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Transforming Growth Factor-β signalling in extraembryonic mesoderm is required for yolk sac vasculogenesis in mice

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SUMMARY

We have analysed the function of transforming growth factor β (TGF-β) in yolk sac development in mice by generating somatic chimaeras in which the extraembryonic mesoderm, which gives rise to the endothelial and haematopoietic cells of the yolk sac vasculature, is derived from embryonic stem (ES) cells. The ES cells were stably transfected and express either the full-length type II binding receptor or a kinase-deficient mutant of this receptor. Examination of yolk sacs from chimaeras between E8.5 and 9.5, and analysis of marker expression in embryoid bodies from these mutant ES cell lines in prolonged suspension culture demonstrated that (1) a major function of TGF-β in yolk sac mesoderm is to regulate production and deposition of fibronectin in the extracellular matrix that maintains yolk sac integrity, (2) TGF-β signalling is not required for differentiation of extraembryonic mesoderm into endothelial cells but is necessary for their subsequent organisation into robust vessels, and (3) TGF-β signalling must be tightly regulated for the differentiation of primitive haematopoietic cells to take place normally. Together, these results show that defective TGF-β signalling in the extraembryonic mesoderm alone is sufficient to account for the extraembryonic phenotype reported previously in *TGF-β1*$^{-/-}$ mice (Dickson, M. C., Martin, J. S., Cousins, F. M., Kulkarni, A. B., Karlsson, S. and Akhurst, R. J. (1995) *Development* 121, 1845-1854).

Key words: Chimaera, ES cell, Fibronectin, Mouse, TGF-β receptor, Vasculogenesis, Yolk sac

INTRODUCTION

The functional analysis of growth factors in early development by conventional gene ablation via embryonic stem cells is complicated by the fact that many of these ligands are available to the conceptus from maternal sources. Maternal TGF-β may be present in the zygote or in the deciduum, or protein circulating in the maternal blood stream may be transferred to the conceptus via the placenta (Letterio et al., 1994). Mutant mice generated to analyse the effect of TGF-β1 deficiency (Kulkarni et al., 1993; Shull et al., 1992) provide probably the best-studied example of how maternal rescue occurs. In the original descriptions of the TGF-β null phenotype on either a 129/sv (Kulkarni et al., 1993) or a C57BL/6J (Shull et al., 1992) genetic background, homozygous *TGF-β1*$^{-/-}$ mice were born alive but died shortly after weaning due to massive inflammatory response. Transplacental and lactational transfer of maternal TGF-β1 from *TGF-β*$^{+/-}$ mothers to their *TGF-β1*$^{-/-}$ foetuses and pups was shown to take place and result in their temporary rescue (Letterio et al., 1994). However, later studies demonstrated that the severity of the *TGF-β1*$^{-/-}$ phenotype was highly mouse strain dependent. When embryos were generated on a mixed background of C57BL6/6J × NIH/Ola about half of the *TGF-β1* homozygous embryos died at E10 due to haematopoietic and vascular defects in the yolk sac (Dickson et al., 1995). The other half were morphologically normal but died 3 weeks postpartum. A modifier element near the *TGF-β1* gene on chromosome 5 was identified that appeared to determine the relative dependence of the embryo on TGF-β in the different mouse strains (Bonyadi et al., 1997).

Here we have taken a different approach to analyse the aberrant yolk sac development in more detail and circumvent the complexity in interpretation caused by maternal rescue. We selected the ligand binding component of the TGF-β signalling complex, the so-called type II receptor or TβRII (reviewed by Heldin et al., 1997), to interfere with endogenous TGF-β signalling since as far as is known TβRII is functionally unique. We have (over)expressed either a full-length (wtTβRII) or a truncated, dominant negative form of the TβRII (ΔTβRII), in the mesodermal component of the yolk sac but not in the endodermal component. This was carried out by stable transfection of these constructs in ES cells and generation of somatic chimaeras (Beddington and Robertson, 1989).

Preaggregation of the (mutant) ES cells in suspension culture before allowing them to attach to morula stage host embryos resulted in consistently high contribution chimaeras, essentially indistinguishable from those described previously using tetraploid host embryos (Nagy et al., 1993). However, in our case, the wild-type (diploid) host embryo provides the endodermal component of the yolk sac, while its mesodermal component and all the embryonic lineages are ES cell derived (Goumans et al., 1998). We demonstrated that overexpression of the wtTβRII in the yolk sac mesoderm mimicked the haematopoietic abnormalities described in the yolk sacs of the *TGF-β1*$^{-/-}$ mice. In addition, expression of the dominant negative receptor, which prevented TGF-β signalling, mimicked defects in yolk sac vasculogenesis. Analysis of the mutant ES cells grown as embryoid bodies demonstrated that overexpression of the wtTβRII inhibited the formation of red blood cells (RBCs) and that, although cells expressing the ΔTβRII could differentiate to endothelial cells, these failed to organise into vessels. These results demonstrate that the level of TGF-β signalling in the extraembryonic mesoderm requires tight regulation to support normal yolk sac development. In addition, the data suggest that the principal function of TGF-β in extraembryonic mesoderm is to control fibronectin production for deposition in the extracellular matrix under the visceral endoderm, essential for maintenance of yolk sac integrity.

MATERIALS AND METHODS

Production of ES-cell-derived embryos by morula aggregation and histological analysis

Aggregation chimaeras between (mutant) ES cells and diploid morulae from a mouse line (ROSA-β-geo-26; Friedrich and Soriano, 1991) expressing *lacZ* ubiquitously were generated as described previously (Zwijsen et al., 1998). To bias towards the generation of high percentage chimaeric embryos, the ES cells were preaggregated in suspension in Buffalo rat liver cell (BRL)-conditioned medium (Mummery et al., 1990), containing 20% FCS at 37°C, 1-2 hours prior to use. This resulted in the formation of compact clumps of 15-20 cells for aggregation with morulae stage embryos. Embryos that had developed into blastocysts following overnight culture were transferred into E2.5 pseudopregnant (C57BL/6 × CBA)F_1 recipients. Chimaeric embryos were isolated in ice-cold phosphate-buffered saline (PBS) at nominal E8.5 and E9.5, fixed in 4% ice-cold paraformaldehyde for 30 minutes and stained overnight with X-Gal to detect *lacZ* activity. The embryos were washed in PBS, fixed overnight in 4% paraformaldehyde at 4°C, dehydrated and embedded in paraffin. Serial sections (6 μm) were cut and stained with hematoxylin and eosin.

Cell culture

E14 ES cells stably transfected with cDNA constructs containing *wtTβRII*, *ΔTβRII* or an empty vector, driven by the PGK promoter and containing a KT3 tag to monitor expression of the protein product (Fig. 1A), were routinely cultured on mitomycin-treated feeder cells in BRL-conditioned medium (Goumans et al., 1998). Two independent cell lines for each construct, selected on the basis of highest ^{125}I-TGF-β1 binding capacity, were used in all experiments. No differences between the lines were observed for the parameters studied (Goumans et al., 1998; Zwijsen et al., 1999). Homogeneous expression of the KT3 tag in the construct was retained over more then 20 passages in monolayer culture and in cell aggregates grown in suspension (Zwijsen et al., 1999). The ΔTβRII construct did not affect BMP signalling (Goumans et al., 1998). Embryoid bodies (EB) were allowed to develop for up to 20 days after aggregation (day 0) in hanging drops as described by Mummery et al. (1990), in the presence of 20% FCS with or without 5 ng/ml TGF-β1. EBs were scored morphologically on days 8, 16, 20 as described by Wang et al. (1992); the number of EBs that were cystic (CEB) was determined and each CEB was examined for the presence of vessels (channels lined by a thin layer of cells in CEBs). CEBs containing dark cells after staining for haemoglobin (see below) were considered to be positive for haematopoietic cells.

