Maintenance of mouse trophoblast stem cell proliferation by TGF-β/activin

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Abstract

Mouse trophoblast stem (TS) cells can be grown indefinitely in vitro with FGF4 and embryonic fibroblast conditioned media (EFCM). Here, we report that the active protein components of EFCM include TGF-β and the related factor activin, and that long-term continuous TS cell proliferation is possible in media supplemented with only serum, FGF4, and TGF-β. As trophoblasts are an epithelial cell type, the promotion of TS cell proliferation represents an unusual function for TGF-β and activin since TGF-β in particular is well known as an inhibitor of nontransformed epithelial cell proliferation. Our data suggest that constitutive FGF signaling in TS cells selectively inhibits the ability of TGF-β to repress c-myc expression, a central component of the TGF-β cytostatic transcriptional response previously observed to be lost in other epithelial cell types upon oncogenic Ras transformation.

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Introduction

Delineating the precise extracellular environments required for mammalian stem cell renewal will both further the experimental study of stem cell behavior as well as advance the application of stem cell technologies in the clinical arena. Recently, in vitro culture conditions have been identified for the continuous growth of mouse trophoblast stem (TS) cells (Tanaka et al., 1998). TS cells can be isolated from blastocysts or extraembryonic ectoderm and retain the capacity to differentiate into all trophoblast subtypes of the placenta when injected into blastocysts. Besides their usefulness as a model stem cell system, TS cells studied in vitro will likely provide insight into the pathogenesis of defects in placental development and physiology. Full use of the TS cell system, however, has been limited by an incomplete understanding of the exact set of factors required for TS cell growth. Currently, TS cells are known to require fibroblast growth factor 4 (FGF4), as well as embryonic fibroblast feeder layers or the embryonic fibroblast conditioned media (EFCM) collected from these feeders (Tanaka et al., 1998). Removal of these factors leads to postmitotic differentiation largely into a trophoblast subtype called trophoblast giant cells.

Here, we identify TGF-β and activin as key protein components of EFCM involved in the maintenance of TS cell proliferation. TGF-β and activin are members of the TGF-β superfamily of secreted polypeptides, which have critical roles in regulating many developmental and physiological processes (Chang et al., 2002). TGF-β itself initiates cellular responses upon binding its heterotetrameric transmembrane receptor complex composed of type I (TβRI) and type II (TβRII) receptors, both serine–threonine kinases, whereas activin binds the ActRIB receptor in conjunction with either ActRIIA or ActRIIB (Derynck and Zhang, 2003). TGF-β and activin receptors both transduce signals largely through the phosphorylation and induced nuclear trans-
location of Smad2 and Smad3, which in association with Smad4 regulate gene expression by interacting with a large group of transcriptional coactivators and corepressors (Derynck and Zhang, 2003; Massague and Wotton, 2000). TGF-β and activin receptor signaling can evoke virtually identical transcriptional responses within the same cell type, in accord with the complete amino acid identity between the L45 loop of their type I receptor cytoplasmic domain and the function of this loop as a major determinant of Smad-dependent signaling specificity (Carcamo et al., 1994; Chen et al., 1998; Feng and Derynck, 1997).

G1 cell cycle arrest is an important epithelial cell type response towards TGF-β, and altered TGF-β responsiveness is thought to be a major factor contributing to epithelial cell tumorigenesis (Ten Dijke et al., 2002). The TGF-β cytostatic response is largely Smad dependent and is mediated by altered transcription of a relatively small set of cell cycle regulatory genes (Kang et al., 2003). In particular, TGF-β induces mRNA expression of p15Ink4b and p21Cip1, which encode two cyclin-dependent kinase (Cdk) inhibitors, and down-regulates the expression of Id1, Id2, and Id3, which encode proteins that both promote cell cycle progression as well as inhibit cell differentiation programs induced by basic helix-loop-helix transcription (bHLH) factors (Kang et al., 2003; Norton, 2000; Ten Dijke et al., 2002). Perhaps most importantly, TGF-β down-regulates mRNA expression of c-myc, which encodes a ubiquitous short-lived transcription factor central to cell cycle progression (Alexandrow and Moses, 1995). c-Myc down-regulation not only deprives cells of a growth stimulus in its own right, but also directly or indirectly promotes p15Ink4b and p21Cip1 mRNA induction upon TGF-β stimulation (Claassen and Hann, 2000; Feng et al., 2002; Seoane et al., 2001; Staller et al., 2001; Warner et al., 1999). These characteristics explain the ability of ectopic c-Myc expression to prevent TGF-β-induced cell cycle arrest (Alexandrow and Moses, 1995; Claassen and Hann, 2000). Furthermore, a selective inability to repress c-myc mRNA expression may explain why some cells transformed with oncogenic Ras are resistant to TGF-β-induced growth inhibition (Chen et al., 2001), although the basis for the phenomenon in general remains unclear (Derynck and Zhang, 2003). In TS cells, which are epithelial in origin, we find that a similar selective inhibition of the TGF-β cytostatic transcriptional program occurs in response to constitutive FGF signaling.

