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Activin promotes differentiation of cultured mouse trophoblast stem cells towards a labyrinth cell fate

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ABSTRACT

Prolonged maintenance of trophoblast stem (TS) cells requires fibroblast growth factor (FGF) 4 and embryonic fibroblast feeder cells or feeder cell-conditioned medium. Previous studies have shown that TGF-β and Activin are sufficient to replace embryonic fibroblast-conditioned medium. Nodal, a member of the TGF-β superfamily, is also known to be important *in vivo* for the maintenance of TS cells in the developing placenta. Our current studies indicate that TS cells do not express the Nodal co-receptor, Cripto, and do not respond directly to active Nodal in culture. Conversely, Activin subunits and their receptors are expressed in the placenta and TS cell cultures, with Activin predominantly expressed by trophoblast giant cells (TGCs). Differentiation of TS cells in the presence of TGC-conditioned medium or exogenous Activin results in a reduction in the expression of TGC markers. In line with TGC-produced Activin representing the active component in TGC-conditioned medium, this differentiation-inhibiting effect can be reversed by the addition of follistatin. Additional experiments in which TS cells were differentiated in the presence or absence of exogenous Activin or TGF-β show that Activin but not TGF-β results in the maintenance of expression of TS cell markers, prolongs the expression of syncytiotrophoblast markers, and significantly delays the expression of spongiotrophoblast and TGC markers. These results suggest that Activin rather than TGF-β (or Nodal) acts directly on TS cells influencing both TS cell maintenance and cell fate, depending on whether the cells are also exposed to FGF4.

Introduction

The placenta is one of the first organs to form during development and is essential for a healthy pregnancy. At the time of implantation, trophoblast cells of the mural trophectoderm in the blastocyst make contact with the uterine epithelium and undergo terminal differentiation. The resulting trophoblast giant cells (TGCs) are post-mitotic, migrate into the uterine tissue, and line the developing implantation site. The bulk of the developing placenta is formed from a subpopulation of trophoblast cells that overlie the inner cell mass, the polar trophectoderm (Gardner and Johnson, 1973; Gardner et al., 1973; Simmons and Cross, 2005; Tanaka et al., 1998; Uy et al., 2002). Following implantation, these trophoblast stem cells proliferate to give rise to the extraembryonic ectoderm (also called chorionic ectoderm) and ectoplacental cone (EPC). As development proceeds, all of the different trophoblast subtypes required to form the mature placenta, including TGCs, glycogen trophoblast cells, spongiotrophoblasts, and syncytiotrophoblasts, are derived from these tissues (Gardner and Johnson, 1973; Gardner et al., 1973; Simmons et al., 2007). However, in order for trophoblast proliferation and differentiation to occur properly, a specific microenvironment must exist to support the maintenance of the trophoblast stem cell population (Guzman-Ayala et al., 2004). Fibroblast growth factor 4 (FGF4) is a component of this microenvironment. FGF4 not only is required for the proliferation of the inner cell mass and development of the embryonic ectoderm following implantation (Feldman et al., 1995) but also is essential for the derivation and maintenance of TS cells *in vitro* (Goldin and Papaioannou, 2003; Gotoh et al., 2005; Tanaka et al., 1998).

In addition to FGF4, members of the TGF-β superfamily of growth factors have been shown to regulate TS cell maintenance and differentiation (Erlebacher et al., 2004; Goumans and Mummery, 2000; Jones et al., 2006). The TGF-β superfamily of growth factors includes TGF-βs, growth differentiation factors (GDFs), bone morphogenetic proteins (BMPs), Nodal, Activins, and Inhibins. These proteins regulate many developmental and physiological processes and, in general, signal through transmembrane serine/threonine kinase receptors and downstream Smad proteins to regulate cellular functions (Chang et al., 2002). Signaling by these molecules can be broadly divided into two distinct pathways, the GDF/BMP pathway,

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which signals through Smads 1/5/8 in coordination with Smad 4 and the TGF-β/Activin/Nodal pathway (Chang et al., 2002; de Caestecker, 2004). TGF-β initiates cellular responses by binding its heterotetrameric transmembrane receptor complex composed of Tgfbr1 (type I) and Tgfbr2 (type II) receptors, whereas Activin and Nodal bind the Activin receptors Acvr1b and Acvr2b (or Acvr2a) (de Caestecker, 2004; Derynck and Zhang, 2003). An additional level of complexity and control exists with other proteins acting as ligand traps such as Follistatin which binds Activin, and with co-receptors such as Cripto which interacts specifically with Nodal (but not Activin) and the Activin receptors (Shi and Massague, 2003). Downstream of the receptor complexes, all three ligands transduce their signals through phosphorylation of Smad2/Smad3, which complexes with Smad4 and is translocated to the nucleus to regulate gene transcription (Shi and Massague, 2003).

TGF-β and Activin have each been shown to be sufficient, together with FGF4, to support the proliferation and pluripotency of undifferentiated TS cells in culture (Erlebacher et al., 2004). However, while TGF-β ligands and receptors are expressed in the uterine tissue as well as in the developing embryonic and extraembryonic tissues, mouse mutants have not revealed an essential role for this pathway in the trophoblast cell lineage (Goumans and Mummery, 2000; Jones et al., 2006; Kitisin et al., 2007). Conversely, mouse mutants for the type I Activin receptor (Acvr1b) result in early embryonic lethality and display a disorganized epiblast and extraembryonic ectoderm at embryonic day (E) 6.5 (Gu et al., 1998). Activin mutants have been made and have no obvious placental phenotypes (Lau et al., 2000; Matzuk et al., 1995; Vassalli et al., 1994). However Activin is encoded by three separate subunit genes, and although single and double mutant mice have been reported, triple mutants have not. Nodal mutants display a phenotype similar to *Acvr1b* mutants, while a hypomorphic mutation in Nodal results in placental defects affecting trophoblast cells of the labyrinth, spongiotrophoblast and TGC layers (Ma et al., 2001). More recently, Nodal has been shown to be important *in vivo* in coordination with FGF4 for the maintenance of the TS cell

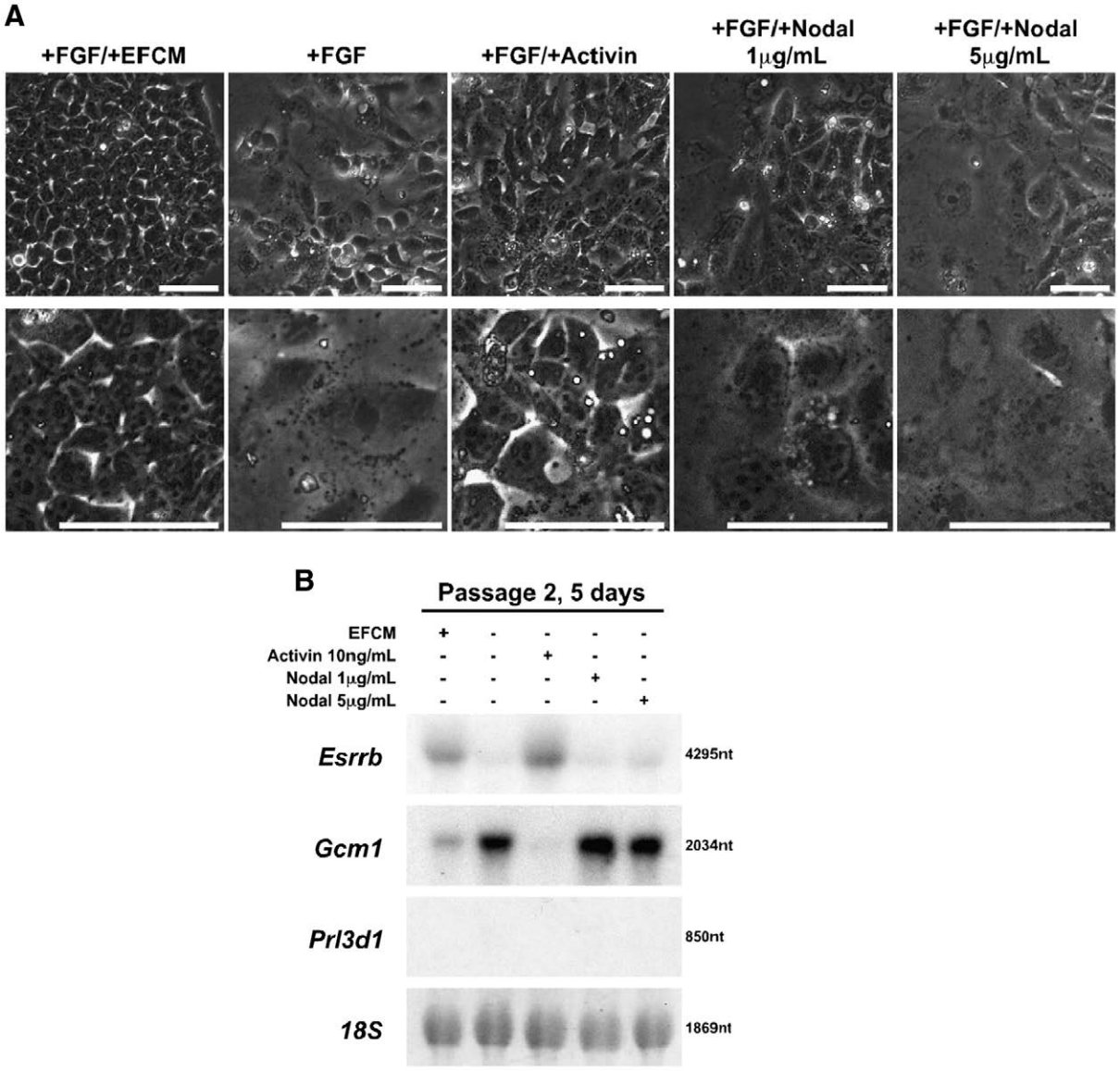

Fig. 1. Nodal cannot maintain the proliferation of undifferentiated TS cells in culture. (A) Morphology of TS cells grown in culture medium + FGF/+ EFCM or + FGF/+ Activin versus + FGF alone, + FGF/+ Nodal (1 or 5 μg/mL); top row, low magnification, bottom row, high magnification. Scale bar = 100 μm. (B) Expression of *Esrrb*, *Gcm1*, and *Prl3d1* were examined by Northern blot analysis of TS cells grown in the presence of FGF plus combinations of embryonic fibroblast feeder cell-conditioned medium (EFCM), Activin (10 ng/mL), or Nodal (1 or 5 μg/mL) as indicated.

microenvironment (Guzman-Ayala et al., 2004). However, given that Nodal is required to maintain FGF4 expression in the epiblast, it has been unclear if the effect of Nodal on trophoblast development *in vivo* is direct or indirect. In addition, because Nodal and Activin signal through the same receptor complex and downstream Smad proteins, it has also been unclear if Activin itself has any role. Our current data suggest that Activin rather than Nodal acts in conjunction with FGF4 directly on TS cells influencing both TS cell maintenance as well as trophoblast cell fate.

