TGFβ inhibition of yolk-sac-like differentiation of human embryonic stem-cell-derived embryoid bodies illustrates differences between early mouse and human development

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Summary
Transforming growth factor β (TGFβ) plays an important role in development and maintenance of murine yolk sac vascular development. Targeted deletions of Tgfβ1 and other components of this signaling pathway, such as Acvrl1, Tgfr1 and Tgfbr2, result in abnormal vascular development especially of the yolk sac, leading to embryonic lethality. There are significant differences between murine and primate development that limit interpretation of studies from mouse models. Thus, to examine the role of TGFβ in early human vascular development we used the model of differentiating human embryonic stem cell-derived embryoid bodies to recapitulate early stages of embryonic development. TGFβ was applied for different time frames after initiation of embryoid body cultures to assess its effect on differentiation. TGFβ inhibited the expression of endodermal, endothelial and hematopoietic markers, which contrasts with findings in the mouse in which TGFβ reduced the level of endodermal markers but increased endothelial marker expression. The inhibition observed was not due to changes in proliferation or apoptosis. This marked contrast between the two species may reflect the different origins of the yolk sac hemangiogenic lineages in mouse and human. TGFβ effects on the hypoblast, from which these cell lineages are derived in human, would decrease subsequent differentiation of hematopoietic, endothelial and endodermal cells. By contrast, TGFβ action on murine hypoblast, while affecting endoderm would not affect the hemangiogenic lineages that are epiblast-derived in the mouse. This study highlights important differences between early human and mouse embryonic development and suggests a role of TGFβ in human hypoblast differentiation.

Key words: TGFβ, Embryonic stem cells, Yolk sac, Endothelial, Endoderm

Introduction
Current understanding of early human embryonic development is based largely on comparison with the mouse, an organism used extensively for studies on the molecular regulation of early mammalian development because of the ease with which genetic manipulation can be undertaken. However, there are significant differences between murine and primate development that limit the usefulness of the mouse as a model. Human embryos, for instance, have two phases of extra-embryonic endoderm formation and limited reliance on the yolk sac circulation. Conversely, the mouse has one phase of extra-embryonic endoderm generation and utilizes the yolk sac until birth (Pera and Trounson, 2004). The derivation of human embryonic stem (ES) cell lines from the inner cell mass of the human blastocyst (Thomson et al., 1998) and manipulation of ES differentiative capacity in vitro (Gerami-Naini et al., 2004; Reppel et al., 2004; Schuldiner and Benvenisty, 2003; Schuldiner et al., 2000; Xu et al., 2002) now permits the validation of knowledge gained from molecular manipulation of the mouse in vivo to the study of human embryogenesis. When allowed to differentiate in suspension, ES cells form cystic embryoid bodies (EB) that have some features of early post-implantation embryos. Importantly, EB formation from mouse ES cells has already been extensively utilized and validated as an in vitro model of early mouse development (Feraud et al., 2003; Gendron et al., 1996; Goumans et al., 1999; Hirashima et al., 1999; Ng et al., 2004).

Our laboratory has been interested in the role played by transforming growth factor β (TGFβ) in vascular development. We and others have shown that TGFβ1 and its signaling components are essential for the early vascular development of the mouse, particularly within the yolk sac where vessels appear de novo from yolk-sac mesoderm by the process of vasculogenesis (Dickson et al., 1995; Goumans et al., 1999). Evolutionary conservation of embryonic TGFβ gene expression patterns between mouse and human suggest
conservation of function between these two organisms (Akhurst et al., 1992; Gatherer et al., 1990). Moreover, the finding of vascular dysplasia and malformation resulting from germ-line loss of TGFβ signaling components in the hereditary hemorraghic telangiectasias (HHTs) (Berg et al., 1997; McAllister et al., 1994), familial thoracic aortic aneurysms and dissections (FTAAD) (Pannu et al., 2005) and other cardiovascular malformations (Loeys et al., 2005; Mizuguchi et al., 2004), emphasizes the importance of TGFβ signaling in vascular remodeling and homeostasis in humans (Akhurst, 2004).

In the current study, we examined the effects of exogenous TGFβ on spontaneous differentiation of human EBs into endothelial cells and resultant vessels (Gerecht-Nir et al., 2003; Levenberg et al., 2002). The results show that mouse and human EBs have quite disparate responses to this ligand that may reflect fundamental differences in cell lineage development of the yolk sac between mouse and human.