Benzidine staining

Haematopoietic cells in EBs and chimaeras were stained for haemoglobin with benzidine as described by O'Brien (1960) with minor modifications. After washing with PBS, the unfixed tissues were incubated in a freshly made staining solution containing 87 mg/ml benzidine (Sigma) in acetate buffer pH 4.8 and 61% ethanol for 15 minutes at 4°C, washed with PBS and postfixed with 4% ice-cold paraformaldehyde.

Separation of yolk sac mesoderm and endoderm and isolation of RNA

The yolk sac mesoderm and endoderm of E8.5 embryos were mechanically dissected after incubation in trypsin/pancreatin as described in Hogan et al. (1994). The layers were collected in 100 μl of Ultraspec^{TM} (Biotecx), and RNA was collected and used for RT-PCR analysis as is described by Roelen et al. (1998).

Semiquantitative RT-PCR analysis

Semiquantitative RT-PCR was performed essentially as described by Keller et al. (1993) and Wilson and Melton (1994). Pools of 20 EBs were collected in 200 μl Ultraspec^{TM} (Biotecx) and RNA was extracted according to the manufacturer's protocol; 15 μg PolyI (Sigma) was added as a carrier. Samples were DNase treated to eliminate genomic DNA and 1 μg RNA was reverse transcribed as described by Roelen et al. (1994). One-tenth of the reverse transcription product served as a template for PCR-amplification. Amplification took place in a total volume of 50 μl containing 75 mM Tris-HCl pH 9.0, 0.1% (v/v) Tween 20, 20 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.2 mM dNTP each (Gibco-BRL), 1 μM of each specific oligonucleotide primer and 1.25 U Goldstar polymerase (Eurogentec). After 5 minutes initial denaturation at 94°C, reactions were cycled through 15 seconds at 94°C, 30 seconds annealing at primer-specific temperature (Table 1) and 45 seconds primer extension at 72°C. After 20 cycles for β-actin, 28 cycles for VEGF and ξ-globin and 24 cycles for other primer sets, final extension was carried out for 5 minutes at 72°C. The PCR amplification of the cDNA remained exponential after this PCR profile (data not shown). One tenth of each reaction product was loaded on a 1.5% agarose gel containing 0.01% (v/v) Vistragreen (Amersham) and quantified using a FluorImager system (Molecular Dynamics) and ImageQuant software (version 4.2a).

Immunofluorescent staining

EBs were plated on gelatinised coverslips 11 days after aggregation and grown for an additional 5 days. After washing with PBS, they were fixed in methanol at –20°C for 30 minutes, washed with PBS and blocked for 1 hour in 10% normal goat serum, then incubated for 1 hour with mAb 390, a rat anti-mouse PECAM-1 antibody (Baldwin et al., 1994). The coverslips were washed three times with PBS and counterstained with Cy3-conjugated goat anti-rat secondary antibody for 1 hour in the dark. The specimens were washed three times with PBS, mounted in Mowiol and photographed using a Zeiss Axioplan fluorescent microscope. 6 μm sections of yolk sacs from aggregation chimaeras were stained for fibronectin as described previously (Zwijsen et al., 1999).

Western blotting

ES cells were aggregated for 20 days as described above and harvested

in buffer containing 50 mM Tris (pH 7.4), 50 mM NaCl, 0.5% Nonidet P-40, 1 mM DTT, 1 mM PMSF, 1 μg/ml aprotinin and 1 μg/ml leupeptin at 4°C. Subsequently, cells were centrifuged for 15 minutes at 4°C and the protein concentration of the supernatant was determined by the BioRad protein assay. Extracts (40 μg of total protein) was separated on SDS-PAGE gels and transferred to Immobilon (Millipore, MA). Blots were blocked in TBST (10 mM Tris (pH 8), 150 mM NaCl, and 0.05% Tween-20) containing 3% non-fat milk powder for 1 hour. Blots were probed with a polyclonal antibody against laminin (Sigma, MO), or fibronectin (Sigma, MO), in TBST containing 0.5% non-fat milk powder. All subsequent steps were carried out with TBST. After vigorous washing, blots were incubated with peroxidase-conjugated antibodies (1:10,000; Amersham). Blots were washed again and immunoreactive bands were visualised with ECL (Amersham).

RESULTS

Aberrant TGF-β signalling in the extraembryonic mesoderm is sufficient to cause defective yolk sac development

Pre-aggregation of E14 ES cells immediately before allowing them to attach to morula stage embryos results in the embryonic part and extraembryonic mesoderm of nearly all of the resulting chimaeras being entirely ES-derived, with the exception of some endoderm cells in the developing gut; this is particularly clearly illustrated if the ROSA-26 transgenic mice strain is used as a host (Zwijsen et al., 1999). *lacZ* is expressed in these mice in all cells throughout gestations so that host-derived cells in the embryo proper and the yolk sac are readily detectable on a background of colourless ES-derived tissues. The visceral endoderm of the yolk sac is thus easily shown to be host derived while the mesodermal component is entirely derived from the ES cells. We have demonstrated that ectopic expression of either wtTβRII or ΔTβRII in the mesodermal but not in the endodermal component of the visceral yolk sac results in defective vasculogenesis and haematopoiesis at E9.5 (Goumans et al., 1998) similar to that described in *TGF-β1*$^{-/-}$ embryos. To identify the basis for these defects and the role TGF-β plays in the development of endothelial and haematopoietic cells, we analysed the yolk sacs of these chimaeric embryos in detail. In particular, we addressed the question of the extent to which 'classical' TGF-β responses (growth inhibition, ECM protein deposition; Roberts and Sporn, 1990) contributed to the phenotype.

Histological and morphological examination at nominal E8.5 demonstrated no obvious differences between embryos expressing ΔTβRII in the mesodermal component of the yolk sac ("ΔTβRII yolk sacs") and controls, either expressing empty vector or wild-type embryos (data not shown); both had expanded yolk sacs containing developing blood islands. At E9.5, the ΔTβRII yolk sacs exhibited a number of distinct abnormalities; most striking were defects in vasculogenesis, ranging from the development of small, disorganised and delicate vessels (Fig. 1C,D open arrowhead) to, in the most severe cases, regions of the yolk sac completely devoid of vessels (Fig. 1B). A higher power magnification of the yolk sac showed that the mesodermal layer was only attached incidentally to the visceral endoderm resulting in 'spikes' in the yolk sac cavity and indentations on the outside (Fig. 1E). Also abnormal was the accumulation of primitive blood cells in the exocoelomic cavity suggesting that these yolk sacs have impaired circulation. Together, these defects implied compromised circulation from the moment the heart started to beat, causing the pericardial sac to enlarge (Fig. 1B arrowhead) and RBC accumulation in the proximal part of the yolk sac (Fig. 1D white arrowhead).

