Stem Cell Research (2011) 6, 262–275

available at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/scr

REGULAR ARTICLE

Activation of FGFR(IIIc) isoforms promotes activin-induced mesendoderm development in mouse embryonic stem cells and reduces Sox17 coexpression in EpCAM$^+$ cells

Janny M.L. Peterslund, Palle Serup*

Department of Stem Cell Biology, Hagedorn Research Institute, Niels Steensens Vej 6, DK-2820 Gentofte, Denmark

Received 5 November 2010; received in revised form 17 February 2011; accepted 18 February 2011
Available online 27 February 2011

Abstract Activin induces the formation of definitive endoderm from mouse ES cells dependent on active fibroblast growth factor (Fgf) signaling. Here we report that *Fgf4* is dispensable for activin A-induced differentiation of mouse ES cells into endoderm. We find that *Fgf4*$^{-/-}$ cells readily differentiate into definitive endoderm without exogenous administration of Fgf4. Additionally, we investigate the spatio-temporal dynamics of Fgf receptor (FGFR) isoform distribution in activin A-treated ES cell cultures and find that FGFR(III)c isoforms are expressed in DE as well as non-DE populations, whereas FGFR2(III)b and FGFR4 are found specifically enriched in the DE fraction. Ligands that preferentially activate the FGFR(III)c isoforms induce mesendoderm markers *T* and *Gsc*, but reduce expression of the DE marker *Sox17* in activin-induced EpCAM$^+$ cells. In contrast, ligands specifically activating FGFR(III)b isoforms have no effect on either population. Activation of FGFR(III)c isoforms results in a strong mitogenic effect on activin A-induced ES cell progeny early in the differentiation period whereas activation of FGFR(III)b isoforms has only a moderate mitogenic effect confined to the late differentiation period. We conclude that FGFR(III)c-isoform activation selectively drives the differentiation of mES cells toward mesendoderm and that Fgf4 is dispensable for the differentiation into definitive endoderm.

Introduction

Knowledge obtained from developmental biology can be used to direct differentiation of mouse embryonic stem cells (mESC) toward specific germ layers and more mature tissues. Recently, differentiation into glucose-responsive beta cell-like insulin-secreting cells has received much attention since such cells could serve as a cure for type I diabetes mellitus if implanted into patients (McCall et al., 2010). For this purpose, the first step is to generate definitive endoderm (DE) with the potential to further differentiate into cells resembling the primitive gut tube (reviewed by (Van Hoof et al., 2009)). Understanding the role of each component used in this directed differentiation is crucial for obtaining the optimal progenitor cell population in each step.

In the late blastocyst stage of the developing mouse embryo (E4.5), the inner cell mass (ICM) is divided into the epiblast and the primitive, later visceral, endoderm. The

* Corresponding author at: Hagedorn Research Institute, Niels Steensens Vej 6, DK-2820 Gentofte, Denmark. Fax: +45 44438000.
E-mail address: pas@hagedorn.dk (P. Serup).

1873-5061/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.scr.2011.02.003

visceral endoderm (VE) is involved in anterior–posterior patterning of the epiblast, restricting the domain where primitive streak (PS) formation can initiate at the posterior end (reviewed by (Rossant, 2004)). In the gastrulating mouse embryo, epiblast cells migrate through the PS, and in this process become determined toward either mesoderm or DE (Tam et al., 1993; Lawson et al., 1991; Carey et al., 1995). The transforming growth factor-β family member nodal, an activator of Smad2/3 signaling, is the main initiator of epiblast patterning and PS formation (Waldrip et al., 1998; Conlon et al., 1994). At high levels, nodal induces anterior PS structures and DE and at low doses it induces more posterior streak fates (Ben-Haim et al., 2006). Close to the posterior-most end of the PS, bone morphogenetic protein 4 (BMP4) is produced by the extraembryonic ectoderm and establishes a signal gradient. BMP4 is critical for formation of the PS and induces mesoderm formation (reviewed by (Gadue et al., 2005)). It is believed that meso- and endodermal cells arise from a common progenitor cell population, the mesendoderm (Lawson et al., 1991; Kinder et al., 2001). *Brachyury* (*T*) is expressed in the PS as well as in nascent mesoderm of the mouse embryo (Kispert and Herrmann, 1994). *Goosecoid* (*Gsc*) is expressed in the anterior streak, from which the DE arises (Blum et al., 1992). *Gsc* is induced by high concentrations of activin A (activin hereafter) in animal cap explants from Xenopus and in mES cells *Gsc* expression is used as a marker of an anterior mesendoderm-like population (Gadue et al., 2006; Kubo et al., 2004). Sry-*related HMG box gene 17* (*Sox17*) is an early marker expressed in the definitive endoderm of the gastrula, and later expands to the endoderm underlying the neural plate of the early-bud-stage embryo (Kanai-Azuma et al., 2002). *Sox17* is also expressed in the extraembryonic visceral endoderm.

In mESC cultures, cells take on a mesendodermal fate before being committed to either mesoderm or DE (Tada et al., 2005). Activin is used as a surrogate for nodal as they both activate Smad2/3 signaling via activation of the Alk4 receptor (Schier, 2003). In the mesendoderm population, high concentrations of nodal/activin-signaling induce anterior streak and DE cells while BMP4 or low concentrations of nodal/activin induce posterior streak and mesoderm (Kubo et al., 2004; Hansson et al., 2009; Willems and Leyns, 2008). The PS genes *T*, *Mix-like 1* (*Mixl1*), and *Gsc* are expressed in this population in response to increasing concentrations of activin. High activin levels further induce the DE markers *Sox17*, *Cadherin1* (*Cdh1* (E-cadherin)), and *Forkhead box a2* (*Foxa2*). In contrast, BMP4 induces *T*, *Mixl1*, and the mesodermal marker *Fetal like kinase 1 (Flk1*; *VEGFR2*/*Kdr*; (Gadue et al., 2005)). During mESC differentiation, *T*-expressing cells give rise to both meso- and endodermal derivatives (Kubo et al., 2004) and we have previously shown that a T-GFP reporter cell line ($T^{Gfp/+}$; (Fehling et al., 2003)) is preferentially activated by BMP4 or by a low concentration of activin (Hansson et al., 2009).

Fibroblast growth factor (Fgf) signaling is required for the differentiation of mesendoderm and DE to occur properly in mES cells (Hansson et al., 2009; Willems and Leyns, 2008; Funa et al., 2008; Morrison et al., 2008). The Fgf family of proteins consists of 22 members named Fgf1–23 (Fgf15 is the mouse ortholog of human Fgf19). They activate one or more of four receptor tyrosine kinases, the Fgf receptors (FGFR)1–4. FGFR1–3 have two splice variants in their Ig-like domain III, the FGFR(III)b or FGFR(III)c isoforms (FGFRb or FGFRc hereafter; (Itoh and Ornitz, 2004; Ornitz and Itoh, 2001)). Fgfs are involved in many functions in the developing embryo such as germ layer formation, cell proliferation, and cell migration (Ornitz and Itoh, 2001). In early mouse development, Fgf signaling is necessary for the migration of epiblast cells through the PS (Ciruna et al., 1997; Guo and Li, 2007). The loss of Fgf4 is lethal at Embryonic Days (E)4–5, due to the inability of epiblast cells to undergo epithelial-to-mesenchymal transition and migrate through the PS (Feldman et al., 1995). $FGFR1^{-/-}$ mice also die at gastrulation and both Fgf4 and FGFR1 are expressed in the ICM and PS (Yamaguchi et al., 1994; Deng et al., 1994). Fgf4 is expressed in pluripotent mES cells and has been shown to be necessary for differentiation into ectoderm and mesoderm lineages, suggesting a crucial role of Fgf4 in the initiation of differentiation (Kunath et al., 2007). Nevertheless, previous work has shown that $Fgf4^{-/-}$ cells can differentiate *in vitro*, albeit at a low frequency, and give rise to tumors consisting of a wide range of differentiated cell types *in vivo* (Wilder et al., 1997).

Here we show that ES cells deficient for Fgf4, a potential FGFRc isoform-activating ligand, were able to differentiate to definitive endoderm cells at levels comparable to wt and $Fgf4^{+/-}$ cells. Furthermore, we extend our previous finding that Fgf signaling is necessary for DE formation (Hansson et al., 2009), and investigate the effects of activating different FGFR isoforms on mesendoderm and DE differentiation. We demonstrate that FGFRc isoforms are up-regulated in both $Sox17^{+}$ and $Sox17^{-}$ populations emerging after activin treatment. By means of reporter cell lines and immunocytochemistry we find that Fgfs which preferentially activate FGFRc isoforms augment the expression of PS and mesendoderm markers *T* and *Gsc* and selectively expand an $EpCAM^{+}Sox17^{-}$ population. In contrast, Fgfs activating FGFRb isoforms have no effect on the expression of these markers nor on the expansion of $EpCAM^{+}$ cells. Fgfs activating the FGFRc isoforms show the highest mitogenic effects early in the differentiation period, while proliferation rates are reduced later in the culture period as expected from increased contact inhibition at higher cell densities. We conclude that FGFRc isoforms promote mesendoderm but not DE formation and that Fgf4 signaling is dispensable for induction of DE in mES cells.

