to the other. In this review, we discuss and compare the role of the relevant signaling pathways and their crosstalk during undifferentiated growth and during the endoderm differentiation of mouse and human ESCs.

KEY WORDS: human embryonic stem cell, mouse embryonic stem cells definitive endoderm, FGF, Nodal/Activin A

Introduction

Mouse embryonic stem cells (mESCs) were first isolated in 1981 and derived from the inner cell mass (ICM) of pre-implantation blastocyst stage embryo (Evans and Kaufman, 1981, Martin, 1981). They are able to self-renew and to differentiate into any somatic cell type in culture or following transplantation in vivo. With regards to these properties, the derivation of human embryonic stem cells (hESCs) (Thomson et al., 1998) was immediately followed by efforts to differentiate them into desired cell types such as neurons (Cho et al., 2008, Di Giorgio et al., 2008, Mueller et al., 2005, Nat and Dechant, 2011), cardiomyocytes (Laflamme et al., 2007, Mummery et al., 2007, Parsons et al., 2011, Xu et al., 2009), hepatocytes (Agarwal et al., 2008, Hay et al., 2008, Touboul et al., 2010) and pancreatic endocrine cells (Assady et al., 2001, Cai et al., 2010, D’Amour et al., 2006, Jiang et al., 2007a, Jiang et al., 2007b, Johannesson et al., 2009, Kroon et al., 2008, Mfopou et al., 2010a, Xu et al., 2011, Zhang et al., 2009) in view of their future use to replace dysfunctional or missing cells in diseases. ESCs are not only used as a potential tool for cell replacement therapy, disease modeling, drug discovery and toxicity testing; they also serve as a model to understand the molecular mechanisms of germ layer formation during early development, a pivotal process that dictates further ontogeny of organ-specific cells. Three germ layers, namely endoderm, mesoderm and ectoderm, are specified during gastrulation in the developing embryo, thereby setting up the landmarks for future tissues and organs. Similarly, the specification of these germ layer equivalents in vitro appears as one of the first and most important events required during ESC differentiation for the generation of desired cell types. Considering the endoderm, a number of signaling pathways have been identified that control its differentiation in lower and higher vertebrates, including Nodal/Activin A, BMP, WNT and FGF (Hansson et al., 2009, McLean et al., 2007, Morrison et al., 2008, Poulain et al., 2006, Rodaway et al., 1999, Rossant, 2008, Slack, 1994, Sumi et al., 2008, Vallier et al., 2009b, Xu et al., 2011, Zhang et al., 2008, Zheng et al., 2010). Although the contribution of these pathways is conserved among many species, their particular functions (inductive or repressive) and their activity timing can vary significantly, explaining in part the contrasting findings that have been described, for instance, between mESCs and hESCs. In the scope of this review, we aim at giving an overview of the current understanding of germ layer

Abbreviations used in this paper: BMP, bone morphogenetic protein; CDM, chemically defined medium; DE, definitive endoderm; ESC, embryonic stem cell; FGF, ﬁbroblast growth factor; KSR, knockout serum replacement; LIF, leukemia inhibitory factor; MEF, mouse embryonic ﬁbroblasts; PS, primitive streak; TGF-beta, transforming growth factor beta; WNT, wingless-type MMTV integration site family member.

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from mESCs and hESCs, with special focus on the definitive endoderm (DE).

The signaling pathways maintaining undifferentiated ESCs status

Mouse or human ESCs are essentially characterized by their ability to make multiple copies of themselves for long periods in culture (self-renewal), and to generate cells belonging to the three germ layers upon differentiation (pluripotency). These essential properties of mESCs and hESCs (Rossant, 2008, Vallier et al., 2009b) are actually under the control of signaling pathways that differ between the two species. Undifferentiated mESCs are mainly maintained under the control of LIF and BMP signaling (Chambers and Smith, 2004, Williams et al., 1988, Ying et al., 2003a), while hESCs require Activin and FGF signaling to sustain long-term self-renewal and pluripotency (Ding et al., 2010, Vallier et al., 2005, Xiao et al., 2006). However, these signaling pathways converge towards the activation of a core transcriptional network supporting self-renewal that is similar in both systems and involves Oct4, Nanog and Sox2 (Schnarch et al., 2010) (Fig. 1).

The discrepancies observed between mESCs and hESCs in their requirements for undifferentiated growth cannot be accounted only to species differences, but they can be largely explained by the different developmental origins of these pluripotent cells. hESCs are derived at a later stage than mESCs, at a point when epiblast differentiation is initiated. It is possible that the derivation of pluripotent stem cells from mouse ICM cells is facilitated by the delayed implantation or embryonic diapause (Hondo and Stewart, 2005, Lopes et al., 2004, Renfree and Shaw, 2000) that exists in this species but is absent in human embryo. Interestingly, pluripotent cells were also derived from mouse and rat post-implantation embryos and shown to share similar gene expression and signalling response patterns with hESCs and epiblast (Brone et al., 2007, Tesar et al., 2007). In addition to sharing signalling pathways for self-renewal with hESCs, epiblast stem cells (EpiSCs) also display similar culture characteristics including the requirement for passaging in clumps, the low clonal capacity and the reduced efficiency of chimera formation. Therefore mouse and rat EpiSCs are the exact developmental counterparts of hESCs and are considered to be “primed”, on the contrary of mESCs that are in a “naïve” pluripotency state (Nichols et al., 2009). Given the ease of manipulation and the stable phenotype of “naïve” pluripotent stem cells in culture, current studies investigate the derivation of such cells from early human embryos (before the epiblast stage) as well as the conversion of current hESCs lines into a “naïve” state (Gu et al., 2012, Hanna et al., 2010, Zhou et al., 2010).