**Results**

**Human ES-derived EB formation appears to recapitulate yolk sac development**

HSF-6 ES cells were allowed to undergo spontaneous differentiation to form EBs as previously described (Abeyta et al., 2004). When cultured in suspension, EBs formed an outer layer of α fetoprotein (AFP)-positive cells and an inner core of AFP-negative cells that, by their relative positions and morphologies, have previously been reported to be equivalent to primitive endoderm (PrE) and primitive ectoderm, respectively (Shen and Leder, 1992). By day 10-13, vessels began to appear and could be detected by light-contrast microscopy (Fig. 1A,B). Vascular potential was assessed by PECAM1 and ENG gene expression. Both are endothelial-specific markers; PECAM1 is a cell adhesion molecule and endoglin is a TGFBR3-related molecule that is mutated in HHT1 (McAllister et al., 1994). PECAM1 and ENG RNA were both detectable 5 days after initiation of differentiation and continued to rise up to day 15, when they reached a plateau (Fig. 1C). PECAM1 protein was not detectable by immunohistochemistry until around day 8, but by day 13-18, when vessels could be seen in EBs by phase-contrast microscopy, PECAM1 staining was clear. PECAM1-stained cells were seldom found in isolation but were usually organized around cavities (Fig. 1D), and sometimes juxtaposed to visceral endoderm (VE)-like cells that stained for α-feto protein (AFP) (Fig. 1E-H). These endothelial cells were also positive for VE-cadherin (data not shown), another endothelial-specific marker commonly found at cell-cell junctions. These findings are consistent with those of Levenberg et al. (Levenberg et al., 2002) who also showed that RNA levels for these endothelial genes peaked between 13-15 days after differentiation. In the present study, the proportion of endothelial cells in day 18 EBs was approximately 0.5%, as identified by PECAM1 expression, compared with 2% in the previous study (Levenberg et al., 2002). Treatment with VEGF throughout the culture period did not augment endothelial differentiation.

**Expression of TGFβ signaling molecules during EB formation**

Expression of TGFβ1 signaling molecules was characterized by RT-PCR during EB differentiation. TGFB1, TGFBRI, SMAD2 and SMAD4 were all expressed at similar levels from day 0 to day 14 (Fig. 2A). TGFB2, TGFB3, TGFBRII, ACVRL1 and SMAD3 were expressed at lower levels on day 0 and were upregulated between day 2 and day 6. ENG expression was undetectable in HESC cells (Fig. 2A), but rapidly increased in expression after 4 days in culture, concomitantly with PECAM1 expression (Fig. 1). The inhibitory SMADs, SMAD6 and SMAD7, were both expressed within HESCs and EBs, but with variable expression levels. In contrast to all the other TGFβ signaling molecules, SMAD6, which is widely accepted as an inhibitor of the canonical BMP signaling pathway, showed diminishing expression levels as EBs differentiated. SMAD7 showed a slight increase in expression, peaking around 9-10 days after EB initiation followed by a slight decrease in expression (Fig. 2A).
TGFβ in human embryoid body development

**Fig. 2.** Expression of the TGFβ pathway during EB formation. (A) Human ES cells were allowed to spontaneously differentiate to form EBs. The expression of components of the TGFβ pathway was examined by RT-PCR. GAPDH primers were used as loading control. (B) The expression of TGFβR2 in undifferentiated ES cells was examined in three independent human ES cell cultures in which the expression of POU5F1 (OCT4) was also verified. A negative control experiment (−) was performed using cDNA from MEFs. The ACVRL1 primers showed some cross-reactivity with mouse Acrv1.

Since TGFβR2 is notably absent in mouse embryonic stem cells, rendering them unresponsive to TGFβ1 signaling (Goumans et al., 1998), TGFβR2 expression was examined by RT-PCR in three independent human ES cell cultures grown under conditions for stem cell maintenance. TGFβR2 transcripts were present in all three cultures (Fig. 2B) and its variable expression level did not correlate with expression of POU5F1 encoding the stem cell marker, OCT4. It is, therefore, unlikely that TGFβR2 transcripts originate from more differentiated cells within the ES cell culture. We conclude that human ES cells, unlike those of the mouse, express TGFβR2, at least at the RNA level. Phospho-SMAD2, a marker of activated TGFβ signaling, was found to be located in the nucleus by immunohistochemistry in both TGFβ-treated and untreated ES and EBs (data not shown), an observation previously made by James et al. (James et al., 2005) and probably indicative of activated nodal signaling that is also propagated via SMAD2.