Results

Fgf4 is dispensable for the formation of endoderm from mES cells

Fgf4 is important during gastrulation where it is responsible for the cell's movement through the PS (Bottcher and Niehrs, 2005), and was recently reported to be required for mESC to leave the pluripotent stage (Kunath et al., 2007). However, our previous work demonstrated that Fgf signaling is required only at a late stage (Days 4–5) during activin-induced DE formation (Hansson et al., 2009). We therefore investigated whether Fgf4 is required for activin-induced differentiation of mESCs into the endoderm lineage. Pluripotent $Fgf4^{-/-}$ mESCs showed different cell morphology than pluripotent E14 and $Fgf4^{+/-}$ cell lines. $Fgf4^{-/-}$ cells grew in small, very dense clusters indicative of pluripotent cells (Fig. 1A) and growth rates were slower than for $Fgf4^{+/-}$ cells, confirming

the mitogenic effect of Fgf4 on mESC. Undifferentiated *Fgf4*$^{-/-}$ cells stained positive for the pluripotency marker Oct4 and negative for the endoderm marker Sox17, similar to the wt and heterozygous cell lines. qPCR analyses revealed 2- to 3-fold higher expression levels of *Nanog* and *Oct4* compared to wild-type cells in the pluripotent state and after 5 days of differentiation in activin-supplemented medium, expression levels of *Nanog* and *Oct4* in the *Fgf4*$^{+/-}$ cell line and *Fgf4*$^{-/-}$ cell line supplemented with Fgf4 were reduced as seen in wt cells. In the *Fgf4*$^{-/-}$ cell line, *Nanog* levels were reduced by approx 50% compared to undifferentiated cells, but *Oct4* levels remained almost as high as in the undifferentiated cells (Fig. 1B), consistent with the notion that Fgf4 is required for the cells to leave the pluripotent state. To rigorously test if Fgf4 was required for endoderm differentiation we treated E14, *Fgf4*$^{+/-}$, and *Fgf4*$^{-/-}$ mESC with 30 ng/ml activin and examined expression of the endoderm markers Sox17, Foxa2, and Cdh1 (Kaestner

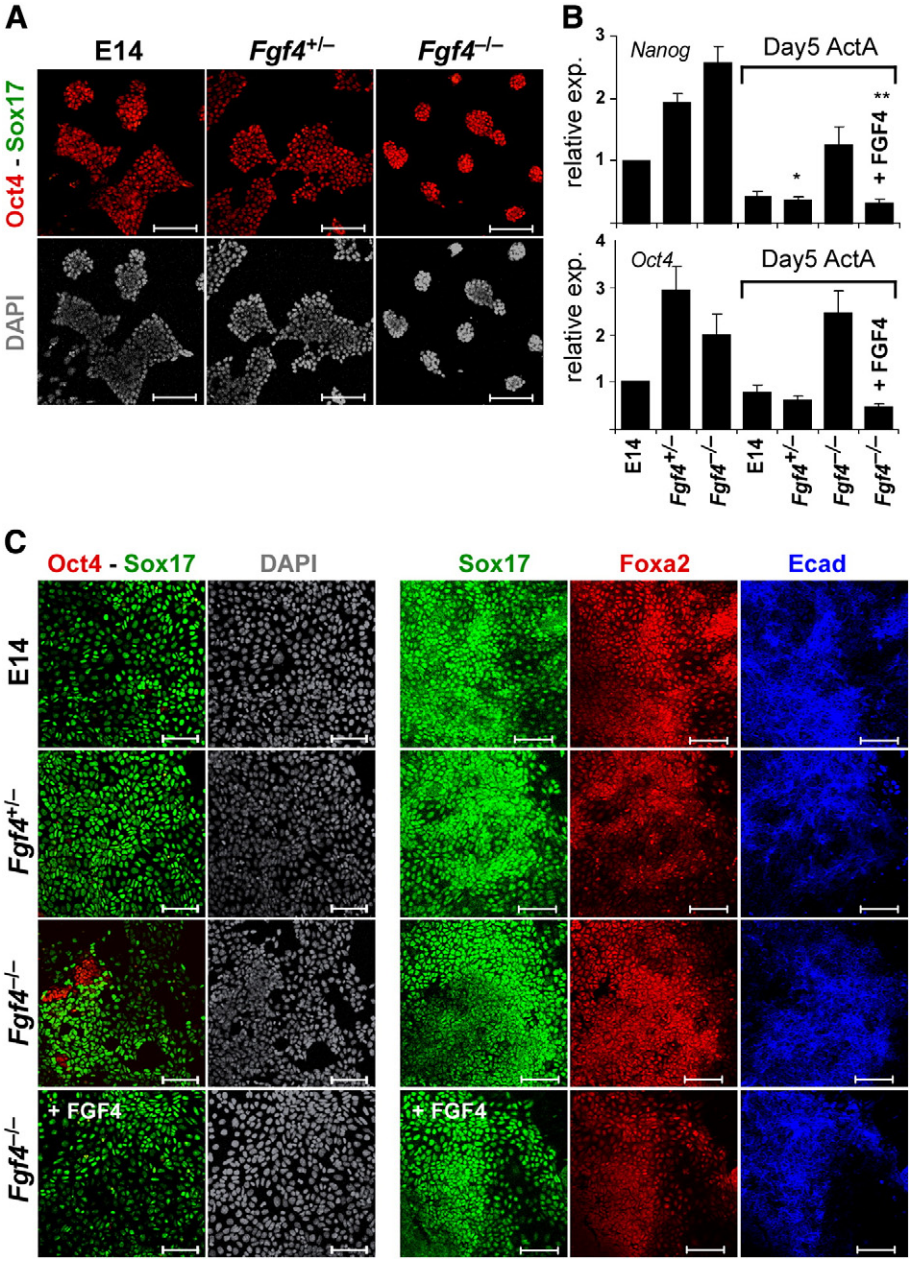


Figure 1 Fgf4 is dispensable for endoderm differentiation. E14, Fgf4$^{+/-}$, and Fgf4$^{-/-}$ cells analyzed for markers of pluripotency and endoderm by immune staining and qPCR before onset of differentiation and on Day 5 of the DE protocol. (A) Undifferentiated cells were analyzed by immune cytochemistry (ICC) for Oct4 and Sox17 and the nuclear stain DAPI. Scale bars: 100 μm. (B) qPCR for pluripotency markers Nanog and Oct4 in undifferentiated cells and at Day 5. The relative expression to E14 ESC conditions is shown, and samples were standardized to the housekeeping gene Tbp. The mean expression ± SEM of 3–4 independent experiments is shown, using a Student's *t* test for the statistical analysis: *$P < 0.05$; **$P < 0.01$ compared with the undifferentiated condition of each cell line. (C) Cells were stained for Oct4 and endoderm markers Sox17, Foxa2, and E-cadherin (Ecad), and DAPI at Day 5 of differentiation. Representative images are shown for each condition. Scale bars: 100 μm.

et al., 1994; Weinstein and Ruiz Test, 1994). *Fgf4*$^{+/-}$ cells behaved much like E14 wt cells, showing high numbers of Oct4^{-}Sox17^{+} and Sox17^{+}Foxa2^{+}Cdh1^{+} cells by Day 5 (Fig. 1C). Remarkably, *Fgf4*$^{-/-}$ cells treated with activin readily differentiated along the endoderm lineage, showing mainly Sox17^{+}Oct4^{-}cells with a few Sox17^{+}Oct4^{+} cells not seen in the wild-type and *Fgf4* heterozygous cell lines (Fig. 1C), but we could confirm the lack of neuroectodermal differentiation in *Fgf4*$^{-/-}$ cells (Fig. S1). Treatment of *Fgf4*$^{-/-}$ cells with Fgf4 restored neuroectodermal differentiation and reduced the number of Oct4^{+} cells to wild-type levels (Fig. S1 and data not shown). There were comparable numbers of Sox17^{+}Foxa2^{+}Cdh1^{+} cells in the *Fgf4*$^{-/-}$ cells and wild-type or heterozygous cell lines, and these did not change by the addition of Fgf4 to the medium (Fig. 1C). We conclude that Fgf4 is dispensable for activin-induced differentiation of mES cells along the endoderm lineage and for mESC to leave the pluripotent state.

Definitive endoderm is formed in the absence of Fgf4

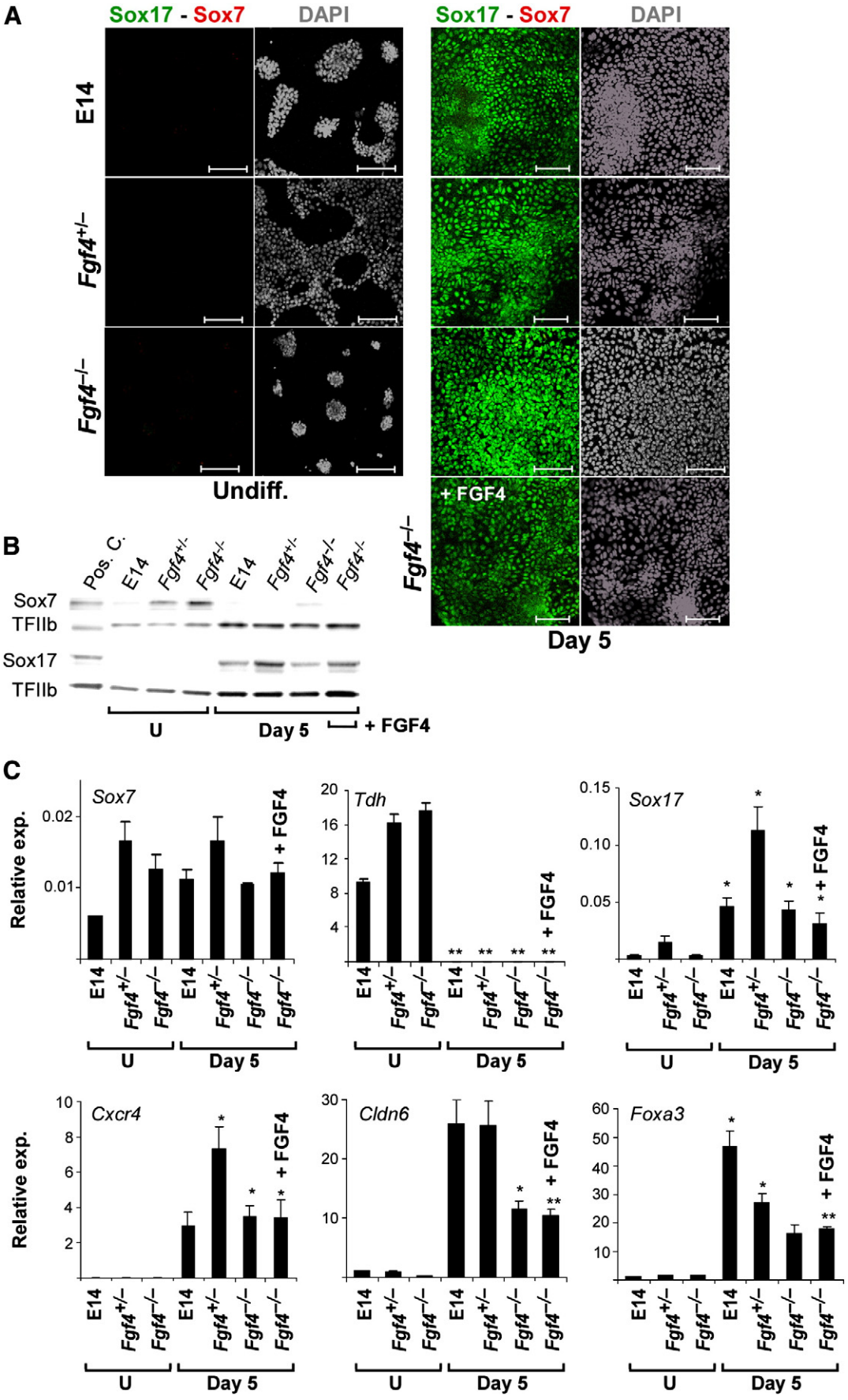
To determine if the endoderm formed was of a definitive or visceral type we analyzed for markers of visceral endoderm: *Sox7* and *Thermostable direct hemolysin gene* (*Tdh*) (Sherwood et al., 2007), and DE: *Sox17*, *CXC chemokine receptor 4 (Cxcr4)*, *Claudin-6 (Cldn6)* and *Forkhead box a3 (Foxa3)* (Sherwood et al., 2007; Monaghan et al., 1993; Gadue et al., 2009). Immune staining of undifferentiated cells showed no expression of Sox17 or Sox7 at this stage (Fig. 2A), but qPCR and Western blot analyses revealed some *Sox7* mRNA and Sox7 protein along with a very low expression of *Sox17* mRNA and no Sox17 protein (Figs. 2C–E). After 5 days of activin-induced differentiation the Sox17^{+} cells that developed from E14 cells as well as *Fgf4*$^{-/-}$ and *Fgf4*$^{+/-}$ cell lines were Sox7^{-}, indicative of DE formation (Fig. 2B). We further tested for expression of DE markers in all three cell lines by Western and qPCR analyses and confirmed expression of Sox17 and absence of Sox7 (Fig. 2C). At the mRNA level, *Sox17*, *Cldn6*, *Foxa3*, and *Cxcr4* transcription were induced after activin treatment (Fig. 2C) indicative of the formation of DE rather than VE. The VE marker protein Sox7 was only present in the *Fgf4*$^{-/-}$ cell line at Day 5, and to a much reduced level compared to the undifferentiated state (Fig. 2C). Notably, Sox17 was induced at Day 5 in *Fgf4*$^{-/-}$ cells to levels comparable to those seen in E14 wild-type cells, *Fgf4*$^{+/-}$ cells and *Fgf4*$^{-/-}$ cells supplemented with exogenous Fgf4. *Sox7* mRNA showed similar levels of expression in the pluripotent and differentiated states for all three cell lines (Fig. 2C) and the absolute amount of mRNA was very low, i.e., similar to *Sox17*-expression levels in undifferentiated mES cells. *Tdh* was expressed at intermediary levels in mES cells but was down-regulated on DE induction (Fig. 2C). In summary, we conclude that Fgf4 signaling is dispensable for induction of DE in *Fgf4*$^{-/-}$ mES cells.