Materials and methods

Cells, cell culture reagents, and media

The TS cell lines EGFP-TS3.5, TSInk4a, and TSCMI have been described previously (Erlebacher et al., 2002; Tanaka et al., 1998). Cells were grown on non-gelatized tissue culture plastic in 37° humidified incubators containing 5% CO2 and were routinely passaged every 2–3 days. TS media were based upon DMEM/F-12 with 15 mM Heps (Invitrogen, Carlsbad, CA, catalog #11330-032) and contained 20% FCS (HyClone, Logan, UT), 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, and 100 μM beta-mercaptoethanol and was supplemented with 25 ng/ml human FGF4 (R&D Systems, Minneapolis, MN) plus 1 μg/ml heparin (Sigma, St. Louis, MO). Recombinant human TGF-β1, recombinant human Activin A, human ActRII/Fc (Sf21-expressed), and recombinant mouse follistatin were purchased from R&D Systems and used at concentrations of 2 ng/ml, 10 ng/ml, 5 μg/ml, and 1 μg/ml, respectively. Neutralizing anti-TGF-β antibodies (clone 1D11) were the generous gift of Genzyme (Cambridge, MA), and control mouse IgG antibodies were purchased from ICN (Aurora, OH); these antibodies were used with a concentration of 1 μg/ml. EFCM was an 80% volume/volume concentration of a low bicarbonate DMEM basal media conditioned for 3 days with embryonic fibroblasts (Specialty Media, Phillipsburg, NJ), with FCS adjusted to a final 20% concentration as described previously (Erlebacher et al., 2002).

Growth curves

EGFP-TS3.5 or TSInk4a cells (passage n = 25–45) were seeded in triplicate at a density of 80,000 or 20,000 cells, respectively, per 12-well tissue culture dish in 1 ml TS media containing FGF4/heparin. Every 3 days, all cells were trypsinized, washed twice in TS media, and passaged equivalently (1:4–1:8 for TSFGP, 1:8–1:20 for TSInk4a). The remaining cells were resuspended in PBS/1% FCS/5 mM EDTA and counted on a BD Biosciences FACSCalibur using CellQuest software for analysis (San Diego, CA). Total cell numbers were back calculated from 1 min of acquisition at the high flow rate (1 μl/s).

RNA analysis

To determine TSCMI mRNA expression levels in response to TGF-β alone, cells growing in TS media containing FGF4/heparin were plated 2 days before the direct addition of TGF-β1. For experiments determining TGF-β transcriptional responses in TSCMI as influenced by the presence or absence of FGF4, cells were plated 2 days before the 0-h time point and the plates were rinsed twice with PBS before the addition of fresh prewarmed TS media with or without FGF4/heparin and with either TGF-β or anti-TGF-β antibodies. For similar experiments with EGFP-TS3.5 (n = 30–40), we took two approaches using cells growing >1.5 months in TS media containing FGF4/heparin and TGF-β. In the first approach, we plated the cells 3 days before the zero time point and at −24 h rinsed the plate twice with PBS and replaced the media with fresh TS media containing only FGF4/heparin. At 0 h, the plates were treated as above. In the second approach, we
trypsinized the cells and plated them directly in TS media containing FGF4/heparin plus TGF-β, TGF-β alone, FGF4/heparin plus anti-TGF-β antibodies, or anti-TGF-β antibodies alone. RNA was prepared at 24 h. Lastly, for the effects of selective FGF4 or TGF-β withdrawal, EGFP-TS3.5 cells growing >1.5 months in TS media containing FGF4/heparin and TGF-β were trypsinized and an aliquot of these cells was immediately used to prepare the day 0 mRNA. The remaining cells were replated for days 1, 2, and 3 time points in appropriately supplemented TS media. Care was taken to ensure that cells were subconfluent for all experiments.

Total RNA was prepared using the Trizol reagent (Invitrogen), and real-time RT-PCR was performed as previously described (Erlebacher et al., 2002) using an ABI Prism 7700 Sequence Detector (Applied Biosystems). All reactions were run in duplicate using cDNA template synthesized from 10 ng RNA, except for the amplification of p15Ink4b, where 100 ng was used. C\textsubscript{T} values were averaged, and ΔC\textsubscript{T} values were calculated relative to β-actin. Transcript abundance relative to β-actin was calculated as \(2^{-\Delta CT}\), and relative transcript abundance between samples was calculated as \(2^{-(\Delta CT, \text{sample} - \Delta CT, \text{reference})}\). Statistical significance was determined by Student’s \(t\) test using groups of ΔC\textsubscript{T} values, and error bars were extrapolated from the standard deviation of the ΔC\textsubscript{T} mean for each group. In cases where an individual experiment is shown, bars representing the error from duplicate sample runs are omitted; C\textsubscript{T} duplicates were typically within 0.5 cycles, corresponding to a standard deviation in expression level of 1.4-fold.

In addition to those described previously (Erlebacher et al., 2002), we used the following primer or probe sets listed 5' to 3' in the order of forward primer, reverse primer, and probe (when employed): Tgfbr1, ggcagactgtatgcttggcag, gcaagtctgcagctgt, acgctgtgccttgaggtgtgc; Tgfbr2, tgcgcctgtcagctcttc, ccatgtctcttctcc, acctggcaca-cagcgcgcagg; Acvr2b, ggcagacaaagctctctctg, gggttggaagacgttgaga; Smad2, cgacaggaattgagccacagagt, tgggattccttttgccg, tgggtgaagttcacgttgaggg; Tgfbr2, gcgaacagaagttaaggccaaa, tcctgggcctactgtccaatg, cctgggcctactgtccaatg; Tgfbr1, gcgaacagaagttaaggccaaa, tcctgggcctactgtccaatg, cctgggcctactgtccaatg. All probes were dual-labeled with FAM and TAMRA; SYBRG was used for amplifications when a primer set was used without a probe. For all reactions, the approximate fold difference in amplification between plus and minus RT reactions was >1000×, except the SYBRG amplifications of β-actin (50×), p15Ink4b (25×), p21Cip1 (50×), PAI-1 (100×), and Smad7 (500×).

