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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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formation 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 (Schnerch *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

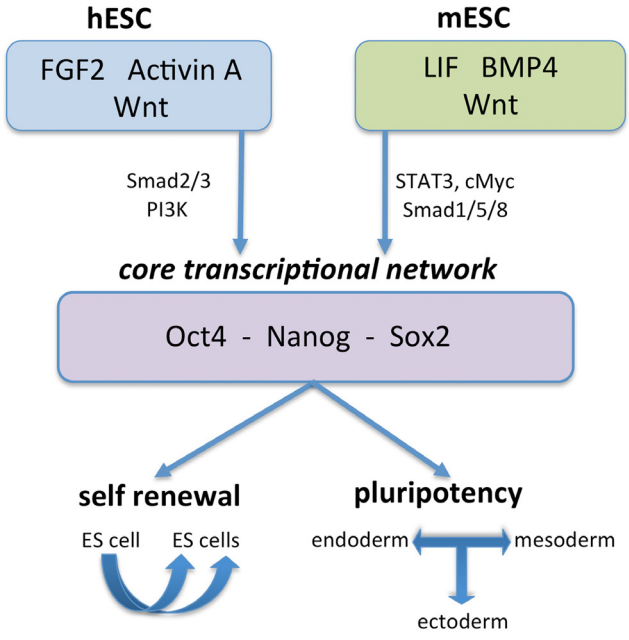

Fig. 1. Schematic representation of the differences in signaling pathways regulating self-renewal and pluripotency of human and mouse ESCs. *Undifferentiated mESCs are mainly maintained under the control of LIF and BMP signaling, whereas hESCs are in need of Activin and FGF signaling to sustain long-term self-renewal and pluripotency. The Wnt signaling also plays a role in self-renewal of hESCs and mESCs, but its underlying mechanism is still to be addressed. These signaling pathways in both systems converge towards the activation of a core transcriptional network that involves Oct4, Nanog and Sox2.*

delayed implantation or embryonic diapause (Hondo and Stewart, 2005, Lopes *et al.*, 2004, Renfree and Shaw, 2000) that exists in this specie 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 (Brons *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 mouse 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 crosstalk 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.

TABLE 1

GROWTH FACTORS REQUIRED FOR SELF-RENEWAL IN PLURIPOTENT STEM CELLS DERIVED FROM THE EMBRYO

Stem cells	Main self-renewal factors	Remarks
mESCs	LIF, BMP4, WNT	None of them could support long-term self-renewal in the absence of others. LIF+BMP4, LIF+WNT/ iGSK3 or WNT/iGSK3+iMAPK maintain long-term pluripotency.
hESCs	FGF, Activin A	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.
m/rEpiSCs	FGF, Activin A	Similar to hESCs

iGSK3, inhibitor of GSK3; iMAPK, inhibitor of MAPK; m/rEpiSCs, mouse or rat epiblast stem cells.

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.*,

2008) 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.*, 2003a) as well as trophoblast-like cells (Hayashi *et al.*, 2010); the latter having been widely thought not to develop from mESCs cultures (Beddington and Robertson, 1989). Moreover, BMP4 supplementation to EBs cultures led to the formation of posterior PS and mesodermal cells (Nostro *et al.*, 2008, Pearson *et al.*, 2008), which recalls its involvement in mesoderm differentiation during gastrulation (Hogan, 1996, Wells and Melton, 1999). This suggests that BMP4 is primarily an inducer of PS-like population expressing T (Brachyury) and Mixl1 (Fujiwara *et al.*, 2001), and explains its supplementation in combination with Activin A at the early stage of DE induction from mESCs (Morrison *et al.*, 2008).

WNT pathway

Beside Nodal/Activin A signaling, the WNT pathway is also important for PS differentiation as revealed by its absence in WNT-/- 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 Mixl1 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

TABLE 2

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

		Effect of pathway inhibition			Effect of pathway activation		
Stem cells	**Pathways/Factors (References)**	**PS**	**DE**	**ME**	**PS**	**DE**	**ME**
mESCs	Activin A/Nodal (Gadue *et al.*, 2006, Hansson *et al.*, 2009, Kubo *et al.*, 2004, Tada *et al.*, 2005, Yasunaga *et al.*, 2005)	X	X	X	Low level ⇨ post. PS High level ⇨ ant. PS	High level ⇨ DE	Low level ⇨ post. ME
	BMP4 (Fujiwara *et al.*, 2001, Hansson *et al.*, 2009, Li *et al.*, 2011, Nostro *et al.*, 2008, Pearson *et al.*, 2008)	N	With ActA and WNT ⇨DE	N	PS	X	post. ME
	WNT (Gadue *et al.*, 2006, Hansson *et al.*, 2009, Nakanishi *et al.*, 2009)	X	X	N	PS	Marginal effect	post. ME
	FGF (Hansson *et al.*, 2009, Kunath *et al.*, 2007, Morrison *et al.*, 2008, Zheng *et al.*, 2010)	X	X	X	with inductive signal	with inductive signal	with inductive signal
	NOTCH (Lowell *et al.*, 2006, Nemir *et al.*, 2006)			ME			
hESCs	Activin A/Nodal (D'Amour et al. 2005; Smith et al. 2008; Sumi et al. 2008; Borowiak et al. 2009)	X	X	X	Low level ⇨ post. PS High level ⇨ ant. PS	High level ⇨ DE	Low level ⇨ post. ME
	BMP4 (Phillips *et al.*, 2007, Sumi *et al.*, 2008, Takei *et al.*, 2009, Zhang *et al.*, 2008)	PS	DE	X	with Activin A	with Activin A	Short time exposure or EB ⇨ ME
	WNT (D'Amour *et al.*, 2005, Sumi *et al.*, 2008)				PS	With Noggin ⇨ DE	post. ME
	FGF (Ding *et al.*, 2010, Na *et al.*, 2010, Vallier *et al.*, 2009b)	X	X	ME	with inductive signal	with inductive signal	with inductive signal
	NOTCH (Hughes *et al.*, 2009, Jang *et al.*, 2008)	X		ME			