Expression of Fgf receptor isotypes during definitive endoderm formation

Although Fgf4 does not appear to be required for activin-induced DE formation, we and others have shown that Fgf signaling is necessary for the differentiation of mouse mESC cultures to definitive endoderm (Hansson et al., 2009; Willems and Leyns, 2008; Funa et al., 2008; Morrison et al., 2008). To further understand the dependence on Fgf signaling during DE formation, we next examined the expression of FGFR isoforms during a 5-day differentiation period by quantitative RT-PCR (qPCR). We sorted activin-treated Sox17$^{Gfp/+}$ cells (Kim et al., 2007) into Sox17-GFP^{Hi} and Sox17-GFP^{Lo} fractions, in order to isolate RNA from the forming DE and the non-DE populations of cells, respectively (Fig. 3A). In general, FGFR1c was expressed at high levels, FGFR2b and 2c at intermediate levels, and FGFR1b, 3c, and 4 at low levels. During the 5-day differentiation period, FGFR2b and 4 were expressed in the unsorted, Sox17-GFP^{Lo}, and Sox17-GFP^{Hi} fractions but their expression was not significantly different from undifferentiated ES cells (Fig. 3B). FGFR1b was down-regulated in both the unsorted and Sox17-GFP^{Lo} fractions on initiation of differentiation, although not significantly different from the undifferentiated culture. These data are consistent with findings in the mouse embryo, showing that FGFR2b and 4 are expressed in endodermal epithelia such as the definitive endoderm (Orr-Urtreger et al., 1993; Elghazi et al., 2002; Stark et al., 1991). FGFR1c was up-regulated in the Sox17-GFP^{Lo} fraction alone, peaking on Day 5, whereas FGFR2c and 3c were up-regulated in both Sox17-GFP^{Lo} and Sox17-GFP^{Hi} fractions, on activin-induced differentiation (Fig. 3B). In summary, FGFRc isoforms are highly up-regulated throughout the cell culture or in the Sox17-GFP^{Lo} fraction alone, suggesting a role for FGFRc isoform activation as a modulator of mesendoderm and/or DE formation in mESCs.

FGFR isoform-specific ligands differentially activate PS and DE markers

Since different Fgfs bind to and activate specific FGFR isoforms, we speculated that addition of b or c isoform-specific Fgfs might have different effects on expression of PS and DE markers in our mESC cultures. We chose to focus on Fgfs that are described to have a function during gastrulation and in the development of the DE. Based on which FGFR they activate (Bottcher and Niehrs, 2005; Ornitz et al., 1996; Zhang et al., 2006) they were divided into three categories: Fgf1, 2, and 9 activate a mixed population of both FGFRb and FGFRc isoforms, with a preference for the latter; Fgf7 and 10 activate FGFRb isoforms only; and Fgf4, 5, 6, 8b, 8c, 8e, and 16 activate one or more FGFRc isoforms and/or FGFR4 (Ornitz et al., 1996; Zhang et al., 2006; MacArthur et al., 1995; Olsen et al., 2006; Mason, 2007). FGFR4 is grouped with the FGFRc type of receptors, as it structurally resembles this group of FGFRs (Vainikka et al., 1992) and most Fgfs-activating FGFRc isoforms also activate FGFR4 (Mason, 2007), making it also functionally FGFRc isoform-like. To evaluate the effect of the different Fgfs in mesendodermal differentiation, we monitored the expression of PS and DE markers by means of reporter cell lines on Days 3 and 5. Accordingly, we treated T$^{Gfp/+}$ cells with 10 ng/ml BMP4 and added different Fgfs to evaluate their effect on


A
Sox17 - Sox7
DAPI
E14
Fgf4+/–
Fgf4–/–
Undiff.
+ FGF4
Day 5
B
Pos. C.
E14
Fgf4+/-
Fgf4-/-
Sox7
TFIIb
Sox17
TFIIb
U
Day 5
+ FGF4
C
Sox7
Tdh
Sox17
Cxcr4
Cldn6
Foxa3
Relative exp.
U
Day 5
+ FGF4

Brachyury expression. Fgf1, 2, 4, 6, and 9, binding a mixed population of FGFRs or FGFRc isoforms only, increased the number of T-GFP$^+$ cells on Day 3 by up to 20% compared to BMP4 treatment alone, i.e., 78–83±3–4 and 69±6%, respectively (mean %±SD, n=3; Fig. 4A). Fgf7 and 10 had no significant effect on T-GFP induction, nor did Fgf8b, 8c, 8e, or 16, but Fgf5 slightly repressed T-induction (Fig. 4A). Looking at the same marker after treatment with 1 ng/ml activin, we saw that Fgf4 and 6 resulted in a 31–42% increase in the number of T-GFP$^+$ cells on Day 3 (Fig. 4B), while Fgf5, 9, and 10 resulted in smaller increases. Fgf1, 2, 4, 6, and 9 induced increased numbers of T-GFP$^+$ cells by up to 34% on Day 5. Fgf7, 8b, 8c, 8e, and 16 showed no effect on the numbers of T-GFP$^+$ cells on either Day 3 or Day 5. Thus, the largest effect was seen when adding Fgfs-binding FGFRc isoforms, which mediated an increase in the measured number of T-GFP$^+$ cells in general and on Day 5 in particular. This Fgf-induced increase in Day 5 T-GFP$^+$ cells may be caused by maintenance of *T* expression from Day 3 to 5, rather than by inducing more cells to express *T*. We next looked at the effect of Fgf treatment on anterior streak/DE induction by 30 ng/ml activin in Gsc$^{Gfp/+}$ mESC (Tada et al., 2005). Addition of Fgf1, 2, 4, 6, 8b, and 9 increased the number of Gsc-GFP$^+$ cells by 22–40% (Fig. 4C). Activation of FGFRb isoforms only, by Fgf7 and 10, had no effect, nor did Fgf5, 8c, 8e, and 16. This finding was not due to the lack of cognate receptors, as they were present in the cell population (Fig. 3B). With the DE marker *Sox17*, we observed a 50% decrease of the Sox17-GFP^{Hi} fraction (from 34±4 to 17±3%) when adding Fgfs activating FGFRc isoforms (Fig. 4D). Treatment with Fgfs that only activate FGFRb isoforms slightly increased the number of Sox17-GFP^{Hi} cells or had no effect (Fgf7 and Fgf10, respectively). In summary, Fgf ligands that predominantly activate FGFR4/FGFRc isoforms, i.e., Fgf1, 2, 4, 6, 8b, and 9, promote differentiation toward a mesendoderm cell population expressing primitive streak markers but reduce the Sox17$^+$ DE population in the culture.

FGFRc activation suppresses Sox17 expression in EpCAM$^+$ cells

The reduction in the number of Sox17-GFP$^+$ cells observed in response to activation of FGFRc isoforms could be the result of a shift in lineage allocation or a more specific inhibitory effect on Sox17 expression. To test between these notions we used triple color flow cytometry to analyze Sox17-GFP cells labeled with antibodies against the mesodermal marker Flk1 (Ema et al., 2006) and EpCAM (epithelial cell adhesion molecule), which is expressed in pluripotent mES cells, transiently in ectoderm, and sustained in the DE epithelium during embryonic development (Sherwood et al., 2007; Balzar et al., 1999). Looking at the three markers separately, we observed a decrease in Sox17-GFP^{Hi} cells in the presence of Fgf1, 2, 4, and 6 in agreement with previous data (Fig. 5A). Very few Flk1$^+$ cells were formed after activin treatment, regardless of the Fgf added, but high numbers of Flk1$^+$ cells were seen in the BMP4-treated samples as expected (Fig. 5B; (Hansson et al., 2009)). Activin strongly induced EpCAM$^+$ cells (87±2%) and addition of Fgfs had no effect on the number of these (Fig. 5C). However, the number of EpCAM$^+$ cells that expressed Sox17-GFP was reduced by ~50% after treatment with Fgf1, -2, -4, and −6 (Fig. 5D). Analysis of EpCAM and Flk1 expression in the Sox17-GFP$^+$ and -GFP$^-$ populations showed that the GFP$^+$ population was uniformly EpCAM+Flk$^-$ while the majority (~80%) of the GFP$^-$ population was EpCAM+Flk$^-$ and the remainder (~20%) was EpCAM$^-$Flk1$^-$, regardless of Fgf treatment (Fig. 5E).