**Analysis of DNA content**

EGFP-TS3.5 cells were plated for 4 days in TS media containing FGF4 and TGF-β or containing TGF-β-neutralizing antibodies. Cells were trypsinized, fixed in 40% ethanol, treated with 50 mg/ml RNAase A (5 Prime, Boulder, CO), and stained with 46 μg/ml propidium iodide (PI, Sigma) dissolved in 38 mM sodium citrate before analysis by flow cytometry as above.

**Blastocyst injections and immunostaining**

Blastocysts derived from C57BL/6 matings were injected with 15–20 trypsinized EGFP-TS3.5 cells previously growing >2 months in TS media supplemented with FGF4 and TGF-β. Blastocysts were transferred into the uterine cavities of pseudopregnant B6CBAF1 females using standard techniques (Nagy et al., 2003). We implanted blastocysts into four mice, which were sacrificed on E7–12. Embryonic day was calculated relative to noon of the vaginal plug of the pseudopregnant recipient (E0.5). Dissected placentas and embryos were photographed using a Zeiss Stemi SV11 stereomicroscope equipped with GFP epifluorescence. All mouse experiments were approved by the Standing Committee on Animals of Harvard Medical School.

Paraffin-embedded sections of placentas were stained with a rabbit anti-GFP antibody (ab6556-25, Abcam, Cambridge, MA; diluted 1/3000), followed sequentially by a biotin-conjugated goat anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA), horseradish peroxidase-conjugated streptavidin, biotin–tyramide amplification (NEN, Boston, MA), and lastly streptavidin-Alexa 594 (Molecular Probes, Eugene, OR). Sections were counterstained with DAPI (Sigma) and digitally photographed at 10× magnification.

**Results and discussion**

TGF-β or activin is necessary and sufficient to maintain trophoblast stem cell proliferation in the presence of FGF4

We identified TGF-β or activin as critical factors for trophoblast stem cell maintenance using the TS1nk4a trophoblast stem cell line we had derived from h1nk4a/Arf\textsuperscript{12,3} mice deficient in both the p16\textsuperscript{Ink4a} and p19\textsuperscript{ARF} tumor suppressor genes (Erlebacher et al., 2002). This line expressed a set of trophoblast markers (Eomes, Esrrb,
Cdx2, Hand1, and Mash2) at levels similar to wild-type TS cells, required both FGF4 and EFCM for continuous growth and showed the same changes in gene expression upon differentiation (Erlebacher et al., 2002). However, it is important to note that the line had lost the tight epithelial morphology typical of TS cells at around passage \( n = 12 \), coinciding with an increase in growth rate (see below). TSInk4a cells \( (n = 25–45) \) cultured in EFCM plus FGF4 ceased dividing within 4 days upon the addition of a monoclonal antibody (1D11) that neutralizes all three TGF-\( \beta \) isoforms (TGF-\( \beta \)1, -\( \beta \)2, and -\( \beta \)3), plus either an activin-neutralizing chimeric protein (ActRIIB/Fc) consisting of the extracellular domain of the ActRIIB receptor fused to a human IgG1 Fc domain (Fig. 1D) or the natural activin antagonist follistatin (Fig. 1F). The cells instead started to assume a morphology typical of trophoblast giant cells in culture (Tanaka et al., 1998), with an increase in nuclear and total cell size, a flattening of the cells, and the appearance of conspicuous perinuclear granules. In contrast, none of these neutralizing reagents alone induced TSInk4a differentiation after 4 days (Figs. 1B, C, and E), with the cells maintaining a similar morphology to that seen with culture in EFCM plus FGF4 (Fig. 1A). Thus, we conclude that TGF-\( \beta \) or activin are required to maintain TSInk4a cell proliferation, and that both of these molecules are present in EFCM at sufficient levels to independently prevent cell differentiation in the short term.

Conversely, TGF-\( \beta \) or activin was sufficient to maintain TSInk4a proliferation in the absence of EFCM, with culture in FGF4 and either TGF-\( \beta \) or activin consistently improving the growth rate after a few days over that seen with continued culture in FGF4 and EFCM (Fig. 2A). In contrast, TSInk4a culture in FGF4 alone with or without control mouse IgG antibodies led to a cessation of cell proliferation (Fig. 2A) and the induction of morphological differentiation (Fig. 2A), implying that a functionally relevant non-EFCM TGF-\( \beta \) source was also present in our culture conditions. This source was likely a combination of that produced by trophoblasts themselves (Adelman et al., 2000), as well as that present in FCS, which