Self-renewal and pluripotency in mESCs

The mESCs were originally isolated from the ICM of early mouse blastocyst and maintained on mitotically inactivated embryo fibroblasts (feeder layer) that contribute by secreting anti-differentiation cytokines such as BMP4 and LIF (Martin, 1981, Qi et al., 2004). LIF receptor, but not the ligand, is normally expressed by the ICM cells and is involved in the maintenance of pluripotency in the mouse embryo (Nichols et al., 1996). Interestingly, exogenous LIF combined with serum or BMP4 sustains self-renewal in feeder-free culture of mESCs. In this setting and similar to its function during gastrulation, BMP4/SMAD pathway represses the default neural differentiation (Di-Gregorio et al., 2007), whereas in the absence of LIF it generates uniform sheets of flat cells (Kunath et al., 2007, Ying et al., 2003a). Together, LIF and BMP4 might suppress differentiation events triggered by autocrine FGF4-mediated ERK phosphorylation. This hypothesis led to the discovery that FGF inhibition (with SU5402 and PD184352) combined with GSK3 inhibition with CHIR99021 maintains pluripotency in serum-free and feeder-free conditions (Ying et al., 2008). These latter findings are concordant with previous observations that inhibition of FGF/MAPK improves self-renewal in mESCs (Burdon et al., 1999, Burdon et al., 2002, Kunath et al., 2007). Beside LIF and BMP4, WNT ligands expressed by mESCs and by feeder cells are also crucial for preventing mESCs differentiation, but similar to WNT stimulation by GSK3 inhibition, the exact mode of action is still a matter of debate (Sato et al., 2004, ten Berge et al., 2011, Wray et al., 2011, Ying et al., 2008). Indeed, the ICM cells also express few WNT ligands and their secreted antagonists, suggesting that this pathway plays a role in preimplantation development (Kemp et al., 2007).

Self-renewal and pluripotency in hESCs

The hESCs are derived from blastocyst stage human embryos; they show activated Nodal/Activin, FGF and WNT pathways and have the potential for long-term maintenance in undifferentiated state and generation of three germ layer derivatives (Sato et al., 2004, Thomson et al., 1998, Xiao et al., 2006). Similar to LIF in mESCs, Activin A is sufficient and necessary for maintaining hESC pluripotency in long-term culture with 20% serum replacer (KSR),
which contributes among others by activating the PI3K pathway (Li et al., 2007, McLean et al., 2007, Xiao et al., 2006), and recalls the function of Nodal signaling in maintaining an undifferentiated status of the epiblast (Camus et al., 2006, Granier et al., 2011, Mesnard et al., 2006). On the contrary, Activin A does not maintain pluripotency in a chemically defined medium (CDM), suggesting that both Activin A and activated PI3K are essential for undifferentiated hESCs (Vallier et al., 2005).

FGF signaling is crucial for embryonic development as revealed by peri-implantation lethality of several mutants and by the expression of FGF4 in the epiblast (reviewed in (Lanner and Rossant, 2010)). FGFs are released by hESCs and are also involved in the maintenance of their pluripotency via intracellular activation of PI3K/AKT and/or MAPK/ERK downstream pathways. On the contrary, FGF receptor inhibitor SU5402 induces hESC differentiation whereas active PI3K/AKT blocks endoderm differentiation induced by Activin A (Armstrong et al., 2006, Ding et al., 2010, Dvorak et al., 2005, Li et al., 2007, McLean et al., 2007). While investigating the molecular mechanisms of FGF-mediated hESC maintenance, MAPK/ERK was suggested as being required for pluripotency (Dvorak et al., 2005, Li et al., 2007); however a study by Ding et al., found that activated PI3K, rather than MAPK, mediates pluripotency in hESCs (Ding et al., 2010). These discrepancies might reside in the experimental models given that FGF2 starvation was performed for either 12h or 5 days (Ding et al., 2010, Li et al., 2007). Since neither FGF nor Activin A alone is sufficient to maintain pluripotency in feeder-free conditions (Vallier et al., 2005), a cross-talk between signaling pathways is likely operative and regulates the growth of undifferentiated hESCs. For instance, Activin A and FGF2 supplementation combined with ERK1/2 inhibition allows for hESC self-renewal in CDM on collagen I coated surface (Na et al., 2010). In this system, the absence of BMP4-like signals (usually present in KSR) and the inhibition of ERK phosphorylation exclude spontaneous differentiation towards mesendoderm and neural fates. This would indicate that a “ground state” (Ying et al., 2008) also exists for hESCs and its requirements are yet to be unravelled in vitro.