**TGFβ1 does not affect EB formation and cavitation**

To examine the function of TGFβ1 in EB formation, EBs were cultured continuously in TGFβ1 for 18 days. EB formation occurred normally and, by day 18, consisted of an outer layer of PrE-like cells, and an inner layer of primitive ectoderm-like cells (Shen and Leder, 1992). More than 90% of EBs treated with TGFβ1 had normal central cavities, similar to the control cultures. This contrasts with observations made during mouse EB differentiation, in which TGFβ inhibits EB cavitation (Goumans et al., 1998). There was no obvious phenotypic or size difference between the TGFβ1 treated and untreated EBs (Fig. 3A,B), and cell types of all three germ layers were present, evident by the detection of markers of the ectoderm (NCAM), endoderm (α-feto-protein, AFP) and mesoderm (KDR and smooth muscle actin, α-SMA) by RT-PCR.

**TGFβ1 attenuates EB vessel formation**

To assess the role of TGFβ1 in vascular development, the expression of PECAM1 and KDR were analyzed by real-time quantitative RT-PCR, using human GAPDH as an internal control. TGFβ1 was found to inhibit the expression of PECAM1, while there was no significant difference in KDR transcript level between treated and untreated samples (Fig. 3C,D). However, KDR/Flik1 expression has previously been demonstrated in undifferentiated human embryonic stem cells, embryonic and extra-embryonic mesoderm, while PECAM1 expression is concentrated in cells further committed to the endothelial lineage (Abeyta et al., 2004; Levenberg et al., 2002; Yamashita et al., 2000; Zambidis et al., 2005).

TGFβ1 may affect differentiation of ES cells into the mesodermal lineage or the emergence of endothelial precursors from ES cells. In addition, or alternatively, TGFβ1 may directly affect the maintenance of endothelial cells. To evaluate these various possibilities, EBs were treated with TGFβ1 for a shorter period (5 days) either at an early stage in EB formation, day 3-8, or later at day 13-18. Day 3-8 of EB formation is a period of many morphological and gene expression changes. The formation of central cavities begins at around day 3 in a process that mimics the formation of the amniotic sac and/or blastocoele. Differentiation of the three germ layers suggests a mechanism similar to gastrulation. PECAM1 is not detectable on day 3 but starts to appear by day 8. Thus, TGFβ1 treatment of day 3 EBs addresses the role of TGFβ1 in early developmental events, such as mesoderm formation and emergence of endothelial precursors. At day 13, endothelial cells are already established, based on morphological examination and expression of endothelial markers. Therefore, treatment after day 13 addresses the effect of TGFβ1 on the endothelial cells per se.

EB treatment with TGFβ1 for 5 days commencing at day 3 inhibited PECAM1 expression in a dose-dependent manner, while there was no statistically significant change in KDR expression between treated and untreated EBs (Fig. 4A,B). Our observation indicates that either endothelial cell differentiation from precursors or maintenance of the established endothelial cells is diminished by TGFβ. NCAM and ACTA2 (α smooth muscle actin) gene expression, markers of epithelial and mesodermal cells, respectively, were unaltered by TGFβ treatment suggesting that there is not a generalized inhibition of all differentiated cell types (Fig. 4C and data not shown). TGFβ, although known to be an inducer of VEGF in some cell systems (Donovan et al., 1997; Kobayashi et al., 2005; Qian et al., 2004; Yamamoto et al., 2001), did not affect VEGF expression in EBs (Fig. 4D).

To examine the action of TGFβ1 on differentiated endothelial cells, the ligand was applied to day 13 EBs. After 5 days, PECAM1 gene expression was downregulated in a dose-dependent manner (Fig. 4E), whereas NCAM, ACTA2 and VEGF were unaffected (Fig. 4G-H, and data not shown).
The expression pattern of PECAM1 in control and TGFβ-treated samples was also examined by immunofluorescence staining (Fig. 4I-N). PECAM1 staining was often seen to line cavities of both controls and TGFβ-treated EBs. However, fewer PECAM1-positive structures were identified in TGFβ-treated EBs than in controls.