Fig. 1. TGF-\( \beta \) and activin are the active components of EFCM that prevent TS cell differentiation. TSInk4a cells were plated in EFCM plus FGF4 containing control mouse IgG antibodies (A, C, and E) or neutralizing anti-TGF-\( \beta \) antibodies (B, D, and F), with or without an activin-neutralizing ActRIIB/Fc fusion reagent (C and D) or activin-neutralizing follistatin (E and F). Cells were photographed after 4 days. Only the combined addition of anti-TGF-\( \beta \) neutralizing antibodies and either ActRIIB/Fc (D) or follistatin (F) inhibited proliferation and induced differentiation in to cells with a typical trophoblast giant cell morphology. Such cells showed a flattened appearance, increased nuclear and total cell size, and perinuclear granules. Arrows in panels D and F highlight two examples. Scale bar shows 200 \( \mu \)m.
at the 20% concentration in TS media contributed in the range of 1–5 ng/ml mature active TGF-β plus unprocessed latent TGF-β (O’Connor and Wakefield, 1987; Wight, 2000). Latent TGF-β was possibly made available to trophoblasts after proteolytic processing by matrix metalloproteinase-9 (MMP-9), a known activator of TGF-β expressed by trophoblast giant cells in vivo (Alexander et al., 1996; Yu and Stamenkovic, 2000) but also at high levels by TS cells in vitro (see below).

TGF-β or activin was also able to maintain wild-type TS cell proliferation in the absence of EFCM. Thus, the enhanced green fluorescent protein-expressing TS cell line EGFP-TS3.5 (Tanaka et al., 1998) cultured with FGF4 and either TGF-β (Fig. 2D) or activin (Fig. 2E) showed the same tight epithelial morphology and growth rate (Fig. 2B) seen with continued culture in FGF4 and EFCM (Figs. 2B and C). In contrast, culture in FGF4 alone with or without control mouse IgG antibodies led to a high degree of morphological differentiation by day 9 (Figs. 2F and G), and although the cells had largely maintained their proliferation rate up until this point (Fig. 2B), they were unable to sustain longer term culture in the absence of exogenous TGF-β or activin. As above, the temporary maintenance of EGFP-TS3.5 proliferation was due to a non-EFCM TGF-β source, as the addition of neutralizing anti-TGF-β antibodies led to a rapid decrease in cell proliferation and assumption of a giant cell morphology by day 9. This process was accelerated in presence of anti-TGF-β antibodies, where by day 9 the culture was largely degenerated (H). Scale bar shows 200 μm.

Fig. 2. TGF-β or activin are sufficient to maintain TS cell proliferation in the presence of FGF4. (A) TSInk4a cells previously growing in EFCM plus FGF4 were plated in EFCM plus FGF4 or TS media plus FGF4 containing either TGF-β, activin, no additional supplementation, control mouse IgG antibodies, or neutralizing anti-TGF-β antibodies. Cells were passaged and counted every 3 days. In the absence of EFCM, TGF-β, or activin, cells ceased proliferating and underwent morphological differentiation. The difference in growth upon culture in control IgG antibodies versus anti-TGF-β antibodies reflects the contribution of non-EFCM TGF-β either present in FCS or produced by trophoblasts themselves. (B) EGFP-TS3.5 cells previously growing in EFCM plus FGF4 were plated and passaged as in panel A. For both growth curves, data points shown mean ± SD of triplicate wells and data are representative of three independent experiments. (C–H) Morphology of EGFP-TS3.5 in various growth conditions. Cells previously growing in EFCM plus FGF4 were photographed after 9 days of culture in EFCM plus FGF4 (C) or TS media plus FGF4 and either TGF-β (D), activin (E), no additional supplementation (F), control mouse IgG antibodies (G), or neutralizing anti-TGF-β antibodies (H). EGFP-TS3.5 maintained its tight epithelial morphology only in the presence of EFCM, TGF-β, or activin. In the absence of further supplementation (F) or in the presence of control mouse IgG antibodies (G), the cells assumed a giant cell morphology by day 9. This process was accelerated in presence of anti-TGF-β antibodies, where by day 9 the culture was largely degenerated (H). Scale bar shows 200 μm.
directly or indirectly result from the Ink4a/Arf mutation in TSInk4a, the overall dependence of both lines on TGF-β/ activin is clearly conserved.

**Maintenance of stem cell characteristics of TS cells grown in FGF4 and TGF-β**

Several criteria support the conclusion that long-term culture in FGF4 and TGF-β is sufficient to maintain the stem cell characteristics of TS cells. First, the expression of a number of trophoblast stem cell markers was maintained after culture >1.5 months in media supplemented with only FGF4 and TGF-β, as compared to their expression during culture in FGF4 and EFCM (Fig. 3A). Specifically, we found similar levels of expression of *Eomes*, which encodes a T-box family transcription factor required in vivo for TS cell lineage determination (Russ et al., 2000); *Esrrb*, which encodes Errh, an orphan nuclear receptor required for continued TS cell proliferation (Luo et al., 1997); *Fgfr2*, which encodes the likely relevant FGF receptor expressed by trophoblasts (Rossant and Cross, 2001); and *Cdx2*, which encodes a caudal-type transcription factor likely also required for TS cell lineage determination in vivo (Chawengsaksophak et al., 1997). Furthermore, there was no change in the expression of *Hand1*, which encodes a bHLH transcription factor required for giant cell differentiation but also expressed by TS cells (Cross et al., 2003; Tanaka et al., 1998). The only statistically significant change in expression was a twofold up-regulation of *Mash2*, which encodes a bHLH transcription factor required for the development of spongiotrophoblasts and their progenitors (Guillemot et al., 1994).

