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A late requirement for Wnt and FGF signaling during activin-induced formation of foregut endoderm from mouse embryonic stem cells

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ABSTRACT

Here we examine how BMP, Wnt, and FGF signaling modulate activin-induced mesendodermal differentiation of mouse ES cells grown under defined conditions in adherent monoculture. We monitor ES cells containing reporter genes for markers of primitive streak (PS) and its progeny and extend previous findings on the ability of increasing concentrations of activin to progressively induce more ES cell progeny to anterior PS and endodermal fates. We find that the number of *Sox17*- and *Gsc*-expressing cells increases with increasing activin concentration while the highest number of *T*-expressing cells is found at the lowest activin concentration. The expression of *Gsc* and other anterior markers induced by activin is prevented by treatment with BMP4, which induces *T* expression and subsequent mesodermal development. We show that canonical Wnt signaling is required only during late stages of activin-induced development of Sox17-expressing endodermal cells. Furthermore, Dkk1 treatment is less effective in reducing development of $Sox17^+$ endodermal cells in adherent culture than in aggregate culture and appears to inhibit nodal-mediated induction of $Sox17^+$ cells more effectively than activin-mediated induction. Notably, activin induction of $Gsc\text{-}GFP^+$ cells appears refractory to inhibition of canonical Wnt signaling but shows a dependence on early as well as late FGF signaling. Additionally, we find a late dependence on FGF signaling during induction of $Sox17^+$ cells by activin while BMP4-induced *T* expression requires FGF signaling in adherent but not aggregate culture. Lastly, we demonstrate that activin-induced definitive endoderm derived from mouse ES cells can incorporate into the developing foregut endoderm in vivo and adopt a mostly anterior foregut character after further culture in vitro.

Introduction

Directed differentiation of embryonic stem (ES) cells into meso- and endodermal derivatives is intensely studied due to their potential clinical applications. Meso- and endoderm is formed by epiblast cells that ingress through the primitive streak (PS) during gastrulation (reviewed in Tam and Loebel, 2007). Fate mapping studies have shown that cells that migrate through different anterior–posterior regions of the streak give rise to different mesodermal and endodermal components (Carey et al., 1995; Lawson, 1999; Lawson and Pedersen, 1992). At early stages, mesodermally fated cells ingress alongside endodermally fated cells but it is unclear when and how ingressing cells acquire their ultimate fate. The definitive endoderm (DE) is derived from progenitors migrating through the anterior PS at early and mid-streak stages (Carey et al., 1995; Lawson, 1999; Lawson and Pedersen, 1987; Lawson and Pedersen, 1992). Moreover, recent evidence suggests that a common progenitor population, the mesendoderm, exists in the PS (Kinder et al., 2001; Lawson et al., 1991; Tada et al., 2005) and that the cumulative exposure to nodal signaling determines mesendodermal fates such that increasing exposure to nodal shifts the fate from posterior mesoderm through anterior mesoderm and posterior endoderm to anterior DE at the largest dose (Ben-Haim et al., 2006).

The use of mouse ES (mES) cell lines with the green fluorescent protein (GFP) targeted to the PS- and early mesodermal-specific genes *Brachyury* (*T*), *Mix1 homeobox-like 1* (*Mixl1*), and *Goosecoid* (*Gsc*) has made it possible to quantify mesendoderm induction and isolate and characterize different mesodermal and endodermal populations (Fehling et al., 2003; Gadue et al., 2006; Kubo et al., 2004; Ng et al., 2005; Tada et al., 2005; Yasunaga et al., 2005). Anterior PS fates and endoderm was induced with high concentrations of activin A (activin hereafter) that activates Smad2/3 signaling through binding to the same receptor as nodal. Recent studies have extended the endoderm-inducing properties of activin to human ES cell differentiation cultures (D'Amour et al., 2005, 2006). However, as nodal signaling in the early

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embryo interacts with other signaling pathways such as the BMP, Wnt, and FGF pathways (reviewed in Tam and Loebel, 2007), we address here the role of these signals and their potential to modulate activin-induced mesendodermal differentiation of mES cells grown under standard conditions in feeder- and serum-free adherent monoculture (Ying et al., 2003a,b). We use ES cells containing reporter genes (*lacZ* or *GFP*) targeted to the *T* (Fehling et al., 2003), *Mixl1* (Hart et al., 2002), *Gsc* (Tada et al., 2005), *Flk1* (Shalaby et al., 1995), *Sox17* (Kim et al., 2007), and *Sox2* (Li et al., 1998) loci to monitor, over time, the effects of different growth factors on the expression of markers specific to different anterior and posterior regions of the PS and derivatives thereof. We confirm and extend previous findings on the ability of increasing concentrations of activin to progressively induce more ES cell progeny to an anterior PS fate. Remarkably, while the number of *Gsc*- and *Sox17*-expressing cells increases with increasing activin concentration, the highest number of *T*-expressing cells is found at the lowest activin concentration, similar to the activin response seen in *Xenopus* animal cap cells. Furthermore, expression of *Gsc* and other anterior markers induced at high activin doses is prevented by simultaneous treatment with BMP4 which redirects development towards mesodermal fates, also similar to results from *Xenopus*. Extending previous work, we find that inhibition of canonical Wnt signaling by treatment with Dkk1 is able to prevent activin-induced development of endodermal cells but only at late stages of differentiation. Dkk1 also inhibits activin-induced Mixl1-expression and consistent with this finding Wnt3a and activin act additively on *Mixl1* expression but not on *Gsc* expression. Wnt3a by itself appears to induce only posterior PS fates depending, however, on endogenous Smad2/3 signaling. Additionally, we demonstrate that induction of anterior and posterior PS fates by activin or BMP4, respectively, is dependent on FGF signaling. Lastly, we demonstrate for the first time that activin-induced DE derived from mES cells can incorporate into the developing foregut endoderm when implanted into chicken embryos but respond only to a limited degree to posteriorizing cues in vitro by initiating expression of regional foregut markers.

Materials and methods

Cell culture and differentiation of ESCs

Mouse ES cells (40,000 cells/cm^2) were kept undifferentiated on gelatin-coated cell culture plastic (Nunc) in serum-free medium; KO-DMEM supplemented with N2, B27, 0.1 mM nonessential amino acids, 2 mM L-glutamine, Penicillin/Streptomycin (all from Invitrogen), 0.1 mM 2-mercaptoethanol (Sigma-Aldrich), 1500 U/ml leukemia inhibitory factor (LIF, Chemicon) and 10 ng/ml BMP4 (R&D Systems), essentially as described by Ying et al. (2003a). ES cells were passaged every second day with daily media changes for at least three passages (6 days) prior to initiation of differentiation studies.

For differentiation experiments cells grown as described above were dissociated to single cells and differentiation was induced by seeding 2000 cells/cm^2 on gelatin-coated cell culture plastic in KO-DMEM supplemented with N2, B27, 0.1 mM nonessential amino acids, 2 mM L-glutamine, Penicillin/Streptomycin (all from Invitrogen), 0.1 mM 2-mercaptoethanol (Sigma-Aldrich) *without* LIF and BMP4. The medium was supplemented with one or more of the following growth factors, soluble receptors, and small molecule compounds: activin (3, 10, 30 or 100 ng/ml), Wnt3a (5 or 100 ng/ml), Nodal (1 μg/ml), BMP4 (10 ng/ml), Dkk1 (320 ng/ml; all from R&D Systems), and FGF2 (100 ng/ml; Invitrogen). Soluble FGF receptors (all from R&D Systems) were first used to achieve inhibition of ligands specific for both b and c splice forms by mixing sFGFR1IIIc, sFGFR2IIIb, and l sFGFR4 (12, 8 and 24 ng/ml, respectively). To achieve selective inhibition of the b or c splice form specific FGFs we mixed sFGFR1IIIb and sFGFR2IIIb (both at 250 ng/ml) or sFGFR1IIIc and sFGFR4 (both at 250 ng/ml), respectively. The medium containing FGF2 or sFGFRs was supplemented with 10 μg/ml heparan sulfate (Sigma-Aldrich), 1 μM SB431542 (Inman et al., 2002), 10 μM SU5402 or 100 nM PD173074 (Calbiochem). The cells were cultured for up to 7 days and the medium was changed daily, beginning at the second day of differentiation. It should be noted that our B27 supplement contain retinyl acetate which is a precursor during RA synthesis. However, experiments using B27 supplement without retinyl acetate (Invitrogen) did not affect the number of activin-induced Sox17-GFP^{Hi} endodermal cells. Further differentiation of day 5 activin-induced cultures was done by 3 days of additional culture in serum-free medium (KO-DMEM, N2, B27, 0.1 mM nonessential amino acids, 2 mM L-glutamine, Penicillin/Streptomycin, 0.1 mM 2-mercaptoethanol) supplemented with Wnt3a (5 ng/ml), FGF4 (10 ng/ml) and/or 0.1 μM all-trans retinoic acid (Sigma).

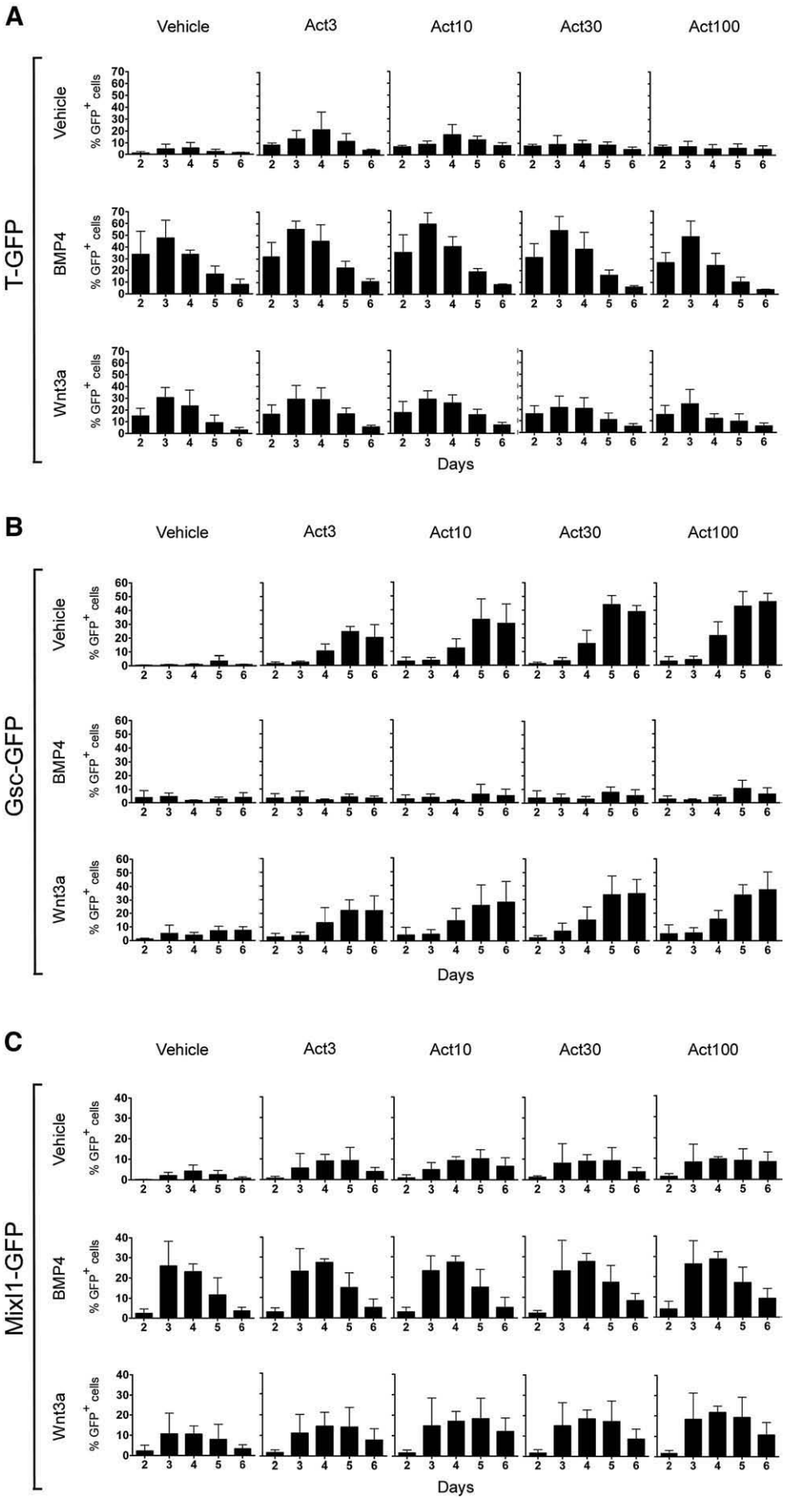
Differentiation in embryoid bodies (EBs) was carried out using the hanging drop method. Cells were dissociated to single cells using non-enzymatic Cell Dissociation Solution (Sigma-Aldrich) and diluted in N2B27 medium containing the relevant growth factors to yield 100 cells per 20 μl drop. Approximately 150 drops were applied to the lid of a 14 cm cell culture dish (Nunc), and placed upside down over autoclaved Millipore water. Drops were left overnight and EBs were washed down with HBSS w/o Ca^{2+} and Mg^{2+} and left to sediment for 3–4 min before removing the supernatant and transferring EBs to 50 mm Petri dishes (Sterilin) containing N2B27 medium with the relevant growth factors. The medium was changed daily. We frequently observed that 3–5 individual aggregates would form in the hanging drop yielding aggregates composed of 20–30 cells.

Flow cytometry

The cells were dissociated in 0.05% Trypsin-EDTA (Invitrogen) and a percentage of GFP$^+$ cells was analyzed on a FACSCalibur flow cytometer (BD Biosciences) at days 2–6 in at least three independent experiments. Mean % GFP$^+$ cells ± standard deviation (S.D.) was calculated and statistical analyses were performed using a two-tailed Student's *t*-test for paired samples, unless we had a clear expectation of the outcome in which case a one-tailed test was used. Sorting of GFP$^+$ cells for RNA extraction was performed on a FACSAria (BD Biosciences). CXCR4 expression was analyzed on a FACSCalibur flow cytometer using a human anti-CXCR4 monoclonal antibody (MAB172; R&D Systems). Cells were dissociated using Collagenase (Sigma-Aldrich) for 5 min. The cell suspension was fixed and stained as described below without permeabilization. Visual inspection of the stained cells by confocal microscopy confirmed surface localization of the antigen.