Materials and methods

Animals

CD-1 male and female mice (Charles River) were crossed to generate embryos and conceptuses for experiments. Blastocyst stage embryos for trophoblast stem (TS) cell derivations were flushed from uteri at embryonic (E) day 3.5 (noon on the day of the vaginal plug is designated E0.5) while conceptuses for RNA extraction and histology were dissected from uteri from E7.5 to E15.5 as previously described (Hogan, 1994; Natale et al., 2006). All animals had free access to food and water and were housed under normal light conditions (12 hours light/12 hours dark). All of the animal procedures were carried out in accordance with the University of Calgary Animal Care Committee.

Cell culture

Cell culture experiments were carried out with two different trophoblast stem (TS) cell lines, one derived from a mouse carrying a LacZ transgene (tgRs26; kindly provided by Janet Rossant) and a new line derived from blastocyst stage embryos from CD-1 matings as previously described (Tanaka et al., 1998). TS cells were cultured at 37 °C under 5% CO_2 in air and maintained in RPMI 1640 culture medium (Invitrogen) supplemented with 20% fetal bovine serum (CanSera), 1 mM sodium pyruvate (Invitrogen), 50 μg/mL penicillin/streptomycin, 5.5×10^{-5} M β-mercaptoethanol (Invitrogen), 25 ng/mL basic fibroblast growth factor (bFGF; Sigma), and 1 μg/mL heparin (Fisher Scientific) with 70% of the medium being preconditioned by incubation with embryonic fibroblasts for 48 hours (Tanaka et al., 1998). For differentiation conditions, TS cell medium was used as described above but without supplementation with bFGF, heparin, and embryonic fibroblast pre-conditioning. TGC-conditioned medium was generated by incubation with differentiated TGCs for 48 hours prior to use in experiments. Recombinant human TGF-β1, recombinant human Activin A, recombinant mouse Follistatin and recombinant mouse Nodal (R&D Systems) were used at concentrations of 5 ng/mL, 10 ng/mL, 1 μg/mL, and 1–5 μg/mL, respectively. In inhibition experiments employing small molecule inhibitors, SB431542 (Sigma) and TGFβR1 kinase inhibitor (Calbiochem) were dissolved in DMSO and used at 20 μM. DMSO alone at similar concentrations was used as a control in these experiments.

RNA isolation, Northern blot, and RT–PCR

Total RNA was collected from ectoplacental cone plus chorion at E7.5 and E8.5 and from placentas at E11.5 and E15.5 by homogenization in TRIzol Reagent (Invitrogen), following the manufacturer's protocol. Total RNA from proliferating or differentiated TS cell cultures was collected by lysis in RLT buffer and Qiashredder column (Qiagen) and processing on RNeasy columns (Qiagen) as outlined in the manufacturer's instructions.

For Northern blot analysis, 10 μg of total RNA was separated by electrophoresis in 1.1% agarose formaldehyde gels, blotted onto GeneScreen nylon membrane (Perkin Elmer) and UV cross-linked. Random-primed DNA labeling of cDNA probes was carried out with 25 μCi ^{32}P-dCTP and isolated on Sephadex G-50 columns (Amersham Biosciences). Hybridizations were at 60 °C overnight as previously described (Church and Gilbert, 1984).

In RT–PCR, for each sample analysed, 1 μg of total RNA was reverse transcribed using 750 ng oligo(dT) primers and 200 U of M-MLV RNaseH-deficient reverse transcriptase (Promega). PCRs were carried out in the presence of 1.5 mM $MgCl_2$ for 30–35 cycles under the following conditions: denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s, followed by a final extension of 5 minutes at 72 °C. Primer sequences were as follows: *Inhba*-F, 5′-GGA GGG CTG GAA GAG GAA AAG GAA, *Inhba*-R, 5′-TGT TGA GTG GAA GGA GAG CGA GGA C; *Inhbb*-F, 5′-GGG TGT TGG GGG AGG GAG AGA A, *Inhbb*-R, 5′-GCC CAC TGC CTC CCC TGT CTC T; *Inhbc*-F, 5′-CTT CGT AGA CTT CCG TGA GAT TGG CT, *Inhbc*-R, 5′-CCG AGA TGT AGG CAC GCA GCA C; *Nodal*-F, 5′-GAA AAG CAG GTG TCC AGT CGA GCA, *Nodal*-R, 5′-CCA GGA GCC CCA GCC AAT CA; *Acvr1b*-F, 5′-TCA TGA TGC GGT CAC TGA CAC C, *Acvr1b*-R, 5′-TCT TTC CCA TCA CTC GCA AGG C; *Acvr2b*-F, 5′-CAT CAC GTG GAA CGA ACT GTG C, *Acvr2b*-R, 5′-AGC ATG TAC TCA TCG ACA GGC C; *Actb*-F, 5′-GTG GGC CGC TCT AGG CAC CAA, *Actb*-R, 5′-CTC TTT GAT GTC ACG CAC GAT TTC; *Gapd*-F, 5′-CCA GGA GCG AGA CCC CAC TAA CA, *Gapd*-R, 5′-TCG GCA GAA GGG GCG GAG.

Western blot and antibodies

Total cell lysates from TS cell cultures were isolated in RIPA buffer and analyzed by SDS-PAGE and immunoblotting using standard procedures. Antibodies recognizing mouse Smad2/3 and the phosphorylated form of mouse Smad2 (Cell Signaling Technology) were used at dilutions suggested by the manufacturer. A horseradish peroxidase-linked donkey anti-rabbit secondary antibody (GE Healthcare) was used in coordination with ECL plus substrate (GE Healthcare) to detect the primary antibody.

Tissue preparation and in situ hybridization

Conceptuses for *in situ* hybridization were dissected at E8.5, fixed overnight in 4% paraformaldehyde (PFA) in PBS at 4 °C, embedded in OCT, and sectioned (9 μm) as previously described (Natale et al., 2006; Simmons et al., 2007, 2008). Digoxigenin-labeled riboprobes were prepared according to the manufacturer's protocol (Roche). Plasmids for the preparation of *Inha*, *Inhba*, *Inhbb*, *Acvr1b*, and *Acvr2b* riboprobes were obtained from Dr. Martin Matzuk (Houston, TX, USA). For the preparation of *Cripto* and *Nodal* riboprobes, plasmids were obtained from Dr. Michael Shen (New York, NY, USA) and Dr. Brigid Hogan (Durham, NC, USA), respectively. Riboprobes for *Gcm1*, *Prl2c2*, *Prl3d1*, and *Tpbpa* have been previously described (Simmons et al., 2008). Riboprobes for *Cryptic* (*Cfc1*), *Jarid1c*, *Ctsb*, *Sdc1*, and *Mmp9* were generated from plasmids following PCR amplification, cloning into pGEM T-easy (Promega) and confirmation of cDNA by sequencing for each gene. For *in situ* hybridization, sections were first rehydrated in PBS, then post-fixed in 4% PFA, treated with proteinase K (15 μg/ml for 5 minutes at room temperature), acetylated for 10 minutes (acetic anhydride, 0.25%; Sigma), and hybridized with DIG-labeled probes overnight at 65 °C. Hybridization buffer contained 1× salts (200 mM NaCl, 13 mM Tris, 5 mM sodium phosphate monobasic, 5 mM sodium phosphate dibasic, 5 mM EDTA), 50% formamide, 10% (wt./vol.) dextran sulfate, 1 mg/ml yeast tRNA (Sigma), 1× Denhardt's [1% (wt./vol.) bovine serum albumin, 1% (wt./vol.) Ficoll, 1% (wt./vol.) polyvinylpyrolidine) and cRNA probe (final dilution of 1:2000 from reaction with 1 μg template DNA). Post-hybridization washes were followed by an RNase treatment [400 mM NaCl, 10 mM Tris (pH 7.5), 5 mM EDTA, 20 μg/ml RNase A]. After blocking, sections were incubated overnight at 4 °C in alkaline phosphatase-conjugated, anti-DIG antibody (Sigma) diluted 1:2500 in blocking solution. Sections were washed and signals were detected using alkaline phosphatase immunohistochemistry as previously

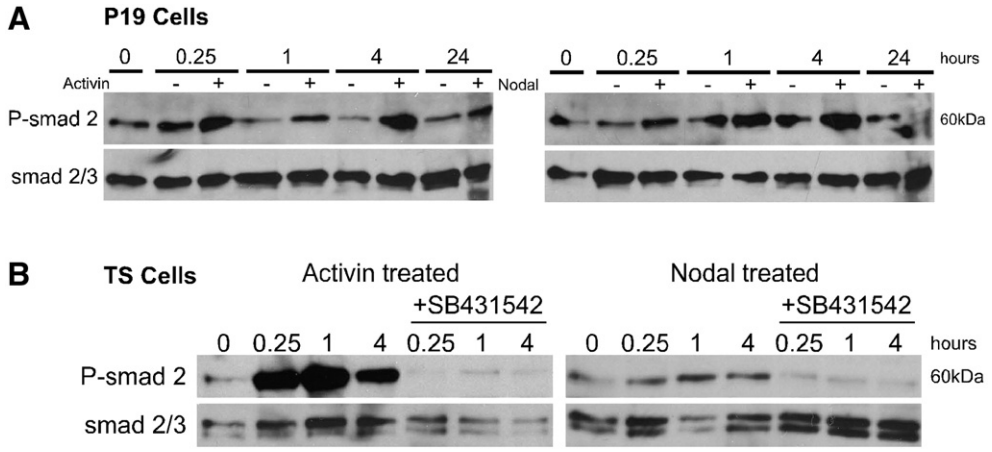

Fig. 2. Nodal is bioactive and results in increased phosphorylation of Smad-2 in P19 but not in TS cells. (A) P19 cells were treated with (+) and without (−) Nodal or Activin for 0, 0.25, 1, 4, or 24 hours. Expression of phosphorylated Smad2 (P-Smad2) versus total Smad2/3 was investigated by Western blot analysis. (B) TS cells in culture were treated with Activin or Nodal for 0, 0.25, 1, or 4 hours in the presence or absence of the Activin type I receptor inhibitor, SB431542. Expression of phosphorylated Smad2 (P-Smad2) versus total Smad2/3 was investigated by Western blot analysis.

described (Simmons et al., 2007, 2008). Sections were counterstained with nuclear fast red.