Second, we found that the capacity for biochemical differentiation of TS cells was preserved after culture for >1.5 months in FGF4 and TGF-β. As described previously for TS cells growing in FGF4 plus EFCM (Tanaka et al., 1998), removal of both factors led to increased expression of the giant cell marker *placental lactogen-1* (*Pl-1*) and the ectoplacental cone and spongiotrophoblast marker *Tpbp* (Fig. 3B). In addition, we found that removal of FGF4 and EFCM up-regulated expression of another giant cell marker *Proliferin* (*Plf*), as well as the cdk inhibitor *p57Kip2*, in line with the role of this cell cycle inhibitor in TS cell differentiation to both trophoblast giant cell and spongio-

![Image of Figure 3](attachment:image.png)

**Fig. 3.** Maintenance of TS cell stem cell characteristics upon long-term culture in FGF4 and TGF-β. EGFP-TS3.5 cells were grown in TS media containing FGF4 plus TGF-β for >1.5 months. (A) Expression of trophoblast stem cell markers. Real-time RT-PCR was performed on RNA isolated on two different occasions and compared to two RNA isolates from EGFP-TS3.5 cells growing in FGF4 plus EFCM. Data show mean ± SD for these two RNA preparations. Only the twofold higher expression of Mash2 in cells grown in FGF4 plus TGF-β was statistically significant (*P* < 0.05). (B) Increased expression of trophoblast differentiation markers upon removal of FGF4 and TGF-β. Cells were cultured for 4 days in media lacking either growth factor and instead containing FGF4 plus TGF-β-neutralizing antibodies. Fold changes in expression were determined relative to the zero time point and represent the mean ± SD of two independent experiments. (C) Increase in DNA content upon removal of FGF4 and TGF-β. Cells growing in FGF4 plus TGF-β showed two prominent peaks, corresponding to 2 N DNA content (diploid cells in G1) or 4 N (diploid cells in G2 or tetraploid cells in G1). A lower number of cells showed >4 N DNA content, consistent with the low level of spontaneous trophoblast differentiation also seen during culture in FGF4 plus EFCM (Tanaka et al., 1998). After a 4-day growth factor withdrawal as in panel B, there was an increase in the number of cells with >4 N DNA content. Data shown are representative of two independent experiments.
trophoblast cell fates in vivo (Takahashi et al., 2000; Zhang et al., 1998). Third, removal of FGF4 and TGF-β increased the number of cells with DNA content greater than 4 N (Fig. 3C), as expected during the generation of polyploid trophoblast giant cells (Tanaka et al., 1998). In these last two experiments, we accentuated the effects of exogenous TGF-β withdrawal by additionally adding TGF-β-neutralizing antibodies.

Lastly, EGFP-TS3.5 cells maintained 2 months in FGF4 plus TGF-β could contribute towards chimeric placentas following their injection into blastocysts, as described previously for TS cells grown in FGF4 plus EFCM (Tanaka et al., 1998). Thus, Fig. 4B shows an E12.5 placenta with a large contribution of EGFP+ cells, whereas its corresponding embryo (Fig. 4D) and other nonplacental structures (yolk sac, allantois, and amnion; not shown) showed no EGFP-TS3.5

Fig. 4. Generation of chimeric placentas following blastocyst injection of EGFP-TS3.5. Cells had been growing ≥2 months in TS media supplemented with FGF4 and TGF-β. (A and B) Whole-mount photographs of the undersides of a nonchimeric (A) and chimeric (B) E12.5 placenta. The chimeric placenta shows an extensive contribution of EGFP+ cells. Corresponding embryos (C and D) show no EGFP+ cells and only background fluorescence. (E and F) Histological analysis of the placentas shown in panels A and B, respectively. EGFP-TS3.5-derived cells were identified by immunostaining with an anti-GFP antibody (red), and all nuclei were stained blue with DAPI. In the chimeric placenta (F), EGFP+ cells are contributing extensively to the labyrinth (L), with a few scattered cells in the spongiosotrophoblast layer (S). The giant cell layer is above the photographic field. (G) An E10.5 chimeric placenta showing an EGFP+ contribution to the secondary trophoblast giant cell layer (arrowheads) at the interface between the placenta (Pl) and the maternal decidua (Dec). Of the 28 E7–12 concepti examined, two showed a high degree of chimerism and three showed a low degree of chimerism. No conceptus showed EGFP+ cells in the embryo proper, yolk sac, allantois, or amnion. Magnification in A–D is 0.8×; scale bar in E shows 500 μm.
contribution. At the histological level (Fig. 4F), visualization of injected cells in this placenta with an anti-GFP antibody showed extensive contribution to the placental labyrinth (L) and sparse contribution to the spongiotrophoblast layer (S). For comparison, a nonchimeric placenta and embryo are shown in Figs. 4A, C, and E. Fig. 4G shows an E10.5 placenta where EGFP+ cells (arrowheads) have contributed to the secondary trophoblast giant cell layer that forms the external shell of the placenta (Pl), directly abutting the maternal decidua (Dec). The variable contribution of these injected TS cells towards different trophoblast subtypes in different placentas is consistent with the prior description of TS cell behavior (Tanaka et al., 1998).