As mentioned above for mESCs, active WNT signaling contributes to the maintenance of pluripotency during hESC culture (Sato et al., 2004, ten Berge et al., 2011). FGF2 can induce phosphorylation of GSK3β (a downstream target of PI3K), thereby activating the WNT pathway in hESCs. However, WNT gradually loses its ability to maintain undifferentiated hESCs in long-term culture, suggesting that it is not an anti-differentiation factor (Ding et al., 2010, Dravid et al., 2005).

Although several studies identified specific pathways that regulate pluripotency in the developing embryo or in ESCs and paved the way for developing “optimal recipes” for undifferentiated growth in vitro (Table 1), the crosstalk between these pathways still needs to be further addressed in order to have a clear understanding of both systems, which will also warrant the high-quality of stem cell cultures by limiting spontaneous differentiation and chromosomal abnormalities.

The signaling pathways in endoderm specification from ESCs

Studies in developmental models such as zebrafish and mouse initially pointed towards the existence of a transit germ layer named mesendoderm that is present during early gastrulation, and is bipotential for endoderm and mesoderm (Lawson et al., 1991, Rodaway and Patient, 2001, Rodaway et al., 1999). The presence of such an intermediate germ layer was also suggested and its characteristics defined during in vitro differentiation of mESCs and hESCs (Tada et al., 2005). Interestingly, several signals involved in the differentiation of mESCs and hESCs into the primitive streak (PS), mesendoderm and then further into endoderm or mesoderm are conserved (Table 2). Therefore, despite the major differences between mESCs and hESCs maintenance, similar pathways control the differentiation into particular germ layers. This would constitute an asset for in vitro differentiation and would help crossing species borders with well-defined universal differentiation protocols. However, this is not always the case in practice. Because of its recent discovery, not enough data are available on the differentiation of EpiSCs into definitive endoderm. We therefore further discuss here the differentiation of DE from mESCs and hESCs in detail, with focus on conserved signaling pathways and their particularities in each system.

Definitive endoderm differentiation in mESCs

Nodal/Activin A pathway

The PS formation is an essential step prior to the generation of endoderm and mesoderm derivatives in vivo. Nodal/Activin A signaling is crucial for the induction of PS formation, given that this structure is absent in Nodal-/- mutant mouse embryo (Conlon et al., 1994, Zhou et al., 1993). In vitro, Nodal/Activin A induces anterior and posterior PS in a concentration-dependent manner both in adherent cultures of ESCs (Hansson et al., 2009, Tada et al., 2005, Yasunaga et al., 2005) and in embryoid bodies (EBs) maintained in suspension (Gadue et al., 2006, Kubo et al., 2004). For instance, high concentration of Activin A (10-100ng/ml) favors anterior PS and further gives rise to DE if the stimulation persists. On the contrary, low concentration (1-3ng/ml) mainly specifies posterior PS and then posterior mesoderm. Although this general principle applies to both adherent and suspension cultures and is concordant with Nodal/Activin A function in embryonic development (Schier, 2003, Tam et al., 2003), the expression of Gsc (mesendoderm and DE marker) induced by high dose of Activin A is lower in EBs or in serum containing condition as compared to cells cultured in monolayer without serum (Tada et al., 2005). However, monolayer induction of DE in the absence of serum is severely restricted by the very poor cell survival (Morrison et al.,

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**Table 1**

<table>
<thead>
<tr>
<th>Stem cells</th>
<th>Main self-renewal factors</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>mESCs</td>
<td>LIF, BMP4, WNT</td>
<td>None of them could support long-term self-renewal in the absence of others. LIF+BMP4, LIF+WNT/iGSK3 or WNT/GSK3+iMAPK maintain long-term pluripotency.</td>
</tr>
<tr>
<td>hESCs</td>
<td>FGF, Activin A</td>
<td>Alone, FGF or Activin A is not sufficient to maintain self-renewal. Their combination is sufficient and efficient. Wnt is also expressed by hESCs but not essential for pluripotency.</td>
</tr>
<tr>
<td>m/EpiSCs</td>
<td>FGF, Activin A</td>
<td>Similar to hESCs</td>
</tr>
</tbody>
</table>

iGSK3, inhibitor of GSK3; iMAPK, inhibitor of MAPK; m/EpiSCs, mouse or rat epiblast stem cells.
and our personal observations). This discrepancy suggests that the undefined factors present in the serum as well as the cellular architecture formed in EB play a pivotal role on mesendoderm/DE formation from mESCs following Activin A induction, primarily by providing survival signals.

**BMP pathway**

Similar to Nodal/Activin A, BMP also belongs to the TGF-beta superfamily of growth and differentiation factors. It blocks the default neural differentiation and maintains the undifferentiated state of mESCs in concert with LIF. In the absence of LIF, BMP4 regains its ability to induce mESCs differentiation, and its effect varies with the cellular spatial organisation. In adherent cultures, BMP4 induces surface ectoderm equivalents (Kunath et al., 2007, Ying et al., 2008), which recalls its involvement in mesoderm differentiation (Burdon et al., 1999, Kunath et al., 2007, Lanner and Rossant, 2010). Activation of the MAPK/ERK1/2 pathway induced by FGF signaling is required for neural specification of mESCs within a short time window, explaining why this has been considered as the default fate of spontaneously differentiating mESCs (Stavridis et al., 2007). Beside its role on ectodermal lineages, FGF signaling from autocrine or paracrine sources is also involved in mesodermal fate commitment (Ciruna and Rossant, 2001, Kunath et al., 2007). However, supplementation of mESCs cultures with FGF ligands does not induce PS and mesoderm formation in the absence of TGF-beta or Wnt signals (Zheng et al., 2010). To this end, the contribution of FGF signaling in DE differentiation is not direct as is the case for Activin A, but is integrated in a signaling network built from the undefined factors present in the serum as well as the cellular architecture formed in EB play a pivotal role on mesendoderm/DE formation from mESCs following Activin A induction, primarily by providing survival signals.