Immunofluorescence and X-gal staining

The cells were cultured on gelatin-coated chamber slides for 3, 5 or 8 days and fixed at room temperature for 30 min in 4% formaldehyde solution (Mallinckrodt Baker) for immunofluorescence or 5 min in 0.2% glutaraldehyde for X-gal staining. For immunofluorescence, the cells were permeabilized in graded ethanol followed by blocking in 10% donkey serum for 1 h and incubation with primary antibody for 1 h at room temperature or overnight at 4 °C. The following antibodies were used: goat anti-Foxa2 (Santa Cruz Biotechnology), goat anti-T (R&D Systems), rat anti-E-cadherin (E-cad; Zymed/Invitrogen), goat anti-Sox17 (R&D Systems), Alexa 448 conjugated rabbit anti-GFP (Molecular Probes/Invitrogen), rabbit anti-β-galactosidase (MP Biomedicals), rabbit anti-Lhx1 (Chemicon), goat and rabbit anti-Pdx1 (a kind gift from C. Wright), mouse and rabbit anti-Nkx6-1 (Jensen et al., 1996; Pedersen et al., 2006), rabbit anti-Sox2 (Chemicon) and mouse anti-Cdx2 (BioGenex). The cells were incubated with Cy2-, Cy3-, Texas Red- or Cy5-conjugated species-


A
Vehicle
Act3
Act10
Act30
Act100
T-GFP
Vehicle
BMP4
Wnt3a
% GFP+ cells
Days
B
Gsc-GFP
C
Mixl1-GFP

specific secondary antibodies (Jackson ImmunoResearch Laboratories) and 4′,6-diamidino-2-phenylindole (DAPI, MP Biomedicals). The Lhx1 staining used tyramide signal amplification (PerkinElmer) according to the manufacturer's recommendations. Negative controls, where the primary antibodies were omitted, were included for all stainings. These controls showed no unspecific staining of the secondary antibodies (data not shown). β-galactosidase activity was visualized by adding X-gal stain solution for 4 h at 37 °C. The slides were analyzed using an LSM 510 META laser scanning microscope (Carl Zeiss) or a BX60 epifluorescence microscope equipped with a DP71 camera (both from Olympus).

RT-PCR and qPCR

Cells were dissociated using 0.05% Trypsin-EDTA and collected by centrifugation. Total RNA was isolated using the RNeasy kit with DNAse treatment (Qiagen) following the manufacturer's protocol. cDNA was prepared from 5 or 100 ng RNA using MMLV Reverse Transcriptase (Invitrogen). PCR reactions were performed using 1 μl cDNA, 1 μl 20 μM primer mix and 23 μl Reddy Mix PCR master mix (Abgene). The PCR was carried out with an initial denaturation step at 96 °C for 2 min, followed by 32–38 cycles of 96 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min. The PCR was finished with a final extension step at 72 °C for 5 min. QPCR was performed using the standard SYBR® Green program with dissociation curve of the Mx3005P (Stratagene). PCR reaction was run in duplicates using 5 μl Brilliant® SYBR® Green QPCR Master Mix (Stratagene), 1 μl cDNA, 1 μl 10 μM primer mix and 3 μl DEPC-treated water. Quantified values for each gene of interest were normalized against the input determined by the housekeeping genes *G6pdh* and *Tbp*. The results are expressed as the relative expression level compared with the vehicle control condition or the scrambled control siRNA in the vehicle condition. Primer sequences are available on request.

siRNA transfection

The sequence effective for mouse β-catenin knock-down was designed using software available at the web site of Invitrogen (http://rnaidesigner.invitrogen.com/rnaiexpress/). The β-catenin (accession number NM_0079614.2) target sequences of the STEALTH siRNAs were 5′-GCCTTCATTATGGACTGCCTGTTGT-3′ (siRNA1) and 5′-GAGCAAGGCTTTTCCCAGTCCTTCA-3′ (siRNA2). The STEALTH negative control siRNA (scrambled; Invitrogen) has been used as negative control and is labeled “scrambled”.

The cells were cultured on gelatin-treated 24-well plates as previously described with a starting density of 4000 cells/cm^2. After 1 day of differentiation the cells were transfected with 100 nM STEALTH siRNAs using Lipofectamine2000 according to the manufacturer's instructions. The transfected cells were grown further and analyzed for β-catenin expression by western blot at days 3 and 5 of differentiation. QPCR and flow cytometry analysis was performed at day 5.

Western blot

Total cell lysates were obtained on days 3 and 5 of differentiation culture using RIPA lysis buffer. 20 μg of each protein sample were loaded and analyzed by western blot using a rabbit anti-β-catenin monoclonal antibody (Lab Vision) and a mouse anti-β-Actin monoclonal antibody (Sigma-Aldrich) as primary antibodies as well as secondary HRP-conjugated antibodies (Santa Cruz Biotechnology).

Generation of stable Wnt-reporter ES cell lines and chimera formation

The SuTOP-CFP construct was generated by cutting the luciferase gene from the Super8XTOPFLASH Wnt reporter (generously provided by Dr. R. Moon, University of Seattle, WA) with Fse and Nco1 and replacing it with Cerulean PCR product from the pmCerulean-C1 vector (Rizzo et al., 2004). To generate stably transfected cell lines, E14 cells (Hooper et al., 1987) of low passage number were co-transfected with SuTOP-CFP and a ploxP-Neo vector, conferring resistance to Neomycin. The relationship between plasmids was 10:1 (reporter: ploxP-Neo). Transfection was carried out using Lipofectamine2000 (Invitrogen) and 24 h after transfection, selection was added (200 μg/ml G418, Invitrogen). Medium was changed daily for 9 days and 0.5 μM BIO (GSK3β inhibitor, Calbiochem), which activates canonical Wnt signaling, was added to the medium for the last 2 days of selection to identify Wnt-responsive colonies. Fluorescent colonies were then picked and expanded and one clone was selected for chimera formation via blastocyst injection.

E3.5 blastocysts were harvested by flushing the uteri of mature, time mated NMRI mice (Taconic). Chimeric embryos were generated by injection of approximately 10 SuTOP-CFP ES cells into the blastocoel using a paraffin-oil driven manual injector (Cell Tram Vario, Eppendorf) and a Narishige micromanipulator. Following 3 h of culture in M16 medium, embryos were transferred to the uterus of E2.5 pseudo-pregnant, 7 week old NMRI foster mothers. Care of the animals was done according to institutional guidelines. Embryos were harvested at E10.5 and fixed in Lilly's fixative (4% phosphate buffered formaldehyde) for 30 min before being analyzed for native Cerulean fluorescence. All animal experiments were performed in accordance with institutional and national regulations.

Chick embryo grafting

Fertilized eggs from white leghorn chicken were purchased from Triova and incubated at 38 °C to Hamburger and Hamilton (HH) stages 8–10 (Hamburger and Hamilton, 1951). The embryos were explanted as previously described (Chapman et al., 2001). E14 ES cell progeny was prepared for grafting by labeling with fluorescent CMTMR CellTracker dye (Molecular Probes/Invitrogen). Clumps of cells were scraped off, washed in PBS and inserted between endoderm and mesoderm of chicken embryos via a small incision in the endoderm. Grafted embryos were incubated for 48 h in a humidified incubator at 38 °C. The embryos were isolated, washed in PBS, fixed in 4% PFA at room temperature for 2 h and stored in methanol at −20 °C until the time of analysis. Whole-mount immunofluorescent analyses of grafted chicken embryos were performed as previously described (Ahnfelt-Ronne et al., 2007).

Results

Dose-dependent effects of activin on the expression of PS genes are modulated by BMP and Wnt signaling

Previous studies have demonstrated induction of *Brachyury* (*T*) and *Goosecoid* (*Gsc*) by activin in mES cells (Gadue et al., 2006; Kubo et al., 2004; Tada et al., 2005; Yasunaga et al., 2005). However, differences in media compositions and the culture methods used make a direct comparison of the response of these two genes to varying doses of activin difficult. Prior to the induction of differentiation by addition of growth factors, we culture the undifferentiated ES cells under defined conditions (Ying et al., 2003a). As activin is known to dose-dependently regulate *T* and *Gsc* expression in *Xenopus* animal cap

Fig. 1. Activin, BMP and Wnt signaling control the dynamic expression of primitive streak genes in differentiating ES cells. Flow cytometric analysis of $T^{Gfp/+}$ (A), $Gsc^{Gfp/+}$ (B), or $Mixl1^{Gfp/+}$ (C) cells grown in adherent culture for up to 6 days in serum-free media containing 0, 3, 10, 30 or 100 ng/ml activin in the presence or absence of 10 ng/ml BMP4 or 100 ng/ml Wnt3a. The mean % GFP^+ cells ± standard deviation of three independent experiments is presented.

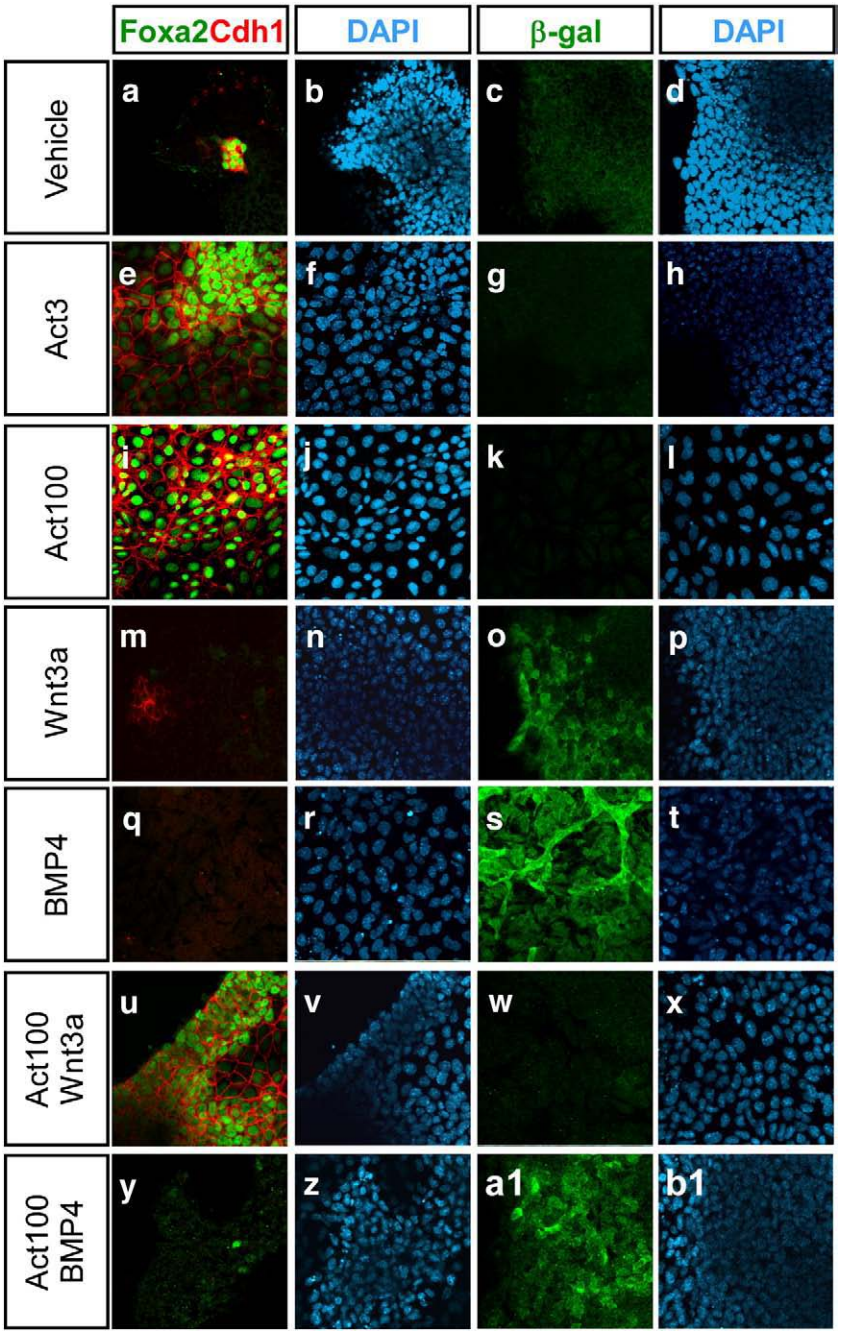


Fig. 2. BMP4 but not Wnt3a inhibits the expression of Foxa2 and E-cadherin, and promotes expression of *Flk1* in the presence of activin. The expression of Foxa2, E-cad (Cdh1), and Flk1 (β-gal) was analyzed by immunofluorescence in Flk1$^{LacZ/+}$ ES cells cultured for 5 days in media containing 0, 3 or 100 ng/ml activin, 100 ng/ml Wnt3a, 10 ng/ml BMP4, 100 ng/ml activin + 100 ng/ml Wnt3a, or 100 ng/ml activin + 10 ng/ml BMP4.

cells such that low doses will activate *T* and high doses will activate *Gsc* (Dyson and Gurdon, 1998; Green et al., 1992; Gurdon et al., 1994, 1999; Latinkic et al., 1997), we initially tested if exposure of mES cells to increasing doses of activin would lead to a shift from *T* to *Gsc* expression. ES cell lines carrying *T-Gfp* (T$^{Gfp/+}$) and *Gsc-Gfp* (Gsc$^{Gfp/+}$) knock-in alleles (Fehling et al., 2003; Tada et al., 2005) were cultured with increasing amounts of activin (from 3 to 100 ng/ml) and the number of GFP$^+$ cells was analyzed by flow cytometry. Examples of primary flow cytometry diagrams are shown in Fig. S1. When GFP$^+$ cells were quantitated we found that 3 ng/ml activin transiently induced 21 ± 15% T-GFP$^+$ cells (mean % ± S.D., $n = 3$) peaking at day 4 (Fig. 1A). However, this induction was not statistically significant. At higher activin concentrations the number of T-GFP$^+$ cells declined gradually such that the highest dose (100 ng/ml) resulted in only 5 ± 4% T-GFP$^+$ cells at day 4, comparable to the control samples cultured in the absence of activin (6 ± 5% T-GFP$^+$ cells, Fig. 1A). In contrast, flow cytometric analyses of Gsc$^{Gfp/+}$ cells showed that the expression of this anterior PS marker (Blum et al., 1992) was induced by activin in a dose-dependent manner with expression peaking at days 5–6 (Fig. 1B). 3 ng/ml activin induced 25 ± 4% Gsc-GFP$^+$ cells at day 5, and this number increased with increasing concentration of activin reaching 43 ± 11% Gsc-GFP$^+$ cells in cultures treated with 100 ng/ml activin (Fig. 1B). Control samples grown in the absence of activin contained 3 ± 4% Gsc-GFP$^+$ cells at this time point. The induction of *Gsc* expression at day 5 was statistically significant for all activin concentrations tested ($p < 0.05$). Thus, similar to the case in *Xenopus* animal cap cells, low doses of activin support *T* expression while high doses stimulate *Gsc* expression. Intriguingly, while

expression of the PS marker *Mixl1* (Pearce and Evans, 1999) was induced by activin, the number of Mixl1-GFP$^+$ cells was independent of the activin concentration (Fig. 1C).

