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FGF Signalling as a Mediator of Lineage Transitions—Evidence From Embryonic Stem Cell Differentiation

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ABSTRACT

The fibroblast growth factor (FGF) signalling pathway is one of the most ubiquitous in biology. It has diverse roles in development, differentiation and cancer. Embryonic stem (ES) cells are in vitro cell lines capable of differentiating into all the lineages of the conceptus. As such they have the capacity to differentiate into derivatives of all three germ layers and to some extent the extra-embryonic lineages as well. Given the prominent role of FGF signalling in early embryonic development, we explore the role of this pathway in early ES cell differentiation towards the major lineages of the embryo. As early embryonic differentiation is intricately choreographed at the level of morphogenetic movement, adherent ES cell culture affords a unique opportunity to study the basic steps in early lineage specification in the absence of ever shifting complex in vivo microenvironments. Thus recent experiments in ES cell differentiation are able to pinpoint specific FGF dependent lineage transitions that are difficult to resolve in vivo. Here we review the role of FGF signalling in early development alongside the ES cell data and suggest that FGF dependent signalling via phospho-Erk activation maybe a major mediator of transitions in lineage specification. J. Cell. Biochem. 110: 10–20, 2010.

KEY WORDS: FGF; EMBRYONIC STEM CELL; DIFFERENTIATION; MESODERM; ENDODERM; NEURAL AND EPIBLAST

Embryonic stem (ES) cells are karyotipically normal, self-renewing and pluripotent stem cell lines derived from the mammalian blastocyst [reviewed in Smith, 2001]. ES cells have the capacity to differentiate into all cell types of the adult organism, but the ability to direct their differentiation to mature and functional cell types cannot be fully exploited until the signals that regulate balance between lineage specification and self-renewal are fully understood. Early blastocysts consists of two cell types, the inner cell mass (ICM) from which ES cells are derived and the extra-embryonic trophoblast that will give rise to the placenta. As development proceeds the ICM will differentiate into both the extra-embryonic primitive endoderm and the embryonic epiblast or primitive ectoderm. The epiblast will then give rise to the three principle germ layers; mesoderm (blood, bone and muscle), endoderm (visceral organs) and ectoderm (skin and neural cells). One of the principle pathways regulating these early specification events is the fibroblast growth factor (FGF) pathway. Signalling downstream of the FGF receptor is an essential determinant of both self-renewal and early differentiation. As this pathway has multi-facetted and sometimes contradictory roles in early development, ES cell differentiation may afford a unique opportunity to dissect out the effect of this pathway on specific populations of cells at defined points in time. In this review we focus on the role of FGFs in both ES cells and the early steps in ES cell differentiation, highlighting places where in vitro studies of ES cell differentiation maybe informing our ideas about lineage specification during development. Based on the increasing amount of ES cell data becoming available, we propose a model whereby FGF signalling is a general regulator of lineage transitions in development.

FGF SIGNALLING

FGFs make up a large cytokine family with diverse biological roles. There are 22 mammalian members of this family and 4 receptor genes, 3 of which can be alternatively spliced to generate multiple receptor isoforms. An FGF receptor consists of an extra-cellular domain with three immunoglobulin-like domains, a transmembrane domain and a split kinase domain. FGFR1-3 encode alternate versions of the third Ig domain via alternate splicing and this region (IIIc) provides variation in the recognition site for different ligands [Johnson and Williams, 1993; Ornitz and Itoh, 2001; Groth and

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Lardelli, 2002; Itoh and Ornitz, 2004]. There is also a fifth receptor (FGFRL1) which does not have a tyrosine kinase domain [Wiedemann and Trueb, 2000]. This truncated receptor could function as a negative regulator of FGF signalling by sequestering the ligand at the level of the membrane. With the exception of FGF11-14, so-called inhibitory or iFGFs, FGFs bind to heparin sulphate proteoglycans (HSPGs) and their cognate receptor [reviewed in Ornitz and Itoh, 2001]. Binding of the ligand and HSPG induces receptor dimerisation and the induction of downstream signalling. Interestingly, mice with a mutation that disrupts the gene encoding UDP-glucose dehydrogenise, an enzyme that is involved in the synthesis of proteoglycan side chains produces an early embryonic phenotype similar to that induced by targeted mutation of either FGF8 or FGFR1 [Garcia-Garcia and Anderson, 2003].

Given the complexity of FGF signalling it is difficult to make generalisations about its role in development and differentiation. The number of receptor–ligand combinations alone is predicted to be on the order of 100 [Zhang et al., 2006]. Moreover, if we include additional interactions between FGF ligands and HSPGs co-receptors (six glypicans and four syndecans in mammals [Jen et al., 2009]) this number would increase further. The number of permutations of this pathway increases even more dramatically when a growing number of non-canonical FGFR co-receptors, such as integrins, NCAM and cadherins (see Polanska et al. [2009] for review) are included.

SIGNALLING DOWNSTREAM OF THE FGF RECEPTOR

Signalling downstream of the receptors appears relatively ubiquitous and involves a defined set of pathways that are depicted in Figure 1. These pathways are important mediators of early embryonic decisions and are therefore pivotal points in the regulation of lineage choice by ES cells. Receptor dimerisation allows the receptor kinase domains to transphosphorylate each other at a number of conserved tyrosines. Activation of these tyrosines leads to the recruitment and tyrosine phosphorylation of membrane anchored docking proteins FRS2 and 3 to recruit Grb2 and SHP2. Grb2 exists in complex with the nucleotide exchange factor Sos and is involved in the activation of the GTP binding protein Ras. In the activated Grb2 complex, Sos catalyzes the activation of Ras by inducing exchange of GDP for GTP. Activated Ras then stimulates