Microarray analysis

Microarray experiments were conducted in triplicate on independent experiments to examine the influence of Activin and TGF-β on differentiating TS cells. RNA was isolated from TS cells differentiated for 3 days under normal differentiation conditions (minus FGF/EFCM), in the presence of Activin (10 ng/mL) or in the presence of TGF-β (5 ng/mL). Gene expression profiling was preformed using Affymetrix MOE-430A chips (Affymetrix, Santa Clara, CA) and conducted by the StemCore Microarray Facility at the Ottawa Health Research Institute (Ottawa, Canada). Briefly, cDNA target preparation, labeling, and hybridization were carried out according to standard

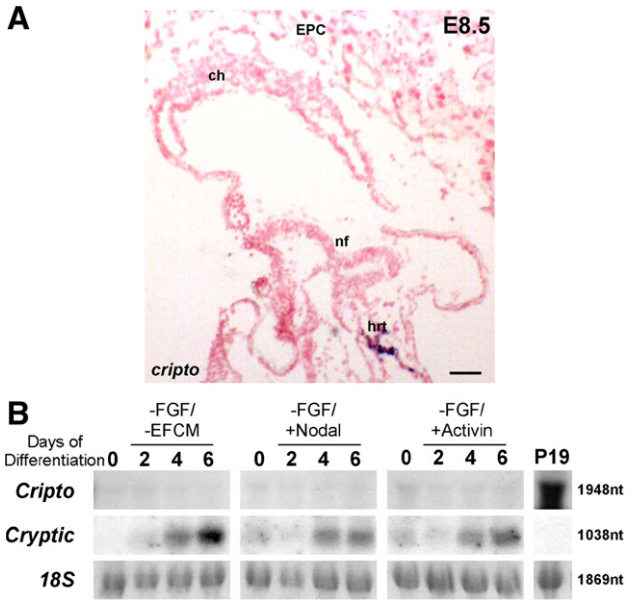

Fig. 3. The Nodal co-receptor, Cripto, is not expressed in the developing placenta or in TS cell cultures. (A) mRNA *in situ* hybridization was performed on E8.5 cryosections of embryo and placenta to detect expression of *Cripto* mRNA. *Cripto* was undetectable in trophoblast cells of the placenta but was expressed in the heart as previously described. (B) *Cripto* mRNA was undetectable by Northern blot in TS cells at 0, 2, 4, or 6 days of differentiation under normal differentiation conditions (−FGF/EFCM) or in the presence of Nodal (−FGF/+Nodal) or Activin (−FGF/+Activin). However, *Cripto* mRNA was detectable in the P19 embryonal carcinoma cell line. Conversely, *Cryptic* mRNA was detectable in TS cells primarily at 4 and 6 days of differentiation but undetectable in P19 cells. EPC, ectoplacental cone; ch, chorion; nf, neural fold; hrt, heart. Scale bar = 100 μm.

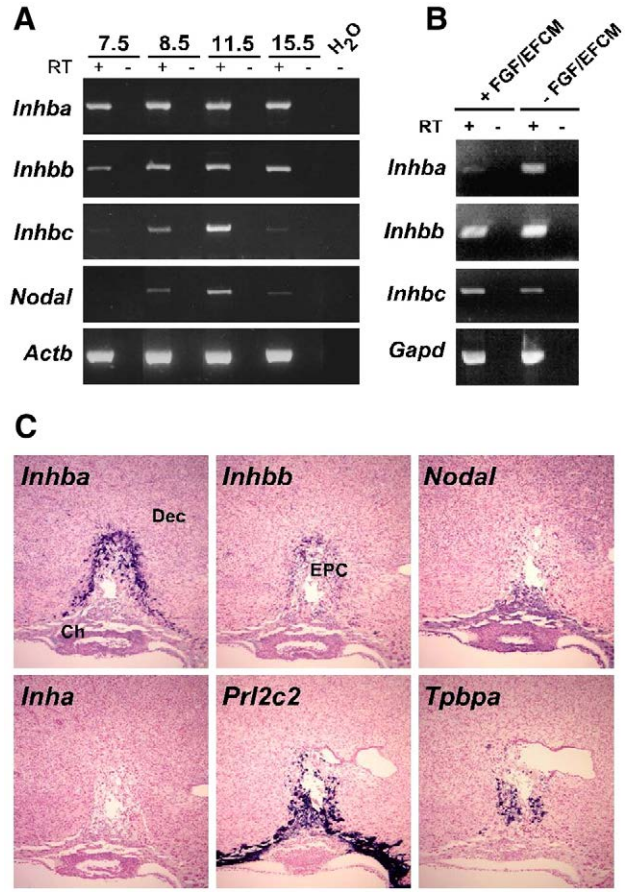

Fig. 4. Messenger RNAs encoding Activin ligand subunits are expressed in TS cells in culture and in trophoblast cells of the mid-gestation placenta. (A) RT–PCR analysis of *Inhba*, *Inhbb*, *Inhbc*, and *Nodal* mRNA at different times throughout gestation in the placenta. (B) RT–PCR analysis of proliferating (+FGF/EFCM) and differentiating (−FGF/EFCM) TS cells in culture. (C) *In situ* hybridization of *Inhba* and *Inhbb* mRNA at E8.5. *Inhba* and *Inhbb* mRNA were detected in the EPC and TGCs while *Nodal* was detected in the EPC and chorion. Expression of *Inha* was undetectable in the placenta while expression of *Prl2c2* and *Tpbpa* indicate parietal TGCs and trophoblast cells of the EPC, respectively. Dec, decidua; EPC, ectoplacental cone; Ch, chorion.

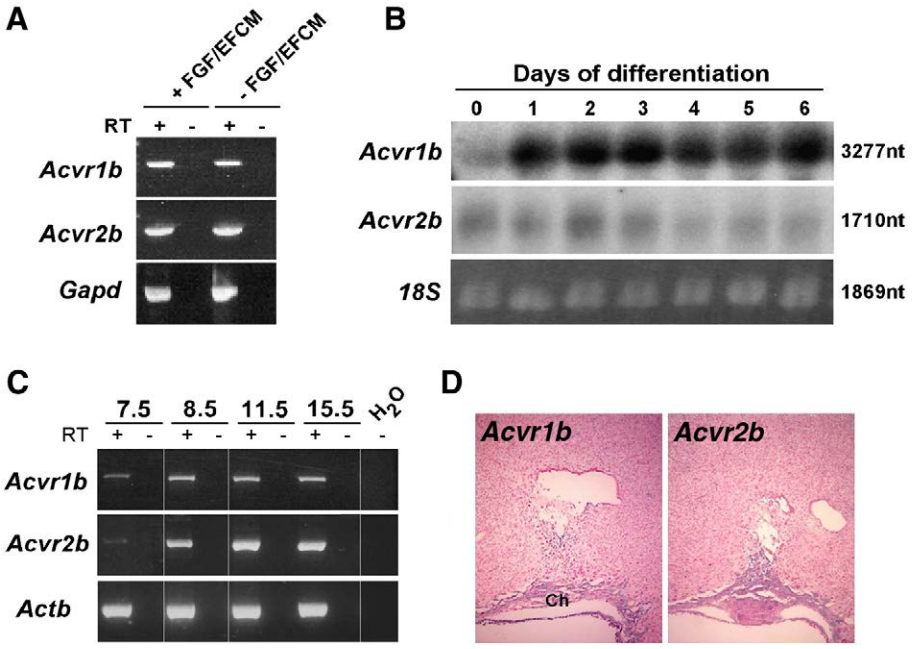

Fig. 5. Messenger RNAs encoding Activin type 1 and 2 receptors are expressed in trophoblast cells of the mid-gestation placenta and in trophoblast stem cells in culture. (A, B) Expression of *Acvr1b* and *Acvr2b* were detected by RT–PCR and Northern blot analysis in proliferating (+FGF/EFCM) and differentiating (−FGF/EFCM) TS cells in culture. (C) *Acvr1b* and *Acvr2b* were also detected by RT-PCR throughout gestation in the placenta as well as observed broadly expressed in trophoblast cells of the chorion and EPC at E8.5 by *in situ* hybridization (D). Ch, chorion.

Affymetrix protocols utilizing Hybridization Oven 640, Fluidics Station 450, and Scanner 3000 (Affymetrix, Santa Clara, CA). Image processing, normalization, and data analysis were performed using the Affymetrix Microarray suite 5.0 software. Genes with a false discovery rate P value of <0.05 and a signal log ratio fold change of greater or less than 1.5 were considered significantly different.