In vivo, BMP4 is a ventralizing agent, acting during gastrulation to induce *T* and repress *Gsc* expression (Fainsod et al., 1994; Jones et al., 1996; Steinbeisser et al., 1995). We found a strong but transient induction of *T* expression in response to BMP4 (Fig. 1A). Peaking at day 3, we observed 48 ± 15% T-GFP$^+$ cells, which was significantly higher than detected in vehicle-treated cells ($p<0.05$). The induction of *T* expression by BMP4 was observed regardless of the presence or absence of activin. However, the activin-mediated induction of Gsc-GFP$^+$ cells at day 5 was strongly inhibited by BMP4 ($p<0.05$), irrespective of the activin concentration (Fig. 1B). Cultures containing BMP4 never contained more than 10 ± 6% Gsc-GFP$^+$ cells, which is comparable to vehicle-treated cells. BMP4 also stimulated *Mixl1* expression peaking at day 3 with 26 ± 12% Mixl1-GFP$^+$ cells but further addition of activin did not affect the number of Mixl1-GFP$^+$ cells (Fig. 1C).

In *Xenopus*, Wnt molecules have both organizer-inducing and posteriorizing activities (Niehrs, 2004) and in mice Wnt3 is required for proper axis formation and induction of the primitive streak (Barrow et al., 2007; Liu et al., 1999). We therefore tested the ability of Wnt3a by itself or in combination with different doses of activin to induce PS markers. 5 ng/ml Wnt3a induced 23 ± 4% T-GFP$^+$ cells (data not shown), whereas 100 ng/ml induced 30 ± 9% T-GFP$^+$ cells at day 3, significantly higher than the control cells (Fig. 1A; $p<0.05$). Activin did not have a significant effect on Wnt3a-induced *T* expression although we observed a tendency to reduced numbers of T-GFP$^+$ cells with the highest doses of activin. The prominent induction of *T* expression seen with both BMP4 and Wnt3a was confirmed by immunofluorescence at day 3 (Fig. S2A). Wnt3a also induced *Mixl1* expression. Cultures containing 100 ng/ml Wnt3a contained 11 ± 4% Mixl1-GFP$^+$ cells at day 4. Notably, the combination of 100 ng/ml activin and 100 ng/ml Wnt3a induced 22 ± 3% Mixl1-GFP$^+$ cells at day 4, approximately twice that achieved by either factor alone (Fig. 1C; $p<0.05$). When examining co-expression of T and GFP at day 3 by immunofluorescence we found that most, if not all, *Mixl1* expressing cells also expressed T, while the converse was not the case (Fig. S2B). While Wnt3a stimulated T and Mixl1 expression, it did not affect the number of Gsc-GFP$^+$ cells (Figs. 1B and S2). The induction of *Gsc* expression by activin in the presence or absence of Wnt3a and its inhibition by BMP4 was confirmed by immunofluorescence at day 5 (Fig. S2C). Notably, the few T-expressing cells present in activin-treated Gsc$^{Gfp/+}$ cells after 5 days did not express GFP. Considering that T is found not only in the PS, but also in the emerging mesoderm at the late gastrula stage (Inman and Downs, 2006), this may indicate that mesoderm is also formed in activin-treated cultures. Collectively, the different ES lines all responded similarly to growth factor treatment (Fig. S2). Overall, our results indicate an anteriorizing role of activin during ES cell differentiation that can be modulated by the posteriorizing factors BMP4 and Wnt3a, consistent with the roles of these factors before and during gastrulation (reviewed in Tam and Loebel, 2007).

BMP4 induces mesoderm and blocks activin-mediated induction of definitive endoderm

To establish if our cultures contained embryonic or extraembryonic cell types we first examined expression of CXCR4 (chemokine (C-X-C motif) receptor-4), which is expressed in embryonic but not in extraembryonic tissues (McGrath et al., 1999; Sherwood et al., 2007). Using FACS analysis we compared surface expression of CXCR4 on cells isolated from dissociated E11 mouse embryo heads with that of differentiated ES cell progeny. Two distinct CXCR4-expression populations could be detected among cells from mouse embryos, a CXCR4^{lo} and a CXCR4^{hi} population (Fig. S3A). When ES cell progeny from either vehicle or activin-treated cultures was analyzed it was clear that the vast

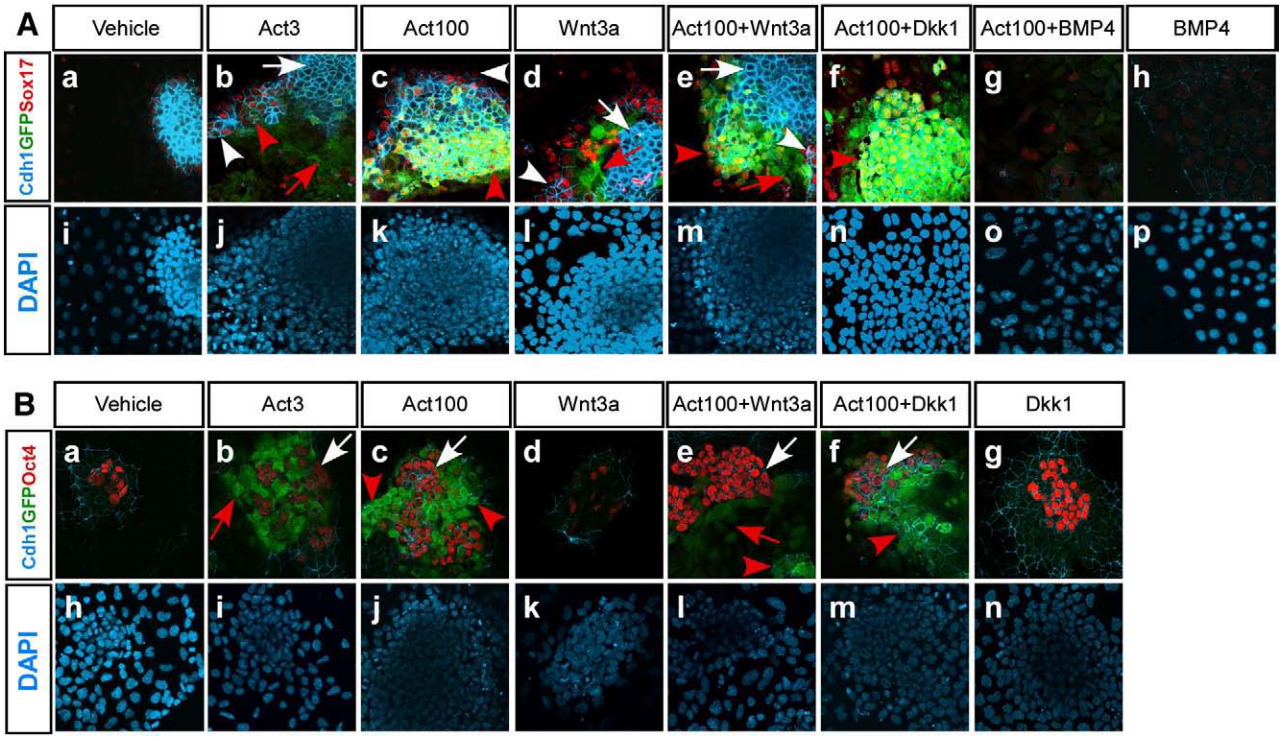


Fig. 3. Activin-induced expression of the anterior primitive streak marker Gsc is inhibited by BMP4 but not by Dkk1. Gsc$^{Gfp/+}$ ES cells were grown in serum-free medium supplemented with one or more of the following growth factors or inhibitor; 10 ng/ml BMP4, 100 ng/ml Wnt3a, 320 ng/ml Dkk1, 3 or 100 ng/ml activin as indicated. (A) Triple-label immunofluorescence was performed to analyze the co-expression of E-cad (Cdh1), Gsc (GFP), and Sox17 in cells grown for 5 days under the indicated conditions. Note Cdh1$^+$GFP$^-$Sox17$^-$ cells (white arrows), Cdh1$^-$GFP$^+$Sox17$^-$ cells (red arrows), Cdh1$^+$GFP$^-$Sox17$^+$ cells (white arrowheads), and Cdh1$^+$GFP$^+$Sox17$^+$ cells (with yellow nuclei, red arrowheads) (B) Triple-label immunofluorescence was also performed to analyze co-expression of Cdh1, Gsc (GFP), and Oct4 on day 5. Note Cdh1$^+$GFP$^-$Oct4$^+$ cells (white arrows), Cdh1$^-$GFP$^+$Oct4$^-$ cells (red arrows), and Cdh1$^+$GFP$^+$Oct4$^-$ cells (red arrowheads).

majority of the cells were CXCR4^{hi} (Fig. S3A), indicating that most cells, in both vehicles and activin-treated cultures, are embryonic rather than extraembryonic in nature. Furthermore, significant levels of *Sox7* (SRY-box containing gene 7) transcripts, which is exclusively expressed in the extraembryonic part of the endoderm in the gastrula stage embryo (Kanai-Azuma et al., 2002), could only be detected in Wnt3a-treated cultures but not in vehicle, BMP4-, or activin-treated cultures (Fig. S3B).

Having established the embryonic nature of the ES cell progeny in our cultures we next examined the expression of a number of germ layer specific markers. We initially analyzed expression of the transcription factor gene *Foxa2* and the epithelial marker E-cadherin (E-cad; *Cdh1*), both of which are expressed in developing endoderm (Ang et al., 1993; Sasaki and Hogan, 1993). Immunofluorescent staining of cells grown in 3 or 100 ng/ml activin showed that these cultures contained many Foxa2$^+$Cdh1$^+$ cells compared to vehicle-treated samples (Fig. 2). Notably, addition of BMP4 (10 ng/ml) but not Wnt3a (100 ng/ml) was able to drastically reduce the number of Foxa2$^+$Cdh1$^+$ cells induced by activin (Fig. 2). Analysis of VEGF receptor-2 (*Kdr* or *Flk1*) expression using *Flk1*-LacZ ES cells revealed that both BMP4 (with or without 100 ng/ml activin) and Wnt3a were capable of inducing *Flk1*-expressing cells (Fig. 2), indicative of mesoderm formation (Ema et al., 2006).

Wnt signaling augments the development of Sox17-expressing definitive endoderm induced by activin

Based on the analysis of Foxa2 and Cdh1 expression it was not clear if the concentration of activin used influenced subsequent differentiation towards DE. Furthermore, analyses of Foxa2 and Cdh1 expression cannot distinguish between DE from different A–P positions. We therefore examined the number of Gsc$^{Gfp/+}$ cells that co-expressed GFP, Cdh1, and Sox17 by ICC as an indicator of anterior DE (ADE), in response to varying doses of activin with or without additional BMP4, Wnt3a, or Dkk1 treatment. Notably, we found that 100 ng/ml activin resulted in higher numbers of Cdh1$^+$GFP$^+$Sox17$^+$ triple positive cells than seen with 3 ng/ml activin (Fig. 3A, compare panels b and c), supporting that efficient formation of ADE depends on the activin concentration (Yasunaga et al., 2005). Treatment with 3 ng/ml activin resulted in many Cdh1$^+$ cells but the majority of these were not co-expressing GFP or Sox17 and most likely represent undifferentiated ES cells (see below). Most of the GFP$^+$ cells generated in response to 3 ng/ml activin were Cdh1$^-$Sox17$^-$, suggesting that they may represent mesoderm (Fig. 3A, panel b). Similarly, after treatment with Wnt3a alone (100 ng/ml) most GFP$^+$ cells were Cdh1$^-$Sox17$^-$ (Fig. 3A, panel d). We tested if Wnt signaling was required for the development of