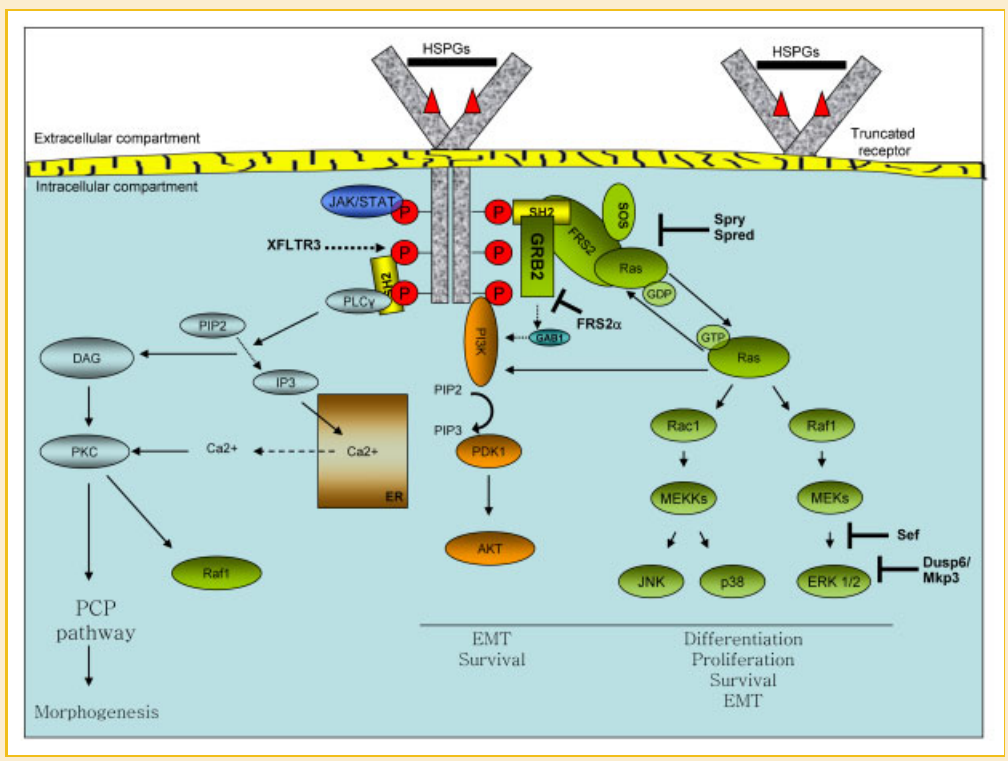

Fig. 1. Diagram illustrating the for intracellular signalling pathways activated downstream of FGF receptor. Only proteins discussed in the text are indicated. Activation by receptor autophosphorylation triggerers a diverse signalling cascades, including the Ras/MAPK, PI3K/Akt, PLCγ/Ca2 and the JAK/STAT pathways. Phosphorylation of the docking protein FRS2 is followed by Grb2 activation which in turns can activate either the Ras/MAPK cascade via SOS, or the PI3K/AKT pathway via Gab1. PI3K can also be activated directly by tyrosine phosphorylation or alternatively by Ras1. The other main transduction pathway involve PLC. The SH2 domain of the PLC interacts directly with the receptor leading to the hydrolysis of PIP2 to IP3 and DAG. IP3 releases Ca2 from the ER while DAG activates PKC that in turns can activate the PCP pathway (not discussed here) and Raf1. Feedback inhibitors such as Dusp6/Mkp3, Spry, FRS2a, Spred and Sef involved in signal attenuation, and enhancers such as XFLRT3 can also contribute to the overall levels of FGF signalling. DAG, diacylglycerol; HSPGs, heparin sulphate proteoglycans; EMT, epithelial-to-mesenchymal transition; IP3, inositol-1,4,5-triphosphate; P, phosphorylation; PIP2, phosphatidyl-inositol-4,5-diphosphate; IP3, inositol-1,4,5-triphophate; PKC, protein kinase C; RE, endoplasmic reticulum.

the Map kinase pathway consisting of Raf1, Rac1, MEK, MEKKs and ERK 1/2 (extra-cellular signal-regulated kinases), JNK and p38. The activated FRS2, Grb2 complex can also recruit Gab1 to activate phosphatidylinositol 3-kinase (PI3K) which then phosphorylates phosphatindylinositol-4,5-diphosphate (PIP2) to generate phosphatidylinositol -3, 4, 5-tripphosphate (PIP3). PIP3 then drives translocation of AKT1 to the membrane where is activated by the phosphoinositide-dependent kinase (PDK1). MAPKs also can negatively feedback on FRS2 via Sprouty [Dailey et al., 2005].

The process of FGF receptor activation culminates in the phosphorylation of an invariant tyrosine (Y766 in FGFR1) in the C terminal tail of the FGF receptor. Activated tyrosines within FGFR are known to bind a variety of intracellular substrates. The activation of the C terminal tail results in the creation of a binding site for proteins containing a Src homology (SH2) domain and in particular binds and activates PLCγ leading to the hydrolysis of PIP2 to IP3 and diacylglycerol (DAG). IP3 induces the release of intracellular Ca+, whereas DAG is a protein kinase C activator that can feedback directly onto MAP kinase via Raf1. More recently, FGF signalling has also been also associated with the JAK/Stat pathway [Ben-Zvi et al., 2006; Schmerer et al., 2006; Citores et al., 2007; Krejci et al., 2008].

While specificity appears to be predominantly controlled at the level of the receptor–ligand interaction, a number of additional factors have been shown to modulate the response to these pathways. These include FRS2a{Lax, 2002 #1774}, Sprouty [Kramer et al., 1999] and Spred [Wakioka et al., 2001], Sef [Furthauer et al., 2002; Tsang et al., 2002], XlFLRTs [Bottcher et al., 2004] and Mkp3/Dusp6 [Groom et al., 1996]. Expression of these factors in embryonic development correlates with sites of FGF activity. Although the majority of these factors have been shown to regulate MAPK signalling, there is some evidence that Sprouty and Spred can directly modulate cellular morphogenesis [Miyoshi et al., 2004; Sivak et al., 2005]. These co-factors are expressed in embryonic development in regions of the embryo where MAPK signalling is active [Lunn et al., 2007] and some are known to respond to FGF stimulation to produce a feedback mode of regulation [Furthauer et al., 2001, 2002; Lax et al., 2002; Tsang et al., 2002; Eblaghie et al., 2003; Sivak et al., 2005].

Despite this canonical set of transduction pathways; FGF signalling has a broad set of physiological responses. The explanation for this diverse set of responses lies in the competence of cells to respond to pathway based both on concurrent signalling via other parallel pathways and the differential presence and/or activity of particular components of FGF signalling machinery. Factors that affect cell type specific responses to the pathway include specific FGF:FGFR subtype affinity, binding of the ligand to dominant negative, truncated or secreted receptor fragments, receptor adaptors, enhancers or attenuators of cell signalling, diffusibility of the ligand, inhibitors of signal transduction and availability of downstream target kinases. Moreover, while there is some context/cell type dependent activation of PI3K, the major response to this diverse set of ligands and receptors is phosphorylation of ERK. However, during embryonic development there are cell types that are exposed to both the ligand and express the correct corresponding receptor, but that do not exhibit Erk activation [Corson et al., 2003].

In addition to regulation by FGFs, ERK activation appears to be the key response downstream of many receptor tyrosine kinases (RTKs) and where RTKs influence differentiation, this appears ERK dependent, despite the evidence that pERK is mainly a mediator of proliferation. Whether ERK regulates lineage specification via promotion of differential rates of proliferation in specific progenitor populations or some other mechanism is unknown. However, despite the regulation of ERK activation by other cytokines (e.g. EGF), activation of this kinase is generally been found to correspond to the regions of the embryo that express FGFs and their receptors [Corson et al., 2003; Lunn et al., 2007]. Whether or not PI3K/AKT activation corresponds to specific domains of FGF:FGFR expression during embryogenesis remains to be determined.

FGF SIGNALLING AND PRE-IMPLANTATION DEVELOPMENT

Murine ES cells are derived from the early mammalian blastocyst. As a result, understanding the role of FGF signalling in ES cell self-renewal and differentiation, requires an understanding of its role in early embryonic development. The earliest decisions in embryonic development concern the segregation of embryonic and extra-embryonic fates (Fig. 2, top panel). At eight cell stage the mouse embryo undergoes compaction, at which point the outside cells go on to form trophoblast and the inner cells form the ICM of the blastocyst. The ICM will go onto form the embryo proper and the trophoblast will develop into placenta. While the initial specification of trophoblast does not appear to depend on FGF signalling, trophoblast stem cells are dependent on this pathway both in vivo and in vitro [Georgiades and Rossant, 2006]. FGF signalling is also required for the induction of the second extra-embryonic lineage, the primitive endoderm. The primitive endoderm is first apparent morphologically at 4.0 days post coitum (dpc), but its specification begins with in the ICM, as cells begin to adapt a primitive endoderm fate as a result of ERK activation [Chazaud et al., 2006]. FGFR2 [Arman et al., 1998], Grb2 [Chazaud et al., 2006] and FGF4 mutant embryos [Feldman et al., 1995] fail to develop primitive endoderm. Moreover, recent imaging studies of the early blastocyst suggest a role for PI3K downstream PDGFRa in this process [Plusa et al., 2008]. The primitive endoderm will later differentiate and give rise to the visceral endoderm, surrounding the epiblast and the parietal endoderm that forms part of Reichert's membrane.