**WNT pathway**

Beside Nodal/Activin A signaling, the WNT pathway is also important for PS differentiation as revealed by its absence in WNT-β mutant mouse embryos (Barrow et al., 2007, Liu et al., 1999). In vitro in serum-free medium, WNT signaling induces a posterior PS population both in monolayer cultures of mESCs and in EBs (Gadue et al., 2006, Nakanishi et al., 2009). Combination of WNT and Activin A improved DE induction as evaluated by Sox17 expression, but this effect is minimal if WNT is supplemented after the initiation of DE differentiation (Hansson et al., 2009). Accordingly, inhibition of WNT with Dkk1 reduced PS and DE formation in the presence of Activin A as indicated by low expression of Mix1 and Sox17 (Hansson et al., 2009). These data are concordant with the in vivo findings that WNT signaling, similar to BMP4, is required for PS formation but is not an inducer of anterior lineages (Kemp et al., 2007).

**FGF pathway**

FGF signaling controls mESCs transition from pluripotency to lineage commitment, and blockade of this pathway results in the maintenance of pluripotency markers expression (Burdon et al., 1999, Kunath et al., 2007, Lanner and Rossant, 2010). Activation of the MAPK/ERK1/2 pathway induced by FGF signaling is required for neural specification of mESCs within a short time window, explaining why this has been considered as the default fate of spontaneously differentiating mESCs (Stavridis et al., 2007). Beside its role on ectodermal lineages, FGF signaling from autocrine or paracrine sources is also involved in mesodermal fate commitment (Ciruna and Rossant, 2001, Kunath et al., 2007). However, supplementation of mESCs cultures with FGF ligands does not induce PS and mesoderm formation in the absence of TGF-beta or Wnt signals (Zheng et al., 2010). To this end, the contribution of FGF signaling in DE differentiation is not direct as is the case for Activin A, but is integrated in a signaling network built from the undefined factors present in the serum as well as the cellular architecture formed in EB play a pivotal role on mesendoderm/DE formation from mESCs following Activin A induction, primarily by providing survival signals.

**TABLE 2**

OVERVIEW OF THE EFFECTS OF INHIBITING OR ACTIVATING RELEVANT PATHWAYS ON GERM LAYER DIFFERENTIATION FROM EMBRYONIC STEM CELLS

<table>
<thead>
<tr>
<th>Stem cells</th>
<th>Pathways/Factors (References)</th>
<th>Effect of pathway inhibition</th>
<th>Effect of pathway activation</th>
</tr>
</thead>
<tbody>
<tr>
<td>mESCs</td>
<td>Activin A/Nodal (Gadue et al., 2006, Hansson et al., 2009, Kubo et al., 2004, Tada et al., 2005, Yasunaga et al., 2005)</td>
<td>X X X</td>
<td>PS DE ME</td>
</tr>
<tr>
<td></td>
<td>BMP4 (Fujikawa et al., 2001, Hansson et al., 2009, Li et al., 2011, Nostro et al., 2008, Pearson et al., 2008)</td>
<td>N With ActA and WNT = DE</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>WNT (Gadue et al., 2006, Hansson et al., 2009, Nakanishi et al., 2009)</td>
<td>X X N</td>
<td>PS</td>
</tr>
<tr>
<td></td>
<td>FGF (Hansson et al., 2009, Kunath et al., 2007, Morrison et al., 2008, Zheng et al., 2010)</td>
<td>X X X</td>
<td>PS Marginal effect</td>
</tr>
<tr>
<td></td>
<td>NOTCH (Lowell et al., 2006, Nemir et al., 2008)</td>
<td>X</td>
<td>ME</td>
</tr>
<tr>
<td>hESCs</td>
<td>Activin A/Nodal (D’Amour et al. 2005; Smith et al. 2008; Sumi et al. 2008; Borowiak et al. 2009)</td>
<td>X X X</td>
<td>Low level (\Rightarrow) post. PS</td>
</tr>
<tr>
<td></td>
<td>BMP4 (Phillips et al., 2007, Sumi et al., 2008, Takei et al., 2009, Zhang et al., 2008)</td>
<td>PS DE X</td>
<td>High level (\Rightarrow) post. PS</td>
</tr>
<tr>
<td></td>
<td>WNT (D’Amour et al. 2005, Sumi et al. 2008)</td>
<td>PS With Activin A</td>
<td>Short time exposure or EB (\Rightarrow) ME</td>
</tr>
<tr>
<td></td>
<td>FGF (Ding et al., 2010, Na et al., 2010, Valiier et al., 2009b)</td>
<td>X X ME</td>
<td>PS With Noggin (\Rightarrow) DE</td>
</tr>
<tr>
<td></td>
<td>NOTCH (Hughes et al., 2009, Jang et al., 2008)</td>
<td>X</td>
<td>ME</td>
</tr>
</tbody>
</table>