FGFRc activation stimulates cell proliferation during the first 3 days of culture

Fgfs were originally discovered as having a mitogenic effect in fibroblast cells, and were later found to have diverse effects in embryonic development, including endoderm formation (Bottcher and Niehrs, 2005; Ornitz et al., 1996; Gospodarowicz and Moran, 1975). We analyzed the mitogenic effect of the Fgfs in wild-type mESCs on Days 3 and 5, and found that activin treatment alone gave a ~3-fold increase in cell numbers by Day 3 (from 2000 cells/cm^2 to 6600 cells/cm^2; Fig. 6A). All Fgfs improved cell growth to varying degrees, Fgf1, 2, 4, and 9 being the most effective (up to 20 400 cells/cm^2 or a 3-fold increase compared to the activin-treated cells at Day 3). We used 5-ethynyl-2′-deoxyuridine (EdU) incorporation (Salic and Mitchison, 2008) to quantify the proliferation of cells (see Fig. S2 for gating and controls) and found that the absolute number of EdU incorporating cells was 4- to 7-fold higher at Day 3 compared to Day 5, but treatment with Fgfs did not increase the relative number of proliferating cells at Day 3 (Fig. 6A). On Day 5, there were ~80 000 cells/cm^2 in activin-treated samples. Addition of Fgf2, which have the largest mitogenic effect in other systems (Ornitz et al., 1996; Zhang et al., 2006), increased total cell numbers ~1.6-fold to ~130 000 cells/cm^2 (Fig. 6B). Moreover, treatment with Fgf1, 4, and 6 increased the total cell number more modestly while other Fgfs did not have any discernable effect. The reduction in Sox17^{Hi}EpCAM$^+$ cells in relation to total EpCAM$^+$ cells seen after Fgf1, 2, 4, or 6 treatment (Fig. 5D) correlates well with the increase in total cell numbers and may therefore be explained by the

Figure 2 Activin induces DE in the absence of Fgf4. Analyses of DE and VE markers in E14, *Fgf4*$^{+/-}$, and *Fgf4*$^{-/-}$ cells before onset of differentiation and on Day 5 of the DE protocol. (A) Cells were stained for Sox17, Sox7, and the nuclear stain DAPI. Scale bar: 100 μm, n=3–4. Representative images are shown. (B) Western blot analysis of Sox7 and Sox17 and the housekeeping protein TFIIB. n=3, representative images are shown. (C) qPCR analyses of VE markers *Sox7* and *Tdh* and DE markers *Sox17*, *Cxcr4*, *Cldn6*, and *Foxa3*. The relative expression to E14 ESC conditions is shown, and samples were standardized to the housekeeping gene *Tbp*. The mean expression±SEM of 3–4 independent experiments is shown, using a Student's t test for the statistical analysis: *P<0.05; **P<0.01 compared to the undifferentiated condition of each cell line. U: undifferentiated cells.

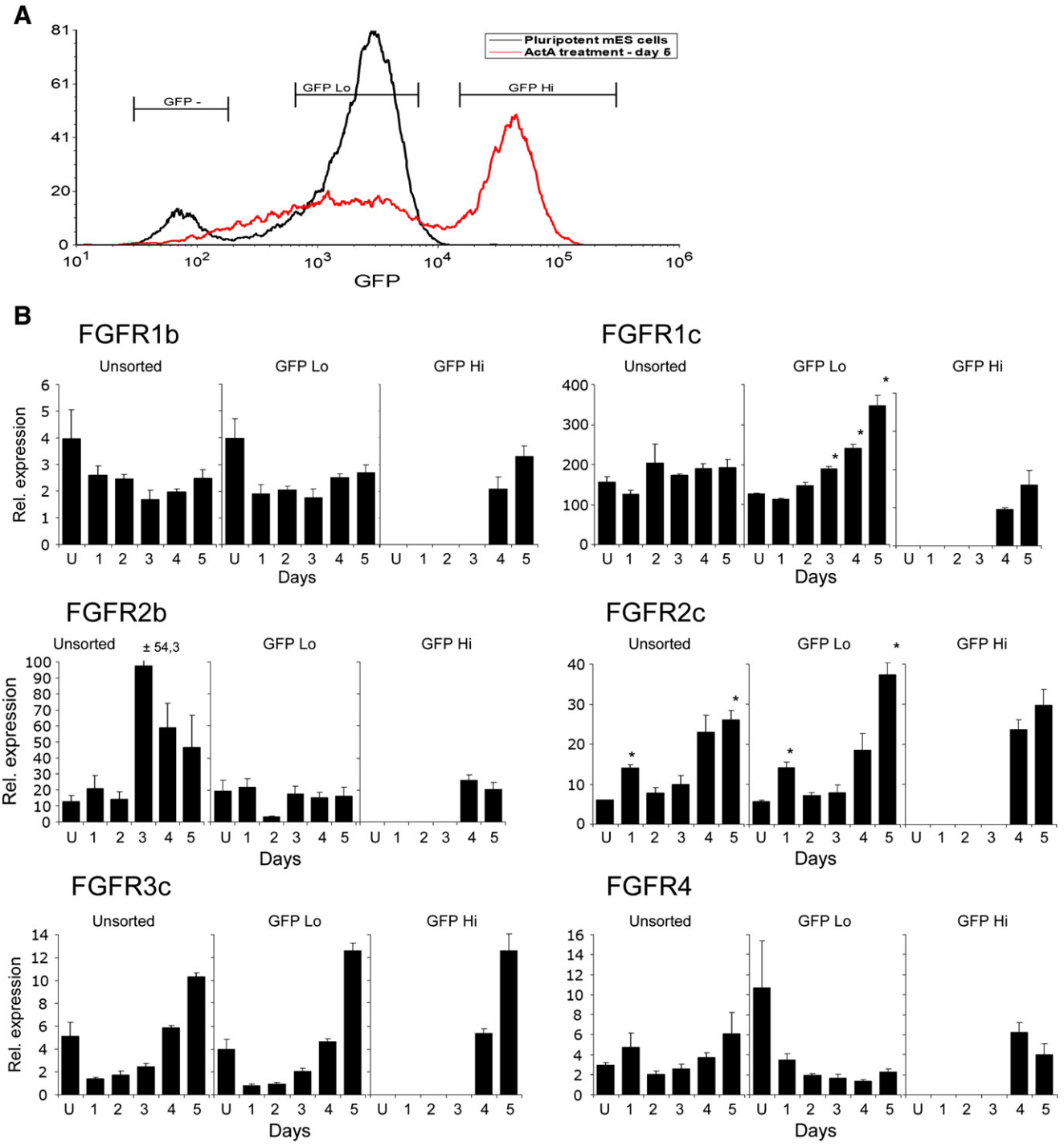


Figure 3 Expression of FGFR isoforms during DE differentiation. The expression of each FGFR isoform was analyzed by qPCR in both sorted and unsorted fractions of Sox17-GFP cells, differentiated in the presence of 30 ng/ml activin for 5 days. (A) Histogram showing sorting gates in GFP^{-}, GFP^{Lo}, and GFP^{Hi} fractions. (B) The relative expression of each FGFR isoform standardized to the housekeeping gene *Tbp*. $Sox17^{Hi}$ fractions are shown only at Days 4 and 5, when they appeared in the culture. The number of Sox17-GFP^{-} cells was too low for RNA extraction. The relative expression (mean ± SEM) of 3 independent experiments is shown, using a Student's paired, two-tailed *t* test for the statistical analysis: $^{*}P<0.05$ compared to the ESC conditions for each fraction (Sox17-GFP^{Hi} fractions were compared to the unsorted ESC sample). U: undifferentiated cells.

selective expansion of $Sox17^{-}EpCAM^{+}$ cells. EdU incorporation at Day 5 revealed a ~67% higher proliferation rate in the vehicle-treated controls compared to cells treated with activin (Fig. 6B). Treatment with Fgf1, -2, -4, and −6 reduced the number of EdU^{+} cells by 40–50% while treatment with Fgf8b, 9, and 10 resulted in more modest reductions. These data indicate that the main effect seen by Fgf1, 2, 4, 6, and 9 on proliferation occurs prior to Day 3, and that most