Interestingly, in contrast with findings from TGFβ-treatment of day 3 EBs, KDR was also downregulated in day 13-18 EBs (Fig. 4F). This could be explained if most KDR+ cells in day 13 EBs are already differentiated endothelial cells and thus diminished after TGFβ treatment. Conversely, KDR expression in day 3 EBs may predominantly be from undifferentiated mesoderm cells that are still the predominant cell type at this time. The discrepancy between KDR downregulation in EBs exposed to TGFβ from day 13 to day 18 versus those exposed continually is difficult to explain. It is possible that TGFβ treatment at early times of EB formation results in persistence of KDR-expressing mesodermal progenitor cells at later stages.

TGFβ1 does not alter proliferation or apoptosis of EB-derived endothelial cells

TGFβ1 may modulate vessel formation by decreasing proliferation of endothelial cells, inducing their apoptosis, inhibiting the maintenance of the endothelial differentiated state and/or inhibiting differentiation of mesenchymal to endothelial cells. To assess the effects of TGFβ1 on endothelial proliferation, day 13 EBs were treated with TGFβ1 for approximately 24 hours, and proliferating cells were identified by Ki67 staining. There was no statistically significant difference between treatment and control groups. The proportion of PECAM1+ endothelial cells in cycle was 18±8% and 22±10% in control and treated EBs respectively. The apoptotic response to TGFβ1 was also assessed using TUNNEL staining, after overnight treatment of day 13 EBs with TGFβ1. 7.7±2.8% and 7.4±0.4% of PECAM1+ cells were apoptotic in control and TGFβ-treated samples, respectively, again showing no statistically significant difference.

TGFβ1 reduces hematopoiesis in EBs

It has been suggested that endothelial cells and hematopoietic cells are derived from a common precursor (Choi et al., 1998; Murray, 1932; Sabin, 1920), and studies of TGFβ signaling gene knockout mice have suggested that both lineages are affected by this ligand (Dickson et al., 1995; Goumans et al., 1998; Goumans et al., 1999). CD34 and GATA2 were selected as markers of cells of the hematopoietic lineage, and both have been found to be expressed at low levels during early EB development and were upregulated from approximately day 7, when hematopoietic potential could be demonstrated by CFU assays (Levenberg et al., 2002; Wang et al., 2004). TGFβ1 treatment of day 13 EBs led to a dose-dependent reduction in CD34 and GATA2 RNA levels as assessed by real-time quantitative RT-PCR (Fig. 5).

TGFβ1 attenuates differentiation of VE and PE

During embryonic development, the endothelial and hematopoietic lineages are derived from the yolk-sac mesoderm, which is in close apposition with the VE. It is known that proteins secreted by the VE modulate endothelial differentiation (Bielsinska et al., 1996; Damert et al., 2002; Palis et al., 1995; Wilt, 1965). Furthermore, it has been suggested that defective vessel formation in Tgfb1-null embryos is related to abnormalities in extracellular matrix deposition between the VE and developing endothelium (Goumans et al., 1999). We thus examined the effect of TGFβ on endodermal differentiation.

AFP is expressed in the VE and fetal liver (Jones et al., 2001; Meehan et al., 1984) and is commonly used as a marker of VE and definitive endoderm (DE) in EB studies (Conley et al.,
The majority of cells positive for AFP were found on the outer surface of EBs, reminiscent of the VE (Fig. 1E). In some EBs, these VE-like structures were juxtaposed to a layer of PECAM1+ cells (Fig. 1G,H). Thus, the organization of the VE and endothelium in these EBs recapitulate that of the embryonic yolk sac, in which the VE is arranged adjacent to the endothelium. TGFβ1 was found to decrease AFP RNA and protein levels in day 13 EBs treated for 5 days (Fig. 6A,B). The expression pattern of AFP in control and treated samples was found to be similar, mostly consisting of ring-like structures (Fig. 6C-F). Occasionally, staining was also seen around an isolated cell cluster. There was no obvious difference between the staining patterns or intensity of control and treated samples. However, fewer AFP-positive (AFP+) structures could be identified in treated cells. AFP expression in day 3 EBs was similarly reduced by the ligand (Fig. 6G). Endodermal inhibition was confirmed by RT-PCR analysis of transthyretin (TTR; Fig. 6H), another marker of VE/DE (Makover et al., 1989; Thomas et al., 1990).