Differential regulation of trophoblast stem cell marker expression by FGF4 and TGF-β

To gain insight into how FGF4 and TGF-β might differentially regulate TS cell behavior, we determined the 3-day temporal expression patterns of several genes involved in trophoblast stem cell maintenance upon selective growth factor withdrawal from EGFP-TS3.5, using cells that had been previously growing >1.5 months in media supplemented with only FGF4 and TGF-β. Since the presence of an autocrine or FCS-derived TGF-β source would moderate the effects of exogenous TGF-β withdrawal, we accentuated the withdrawal response by simultaneously adding anti-TGF-β neutralizing antibodies as above. As shown in Fig. 5, FGF4 withdrawal led to rapid down-regulation of both *Eomes* and *Esrrb*, suggesting that these two genes might be primary FGF4 targets critical for TS cell maintenance in vitro. Although down-regulation of both genes also occurred upon selective TGF-β withdrawal, the effect was delayed and less dramatic than that seen with FGF4 withdrawal. These more mild decreases in expression might reflect a requirement for TGF-β to secondarily maintain optimal FGF signaling since TGF-β withdrawal also led to decreased expression of *Fgfr2* (Fig. 5).

In contrast to *Eomes* and *Esrrb*, selective FGF4 or TGF-β withdrawal led to a similar and rapid down-regulation of *Cdks2* as well as *Mmp9* (Fig. 5). Thus, these genes might represent common targets for FGF4 and TGF-β, and their maintained expression by TGF-β might be important mechanisms for TS cell maintenance. Interestingly, FGF4 withdrawal led to strongly decreased *Id2* mRNA expression only after 48 h, which is after the initial down-regulation of *Eomes* and *Esrrb*, while TGF-β withdrawal led to *Id2* down-regulation even later (Fig. 5). Thus, down-regulation of *Id2*, which is expressed at very high levels in undifferentiated TS cells (near the level of β-actin and 1000-fold greater than the expression level of *Id1*), is unlikely a primary event in trophoblast differentiation. Conversely, *Id1* (data not shown) and *Id2* (see below) were not rapidly induced by de novo addition of TGF-β to TS cells. This stands in contrast to the rapid induction of *Id* gene expression by BMP4 in embryonic stem (ES) cells and the key requirement for this induction to maintain ES cells in an undifferentiated state (Ying et al., 2003).

**Inhibition of cytostatic TGF-β signaling by FGF4**

Since TS cells are epithelial and nontransformed, it was surprising that they were not growth inhibited by TGF-β. To explore the basis of this phenotype, we characterized the transcriptional responses of TS cells towards TGF-β, taking
advantage of a TSInk4a subline (TSCMI) that we had selected on the basis of continued growth in the absence of EFCM (Erlebacher et al., 2002). This line maintained high expression of all trophoblast stem cell markers tested and retained the capacity to undergo morphological and biochemical differentiation upon FGF4 withdrawal (Erlebacher et al., 2002). The use of TSCMI, however, allowed us to determine de novo TGF-β transcriptional responses since TSCMI growth did not require the continuous addition of exogenous TGF-β. Nonetheless, these cells still required some level of extracellular TGF-β stimulation since they differentiated upon addition of anti-TGF-β antibodies (data not shown). Presumably, TSCMI had undergone genetic or epigenetic alterations that either up-regulated autocrine TGF-β expression or led to increased activation of FCS-derived TGF-β, amplified the TGF-β signaling induced by these potential TGF-β sources, or accommodated reduced TGF-β signaling more distally.

Addition of TGF-β to TSCMI previously growing >1.5 months in media supplemented only with FGF4 rapidly down-regulated mRNA expression of Id2 and induced expression of Mmp9 as well as the well-characterized TGF-β target genes plasminogen activator inhibitor-1 (PAI-1) and Smad7 (Massague and Wotton, 2000) (Fig. 6A). In contrast, TGF-β had no significant sustained effect on the mRNA levels of p15Ink4b, p21Cip1 and, most importantly c-myc. Thus, while the overall transcriptional response towards TGF-β was clearly intact, critical components of the cytostatic response were absent, a situation reminiscent of the selective alterations in TGF-β signaling seen in some cell lines following transformation with oncogenic Ras (Chen et al., 2001). Since FGF receptors are receptor tyrosine kinases and thus activate the Ras/Mek/Erk mitogen-activated kinase (MAPK) pathway, it was possible that constitutive engagement of the FGF receptor by FGF4 was having a similar effect on TGF-β signaling in TS cells.

To test this idea, we determined the de novo transcriptional response of TSCMI towards TGF-β in the presence or absence of FGF4. We accentuated TGF-β-dependent effects by comparing media supplemented with TGF-β to media without TGF-β supplement and instead containing anti-TGF-β neutralizing antibodies to reduce the effects of autocrine or FCS-derived TGF-β. As shown in Fig. 6B, addition of TGF-β to TSCMI while simultaneous removing the FGF4 led to a rapid and sustained four- to fivefold down-regulation of c-myc expression that persisted for up to 48 h. The c-myc down-regulation was TGF-β dependent and not simply due to FGF4 withdrawal since it was not seen in media containing neutralizing anti-TGF-β antibodies. In contrast, and in agreement with the above results, TGF-β added in the continued presence of FGF4 had no sustained effect on c-myc mRNA levels. The inhibitory effect of FGF4 on TGF-β signaling appeared gene specific since the induction of PAI-1 or Smad7 by TGF-β was not inhibited in the presence of FGF4 (Fig. 6B).