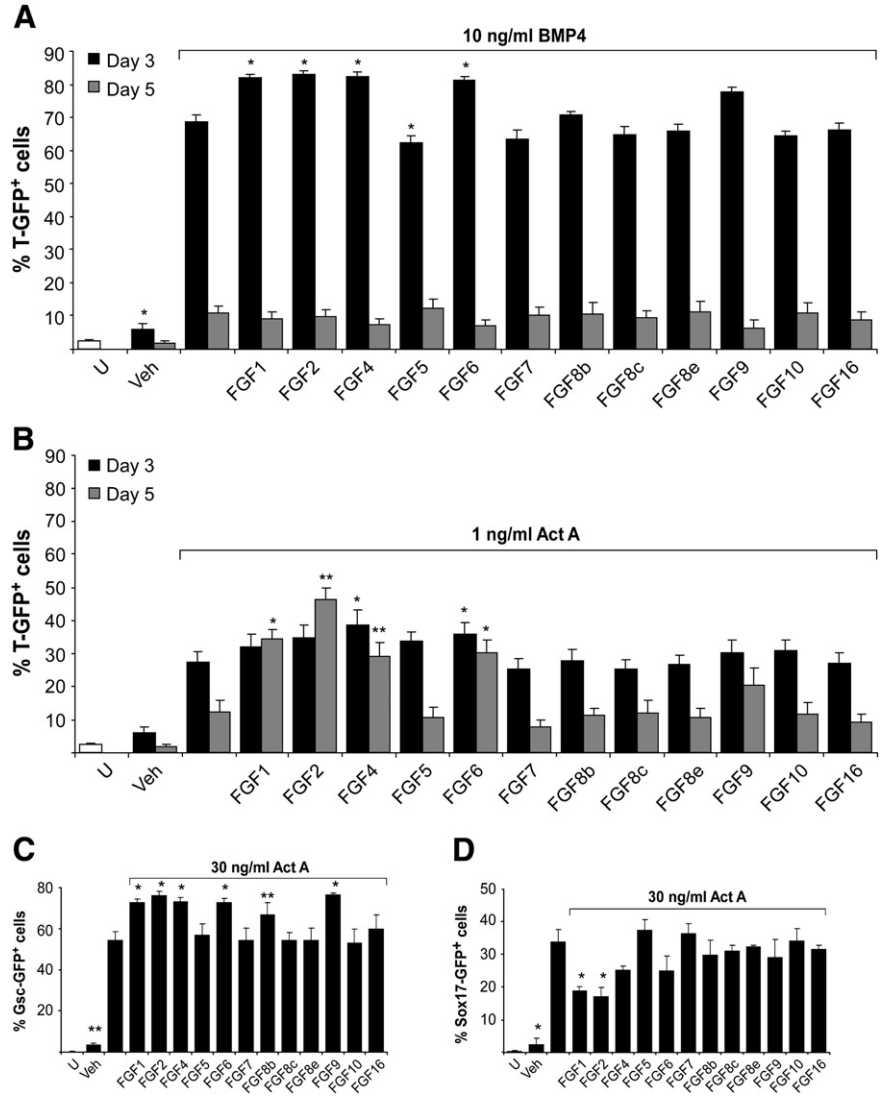


Figure 4 Activation of FGFRc isoforms promotes mesendoderm formation and inhibits Sox17 expression. Analyzing GFP-reporter cell lines T-GFP, Gsc-GFP, and Sox17-GFP, cells were differentiated for 3 (T-GFP cell line only) and 5 days in BMP4 or activin-containing media ± a range of Fgfs. (A and B) T-GFP cells were differentiated in 10 ng/ml BMP4 (A) or 1 ng/ml activin (B) ± Fgfs, and expression of GFP was analyzed by FACS at Days 3 and 5. (C and D) Gsc-GFP cells (C) or Sox17-GFP cells (D) were differentiated in 30 ng/ml activin ± Fgfs, and expression of GFP was measured by FACS at Day 5. The mean expression ± SEM of 3 independent experiments is shown, using a Student's t test for the statistical analysis: $*P<0.05$; $**P<0.01$ compared to the BMP4 or activin conditions. U, Undifferentiated cells, Veh., vehicle.

of the cells in these cultures have left the proliferative state by Day 5.

Discussion

We have previously shown that Fgf signaling augments differentiation toward PS-like cells and is required at Days 3–5 for efficient differentiation of mESC into DE in response to activin treatment. However, addition of Fgf2 attenuated formation of activin-induced Sox17-GFP$^+$ DE cells (Hansson et al., 2009)), suggesting that levels of signaling must be tightly regulated. Recently, Fgf4 was shown to be necessary for mESCs to leave the pluripotent state and differentiate into either ectoderm or mesoderm lineages (Kunath et al., 2007; Stavridis et al., 2007). *Fgf4*$^{-/-}$ mESCs could not differentiate into either lineage, except when supplementing the growth medium with exogenous Fgf4 protein. Fgf4 is important during gastrulation where it is responsible for movement of gastrulating cells through the PS (Bottcher and Niehrs, 2005) and *Fgf4* knock-out mice die during gastrulation (Feldman et al., 1995).

Using culture conditions similar to those Kunath and co-workers, we found that addition of exogenous Fgf4 was dispensable when differentiating *Fgf4*$^{-/-}$ cells into DE by treatment with activin. We readily obtained Sox17$^+$/E-cadherin$^+$/Foxa2$^+$/Sox7$^-$ DE cells which by qPCR were shown to express the additional DE markers *Cxcr4*, *Cldn6*, and *Foxa3*, but not the VE markers *Sox7* and *Tdh*. This supports our previous finding that induction of DE formation is not dependent on early Fgf signaling, as cells readily

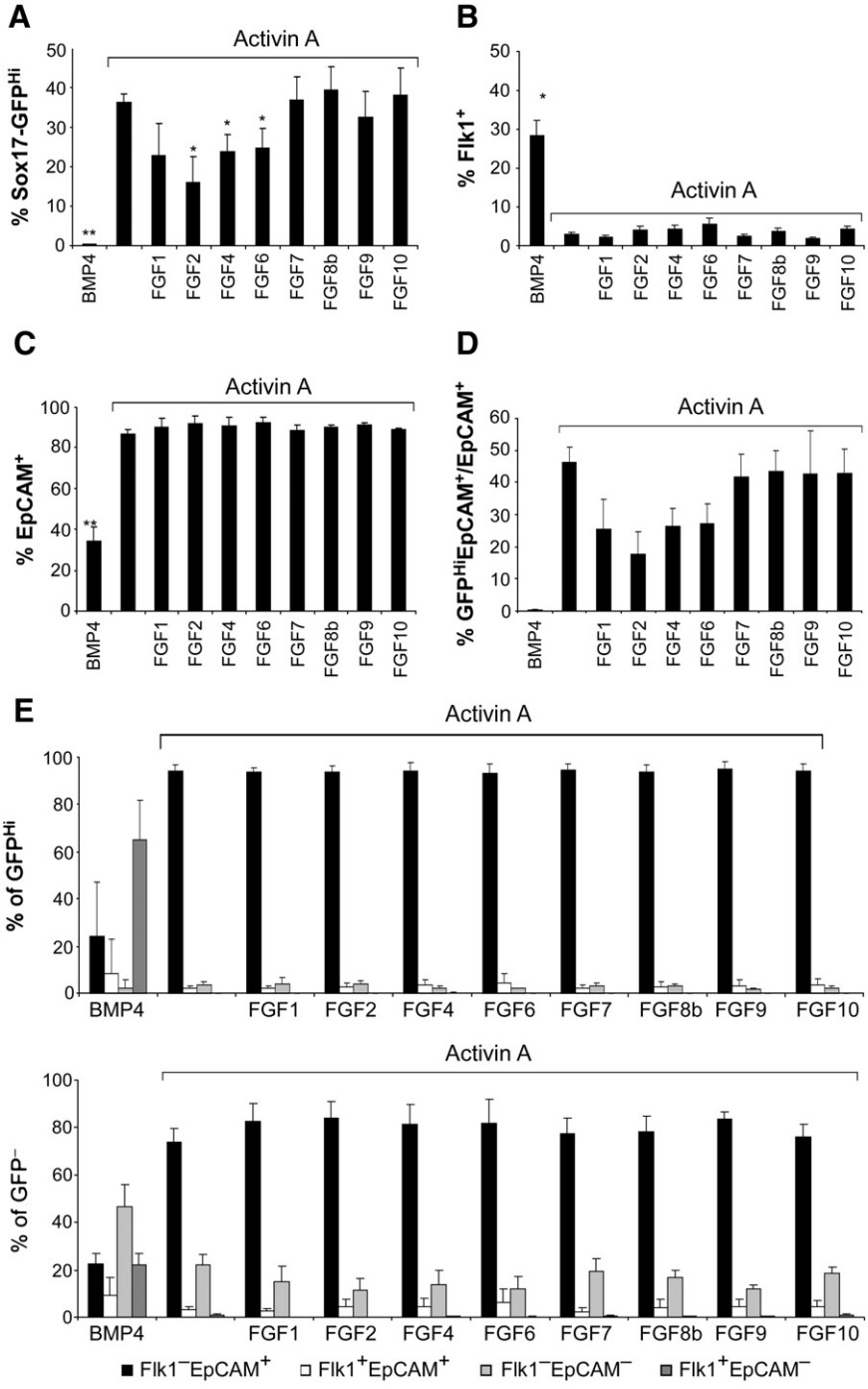


Figure 5 Activation of FGFRb and FGFRc isoforms differentially affects the expression of DE and mesoderm markers. Sox17-GFP cells were differentiated in media containing BMP4 or activin ± Fgfs for 5 days before analysis. Cells were stained for markers of DE or mesoderm and analyzed. (A–C) *Sox17*$^{Gfp/+}$ cells stained for Flk1 and EpCAM were analyzed individually for (A) GFP, (B) Flk1, and (C) EpCAM. (D) Percentage Sox17-GFP^{Hi}EpCAM$^+$ cells of the total EpCAM$^+$ population. (E) Multichannel analysis for Flk1 and EpCAM in the Sox17-GFP^{Hi} or Sox17-GFP$^{-/Lo}$ populations. The percentage Flk1$^-$EpCAM$^+$, Flk1$^+$EpCAM$^+$, Flk1$^-$EpCAM$^-$, and Flk1$^+$EpCAM$^-$ is indicated. The mean expression ± SEM of 3–4 independent experiments is shown, using a Student's paired, two-tailed *t* test for the statistical analysis: *$P<0.05$; **$P<0.01$ compared to the activin-treated conditions.

become Sox17-GFP$^+$ when the FGFR-inhibitor PD173074 is present at early stages of differentiation (Hansson et al., 2009). We propose that exogenous Fgf4 is only necessary for differentiation into ectoderm and mesoderm lineages but not for leaving the pluripotent state per se (Kunath et al., 2007; Stavridis et al., 2007). Although *Fgf4* knockout mice have been shown to be embryonic lethal at the stage of gastrulation (Feldman et al., 1995; Wilder et al., 1997), their dependence on Fgf4 signaling may lie at an earlier time point, namely in the area of embryonic ectoderm where later