After staining the embryos for *lacZ* activity at E9.5 it became

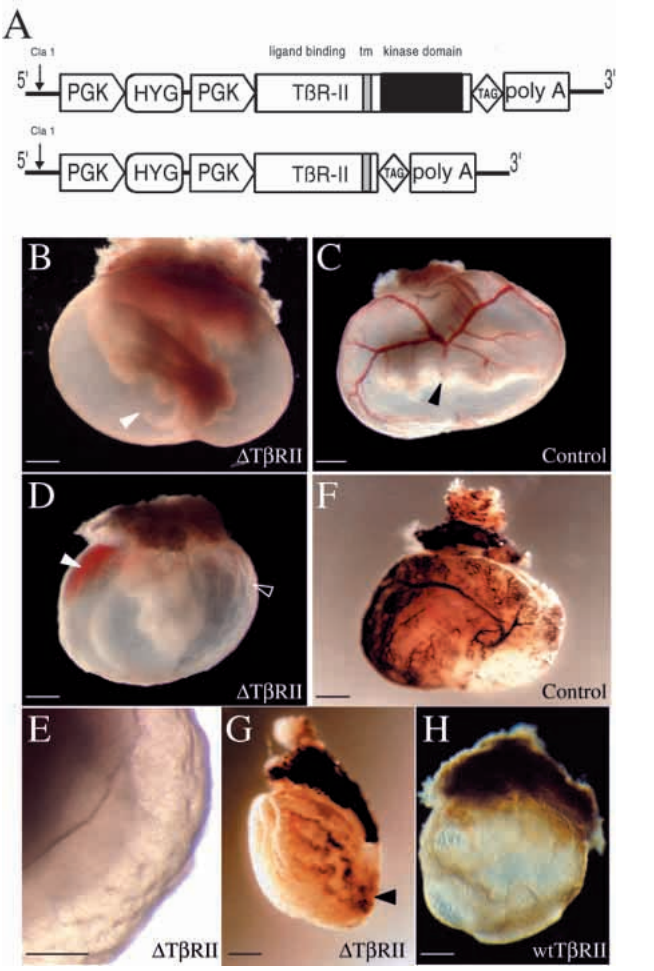

Fig. 1. (A) The plasmid constructs used to introduce the wtTβRII and ΔTβRII into ES cells. TβRII carboxyterminally flanked by a KT3 epitope tag, was cloned into the PGK-CAS vector, containing the PGK-1 promoter and a polyadenylation signal. Hygromycin was used as a selection marker. The ΔTβRII construct was made by truncating the juxta-membrane region from amino acid 700, but was otherwise similar. Tm, transmembrane domain. (B) Morphology of embryos resulting from morula aggregation with embryonic stem cells stably transfected with the ΔTβRII (B,D,E,G), wtTβRII (H) or empty vector (Control; C,F). (B) E9.5 ΔTβRII chimaera with yolk sac anaemia and defective vasculogenesis. The arrowhead indicates the enlarged pericardial sac. (C) E9.5 control embryo showing a well developed blood filled vasculature. The arrowhead points to the pericardial sac. (D) E9.5 ΔTβRII chimaera with defective vasculogenesis. The white arrowhead shows accumulation of red blood cells. The open arrowhead points at the formation of a primitive plexus. (E) Magnification of a ΔTβRII yolk sac. (F) E9.5 control embryo and (G) ΔTβRII chimaera stained with benzidine to identify primitive red blood cells. The arrowhead in G indicates the primitive plexus. (H) E9.5 wtTβRII chimaera with a blistered appearance. Scale bar: 100 μm.

Table 1. Oligonucleotide primers used for gene expression analysis

Gene	Primer sequence	Reference	Annealing temp. (°C)	Size (bp)
AFP	5′-ATACTCAAGAACTCACCCCAACCT-3′ 5′-CTCACACCAAAGCGTCAACACATT-3′	Law and Dugaixzyk, 1981	58	812
Endo-A	5′-TTCAGCAGCCGCTCGTTCAC-3′ 5′-TTCTCCTGAGTGCGCACAGC-3′	Tamai et al., 1991	58	341
PECAM-1	5′-GTCATGGCCATGGTCGAGTA-3′ 5′-CTCCTCGGCATCTTGCTGAA-3′	Xie and Muller, 1992	58	260
Flt-1	5′-TGACCTTCGCATACTGCTCACG-3′ 5′-TTAGCTCCTCTCAGACTGCCTT-3′	Finnerty et al., 1993	55	364
ζ-globin	5′-GCTTCAAGATCATGACCGCCGT-3′ 5′-CGGTCCAGCTTAGCGGTACTT-3′	Alonso et al., 1986	58	259
VEGF	5′-ACCAGCGAAGCTACTGCCGT-3′ 5′-TAACTCAAGCTGCCTCGCCT-3′	Claffey et al., 1992	55	398
TβRI	5′-CGTTACAGTGTTTCTGCCACCT-3′ 5′-AGACGAAGCACACTGGTCCAGC-3′	Roelen et al., 1994	52	314
ALK-1	5′-CTTGGGGAGCTTCAGAAGGGG-3′ 5′-TGCCTGTTCAGATGCCTTCAG-3′	Roelen et al., 1997	60	501
TβRII	5′-GGAAGTCTGCGTGGCCGTGTGG-3′ 5′-CTATGGCAATCCCCAGCGGAGG-3′	Roelen et al., 1994	52	298
β-actin	5′-TGAACCCTAAGGCCAACCGTG-3′ 5′-GCTCATAGCTCTTCTCCAGGG-3′	Roelen et al., 1994	58	398

Set of primer pairs used for amplification of indicated genes from reverse-transcribed RNA derived from EBs and yolk sac layers.

evident that the visceral endoderm from the ΔTβRII-expressing chimaeras was different from that in control chimaeras despite it being derived from the host morula. The blue staining was less intense than in the control chimaeras or the wtTβRII-expressing chimaeras (Fig. 2D-F), although one day earlier (at E8.5), the *lacZ* activity in the visceral endoderm in all three types of chimaera was of equal intensity (Fig. 2A,B). Histological sections revealed that the morphology of the endodermal layer of the ΔTβRII yolk sacs was different from control yolk sacs (Fig. 3A,B). The cells were more columnar and the nuclei were aligned centrally in the cell rather than on the basal side (Fig. 3B). The expression of α-fetoprotein, a marker for differentiated visceral endoderm, was normal, indicating that the differences in morphology seen in the control and ΔTβRII-expressing yolk sac were not due to an overall impairment of differentiation towards visceral endoderm (Fig. 3C,D). Histological sections also showed that contact between the mesodermal and endodermal layers of the yolk sacs expressing ΔTβRII was very limited (Fig. 3K,N) or that they had separated entirely (Fig. 3J,M); this was never observed in controls after fixation. Haematopoietic cells were present between the separated layers of the yolk sac mostly in the proximal part (Fig. 3J,K).

The difference in haematopoiesis between yolk sacs from control chimaeras and those expressing ΔTβRII in the mesodermal compartment became particularly evident after benzidine staining (Fig. 1F,G). While the yolk sac of the control chimaera at this stage had formed a complete network of blood-filled vessels (Fig. 1F), the ΔTβRII yolk sac contained RBCs captured in a primitive plexus (Fig. 1G arrowhead). The aberrant distribution of RBCs in these mutant yolk sacs is probably due to their leakage from the delicate and fragile vessels that had formed.

In a series of parallel experiments, we also analysed chimaeric embryos in which the mesodermal component of the yolk sac was derived from ES cells overexpressing wtTβRII (“wtTβRII yolk sacs”). At nominal E8.5, the wtTβRII yolk sacs were already distinguishable from controls since they had distinctly fewer RBCs (data not shown). At E9.5 the mesodermal layer was undersized compared with the visceral endoderm (Fig. 3L) resulting in a blistered appearance (Fig. 1H) and there was a delay in vessel formation (Figs 1H, 3L). Furthermore, the wtTβRII yolk sacs were still severely anaemic with few or, in some cases, no RBCs evident (Fig. 1H).