Since they need exogenous TGF-β (or EFCM) for continuous growth, we could not perform a directly parallel experiment with wild-type TS cells. Thus, we first tried culturing EGFP-TS3.5 in the absence of TGF-β for 24 h before the 0-h time point so that we could detect TGF-β responses upon restimulation as above. Under these conditions, TGF-β addition concurrent with FGF4 removal led to a sustained twofold c-myc mRNA down-regulation not seen in the presence of FGF4 (data not shown). This more attenuated response compared to TSCMI was also apparent in the less robust transcriptional induction of PAI-1 and Smad7 (data not shown) and may reflect a differential sensitivity towards TGF-β in the two cell lines. Since 24 h culture in the absence of TGF-β before restimulation may have altered subsequent gene regulation, however, we also sought independent evidence of the c-myc transcriptional effect during primary growth factor withdrawal. Thus, we cultured EGFP-TS3.5 previously growing in FGF4 plus TGF-β in media containing both factors, media selectively removing FGF4, media selectively removing TGF-β, or media removing both growth factors. For conditions removing TGF-β, we additionally added TGF-β-neutralizing antibodies to attenuate the effect of FCS-derived or autocrine TGF-β. As shown in Fig. 6C, selective removal of FGF4 caused a reproducible threefold decrease in c-myc transcript levels after 24 h, whereas selective removal of TGF-β or removal of both growth factors had no effect on c-myc mRNA expression levels. Thus, c-myc down-regulation required both the presence of TGF-β and the absence of FGF4. This strongly suggests that constitutive FGF4 signaling in wild-type TS cells prevents the cytostatic TGF-β transcriptional response and may explain why TGF-β acts as growth promoter rather than a growth inhibitor in TS cells.

Maintenance of TS cell proliferation by FGF4 and TGF-β/activin: in vivo correlations

During mouse development, trophoblast stem cells formed in the polar trophectoderm by E4.5 are thought to proliferate within the extraembryonic ectoderm up until about E7.5 (Cross et al., 2003; Rossant and Cross, 2001). This process is thought to require the actions of FGF4 secreted by the underlying inner cell mass and its derivative the epiblast. Our demonstration that TGF-β or activin is also required for TS cell proliferation in vitro raises the question of what TGF-β superfamily ligand and receptor pairs are relevant for TS cell growth in vivo. Despite expression of the three TGF-β isoforms in varying patterns within the maternal decidua or ectoplacental cone (Adelman et al., 2000; Manova et al., 1992), TGF-β is not absolutely required for TS cell development since embryos deficient in either Tgfb1 or Tgfb2, which encode the two TGF-β receptors, die at mid-gestation (Chang et al., 2002). In contrast, embryos deficient in ActRIB, which encodes ActRIB, die by E8.5 with defects including a disorganized
extraembryonic ectoderm apparent by E6.5 (Gu et al., 1998), and embryos doubly deficient in \(\text{Acvr2}\) and \(\text{Acvr2b}\), which encode the two type II activin receptors, die by E8.5 with a severe growth retardation of both embryonic and extraembryonic tissue apparent by E7.5 (Song et al., 1999). It is possible that these phenotypes might in part involve loss of the direct actions of activin on the extraembryonic ectoderm since this structure expresses all three activin receptors (Gu et al., 1998; Manova et al., 1995). If activin is a relevant ligand, however, its source is unlikely to be exclusively embryonic since ablation of the two activin gene isoforms (\(\beta_A\) and \(\beta_B\), which as homo- and heterodimers, encode mature activin) causes death in the perinatal period (Matzuk et al., 1995). The death of these embryos, however, precludes testing the relevance of a maternal activin source, although the expression of activin \(\beta_A\) and \(\beta_B\) in the decidual tissue surrounding the embryo starting by E5.0–5.5 is suggestive (Albano et al., 1994; Manova et al., 1992).