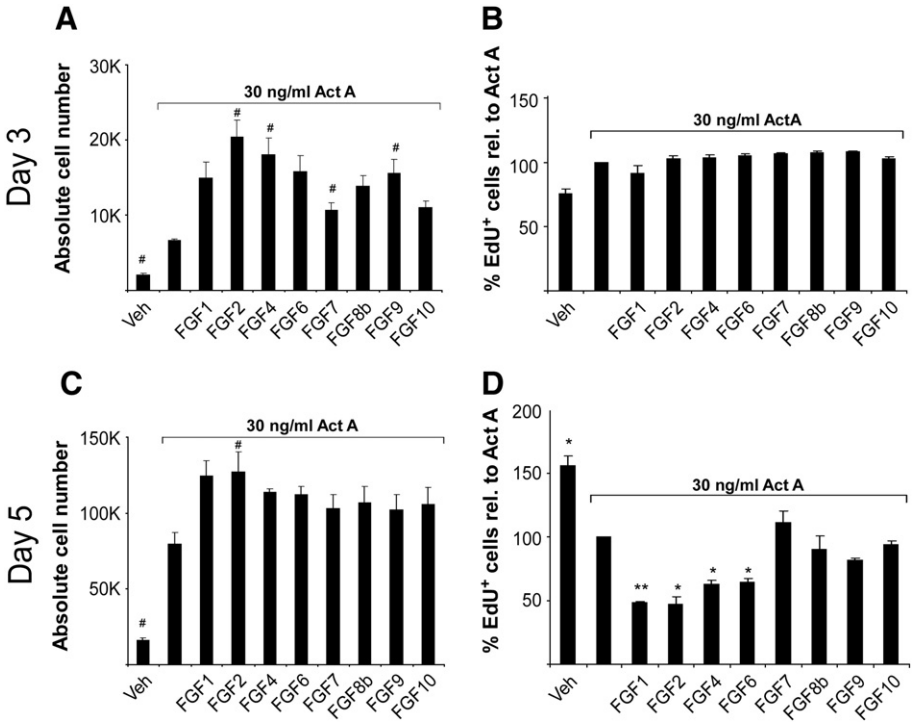


Figure 6 Activation of FGFRc isoforms affects early cell growth and proliferation. A wt mES cell line, E14, was grown in media containing activin ± Fgfs and harvested for analysis of total cell number and proliferation on Days 3 and 5. An absolute cell number and the relative proliferation (% EdU$^+$ cells) of cells are shown for Day 3 (A and B) and Day 5 (C and D). The mean expression ± SEM of 3 independent experiments is shown, using a ratio t test for absolute cell numbers: $^{\#}P<0.05$; $^{\#\#}P<0.01$; and a Student's *t* test for the relative proliferation: $^{*}P<0.05$; $^{**}P<0.01$; both compared to the activin conditions.

the PS forms. This would render Fgf4 necessary for the formation of the PS rather than its function (Tam et al., 1993; Niswander and Martin, 1992). This substantiates that Fgf4 is not necessary for cells to leave the pluripotent state when the differentiation protocol applied includes activin.

Most studies on endoderm formation from mES cells rely on culturing conditions using either embryoid bodies as starting material or high cell densities (Willems and Leyns, 2008; Funa et al., 2008; Morrison et al., 2008). Compared to Kunath and co-workers, we seed cells at a low density. Possibly, a high cell density to some degree inhibits differentiation, a common phenomenon seen in ES cell differentiation systems. Indeed, when applying the ectoderm differentiation protocol as described by Kunath and co-workers to cells at low density, we saw an increase in neural differentiation. We speculate that high cell densities retain mES cells in the pluripotent state to a higher degree than cells at low densities and that Fgf4 signaling may be necessary for leaving the pluripotent state at high cell densities only.

Nevertheless, formation of Sox17$^+$ DE from mESC in response to activin treatment is influenced by Fgf signaling. Treatment with the FGFR inhibitor PD173074 at Days 3–5 prevents differentiation of Sox17$^+$ DE but addition of exogenous Fgf2 also inhibits formation of these cells (Hansson et al., 2009). Here we find that activation of FGFRc but not FGFRb isoforms changes the fraction of EpCAM$^+$ cells that coexpress Sox17. While activin alone induces a nearly uniform population of EpCAM$^+$Sox17^{Hi} cells addition of, e.g., Fgf2 reduces the fraction of EpCAM$^+$ cells that coexpress Sox17. This shift may be caused by a selective expansion of an EpCAM$^+$Sox17$^-$ population developing in response to activin treatment, without affecting the total number of EpCAM$^+$Sox17$^+$ DE cells. Alternatively, Fgf2 may inhibit Sox17 expression in a subset of the EpCAM$^+$ cells. During embryonic development, epithelial tissues generally express b but not c isoforms while mesenchymal tissues express mainly c isoforms (Ornitz and Itoh, 2001). Fgfs specifically activating FGFRb isoforms (e.g., Fgf7 and 10) are mainly expressed in the mesenchyme and Fgfs activating FGFRc isoforms (e.g., Fgf4, 8, and 9) are mainly expressed in epithelia, resulting in specificity during reciprocal epithelial-mesenchymal signaling in developing organs such as the lung, cecum, salivary glands, and pancreas (Ornitz and Itoh, 2001; Orr-Urtreger et al., 1993; Elghazi et al., 2002; Stark et al., 1991; Colvin et al., 2001; Manfroid et al., 2007). In the present report we show that both FGFRb and -c isoforms are expressed in the Sox17-GFP^{Hi} fraction after activin treatment. The expression of FGFRb as well as c isoforms in the Sox17-GFP^{Hi} fraction may be explained if the Sox17-GFP^{Hi} fraction is heterogeneous and contains a pool of cells slated to become DE but not yet committed to an epithelial fate or cells that are undergoing mesodermal differentiation. However, the Sox17-GFP^{Hi} fraction is also positive for EpCAM, indicating that they represent epithelial endoderm. When factors capable of activating the c isoforms for the FGFRs are added to the activin induction we still observe the formation of EpCAM$^+$ cells but the percentage of these coexpressing Sox17-GFP^{Hi} is reduced. These cells may represent undifferentiated ES cells, but we consider this

unlikely as activin-induced expression of the early mesendoderm markers *T* and *Gsc* is enhanced by FGFR c-isoform ligands and the concomitant reduction in Oct4 expression is not affected. Alternatively, the EpCAM^{+}Sox17^{-} cells may represent endoderm which has yet to activate Sox17 expression or alternatively have passed through the transient Sox17-expressing phase. Regardless of their true identity, this EpCAM^{+}Sox17^{-} population appears to be selectively expanded by Fgfs that activate the c isoforms for the FGFRs, although the precise mechanism causing this change remains to be determined. Examining EpCAM and Sox17-GFP at later time points as well as qPCR analyses of purified cell populations could determine if Fgf signaling is delaying the kinetics of development of EpCAM^{+}Sox17^{Hi} DE cells.

Fgfs activating only FGFRb isotypes had no effect on the number of EpCAM^{+}Sox17-GFP^{Hi} cells developing in response to activin or on the number of EdU-incorporating cells. Since FGFR inhibitors reduce the numbers of Sox17-GFP^{Hi} cells (Hansson et al., 2009) we suspect that endogenous Fgf signaling is sufficient and therefore the further addition of b-isoform-specific factors has no effect. A putative endogenous Fgf may be Fgf3, which is expressed in the PS during gastrulation and which only activates FGFRb isoforms (Ornitz et al., 1996; Zhang et al., 2006; Wilkinson et al., 1988). Alternatively, induction of EpCAM^{+}Sox17-GFP^{Hi} cells may rely on precise levels of FGFRc signaling and increasing this signaling by addition of exogenous Fgf2 may be detrimental to formation of EpCAM^{+}Sox17-GFP^{Hi} cells. The FGFR1b and -2b expression we observe may render the cells competent to respond to signals occurring later during organogenesis. Optimal induction of DE may be supported by a combination of factors, with initial activation of FGFRc isoforms during early differentiation to promote mesendoderm formation followed by a second step where activation of FGFRb isoforms maintains definitive endoderm.

Materials and methods

Cell culture and differentiation of mESCs

We used the following mouse ES cell lines: E14 (Hooper et al., 1987), *T*$^{Gfp/+}$ (Fehling et al., 2003), *Gsc*$^{Gfp/+}$ (Tada et al., 2005), *Sox17*$^{Gfp/+}$ (Kim et al., 2007), *Fgf4*$^{+/-}$, and *Fgf4*$^{-/-}$ ES cells (Wilder et al., 1997). Cells were grown as previously described (Hansson et al., 2009; Ying et al., 2003) on cell culture plastic ware (Nunc) coated with 0.1% gelatine (Sigma), using 0.05% trypsin-EDTA (Invitrogen) for dissociation of cells during passage. Trypsin was inactivated by N2B27 medium: KO-DMEM supplemented with N2, B27, 0.1 mM nonessential amino acids, 2 mM L-glutamine, penicillin/streptomycin (all from Invitrogen), and 0.1 mM 2-mercaptoethanol (Sigma-Aldrich). Cells were cultured for at least 3 passages, i.e., 6 days, before onset of differentiation.

For differentiation purposes, cells were dissociated into single cells and seeded at 2000 cells/cm^2 in N2B27 medium containing one or more of the following growth factors: BMP4 (10 ng/ml), activin A (1 or 30 ng/ml; both from R&D Systems), Fgf1 (100 ng/ml; Chemicon International), Fgf2 (100 ng/ml; Invitrogen), Fgf4, Fgf5, Fgf6, Fgf7, Fgf8b, Fgf8c, Fgf8e, Fgf9, Fgf10, and FGF16 (5 or 100 ng/ml; all from R&D Systems). Media containing Fgfs were supplemented with 10 μg/ml heparan sulfate (Sigma-Aldrich).

Flow cytometry

For analysis of GFP expression in reporter cell lines, live cells were dissociated into single cells by 0.05% trypsin-EDTA (Invitrogen) and analyzed by FACS Calibur flow cytometer (BD Biosciences). For analysis of cells stained with antibodies, cells were fixed in Lilly's fixative (Bie & Berntsen), and resuspended in 0.1% BSA in PBS. Cells were stained in 0.1% BSA in PBS for 2 h at 4 °C with anti-Flk1-PE (BD Pharmigen, No. 555308) and anti-EpCAM-PE-Cy7 (eBioscience, No. 25-5791-80). Alternatively, cells were permeabilized in dilution buffer (0.3% Triton X-100+0.1% BSA in PBS); unspecific binding sites were blocked by 10% Normal Donkey Serum (Jackson Immunoresearch Laboratories) for 30 min at RT and stained for Brachyury (R&D Systems, No. AF2085) for 2 h at RT in dilution buffer, followed by a Cy3-conjugated secondary antibody (Jackson Immunoresearch Laboratories, No. 705-165-147) for 1 h at RT. Cells were analyzed by FACS Aria flow cytometer (BD Biosciences). For sorting experiments the cells were dissociated by 0.05% trypsin-EDTA (Invitrogen), washed, and resuspended in N2B27 medium before sorting by FACS Aria flow cytometer (BD Biosciences).