Results

Nodal does not maintain proliferation of trophoblast stem cells in culture

In order to investigate if Nodal has a direct effect on TS cells, similar to that reported for Activin and TGF-β, we cultured TS cell lines in medium containing FGF4 alone (+FGF), FGF4 plus embryonic feeder cell-conditioned medium (+FGF/+EFCM), FGF4 plus Activin or FGF4 plus Nodal (1 and 5 μg/mL). TS cells cultured in either FGF4 plus EFCM, or FGF4 plus Activin continued to proliferate, formed colonies with tight epithelial morphology and could be serially passaged every 5 days for several weeks (Fig. 1A). When examined by Northern blot after 10 days in culture, both of these groups of cells expressed the trophoblast stem cell marker gene, *Esrrb*, whereas expression of *Gcm1*, a marker of differentiating syncytiotrophoblast, was expressed at very low levels or was undetectable (Fig. 1B). In contrast, cells cultured in FGF4 alone or FGF4 plus 1 or 5 μg/mL of Nodal began to differentiate immediately and showed changes in morphology, including increases in cell and nuclear size and appearance of TGC-like cells (Fig. 1A). In addition, the cells failed to proliferate and could not be maintained past the second passage. Their expression of *Essrb* was dramatically reduced and expression of *Gcm1* was induced, indicating trophoblast differentiation (Fig. 1B). In order to ensure that the Nodal was bioactive, we tested the effect in P19 mouse embryonal carcinoma cells which respond to Nodal (Kumar et al., 2001). P19 cells cultured in the presence of Activin or Nodal both showed increased levels of phosphorylated Smad2 compared to untreated controls (Fig. 2A). When a similar experiment was conducted in TS cell cultures, a robust increase in phosphorylated Smad2 was observed in response to Activin but not to Nodal (Fig. 2B). This response could be blocked when cells were treated with SB431542 (Fig. 2B), a small molecule inhibitor of TGF-β/Activin/Nodal type 1 receptors (Laping et al., 2002; Ogawa et al., 2007; Watabe et al., 2003).

The Nodal co-receptor Cripto is not expressed in trophoblast stem cells

In order to address why TS cells were non-responsive to Nodal, we examined the expression of Cripto, a co-receptor required for Nodal, but not Activin, signaling (Gritsman et al., 1999; Kumar et al., 2001; Yeo and Whitman, 2001). In cryosections of whole E8.5 mouse implantation sites examined by RNA *in situ* hybridization, *Cripto* mRNA expression was undetectable in the placenta (Fig. 3A) but was observed in the heart as previously described (Dono et al., 1993). In addition, while *Cripto* mRNA was strongly expressed in P19 cells, it was virtually undetectable in TS cells by Northern blot analysis (Fig. 3B). Interestingly, analysis of the closely related molecule Cryptic (Shen et al., 1997) revealed that, while no expression was detected in the placenta at E8.5 by *in situ* hybridization (data not shown) nor in TS cells in the presence of FGF, *Cryptic* was strongly induced upon differentiation of TS cells (Fig. 3B).

Activin ligand and receptor mRNAs are expressed in placenta and TS cell cultures

To investigate the potential importance of Activin in supporting trophoblast proliferation both *in vitro* and *in vivo*, we characterized the expression of Activin ligand and receptor mRNAs in the developing placenta and TS cell cultures. Activin constitutes a dimer of combinations of three different subunits: βA, βB, and βC, encoded by the *Inhba*, *Inhbb*, and *Inhbc* genes, respectively (Shi and Massague, 2003). These βA, βB, and βC subunits can also form heterodimers with an α-subunit, encoded by *Inha*, to form the hormone Inhibin. Using RT–PCR we detected expression of β subunits in trophoblast tissues and the developing placenta beginning at E7.5 as well as in TS cells both in the presence and absence of FGF4/EFCM (Figs. 4A and B). When examined by *in situ* hybridization, *Inhba* and *Inhbb* were localized to cells of the ectoplacental cone and parietal TGCs surrounding the implantation site as suggested by co-localization with *Tpbpa* and *Proliferin* (*Prl2c2*), respectively (Fig. 4C). By contrast,

Inha was not detectable in these cell types. *Nodal* expression was observed at the base of the ectoplacental cone as well as the chorion from E8.5 onwards, but was absent from the trophoblast compartment at earlier stages (Fig. 4).

Activin and Nodal (but not TGF-β) signaling are transduced through a receptor complex made up of Activin receptor type 1 (Acvr1b/ALK4) and Activin receptor type 2 (Acvr2b) subunits (Shi and Massague, 2003). In TS cell cultures, mRNAs for both *Acvr1b* and *Acvr2b* were detectable by RT–PCR (Fig. 5A). When examined by Northern blotting, *Acvr1b* mRNA expression increased in differentiating TS cells while *Acvr2b* mRNA expression remained relatively constant (Fig. 5A). *In vivo*, *Acvr1b* and *Acvr2b* mRNAs were detectable by RT–PCR at E7.5 through E15.5 and were observed by *in situ* hybridization to be broadly expressed in all trophoblast cell subtypes at E8.5 (Fig. 5B).

Activin produced by parietal trophoblast giant cells suppresses TS cell differentiation

Given that Activin ligand and receptor subunits were expressed in trophoblast tissues of the developing placenta *in vivo* and in TS cell cultures, we tested if Activin produced by parietal TGCs could affect the differentiation of TS cells. We first examined the effect of blocking all TGF-β/Activin/Nodal signaling by using SB431542 to inhibit TGF-β/Activin/Nodal type I receptor activity. TS cells cultured in the presence of FGF4 but in the absence of EFCM (to reduce feeder cell-derived TGF-β/Activin) were exposed to 20 μM SB431542, as well as 1 μg/mL Follistatin or 20 μM TGFbR1 kinase inhibitor to specifically block Activin or TGF-β, respectively. We found that SB431542 promoted differentiation, even in the presence of exogenous Activin, as expression of trophoblast stem cell marker genes (*Eomes* and *Essrb*) was decreased while markers of TGCs (*Prl3d1* and *Prl3b1*) were increased in comparison to controls (Fig. 6A). Changes in cell morphology were also observed as a result of SB431542 treatment as cells in this group were fewer in number and showed an increase in frequency of TGCs compared to control (Fig. 6B). In contrast, treatment with either Follistatin or TGFbR1 kinase inhibitor alone resulted in minimal decreases in *Eomes* and *Essrb* expression and moderate changes in morphology (Figs. 6A and B). These data suggest that Activin and TGF-β were both present in the cultures and had paracrine effects on TS cell differentiation. To investigate this further, TS cells were cultured in the absence of FGF4 but in the presence of TGC-conditioned medium (TGC-CM). Compared to TS cells cultured in the absence of FGF4 and EFCM, TGC-CM caused a reduction in expression of differentiated markers *Prl3b1* and *Prl2c2* (Fig. 6C). When Follistatin was included with TGC-CM, the effect was decreased, although not completely (Fig. 6C).

Activin promotes differentiation of labyrinth trophoblast subtypes in the absence of FGF4

Our results indicated that together with FGF4, Activin (and/or TGF-β) is necessary and sufficient to maintain undifferentiated TS cells in culture. To further investigate its role in controlling trophoblast cell differentiation, we tested the effect of Activin after withdrawal of FGF4, which alone normally results in differentiation towards both TGC and syncytiotrophoblast fates (Hughes et al., 2004; Tanaka et al., 1998). In general, TGF-β treatment had only minor effects on the expression of marker genes; a moderate increase in the expression of *Esrrb* as well as in the syncytiotrophoblast cell gene *Syncytin B* (*Synb*) was observed. In addition, the ectoplacental cone (EPC)/spongiotrophoblast gene, *Tpbpa*, and the TGC genes, *Prl3d1*, *Prl3b1*, and *Prl2c2* (Fig. 7A) exhibited an earlier onset and increased level of expression (Fig. 7A). In contrast, TS cells differentiated in the presence of Activin displayed gene expression patterns quite different from both control and TGF-β-treated groups. Specifically, *Esrrb* was detectable until day 5 and the onset of *Mash2* was delayed until day 3 of differentiation. Most significantly, expression of the syncytiotrophoblast gene *Gcm1* began at day 2 and was detectable until day 8 of differentiation (Fig. 7A). This prolongation of *Gcm1* expression was also accompanied by an increase in *Synb* expression as well as a significant decrease and/or delay in the expression of *Tpbpa*, *Prl3d1*, *Prl3b1*, and *Prl2c2* (Fig. 7A).

The difference in the effects of Activin and TGF-β was assessed in more detail by using Affymetrix microarray analysis of RNA collected from TS cells differentiated in the presence of Activin or TGF-β for 3 days. When compared to controls, several genes were found to be up- or down-regulated by either Activin or TGF-β. Importantly, however, the overlap of genes exhibiting altered expression in response to Activin and TGF-β was relatively minimal with only 1157 genes commonly de-regulated by both Activin and TGF-β. By contrast, 2098 genes were differentially expressed only in response to Activin and 681 were changed specifically to TGF-β (Fig. 7B, Supplemental Tables). These data indicate that TS cells respond differentially to Activin and TGF-β.

To further address the requirement for Activin and TGF-β during TS cell differentiation, we again used the small molecule inhibitor, SB431542, to block Activin and TGF-β signaling during this process. In TS cells differentiated in the presence of SB431542, we were unable to detect expression of *Gcm1* (Fig. 7C). In addition, expression of *Tpbpa* and *Prl3b1* were both delayed and reduced compared to controls (Fig. 7C). These data suggest that Activin and/or TGF-β signaling is necessary for TS cell differentiation.