ActA, Activin A; ant., anterior; DE, definitive endoderm; ME, mesoderm; N, not determined; post., posterior; PS, primitive streak; X, not induced.
Crosstalk between signaling pathways during definitive endoderm differentiation from mESCs

Using monolayer and EB culture conditions, Morrison et al., showed that mESCs treated with Activin A plus BMP4 for the first 2 days and then with Activin A plus EGF for 5 days generated the highest proportion (up to 20%) of DE cells (Morrison et al., 2008). Inhibition of Activin A signal blocks BMP4 induced PS differentiation, whereas continuous exposure to BMP4 after PS formation potentiates mesoderm formation over endoderm. Once specified, the mesoderm cannot be shifted back to DE by addition of Activin A (Pearson et al., 2008). On the contrary, combination of BMP4 and Activin A prevented anterior PS formation but favored the posterior PS lineage differentiation. (Hansson et al., 2009, Tada et al., 2005). All together, Activin A and BMP4 signals induce PS in a cooperative manner and the effect of BMP4 on PS induction appears to be Activin A-dependent. After PS formation, Activin A directs cells into the anterior endoderm lineage whereas BMP4 favors the posterior mesoderm lineage. An interplay between these TGF-beta ligands and the WNT pathway certainly operates in the specification of PS, considering that Activin A can induce Wnt3a expression and that continuous stimulation of the WNT pathway alone in mESCs induces a PS-like phenotype and further gives rise to mesoderm cells (Bakre et al., 2007, Nakanishi et al., 2009, Xiao et al., 2006).

Inhibition of FGF receptor signaling reveals a role for FGF on DE formation induced by Activin A (Hansson et al., 2009, Morrison et al., 2008). FGF2 enhances Activin A-induced Gsc expression and this effect is antagonized by FGF receptor inhibition, suggesting that Gsc positive mesendoderm derived from anterior PS requires FGF signaling and that anterior PS fate is further improved by active FGF signaling. Furthermore, DE formation as assessed by Sox17 expression has a late dependence on FGF signaling (Hansson et al., 2009), however further stimulation of the pathway by exogenous FGF does not increase Activin A-induced DE marker expression. This result is not consistent with the observations from another study that addition of FGF ligands at later stages increased the resulting DE population (Morrison et al., 2008), although different induction protocols were applied. Nevertheless, they suggest the involvement of FGF signaling in essential steps of mesendoderm and DE induction.

Whereas all these pathways are known to contribute to a certain extent to PS and DE formation in vitro, it remained for long challenging to obtain more than 30% DE differentiation efficiency or to maintain adequate survival of DE precursors in monolayer cultures. It is only recently that the combination of TGF-beta stimulation, WNT stimulation and BMP inhibition was found to efficiently induce DE cells (more than 75% CxCR4+ cells) in EBs from mESCs (Li et al., 2011). Our personal observations confirmed these findings on monolayer cultures and therefore significantly improved the previously reported efficiencies (Hansson et al., 2009, Morrison et al., 2008). Taken together, Nodal/Activin A, WNT, BMP4 and FGF signals also crosstalk to regulate PS formation and DE specification from mESCs in vitro (Fig. 2). However, we might still be far from understanding how faithfully the current in vitro differentiation systems reproduce all the critical aspects of DE differentiation in vivo, including the cellular spatial organization and intercellular contacts, the timing of growth factors activity and the morphogen effects.

Definitive endoderm differentiation in human ESCs

The differentiation of DE lineages from hESCs was facilitated by the initial reports on mouse embryo development and on endoderm differentiation from mESCs. It was therefore obvious from these previous models that the four main pathways (Activin/Nodal, BMP,
FGF and WNT) that operate in mouse endoderm differentiation might also be implicated in humans. As described in the following paragraphs, these pathways control DE differentiation from hESCs and also function in a signaling network that operates via molecular and temporal crosstalks between them.

**Nodal/Activin A pathway**

In addition to maintaining the undifferentiated state of hESCs, Activin A plays a similar role in germ layer formation as in mESCs by inducing BRY positive mesendoderm formation and by generating DE and mesoderm in a concentration dependent manner (D’Amour et al., 2005, Gadue et al., 2006, Hansson et al., 2009, Kubo et al., 2004). This implies that the functions of TGF-beta signaling in mesoderm and endoderm formation are well conserved from lower to higher vertebrates (Tam et al., 2003, Thise et al., 2000, Whitman, 2001). However, DE induction by Activin A in monolayer cultures of hESCs is much more efficient than currently reported in mESCs (D’Amour et al., 2005, Hansson et al., 2009, Morrison et al., 2008) and personal observations). Although FGF and WNT pathways can modulate the extent of DE differentiation, it is remarkable that in the absence of Activin signaling, they could neither maintain hESC pluripotency, nor drive mesendoderm commitment in chemically defined medium (Sui et al., 2012b, Vallier et al., 2009b), (Fig. 3). Therefore, Activin A acts upstream of the other factors that modulate endoderm differentiation, and is absolutely required for the generation of this germ layer. Indeed, inhibition of Activin signaling promotes neuroectoderm differentiation instead of PS/DE (Smith et al., 2008). To this end, Activin A has been extensively used as an essential component in the induction of DE from hESCs in a number of protocols wherein DE-derived pancreatic cells and hepatocytes were generated (Agarwal et al., 2008, D’Amour et al., 2006, Hay et al., 2008, Kroon et al., 2008, Mlopou et al., 2010a, Xu et al., 2011).