Fig. 6. Regulation of TGF-\(\beta\) transcriptional responses in TS cells by FGF4. (A) Time course of the transcriptional responses of TSCMI towards TGF-\(\beta\). mRNA levels relative to \(\beta\)-actin (\(\Delta\Delta C_T\) values) were determined by real-time RT-PCR, then normalized to each 0-h time point, respectively. The 0-h \(\Delta C_T\)s were 2.58 (\(\text{Id2}\)), 4.20 (\(\text{Mmp9}\)), 6.74 (\(\text{P4I-1}\)), 5.53 (\(\text{Smad7}\)), 6.31 (\(\text{c-myc}\)), 19.11 (\(\text{p15Ink4b}\)), and 11.53 (\(\text{p21Cip1}\)). Data are representative of two independent experiments. (B) Influence of FGF4 on the transcriptional responses of TSCMI towards TGF-\(\beta\). Cells previously growing in FGF4 were stimulated with TGF-\(\beta\) or TGF-\(\beta\)-neutralizing antibodies in the presence or absence of FGF4. RNA was isolated at 0, 4, 24, and 48 h. The 0-h \(\Delta C_T\) relative to \(\beta\)-actin were 6.07 (\(\text{c-myc}\)), 6.32 (\(\text{P4I-1}\)), and 6.71 (\(\text{Smad7}\)). Data are representative of two independent experiments. (C) Regulation of \(\text{c-myc}\) expression in EGFP-TS3.5. Cells previously growing in FGF4 and TGF-\(\beta\) were plated in TS media containing both factors, media selectively removing FGF4, media selectively removing TGF-\(\beta\), or media removing both growth factors. TGF-\(\beta\)-neutralizing antibodies were added in conditions removing TGF-\(\beta\). RNA was prepared after 24 h, and \(\text{c-myc}\) mRNA expression levels were determined by real-time RT-PCR. Fold changes in expression were calculated relative to the \(\text{c-myc}\) expression level in cells plated in FGF4 and TGF-\(\beta\). Removal of FGF4 in the continued presence of TGF-\(\beta\) led to a threefold decrease in \(\text{c-myc}\) abundance (*\(P < 0.05\)). Data represent the mean ± SEM of all data from three independent experiments with all four treatment groups, plus an additional experiment that did not involve plating cells in the absence of both growth factors.
The TGF-β superfamily member Nodal is an excellent candidate for a factor maintaining TS cell proliferation in vivo, perhaps one that acts a bit later than activin. Nodal is expressed in the epiblast and in conjunction with the epidermal growth factor-related molecule Cripto signals via activin receptors and Smad2 and Smad3 (Schier, 2003). Defects observed in Nodal−/− embryos by E9.5 include a loss of the spongiotrophoblast and placental labyrinth and an overgrowth of trophoblast giant cells, consistent with a failure of pluripotential TS cell maintenance (Iannaccone et al., 1992; Ma et al., 2001). Furthermore, transfection of TS cells with a nodal expression construct partially inhibits differentiation upon FGF4 and EFCM removal (Ma et al., 2001).

Although we have described some trophoblast-specific transcriptional responses to TGF-β and FGF4, how these factors prevent TS cell differentiation is unclear and may involve complex interactions between Smad-signaling pathways activated by TGF-β/activin/Nodal and Ras/Mek/Erk pathways activated by FGF4. Furthermore, TGF-β/activin/Nodal might inhibit TS cell differentiation through non-Smad-signaling pathways (Derynck and Zhang, 2003) or through Smad-dependent mechanisms aside from the direct transcriptional regulation of Smad target genes. For example, activated Smad proteins inhibit myoblast, osteoblast, and adipocyte differentiation by interfering via direct physical interaction with the function of more cell-type-specific transcription factors (Alliston et al., 2001; Choy and Derynck, 2003; Liu et al., 2001).

Our data suggest that in contrast to the possible requirement of TGF-β for optimal FGF signaling (see above), FGF4 was not conversely required for global TGF-β signaling. Thus, FGF4 withdrawal from EGFP-TS3.5 had no acute effects on the mRNA levels of Tgfb1, Tgfb2, Acvr1b, Acvr2, Acvr2b, Smad2, Smad3, or Smad4, with two- to fivefold decreases in mRNA levels for some of these genes seen on day 3 (data not shown). Furthermore, only withdrawal of TGF-β, but not FGF4, led to the acute down-regulation of the Smad target genes PAI-1 and Smad7 (data not shown). However, FGF4 may have an influence on TGF-β availability in vivo by regulating expression of MMP-9, a proteolytic activator of latent TGF-β, and may modify the regulation of Smad target genes on a selective basis. One important example of this comes from our data suggesting that FGF signaling constitutively attenuates the cytostatic TGF-β transcriptional response in a manner similar to that previously seen in some Ras-transformed tumor cells of epithelial origin (Chen et al., 2001). Additional experiments are needed to determine how this pathway relates to the other more general Ras/Mek/Erk-dependent mechanisms described for preventing TGF-β-induced growth arrest or modifying TGF-β transcriptional responses (Derynck and Zhang, 2003). However, it is likely that the pathway is active in vivo since FGF-dependent Erk activity is constitutive in the extraembryonic ectoderm containing TS cells (Corson et al., 2003). It is conceivable that similar pathways may also play a role elsewhere during development or as a component of epithelial stem cell maintenance in adults. For example, high circulating levels of activin have been shown to expand gastric epithelial progenitors in vivo (Li et al., 1998). Lastly, these intersecting pathways might provide a physiological mechanism for rapid and synchronous TS cell differentiation upon exposure to decreased FGF4 concentrations in the presence of constant TGF-β/activin/Nodal levels, as the requirement for TGF-β signaling to maintain stem cell proliferation would now switch to a stimulus for cell cycle arrest.

Note added in proof

We have been able to derive two new independent TS cell lines following the individual culture of twelve blastocysts in DMEM/F12-based TS media supplemented with only FGF4, activin, and anti-TGF-β antibodies. At passage n = 7–8, these lines showed the epithelial morphology and trophoblast stem cell marker expression profile typical of other established TS cell lines, and showed a similar pattern of stem cell marker gene downregulation and morphological differentiation upon removal of either FGF4 or activin. These results suggest that activin is sufficient to establish TS cell lines de novo in the absence of EFCM.

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