Activin and TGF-β-regulated genes have different patterns of expression in the developing placenta

To confirm and characterize the expression of genes identified in the microarray experiments, we performed further analysis on a subset of genes that were strongly de-regulated by either Activin or TGF-β. A total of 15 genes were investigated and confirmed to be expressed in TS cells by Northern blotting (data not shown). Expression of these genes was then examined by *in situ* hybridization on implantation sites at E8.5 and compared to that of *Gcm1* and *Prl3d1*, genes which were observed to be differentially expressed in response to Activin and TGF-β, respectively (Fig. 7A). Of the 15 genes examined, 11 were undetectable by *in situ* hybridization while the remaining 4 were expressed in distinct regions of the developing trophoblast tissues. *Gcm1* and *Jarid1c* were observed to be up-regulated by Activin in TS cells. *Gcm1* was expressed as previously described (Anson-Cartwright et al., 2000; Basyuk et al., 1999; Simmons et al., 2008) in trophoblast cells associated with sites of initiation of chorio-allantoic branching while *Jarid1c* was expressed in subsets of trophoblast throughout the chorion but was undetectable in trophoblast cells of the ectoplacental cone (Fig. 8A). Conversely, while both *Ctsb* and *Sdc1*, which were down-regulated by Activin in TS cells, were expressed in the chorion, their expression was also detectable in the ectoplacental cone (Fig. 8B). Expressions of *Mmp9* and *Prl3d1*, which were up-regulated in TS cells by TGF-β, were both restricted to parietal trophoblast giant cells at the interface between the developing placenta and the maternal decidua and were not detected in trophoblast cells of the ectoplacental cone or the chorion (Fig. 8C).

Discussion

The aim of the current study was to investigate the role of TGF-β/Activin/Nodal signaling in maintaining the pluripotency and controlling the differentiation of mouse TS cells. Our results indicate that proliferating, multipotent TS cells are not responsive to the mature form of Nodal and that Nodal is not capable of maintaining undifferentiated TS cells in culture. Activin on the other hand is

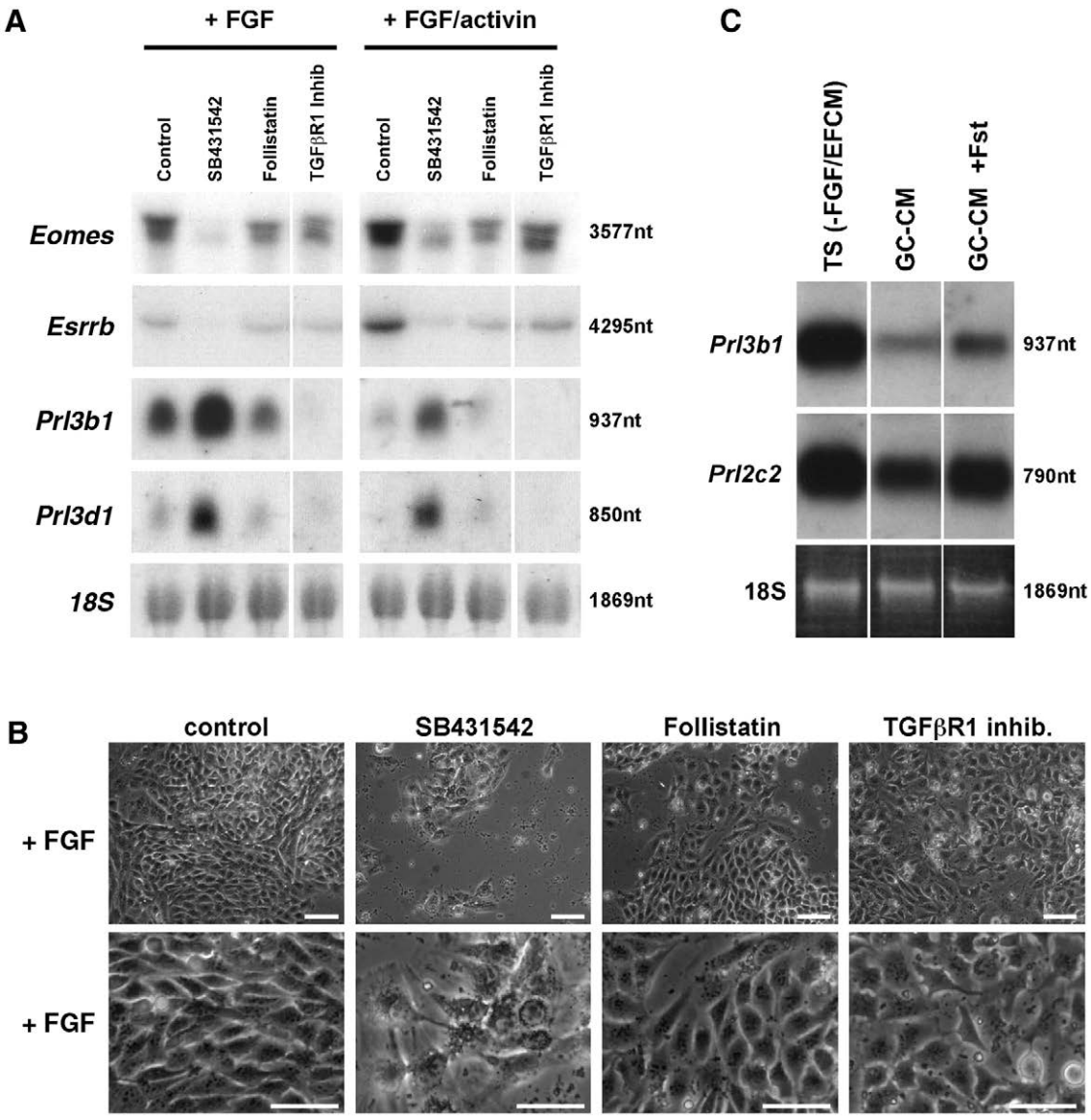

Fig. 6. Activin produced by parietal TGCs suppresses TS cell differentiation in culture. (A) Expression of trophoblast stem cell (*Eomes*, *Esrrb*) and TGC (*Prl3d1*, *Prl3b1*) marker genes in cultured TS cells. (B) Morphology of cells at low and higher magnification (scale bar = 100 μm). Cells were grown in the presence of FGF or FGF/Activin, with or without (Control) inhibitors of all type I receptors for TGF-β/Activin/Nodal (SB431542), Activin (Follistatin), or TGF-β (TGF-βR1 inhibitor). Treatment with SB431542 resulted in a reduction of *Eomes* and *Esrrb*, an increase in *Prl3d1* and *Prl3b1*, and an increase in the presence of TGCs. Inhibition of Activin or TGF-β alone also resulted in a slight reduction of *Eomes* and, in the case of Follistatin treatment, an increase in *Prl3b1*. These changes in gene expression were accompanied by moderate changes in morphology, suggesting differentiation, of TS cells cultured in Follistatin or TGF-βR1 inhibitor. (C) Expression of TGC markers (*Prl3b1* and *Prl2c2*) examined by Northern blot analysis in TS cell cultures. Cells were differentiated as usual (−FGF/EFCM), or in the presence of TGC-conditioned medium (GC-CM) or TGC-conditioned medium plus Follistatin (GC-CM + Fst). Expression of *Prl3b1* and *Prl2c2* were markedly reduced in response to GC-CM, an effect that was reversed by the addition of Follistatin.

expressed by trophoblast cells both in culture and *in vivo* and affects TS cell maintenance when FGF4 is present and TS cell fate upon differentiation after FGF4 withdrawal. Based on our results, we propose the hypothesis that Activin, through paracrine signaling from TGCs, acts to regulate TS cell differentiation and promotes the expression of genes required for the formation of the labyrinth layer.

TGF-β/Activin/Nodal signaling is one branch of the greater TGF-β superfamily of growth factors and has been extensively studied in early embryonic development (Kitisin et al., 2007; Shi and Massague, 2003). Acting through similar receptor complexes and sharing downstream effector molecules (Smad2/3 and Smad 4), unique roles for TGF-β and Activin/Nodal signaling have been elucidated by gene knockout studies in mice (Goumans and Mummery, 2000). With respect to early embryonic development and placenta formation, TGF-β signaling has been shown to be indispensable around mid-gestation for extraembryonic endothelial cell differentiation and yolk sac vasculogenesis (Dickson et al., 1995; Larsson et al., 2001; Oshima et al., 1996). By contrast, Nodal signaling is required for development of embryonic mesoderm (Iannaccone et al., 1992), primitive streak (Conlon et al., 1994), and placenta (Guzman-Ayala et al., 2004; Iannaccone et al., 1992), and similar phenotypes are observed for mouse embryos with targeted mutations in type I or type II Activin receptors (Gu et al., 1998; Song et al., 1999). Further studies have established that Nodal is essential for maintaining the pluripotency of TS cells in early post-implantation stage embryos working in concert with FGF4 (Guzman-Ayala et al., 2004). Both FGF4 and Nodal are expressed by the embryonic ectoderm. The FGF receptor, Fgfr2, is expressed in trophoblast cells (extraembryonic ectoderm) adjacent to the FGF signal and is essential for maintaining pluripotency (Guzman-Ayala et al., 2004).

The mechanism of Nodal action has been less clear. When TS cell lines were first isolated, feeder cells in the form of embryonic

fibroblasts (or conditioned medium from embryonic fibroblasts) were required to establish and maintain the cells together with FGF4. We (this study) and others (Erlebacher et al., 2004) have found that Activin (which works through the same receptors as Nodal) and TGF-β can replace feeder cells and maintain the proliferation and pluripotency of cultured TS cells in conjunction with FGF4. Based on these data, it has been assumed that Nodal acts directly on trophoblast cells *in vivo*. However, Nodal is also known to have autocrine effects on the embryonic ectoderm (Guzman-Ayala et al., 2004), and therefore, the effects of Nodal *in vivo* could be indirect through maintenance of FGF4 production. We addressed this question by culturing TS cells in Nodal to determine if it was sufficient to maintain the self-renewing state of TS cells and hence if it could replace the feeder cell requirement. To our surprise, while we could maintain TS cell lines in the presence of FGF4 plus Activin, FGF4 plus Nodal was without effect even at very high doses. In pursuing this further, we examined the expression of the closely related molecules Cripto and Cryptic, co-receptors that are essential for Nodal but not Activin binding to their common receptor complex (Acvr2b/Acvr1b). We found that TS cells, both *in vitro* and *in vivo*, do not express either Cripto or Cryptic, although Cryptic expression was detected in trophoblast differentiated from TS cells *in vitro*. Taken together, these data pointed towards a critical role of Activin as opposed to Nodal in trophoblast stem cell maintenance.