ActA, Activin A; ant., anterior; DE, definitive endoderm; ME, mesoderm; N, not determined; post., posterior; PS, primitive streak; X, not induced.

above described pathways (Activin, BMP, WNT) and involves the generation of the transient population of PS/mesendoderm cells as well as the commitment of these cells towards a DE fate (Hansson *et al.*, 2009, Morrison *et al.*, 2008).

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,

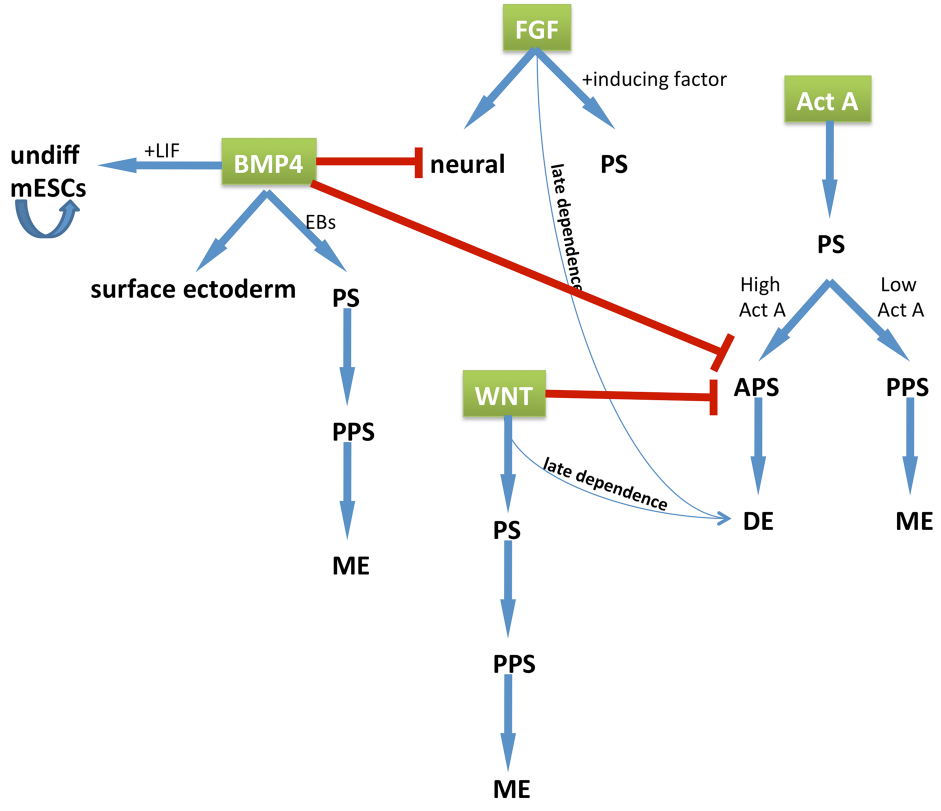

Fig. 2. Schematic representation of the roles played by several signaling pathways on germ layer specification in mESCs. *BMP4 induces surface ectoderm. The presence of other factors converts the role of BMP4 into mESCs maintenance and mesoderm induction. FGF induces neural ectoderm by default and augments PS formation synergistically with PS specific inducing factor, like Activin A. WNT contributes to PS induction followed by mesendoderm specification if continuously activated. The low level of Activin A favors mesendoderm formation. The definitive endoderm induced by high Activin A has a late dependence on FGF and WNT signaling. The specification of the anterior PS, which gives rise to the definitive endoderm, is blocked by BMP4 and WNT.*

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, Thisse *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, Mfopou *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.*, 2009). 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