Immunofluorescent staining

Cells were grown in 9 cm^2 slide flasks (Nunc) coated with 0.1% gelatine (Sigma) and fixed in Lilly's fixative (Bie & Berntsen), permeabilized in dilution buffer (see above), and blocked for 30 min at RT in 10% Normal Donkey Serum (Jackson Immunoresearch Laboratories) in dilution buffer. They were stained overnight at 4 °C with primary antibodies: mouse anti-Oct3/4 (C-10), goat anti-Foxa2 (both Santa Cruz Biotechnology), goat anti-Brachyury (R&D Systems), rat anti-E-cadherin (Zymed/Invitrogen), goat anti-Sox17 (R&D Systems), and rabbit anti-Sox7 (ab22584, Biozol). After several washes the cells were incubated for 1 h with Cy2-, Cy3-, or Cy5-conjugated species-specific secondary antibodies (Jackson ImmunoResearch Laboratories) to visualize expression and 4′,6-diamidino-2-phenylindole (DAPI; MP Biomedicals) was used as counter stain. Slides were mounted in fluorescent mounting medium (KPL). Negative controls, where the primary antibodies were omitted, were included for all stains and showed no unspecific staining of the secondary antibodies (data not shown). The slides were analyzed using an LSM 510 META laser scanning microscope (Carl Zeiss).

qPCR

Cells were harvested in lysis solution (Invitek), supplemented with 10 mM dithiothreitol (DTT). Total RNA was isolated using the Invisorb Spin RNA kit (Invitek) with DNAse treatment (Promega) following the manufacturer's protocol. cDNA was prepared from 250 ng RNA using MMLV reverse transcriptase (Invitrogen) with random oligos or oligo(dT)$_{12-18}$ primers (both Invitrogen). qPCR was performed using the standard

SYBR Green program with dissociation curve on Mx3005P (Stratagene). PCR were run in duplicates using 10 μl Brilliant SYBR Green qPCR Master Mix (Stratagene), 1 μl cDNA, 1 μl 20 μM primer mix, and 8 μl dH_2O. Quantified values for each gene were normalized against the housekeeping gene TATA-binding protein (TBP). Primer sequences are: FGFR1c F_CCGTATGTCCAGATCCTGAAGA, R_GATAGAGTTACCCGCCAAGCA; FGFR2c F_GCCCTACCTCAAGGTTCTGAAAG R_GATAGAATTACCCGCCAAGCA; FGFR3c F_CCCTACGTCACTGTACTCAAGACTG R_GTGACATTGTGCAAGGACAGAAC; FGFR4 F_CGACGGTTTCCCCTACGTACA R_TGCCCGCCAGACAGGTATAC (all from (Woei Ng et al., 2007); FGFR1b F_CTTGACGTCGTGGAACGATCT, R_CACGCAGACTGGTTAGCTTCAC (Nakayama et al., 2007); FGFR2b F_AACGGGAAGGAGTTTAAGCAG, R_GGAGCTATTTATCCCCGAGTG (Yamanaka et al., 2000); Nanog F_CTCTTCAAGGCAGCCCTGAT, R_CCATTGCTAGTCTTCAACCAC (Storm et al., 2007); Oct4 F_AGAGGGAACCTCCTCTGAGC, R_TGATTGGCGATGTGAGTGAT; Sox17 F_GGAGGGTCACCACTGCTTTA, R_TCAGATGTCTGGAGGTGCTG; Cxcr4 F_AGGTACATCTGTGACCGCCTTT, R_ AGACCCACCATTATATGCTGGAA (Kim et al., 2008); FoxA3 F_GGA ACA TGT TTG AGA ACG GCT, R_CGA TGT GGC GCT GTT TCC TTT (Gadue et al., 2009); Cldn6 F_TATCCTGTCCCAGTCCCAAG, R_CAGGGCTGGAGAGAAGTCTG (Nakazawa et al., 2008); Sox7 F_GGCAGTGCAGAACCCGGACC, R_TGCAGAGGCGCTTGCCTTGT; Tdh F_CCTGGAGGAGGAACAACTGACTA, R_ ACTCGAATGTGCCGTTCTTTG (Wang et al., 2009); TBP F_TCTGAGAGCTCTGGAATTGT, R_GAAGTGCAATGGTCTTTAGG.

Cell count and proliferation assay

Cells were fixed in Lilly's fixative (Bie & Berntsen) and counted in a NucleoCassette by the NucleoCounter (ChemoMetec A/S) according to the manufacturer's protocol. For the proliferation assay, EdU incorporation by the Click-iT EdU HCS Assay (Invitrogen) was used (Salic and Mitchison, 2008). Cells were incubated for 15 min in their respective media containing 10 μM EdU, then washed, fixed, and stained by the Click-iT reaction cocktail according to the manufacturer's protocol, using an Alexa Fluor 488-conjugated antibody to detect incorporation. Stained cells were quantified using a FACS Aria flow cytometer (BD Biosciences).

Western blot

Total cell lysates were harvested at experimental onset and Day 5 of differentiation using Cell Lysis Buffer (Cell Signaling Technology). Twenty micrograms of each protein sample was loaded and analyzed by Western blot on PVDF membranes using rabbit anti-Sox7 (ab22584, Biozol), goat anti-Sox17 (R&D Systems), or TFIIb (C-18; Santa Cruz) as primary antibodies and HRP-conjugated goat anti-rabbit or donkey anti-goat secondary antibodies (both Santa Cruz Biotechnology).

Statistics

Mean percentage of the cells of interest ± standard error of the mean (SEM) was calculated and statistical analyses by Student's paired, two-tailed *t* test or ratio *t* test were performed.

Supplementary data associated with this article can be found, in the online version, doi:10.1016/j.scr.2011.02.003.

Acknowledgments

We thank Drs. G. Keller, S. Nishikawa, and S. J. Morrison for the T-GFP, Gsc-GFP, and Sox17-GFP cell lines, respectively, and A. Rizzino and T. Kunath for $Fgf4^{+/-}$ and $Fgf4^{-/-}$ cell lines. We thank Søren Refsgaard Lindskog for excellent technical assistance. This work was made possible by funding from the Juvenile Diabetes Research Foundation (to P.S.).

References

Balzar, M., Winter, M.J., de Boer, C.J., Litvinov, S.V., 1999. The biology of the 17-1A antigen (Ep-CAM). J. Mol. Med. 77, 699–712.

Ben-Haim, N., Lu, C., Guzman-Ayala, M., Pescatore, L., Mesnard, D., Bischofberger, M., Naef, F., Robertson, E.J., Constam, D.B., 2006. The nodal precursor acting via activin receptors induces mesoderm by maintaining a source of its convertases and BMP4. Dev. Cell 11, 313–323.

Blum, M., Gaunt, S.J., Cho, K.W., Steinbeisser, H., Blumberg, B., Bittner, D., De Robertis, E.M., 1992. Gastrulation in the mouse: the role of the homeobox gene goosecoid. Cell 69, 1097–1106.

Bottcher, R.T., Niehrs, C., 2005. Fibroblast growth factor signaling during early vertebrate development. Endocr. Rev. 26, 63–77.

Carey, F.J., Linney, E.A., Pedersen, R.A., 1995. Allocation of epiblast cells to germ layer derivatives during mouse gastrulation as studied with a retroviral vector. Dev. Genet. 17, 29–37.

Ciruna, B.G., Schwartz, L., Harpal, K., Yamaguchi, T.P., 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.

Colvin, J.S., White, A.C., Pratt, S.J., Ornitz, D.M., 2001. Lung hypoplasia and neonatal death in Fgf9-null mice identify this gene as an essential regulator of lung mesenchyme. Development 128, 2095–2106.

Conlon, F.L., Lyons, K.M., Takaesu, N., Barth, K.S., Kispert, A., Herrmann, B., Robertson, E.J., 1994. A primary requirement for nodal in the formation and maintenance of the primitive streak in the mouse. Development 120, 1919–1928.

Deng, C.X., Wynshaw-Boris, A., Shen, M.M., Daugherty, C., Ornitz, D.M., Leder, P., 1994. Murine FGFR-1 is required for early postimplantation growth and axial organization. Genes Dev. 8, 3045–3057.

Elghazi, L., Cras-Meneur, C., Czernichow, P., Scharfmann, R., 2002. Role for FGFR2IIIb-mediated signals in controlling pancreatic endocrine progenitor cell proliferation. Proc. Natl Acad. Sci. U.S.A. 99, 3884–3889.

Ema, M., Takahashi, S., Rossant, J., 2006. Deletion of the selection cassette, but not cis-acting elements, in targeted Flk1-lacZ allele reveals Flk1 expression in multipotent mesodermal progenitors. Blood 107, 111–117.

Fehling, H.J., Lacaud, G., Kubo, A., Kennedy, M., Robertson, S., Keller, G., Kouskoff, V., 2003. Tracking mesoderm induction and its specification to the hemangioblast during embryonic stem cell differentiation. Development 130, 4217–4227.

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

Funa, N.S., Saldeen, J., Akerblom, B., Welsh, M., 2008. Inter-dependent fibroblast growth factor and activin A signaling