FGF SIGNALLING AND EXTRA-EMBRYONIC DIFFERENTIATION IN VITRO

As ES cells are derived from the mammalian blastocyst, they readily can be differentiated to form primitive endoderm. While, the induction of trophoblast in the absence of genetic manipulation is difficult, trophoblast stem cells can be derived from the mammalian blastocyst. Moreover, if the trophoblast transcription factors Cdx2 or TEAD4 [Yagi et al., 2007; Nishioka et al., 2008] are expressed in ES cells, they can develop into trophoblast stem cells in an FGF

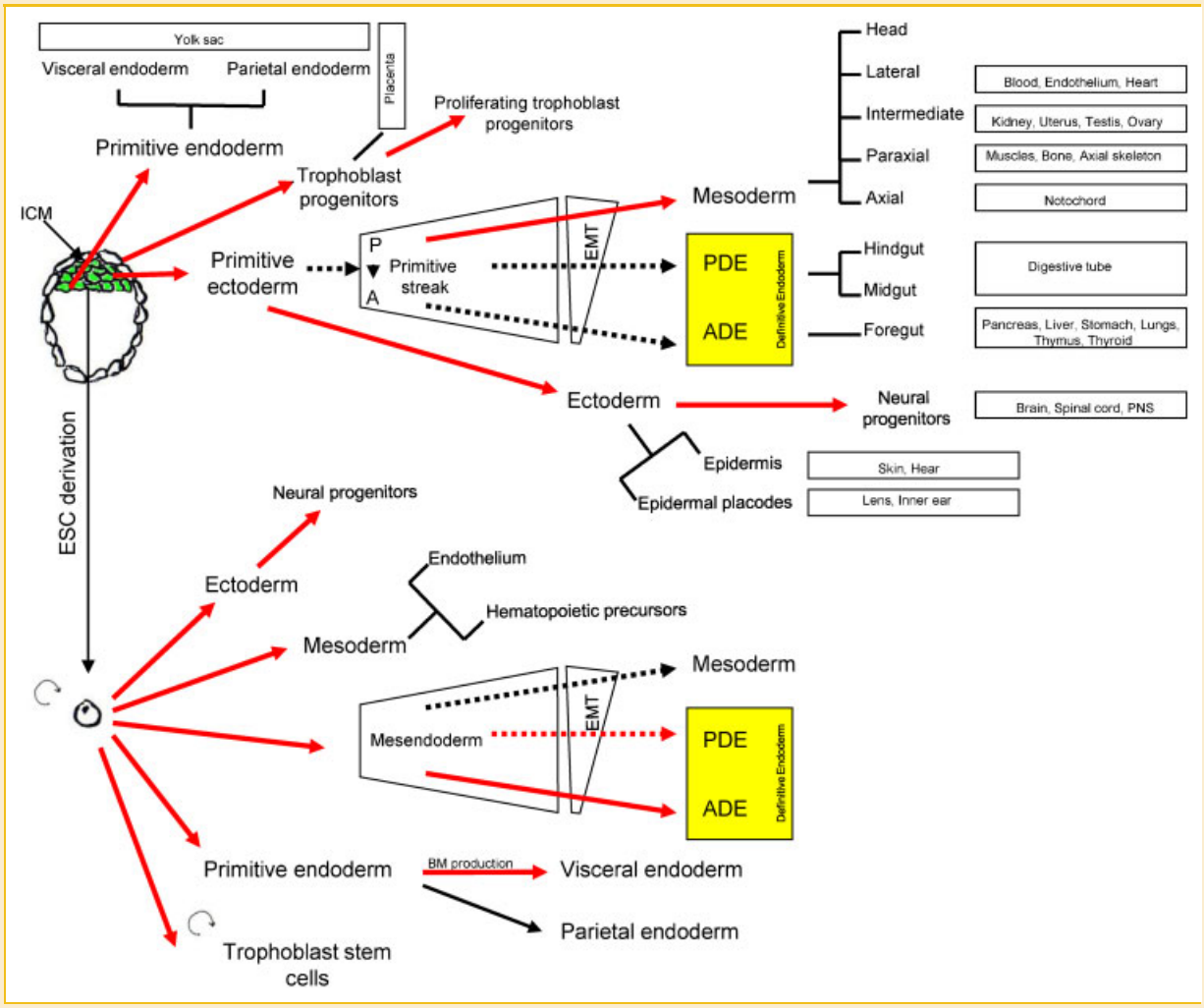

Fig. 2. Schematic representation of the suggested role for FGF signalling pathway both in vivo and in vitro during early lineage specification. Upper side depicts lineage specification in embryonic development and bottom side ES cell differentiation. FGF signalling is required for the generation of the Primitive ectoderm (embryonic epiblast) from the ICM of the blastocyst, and for the induction and maintenance of the extra-embryonic lineages (primitive endoderm and trophectoderm). The primitive endoderm will later give rise to both visceral and parietal endoderm, while the trophoblast will develop in placenta. The position in the PS (anterior–to–posterior) and the length of the EMT regulated by FGF is predicted to determine the fate of the epiblast cells transiting through the PS, segregating them into mesoderm and anterior–posterior definitive endoderm. Neural progenitors will also arise from the Primitive ectoderm in a FGF dependent manner. ESC can be derived from the blastocyst ICM and differentiated to form trophoblast progenitors, primitive endoderm, mesoderm, ectoderm and mesendoderm (PS–like cells). Mesendodermal cells will differentiate towards ADE in the presence of exogenous FGF while all endoderm differentiation requires FGF signal. As a result the red arrow to ADE (see below) is depicted as a continuous line. The FGF role in the generation of visceral endoderm appear to be driven by the production of components of the basal membrane. A, anterior; ADE, anterior definitive endoderm; BM, basal membrane; EMT, epithelial–to–mesenchymal transition; ESC, embryonic stem cells; ICM, inner cell mass; P, posterior; PDE, posterior definitive endoderm; PS, Primitive streak.

dependent process. The formation of primitive endoderm from ES cells can be accomplished in different defined protocols [Hamazaki et al., 2004; Yasunaga et al., 2005] or via simple suspension culture [Abe et al., 1996]. When differentiated in suspension culture, ES cells form embryo like cavitated structures known as embryiod bodies (EBs) that contain extra-embryonic visceral endoderm on the outside and epiblast-like cells on the inside [Robertson, 1987]. The formation of these structures appears to be itself dependent signalling downstream of the FGF receptor as when Grb2 (−/−) ES cells are differentiated in EBs they neither produce visceral endoderm nor do they cavitate [Cheng et al., 1998]. Similar results have been obtained through the misexpression of dominant negative FGF receptors [Chen et al., 2000; Li et al., 2001] and the culture of EBs in FGF antagonist SU5402 [Li et al., 2004]. Interestingly, the response of this particular dnFGF receptor appears to be directed towards the PI3K pathway [Chen et al., 2000] and similar phenotypes were obtained by targeting PI3K signalling with specific antagonists. Moreover, a number of basement membrane (BM) components normally produced by the primitive endoderm appear to be FGF dependent via the PI3K pathway [Li et al., 2001]. BM components can also be induced by the expression of GATA6 in ES cells and the production of BM can cell non-autonomously

rescue cavitation and epiblast differentiation in mixed EBs made from cells expressing the dominant negative FGF receptor [Li et al., 2004]. Interestingly, disruption of VE formation in ES cells mutant for GATA 4 and 6 can also be rescued by addition of either VEGFa or Indian Hedgehog (IHH) [Pierre et al., 2009]. As VEGFa also signals trough PI3K, this suggests that the initial induction of PI3K through FGF is then maintained via a GATA dependent paracrine regulatory loop.

While activation of PI3K downstream of FGF appears required for aspects of visceral endoderm formation in EBs, it is neither sufficient nor absolutely required for extra-embryonic endoderm differentiation from ES cells. The endoderm differentiation phenotype in Grb2 (−/−) mutant ES cells can be rescued by activated H-Ras and chimeric fusion proteins directed specifically towards Ras activation (e.g. a Sos–Grb2–SH2 domain fusion) [Cheng et al., 1998]. Interestingly, activated H-Ras can induce endoderm differentiation even in the presence of LIF. Moreover, activated Ras mutants specific for the activation of pERK, induced ES cell differentiation to primitive endoderm [Yoshida-Koide et al., 2004] and the expression of an activated MEK also drove cells in this direction [Hamazaki et al., 2006] This role for Ras-ERK signalling is also supported by earlier work in embryonic carcinoma cells (EC). In EC cells, activated c-Ha-ras induced endoderm-like phenotypes [Yamaguchi-Iwai et al., 1990; Verheijen et al., 1999b] in a MEK dependent manor [Verheijen et al., 1999a,b]. Moreover, sustained and elevated levels of ERK activity also appear to block the further differentiation of primitive endoderm into parietal endoderm [Verheijen et al., 1999a].