The decrease in endodermal cell number after TGFβ1 treatment was not due to decreased proliferation or increased apoptosis. AFP+ cells of day 18 EBs showed similar levels of Ki67 staining (13.9±3.1 and 13.1±2.3%) and TUNNEL staining (8.1±0.52 and 6.8±1.8%), whether or not they had been cultured in TGFβ1. Since TGFβ1 did not affect either proliferation or apoptosis of AFP+ cells, we hypothesize that TGFβ1 may modulate differentiation of AFP+ cells from their precursors. The VE is derived from the

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**Fig. 4. Expression of lineage markers in response to TGFβ treatment.** EBs were treated with different concentrations of TGFβ: (A-D) from day 3 to day 8, or (E-F) from day 13 to day 18. Expression was measured by qRT-PCR, normalized to GAPDH. Results were presented relative to expression in untreated samples. The expression of PECAM1 in control (I,K) and treated (L,N) samples was examined by immunofluorescence staining. DAPI-stained nuclei are also shown (J,M). K and N are high power magnifications of the boxed areas within I and L, respectively.
PrE, which also gives rise to the parietal endoderm (PE). TGFβ1 may block differentiation from PrE to VE or it may divert differentiation towards the PE lineage. To address this question, we examined the expression level of markers of both the PrE and PE.

EndoA/KR/TROMA-1 is expressed in all early endoderm cells (Duprey et al., 1985), whereas THBD, encoding thrombomodulin, is highly expressed specifically in PE and has been used as a cell type marker for PE in EBs (Thompson and Gudas, 2002; Weiler-Guetttler et al., 1992). In day 13 EBs, EndoA is highly expressed and is present in the majority of EBs. Five days of TGFβ1 treatment produced no difference in EndoA protein level (Fig. 6I). Conversely, THBD RNA level is strongly inhibited by TGFβ, suggesting that TGFβ1 inhibits both VE and PE (Fig. 6J). Unfortunately, there are no specific markers for PrE/hypoblast since Endo-A is also expressed in primitive epithelial cells. It was, therefore, not possible to determine whether TGFβ1 attenuated appearance of VE and PE by reducing initial PrE differentiation, or whether this resulted from reduced differentiation of PrE into VE and PE.

**Discussion**

To examine the role of TGFβ1 in early development, the expression of components of the TGFβ1 signaling pathway was examined by RT-PCR at different stages of EB differentiation. TGFβ1 itself, its receptors, TGFBR1 and TGFBR2, as well as SMAD2 and SMAD4 are all expressed throughout EB formation. It is of interest that TGFBR2 transcripts could be detected in undifferentiated human ES cells. This is in contrast to published results, which show that Tgfb2 RNA is absent from undifferentiated mouse ES cells (Goumans et al., 1998). The differential expression of TGFBR2 in human and mouse cells is consistent with reports suggesting that TGFβ1 can contribute to support of human ES cell culture in serum- and feeder-free conditions (Amit et al., 2004), while mouse ES cells are unresponsive to TGFβ1 (Goumans et al., 1998). This may point to fundamental differences between human and mouse ES cells with regards to the importance of TGFβ1 signaling in early development. Furthermore, several members of the TGFβ family have been implicated in stem cell maintenance (Ying et al., 2003; Beattie et al., 2005), and species-specific differences have also been observed. BMP and LIF signaling are sufficient to promote the growth of undifferentiated mouse ES cells in serum-free conditions (Ying et al., 2003). Conversely, BMPs caused human ES cell differentiation either to trophoblast or PrE in conditions that would otherwise support their undifferentiated proliferation (Pera et al., 2004; Xu et al., 2002; Xu et al., 2005). In direct contrast to findings in mouse, the inhibition (rather than the activation) of BMP signaling was shown to promote the undifferentiated growth of human ES cells (Pera et al., 2004; Xu et al., 2005). Together, these data demonstrate that the TGFβ1 signaling pathway may have important roles in human ES cell maintenance and early development that are not conserved in mouse.

A common theme that emerges from the current studies on human EBs and previous studies on mouse EBs (Goumans et al., 1999) is that TGFβ1 inhibits extraembryonic endodermal differentiation in both systems. These results in EBs are somewhat contrary to expectations based on transgenic mouse experiments that have demonstrated the importance of TGFβ and its signaling components in promoting endodermal development (Henry et al., 1996; Liu et al., 2004; Nomura and Li, 1998; Tremblay et al., 2000; Vallier et al., 2004; Waldrip et al., 1998; Weinstein et al., 1998). Smad2 mutant mice die early during development and fail to form embryonic endoderm (Nomura and Li, 1998; Tremblay et al., 2000; Waldrip et al., 1998; Weinstein et al., 1998). Liu et al. also suggested that Smad2 and Smad3 function cooperatively to regulate liver development (Liu et al., 2004). Nodal, a member of the TGFβ superfamily, has traditionally been implicated in VE development (Schier, 2003; Vallier et al., 2004). The general inhibition of endothelial and endodermal differentiation seen in the current study may relate to the role of the TGFβ/nodal/activin axis in maintenance of ‘stemness’ of human ES cells and EBs (James et al., 2005; Vallier et al., 2004; Waldrip et al., 1998). Smad2 signaling pathway is, nevertheless, also necessary for endodermal development (Nomura and Li, 1998; Waldrip et al., 1998; Weinstein et al., 1998).