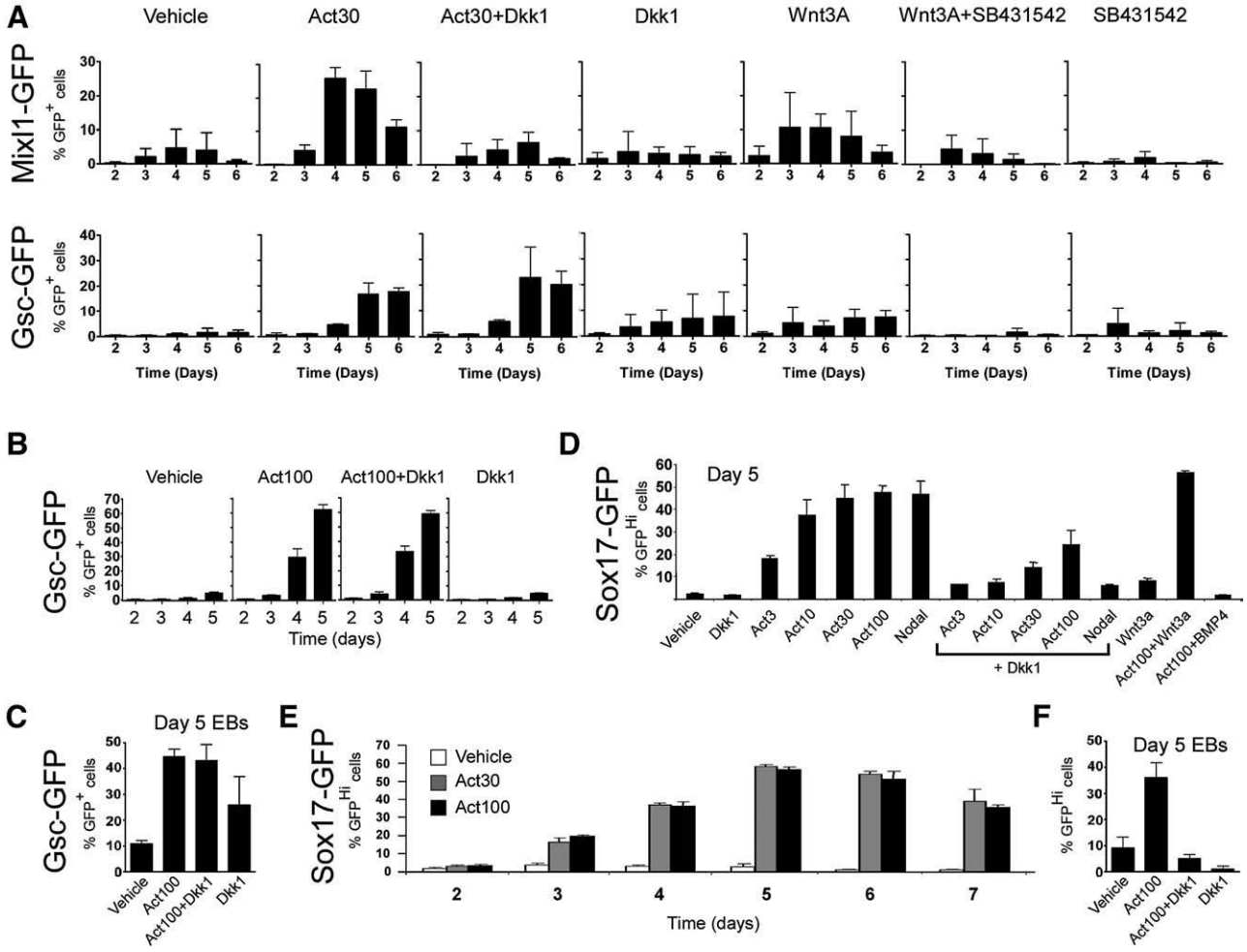


Fig. 4. The requirement for canonical Wnt signaling during activin-induced *Sox17* expression is more pronounced in aggregate culture than in adherent culture, and Dkk1 inhibits nodal-induced Sox17 expression more than activin-induced Sox17 expression. (A) Nodal/activin and Wnt signaling interactions were analyzed in Mixl1$^{Gfp/+}$ and Gsc$^{Gfp/+}$ cells at days 2–6 of differentiation using flow cytometry. (B) Gsc$^{Gfp/+}$ cells cultured in the presence of Dkk1 prior to and during activin induction were analyzed by flow cytometry. (C) Gsc$^{Gfp/+}$ cells were induced to form embryoid bodies in the presence of the indicated growth factors and analyzed for GFP expression by flow cytometry after 5 days of culture. (D) Sox17$^{Gfp/+}$ cells cultured for 5 days were analyzed for GFP expression by flow cytometry. The mean % GFP$^+$ cells ± S.E.M. of three independent experiments is presented. (E) Sox17$^{Gfp/+}$ cells cultured for 2–7 days in the presence of activin (30 or 100 ng/ml) were analyzed for GFP expression by flow cytometry at the indicated time points. (F) Sox17$^{Gfp/+}$ cells were induced to form embryoid bodies in the presence of the indicated growth factors and analyzed for GFP expression by flow cytometry after 5 days of culture. The mean % GFP$^+$ cells ± standard deviation of three independent experiments is presented for all flow cytometric analyses unless otherwise noted.

Cdh1$^+$GFP$^+$Sox17$^+$ triple positive cells in response to activin and found that such cells were still generated efficiently in response to 100 ng/ml activin if Dkk1 was included (Fig. 3A, panel f). In contrast, addition of BMP4 completely prevented the development of such cells (Fig. 3A, panel g). We often found clusters of Cdh1$^+$GFP$^-$Sox17$^-$ cells in cultures treated with different combinations of activin and Wnt3a (Fig. 3A, panels b–e). Immunocytochemistry revealed that many Cdh1$^+$Gsc$^-$ cells were Oct4$^+$ and thus likely represent undifferentiated ES cells (Fig. 3B). Attempts to quantify the relative number of Gsc-GFP$^+$Cdh1$^+$ and Gsc-GFP$^-$Cdh1$^+$ cells by FACS under the various conditions failed due to problems with achieving reliable FACS data using the Chd1 monoclonal antibodies available.

Although it was not evident from the above experiments with the Gsc$^{Gfp/+}$ cells that canonical Wnt signaling influenced the expression of PS markers or the formation of DE in response to activin we wanted to examine closer if Wnt activity was required for expression of other PS markers and DE formation in our ES cell cultures since gastrulation and thereby also endoderm formation requires Wnt3 activity in vivo (Barrow et al., 2007; Liu et al., 1999) and because we detected expression of Wnt3 and Wnt3a in our differentiating ES cell cultures (Fig. S4). To test this notion, we first cultured Mixl1$^{Gfp/+}$ cells with activin or Wnt3a in the presence or absence of 320 ng/ml Dkk1 or 1 μM of the ALK4/5/7-specific inhibitor SB431542, respectively. The concentration of the inhibitors was titrated by using a Wnt- or activin-responsive luciferase assay (data not shown), choosing the concentration that blocked the response to exogenous Wnt3a or activin, respectively, without causing non-specific toxicity in ES cells. When analyzing the number of Mixl1-GFP$^+$ cells after 4 days of activin treatment (30 ng/ml) we found that these were significantly reduced when Dkk1 was included (26 ± 3% vs. 4 ± 3%; $p<0.05$; Fig. 4A). Similarly, the number of Mixl1-GFP$^+$ cells induced by 100 ng/ml Wnt3a was reduced from 11 ± 4% on day 4 to 3 ± 4% by simultaneous SB431542 treatment (Fig. 4A). Thus, activin and Wnt3a act cooperatively to induce *Mixl1* expression and both signaling pathways are required for *Mixl1* expression in ES cell progeny. Although Wnt3a only induced low numbers of *Gsc*-expressing cells, these were dependent on endogenous nodal/activin signaling as SB431542 significantly inhibited the development of Gsc-GFP$^+$ cells in response to Wnt3a (Fig. 4A). In contrast, FACS analyses confirmed that activin-induced *Gsc* expression was not inhibited by Dkk1 treatment. Cultures of Gsc$^{Gfp/+}$ cells contained 17 ± 4% Gsc-GFP$^+$ cells at day 5 when grown in 30 ng/ml activin and 23 ± 12% when cultured in the presence of activin and Dkk1 (Fig. 4A) consistent with the above mentioned immunofluorescent analysis that demonstrated that Gsc-GFP$^+$Sox17$^+$ Cdh1$^+$ triple positive cells were efficiently generated in response to activin treatment, regardless of the presence of Dkk1. Undifferentiated ES cells have a low level of active canonical Wnt signaling despite the lack of exogenously added Wnt factors (Sato et al., 2004). To test if this low level of Wnt signaling has any influence on the later activation of *Gsc* expression we cultured undifferentiated Gsc$^{Gfp/+}$ cells for three passages in media containing Dkk1 before differentiation was induced by removing LIF and BMP4 and adding activin and Dkk1 for 5 days. We found that inclusion of Dkk1 prior to differentiation did not prevent activin from efficiently inducing *Gsc* expression (Fig. 4B). Furthermore, Dkk1 failed to prevent activin from inducing Gsc-GFP$^+$ cells in aggregate culture (Fig. 4C).

To obtain quantitative data on the number of DE cells after growth factor treatment we subjected a Sox17$^{Gfp/+}$ reporter line (Kim et al., 2007) to our differentiation protocol. At mid-streak stage *Sox17* expression marks the definitive endoderm forming adjacent to the anterior end of the PS. Simultaneous with the movement of DE to the anterior region of the gastrula, the *Sox17* expression domain expands to include the endoderm underlying the neural plate of the early tail-bud-stage embryo (Kanai-Azuma et al., 2002). Thus, *Sox17* is an early marker that similar to genes such as *Hex*, *Foxa2*, and *Cer1*, are expressed simultaneously in the anterior visceral endoderm and the DE in the embryonic part of the gastrulating embryo. Sox17$^{Gfp/+}$ cells express a low level of GFP (Sox17-GFP^{Lo}) when kept undifferentiated in the presence of LIF and BMP4 (not shown). Differentiation under neural promoting conditions (Ying et al., 2003a) results in the development of a Sox17-GFP$^-$ population and some remaining Sox17-GFP^{Lo} cells while treatment with activin for 5 days induces Sox17-GFP^{Hi} and Sox17-GFP$^-$ populations in addition to a remaining Sox17^{Lo} population (Fig. S1). Increasing concentrations of activin resulted in development of progressively more Sox17-GFP^{Hi} cells with the highest numbers reached with 30 and 100 ng/ml of activin (Fig. 4D). We observed an increase in Sox17-GFP^{Hi} cells over time, peaking at day 5, followed by a modest decrease at days 6 and 7 (Fig. 4E). The induction of Sox17-GFP^{Hi} cells was at least partly dependent on Wnt signaling as treatment with Dkk1 reduced the number of GFP^{Hi} cells by ~50% at the highest activin concentration (Fig. 4D). Notably, the number of Sox17-GFP^{Hi} cells induced by 1 μg/ml nodal appeared more strongly reduced in response to Dkk1 treatment than did a similar number of Sox17-GFP^{Hi} cells induced by 30 and 100 ng/ml of activin (Fig. 4D). Furthermore, when differentiation was performed in aggregate culture, which may rely more on endogenous signaling (Sachlos and Auguste, 2008; ten Berge et al., 2008), the number of activin-induced Sox17-GFP^{Hi} cells were strongly reduced by Dkk1 treatment (Fig. 4F). As expected, the addition of BMP4 prevented activin-induced formation of Sox17-GFP^{Hi} cells ($p<0.001$), while addition of Wnt3a resulted in a marginal, but significant ($p<0.05$) increase in the development of Sox17-GFP^{Hi} cells (Fig. 4D).

To further define the time at which canonical Wnt signaling was required for the formation of T-, Gsc- and Sox17-GFP^{Hi} cells, we cultured the cells with activin for three (T$^{Gfp/+}$) or five (Gsc$^{Gfp/+}$ and Sox17$^{Gfp/+}$) days and added either Wnt3a or Dkk1 for shorter periods

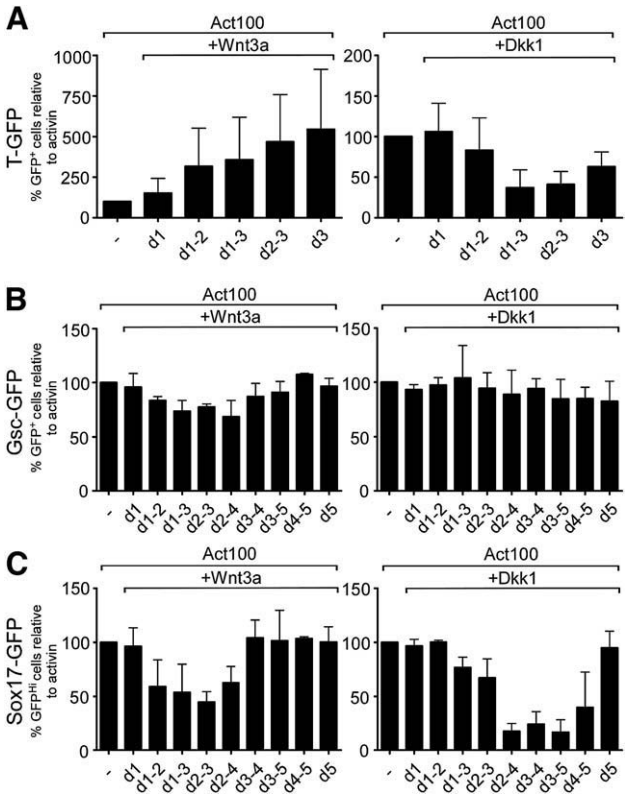


Fig. 5. Wnt signaling is required during late stages of activin-induced definitive endoderm formation. ES cells were cultured in serum-free medium with 100 ng/ml activin and supplemented with 100 ng/ml Wnt3a or 320 ng/ml Dkk1 for a variable number of days. T$^{Gfp/+}$ cells (A) were cultured for 3 days and Gsc$^{Gfp/+}$ (B) and Sox17$^{Gfp/+}$ (C) cells were cultured for 5 days before being analyzed for GFP expression by flow cytometry. The mean % GFP$^+$ cells ± standard deviation of three independent experiments is presented.

during the 5 day activin treatment. To monitor the effectiveness of Dkk1 treatment in reducing canonical Wnt signaling we first generated an ES cell line stably transfected with a SuperTOP-Cerulean reporter (SuTOP-CFP) and established its ability to report Wnt signaling in E10.5 chimeric embryos after injection into E3.5 blastocysts and implantation into pseudo-pregnant females. As shown in Fig. S5A, native Cerulean fluorescence can be observed in several sites known to harbor active Wnt signaling at this stage, including the peripheral aspects of the otic vesicles (Maretto et al., 2003). When SuTOP-CFP cells were cultured in activin we found that CFP^+ colonies developed at day 3 even in the absence of exogenous Wnt addition and that additional treatment with Dkk1 reduced or abolished reporter activity depending on the duration of treatment (Fig. S5B). Similarly, when SuTOP-CFP cells were assayed at day 5, we found that Dkk1 treatment at days 3–4 or 4–5 inhibited reporter activity (Fig. S5C). Thus, treatment with Dkk1 for as little as 1 to 2 days at the concentration used appeared quite effective in suppressing SuTOP reporter activity.

We then analyzed the number of $T\text{-}GFP^+$ cells developing in response to activin when Wnt signaling was experimentally perturbed. $T\text{-}GFP^+$ cell numbers were augmented by Wnt3a with the largest effect reached when Wnt3a was added only at day 3. Treatment with Dkk1 on the other hand reduced the number of $T\text{-}GFP^+$ cells most effectively when included in that last part of the three day period (Fig. 5A). Neither stimulating nor inhibiting Wnt signaling in $Gsc^{Gfp/+}$ cells, by treatment with Wnt3a and Dkk1, respectively, resulted in a significant change in the number of activin-induced GFP^+ cells irrespective of the treatment period (Fig. 5B).

However, treatment of $Sox17^{Gfp/+}$ cells with Wnt3a prior to appearance of GFP^+ cells reduced the number of $Sox17\text{-}GFP^{Hi}$ cells, while later Wnt3a treatment had no effect. Conversely, treatment with Dkk1 reduced the number of $Sox17\text{-}GFP^{Hi}$ cells only if present after the first appearance of these (Fig. 5C).