These defects indicate that a type I TGF-β receptor is normally present since TβRII cannot transduce a signal on its own, and the ΔTβRII chimaera results imply that TβRII is endogenously expressed in the mesodermal component of the yolk sac. In most cell types, TβRI/ALK-5 is the signalling receptor for TGF-β. In endothelial cells, it has been suggested that ALK-1 may also function as a type I receptor for TGF-β during embryogenesis (Roelen et al., 1997). We therefore examined the expression of both TGF-β type I receptors and TβRII in the separated layers of the yolk sac by RT-PCR. Fig. 4 demonstrates that mRNA for *TβRI/ALK-5*, *ALK-1* and the *TβRII* is expressed in the extraembryonic mesoderm at E8.5, while none of them were detected in the visceral endoderm, which did express AFP as expected. This confirms that TGF-β signalling can take place in the mesodermal cells of the yolk sac via TβRI/ALK-5 and/or ALK-1 in combination with TβRII and that ectopic expression of ΔTβRII could interfere with TGF-β signalling.

Endothelial and haematopoietic development in embryoid bodies of wtTβRII- and ΔTβRII-expressing ES cells

To address the question of whether ES cells expressing ΔTβRII

or wtTβRII ectopically were intrinsically defective in their capacity to differentiate to endothelial and/or haematopoietic cells, we examined their ability to form these cell types in EBs in culture compared with their negative control counterparts (empty vector). ES cells have the ability to differentiate into multiple cell types in vitro. When grown in suspension culture, ES cells form a structure called a cystic embryoid body (CEB), which resembles the yolk sac in overall morphology and gene expression pattern (Doetschman et al., 1985; Keller et al., 1993; Wang et al., 1992). The use of an in vitro model system facilitates accessibility to the cells and exogenous growth factors can be added at all stages of differentiation.

Endothelial cells

Morphological examination showed that, by day 20 after aggregation, 84% of control and 89% of ΔTβRII ES cells (Table 2) had formed CEBs with a prominent cystic cavity surrounded by visceral yolk sac endoderm (Fig. 5A,C). By contrast, wtTβRII-expressing cells had failed to form CEBs (Table 2; Fig. 5B). These EBs were significantly smaller in diameter than controls (Table 2) and remained a dense clump of cells with no clear layer of visceral endoderm. When control EBs were cultured in the presence of TGF-β1, the majority failed to form a clear cystic cavity (Table 2; Fig. 5D). Although ΔTβRII ES cells formed the same percentage of CEBs as control ES cells, the CEBs were significantly larger and failed to form a network of vessels that was evident in control EBs by this time (Table 2; Fig. 5A arrowhead, C). Addition of TGF-β1 to the ΔTβRII cell cultures had no obvious morphological effect.

Analysing the localisation of PECAM-1 protein in EB outgrowth confirmed that ΔTβRII cells were defective in vessel formation. PECAM-1 is expressed by early endothelial precursors in the yolk sac at E7.0 (Baldwin et al., 1994) and expression persists at later stages in development. While the control outgrowths formed a network of PECAM-1-positive cells into vessels (Fig. 6A,B), the ΔTβRII outgrowth contained only sporadic PECAM-1-positive cells (Fig. 6E,F). Outgrowth of EBs expressing the wtTβRII did stain with anti-PECAM-1 but the positive cells remained as a dense clump with no clear endothelial organisation (Fig. 6C,D). However, when the wtTβRII EBs formed a sporadic CEB, it was capable of forming a network of vessels (data not shown).

Haematopoietic cells

To determine whether EBs contained haematopoietic cells, they were stained with benzidine (Fig. 5G), squashed on microscope slides (Fig. 5H) and scored for the presence of brown cells (Table 2). On day 20 after aggregation, 91% of the control CEBs contained benzidine-positive cells (Fig. 5G; Table 2). Some ΔTβRII CEBs also generated benzidine-positive RBCs although fewer than controls (35%). None of the compact EBs from the wtTβRII ES cells ever formed RBCs and of those that were cystic and formed vessels hardly any contained RBCs (5%). This indicates that wtTβRII-expressing ES cells are intrinsically defective in their capacity to form RBCs.

These observations demonstrate that, although ΔTβRII ES cells formed CEBs, i.e. formed a functional epithelium of visceral endoderm-like cells on the outside of the aggregate, this did not appear to be sufficient to result in the formation of a vascular network. wtTβRII cells, in contrast, remained as precystic embryoid bodies and were incapable of generating haematopoietic cells under conditions that support this differentiation pathway in control cells. Although undifferentiated ES cells do not express TβRII, expression is upregulated within 3 days of EB formation (Thorsteinsdóttir et al., 1999) and ES cells secrete active TGF-βs (Slager et al., 1993). Furthermore, undifferentiated ES cells express both *TβRI/ALK-5* (Fig. 4) and *ALK-1* (data not shown). Control cells

Table 2. Differentiation parameters of embryoid bodies formed from ES cells expressing empty vector (Control), truncated TβRII (ΔTβRII) or full-length receptor (wtTβRII)

Day	Cell type	Number of aggregates*	% CEB‡	Diameter (mm)	% CEB with vessels‡	% CEB with RBCs‡
8						
pre-cystic	Control	95	41±2	0.69±0.05	nd	nd
	+TGF-β	87	9±2	0.71±0.03	nd	nd
	ΔTβRII	97	56±4	0.74±0.03	nd	nd
	+TGF-β	78	45±4	0.75±0.03	nd	nd
	wtTβRII	83	3±2	0.49±0.03	nd	nd
	+TGFβ	76	0	0.48±0.03	nd	nd
16						
cystic	Control	79	81±2	0.90±0.03	65±4	66±5
	+TGF-β	77	34±3	0.78±0.02	47±3	19±4
	ΔTβRII	84	85±3	1.14±0.04	23±5	28±2
	+TGF-β	81	86±2	1.10±0.04	31±3	18±1
	wtTβRII	82	20±2	0.74±0.03	56±2	5±2
	+TGF-β	85	17±3	0.72±0.03	59±3	0
20						
cystic	Control	81	84±2	nd	82±3	91±2
	+TGF-β	82	41±4	nd	64±3	27±4
	ΔTβRII	79	89±2	nd	23±2	35±5
	+TGF-β	85	84±4	nd	25±4	20±3
	wtTβRII	79	28±6	nd	79±3	5±3
	+TGF-β	87	25±3	nd	86±2	0

*Total number of EBs scored in 4 experiments.
‡Mean percentage ± s.e.m. from 4 experiments.

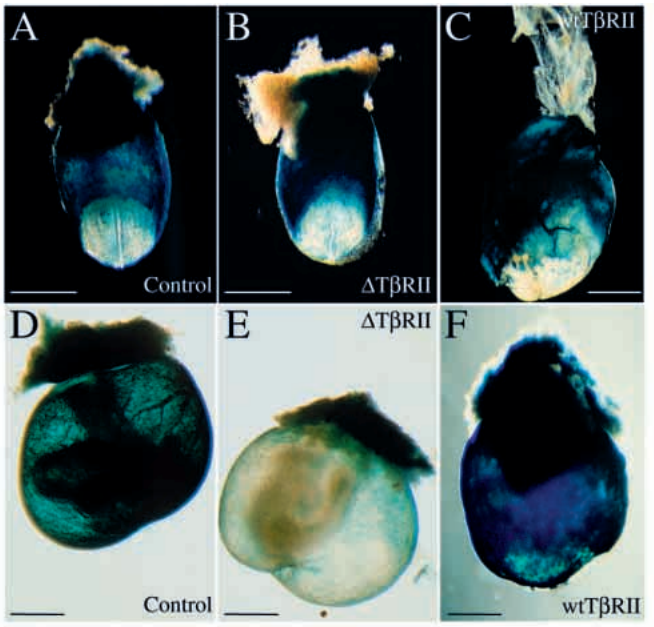

Fig. 2. Morphological analysis of β-gal-stained yolk sacs from embryos collected at E8.5 (A-C) or E9.5 (D-F) obtained from morula aggregation with embryonic stem cells expressing an empty vector (control; A,D), ΔTβRII (B,E) or wtTβRII (C,F). Scale bar, 200 μm.

could then, in principle, undergo some degree of autocrine growth regulation. Increased or precocious TGF-β signalling by ectopic TβRII expression appeared to inhibit haematopoiesis. Addition of exogenous TGF-β1 to EBs from control ES cells had a similar effect in that fewer CEBs formed than in the absence of TGF-β1 (Table 2) and of these fewer contained benzidine-positive cells (Table 2). The TGF-β1-treated EBs derived from control ES cells were virtually indistinguishable from EBs derived from wtTβRII-expressing cells after benzidine staining (Table 2).