Taken together, these data suggest that FGF signalling acts through both PI3K and pERK to specify extra-embryonic endoderm differentiation from ES cells. Signalling through PI3K would be required during visceral endoderm differentiation to regulate the production of essential extra-cellular matrix by primitive endoderm. This matrix is required to support the development of both the visceral endoderm and epiblast lineages in EB differentiation and further signalling via the FGF pathway [Liu et al., 2009]. However, the initial specification of primitive endoderm requires activation of ERK and this pathway may also suppress the differentiation of primitive endoderm to parietal. Because primitive endoderm is very close developmentally to the early blastocyst and ES cells produce FGF4, culturing ES cells in the presence of small molecule inhibitors of ERK1/2 can augment ES cell self-renewal in part by inhibiting primitive endoderm differentiation [Burdon et al., 1999; Ying et al., 2008]. However, the centrality of the pERK signalling to early lineage commitment from ES cells is also apparent from its requirement in embryonic germ layer differentiation as well.

FGF SIGNALLING AND ES CELL DIFFERENTIATION TO NEURAL FATES

The specification of the three definitive germ layers during embryonic development occurs once the ICM has progressed beyond the capacity to make extra-embryonic endoderm and formed the epiblast or primitive ectoderm (see Fig. 2). Epiblast stage naïve ectoderm is still multi-potent and is the raw material from which the all the cell types in the embryo are derived. While early extra-embryonic development is unique to mammals, the ability of pre-gastrulation stage ectoderm to differentiate into neural tissue has been studied extensively in multiple vertebrate model systems. Thus neural differentiation was first induced ectopically through embryonic transplantation experiments over 80 years ago [Spemann and Mangold, 1924] leading to the discovery of an embryonic signalling centre known as the organiser [reviewed in Hemmati-Brivanlou and Melton, 1997; Streit and Stern, 1999]. The organiser produces antagonist(s) of BMP signalling and in some model systems, the antagonism of BMP signalling has been reported to be sufficient to induce neural differentiation in naïve ectoderm [Wilson and Hemmati-Brivanlou, 1995; Di-Gregorio et al., 2007; Wills et al., 2010]. This has lead to the notion that ectoderm will default differentiate into neural tissue in the absence of BMP signalling. However, this so-called, 'Default model', for neural induction is somewhat controversial and overlooks reported requirements for a pro-inductive competence signal via the FGF pathway [Streit et al., 2000; Linker and Stern, 2004]. As a number of these experiments were done in different vertebrate models, where the stages at which experimental manipulations are possible varies and differences in their dependence on FGF signalling more likely reflects a transient requirement for this pathway very early in the progression towards neural differentiation rather than fundamental evolutionary differences in the mechanism of neural induction.

When murine ES cells are placed into defined media in the absence of cytokines they differentiate towards neural progenitors, supporting the notion of a default model for neural induction. Moreover, human ES cells can be converted to neural precursors at efficiencies approaching 80% through the simultaneous inhibition of both BMP and TGF-β signalling [Chambers et al., 2009]. However, while exogenous FGF is not required for this process, autocrine FGF signalling is and inhibitors of FGF signalling can block neural differentiation [Ying et al., 2003b]. The FGF requirement in ES cell differentiation towards neural progenitors is mediated via pERK signalling [Kunath et al., 2007; Stavridis et al., 2007] and this signal maybe required at multiple points during neural differentiation. Stavridis et al. [2007], maintain that the requirement for ERK dependent FGF signalling is in the transition of epiblast-like cells to neural progenitors, while Kunath et al. [2007] argue that it is required for an ICM like state to differentiate towards epiblast ectoderm. As ERK2 (−/−) ES cells are defective in differentiation towards neural, mesodermal and non-neural cell types generated in the presence BMP [Kunath et al., 2007], it would appear that there is a minimal requirement for FGF signalling in ES cell differentiation towards multi-potent epiblast. Thus FGF signalling maybe essential for ES cell differentiation towards all embryonic lineages.

INHIBITION OF FGF SIGNALLING AND MOUSE ES CELL PLURIPOTENCY

As ERK signalling was required for ES cell differentiation towards primitive endoderm, inhibition of ERK has been exploited to further define the conditions for murine ES cell culture [Ying et al., 2008].

Moreover, ES cells have recently been shown to be a heterogeneous mixture a central ICM-like state and reversible intermediates that represent putative early steps in differentiation [Chambers et al., 2007; Hayashi et al., 2008; Kalmar et al., 2009]. The two cell types most readily formed by ICM-like cells are primitive endoderm and epiblast and spontaneous differentiation to these two lineages is readily observed in ES cell culture. As both of these pathways require FGF, it has been suggested that pERK mediates a transition between a stable self-renewing ICM-like state and a differentiation prone state [Kunath et al., 2007; Silva et al., 2008]. The ICM-like state also produces high levels of FGF4 that can in turn drive early differentiation, but this can readily be suppressed by the inclusion of an FGF antagonist in the culture media [Burdon et al., 1999; Silva and Smith, 2008; Ying et al., 2008].

Mouse ES cells are normally cultured in the presence of LIF and BMP4 or serum [Ying et al., 2003a], but as all murine ES cell cultures also produce FGF4, these cultures will always contain a background level of differentiation down the primitive endoderm and ectoderm lineages. However, if cultured in the presence of a complete blockage on ERK activation, ES cells appear more homogenously ICM-like. Interestingly, it has been suggested that the main role of LIF and BMP in supporting mouse ES cells maybe the shielding of cells from ERK activation [Ying et al., 2008]. Inhibition of ERK signalling has also been shown to be an essential component of rat ES cell derivation [Buehr et al., 2008; Li et al., 2008].

While FGF signalling acting via pERK promotes differentiation; AKT/PI3K acts downstream of LIF signalling to support ES cell self-renewal [Jirmanova et al., 2002; Paling et al., 2004]. Moreover, a fusion of AKT to a modified form of the hormone binding domain of the oestrogen receptor can conveys a significant degree of LIF independent self-renewal on murine ES cells [Watanabe et al., 2006]. Murine ES cells also express a variant Ras, ERas that promotes self-renewal rather than primitive endoderm differentiation. The crucial difference between ERas and HRas is that, ERas does not change pERK levels, but rather acts solely on AKT/PI3K signalling [Takahashi et al., 2003].

SELF-RENEWAL IN HUMAN AND EPIBLAST STEM CELLS

While FGF signalling is required for the early stages of differentiation in a number of lineages, it is also an essential mediator of self-renewal in human and epiblast stem cells. While both human and mouse ES cells are derived from the blastocyst stage of development, human ES cells have similar culture conditions to stem cell lines derived from the murine epiblast [Brons et al., 2007; Tesar et al., 2007]. Both of these cell lines depend on FGF signalling for their ability to expand in culture, although the action of FGF signalling may target partially differentiated cells that co-exist in culture with human ES cells [Bendall et al., 2007]. FGF signalling in these stromal cells results in the production of Insulin Growth Factor II (IGFII) and this factor appears an essential cytokine for human ES maintenance and by extension, although this has not been formally shown, for epiblast stem cells.

DIFFERENTIATION TOWARDS THE MESODERM AND ENDODERM LINEAGES

During embryonic development in mouse, embryonic mesoderm and endoderm are induced in the primitive streak region at the onset of gastrulation. As both lineages are induced by similar signalling pathways acting on a single region of the embryo, it has been suggested that they may be derived from a common precursor, so-called mesendoderm [Rodaway and Patient, 2001]. As ES cell differentiation affords the ability to purify single cells during the process of differentiation this hypothesis can be directly tested.