The difference in response to Nodal and Activin implies that spatial differences in their pattern of expression and activity may be important. An alternative is that trophoblast cells may respond only to the preprocessed, uncleaved form of Nodal. The. Nodal precursor,

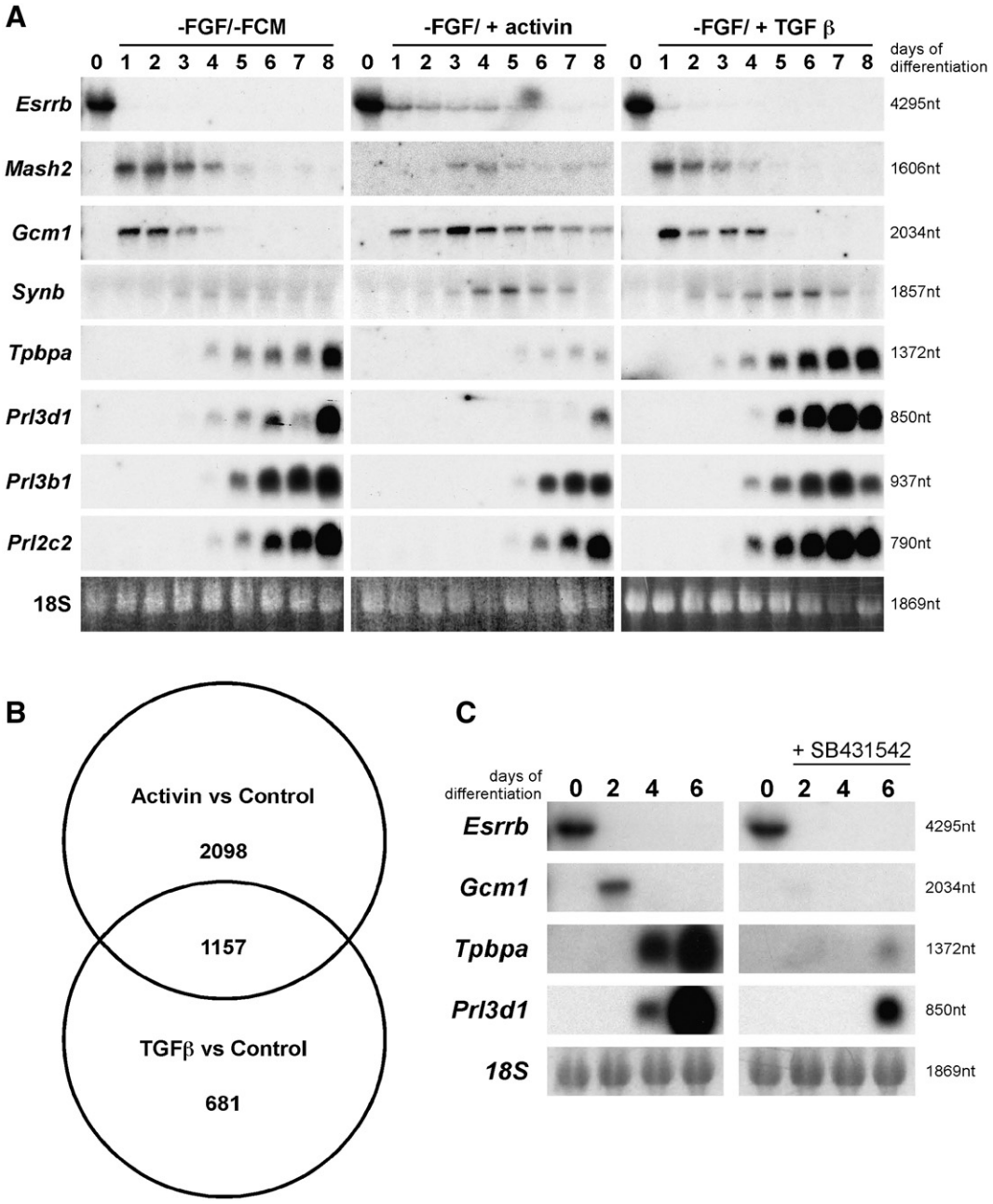

Fig. 7. Activin promotes differentiation of labyrinth trophoblast subtypes in the absence of FGF4 in culture. (A) Northern blot analysis for markers of proliferating TS cells and differentiating trophoblast subtypes in cultures treated with Activin or TGF-β versus controls. (B) Summary of Affymetrix microarray experiments showing the number of genes significantly up- or down-regulated (1.5-fold) versus control in TS cells that were differentiated for 3 days in the presence of Activin or TGF-β. (C) Treatment of TS cells with SB431542 during differentiation resulted in a reduction and/or delay of expression of *Gcm1*, *Tpbpa*, and *Prl3d1*.

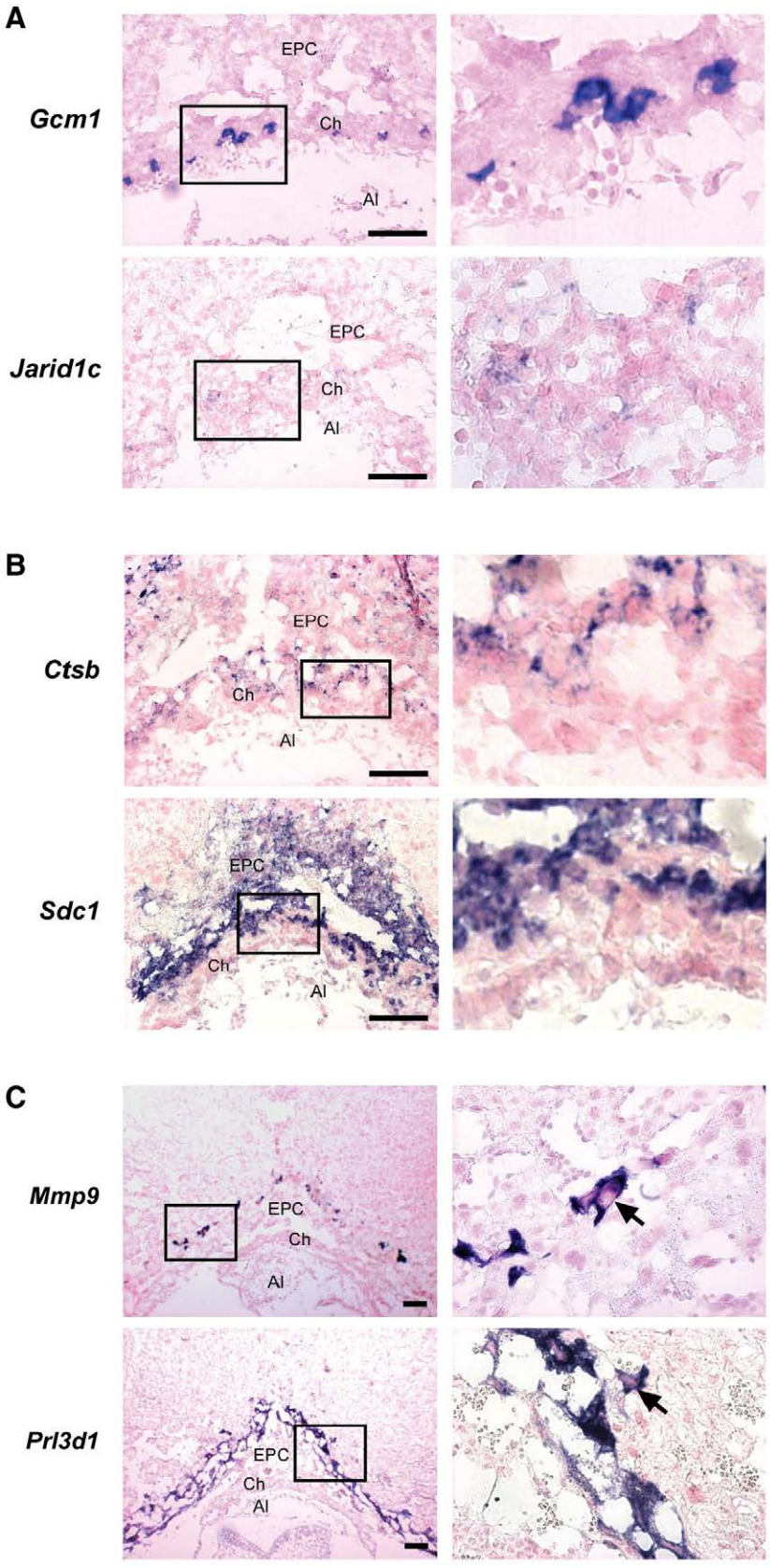

Fig. 8. Activin and TGF-β-regulated genes have different expression patterns *in vitro* and *in vivo*. The expression of genes identified as up-regulated (A; *Gcm1*, *Jarid1c*) or down-regulated (B; *Ctsb*, *Sdc1*) by Activin or up-regulated by TGF-β (C; *Mmp9*) were investigated by *in situ* hybridization on sections of placenta tissue at E8.5. *Gcm1* and *Jarid1c* were restricted to the chorion while *Ctsb* and *Sdc1* were expressed in the top of the chorion as well as the ectoplacental cone. *Mmp9* expression overlapped with expression of *Prl3d1* and was restricted to parietal TGCs (arrow). Boxed regions are shown in higher magnification to the right of each micrograph. Scale bars = 100 μm. EPC, ectoplacental cone; Ch, chorion; Al, allantois.

which is produced by the epiblast, acts on the extraembryonic ectoderm to maintain expression of the subtilisin-like proprotein convertases (SPC) Furin and PACE4 (Ben-Haim et al., 2006). In turn, Furin and PACE4 cleave the Nodal proprotein into its mature, active form thereby providing a source of active Nodal that acts on the epiblast to maintain FGF4 expression and to specify anterior visceral endoderm and mesoderm in the embryo (Ben-Haim et al., 2006; Guzman-Ayala et al., 2004). Taken together, TS cells appear to be able to respond only to the unprocessed form of Nodal; however, this does not exclude the possibility that differentiated trophoblast may, in fact, respond to processed Nodal.