Marker expression

To specify the differences in morphology of the embryoid bodies more precisely, we examined the expression of various cell-type-specific markers by semiquantitative RT-PCR (Keller et al., 1993; Wilson and Melton, 1994) throughout their development (Fig. 7; Table 1). Equal amounts of cDNA were used in each reaction, as shown by an equivalent amount of product generated by β-actin primers (Fig. 7A). To determine the extent of haematopoiesis in EBs, they were analysed for the expression of *ξ-globin*, a marker gene for developing RBCs (Leder et al., 1992). *ξ-Globin* mRNA was expressed in control and ΔTβRII cells (Fig. 7A,F), although, in the latter, in a delayed and decreased fashion. No expression was detected in the wtTβRII EBs. Addition of TGF-β1 to the control EBs decreased the levels of *ξ-globin*. This confirmed that differentiation towards the haematopoietic cell lineage was inhibited by early ectopic TGF-β signalling.

EBs become cystic as the outer (epithelial) endoderm layer begins unidirectional transport of fluid to a central cavity. From their morphology, the embryoid bodies appear to differ in the

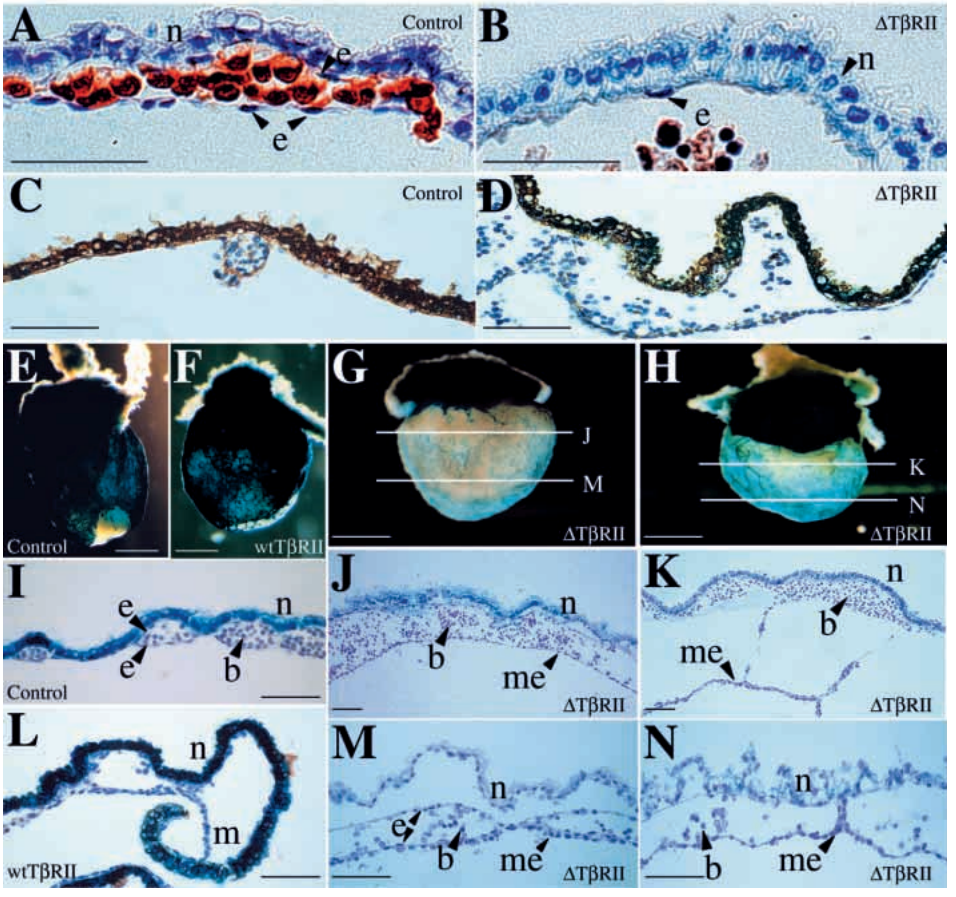

Fig. 3. Histological analysis of E9.5-dissected yolk sacs from embryos obtained by morula aggregation. (A) Control yolk sac stained with benzidine and counterstained with haematoxilin showing endothelial cells forming channels and RBCs captured within them. (B) Visceral endodermal layer of a ΔTβRII yolk sac with a sporadic endothelial cell attached to it. Note the difference in morphology compared to the endodermal layer in control yolk sacs. (C,D) Control and ΔTβRII yolk sac stained for α-fetoprotein, a marker for visceral endoderm. (E-H) Morphology of sectioned embryos; (I-N) sections through yolk sacs; (I,L) sections through E and F, respectively; (J,K,M,N) sections as indicated in G and H. (I) Control yolk sac showing the host-derived blue visceral endoderm and the vessels formed by the ES cells derived extraembryonic mesoderm. (J) Both layers of the yolk sac are completely separated. (K,N) The mesodermal layer of the yolk sac is sporadically attached to the endoderm resulting in the formation of 'spikes'. (M) Both layers of the yolk sac are completely separated. b, blood cells; e, endothelial cell; m, extraembryonic mesoderm; me, mesothelial cell layer; n, endoderm. Scale bar, (A-D) 50 μm, (E-H) 200 μm, (I-N) 50 μm.

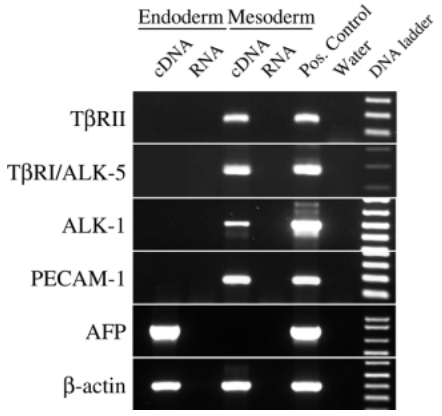

Fig. 4. Detection of TGF-β receptors in the endodermal and mesodermal layer of the visceral yolk sac at E8.5 by RT-PCR. Amplified products of the type II receptor (TβRII), type I receptors (ALK-1 and ALK-5), PECAM-1 (expressed in endothelial cells), α-fetoprotein (AFP; expressed in visceral endoderm) and β-actin are indicated.

extent to which the visceral endoderm-like cell layer formed. Therefore we determined the expression of two endodermal marker genes, *Endo-A* and *α-fetoprotein* (*AFP*). *Endo-A* is a marker for both primitive and visceral endoderm (Duprey et al., 1985) while *AFP* is a marker specific for differentiated visceral endoderm (Dziadek and Adamson, 1978) in early development. In all EBs, *Endo-A* is expressed at day 8 and day 16 although the expression levels of *Endo-A* are elevated in the ΔTβRII EBs (Fig. 7A,B). In the wtTβRII EBs, the expression of *AFP* is significantly lower (Fig. 7A,C), as in the control EBs grown in the presence of TGF-β1. This suggests a defect in terminal differentiation to visceral endoderm in the wtTβRII EBs, although differentiation to primitive endoderm did appear to take place.