We next used siRNA mediated knock-down of β-catenin (encoded by *Ctnnb1*) to confirm that inhibition of canonical Wnt signaling at the latter part of the 5 day activin stimulation period could suppress the appearance of $Sox17\text{-}GFP^{Hi}$ cells and to further test the $Gsc^{Gfp/+}$ cell line which appeared refractory to Wnt inhibition in previous experiments. We transfected $Gsc^{Gfp/+}$ and $Sox17^{Gfp/+}$ cells with control and two different *Ctnnb1* siRNAs at day 2 of differentiation and assayed β-catenin expression by western blotting at days 3 and 5. In $Gsc^{Gfp/+}$ cells we found a reduction of β-catenin expression at day 3 which was normalized at day 5. siRNA treatment appeared more effective in $Sox17^{Gfp/+}$ cells with strong inhibition of β-catenin expression at day 3 which was only partly recovered by day 5 (Fig. 6A). FACS analysis at day 5 showed a reduction in the number of $Gsc\text{-}GFP^+$ cells after β-catenin knock-down, although this only reached significance in *Ctnnb1* siRNA2 treated samples ($p<0.05$, Fig. 6B). Furthermore, Q-RT-PCR analysis of $Gsc^{Gfp/+}$ cells after β-catenin knock-down did not reveal significant changes in expression of *Lhx1* and *Chrd* (Fig. S6).

However, in agreement with the results obtained with Dkk1 treatment, we found a prominent reduction in the number of $Sox17\text{-}GFP^{Hi}$ cells at day 5, in both siRNA1 and siRNA2 treated samples ($p<0.05$, Fig. 6B).

Activin dose-dependently induces an anterior gene expression pattern

To determine whether the DE formed in the presence of high concentrations of activin was anterior or posterior in character we analyzed the expression of a number of markers displaying differential expression depending on the anterior–posterior position of the cells. RT-PCR analyses at day 5 showed that activin, regardless of concentration, could induce expression of genes associated with anterior cell fates, including *Lefty1*, *Hex*, (Martinez-Barbera et al., 2000) and *Otx2*, (Rhinn et al., 1998). However, robust expression of

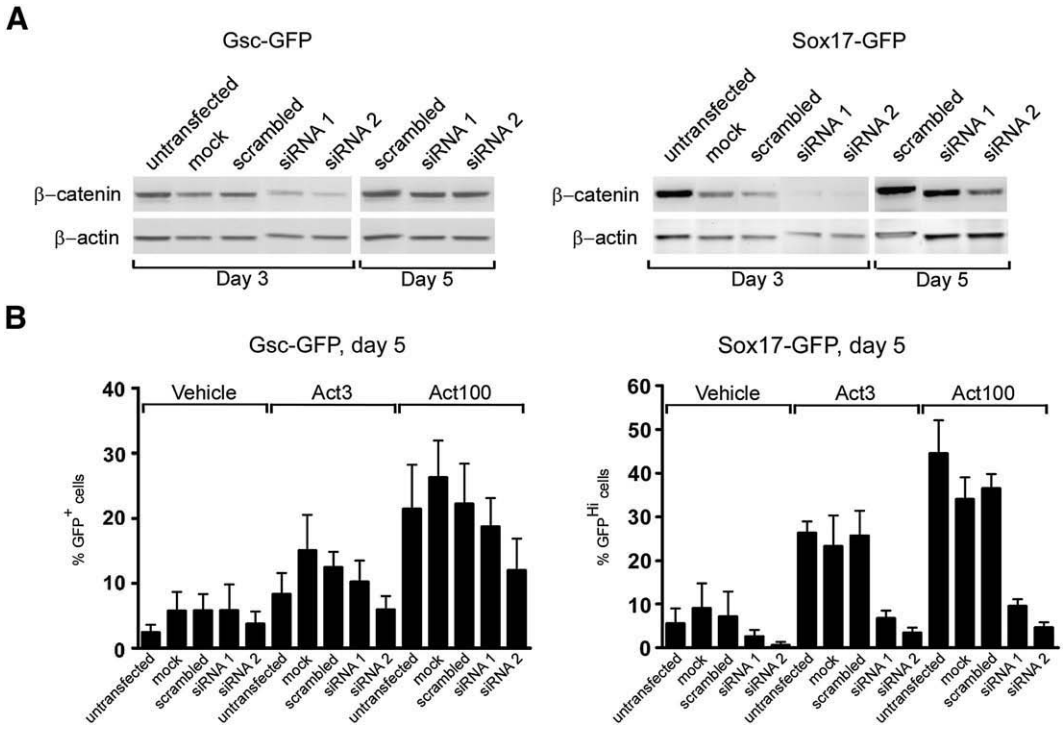


Fig. 6. Inhibition of canonical Wnt signaling inhibits activin-induced $Sox17\text{-}GFP^{Hi}$ definitive endoderm formation, but has little effect on *Gsc* expression. (A) Western blot analysis of β-catenin expression after siRNA mediated knock-down. Two different siRNAs targeting β-catenin (siRNA1 and siRNA2) or a scrambled control siRNA were introduced to $Gsc^{Gfp/+}$ and $Sox17^{Gfp/+}$ cell lines on day 2 and β-catenin levels assayed at day 3 and 5. An anti-β-actin western blot served as loading control. (B) siRNA treated $Gsc^{Gfp/+}$ and $Sox17^{Gfp/+}$ cells were analyzed for GFP expression by flow cytometry after 5 days of serum-free culture supplemented with 0, 3 or 100 ng/ml activin. The mean % GFP^+ cells ± standard deviation of three independent experiments is presented. Untransfected and mock transfected controls are also shown.

Cer1 as well as repression of the posterior marker *Cdx2* was only seen in the presence of the high activin concentration (Fig. 7A). In contrast, *Cdx2* (Beck et al., 1995) was expressed in samples treated with 10 ng/ml BMP4 or 100 ng/ml Wnt3a, as well as cells treated with the low dose of activin (Fig. 7A). We next isolated Gsc-GFP^{+} and Gsc-GFP^{-} cells as well as Sox17-GFP^{Hi}, Sox17-GFP^{Lo} and Sox17-GFP^{-} cells from activin-stimulated cultures at day 5 by FACS and prepared RNA for gene expression analysis. Message for *Otx2* and *Cer1* was detected in both Gsc-GFP^{+} and Gsc-GFP^{-} fractions, but with an enrichment in the GFP^{+} fraction (Fig. 7B). Cer1 was also enriched in Sox17-GFP^{Hi} cells compared to Sox17-GFP^{-} and Sox17-GFP^{Lo} cells (Fig. 7B). Furthermore, when analyzing the expression of the respective DE and VE markers, *Pyy* and *Tdh* (Hou et al., 2007; Sherwood et al., 2007) we only found *Pyy* in the Sox17-GFP^{Hi} cells while *Tdh* was found in the Sox17-GFP^{-} and, to a lesser degree, Sox17-GFP^{Lo} populations (Fig. 7B). However, it should be noted that FACS sorted populations are probably not completely pure. For example, the Sox17^{Hi} and Sox17^{Lo} populations are likely displaying "cross-contamination" to some extent. Finally, as Lhx1 is expressed in the anterior part of the PS in the late-streak embryo and expected to co-localize with Gsc (Shawlot and Behringer, 1995; Tada et al., 2005) we performed GFP-Lhx1 double immunofluorescent stainings on Gsc$^{Gfp/+}$ cells after 5 days of differentiation. As expected, we found Gsc-GFP^{+}Lhx1^{+} double positive cells in activin-treated cultures and the formation of these cells was inhibited by the addition of BMP4 but not Wnt3a (Fig. 7C).

FGF receptor signaling is required for mesendoderm and endoderm differentiation

If FGF signaling is compromised during early development as in FGF8, FGFR1, or UDP-glucose dehydrogenase mutants, the embryos' arrest at gastrulation and no embryonic mesoderm- or endoderm-derived tissues develop (Ciruna et al., 1997; Deng et al., 1994; Garcia-Garcia and Anderson, 2003; Sun et al., 1999; Yamaguchi et al., 1994). The phenotype is associated with a failure of migration and it is unclear to what degree FGF signaling regulates allocation of mesodermal and endodermal fates. However, studies in zebrafish indicate that FGF signaling promotes mesodermal development at the expense of endodermal development (Mathieu et al., 2004; Mizoguchi et al., 2006; Poulain et al., 2006). We first examined the

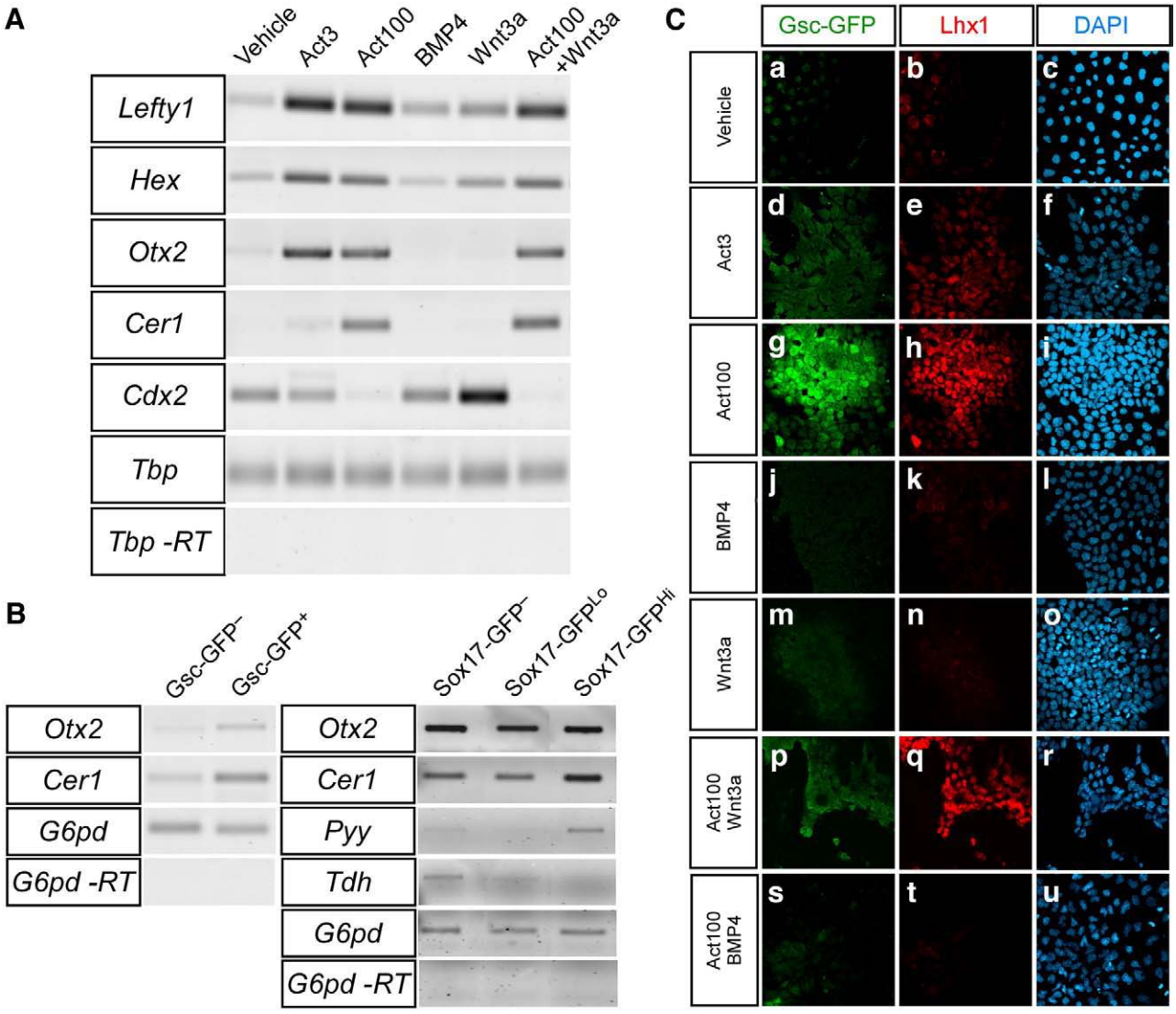


Fig. 7. Activin, BMP and Wnt signaling influence anterior–posterior patterning during differentiation of ES cells. ES cells were grown for 5 days in serum-free medium supplemented with indicated growth factors. (A) The expression of *Lefty1*, *Hex*, *Otx2*, *Cer1* and *Cdx2* was analyzed in T$^{Gfp/+}$ cells by RT-PCR. The expression of TATA binding protein (*Tbp*) was used as internal standard. (B) ES cells were cultured with 100 ng/ml activin and FACS sorted based on native GFP fluorescence after 5 days of culture. The expression of *Otx2* and *Cer1* was analyzed in the GFP^{+} and GFP^{-} fraction of Gsc$^{Gfp/+}$ cells, and *Otx2*, *Cer1*, *Pyy* and *Tdh* expression was analyzedin the GFP^{Hi}, GFP^{Lo} and GFP^{-} fraction in Sox17 $^{Gfp/+}$ cells. Glucose-6-phosphate dehydrogenase (*G6pd*) was used as internal standard. (C) The expression of Lhx1 and GFP was examined in Gsc$^{Gfp/+}$ cells by immunofluorescence after 5 days in the presence of the indicated factors.

expression of members of the FGF family in differentiating ES cell cultures treated with activin and BMP4 (alone or in combination). We found that FGF4 and FGF8 were expressed in ES cell cultures treated with activin or BMP4 (Fig. S4). We therefore addressed the role of FGF signaling by culturing the cells in activin (100 ng/ml) or BMP4 (10 ng/ml) with or without the FGF receptor inhibitor SU5402 (Mohammadi et al., 1997) or in the absence or presence of exogenously added FGF2. Consistent with a recent report (Kunath et al., 2007), flow cytometric analyses of undifferentiated cells (day 0) and at days 3 and 5 of differentiation revealed that both BMP4-induced *T* and *Mixl1* expression were strongly inhibited by 10 μM SU5402 (Fig. 8A), a concentration of SU5402 that did not compromise cell viability (data not shown). As BMP4-induced T expression has been shown to be insensitive to SU5402 in EB culture (Willems and Leyns, 2008), we repeated this experiment to determine if choice of culture system could explain this difference. Indeed we also find that BMP4-induced T expression is unaffected by addition of SU5402, and even by itself SU5402 is able to induce T expression (Fig. 8B). Nevertheless, addition of soluble FGF receptors (sFGFRs, b and c splice forms) failed to inhibit BMP4-induced *T* or *Mixl1* expression at day 3 of adherent culture (Fig. 8A). Conversely, addition of FGF2 augmented the number of T-GFP$^+$ and Mixl1-GFP$^+$ cells formed in response to BMP4 treatment (from 39 ± 23% to 68 ± 6% and from 41 ± 8% to 53 ± 8%, respectively). However, this induction was only significant for Mixl1-GFP ($p<0.01$).