While previous studies also implied that Activin and TGF-β have similar effects on TS cells (Erlebacher et al., 2004), our cumulative data indicate that these two factors have different effects and roles in regulating trophoblast development. Both Activin and TGF-β, when combined with FGF4, are capable of maintaining undifferentiated TS cell proliferation *in vitro* (Erlebacher et al., 2004). However, they have very different effects on differentiating TS cells after withdrawal from FGF4 as shown by both microarray analysis of global gene expression and expression studies of specific differentiation markers. The spatial expression patterns of TGF-β ligands (Slager et al., 1991; Tamada et al., 1990) and receptors have been documented (Mariano et al., 1998; Roelen et al., 1994). While TGF-β ligands and receptors are broadly expressed in the maternal decidua, and developing embryo, the primary sites of receptor expression (and therefore signaling activity) in the placenta at mid-gestation are in the EPC and TGC layer (Mariano et al., 1998; Roelen et al., 1994). Based on the response of TS cells *in vitro* to TGF-β (this study and (Erlebacher et al., 2004), we expect that cells in the chorion would also be responsive to this ligand. However, while Acvr1b mutants (therefore deficient in Activin/Nodal signaling) have trophoblast defects, none of the targeted mutants in TGF-β ligands or receptors show a trophoblast phenotype (Goumans and Mummery, 2000; Jones et al., 2006). This suggests that TGF-β signaling is not likely to be important for maintaining the TS cell environment *in vivo*.

In contrast to TGF-β, our data support a role for Activin *in vivo* such that it mediates a paracrine signaling interaction between TGCs and TS cells. Using *in situ* hybridization and RT–PCR, we could not detect expression of *Inha* (encoding the α subunit) mRNA, but detected all of the β subunits (*Inhba*, *Inhbb*, and *Inhbc*). The strongest expression was in parietal TGCs, which surround the implantation site and are the first differentiated type of trophoblast cells to form during development (Muntener and Hsu, 1977). In support of the idea that Activin is produced *in vivo* and feeds back to TS cells, TGC-conditioned medium had an activity that could maintain the expression of stem cell markers and suppress the expression of differentiated markers that was at least partially reversible by Follistatin (a binding protein inhibitor of Activin but not TGF-β or Nodal) (Shi and Massague, 2003). TGF-β is present in the serum of cultured cells, and this may have limited the reversibility of the effect. This is supported by the fact that inhibition of both TGF-β and Activin with SB431542 was very effective in promoting TS cell differentiation, whereas inhibition of TGF-β signaling on its own had little or no effect.

Activin receptor expression was detectable throughout the trophoblast lineage at E8.5, not just in the chorion where trophoblast progenitor cells reside. These data implied that Activin could act on various different cell types including those that are beginning to differentiate. To determine if this was the case, we removed FGF4 from TS cell cultures but maintained them in the presence of Activin. Compared to control cells, TGF-β, if anything, promoted TGC differentiation based on the expression of TGC-specific genes, although some transient *Synb* expression, indicative of syncytiotrophoblast, was detected. This is consistent with our finding that *Mmp9*, a gene expressed by parietal TGCs, is up-regulated in response to TGF-β. By contrast, Activin maintained the expression of *Esrrb* (stem cell marker), delayed the onset of *Mash2* expression (spongiotrophoblast and chorion), suppressed onset and level of TGC markers (*Prl3d1*, *Prl3b1*, and *Prl2c2*), and maintained the expression of *Gcm1* throughout the time course. In addition, when we examined the *in vivo* expression patterns of genes found to be responsive to Activin, those that were up-regulated (*Gcm1* and *Jarid1c*) were expressed exclusively in trophoblast cells of the chorion. We have previously presented a model that trophoblast cells in the chorion contain progenitors of the labyrinth layer, including syncytiotrophoblast (Simmons et al., 2008). Those genes that were down-regulated by activin (*Sdc1* and *Ctsb*) were expressed in the chorion, but also in the ectoplacental cone, which gives rise to spongiotrophoblast and TGCs (Simmons et al., 2007, 2008). These data suggest that when TS cells begin to differentiate upon withdrawal of FGF4, Activin promotes differentiation away from the TGC fate and toward syncytiotrophoblast cell fate. Because TGCs produce Activin, it implies that Activin acts as part of a negative feedback loop to regulate further TGC differentiation (Fig. 9). This effect is similar to that observed in retinal development, where retinal ganglion cells (RGCs) and amacrine cells secrete factors to inhibit the differentiation of additional cells of that type. In RGCs, the factor is growth and differentiation factor-11 (GDF11) (Kim et al., 2005; Liem et al., 1997), whereas in amacrine cells, it is TGF-βII (Ma et al., 2007), both of which are members of the TGF-β superfamily (Shi and Massague, 2003). In addition, however, in the trophoblast cell lineage, Activin has a permissive and/or instructional effect in promoting an alternative fate, namely differentiation towards *Gcm1*-positive syncytiotrophoblast. The increase in *Gcm1* expression was accompanied by an increase in *Synb* expression, which we have shown to be co-expressed with *Gcm1* in syncytiotrophoblast (Simmons et al., 2008) and is known to promote cell–cell fusion (Dupressoir et al., 2005). It is notable that while the effect on

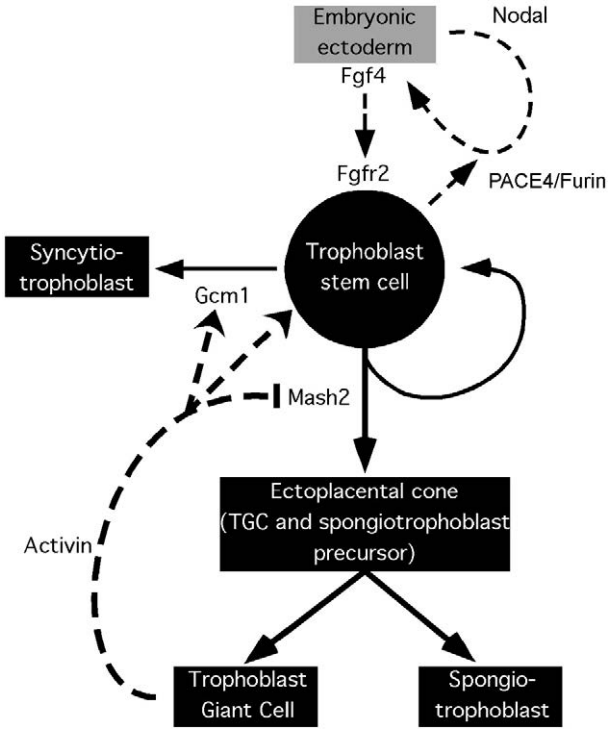

Fig. 9. A model for paracrine signaling, in which Activin produced by TGCs, controls fate decisions of TS cells. In the presence of FGF4 (produced by the embryonic ectoderm) and Activin (produced by TGCs), TS cells proliferate and self-renew. This process is maintained, as previously described, by Nodal produced by the embryonic ectoderm that is cleaved and activated by serine proteases (PACE4/Furin) produced by the extraembryonic ectoderm/TS cell compartment. Activated Nodal, in turn, maintains the production of FGF4 by the embryonic ectoderm. As TS cells differentiate, paracrine Activin signaling inhibits the differentiation of spongiotrophoblast and TGCs while promoting the differentiation of syncytiotrophoblast.

Gcm1 expression was dramatic, we did not see morphological evidence of extensive trophoblast cell–cell fusion. It may be that other factors are required to promote that final step in differentiation such as interactions with the allantois (Cross et al., 2006).

Acknowledgments

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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.2009.08.022.

References

Anson-Cartwright, L., Dawson, K., Holmyard, D., Fisher, S.J., Lazzarini, R.A., Cross, J.C., 2000. The glial cells missing-1 protein is essential for branching morphogenesis in the chorioallantoic placenta. Nat. Genet. 25, 311–314.

Basyuk, E., Cross, J.C., Corbin, J., Nakayama, H., Hunter, P., Nait-Oumesmar, B., Lazzarini, R.A., 1999. Murine Gcm1 gene is expressed in a subset of placental trophoblast cells. Dev. Dyn. 214, 303–311.

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.

Chang, H., Brown, C.W., Matzuk, M.M., 2002. Genetic analysis of the mammalian transforming growth factor-beta superfamily. Endocr. Rev. 23, 787–823.

Church, G.M., Gilbert, W., 1984. Genomic sequencing. Proc. Natl. Acad. Sci. U. S. A 81, 1991–1995.

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.

Cross, J.C., Nakano, H., Natale, D.R., Simmons, D.G., Watson, E.D., 2006. Branching morphogenesis during development of placental villi. Differentiation 74, 393–401.

de Caestecker, M., 2004. The transforming growth factor-beta superfamily of receptors. Cytokine Growth Factor Rev. 15, 1–11.

Derynck, R., Zhang, Y.E., 2003. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. Nature 425, 577–584.

Dickson, M.C., Martin, J.S., Cousins, F.M., Kulkarni, A.B., Karlsson, S., Akhurst, R.J., 1995. Defective haematopoiesis and vasculogenesis in transforming growth factor-beta 1 knock out mice. Development 121, 1845–1854.

Dono, R., Scalera, L., Pacifico, F., Acampora, D., Persico, M.G., Simeone, A., 1993. The murine cripto gene: expression during mesoderm induction and early heart morphogenesis. Development 118, 1157–1168.

Dupressoir, A., Marceau, G., Vernochet, C., Benit, L., Kanellopoulos, C., Sapin, V., Heidmann, T., 2005. Syncytin-A and syncytin-B, two fusogenic placenta-specific murine envelope genes of retroviral origin conserved in Muridae. Proc. Natl. Acad. Sci. U. S. A. 102, 725–730.

Erlebacher, A., Price, K.A., Glimcher, L.H., 2004. Maintenance of mouse trophoblast stem cell proliferation by TGF-beta/activin. Dev. Biol. 275, 158–169.

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.

Gardner, R.L., Johnson, M.H., 1973. Investigation of early mammalian development using interspecific chimaeras between rat and mouse. Nat. New Biol. 246, 86–89.