Although vessels were not evident or were sparse in EBs containing wtTβRII or ΔTβRII, expression of two marker genes for endothelial cells, *flt-1* and *PECAM-1*, was determined. *Flt-1* is a marker for haemangioblasts in the early embryo and later becomes restricted to the endothelial cells of the developing yolk sac vasculature at E8.5, decreasing by E11.5. In all three types of EBs, both endothelial cell markers were expressed at day 8 and 16 of culture (Fig. 7A,D,E). In the ΔTβRII EBs examined, there was a slight decrease in *flt-1* mRNA expression (Fig. 7A,E) compared to control cells, as was the expression of *PECAM-1* (Fig. 7A,D), suggesting that indeed endothelial cells could form in ΔTβRII EBs, but they failed to differentiate fully. Levels of *PECAM-1* mRNA were increased in wtTβRII (Fig. 7A,D), but in contrast *flt-1* expression was decreased (Fig. 7A,E). *PECAM-1* mRNA was also increased in the control EBs cultured in the presence of TGF-β1. Since *flt-1* is present in both the early precursors of endothelial cells as well as haematopoietic cells, this could reflect the difference in capacity of forming RBCs cells between wtTβRII and ΔTβRII EBs.

VEGF is a crucial growth factor for vasculogenesis. The mRNA is normally expressed in the visceral endoderm of the yolk sac, whereas its receptors (flt-1 and flk-1) are localised in the extraembryonic mesoderm. We examined the expression of *VEGF* mRNA in EBs of the different ES cell lines (Fig. 7A,G). The expression of *VEGF* is upregulated in the wtTβRII EBs and addition of TGF-β1 to the culture medium of wtTβRII and control ES cells further increases this expression; this was somewhat unexpected since, under these conditions, morphological examination and *AFP* expression had suggested that fewer visceral endoderm cells were present. However, there is evidence suggesting that VEGF is in fact a target gene for TGF-β1 (Pertovaara et al., 1994; Hatzopoulos et al., 1998), which might account for the increase in mRNA, since no changes in mRNA levels were detectable in the ΔTβRII EBs treated with TGF-β1. Alternatively, or in addition, VEGF could be expressed in mesoderm; after 40 cycles of amplification, we in fact detected VEGF transcripts by RT-PCR in 11 of 14 samples of mesoderm isolated from E8.5 visceral yolk sac, suggesting that VEGF is indeed expressed by these cells, albeit at low levels (data not shown).

Although altered VEGF levels could have contributed to the failure of ΔTβRII yolk sac endothelial cells to organise into vessels, the above results would suggest this is not the case. In addition, although *lacZ* expression in the visceral endoderm in chimaeras precluded direct examination of *VEGF* expression by in situ hybridisation, Palis et al. (1995) have demonstrated that exogenous VEGF addition to extraembryonic mesoderm in culture does not circumvent the requirement for direct contact with visceral endoderm and/or its associated ECM.

ΔTβRII embryoid bodies are defective in extracellular matrix production

The vascular phenotype observed in the yolk sac of the

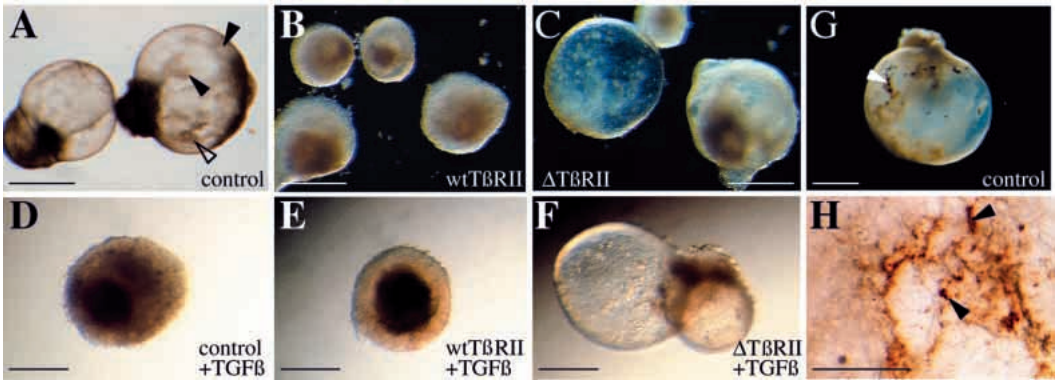

Fig. 5. Morphological analysis of embryoid bodies formed from embryonic stem cells expressing an empty vector (A,D), wtTβRII (B,E) or ΔTβRII (C,F) cultured for 20 days in the absence (A-C) or presence (D-F) of 5 ng/ml TGF-β1. The arrowhead in A indicates formed vessels. The open arrowhead points at primitive blood cells. (G) Benzidine-stained control embryoid body. The arrowhead indicates benzidine-stained cells. (H) Squash preparation from the embryoid body shown in (G). Scale bar, 200 μm.

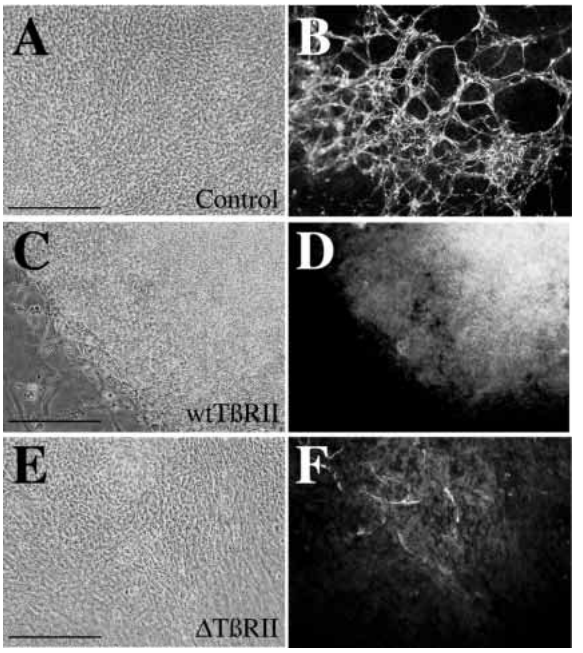

Fig. 6. Staining with anti-PECAM-1 in the outgrowth of plated embryoid bodies from control ES cells (A,B), ES cells expressing wtTβRII (C,D) or ΔTβRII (E,F). Scale bar, 50 μm.

ΔTβRII chimaeras resembles that described for embryos in which fibronectin or one of its receptor subunits, α5-integrin had been ablated (George et al., 1993; Yang et al., 1993). Since TGF-β is known to regulate extracellular matrix deposition in many cell types in culture, it was possible that TGF-β affects endothelial cells by altering the expression of extracellular matrix molecules that regulate adhesion. We therefore analysed protein extracts of embryoid bodies allowed to develop for 20 days, for the presence of laminin and fibronectin. Control embryoid bodies produced easily detectable levels of laminin and fibronectin (Fig. 8), but these proteins were undetectable or strongly decreased in ΔTβRII EBs. wtTβRII EBs, in contrast, contained comparable amounts of laminin as control EBs and increased levels of fibronectin. This observation indicates that one function of TGF-β in the yolk sac could be the regulation of extracellular matrix protein production and deposition between its two cell layers.