From studies in zebrafish we would expect that FGF signaling would inhibit endoderm development and redirect cells towards mesoderm (Mizoguchi et al., 2006; Poulain et al., 2006). When Gsc$^{Gfp/+}$ cells were cultured in the presence of activin with addition of FGF2, SU5402, PD173074, or soluble FGF receptors we observed a strong dependence on FGF signaling for the development of Gsc-GFP$^+$ cells (Figs. 9A and B). The number of Gsc-GFP$^+$ cells appearing at day 5 in the presence of activin was significantly inhibited by the addition of SU5402 ($p<0.05$), PD173074 ($p<0.001$) or sFGFRs ($p<0.05$). Furthermore, both the b and c splice forms of soluble FGF receptors reduced the number of Gsc-GFP$^+$ cells ($p<0.05$ using Student's one-tailed *t*-test). Moreover, the addition of FGF2 significantly enhanced the amount of Gsc-GFP$^+$ cells seen at day 5 in activin-treated cultures ($p<0.01$). These results demonstrate that activin-induced *Gsc* expression depends on FGF signaling and suggest that anterior PS fate is augmented by FGF signaling. However, as *Gsc*-expressing cells develop further into both meso- and endoderm we next asked whether development of definitive endoderm is also FGF dependent. Hence we examined if activin-induced formation of Sox17-GFP^{Hi} cells was influenced by FGF signaling. Culture of Sox17$^{Gfp/+}$ cells in activin together with SU5402 or PD173074 resulted in a ~50% and ~90% reduction in the number of Sox17-GFP^{Hi} cells compared to activin alone ($p<0.001$; Fig. 9B), demonstrating that FGFR signaling is required for normal formation of definitive endoderm. However, in contrast to results obtained with the *Gsc* reporter, addition of sFGFR did not affect Sox17 expression. Moreover, where the addition of FGF2 boosted the formation of Gsc-GFP$^+$ cells it resulted in an almost 50% reduction in the number of Sox17-GFP^{Hi} cells ($p<0.005$; Fig. 9B), suggesting that precise control of FGF levels is important for regulation of Sox17 promoter activity.

To better define the time where FGF signaling acted during induction of Gsc- and Sox17 expression we cultured Gsc- and Sox17-GFP cells with activin for 5 days and added either FGF2, SU5402, or PD173074 for shorter periods. The (modest) stimulatory effect of FGF2 upon Gsc-GFP expression required that FGF2 was present early in the 5 day culture period, prior to the initial appearance of GFP$^+$ cells (Fig. 9C). In contrast, SU5402 and PD173074 reduced the number of Gsc-GFP$^+$ cells both when added early and late in the culture period (Fig. 9C). In contrast, the number of Sox17-GFP^{Hi} cells was reduced when FGF2 and FGFR inhibitors were present after the initial appearance of GFP^{Hi} cells (Fig. 9C).

We used RT-PCR to analyze whether the expression of additional markers, informative in relation to anterior–posterior positional

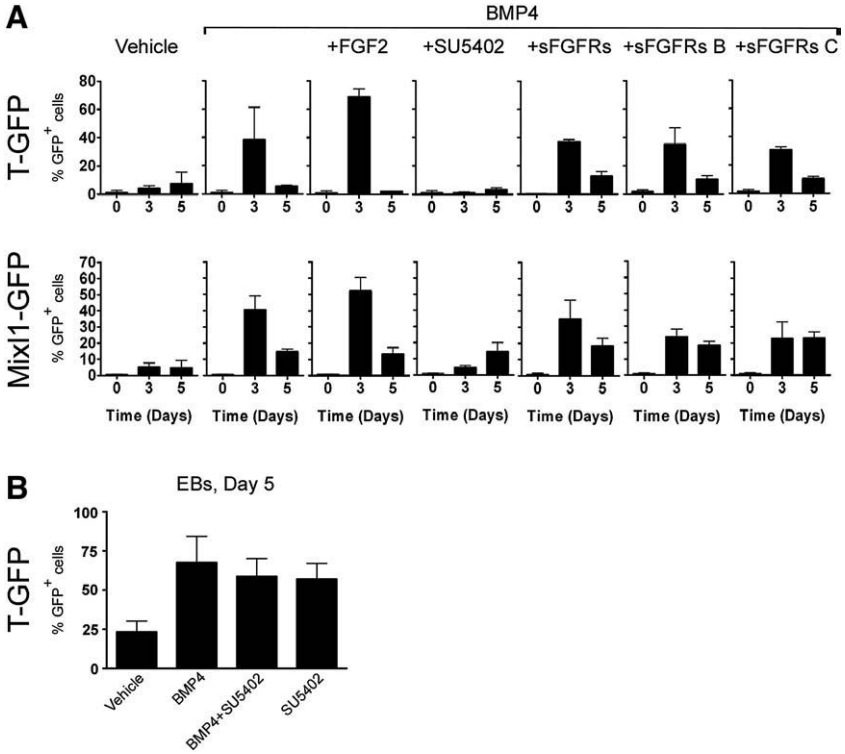


Fig. 8. FGF receptor signaling is required for BMP4-induced mesendoderm formation in adherent, but not in aggregate culture. (A) T$^{Gfp/+}$ and Mixl1$^{Gfp/+}$ cells were cultured in medium containing 10 ng/ml BMP4, with or without 100 ng/ml FGF2, 10 μM SU5402, or different concentrations of soluble forms of FGF receptors (b or c splice forms, or a combination of both). Cells were analyzed for GFP expression by flow cytometry on days 0, 3 and 5. (B) T$^{Gfp/+}$ were induced to form embryoid bodies with the indicated growth factors and analyzed for GFP expression by flow cytometry on day 3. The mean % GFP$^+$ cells ± standard deviation of three independent experiments is presented.

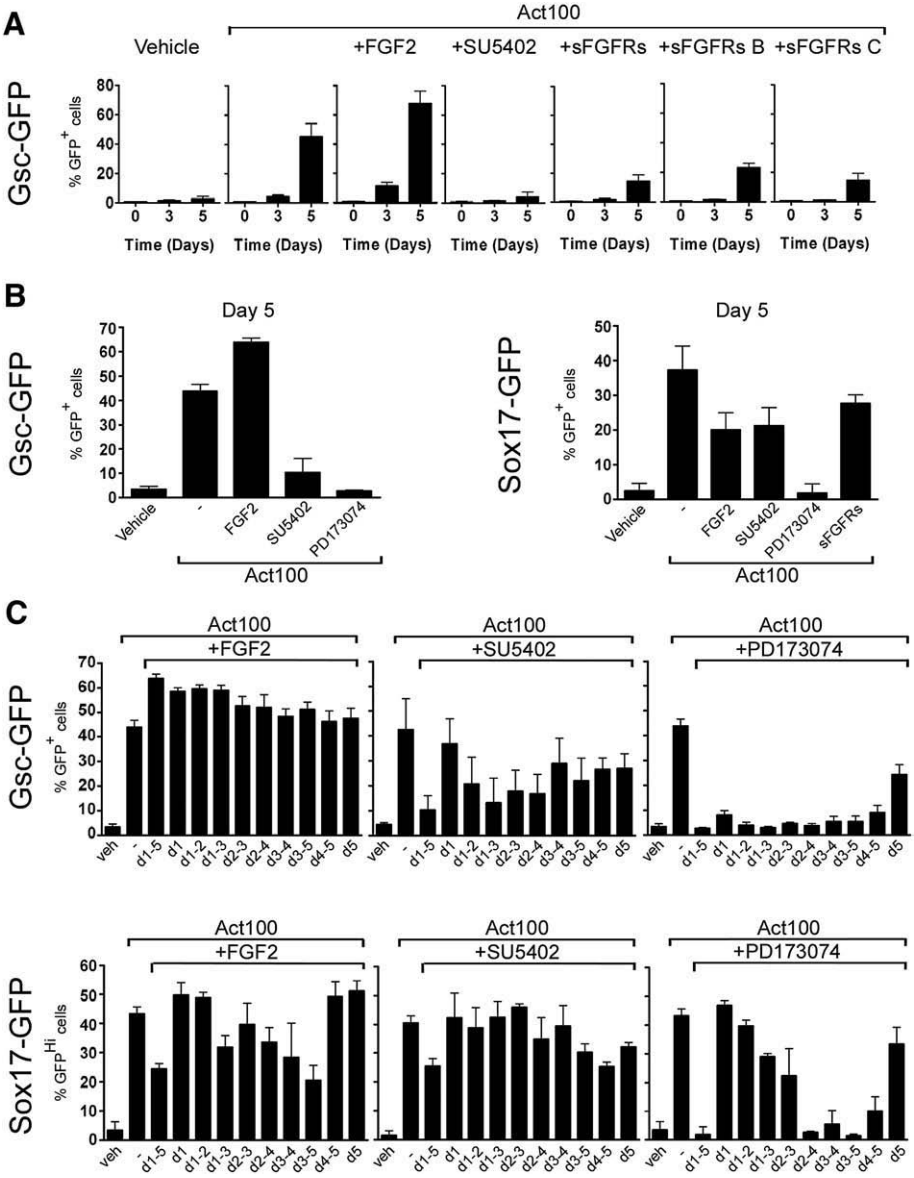


Fig. 9. FGF receptor signaling is required throughout the five day period for activin-induced *Gsc* expression but only required late for activin induction of *Sox17*-expressing definitive endoderm. $Gsc^{Gfp/+}$ and $Sox17^{Gfp/+}$ cells were cultured in medium containing 100 ng/ml activin, in combination with 100 ng/ml FGF2, 10 μM SU5402, 100 nM PD173074, or soluble forms of FGF receptors as indicated. (A) Time course analysis of appearance of Gsc-GFP$^+$ cells by flow cytometry at time 0 and after 3 and 5 days in culture. (B) $Gsc^{Gfp/+}$ and $Sox17^{Gfp/+}$ cells were analyzed for GFP expression at day 5 after culture with the indicated factors. (C) $Gsc^{Gfp/+}$ and $Sox17^{Gfp/+}$ cells were analyzed for GFP expression at day 5 after culture with activin and either FGF2, SU5402, or PD173074 for shorted periods as indicated. The mean % GFP$^+$ cells ± standard deviation of three independent experiments is presented.

identity, were affected by blocking FGF receptor signaling in activin-treated cells. Expression of *Otx2*, *Chrd*, and *Cer1*, genes that are repressed by BMP4, is not affected by the addition of SU5402 after 3 days (Fig. 10). Among the markers analyzed only *Bmp2*, which is first expressed in the embryo proper at E7.5 just lateral to the anterior neural folds and in precardiac mesoderm and slightly later in foregut endoderm (Winnier et al., 1995), is repressed both by BMP4 and SU5402 at day 3. *Chrd* expression appears at day 3 in vehicle-treated cultures and at day 5 in BMP4-treated cultures but in both cases it is sensitive to SU5402. Also BMP4-induced expression of mesoderm markers *Chrd*, and *Nog* at day 5, as well as the posterior markers *Bmp4* and *Cdx2*, is sensitive to SU5402 (Fig. 10 and Fig. S4).

Foregut and pancreatic competence of ES cell-derived endoderm

To test if the DE-like cells had potential to functionally integrate into developing embryonic endoderm, we implanted approximately 50 cells labeled with a fluorescent dye into 6 to 10 somite stage chicken embryos at the level of the prospective pancreatic endoderm and incubated for 48 h. When ES cell progeny from activin-treated cultures were grafted, 15 out of 21 transplanted embryos contained dye-labeled, Foxa2$^+$ cells incorporated into the endoderm. In contrast, only 3 out of 20 embryos receiving cells cultured without activin and 0 of the 8 embryos receiving activin- and BMP4-treated cells contained dye-labeled cell in the endoderm (Figs. 11A, D, G). Frequently, the activin-treated cells incorporated in the Nkx6-1$^+$Pdx1$^+$ pancreatic endoderm (Figs. 11E, F) (Pedersen et al., 2005). In a second series of grafting experiments we tested if the Sox17-GFP^{Hi} cells obtained after activin treatment for 5 days with or without additional Dkk1 treatment were capable of incorporating into chick foregut endoderm. Such cells were equally capable of contributing to the foregut endoderm (Figs. 11H, I, K–M), although the number of Sox17-GFP^{Hi} cells were reduced in cultures containing Dkk1. Orthogonal projections of the confocal stacks obtained from grafted embryos suggested expression of Nkx6-1 in some of the grafted cells (Fig. 11J). Considering the anterior markers expressed by activin-treated cells, and the absence of pancreatic or

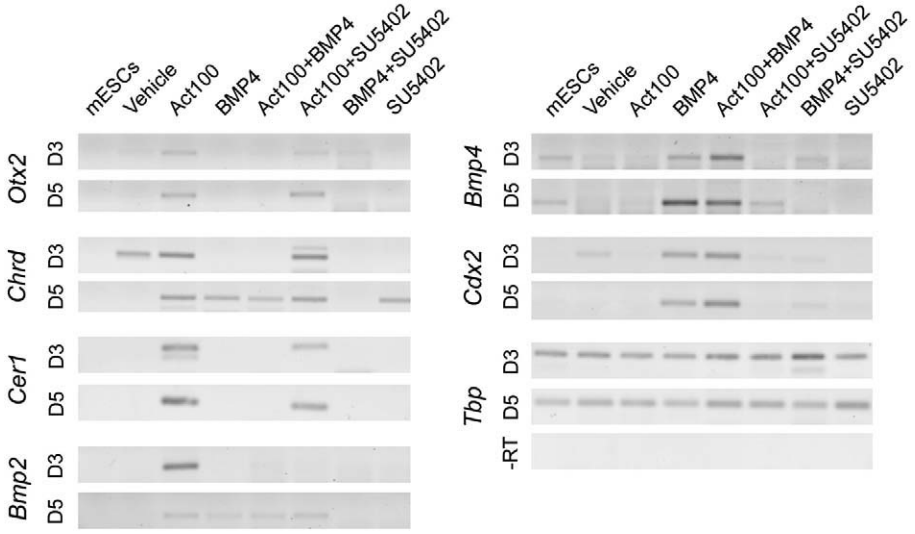


Fig. 10. TGF-β, Wnt and FGF signaling control the dynamic gene expression in differentiating ES cells. $T^{Gfp/+}$ cells cultured for 3 or 5 days in serum-free medium supplemented with 100 ng/ml activin, 10 ng/ml BMP4 and/or 10 μM SU5402, as indicated, were analyzed by RT-PCR. The expression of *Otx2*, *Chrd*, *Cer1*, *Bmp2*, *Bmp4* and *Cdx2* was analyzed. *Tbp* was used as internal standard.