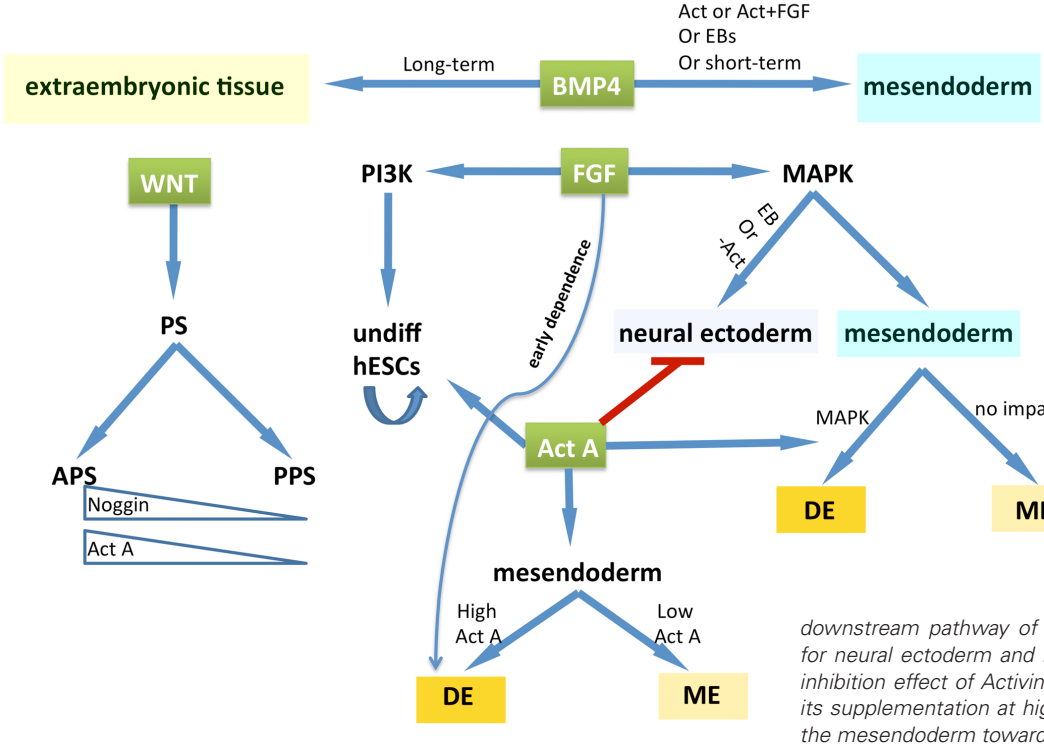

Fig. 3. Schematic representation of the roles played by several signaling pathways on germ layer specification in hESCs. *The mesendoderm and extraembryonic tissue specification induced by BMP4 are dependent on the culture time periods and factors indicated in the graph. The WNT contributes to PS formation and anterior PS is further induced with Activin A activation and BMP signaling inhibition. Interaction of Activin A and PI3K, one of the FGF downstream pathways, allows for hESCs self-renewal. The MAPK pathway, the other downstream pathway of FGF signaling, is mainly responsible for neural ectoderm and mesendoderm derivation. Beside the inhibition effect of Activin A on neural ectoderm development, its supplementation at high concentration could further induce the mesendoderm towards definitive endoderm.*

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 trophoectoderm (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 mesendoderm/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. Concordant with the finding 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/mesendoderm 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

OVERVIEW OF FEW SYSTEMS USED FOR DEFINITIVE ENDODERM DIFFERENTIATION FROM HESCS

Parameters in the DE protocol	Efficiency / Advantage	Disadvantage	Differentiated progenitors	References
RPMI – Activin A – WNT – FBS – Feeders or Matrigel – 3-4 days	80%CxCR4+ cells; 70% FOXA2+ SOX17+ cells	Unknown factors in FBS and Matrigel; feeders variability	Pancreas	(Ameri *et al*., D'Amour *et al*., 2006, Johannesson *et al*., 2009, Kroon *et al*., 2008, Mfopou *et al*., 2010a)
SFD – EBs – Activin A – BMP4 – VEGF – bFGF – WNT – 4 days	70% SOX17+ cells; Defined medium	Stochastic differentiation in EBs	Pancreas	(Nostro et al., 2011)
CDM – Activin A – bFGF – BMP4 – Ly294002 – 3 days	Defined medium; free of animal products		Pancreas and liver	(Cho et al., 2012)
CDM – Activin A – BMP4 – bFGF – Fibronectin – (Ly294002) – 3-4 days	70% CxCR4+ cells; Defined medium	–	–	(Vallier *et al*., 2009a, Vallier *et al*., 2009b)
RPMI – Activin A – NaB – 3-5 days	70% CxCR4+ cells; Feeder-free and serum-free	Unknown molecular effects of NaB	Liver	(Hay et al., 2008)
DMEM/F12 – Activin A – bFGF – FBS – EBs on Matrigel	?	Unkwon factors in Matrigel	Liver	(Basma et al., 2009)
CDM – Activin A – Ly294002 – bFGF – BMP4 – FBS – 5 days	80% CxCR4+ cells; CDM Feeder-free	Unknown factors in FBS	Liver	(Touboul et al., 2010)
DMEM – Activin A – WNT – Collagen IV – 4 days	87% CxCR4+ cells;	–	Lung	(Wong et al., 2012)
RPMI – Activin A – FBS – 3 days	90% FOXA2+ SOX17+ cells; Feeder free	Unknown factors in FBS	Intestine	(Spence et al., 2011)

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.

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