promotes the expression of endodermal genes in differentiating mouse embryonic stem cells expressing Src Homology 2-domain inactive Shb. Differentiation 76, 443–453.
Gadue, P., Huber, T.L., Nostro, M.C., Kattman, S., Keller, G.M., 2005. Germ layer induction from embryonic stem cells. Exp. Hematol. 33, 955–964.
Gadue, P., Huber, T.L., Paddison, P.J., Keller, G.M., 2006. Wnt and TGF-beta signaling are required for the induction of an in vitro model of primitive streak formation using embryonic stem cells. Proc. Natl Acad. Sci. U.S.A. 103, 16806–16811.
Gadue, P., Gouon-Evans, V., Cheng, X., Wandzioch, E., Zaret, K.S., Grompe, M., Streeter, P.R., Keller, G.M., 2009. Generation of monoclonal antibodies specific for cell surface molecules expressed on early mouse endoderm. Stem Cells 27, 2103–2113.
Gospodarowicz, D., Moran, J.S., 1975. Mitogenic effect of fibroblast growth factor on early passage cultures of human and murine fibroblasts. J. Cell Biol. 66, 451–457.
Guo, Q., Li, J.Y., 2007. Distinct functions of the major Fgf8 spliceform, Fgf8b, before and during mouse gastrulation. Development 134, 2251–2260.
Hansson, M., Olesen, D.R., Peterslund, J.M., 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.
Hooper, M., Hardy, K., Handyside, A., Hunter, S., Monk, M., 1987. HPRT-deficient (Lesch-Nyhan) mouse embryos derived from germline colonization by cultured cells. Nature 326, 292–295.
Itoh, N., Ornitz, D.M., 2004. Evolution of the Fgf and Fgfr gene families. Trends Genet. 20, 563–569.
Kaestner, K.H., Hiemisch, H., Luckow, B., Schutz, G., 1994. The HNF-3 gene family of transcription factors in mice: gene structure, cDNA sequence, and mRNA distribution. Genomics 20, 377–385.
Kanai-Azuma, M., Kanai, Y., Gad, J.M., Tajima, Y., Taya, C., Kurohmaru, M., Sanai, Y., Yonekawa, H., Yazaki, K., Tam, P.P., Hayashi, Y., 2002. Depletion of definitive gut endoderm in Sox17-null mutant mice. Development 129, 2367–2379.
Kim, I., Saunders, T.L., Morrison, S.J., 2007. Sox17 dependence distinguishes the transcriptional regulation of fetal from adult hematopoietic stem cells. Cell 130, 470–483.
Kim, S.Y., Lee, C.H., Midura, B.V., Yeung, C., Mendoza, A., Hong, S.H., Ren, L., Wong, D., Korz, W., Merzouk, A., Salari, H., Zhang, H., Hwang, S.T., Khanna, C., Helman, L.J., 2008. Inhibition of the CXCR4/CXCL12 chemokine pathway reduces the development of murine pulmonary metastases. Clin. Exp. Metastasis 25, 201–211.
Kinder, S.J., Tsang, T.E., Wakamiya, M., Sasaki, H., Behringer, R.R., Nagy, A., Tam, P.P., 2001. The organizer of the mouse gastrula is composed of a dynamic population of progenitor cells for the axial mesoderm. Development 128, 3623–3634.
Kispert, A., Herrmann, B.G., 1994. Immunohistochemical analysis of the Brachyury protein in wild-type and mutant mouse embryos. Dev. Biol. 161, 179–193.
Kubo, A., Shinozaki, K., Shannon, J.M., Kouskoff, V., Kennedy, M., Woo, S., Fehling, H.J., Keller, G., 2004. Development of definitive endoderm from embryonic stem cells in culture. Development 131, 1651–1662.
Kunath, T., Saba-El-Leil, M.K., 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.
Lawson, K.A., Meneses, J.J., Pedersen, R.A., 1991. Clonal analysis of epiblast fate during germ layer formation in the mouse embryo. Development 113, 891–911.
MacArthur, C.A., Lawshe, A., Xu, J., Santos-Ocampo, S., Heikinheimo, M., Chellaiah, A.T., Ornitz, D.M., 1995. FGF-8 isoforms activate receptor splice forms that are expressed in mesenchymal regions of mouse development. Development 121, 3603–3613.
Manfroid, I., Delporte, F., Baudhuin, A., Motte, P., Neumann, C.J., Voz, M.L., Martial, J.A., Peers, B., 2007. Reciprocal endoderm-mesoderm interactions mediated by fgf24 and fgf10 govern pancreas development. Development 134, 4011–4021.
Mason, I., 2007. Initiation to end point: the multiple roles of fibroblast growth factors in neural development. Nat. Rev. Neurosci. 8, 583–596.
McCall, M.D., Toso, C., Baetge, E.E., Shapiro, A.M., 2010. Are stem cells a cure for diabetes? Clin. Sci. (Lond.) 118, 87–97.
Monaghan, A.P., Kaestner, K.H., Grau, E., Schutz, G., 1993. Postimplantation expression patterns indicate a role for the mouse forkhead/HNF-3 alpha, beta and gamma genes in determination of the definitive endoderm, chordamesoderm and neuroectoderm. Development 119, 567–578.
Morrison, G.M., Oikonomopoulou, I., Migueles, R.P., Soneji, S., Livigni, A., Enver, T., Brickman, J.M., 2008. Anterior definitive endoderm from ESCs reveals a role for FGF signaling. Cell Stem Cell 3, 402–415.
Nakayama, T., Mutsuga, N., Tosato, G., 2007. FGF2 posttranscriptionally down-regulates expression of SDF1 in bone marrow stromal cells through FGFR1 IIIc. Blood 109, 1363–1372.
Nakazawa, M., Matsunaga, K., Asamura, S., Kusuhara, H., Isogai, N., Muragaki, Y., 2008. Molecular mechanisms of cleft lip formation in CL/Fr mice. Scand. J. Plast. Reconstr. Surg. Hand Surg. 42, 225–232.
Niswander, L., Martin, G.R., 1992. Fgf-4 expression during gastrulation, myogenesis, limb and tooth development in the mouse. Development 114, 755–768.
Olsen, S.K., Li, J.Y., Bromleigh, C., Eliseenkova, A.V., Ibrahimi, O.A., Lao, Z., Zhang, F., Linhardt, R.J., Joyner, A.L., Mohammadi, M., 2006. Structural basis by which alternative splicing modulates the organizer activity of FGF8 in the brain. Genes Dev. 20, 185–198.
Ornitz, D.M., Itoh, N., 2001. Fibroblast growth factors. Genome Biol. 2 REVIEWS3005.
Ornitz, D.M., Xu, J., Colvin, J.S., McEwen, D.G., MacArthur, C.A., Coulier, F., Gao, G., Goldfarb, M., 1996. Receptor specificity of the fibroblast growth factor family. J. Biol. Chem. 271, 15292–15297.
Orr-Urtreger, A., Bedford, M.T., Burakova, T., Arman, E., Zimmer, Y., Yayon, A., Givol, D., Lonai, P., 1993. Developmental localization of the splicing alternatives of fibroblast growth factor receptor-2 (FGFR2). Dev. Biol. 158, 475–486.
Rossant, J., 2004. Lineage development and polar asymmetries in the peri-implantation mouse blastocyst. Semin. Cell Dev. Biol. 15, 573–581.
Salic, A., Mitchison, T.J., 2008. A chemical method for fast and sensitive detection of DNA synthesis in vivo. Proc. Natl Acad. Sci. U.S.A. 105, 2415–2420.
Schier, A.F., 2003. Nodal signaling in vertebrate development. Annu. Rev. Cell Dev. Biol. 19, 589–621.
Sherwood, R.I., Jitianu, C., Cleaver, O., Shaywitz, D.A., Lamenzo, J. O., Chen, A.E., Golub, T.R., Melton, D.A., 2007. Prospective isolation and global gene expression analysis of definitive and visceral endoderm. Dev. Biol. 304, 541–555.
Stark, K.L., McMahon, J.A., McMahon, A.P., 1991. FGFR-4, a new member of the fibroblast growth factor receptor family, expressed in the definitive endoderm and skeletal muscle lineages of the mouse. Development 113, 641–651.
Stavridis, M.P., Lunn, J.S., Collins, B.J., Storey, K.G., 2007. A discrete period of FGF-induced Erk1/2 signalling is required for vertebrate neural specification. Development 134, 2889–2894.
Storm, M.P., Bone, H.K., Beck, C.G., Bourillot, P.Y., Schreiber, V., Damiano, T., Nelson, A., Savatier, P., Welham, M.J., 2007. Regulation of Nanog expression by phosphoinositide 3-kinase-dependent signaling in murine embryonic stem cells. J. Biol. Chem. 282, 6265–6273.
Tada, S., Era, T., Furusawa, C., Sakurai, H., Nishikawa, S., Kinoshita, M., Nakao, K., Chiba, T., 2005. Characterization of mesendoderm: a diverging point of the definitive endoderm and mesoderm in embryonic stem cell differentiation culture. Development 132, 4363–4374.

Tam, P.P., Williams, E.A., Chan, W.Y., 1993. Gastrulation in the mouse embryo: ultrastructural and molecular aspects of germ layer morphogenesis. Microsc. Res. Tech. 26, 301–328.

Vainikka, S., Partanen, J., Bellosta, P., Coulier, F., Birnbaum, D., Basilico, C., Jaye, M., Alitalo, K., 1992. Fibroblast growth factor receptor-4 shows novel features in genomic structure, ligand binding and signal transduction. EMBO J. 11, 4273–4280.

Van Hoof, D., D'Amour, K.A., German, M.S., 2009. Derivation of insulin-producing cells from human embryonic stem cells. Stem Cell Res. 3, 73–87.

Waldrip, W.R., Bikoff, E.K., Hoodless, P.A., Wrana, J.L., Robertson, E.J., 1998. Smad2 signaling in extraembryonic tissues determines anterior-posterior polarity of the early mouse embryo. Cell 92, 797–808.

Wang, J., Alexander, P., Wu, L., Hammer, R., Cleaver, O., McKnight, S.L., 2009. Dependence of mouse embryonic stem cells on threonine catabolism. Science 325, 435–439.

Weinstein, D.C., Ruiz Test, A., 1994. The winged-helix transcription factor HNF-3 beta is required for notochord development in the mouse embryo. Cell 78, 575–588.

Wilder, P.J., Kelly, D., Brigman, K., Peterson, C.L., Nowling, T., Gao, Q. S., McComb, R.D., Capecchi, M.R., Rizzino, A., 1997. Inactivation of the FGF-4 gene in embryonic stem cells alters the growth and/or the survival of their early differentiated progeny. Dev. Biol. 192, 614–629.

Wilkinson, D.G., Peters, G., Dickson, C., McMahon, A.P., 1988. Expression of the FGF-related proto-oncogene int-2 during gastrulation and neurulation in the mouse. EMBO J. 7, 691–695.

Willems, E., Leyns, L., 2008. Patterning of mouse embryonic stem cell-derived pan-mesoderm by activin A/Nodal and Bmp4 signaling requires fibroblast growth factor activity. Differentiation 76, 745–759.

Woei Ng, K., Speicher, T., Dombrowski, C., Helledie, T., Haupt, L. M., Nurcombe, V., Cool, S.M., 2007. Osteogenic differentiation of murine embryonic stem cells is mediated by fibroblast growth factor receptors. Stem Cells Dev. 16, 305–318.

Yamaguchi, T.P., Harpal, K., Henkemeyer, M., Rossant, J., 1994. fgfr-1 is required for embryonic growth and mesodermal patterning during mouse gastrulation. Genes Dev. 8, 3032–3044.

Yamanaka, T., Sakamoto, A., Tanaka, Y., Zhang, Y., Hayashido, Y., Toratani, S., Akagawa, Y., Okamoto, T., 2000. Isolation and serum-free culture of epithelial cells derived from epithelial rests of Malassez in human periodontal ligament. In Vitro Cell. Dev. Biol. Anim. 36, 548–553.

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

Zhang, X., Ibrahimi, O.A., Olsen, S.K., Umemori, H., Mohammadi, M., Ornitz, D.M., 2006. Receptor specificity of the fibroblast growth factor family. The complete mammalian FGF family. J. Biol. Chem. 281, 15694–15700.