Activin A functions by activating the intracellular SMAD2/3 signal transducers. A recent chemical library screening identified small molecules (IDE1, IDE2) that also activate this pathway, resulting in the formation of DE from mESCs and hESCs (Borowiak et al., 2009b). In the future the use of such chemical strategies would eliminate the financial and biological hurdles of working with recombinant proteins.

**BMP pathway**

BMP4-treated hESCs are unable to generate BRY positive mesendoderm/mesoderm progenitors (Sumi et al., 2008) but give rise to trophectoderm or primitive endoderm in long-term culture (Pera et al., 2004, Vallier et al., 2009b, Xu et al., 2002). This effect of BMP4 is actually dependent on the concomitant decrease in Activin and FGF signaling that normally maintain NANOG expression, given that addition of FGF2 or forced expression of NANOG switches the BMP4-induced differentiation from extraembryonic lineages into mesendoderm (Yu et al., 2011, Zhang et al., 2008). On the contrary, hESCs exposure to BMP4 for a short time (no more than 24h) or after EBs formation generates mesendoderm/mesoderm progenitors capable of further differentiation into hematopoietic and cardiac lineages (Takei et al., 2009, Zhang et al., 2008), (Fig. 3). Furthermore, BMP4 combined with Activin A synergistically generated endoderm cells expressing FOXA2 and SOX17 from hESCs. These cultures further gave rise to PDX1 and NKX6.1 double positive pancreatic endoderm, indicating the DE nature of the FOXA2+ SOX17+ cells generated by these means (Phillips et al., 2007, Teo et al., 2012). Transient stimulation of hESCs with Activin A for one day in combination with BMP4, VEGF and FGF2 was shown to generate multipotent mesoderm progenitors at day 3.5 that have the potential to generate all mesodermal lineages (Evseenko et al., 2010). Therefore, BMP4 can modulate mesendoderm and mesoderm specification from hESCs, and a
precise control of its signaling time window and the interplay with additional pathways are pivotal for cell fate determination.

**WNT pathway**

The induction of PS and mesendoderm cell types by Activin A is at least in part related to the subsequent activation of WNT3a transcription by this growth factor (Bakre et al., 2007, Kemp et al., 2007, Sumi et al., 2008, Xiao et al., 2006). Indeed, WNT signaling can induce BRY-positive PS formation in serum free condition (Fig. 3); and similar to mESC cultures, continuous exposure to WNT promotes the formation of posterior mesoderm (Sumi et al., 2008). This effect of WNT signaling on posterior PS/mesoderm formation can be shifted towards anterior PS/DE and anterior mesoderm by addition of the BMP antagonist Noggin (Sumi et al., 2008). The PS cells initially induced by WNT can be further directed towards the DE by addition of Activin A and removal of WNT ligands.

Taken together, these data led to the development of an efficient DE induction protocol wherein Activin A and Wnt3a are supplemented to undifferentiated hESCs for a short period (1 or 2 days), followed by Activin A and serum (low concentration) for 2 days (D’Amour et al., 2005). This results in an optimal synchrony in the generation of the mesendoderm population and its further conversion into DE cells, and is considered nowadays as the standard protocol for DE induction from hESCs (Mfopou et al., 2010b).

This strategy has been successfully used by several investigators on multiple cell lines including human induced pluripotent stem cells, and the resulting DE cells could be differentiated further into hepatic and pancreatic lineages (D’Amour et al., 2005, D’Amour et al., 2006, Johannesson et al., 2009, Kelly et al., 2011, Kroon et al., 2008, Mfopou et al., 2010a, Nostro et al., 2011, Sui et al., 2012a, Thatava et al., 2011), (Table 3). However, the use of serum supplementation constitutes a disadvantage for the development of clinical grade progenies from this protocol, which stimulated the search for many other alternatives (Table 3).