The induction of mesoderm and endoderm during gastrulation involves signalling through both the Nodal related TGF-β and Wnt pathways [see for review Stern, 2006; Tam et al., 2006]. Based on work in lower vertebrates, FGF signalling was thought to have a role in resolving early bipotent mesendoderm, promoting the differentiation of the mesoderm lineage over that of endoderm [Rodaway et al., 1999], posteriorising cell types being induced in the streak or marginal zone region and, in particular, triggering the initiation of cell migration [Griffin et al., 1995; Isaacs, 1997]. Moreover, genetic studies suggest that in the absence of either FGF4 or 8 [Sun et al., 1999], the FGF receptor 1 [Ciruna et al., 1997] or appropriately modified proteoglycans [Garcia-Garcia and Anderson, 2003] progenitor cells fail to migrate through the primitive streak. In chimera experiments, ES cells mutant for FGFR1 fail to undergo epithelial to mesenchymal transition and they do not down regulate E-cadherin. These high levels of E-cadherin can sequester β-catenin, damping down the response of cells to the mesoderm inducing properties of Wnt signalling [Ciruna and Rossant, 2001]. Interestingly, while the FGFR1 null ES cells will not contribute to endoderm in chimeras, this has been viewed in terms of the failure of these cells to transgress the primitive streak [Ciruna et al., 1997]. Thus, the prevailing evidence from embryonic development is that FGFs are mediators of mesoderm specification and gastrulation movements, although in zebrafish there is an example of FGF signalling feeding back onto Nodal signalling through FGF dependent stimulation of expression of the one eyed pinhead co-receptor [Mathieu et al., 2004].

As ES cells apparently require FGF signalling to differentiate to an epiblast stage, the activity of this pathway is clearly a prerequisite to the induction of either mesoderm or endoderm (Fig. 2). Consistent with the observations in the embryo, FGF signalling is required for murine [Kunath et al., 2007; Hansson et al., 2009] and human [Zhang et al., 2008] ES cell differentiation to mesoderm. Moreover, under defined conditions FGF2 has been shown to have a general stimulatory role in mesendoderm differentiation, but this effect is particularly pronounced with respect to the generation of mesodermal precursors [Era et al., 2008]. However, what is more surprising is that FGF signalling is required for embryonic endoderm specification from ES cells. Under defined conditions in mouse ES cells, a blockade of the FGF receptor inhibits all definitive endoderm differentiation. Moreover, while inhibition of FGF signalling early in differentiation blocks progression towards both the mesoderm and endoderm lineage, FGF signalling appears specifically required after the specification of epiblast-like cells for endoderm and not

mesoderm specification [Morrison et al., 2008]. FGF signalling appears required for both efficient definitive endoderm differentiation and for the further specification of anterior definitive endoderm (ADE). The kinetics of this process suggests that FGF signalling is also required at the stage when these cultures are already at the primitive streak stage of differentiation for further differentiation towards the endoderm lineage [Morrison et al., 2008; Hansson et al., 2009].

A number of ES cell studies suggest that differentiation to endoderm passes through an intermediate with the potency to form both mesoderm and endoderm [Kubo et al., 2004; Tada et al., 2005]. While there is little evidence for an actual bipotent intermediate of the mesoderm and endoderm during embryonic development [Tzouanacou et al., 2009], time lapse studies suggest they may exist for a very brief period of time [Burtscher and Lickert, 2009]. Mesoderm and endodermal progenitors originate in the epiblast, loose their polarity by undergoing a process reminiscent of epithelial to mesenchymal transitions (EMT-like) as they enter the primitive streak. Once in the primitive streak, these lineages rapidly segregate, with the endoderm intercalating with the existing outside visceral endodermal epithelium to regain polarity and the mesoderm cells becoming migratory and truly mesenchymal [Burtscher and Lickert, 2009]. Thus there maybe a short window of time in the primitive streak when a 'bipotent precursor', maybe be represented by a partially depolarised cell capable of either re-epitheliarising or adopting a mesenchymal fate. Genetic studies in mouse suggest that FGF signalling is required for this EMT-like event in the primitive streak [Ciruna and Rossant, 2001] and any requirement for FGF signalling in endoderm specification has been viewed as indirect effect due to a defect in this EMT-like event. Differentiating ES cells appear to transit into a primitive streak like state undergoing a degree of EMT that recapitulates a component of gastrulation in vitro [D'Amour et al., 2005; Morrison et al., 2008; Livigni et al., 2009; Unpublished observations]. Moreover, ES cells differentiating towards endoderm rapidly down regulate markers of mesenchyme and upregulate E-cadherin, apparently regaining a degree of epithelial morphology [Villegas and Brickman, unpublished observations; Tada et al., 2005; Yasunaga et al., 2005]. Interestingly, there is no in vivo evidence for FGF playing a part in this later step in endodermal epithelialisation. However, in both human and mouse ES cell differentiation, FGF signalling appears absolutely required for the specification of endoderm [Morrison et al., 2008; Shiraki et al., 2008; Hansson et al., 2009; Livigni et al., 2009] and reduced levels of FGF signalling from the primitive streak stage appears to favour mesoderm differentiation [Morrison et al., 2008]. Thus FGF signalling maybe required for multiple morphogenetic changes in this lineage and ES cell differentiation may afford a unique tool to dissect the role of this pathway at these subsequent stages of differentiation. Moreover, while genetic studies in mouse all point to a dependence of gastrulation stage cell migration on FGF signalling, it is difficult to separate the role of this pathway on signalling, migration or migration of a cell to a new signal. ES cell differentiation provides a model system in which the effect of this pathway on induction can be segregated from the way in which it affects the evolution of ever changing in vivo microenvironments.

While some of these early inductive events in the generation of primitive streak-like mesendoderm presumable rely on ERK activation, some insight into the lineage determining role of FGF signalling comes from recent work in human ES cells. Inhibition of PI3K blocks the differentiation of human ES cells towards endoderm, while inhibition of ES cell differentiation with the MEK inhibitor U0126 augments endoderm differentiation and blocks mesoderm [Sumi et al., 2008]. While these data are interesting, they are somewhat at odds with other studies that seem to indicate that endoderm differentiation in the presence of activin requires the suppression of signalling through PI3K [McLean et al., 2007]. The cells differentiated by Sumi et al. [2008] employed transgenic activation of the Wnt pathway as a means to circumvent activin mediated induction, indicating downstream stimulation of PI3K might only block ES cell differentiation in the presence of activin, implying that this signal could feedback on activin/Nodal pathway. It is also interesting to note, that the addition of the PI3K inhibitor LY294002 shifts human ES cells from activin dependent self-renewal into mesendoderm and later endoderm differentiation, suggesting that the interaction might be at the level of insulin signalling [McLean et al., 2007]. While the levels of LY294002 applied in this study are extremely high and the markers examined limited, this maybe related to the previously mentioned role of IGFII in supporting human ES cell self-renewal [Bendall et al., 2007].

While the precise mechanism by which FGF signalling regulates differentiation in the mesoderm and endoderm lineages is still unclear, sequential roles for both ERK activation and PI3K suggest that the underlying role of FGF in regulating endoderm specification maybe the same in both the visceral and definitive endoderm. The similarity of this extra-embryonic endodermal cell type, with the definitive endoderm that will go onto make up the gut, is a notion that may be supported in recent fate mapping and potency studies that indicate that some visceral endoderm contributes to the embryonic gut [Kwon et al., 2008].

FGF SIGNALLING AS A REGULATOR OF EMBRYONIC TRANSITIONS

While we have only explored the role of FGF signalling in the early events surrounding lineage specification from ES cells, these patterns appear to repeat themselves and suggest a general role for this pathway in cell fate decisions. The involvement of this pathway at repeated transitions could imply that signalling downstream of the FGF receptor maybe required for ES cells to continually progress through differentiation. Does this suggest that FGF signalling, at least via MAPK/Erk1/2, is a requirement for cells to progress from one step in lineage specification to the next? To date the majority of analysis on FGF signalling in development has been based on embryo phenotypes that examine the downstream cell types, rather than all the intermediates formed during lineage specification. Defects in specific lineages maybe a result of a failure of embryos to generate lineage restricted progenitors or a failure of those progenitor cells to progress into differentiation or both. Distinguishing between these possibilities in vivo is difficult. ES cell differentiation affords the unique opportunity to identify inter-

mediates, purify them and assess their ability to progress into differentiation.