Gardner, R.L., Papaioannou, V.E., Barton, S.C., 1973. Origin of the ectoplacental cone and secondary giant cells in mouse blastocysts reconstituted from isolated trophoblast and inner cell mass. J. Embryol. Exp. Morphol. 30, 561–572.

Goldin, S.N., Papaioannou, V.E., 2003. Paracrine action of FGF4 during periimplantation development maintains trophectoderm and primitive endoderm. Genesis 36, 40–47.

Gotoh, N., Manova, K., Tanaka, S., Murohashi, M., Hadari, Y., Lee, A., Hamada, Y., Hiroe, T., Ito, M., Kurihara, T., Nakazato, H., Shibuya, M., Lax, I., Lacy, E., Schlessinger, J., 2005. The docking protein FRS2alpha is an essential component of multiple fibroblast growth factor responses during early mouse development. Mol. Cell Biol. 25, 4105–4116.

Goumans, M.J., Mummery, C., 2000. Functional analysis of the TGFbeta receptor/Smad pathway through gene ablation in mice. Int. J. Dev. Biol. 44, 253–265.

Gritsman, K., Zhang, J., Cheng, S., Heckscher, E., Talbot, W.S., Schier, A.F., 1999. The EGF-CFC protein one-eyed pinhead is essential for nodal signaling. Cell 97, 121–132.

Gu, Z., Nomura, M., Simpson, B.B., Lei, H., Feijen, A., van den Eijnden-van Raaij, J., Donahoe, P.K., Li, E., 1998. The type I activin receptor ActRIB is required for egg cylinder organization and gastrulation in the mouse. Genes Dev. 12, 844–857.

Guzman-Ayala, M., Ben-Haim, N., Beck, S., Constam, D.B., 2004. Nodal protein processing and fibroblast growth factor 4 synergize to maintain a trophoblast stem cell microenvironment. Proc. Natl. Acad. Sci. U. S. A. 101, 15656–15660.

Hogan, B., 1994. Manipulating the Mouse Embryo : A Laboratory Manual. Cold Spring Harbor Laboratory Press, Plainview, N.Y.

Hughes, M., Dobric, N., Scott, I.C., Su, L., Starovic, M., St-Pierre, B., Egan, S.E., Kingdom, J.C., Cross, J.C., 2004. The Hand1, Stra13 and Gcm1 transcription factors override FGF signaling to promote terminal differentiation of trophoblast stem cells. Dev. Biol. 271, 26–37.

Iannaccone, P.M., Zhou, X., Khokha, M., Boucher, D., Kuehn, M.R., 1992. Insertional mutation of a gene involved in growth regulation of the early mouse embryo. Dev. Dyn. 194, 198–208.

Jones, R.L., Stoikos, C., Findlay, J.K., Salamonsen, L.A., 2006. TGF-beta superfamily expression and actions in the endometrium and placenta. Reproduction 132, 217–232.

Kim, J., Wu, H.H., Lander, A.D., Lyons, K.M., Matzuk, M.M., Calof, A.L., 2005. GDF11 controls the timing of progenitor cell competence in developing retina. Science 308, 1927–1930.

Kitisin, K., Saha, T., Blake, T., Golestaneh, N., Deng, M., Kim, C., Tang, Y., Shetty, K., Mishra, B., Mishra, L., 2007. Tgf-beta signaling in development. Sci STKE. 2007 cm1.

Kumar, A., Novoselov, V., Celeste, A.J., Wolfman, N.M., ten Dijke, P., Kuehn, M.R., 2001. Nodal signaling uses activin and transforming growth factor-beta receptor-regulated Smads. J. Biol. Chem. 276, 656–661.

Laping, N.J., Grygielko, E., Mathur, A., Butter, S., Bomberger, J., Tweed, C., Martin, W., Fornwald, J., Lehr, R., Harling, J., Gaster, L., Callahan, J.F., Olson, B.A., 2002. Inhibition of transforming growth factor (TGF)-beta1-induced extracellular matrix with a novel inhibitor of the TGF-beta type I receptor kinase activity: SB-431542. Mol. Pharmacol. 62, 58–64.

Larsson, J., Goumans, M.J., Sjostrand, L.J., van Rooijen, M.A., Ward, D., Leveen, P., Xu, X., ten Dijke, P., Mummery, C.L., Karlsson, S., 2001. Abnormal angiogenesis but intact hematopoietic potential in TGF-beta type I receptor-deficient mice. EMBO J. 20, 1663–1673.

Lau, A.L., Kumar, T.R., Nishimori, K., Bonadio, J., Matzuk, M.M., 2000. Activin betaC and betaE genes are not essential for mouse liver growth, differentiation, and regeneration. Mol. Cell Biol. 20, 6127–6137.

Liem Jr., K.F., Tremml, G., Jessell, T.M., 1997. A role for the roof plate and its resident TGFbeta-related proteins in neuronal patterning in the dorsal spinal cord. Cell 91, 127–138.

Ma, G.T., Soloveva, V., Tzeng, S.J., Lowe, L.A., Pfendler, K.C., Iannaccone, P.M., Kuehn, M.R., Linzer, D.I., 2001. Nodal regulates trophoblast differentiation and placental development. Dev. Biol. 236, 124–135.

Ma, L., Cantrup, R., Varrault, A., Colak, D., Klenin, N., Gotz, M., McFarlane, S., Journot, L., Schuurmans, C., 2007. Zac1 functions through TGFbetaII to negatively regulate cell number in the developing retina. Neural. Develop. 2, 11.

Mariano, J.M., Montuenga, L.M., Prentice, M.A., Cuttitta, F., Jakowlew, S.B., 1998. Concurrent and distinct transcription and translation of transforming growth factor-beta type I and type II receptors in rodent embryogenesis. Int. J. Dev. Biol. 42, 1125–1136.

Matzuk, M.M., Kumar, T.R., Vassalli, A., Bickenbach, J.R., Roop, D.R., Jaenisch, R., Bradley, A., 1995. Functional analysis of activins during mammalian development. Nature 374, 354–356.

Muntener, M., Hsu, Y.C., 1977. Development of trophoblast and placenta of the mouse. A reinvestigation with regard to the *in vitro* culture of mouse trophoblast and placenta. Acta Anat. (Basel) 98, 241–252.

Natale, D.R., Starovic, M., Cross, J.C., 2006. Phenotypic analysis of the mouse placenta. Methods Mol. Med. 121, 275–293.

Ogawa, K., Saito, A., Matsui, H., Suzuki, H., Ohtsuka, S., Shimosato, D., Morishita, Y., Watabe, T., Niwa, H., Miyazono, K., 2007. Activin–Nodal signaling is involved in propagation of mouse embryonic stem cells. J. Cell Sci. 120, 55–65.

Oshima, M., Oshima, H., Taketo, M.M., 1996. TGF-beta receptor type II deficiency results in defects of yolk sac hematopoiesis and vasculogenesis. Dev. Biol. 179, 297–302.

Roelen, B.A., Lin, H.Y., Knezevic, V., Freund, E., Mummery, C.L., 1994. Expression of TGF-beta s and their receptors during implantation and organogenesis of the mouse embryo. Dev. Biol. 166, 716–728.

Shen, M.M., Wang, H., Leder, P., 1997. A differential display strategy identifies *Cryptic*, a novel EGF-related gene expressed in the axial and lateral mesoderm during mouse gastrulation. Development 124, 429–442.

Shi, Y., Massague, J., 2003. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. Cell 113, 685–700.

Simmons, D.G., Cross, J.C., 2005. Determinants of trophoblast lineage and cell subtype specification in the mouse placenta. Dev. Biol. 284, 12–24.

Simmons, D.G., Fortier, A.L., Cross, J.C., 2007. Diverse subtypes and developmental origins of trophoblast giant cells in the mouse placenta. Dev. Biol. 304, 567–578.

Simmons, D.G., Natale, D.R., Begay, V., Hughes, M., Leutz, A., Cross, J.C., 2008. Early patterning of the chorion leads to the trilaminar trophoblast cell structure in the placental labyrinth. Development 135, 2083–2091.

Slager, H.G., Lawson, K.A., van den Eijnden-van Raaij, A.J., de Laat, S.W., Mummery, C.L., 1991. Differential localization of TGF-beta 2 in mouse preimplantation and early postimplantation development. Dev. Biol. 145, 205–218.

Song, J., Oh, S.P., Schrewe, H., Nomura, M., Lei, H., Okano, M., Gridley, T., Li, E., 1999. The type II activin receptors are essential for egg cylinder growth, gastrulation, and rostral head development in mice. Dev. Biol. 213, 157–169.

Tamada, H., McMaster, M.T., Flanders, K.C., Andrews, G.K., Dey, S.K., 1990. Cell type-specific expression of transforming growth factor-β1 in the mouse uterus during the periimplantation period. Mol. Endocrinol. 4, 965–972.

Tanaka, S., Kunath, T., Hadjantonakis, A.K., Nagy, A., Rossant, J., 1998. Promotion of trophoblast stem cell proliferation by FGF4. Science 282, 2072–2075.

Uy, G.D., Downs, K.M., Gardner, R.L., 2002. Inhibition of trophoblast stem cell potential in chorionic ectoderm coincides with occlusion of the ectoplacental cavity in the mouse. Development 129, 3913–3924.

Vassalli, A., Matzuk, M.M., Gardner, H.A., Lee, K.F., Jaenisch, R., 1994. Activin/inhibin beta B subunit gene disruption leads to defects in eyelid development and female reproduction. Genes Dev. 8, 414–427.

Watabe, T., Nishihara, A., Mishima, K., Yamashita, J., Shimizu, K., Miyazawa, K., Nishikawa, S., Miyazono, K., 2003. TGF-beta receptor kinase inhibitor enhances growth and integrity of embryonic stem cell-derived endothelial cells. J. Cell Biol. 163, 1303–1311.

Yeo, C., Whitman, M., 2001. Nodal signals to Smads through Cripto-dependent and Cripto-independent mechanisms. Mol. Cell 7, 949–957.