**FGF pathway**

FGF signaling has diverse roles during embryo development depending on the temporal and spatial contexts. Active FGF signaling is required to maintain hESC pluripotency. Blockade of its downstream effector MAPK results in the loss of pluripotency markers and differentiation of primitive endoderm and trophoderm (Dvorak et al., 2005, Li et al., 2007). However, recent studies suggest that inhibition of MAPK has no influence on self-renewal of hESCs, but prevents mesendodermal differentiation (Ding et al., 2010, Na et al., 2010). These observations suggest that MAPK pathway controls the differentiation whereas PI3K, another downstream effector of FGF signaling, is mainly devoted to the regulation of hESC pluripotency. Similarly, the role of FGF on germ layer specification is variable with regards to the context. In the absence of anti-differentiation factors, for instance following depletion of the self-renewal factor Activin A or after formation of EBs, FGF activity induces neuroectodermal differentiation (Cohen et al., 2010, Vallier et al., 2009b). The impact of FGF signaling has been broadly and clearly defined in the maintenance of self-renewal and in the commitment of neural lineages from hESCs (Fig. 3). However, only few studies reported on the role of FGF in mesendoderm, endoderm and mesoderm derivation from hESCs. It is only recently that independent data from Na et al., and from our group clearly showed that FGF signaling promotes Activin A-induced DE differentiation in serum free condition, and that its antagonism severely reduces mesendoderm and DE commitment (Na et al., 2010, Sui et al., 2012b). Interestingly, FGF alone also failed to drive DE formation from hESCs (Sui et al., 2012b, Vallier et al., 2009b), indicating that similar to what occurs in mESCs, TGF-beta signaling is required upstream of FGF pathway during mesoderm/DE differentiation. This suggests that FGF is not a major DE inducing factor, but a synergistic factor that has to be activated to support Activin A-induced DE formation. Concordantly with the findings by Na et al., (Na et al., 2010), we also demonstrated that the FGF downstream effector MAPK/ERK, rather than PI3K/AKT, is crucial for mesendoderm/DE differentiation in the presence of Activin A. However, anterior PS/mesoderm formation induced by combined WNT activation and BMP inhibition appears to involve PI3K/AKT signaling (Sumi et al., 2008). Although all these studies

### TABLE 3

<table>
<thead>
<tr>
<th>Parameters in the DE protocol</th>
<th>Efficiency / Advantage</th>
<th>Disadvantage</th>
<th>Differentiated progenitors</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI – Activin A – WNT – FBS – Feeders or Matrigel – 3-4 days</td>
<td>80% CxCR4+ cells; 70% SOX2+ SOX17+ cells</td>
<td>Unknown factors in FBS and Matrigel; feeders variability</td>
<td>Pancreas</td>
<td>(Ameri et al., D’Amour et al., 2006, Johannesson et al., 2009, Kroon et al., 2008, Mfopou et al., 2010a)</td>
</tr>
<tr>
<td>SFD – E Bs – Activin A – BMP4 – VEGF – bFGF – WNT – 4 days</td>
<td>70% SOX17+ cells; Defined medium</td>
<td>Stochastic differentiation in EBs</td>
<td>Pancreas</td>
<td>(Nostro et al., 2011)</td>
</tr>
<tr>
<td>CDM – Activin A – bFGF – BMP4 – Ly294002 – 3 days</td>
<td>Defined medium; free of animal products</td>
<td>–</td>
<td>Pancreas and liver</td>
<td>(Cho et al., 2012)</td>
</tr>
<tr>
<td>CDM – Activin A – BMP4 – bFGF – Fibronectin – (Ly294002) – 3-4 days</td>
<td>Defined medium; free of animal products</td>
<td>–</td>
<td>–</td>
<td>(Valier et al., 2009a, Vallier et al., 2009b)</td>
</tr>
<tr>
<td>RPMI – Activin A – NaB – 3-5 days</td>
<td>70% CxCR4+ cells; Defined medium; Feeder-free and serum-free</td>
<td>Unknown molecular effects of NaB</td>
<td>Liver</td>
<td>(Hay et al., 2008)</td>
</tr>
<tr>
<td>DMEM/F12 – Activin A – bFGF – FBS – EBs on Matrigel</td>
<td>?</td>
<td>Unkown factors in Matrigel</td>
<td>Liver</td>
<td>(Basma et al., 2009)</td>
</tr>
<tr>
<td>CDM – Activin A – Ly294002 – bFGF – BMP4 – FBS – 5 days</td>
<td>80% CxCR4+ cells; CDM Feeder-free</td>
<td>Unknown factors in FBS</td>
<td>Liver</td>
<td>(Touboul et al., 2010)</td>
</tr>
<tr>
<td>DMEM – Activin A – WNT – Collagen IV – 4 days</td>
<td>87% CxCR4+ cells; SOX2+ SOX17+ Feeder free</td>
<td>–</td>
<td>Lung</td>
<td>(Wong et al., 2012)</td>
</tr>
<tr>
<td>RPMI – Activin A – FBS – 3 days</td>
<td>90% SOX2+ SOX17+ Feeder free</td>
<td>Unknown factors in FBS</td>
<td>Intestine</td>
<td>(Spence et al., 2011)</td>
</tr>
</tbody>
</table>

Despite the large variations in culture conditions and supplements, the common denominator of all these protocols (Nodal/Activin A, WNT and FGF signals) ensures that at least 70% DE cells are generated. CDM, chemically defined medium; EBs, embryoid bodies; NaB, sodium butyrate; SFD, serum free differentiation medium.
point towards a role for FGF in Activin A-induced DE differentiation, there are conflicting data about the time window in which this effect occurs. We demonstrated that DE differentiation has an early dependence on FGF signaling on the basis of reduced SOX17 expression after FGF inhibition at early stages (Sui et al., 2012b). In contrast, a late dependence on FGF signaling in both mESCs (Hansson et al., 2009, Morrison et al., 2008) and hESCs (Vallier et al., 2009b) were initially reported. These discrepancies likely result from differences in the models being used in these studies. Worthy to note, one can declare in light of current findings that FGF signaling is necessary but not sufficient for mesendoderm and DE differentiation, and it acts as a complementary factor to support the stimulatory effect of Activin A on DE induction.