So how would a more general role for FGF/MAPK/Erk1/2 signalling in differentiation fit with its role in development? The expression of these pathways and their phenotypes in embryonic development suggest a widespread, but not global role for this pathway indicating that requirements for FGF signalling in differentiation might depend on the local embryonic context [Corson et al., 2003; Lunn et al., 2007]. Is any potential global requirement for this pathway in ES cells a result of removing cells from their normal in vivo context and creating some form deficiency that can be compensated for through the activation of FGF/MAPK/Erk1/2? Or has ES cell differentiation simply uncovered additional roles for FGF/MAPK/Erk1/2 in differentiation? The resolution of this issue will require careful analysis of inducible conditional mutants in vivo.

How could FGF/MAPK/Erk1/2 signalling render cells competent to progress to the next stage of differentiation? Does it regulate the competence of cells to respond to inducing signals? Given the relatively ubiquitous role of this pathway in development and differentiation, the transcription factors downstream of MAPK/Erk1/2 may serve as a synergistic platform to amplify the transcriptional response to other pathways. Alternatively, exposure to FGF signalling could alter the accessibility or expression levels of receptors for other pathways. However, as FGF/MAPK/Erk1/2 is also a strong mitogen, perhaps its effect on differentiation is merely to stimulate division. Thus as new cell types are induced during lineage specification, FGF/MAPK/Erk1/2 could support the proliferation of these new populations selectively.

While we have focused heavily on MAPK/Erk1/2 signalling, there are clearly instances in vitro that require FGF mediated stimulation of PI3K. This is particularly fascinating as indicates that FGF mediated changes in polarity maybe as important for in vitro differentiation as they are in embryonic development.

DIAGRAMMATIC KEY

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REFERENCES

Abe K, Niwa H, Iwase K, Takiguchi M, Mori M, Abe SI, Yamamura KI. 1996. Endoderm-specific gene expression in embryonic stem cells differentiated to embryoid bodies. Exp Cell Res 229:27–34.

Arman E, Haffner-Krausz R, Chen Y, Heath JK, 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.

Bendall SC, Stewart MH, Menendez P, George D, Vijayaragavan K, Werbowetski-Ogilvie T, Ramos-Mejia V, Rouleau A, Yang J, Bosse M, Lajoie G, Bhatia M. 2007. IGF and FGF cooperatively establish the regulatory stem cell niche of pluripotent human cells in vitro. Nature 448:1015–1021.

Ben-Zvi T, Yayon A, Gertler A, Monsonego-Ornan E. 2006. Suppressors of cytokine signaling (SOCS) 1 and SOCS3 interact with and modulate fibroblast growth factor receptor signaling. J Cell Sci 119:380–387.

Bottcher RT, Pollet N, Delius H, Niehrs C. 2004. The transmembrane protein XFLRT3 forms a complex with FGF receptors and promotes FGF signalling. Nat Cell Biol 6:38–44.

Brons IG, Smithers LE, Trotter MW, Rugg-Gunn P, Sun B, Chuva de Sousa Lopes SM, Howlett SK, Clarkson A, Ahrlund-Richter L, Pedersen RA, Vallier L. 2007. Derivation of pluripotent epiblast stem cells from mammalian embryos. Nature 448:191–195.

Buehr M, Meek S, Blair K, Yang J, Ure J, Silva J, McLay R, Hall J, Ying QL, Smith A. 2008. Capture of authentic embryonic stem cells from rat blastocysts. Cell 135:1287–1298.

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

Burtscher I, Lickert H. 2009. Foxa2 regulates polarity and epithelialization in the endoderm germ layer of the mouse embryo. Development 136:1029–1038.

Chambers I, Silva J, Colby D, Nichols J, Nijmeijer B, Robertson M, Vrana J, Jones K, Grotewold L, Smith A. 2007. Nanog safeguards pluripotency and mediates germline development. Nature 450:1230–1234.

Chambers SM, Fasano CA, Papapetrou EP, Tomishima M, Sadelain M, Studer L. 2009. Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. Nat Biotechnol 27:275–280.

Chazaud C, Yamanaka Y, Pawson T, Rossant J. 2006. Early lineage segregation between epiblast and primitive endoderm in mouse blastocysts through the Grb2-MAPK pathway. Dev Cell 10:615–624.

Chen Y, Li X, Eswarakumar VP, Seger R, Lonai P. 2000. Fibroblast growth factor (FGF) signaling through PI 3-kinase and Akt/PKB is required for embryoid body differentiation. Oncogene 19:3750–3756.

Cheng AM, Saxton TM, Sakai R, Kulkarni S, Mbamalu G, Vogel W, Tortorice CG, Cardiff RD, Cross JC, Muller WJ, Pawson T. 1998. Mammalian Grb2 regulates multiple steps in embryonic development and malignant transformation. Cell 95:793–803.

Ciruna B, Rossant J. 2001. FGF signaling regulates mesoderm cell fate specification and morphogenetic movement at the primitive streak. Dev Cell 1:37–49.

Ciruna BG, Schwartz L, Harpal K, Yamaguchi TP, Rossant J. 1997. Chimeric analysis of fibroblast growth factor receptor-1 (FGFr1) function: A role for FGFR1 in morphogenetic movement through the primitive streak. Development 124:2829–2841.

Citores L, Bai L, Sorensen V, Olsnes S. 2007. Fibroblast growth factor receptor-induced phosphorylation of STAT1 at the Golgi apparatus without translocation to the nucleus. J Cell Physiol 212:148–156.

Corson LB, Yamanaka Y, Lai KM, Rossant J. 2003. Spatial and temporal patterns of ERK signaling during mouse embryogenesis. Development 130:4527–4537.

Dailey L, Ambrosetti D, Mansukhani A, Basilico C. 2005. Mechanisms underlying differential responses to FGF signaling. Cytokine Growth Factor Rev 16:233–247.

D'Amour KA, Agulnick AD, Eliazer S, Kelly OG, Kroon E, Baetge EE. 2005. Efficient differentiation of human embryonic stem cells to definitive endoderm. Nat Biotechnol 23:1534–1541.

Di-Gregorio A, Sancho M, Stuckey DW, Crompton LA, Godwin J, Mishina Y, Rodriguez TA. 2007. BMP signalling inhibits premature neural differentiation in the mouse embryo. Development 134:3359–3369.

Eblaghie MC, Lunn JS, Dickinson RJ, Munsterberg AE, Sanz-Ezquerro JJ, Farrell ER, Mathers J, Keyse SM, Storey K, Tickle C. 2003. Negative feedback regulation of FGF signaling levels by Pyst1/MKP3 in chick embryos. Curr Biol 13:1009–1018.

Era T, Izumi N, Hayashi M, Tada S, Nishikawa S. 2008. Multiple mesoderm subsets give rise to endothelial cells, whereas hematopoietic cells are differentiated only from a restricted subset in embryonic stem cell differentiation culture. Stem Cells 26:401–411.

Feldman B, Poueymirou W, Papaioannou VE, DeChiara TM, Goldfarb M. 1995. Requirement of FGF-4 for postimplantation mouse development. Science 267:246–249.

Furthauer M, Reifers F, Brand M, Thisse B, Thisse C. 2001. Sprouty4 acts in vivo as a feedback-induced antagonist of FGF signaling in zebrafish. Development 128:2175–2186.

Furthauer M, Lin W, Ang SL, Thisse B, Thisse C. 2002. Sef is a feedback-induced antagonist of Ras/MAPK-mediated FGF signalling. Nat Cell Biol 4:170–174.

Garcia-Garcia MJ, Anderson KV. 2003. Essential role of glycosaminoglycans in FGF signaling during mouse gastrulation. Cell 114:727–737.

Georgiades P, Rossant J. 2006. Ets2 is necessary in trophoblast for normal embryonic anteroposterior axis development. Development 133:1059–1068.

Griffin K, Patient R, Holder N. 1995. Analysis of FGF function in normal and no tail zebrafish embryos reveals separate mechanisms for formation of the trunk and the tail. Development 121:2983–2994.