A major difference between human and mouse EBs was found in the influence of TGFβ on EB cavitation. In the current study, it was found that EB formation occurred even when human ES cells were cultured continuously in the presence of TGFβ1. Cavitation occurred normally and cells of all three germ layers could be observed. In contrast TGFβ1 inhibited murine EB cavitation (Goumans et al., 1999).

Another contrasting effect of TGFβ on mouse and human EBs was in endothelial cell outgrowth. Application of TGFβ during three time frames: day 0-18, day 3-8 and day 13-18, inhibited the expression of markers of endothelial cells. This contrasts with observed TGFβ effects in mouse EBs (Goumans et al., 1999). Goumans et al. found upregulation of endothelial cell markers when TGFβ1 signaling was augmented either by over-expressing the TGFβ receptor, Tgfr2, or by applying the cytokine directly to EBs. We propose that this contrasting...
effect on endothelial differentiation could be due to species differences with regards to lineage development of the yolk sac. Considerable differences exist between human and mouse ES cells (Matsuda et al., 1999; Thomson et al., 1998; Xu et al., 2001). Mouse ES cells remain in the undifferentiated state simply by culturing in LIF and BMP, while human ES cells cannot be maintained by these two ligands and additionally need to be cultured on irradiated MEFs, in media conditioned by MEFs, or in the presence of BMP antagonists (James et al., 2005). In addition, differences found in the expression of embryonic antigens (Ginis et al., 2004; Park et al., 2004) and the ability of human ES cells to differentiate along the trophoectoderm lineage (Gerami-Naini et al., 2004; Xu et al., 2002), have led to the suggestion that human ES cells correspond to an earlier stage of embryonic development than their mouse counterparts (Pera and Trounson, 2004). Together, this evidence demonstrates that significant differences exist between mouse and human ES cells and that the TGFβ1 signaling pathway may underlie some of these differences.

Both human and mouse endothelium are thought to be derived from yolk-sac mesoderm via a common endothelial/hematopoietic precursor, commonly termed the hemangioblast. Murine yolk-sac mesoderm is derived from the primitive ectoderm (epiblast) during gastrulation (Lawson et al., 1991), while descriptive studies suggest that primate yolk-sac mesoderm cells may arise from PrE (hypoblast) (Bianchi et al., 1993; Enders and King, 1988), and reviewed by Enders and King (Enders and King, 1993). Emergence of human and rhesus yolk-sac mesoderm cells occurs prior to formation of the primitive streak, diminishing the likelihood that yolk-sac mesoderm cells arise from the epiblast. This difference in the origin of the yolk-sac mesoderm may account for the differing responses of human and mouse EBs to TGFβ (Fig. 7).

We propose that TGFβ exerts an inhibitory effect on the PrE lineage. In human EBs, TGFβ1 inhibition of PrE differentiation would reduce the emergence of its derivatives, including VE and PE, plus its descendants, the hematopoietic and endothelial lineages. This was manifested in the reduction of AFP, TTR, THBD, PECAM1, CD34 and GATA2 expression (Fig. 7). Epiblast-derived lineages, such as embryonic ectoderm and mesoderm would be unaffected by TGFβ1, thus the RNA levels of NCAM and ACTA2 were unchanged. Conversely, since murine yolk-sac mesoderm is derived from the primitive ectoderm, inhibition of PrE differentiation would only decrease the establishment of VE endoderm, but not endothelial differentiation, which is ultimately derived from the mouse epiblast. Consistent with the above theory, Pinar et al. (Pinar et al., 1992) investigated the distribution of TGFβ1 using immunohistochemical methods in a two-week-old