**Other signaling pathways in germ layer commitment**

In addition to the well-studied pathways described above, Notch and RA pathways might also be implicated in germ layer commitment. The role of Notch signaling in early gastrulation-like events from ESCs is controversial. Loss of Notch signaling promotes mesoderm formation and represses neural lineage specification in mESC culture (Lowell et al., 2006, Nemir et al., 2006). Although Notch pathway antagonism with a gamma-secretase inhibitor does not affect self-renewal and differentiation in standard cultures of hESCs (Fox et al., 2008, Noggle et al., 2006), it induced mesoderm lineages and attenuated neural and hematopoietic commitment in small-volume culture conditions (Jang et al., 2008). However, it was also shown that Notch inhibition downregulates expression of PS markers even in the presence of BMP4, thereby establishing surface ectoderm formation in hESCs and neural formation in mESCs (Hughes et al., 2009). This suggests that culture conditions of ESCs affect their responses to Notch inhibitors, and that blocking this pathway can in certain circumstances induce a lineage that is known to originate from the anterior PS/mesendoderm.

The major findings on the role of RA in germ layer commitment focus on neural ectoderm differentiation in mESCs and point towards a crosstalk of this pathway with FGF signaling (Stavridis et al., 2010, Ying et al., 2003b). Similarly, a study reported the differentiation of pancreatic cells from DE induced in monolayer cultures of mESCs by a combination of RA and FGF2 (Kim et al., 2010). However, the supplementation of 15% FBS during DE induction does not allow to draw firm conclusions on the contribution of RA signaling.

**Concluding remarks**

During long-term culture in vitro, mESCs and hESCs maintain their self-renewal and pluripotency properties via integration of different signaling pathways. Although several common growth factors are expressed in both cell types including WNT and FGF ligands, their role on the regulation of the pluripotent state is quite different (Cohen et al., 2010, Kunath et al., 2007, Sato et al., 2004, ten Berge et al., 2011, Xiao et al., 2006, Ying et al., 2008). For instance, WNT is essential to sustain mESCs pluripotency whereas in hESCs it is only responsible for the proliferation (Dravid et al., 2005, Sato et al., 2004, ten Berge et al., 2011, Wray et al., 2011, Ying et al., 2008). FGF signal in mESCs has to be inhibited to maintain pluripotency whereas in hESCs it has to be activated (Armstrong et al., 2006, Dvorak et al., 2005, Li et al., 2007, Ying et al., 2008). These dramatic discrepancies between mESCs and hESCs regarding the maintenance of pluripotency are now uncovered and are related to the developmental origins of these pluripotent cells. With regards to this, pluripotent cells derived from rodent embryos at the epiblast stage (EpiSCs, epiblast stem cells) display similar characteristics and requirements with hESCs (Brons et al., 2007, Rossant, 2008, Tesar et al., 2007, Vallier et al., 2009b). This led to the suggestion that hESCs are much closer or equivalent to the postimplantation epiblast rather than to the inner cell mass cells.

Whereas the functions played by Activin A, WNT, FGF and BMP4 pathways during undifferentiated growth are quite different among hESCs and mESCs, their role during early development is conserved to some extent. For instance, Activin A induces DE and mesoderm in a dose-dependent manner in both mouse and human ESCs, with low concentrations driving mesoderm fate and high concentrations favoring anterior endoderm fate. With regards to mesendoderm and DE differentiation, FGF is mainly a competence factor that improves germ layer formation in concert with specific commitment factors and in a particular competence window. Specific protocols developed for each system allow for efficient generation of DE cells that are competent for further differentiation into endoderm progenies such as liver, pancreas, lungs and intestine (Table 3). While they sometimes significantly differ in the use of feeder cells, extracellular matrix, serum or growth factors combinations, these protocols have as common denominator the requirement for Activin/Nodal, WNT and FGF (MAPK/ERK) signaling. These signals can be provided via growth factors or small molecules supplementation, and in certain models are also provided by the feeder cells or serum. Whereas the mesendoderm stage is well described in both systems, it remains unclear for now whether the passage from mESCs to DE involves an earlier EpiSC-like intermediate stage.

Although we aimed at giving an overview of the main differences in the signaling pathways that control pluripotency and early DE commitment in mESCs and hESCs, we have made an effort to keep it short and for this reason we would like to apologize for the investigators whose works have not been covered owing to space limitations. We also have not discussed DE differentiation from EpiSCs, given the recent discovery of these lines. Further work will be needed to foster the integrated understanding of the complex interactions and crosstalk between different pathways involved in endoderm differentiation, and to estimate how faithfully the in vitro implementation of this knowledge recapitulates the in vivo events (Wang et al., 2012). The current development of defined culture conditions and the increasing interest in using small molecules for pathways modulation will certainly affect the future of stem cell differentiation. They will be valuable for further understanding the basics of human embryo development and more interestingly, for the implementation of this knowledge in the development of clinical grade progenies from human pluripotent stem cells.

**References**


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