We confirmed this implication in vivo, by staining sections of the yolk sacs from control ΔTβRII and wtTβRII chimaeras with antibodies against fibronectin. Fig. 9 shows that the matrix between the visceral endoderm and extraembryonic mesoderm is a continous layer in control embryos (Fig. 9A,B) and in embryos expressing the wtTβRII (data not shown). In the ΔTβRII-expressing yolk sacs, the amount of fibronectin deposited is decreased and there is no continous layer visible (Fig. 9C,D). We observed no differences in α5 integrin expression and distribution between control, ΔTβRII, and wtTβRII chimaeras (data not shown).

Yolk sac development and the influence of the embryo proper

It is conceivable that the embryo proper had defects at the stages investigated which could influence yolk sac development (Tanaka et al., 1999). We therefore removed the yolk sacs from the chimaeric embryos and examined their morphology in more detail. Apart from the pericardial effusion (Fig. 10A) the embryonic part of the ΔTβRII chimaeras exhibited two specific defects not regarded as secondary to poor yolk sac circulation (Copp, 1995), namely an undulating neural tube and a failure in proper epithelialization of somites (Fig. 10B,C). These defects were rescued in "balanced" chimaeras occasionally recovered using the ES cell/morula aggregation procedure we described (Fig. 10D) although yolk sac development in this case remained abnormal (Fig. 1B; Fig. 10D insert) and pericardial effusion was still observed (Fig. 10D arrowhead). We therefore conclude that the defect in yolk

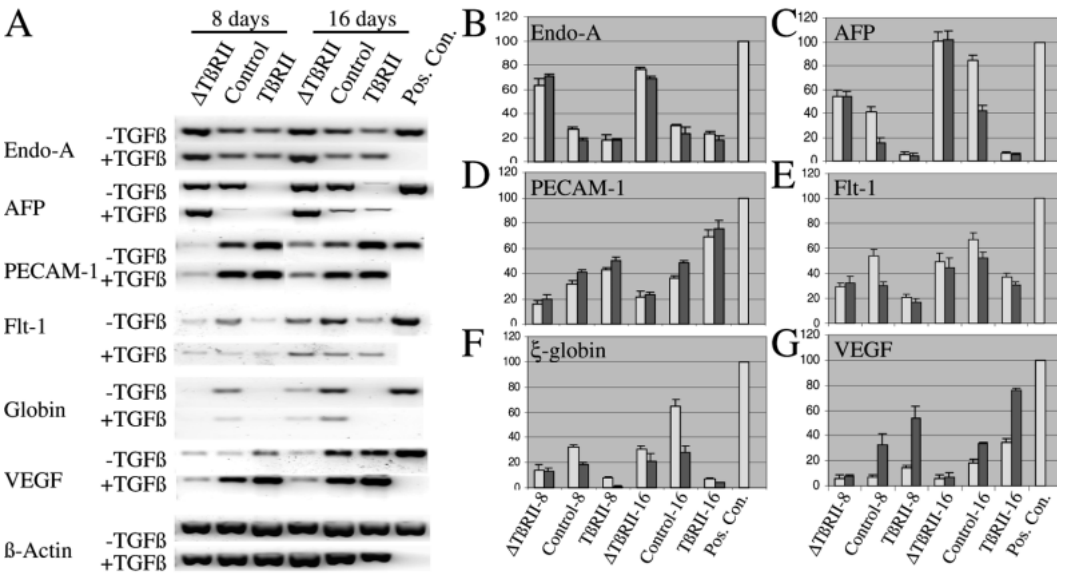

Fig. 7. Semiquantitative RT-PCR analysis of markers for endothelial and haematopoietic cells and endoderm differentiation. RNA was extracted form embryoid bodies at day 8 and 16 of culture in the presence or absence of 5 ng/ml TGF-β1 and analysed for the expression of early (Endo-A) or late (AFP) endoderm markers. The expression of ξ-globin was examined for haematopoietic development, and flt-1 and PECAM-1 for the endothelial cell development. To normalise for the amount of mRNA used as starting material, the cDNA of β-actin was amplified. E8.5 yolk sac mRNA was used as a positive control. (A) Example of one experiment for the different marker genes. (B-G) The PCR products were loaded on an agarose gel, stained with Vistra green and quantified using a Fluorimager; data shown as % positive control. Left bar: without TGF-β. Right bar: with TGF-β. The average + s.e.m. of 4 experiments is shown.

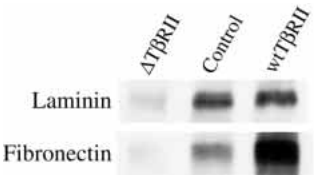

Fig. 8. Analysis of laminin and fibronectin production in embryoid bodies from ES cells expressing empty vector (Control), truncated TβRII (ΔTβRII) or full-length TβRII (wtTβRII). Whole-cell extracts were fractionated on a 6% SDS-PAGE and blotted. The filter was incubated with a polyclonal antibody to laminin or fibronectin. The proteins were visualised after incubation with a peroxidase-conjugated second antibody and ECL.

sac vasculogenesis is the direct result of the expression of ΔTβRII in the mesodermal component of the yolk sac.

DISCUSSION

Mouse embryos lacking functional *TGF-β1* or *TβRII* genes show defects in yolk sac vasculogenesis and haematopoiesis and die around day 10 of gestation (Dickson et al., 1995; Oshima et al., 1996). In the present and previous studies (Goumans et al., 1998), we have addressed this problem more precisely and demonstrated that selective overexpression of either a wtTβRII or ΔTβRII in the mesodermal cells and not the endodermal cells of the yolk sac, results in similar phenotypes. This demonstrates that aberrant TGF-β signalling in the extraembryonic mesoderm compartment can entirely account for the yolk sac phenotypes in TGF-β1 and TβRII null embryos.

Yolk sacs of chimaeric embryos expressing ectopic wtTβRII were deficient in extraembryonic mesoderm underlying the visceral endoderm and so had a blistered appearance. Since TGF-β is known to be a growth inhibitor of many cell types, ectopic expression of the TβRII in cells exposed to ligand may result in cell cycle arrest provided that downstream components of the signal transduction pathway are present. We have recently demonstrated that mesoderm cells in and near the primitive streak of chimaeric embryos expressing wtTβRII indeed no longer express proliferating cell nuclear antigen, a marker for cycling cells (Zwijsen et al., 1999). We therefore assume that classical, but precocious, growth inhibition by TGF-β also causes the paucity of extraembryonic mesoderm in the wtTβRII yolk sacs. This assumption is strengthened by the dramatic reduction in size of the wtTβRII EBs at day 8.

Despite the failure of the wtTβRII-expressing yolk sacs to form an organised vasculature, in vitro studies, using EBs as a model for yolk sac development, demonstrated that wtTβRII-expressing ES cells are capable of forming differentiated endothelial cells expressing both *flt-1* and *PECAM-1*. They however fail to form differentiated visceral endoderm. If direct contact between the visceral endoderm and endothelial cells is necessary for their organisation into vessels, the absence of the visceral endoderm layer in this case would be the reason that any endothelial like cells that did form may not organise. Palis et al. (1995) have demonstrated in tissue recombination experiments that close contact between the extraembryonic mesoderm and visceral endoderm or its extracellular matrix is

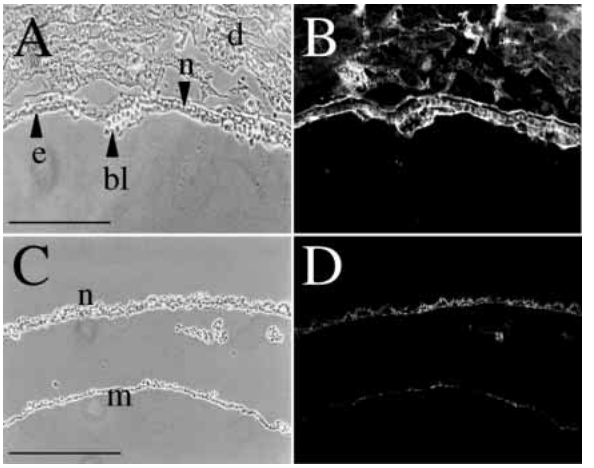

Fig. 9. Expression of fibronectin in sections of yolk sacs from control chimaeras (A,B) or chimaeras expressing ΔTβRII (C,D). Pictures are taken with the same exposure time. Bl, blood islands; d, deciduum; e, endothelial cell; m, extraembryonic mesoderm; n, endoderm. Scale bar, 50 μm.

required for yolk sac vasculogenesis, but not for the specification of the endothelium; this is entirely in agreement with our observations in EBs expressing wtTβRII. In addition, in the yolk sacs of the ΔTβRII-expressing chimaeras, although the visceral endoderm and the extraembryonic mesoderm layer were both present, they were separated and developing endothelial cells were only infrequently in contact with the visceral endoderm. This lack of close contact would again be the reason that there is no formation of a vitelline network.