**Fig. 6.** The effect of TGFβ on endoderm. Day 13 EBs were treated with various concentrations of TGFβ1 for 5 days. (A) The expression of AFP was assessed by qRT-PCR and normalized to GAPDH. Results are presented relative to expression in untreated samples. (B) AFP protein expression in untreated and TGFβ1-treated (1 ng/ml) EBs was compared by western blotting. An anti-β-actin antibody was used as a loading control. The expression of AFP (C,D) in control and treated samples was examined by immunofluorescence staining. DAPI-stained nuclei are also shown (E,F). Day 3 EBs were treated with various concentrations of TGFβ1 for 5 days. The RNA level of (G) AFP and (H) TTR and (J) THBD was measured relative to GAPDH. Results are presented relative to expression in untreated samples. (I) Endo A protein expression in untreated and TGFβ1-treated (1 ng/ml) EBs was compared by western blotting.
Fig. 7. Model for differential responses of mouse and human EBs to TGFβ. The schematic shows the alternative lineages leading to the hematopoietic and endothelial lineages of the human and mouse yolk sac. The key species difference is highlighted as a bold connecting arrow to the yolk-sac mesoderm (solid for human, dashed for mouse). TGFβ inhibition of hypoblast formation would thus affect descendants of the hypoblast in human but not mouse. Cell types downregulated by TGFβ in both species are indicated as solid gray ovals, those down-regulated by TGFβ in human but not in mouse are indicated as hatched gray ovals.

bilateral human embryo, and found that antibodies to mature TGFβ1 peptide localized preferentially to the hypoblast with only weak staining in the epiblast, although its precursor was seen in both the epiblast and the hypoblast. Shi et al. (Shi et al., 1990) also reported the strong presence of TGFβ1 peptide in mouse hypoblast and speculated the TGFβ1 has an important role in the differentiation of endoderm and mesoderm, particularly in the development of extraembryonic tissues.

In conclusion, examining the effects of TGFβ on human EB formation we have highlighted differences in the differentiative properties of human EBs compared with those of mice, as they develop from pluripotent ES cells. In particular, the differentiation of all cell lineages that contribute to the yolk sac appear to be down-modulated by TGFβ whereas, in the mouse, endothelial differentiation of EBs is actually stimulated by this cytokine. It is likely that these differences reflect fundamental differences in early development between human and mouse.

Materials and Methods

Culture and maintenance of undifferentiated hESCs

Human ES cells (HSF-6) were grown on mouse CF-1 embryonic fibroblasts in knockout medium supplemented with NFGF, as described previously (Abeyta et al., 2004). Mouse embryonic fibroblasts (MEFs) were isolated from CF-1 mice at day 13 of gestation (E13.5-14.5) and were used as feeders between passages 3 and 20. Feeder cells were irradiated with 5,000 rad just before use. Mouse MEFs were cultured as described (Yvon et al., 2000). Mouse embryo fibroblasts (MEFs) were isolated from CF-1 mice at day 13 of gestation (E13.5-14.5) and were used as feeders between passages 3 and 20. Feeder cells were irradiated with 5,000 rad just before use. Mouse MEFs were cultured as described (Yvon et al., 2000).

Differentiation of human ESC

To differentiate human ES cells into embryoid bodies (EBs), colonies were detached from the tissue culture plate with collagenase type IV and cultured on low-adherence 6-well plate (Corning) at 5% CO2 in 5 ml medium containing DMEM, 20% fetal calf serum, 1 mM glutamine, and 0.1 mM β-mercaptoethanol (and TGFβ1). Over the next 20 days, human ES cells were allowed to grow in suspension to form embryoid bodies. Cultured medium was refreshed every 3-4 days.

RT-PCR analysis

Total RNA from EBs was isolated using RNeasy Mini kit (Qiagen). cDNA was generated from 1 μg of total RNA using the iScript cDNA synthesis kit (Bio-Rad Laboratories). Primers used were as follows: TGFβ1, GCAGCTGCCAGAG-TGGTAT, TTCCCTTGCGGAAAGTCATGT; TGFβ2, GCAGCCAGGAGGTTTACAAAAA, TGGGACGAGCAGTGTGAAG; TGFβ3, GATCAAACCAACAGCCTC-ATC, CATTTGACCAACACATCTCA; TGFβR1, AGAAT ACCAA CCGCC TTTAT, TATCC TCTCG CTCGTC TCTCA; TGFβR2, TTITCCACCCTGTGACA-ACCA, GCTGATGCGTTGCTACCATGA; SMAD2, CTCTGGTCTGTCGAGTAAT, GAGGTTGCGGTTCGCCATA; SMAD3, GCTTTGAGGCTGTTCTAC- CAGT, TGGTTTGCTCCTGGTGTT; SMAD4, GCACGCAGACAGAGACAGACA TACAG, CAACA GTAAAC TAAAG GCCGAC; SMAD6, GGCGCTCCACAGA CATTACA, GCAGTGTAGGAGGATGGTGTG; SMAD7, AGGGCGACAGAATTA TCTTG; AGCAAGCACCTACGGAGGAA; ENG, AGAGGTGGCTTGCCTGCACA TCA, GATCTCGACTGTTGTTGGTG; AVCL1, ATTTACGGTAGACATGGC AAC, TCCACAACACCACCTTTCT; PECAM1, AGACAGCTACAGGAATGCTCG; GAPDH, TCACTGTTGGAGCTCA; CCT, AGGGCAGATTCAGTTGTTG.