Groom LA, Sneddon AA, Alessi DR, Dowd S, Keyse SM. 1996. Differential regulation of the MAP, SAP and RK/p38 kinases by Pyst1, a novel cytosolic dual-specificity phosphatase. EMBO J 15:3621–3632.

Groth C, Lardelli M. 2002. The structure and function of vertebrate fibroblast growth factor receptor 1. Int J Dev Biol 46:393–400.

Hamazaki T, Oka M, Yamanaka S, Terada N. 2004. Aggregation of embryonic stem cells induces Nanog repression and primitive endoderm differentiation. J Cell Sci 117:5681–5686.

Hamazaki T, Kehoe SM, Nakano T, Terada N. 2006. The Grb2/Mek pathway represses Nanog in murine embryonic stem cells. Mol Cell Biol 26:7539–7549.

Hansson M, Olesen DR, Peterslund JM, Engberg N, Kahn M, Winzi M, Klein T, Maddox-Hyttel P, Serup P. 2009. A late requirement for Wnt and FGF signaling during activin-induced formation of foregut endoderm from mouse embryonic stem cells. Dev Biol 330:286–304.

Hayashi K, Lopes SM, Tang F, Surani MA. 2008. Dynamic equilibrium and heterogeneity of mouse pluripotent stem cells with distinct functional and epigenetic states. Cell Stem Cell 3:391–401.

Hemmati-Brivanlou A, Melton D. 1997. Vertebrate neural induction. Annu Rev Neurosci 20:43–60.

Isaacs HV. 1997. New perspectives on the role of the fibroblast growth factor family in amphibian development. Cell Mol Life Sci 53:350–361.

Itoh N, Ornitz DM. 2004. Evolution of the FGF and FGFr gene families. Trends Genet 20:563–569.

Jen YH, Musacchio M, Lander AD. 2009. Glypican-1 controls brain size through regulation of fibroblast growth factor signaling in early neurogenesis. Neural Dev 4:33.

Jirmanova L, Afanassieff M, Gobert-Gosse S, Markossian S, Savatier P. 2002. Differential contributions of ERK and PI3-kinase to the regulation of cyclin D1 expression and to the control of the G1/S transition in mouse embryonic stem cells. Oncogene 21:5515–5528.

Johnson DE, Williams LT. 1993. Structural and functional diversity in the FGF receptor multigene family. Adv Cancer Res 60:1–41.

Kalmar T, Lim C, Hayward P, Munoz-Descalzo S, Nichols J, Garcia-Ojalvo J, Martinez Arias A. 2009. Regulated fluctuations in nanog expression meidate cell fate decisions in embryonic stem cells. PLoS Biol 7:e1000149.

Kramer S, Okabe M, Hacohen N, Krasnow MA, Hiromi Y. 1999. Sprouty: A common antagonist of FGF and EGF signaling pathways in Drosophila. Development 126:2515–2525.

Krejci P, Salazar L, Goodridge HS, Kashiwada TA, Schibler MJ, Jelinkova P, Thompson LM, Wilcox WR. 2008. STAT1 and STAT3 do not participate in FGF-mediated growth arrest in chondrocytes. J Cell Sci 121:272–281.

Kubo A, Shinozaki K, Shannon JM, Kouskoff V, Kennedy M, Woo S, Fehling HJ, Keller G. 2004. Development of definitive endoderm from embryonic stem cells in culture. Development 131:1651–1662.

Kunath T, Saba-El-Leil MK, Almousailleakh M, Wray J, Meloche S, 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.

Kwon GS, Viotti M, Hadjantonakis AK. 2008. The endoderm of the mouse embryo arises by dynamic widespread intercalation of embryonic and extraembryonic lineages. Dev Cell 15:509–520.

Lax I, Wong A, Lamothe B, Lee A, Frost A, Hawes J, Schlessinger J. 2002. The docking protein FRS2alpha controls a MAP kinase-mediated negative feedback mechanism for signaling by FGF receptors. Mol Cell 10:709–719.

Li X, Chen Y, Scheele S, Arman E, Haffner-Krausz R, Ekblom P, Lonai P. 2001. Fibroblast growth factor signaling and basement membrane assembly are connected during epithelial morphogenesis of the embryoid body. J Cell Biol 153:811–822.

Li L, Arman E, Ekblom P, Edgar D, Murray P, Lonai P. 2004. Distinct GATA6- and laminin-dependent mechanisms regulate endodermal and ectodermal embryonic stem cell fates. Development 131:5277–5286.

Li P, Tong C, Mehrian-Shai R, Jia L, Wu N, Yan Y, Maxson RE, Schulze EN, Song H, Hsieh CL, Pera MF, Ying QL. 2008. Germline competent embryonic stem cells derived from rat blastocysts. Cell 135:1299–1310.

Linker C, Stern CD. 2004. Neural induction requires BMP inhibition only as a late step, and involves signals other than FGF and Wnt antagonists. Development 131:5671–5681.

Liu J, He X, Corbett SA, Lowry SF, Graham AM, Fassler R, Li S. 2009. Integrins are required for the differentiation of visceral endoderm. J Cell Sci 122:233–242.

Livigni A, Villegas SN, Rahman A, Oikonomopoulou I, Morrison GM, Brickman JM. 2009. Differentiation of Embryonic Stem Cells into Anterior Definitive Endoderm. Curr Protoc Stem Cell Biol Chapter 1:Unit 1G.3.

Lunn JS, Fishwick KJ, Halley PA, Storey KG. 2007. A spatial and temporal map of FGF/Erk1/2 activity and response repertoires in the early chick embryo. Dev Biol 302:536–552.

Mathieu J, Griffin K, Herbomel P, Dickmeis T, Strahle U, Kimelman D, Rosa FM, Peyrieras N. 2004. Nodal and FGF pathways interact through a positive regulatory loop and synergize to maintain mesodermal cell populations. Development 131:629–641.

McLean AB, D'Amour KA, Jones KL, Krishnamoorthy M, Kulik MJ, Reynolds DM, Sheppard AM, Liu H, Xu Y, Baetge EE, Dalton S. 2007. Activin a efficiently specifies definitive endoderm from human embryonic stem cells only when phosphatidylinositol 3-kinase signaling is suppressed. Stem Cells 25:29–38.

Miyoshi K, Wakioka T, Nishinakamura H, Kamio M, Yang L, Inoue M, Hasegawa M, Yonemitsu Y, Komiya S, Yoshimura A. 2004. The

Sprouty-related protein, Spred, inhibits cell motility, metastasis, and Rho-mediated actin reorganization. Oncogene 23:5567–5576.

Morrison GM, Oikonomopoulou I, Migueles RP, Soneji S, Livigni A, Enver T, Brickman JM. 2008. Anterior definitive endoderm from ESCs reveals a role for FGF signaling. Cell Stem Cell 3:402–415.

Nishioka N, Yamamoto S, Kiyonari H, Sato H, Sawada A, Ota M, Nakao K, Sasaki H. 2008. Tead4 is required for specification of trophectoderm in pre-implantation mouse embryos. Mech Dev 125:270–283.

Ornitz DM, Itoh N. 2001. Fibroblast growth factors. Genome Biol 2: REVIEWS3005.

Paling NR, Wheadon H, Bone HK, Welham MJ. 2004. Regulation of embryonic stem cell self-renewal by phosphoinositide 3-kinase-dependent signaling. J Biol Chem 279:48063–48070.

Pierre M, Yoshimoto M, Huang L, Richardson M, Yoder MC. 2009. VEGF and IHH rescue definitive hematopoiesis in Gata-4 and Gata-6-deficient murine embryoid bodies. Exp Hematol 37:1038–1053.

Plusa B, Piliszek A, Frankenberg S, Artus J, Hadjantonakis AK. 2008. Distinct sequential cell behaviours direct primitive endoderm formation in the mouse blastocyst. Development 135:3081–3091.