other regional foregut markers at day 5 (Fig. 12), the grafting experiments suggest that DE formed in our cultures can respond to posteriorizing cues from the embryonic environment and progress further in differentiation. We therefore tested if the cells were able to respond to suspected posteriorizing cues in vitro. After 5 days of culture in activin, the cells were shifted to conditions where activin was replaced by candidate posteriorizing factors Wnt3a, FGF4, and retinoic acid (RA) (Grapin-Botton and Constam, 2007) and analyzed for expression of Sox2, Pdx1, and Cdx2, markers of fore-, mid-, and hindgut, respectively (Grapin-Botton and Melton, 2000). Remarkably, we found large numbers of $Sox2^+Foxa2^+$ cells in cultures treated with posteriorizing factors (Fig. 12). We confirmed *Sox2* expression using the OS25 cell line which carries a β-*geo* reporter gene in the *Sox2* locus (Li et al., 1998). β-galactosidase activity was visualized with X-gal staining (Fig. 12). The appearance of $Sox2^+$ cells was depending on neither FGF4 nor RA. Scattered $Pdx1^+$ cells also appeared but in contrast to $Sox2^+$ cells these only appeared in the presence of RA. Cdx2 positive cells were not detected under any of the conditions tested. Together, our data demonstrate that DE formed from mES cells in adherent monoculture is capable of differentiation toward foregut endoderm in vivo and in vitro but that only a limited number of these cells appear to respond to posteriorizing cues in vitro.

Discussion

A recent work from Smith et al. has demonstrated that mES cells can be kept pluripotent under defined serum- and feeder-free conditions (Ying et al., 2003a), and be efficiently converted to $Sox1^+$ neuroectodermal progenitors when kept in adherent monoculture in the absence of LIF and BMP4 (Ying et al., 2003a,b). In the present work, we extend this defined system to demonstrate that mES cells kept in serum- and feeder-free adherent monoculture respond dose-dependently to inducers of primitive streak formation by developing mesendoderm with capacity to differentiate further into cells resembling foregut endoderm, in vivo and in vitro. Although several recent studies have established that the TGF-β family members BMP4 and activin induce mesodermal and endodermal gene expression in differentiating mES cells (Gadue et al., 2006; Kubo et al., 2004; Mossman et al., 2005; Ng et al., 2005; Tada et al., 2005; Yasunaga et al., 2005), the varied conditions under which differentiation was induced; e.g. adherent vs. suspension culture, differences in basal cell culture media and supplements, cell density, absence or presence of serum; as well as the different reporter lines utilized make a direct comparison of these studies difficult. Here we use a series of GFP-based reporters to study the dynamic expression of *T*, *Mixl1*, *Gsc*, and *Sox17* after manipulating one or more of the FGF, TGF-β, and Wnt signaling pathways. Remarkably, we find that the lowest concentration of activin used (3 ng/ml) induced the highest number of $T\text{-}GFP^+$ cells, whereas higher concentrations (10–100 ng/ml) resulted in progressively fewer $T\text{-}GFP^+$ cells. These data apparently conflict with results obtained by Keller et al. who observed increasing numbers of $T\text{-}GFP^+$ cells with increasing activin concentration until a plateau of about 50–60% $T\text{-}GFP^+$ cells was reached at 30 ng/ml of activin (Kubo et al., 2004). It is not entirely clear why a direct correlation between activin concentration and the number of $T\text{-}GFP^+$ cells is observed by Kubo et al. while we observe an inverse correlation. One possible explanation relates to the embryoid body formation used by Kubo et al. in which only cells located at the periphery, directly exposed to the cell culture medium, may experience the full concentration of added growth factor as recently demonstrated (Sachlos and Auguste, 2008). Cells located in the interior of the embryoid body are most likely experiencing a lower concentration and the overall dose–response curve may therefore appear shifted towards higher concentrations. It is also possible that the environment of the embryoid body is more conducive to secondary signaling events that may influence the number of $T\text{-}GFP^+$ cells. A recent study did however report that *T* mRNA levels in differentiating EBs were inversely correlated with activin concentration within the 5–50 ng/ml range (Willems and Leyns, 2008). Moreover, our results are strikingly similar to data from *Xenopus* animal cap explants, where activin dose-dependently controls cell fate specification. The expression of the *Xenopus T* homolog *Xbra* is induced by low concentrations of activin while higher concentrations induce the expression of the *Gsc* homolog *Xgsc* (Green et al., 1992; Gurdon et al., 1994; Latinkic et al., 1997). However, even *Xgsc*-expressing cells induced by high doses of activin have undergone a transient *Xbra*-expressing phase, but direct repression of *Xbra* transcription by binding of Xgsc to the *Xbra* promoter limits the duration of *Xbra* expression (Latinkic et al., 1997). In this regard, analyses of the duration of *T* expression by time-lapse microscopy under different conditions would be highly informative. The notion of activin as a dose-dependent inducer of anterior fate in ES cell progeny is indicated by several observations: the differential effect of 3 and 100 ng/ml activin on gene expression such that only the high dose induces the anterior marker *Cer1* and represses the posterior marker *Cdx2*. Also, extensive co-localization of E-cadherin, Gsc-GFP and Sox17 was only observed after treatment with 100 ng/ml of activin. Additional marker analyses

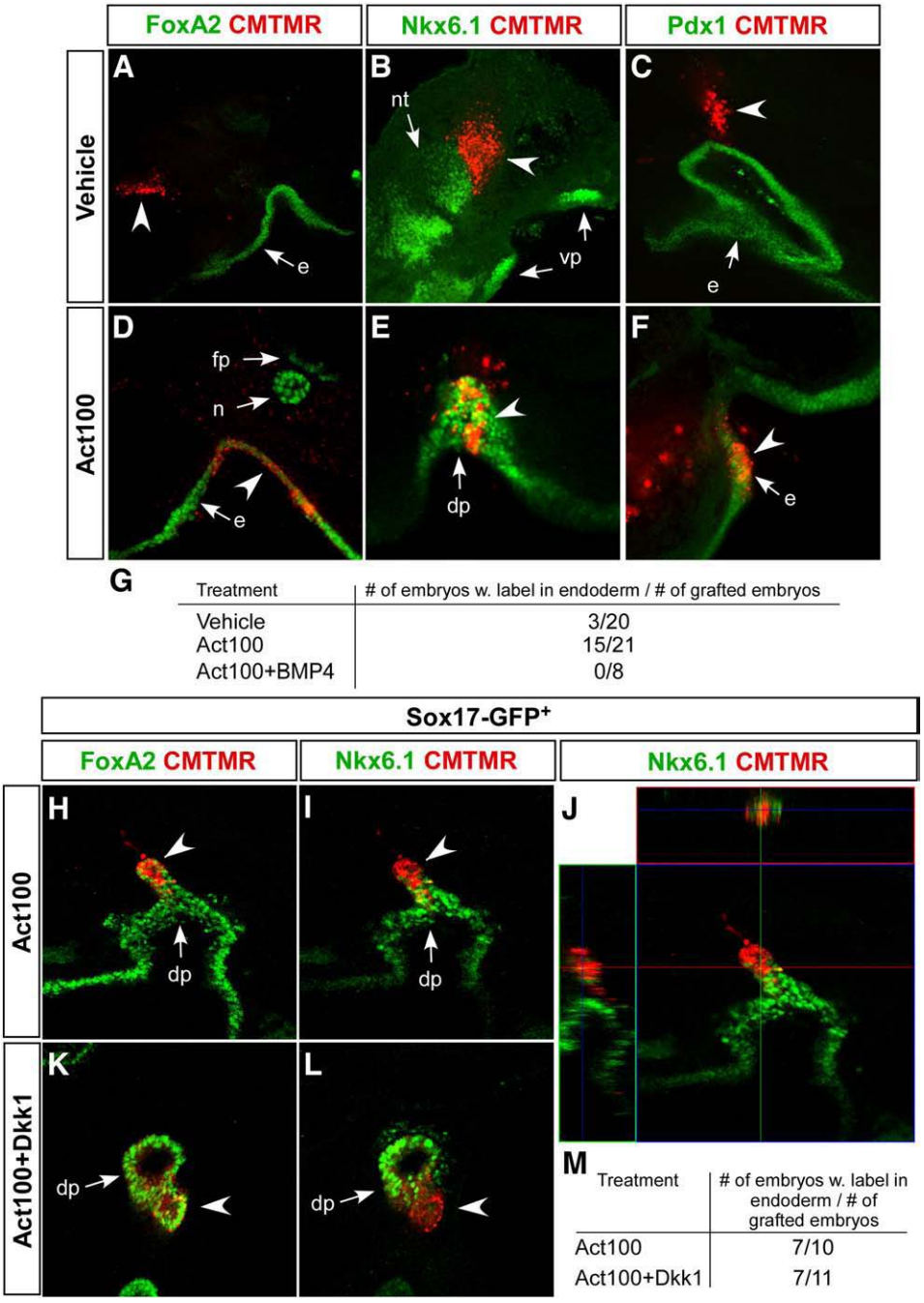


Fig. 11. mES cell-derived endoderm grafts contribute to the developing chicken endoderm. (A–F) ES cells were cultured for 5 days in serum-free medium containing vehicle, 100 ng/ml activin or 100 ng/ml activin and 10 ng/ml BMP4 as indicated. Small clusters of cells were labeled with the red fluorescent cell tracker dye CMTMR and grafted between the endoderm and mesoderm of explanted chicken embryos and allowed to develop for 2 days before being processed for confocal immunohistochemistry. (H–M) Sox17$^{Gfp/+}$ cells were cultured for 5 days in serum-free medium containing either 100 ng/ml activin or 100 ng/ml activin plus 320 ng/ml Dkk1. Small clumps of GFP^{+} cells were labeled with the red fluorescent cell tracker dye CMTMR and grafted between the endoderm and mesoderm of explanted chicken embryos and allowed to develop in ovo for 2 days before being processed for confocal immunohistochemistry. (A–F, H, I, K, L) Optical sections of chicken embryos whole-mount stained with the indicated antibodies and transplanted with ES cell progeny developing after 5 days with the indicated growth factors. (J) Orthogonal view of a Z-stack revealing an Nkx6-1-expressing CMTMR-labeled cell. (G, M) Tabulated results of the grafting experiments. Arrowheads in A–F and H–L indicate implanted cells. dp: dorsal pancreas, e: endoderm, fp: floor plate, n: notochord, nt: neural tube, vp: ventral pancreas.

revealed that such cells were also *Pyy*$^{+}$, Foxa2^{+} and CXCR4^{+} but *Sox7*$^{-}$ and *Tdh*$^{-}$, suggesting embryonic rather than extraembryonic endoderm had formed (Kanai-Azuma et al., 2002; Tada et al., 2005; Yasunaga et al., 2005). Conversely, BMP4- and Wnt3a-treated cells expressed low levels of anterior markers and instead expressed the posterior markers *Bmp4* and *Cdx2*. Furthermore, BMP4 prevented activin-induced gene expression and stimulated the expression of *T* and *Mixl1*. These observations are consistent with previous in vivo data showing that BMP4 is necessary for *T* expression (Winnier et al., 1995) and that activin-induced *Gsc* expression can be counteracted by BMP4 in vivo (Imai et al., 2001; Jones et al., 1996; Sander et al., 2007; Shapira et al., 1999; Steinbeisser et al., 1995). Additionally, Keller et al. also noted a posteriorizing effect of BMP4 upon mouse ES cells derived, activin-induced PS populations (Nostro et al., 2008) and Suemori et al. found that inhibition of BMP signaling redirected human ES cell-derived mesodermal cells (induced by forced expression of stabilized β-catenin) towards an anterior PS/DE lineage, in a process dependent on activin/nodal signaling (Sumi et al., 2008).