Although, in ΔTβRII chimaeras, insufficient mesoderm to line the visceral endoderm could contribute to the separation of the endodermal and mesodermal layer, we thought it more likely that insufficient deposition of extracellular matrix proteins, such as fibronectin, in matrix between the visceral endoderm and extraembryonic mesoderm would be involved. This is supported by the facts that (1) several of these genes are known as direct targets of TGF-β and (2) the yolk sac phenotype that we observed in the ΔTβRII chimaeras has a number of similarities with the fibronectin and α5 integrin knockout embryos (George et al., 1993; Yang et al., 1993). Fibronectin is synthesised by the mesodermal cells and assembled into a matrix between the two cell layers (George et al., 1993). These extracellular matrix proteins may therefore not only be important for the formation of a vascular network, but may also be necessary to keep the two layers of the visceral yolk sac closely apposed. Our results support this hypothesis since they demonstrated a significant reduction in fibronectin deposition between the two layers of the yolk sac in the ΔTβRII chimaeras.

TGF-β1 has been shown to induce the differentiation of capillary tube-like structures in three-dimensional cultures of endothelial cells (Madri et al., 1989) with a concomitant increase of PECAM-1, fibronectin and certain integrins (Merwin et al., 1990; Basson et al., 1992; Madri et al., 1992). Using EBs, we demonstrated that the amount of fibronectin produced by ΔTβRII ES cells was greatly reduced compared to control EBs. By extrapolation, TGF-β signalling in the extraembryonic mesoderm may (up)regulate the synthesis and

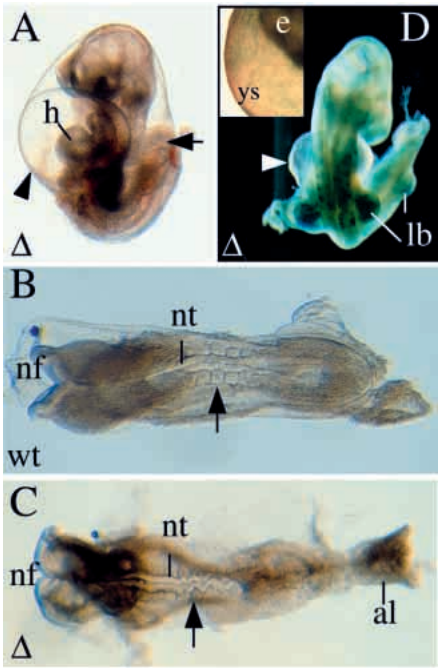

Fig. 10. Morphological analysis of the embryo proper in chimaeras derived from morula aggregation with ES cells expressing ΔTβRII. (A) Entirely ES-derived chimaera (E9.5) showing pericardial effusion (arrowhead) and posterior reduction (arrow). (B) Dorsal view of wild-type embryo (E8.5). (C) Dorsal view of entirely ES-derived ΔTβRII chimaera (E9.5) showing undulating neural tube (nt) and rounded somites (arrow). (D) "Balanced" chimaera rescued by host tissue (blue) showing normal neural tube and somite development (arrow). Note pericardial effusion (arrowhead). Insert: enlargement of the yolk sac after staining for β-gal. al, allantois; e, embryo; h, heart; lb, limb bud; nf, neural fold.

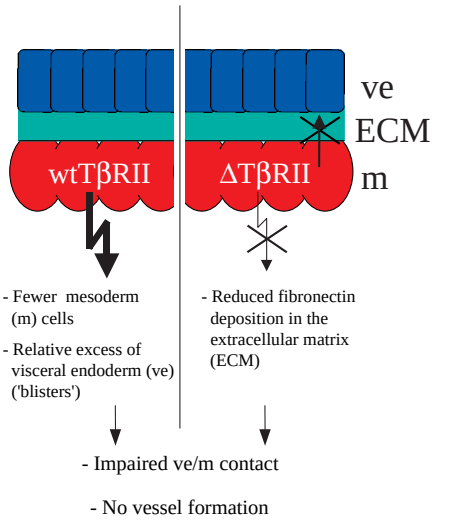

Fig. 11. Schematic representation of the defects observed in chimaeric embryos with aberrant TGF-β signalling. Expression of wtTβRII (left) or ΔTβRII (right) in the mesodermal layer of the yolk sac either increased of inhibited TGF-β signalling respectively and resulted in both cases in a defect in vasculogenesis. M, mesoderm; ve, visceral endoderm; ECM, extracellular matrix.

deposition of fibronectin in the matrix under the visceral endoderm of the yolk sac (Fig. 11), which explains the similarity in phenotype of the ΔTβRII chimaeras and the fibronectin- and α5-integrin-deficient embryos (George et al., 1993; Yang et al., 1993). We also conclude that an intact basement membrane is essential for the integrity and survival of the visceral endoderm layer, since in the ΔTβRII chimaeras β-gal staining of (host) visceral endoderm decreased during the period that fibronectin deposition should have increased, and the morphology of the cells became similar to that illustrated in the yolk sacs of fibronectin-deficient mice (George et al., 1993). We propose that the normal function of TGF-β in yolk sac development is to regulate synthesis and deposition of fibronectin by the extraembryonic mesoderm.

TGF-β signalling in primitive haematopoietic cells must also be tightly regulated since TGF-β has the potential to inhibit their differentiation. TGF-β has been reported to be an inhibitor of haematopoiesis in adult and foetal haematopoietic cells in vitro (Ohta et al., 1987; Ottman and Pelus, 1988), but to promote haematopoiesis in the visceral yolk sac (Dickson et al., 1995). We observe that ES cells expressing wtTβRII were defective in the generation of RBCs in vitro even when endothelial vessels were formed demonstrating that these processes are independent.

Together the results suggest that perturbation of TGF-β signalling results in failure of differentiating endothelial cells to assemble into robust vessels, suggesting that thresholds of TGF-β activity in the extraembryonic mesoderm are essential for precise regulation of yolk sac haematopoiesis and vasculogenesis at this time of development. It is of particular interest that the defects in yolk sac development described for the *TGF-β1*$^{-/-}$ embryos fall into the two separate categories we have also distinguished using the wtTβRII and ΔTβRII ES cells in chimaeric mice. We conclude that TGF-β signalling must be at an optimum to support normal yolk sac development. At what level this takes place, for example through the local availability of active ligand, through different candidate type I receptors, such as ALK-1 (Roelen et al., 1997) or ALK-5 (Roelen et al., 1998), or distinct Smad pathways in endothelial cells and their precursors, remains to be established.

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