Polanska UM, Fernig DG, Kinnunen T. 2009. Extracellular interactome of the FGF receptor-ligand system: Complexities and the relative simplicity of the worm. Dev Dyn 238:277–293.

Robertson EJ. 1987. Teratocarcinomas and embryonic stem cells: A practical approach. Oxford; Washington, D.C.: IRL Press. p xii 254.

Rodaway A, Patient R. 2001. Mesendoderm. an ancient germ layer? Cell 105:169–172.

Rodaway A, Takeda H, Koshida S, Broadbent J, Price B, Smith JC, Patient R, Holder N. 1999. Induction of the mesendoderm in the zebrafish germ ring by yolk cell-derived TGF-beta family signals and discrimination of mesoderm and endoderm by FGF. Development 126: 3067–3078.

Schmerer M, Torregroza I, Pascal A, Umbhauer M, Evans T. 2006. STAT5 acts as a repressor to regulate early embryonic erythropoiesis. Blood 108:2989–2997.

Shiraki N, Yoshida T, Araki K, Umezawa A, Higuchi Y, Goto H, Kume K, Kume S. 2008. Guided differentiation of embryonic stem cells into Pdx1-expressing regional-specific definitive endoderm. Stem Cells 26:874–885.

Silva J, Smith A. 2008. Capturing pluripotency. Cell 132:532–536.

Silva J, Barrandon O, Nichols J, Kawaguchi J, Theunissen TW, Smith A. 2008. Promotion of reprogramming to ground state pluripotency by signal inhibition. PLoS Biol 6:e253.

Sivak JM, Petersen LF, Amaya E. 2005. FGF signal interpretation is directed by Sprouty and Spred proteins during mesoderm formation. Dev Cell 8:689–701.

Smith AG. 2001. Embryo-derived stem cells: Of mice and men. Annu Rev Cell Dev Biol 17:435–462.

Spemann H, Mangold H. 1924. Über induktion von embryonalanlagen durch implantation artfremder organisatoren. Roux Arch Entwick Mech Organ 100:599–683.

Stavridis MP, Lunn JS, Collins BJ, Storey KG. 2007. A discrete period of FGF-induced Erk1/2 signalling is required for vertebrate neural specification. Development 134:2889–2894.

Stern CD. 2006. Evolution of the mechanisms that establish the embryonic axes. Curr Opin Genet Dev 16:413–418.

Streit A, Stern CD. 1999. Neural induction. A bird's eye view. Trends Genet 15:20–24.

Streit A, Berliner AJ, Papanayotou C, Sirulnik A, Stern CD. 2000. Initiation of neural induction by FGF signalling before gastrulation. Nature 406: 74–78.

Sumi T, Tsuneyoshi N, Nakatsuji N, Suemori H. 2008. Defining early lineage specification of human embryonic stem cells by the orchestrated balance of canonical Wnt/beta-catenin, Activin/Nodal and BMP signaling. Development 135:2969–2979.

Sun BI, Bush SM, Collins-Racie LA, LaVallie ER, DiBlasio-Smith EA, Wolfman NM, McCoy JM, Sive HL. 1999. Derriere: A TGF-beta family member required for posterior development in Xenopus. Development 126:1467–1482.

Tada S, Era T, Furusawa C, Sakurai H, Nishikawa S, Kinoshita M, Nakao K, Chiba T, Nishikawa S. 2005. Characterization of mesendoderm: A diverging point of the definitive endoderm and mesoderm in embryonic stem cell differentiation culture. Development 132:4363–4374.

Takahashi K, Mitsui K, Yamanaka S. 2003. Role of ERas in promoting tumour-like properties in mouse embryonic stem cells. Nature 423:541–545.

Tam PP, Loebel DA, Tanaka SS. 2006. Building the mouse gastrula: Signals, asymmetry and lineages. Curr Opin Genet Dev 16:419–425.

Tesar PJ, Chenoweth JG, Brook FA, Davies TJ, Evans EP, Mack DL, Gardner RL, McKay RD. 2007. New cell lines from mouse epiblast share defining features with human embryonic stem cells. Nature 448:196–199.

Tsang M, Friesel R, Kudoh T, Dawid IB. 2002. Identification of Sef, a novel modulator of FGF signalling. Nat Cell Biol 4:165–169.

Tzouanacou E, Wegener A, Wymeersch FJ, Wilson V, Nicolas JF. 2009. Redefining the progression of lineage segregations during mammalian embryogenesis by clonal analysis. Dev Cell 17:365–376.

Verheijen MH, Wolthuis RM, Bos JL, Defize LH. 1999a. The Ras/Erk pathway induces primitive endoderm but prevents parietal endoderm differentiation of F9 embryonal carcinoma cells. J Biol Chem 274:1487–1494.

Verheijen MH, Wolthuis RM, Defize LH, den Hertog J, Bos JL. 1999b. Interdependent action of RalGEF and Erk in Ras-induced primitive endoderm differentiation of F9 embryonal carcinoma cells. Oncogene 18:4435–4439.

Wakioka T, Sasaki A, Kato R, Shouda T, Matsumoto A, Miyoshi K, Tsuneoka M, Komiya S, Baron R, Yoshimura A. 2001. Spred is a Sprouty-related suppressor of Ras signalling. Nature 412:647–651.

Watanabe S, Umehara H, Murayama K, Okabe M, Kimura T, Nakano T. 2006. Activation of Akt signaling is sufficient to maintain pluripotency in mouse and primate embryonic stem cells. Oncogene 25:2697–2707.

Wiedemann M, Trueb B. 2000. Characterization of a novel protein (FGFRL1) from human cartilage related to FGF receptors. Genomics 69:275–279.

Wills AE, Choi VM, Bennett MJ, Khokha MK, Harland RM. 2010. BMP antagonists and FGF signaling contribute to different domains of the neural plate in Xenopus. Dev Biol 337:335–350.

Wilson PA, Hemmati-Brivanlou A. 1995. Induction of epidermis and inhibition of neural fate by Bmp-4. Nature 376:331–333.

Yagi R, Kohn MJ, Karavanova I, Kaneko KJ, Vullhorst D, DePamphilis ML, Buonanno A. 2007. Transcription factor TEAD4 specifies the trophectoderm lineage at the beginning of mammalian development. Development 134: 3827–3836.

Yamaguchi-Iwai Y, Satake M, Murakami Y, Sakai M, Muramatsu M, Ito Y. 1990. Differentiation of F9 embryonal carcinoma cells induced by the c-jun and activated c-Ha-ras oncogenes. Proc Natl Acad Sci USA 87:8670–8674.

Yasunaga M, Tada S, Torikai-Nishikawa S, Nakano Y, Okada M, Jakt LM, Nishikawa S, Chiba T, Era T, Nishikawa S. 2005. Induction and monitoring of definitive and visceral endoderm differentiation of mouse ES cells. Nat Biotechnol 23:1542–1550.

Ying QL, Nichols J, Chambers I, Smith A. 2003a. BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. Cell 115:281–292.

Ying QL, Stavridis M, Griffiths D, Li M, Smith A. 2003b. Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture. Nat Biotechnol 21:183–186.

Ying QL, Wray J, Nichols J, Batlle-Morera L, Doble B, Woodgett J, Cohen P, Smith A. 2008. The ground state of embryonic stem cell self-renewal. Nature 453:519–523.

Yoshida-Koide U, Matsuda T, Saikawa K, Nakanuma Y, Yokota T, Asashima M, Koide H. 2004. Involvement of Ras in extraembryonic endoderm differentiation of embryonic stem cells. Biochem Biophys Res Commun 313:475–481.

Zhang X, Ibrahimi OA, Olsen SK, Umemori H, Mohammadi M, Ornitz DM. 2006. Receptor specificity of the fibroblast growth factor family. The complete mammalian FGF family. J Biol Chem 281:15694–15700.

Zhang P, Li J, Tan Z, Wang C, Liu T, Chen L, Yong J, Jiang W, Sun X, Du L, Ding M, Deng H. 2008. Short-term BMP-4 treatment initiates mesoderm induction in human embryonic stem cells. Blood 111:1933–1941.