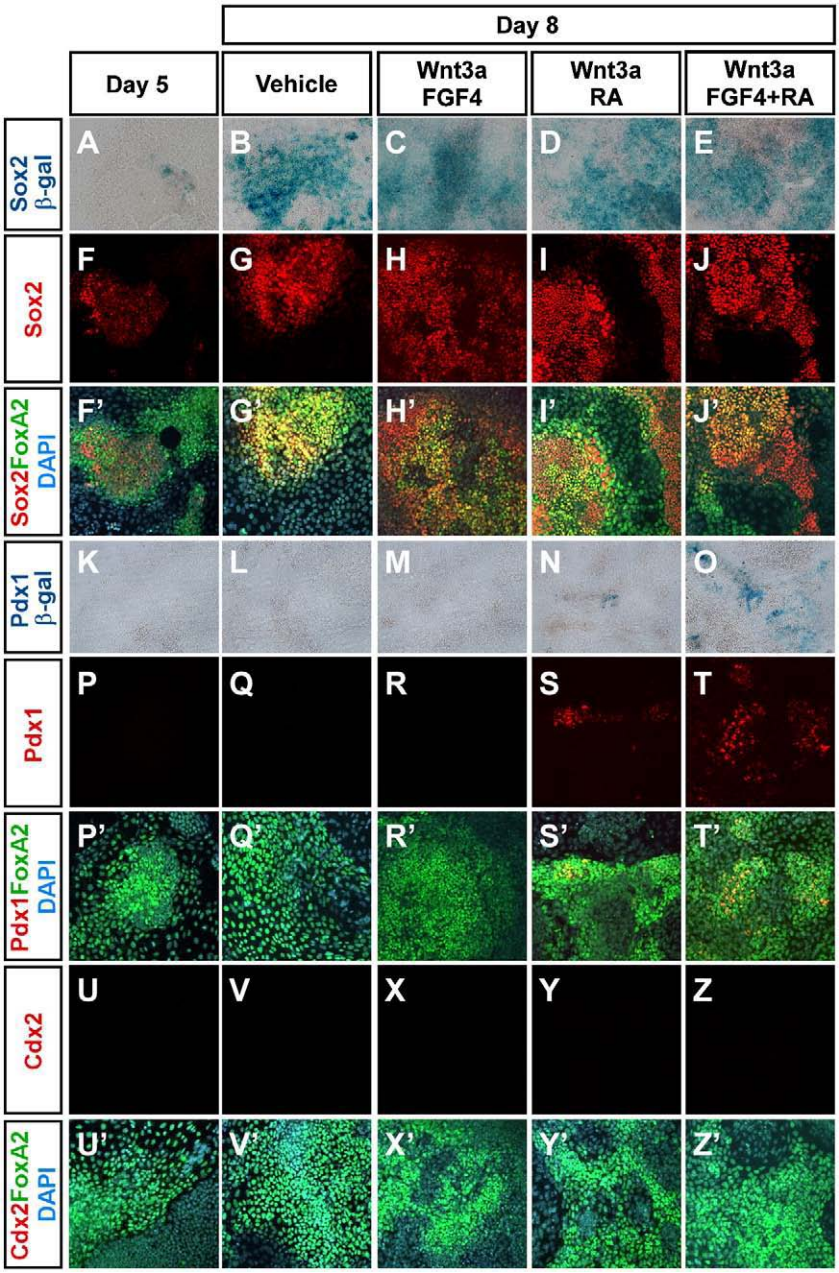


Fig. 12. ES cell-derived endoderm acquire foregut cell fates. (A–J′ and P–Z′) OS25 (Sox2$\beta^{geo/+}$) or (K–O) Pdx1$^{LacZ/+}$ cells were cultured for 5 days in 100 ng/ml activin and subsequently treated with 5 ng/ml Wnt3a, 10 ng/ml FGF4 and/or 0,1 μM RA for 3 days as indicated. The co-expression of Foxa2 and Sox2 (foregut), Pdx1 (midgut) or Cdx2 (hindgut) was analyzed by immunofluorescence. The expression of *Sox2* (A–E) and *Pdx1* (K–O) was confirmed by analyzing β-galactosidase activity using X-gal staining.

Further analysis of BMP4- (and Wnt3a-) treated cells revealed the presence of *Flk1*$^+$ cells demonstrating that mesodermal differentiation had occurred. Interestingly, Smith et al. recently reported that BMP4 treatment of mES cells, under conditions nearly identical to ours, resulted in cells of unknown identity that were unlikely to be mesodermal based on presence of E-cad immunoreactivity (Kunath et al., 2007). While we occasionally detect a faint plasma membrane staining in BMP4-treated cells (see for example Fig. 3), the intensity of the staining is strongly reduced compared to the signal seen in activin-treated cells, and most cells appear E-cad$^-$ in our experiments. Furthermore, the presence of Flk1 expressing cells would appear to conclusively demonstrate mesodermal differentiation. This is also consistent with a number of studies where embryoid bodies are stimulated with BMP4 (Czyz and Wobus, 2001; Finley et al., 1999; Johansson and Wiles, 1995; Lengerke et al., 2008; Ng et al., 2005; Willems and Leyns, 2008).

Our examination of the role of Wnt signaling during mesendoderm differentiation seems to indicate a major difference between differentiation in embryoid bodies and in adherent monoculture. Apparently, the extent to which Wnt signaling is required to induce anterior PS formation is different in the two systems. Gadue et al. found that 100 ng/ml Wnt could induce formation of Foxa2-CD4$^+$T-GFP$^+$ anterior PS-like cells, and that this was dependent on endogenous ALK4/5/7 signaling. Moreover, the ability of activin to induce the same fate was dependent on Wnt signaling since Dkk1 could prevent the effect of activin (Gadue et al., 2006). At first glance these findings may appear to contrast with our results, namely that the same concentration of Wnt3a could not induce significant numbers of Gsc-GFP$^+$ in adherent culture and that Dkk1 could not prevent induction of Gsc-GFP$^+$ cells by activin. However, the Gsc-GFP$^+$ cells in this work do not express Brachyury and very likely represent a mixed population and are thus difficult to compare to the Foxa2/T double positive cells

studied by Gadue et al. Furthermore, although siRNA mediated knock-down of β-catenin significantly reduced the number of Gsc-GFP$^+$ cells developing in response to activin the effect never exceeded 50%. In contrast, the formation of Sox17-GFP^{Hi} DE cells was more sensitive to inhibition of canonical Wnt signaling, but only when signaling was inhibited at a relatively late stage of development, suggesting that the requirement for Wnt signaling is not during the formation of PS cells but may rather represent a Wnt-mediated lineage choice by a common mesendodermal precursor or alternatively, a requirement for Wnt signaling in the maintenance of Sox17-expressing DE cells. Indeed, our observation that Dkk1 only reduces the number of Sox17^{Hi} cells if present at day 4, i.e. after Sox17 expression is initiated, is consistent with a requirement for Wnt signaling in the maintenance of DE. Also, our observations are consistent with the requirement for β-catenin function in DE to acquire or maintain DE fate (Lickert et al., 2002) as well as the observation of synergy between Sox17 and β-catenin in the activation of DE gene expression (Sinner et al., 2004).

We also speculate that the proposed action of Wnt3 during gastrulation might offer at least a partial explanation for the difference in sensitivity towards Dkk1 treatment observed between activin and nodal as well as between adherent and aggregate culture. Wnt3 is required in vivo for nodal to initiate its auto-stimulatory cascade. In the absence of Wnt3, nodal expression is initiated but then regresses rather than expands (Barrow et al., 2007; Liu et al., 1999). Wnt3 is thought to induce expression of *Cfc1* which encodes the obligate nodal co-receptor Cripto (Morkel et al., 2003). It was recently reported that the three dimensional environment in embryoid bodies prevent exogenously added growth factors from diffusing more than a limited distance into the embryoid body (Sachlos and Auguste, 2008) possibly making these more reliant on endogenous relay signaling via nodal which subsequently would require Wnt activity to induce Cripto expression. This notion is supported by our observation that nodal-induced Sox17-GFP^{Hi} cells appear more sensitive to Dkk1-mediated suppression than activin induced Sox17-GFP^{Hi} cells, and by our observation that activin-induced Sox17-GFP^{Hi} cells are more sensitive to Dkk1 treatment in aggregate culture compared to adherent culture. The apparent absence of such an autocrine nodal/Wnt signaling loop in adherent culture is not surprising given the remarkable instability of nodal when secreted by cultured cells (Le Good et al., 2005). Nodal may simply be degraded before it can reach other cells in the dish whereas in the confined environment of an embryoid body it may well signal to nearby cells and such signaling might propagate in a relay fashion. Thus, Wnt3a treatment would not be sufficient to induce this loop in adherent culture. Conversely, since all the cells in adherent culture are exposed evenly to exogenous activin there may be no requirement for Wnt signaling to facilitate ALK4 signaling. Nevertheless, Gsc-GFP$^+$ cells appear largely refractory to the inhibitory effects of Dkk1 treatment. Since Wnt induced expression of *Xgsc* is mediated by two homeodomain proteins (Siamois and Twin which have no apparent homologs in the mouse) binding to the PE-element, this finding raises the question of whether the conserved Wnt-responsive element in the mouse *Gsc* promoter (Watabe et al., 1995) is functional in all contexts. It is possible that *Gsc* is not always induced by Wnt as *Gsc* expression is reduced rather than increased in E8.5 *Dkk1* mutant embryos (Lewis et al., 2008). Lastly, we cannot rule out a more trivial explanation related to the particular Gsc-GFP cell line, which may potentially harbor a defect in its responsiveness to Dkk1-mediated Wnt inhibition. It is possible that more efficient knock-down of β-catenin than we achieved might further reduce or even prevent the formation of Gsc-GFP$^+$ cells.

In contrast, we do observe a requirement for Wnt signaling for induction of *Mixl1* expression. Our experiments revealed the simultaneous requirement of nodal/activin and Wnt signaling to induce expression of *Mixl1* in ES cell progeny. Stimulation of one pathway while inhibiting the other reduced *Mixl1* induction, which corroborates previous findings (Gadue et al., 2006). The *Mixl1* promoter has previously been shown to be nodal/activin-responsive via the presence of Foxh1 and Smad binding sites in the promoter (Hart et al., 2005) and inspection of the published sequence reveals the presence of consensus TCF/LEF binding sites in the promoter as well. In vivo, Wnt/β-catenin signaling is upstream of two distinct gene expression programs acting during anteroposterior axis and mesoderm formation, respectively (Morkel et al., 2003). We suspect that we observe a requirement for Wnt signaling in mesoderm formation but did not pursue this aspect further.

The two differentiation systems, adherent and aggregate culture, also differ strikingly in the requirement for FGF signaling. In agreement with our results a recent study using embryoid body formation found that FGF signaling was not required for BMP4-induced *T* expression (Willems and Leyns, 2008), which is unlike the situation in adherent culture where BMP4-induced *T* expression is completely prevented by SU5402. Moreover, consistent with our results Kunath et al., also using adherent culture, recently reported that FGF4 deficiency or treatment with PD173074 prevented the switch from BMP4-mediated support of pluripotency to BMP4-induced differentiation (Kunath et al., 2007). The requirement for FGF signaling in induction of mesodermal gene expression is also consistent with the in vivo requirement for FGF signaling in *Xenopus* and zebrafish mesodermal induction (Cornell et al., 1995; Mathieu et al., 2004). In zebrafish, FGF signaling is required downstream of Nodal for induction of One-eyed-pinhead, the zebrafish homolog of Cripto (Mathieu et al., 2004). However, as discussed above activin does not require Cripto to activate its receptors, thus activin induced Sox17 expression should not necessarily be inhibited by FGFR inhibitors. This is indeed what we find when inhibiting FGF signaling prior to appearance of Sox17-GFP^{Hi} cells, the time where one would expect to find a requirement for Cripto function if Nodal was the inducer. It might be illuminating to determine if Nodal induced Sox17 expression would be sensitive to these inhibitors. Nevertheless, we do observe that Sox17 expression depends on FGF signaling after the initial appearance of Sox17-GFP^{Hi} cells at the same time where we observe a dependency for Wnt signaling. It thus appears that maintenance of Sox17 expression and/or propagation of Sox17 expressing cells depend on both FGF and Wnt signaling. Consistent with our results, Brickman et al. also observed an absolute requirement for FGF signaling at days 3–7 for the formation of Hex$^+$CXCR4$^+$ADE cells when differentiating ES cells in monolayer culture under defined conditions (Morrison et al., 2008).

In some cases we noticed what appeared to be conflicting results when inhibiting FGF and FGFR signaling by soluble FGF receptors and SU5402, respectively. It is possible that this is a reflection of FGF independent FGFR activation. FGF receptors are known to form complexes with N-cadherin and N CAM in neurons and in pancreatic β-cells, resulting in ligand independent receptor activation (Cavallaro et al., 2001; Saffell et al., 1997; Williams et al., 1994), and this would not be expected to be sensitive to the addition of soluble FGF receptors. More trivial explanations are also possible. We cannot rule out that SU5402 may have unknown non-FGFR-mediated effects or be certain that our soluble FGFR preparations are capable of inhibiting all FGF family members. Additional experiments with dominant negative receptors and cell lines mutated in genes coding for FGF signaling components will likely shed more light on these questions.

The expression of anterior markers such as *Otx2* and *Cer1* in Sox17-GFP^{Hi} cells suggest that these could be anterior definitive endoderm. This notion is supported by the selective presence of *Pyy* transcripts in this population, but the lack of good ADE specific markers prevents us from categorically making this conclusion. However, in vivo ADE forms under conditions of high nodal signaling (Ben-Haim et al., 2006; Lu and Robertson, 2004; Vincent et al., 2003) which we believe we mimic with culture conditions containing 30–100 ng/ml activin or 1 μg/ml nodal. Thus, it is likely that the Sox17-GFP^{Hi} cells formed in our cultures represent ADE. However, it is also likely that mesoderm is formed to some extent in cultures treated with high doses of activin or

nodal. The presence of *Otx2* transcripts in Sox17-GFP^{Lo} and -GFP^{-} populations suggest that these also contain anterior cell types including perhaps small numbers of AVE cells. The latter is indicated by the presence of the VE marker *Tdh* and the presence of *Otx2* and *Cer1* is consistent with this idea. Lastly, we cannot exclude that Sox17-negative endoderm is present in our cultures.

Chick embryo xenografting has previously been used to investigate the developmental potential of embryonic mouse cells (Fontaine-Perus, 2000; Fontaine-Perus et al., 1996, 1997, 1995) as well as mouse and human ES cell derivatives (Lee et al., 2007; Wichterle et al., 2002) but to our knowledge we report the first functional assay for mES cell-derived endoderm. Previous studies have relied almost exclusively on expression of cell-specific markers for the characterization of in vitro generated endoderm. Although the ES cell-derived endoderm did not express transcription factors associated with the regionalization of the primitive gut tube after 5 days of activin treatment, the cells were capable of turning on these genes when integrating into endodermal epithelium in vivo. Moreover, some embryos contained grafted cells in the Nkx6-1^{+} and Pdx1^{+} pancreatic endoderm. Overall, our data suggest that ES cell-derived DE, formed under defined conditions, can contribute to the developing gut tube and further suggests that such cells are, at least partly, capable of responding to patterning cues from the in vivo environment. The latter notion is corroborated by the induction of pancreas markers in a limited number of cells when such cells are cultured further in the presence of known and suspected posteriorizing factors.

It is remarkable how the requirement for certain signaling events are strikingly different when comparing aggregate culture (i.e. embryoid body formation) and adherent culture. However, we do not think that one system is superior to the other but rather that comparison of directed differentiation under adherent and aggregate culture conditions will prove valuable when attempting to decipher the extent of secondary signaling events and their role in lineage selection. In this regard it is also noteworthy that endogenous signaling likely plays a prominent role also in adherent culture. This notion is based on several of our observations, including that many SuTOP-CFP^{+} cells formed in cultures that received activin as the only exogenous factor, the interdependence between activin and Wnt signaling for generation of Mixl1-GFP^{+} cells, and on the dependence on Wnt and FGF signaling for efficient activin-induced formation of Sox17-GFP^{+} cells.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2009.03.026.

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