Quantitative PCR

Quantitative-PCR analysis was performed on an ABI Prism 7900 or 7700 Sequence Detection System (Applied Biosystems). Quantitative detection of specific nucleotide sequences was based on the fluorogenic 5’ nucleotide assay (Ginzinger, 2000), and expression was quantified relative to GAPDH. For APP, TTR, NCAM, KDR and VEGF, assays were designed using Primer Express software v1.5 (Applied Biosystems) with 6-FAM fluorophore on the 5’ end and the quencher BHQ1 on the 3’ end. Reactions were optimized to have >90% efficiency. For PECAM1, CD34, GATA2 and THBD, quantitative PCR was performed using the Assay-on-Demand technology (Applied Biosystems) as per manufacturer’s instructions. Primer and probe concentrations of 500 nM and 200 nM, were used, respectively. The cDNA equivalent to 3-5 ng of RNA was measured in triplicate by real-time PCR using qPCR master mix with final concentrations 5.5 mM MgCl2, 200 μM dNTPs and 0.5 units Hotstart AmpliTag Gold (Applied Biosystems) in 20 μl volume of 384 well plate or 50 μl volume for 96 well plates. For normalization, cDNA equivalent to 3-5 ng input RNA was measured for GAPDH. All experiments included negative controls with no cDNA and/or with cDNA extracted from feeder cells. Primers were designed to be human-specific, and to span introns to distinguish cDNA from genomic DNA products. Primers and probes used were as follows, with probe sequence designated after the primer pair.

Western blotting

EBs were lysed in RIPA buffer (50 mM Tris (pH 7.4), 150 mM sodium chloride, 0.5% sodium deoxycholate, 1% NP-40, 0.1% SDS, 1% Triton X-100, 1 mM EDTA). Soluble protein extract was separated on 4-10% Nupage bis-Tris gels (Invitrogen) and electrophoretically transferred onto PVDF membranes (Millipore). After blocking the membranes for 1 hour with 5% milk in TBST, primary antibodies were applied for 1 hour. The membranes were washed three times with TBST, and horseradish peroxidase-conjugated donkey anti-rabbit (Jackson Immuno Research Laboratories) and horseradish peroxidase-conjugated donkey anti-mouse antibodies (Sigma) were applied for 1 hour. After washing with TBST, the membranes were developed using ECL Plus (Amersham Biosciences) following the manufacturer’s instructions.
Immunofluorescent and immunohistochemical analysis
For immunofluorescence/immunohistochemical studies, EBs were fixed with 4% paraformaldehyde for 30 minutes, processed to paraffin, and cut at 5 μm serial sections onto slides. For staining, sections were de-paraffinized and blocked in 10% FBS for 30 minutes at room temperature. Primary antibodies were diluted in 5% serum and applied to the sections for 1 hour at room temperature. Antibodies used include anti-PECAM (Vector Laboratories), anti-AFP (Zymed), anti-VE-Cadherin (Santa Cruz biotechnologies), anti-Ki67 (Lab Vision). Negative control experiments were performed by omitting the primary antibody and/or by using serum from the same lot as the primary. The secondary antibodies were washed twice with PBS and incubated for a further hour with donkey anti-rabbit Alexa-488 antibody or donkey anti-mouse Alexa-555 antibody (Molecular Probes). Samples were washed twice with PBS and mounted with Vectashield with DAPI (Vector Laboratories) and anti-mouse Alexa-555 antibody (Molecular Probes). Samples were washed twice with PBS and mounted with Vectashield with DAPI (Vector Laboratories).

Tunnel assay
Sections of EBs were de-paraffinized. The Tunnel assay was performed according to manufacturer’s instructions (Intergen).

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