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Developmentally regulated gene expression of thrombomodulin in postimplantation mouse embryos

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SUMMARY

Embryonic lethality of thrombomodulin-deficient mice has indicated an essential role for this regulator of blood coagulation in murine development. Here, the embryonic expression pattern of thrombomodulin was defined by surveying β-galactosidase activity in a mouse strain in which the reporter gene was placed under the regulatory control of the endogenous thrombomodulin promoter via homologous recombination in embryonic stem cells. The murine trophoblast was identified as a previously unrecognized anatomical site where TM expression is conserved between humans and mice and may exert a critical function during postimplantation development. Targeted reporter gene expression in mesodermal precursors of the endothelial cell lineage defined thrombomodulin as an early marker of vascular differentiation. Analysis of the thrombomodulin promoter in differentiating ES cells and in transgenic mice provided evidence for a disparate and cell type-specific gene regulatory control mechanism in the parietal yolk sac. The thrombomodulin promoter as defined in this study will allow the targeting of gene expression to the parietal yolk sac of transgenic mice and the initiation of investigations into the role of parietal endoderm in placental function.

Key words: endothelial cell, trophoblast, parietal endoderm, knock-out mice, embryonic lethality, *lacZ* gene targeting, gene regulation, transgenic mice

INTRODUCTION

Thrombomodulin (TM) is a thrombin receptor on the surface of endothelial cells that was characterized initially by virtue of its cofactor-activity in the thrombin-dependent generation of activated protein C (APC). TM serves to limit excessive thrombin generation through the proteolytic degradation of the activated coagulation factors V and VIII by APC, and in addition directly inhibits thrombin's procoagulant activities (Esmon and Owen, 1981; Esmon et al., 1982, 1983; Polgar et al., 1986; Preissner et al., 1987; Esmon, 1987, 1989). The receptor is localized on the luminal surface of all vascular endothelial cells except those lining hepatic sinusoids (Maruyama et al., 1985; DeBault et al., 1986; Boffa et al., 1987). It is also expressed in a variety of other cell types, including brain meninges, mesothelial surfaces and keratinocytes. In previous immunohistochemical analyses of mouse embryos, TM was found in the developing lung and cartilage, the midline of the embryonic mesencephalic flexure neuroepithelium and the parietal endoderm of the yolk sac (Imada et al., 1987, 1990; Ford et al., 1993). While the presence of TM in vascular endothelium is consistent with its role as a natural anticoagulant, its expression in non-vascular cells during murine development may indicate that TM exerts other or related functions in these locations. This hypothesis was substantiated by our recent observation that homozygous TM deficient ($TM^{-/-}$) embryos die in utero before the development of a functional cardiovascular system (Healy et al., 1995). $TM^{-/-}$ embryos failed to survive beyond embryonic day 9.5 (E9.5) and could only be recovered in a state of progressed resorption at that time. By day 8.5, mutant embryos were slightly smaller than their normal or heterozygous littermates and were found in a developmental stage characteristic for slightly younger embryos (approx. E8.0). The development of the cardiovascular system in homozygous TM-null mice, as analyzed until E8.5, was indistinguishable from heterozygous and wild-type littermates and proceeded further in embryos that had been removed from the uterus and cultured in vitro. This observation led us to conclude that the proximal cause for lethality of mice with an ablated TM gene is not related to a defect in the morphogenetic differentiation of the vascular endothelium.

The only other embryonic tissue previously reported to express TM between E8 and 9 was the endoderm layer of the parietal yolk sac (Imada et al., 1987; Ford et al., 1993), which secretes a thick layer of extracellular matrix surrounding the embryo, called Reichert's membrane. The expression of TM at this interface between fetal and maternal tissue may indicate the need to counter the activation of maternally derived coagulation proteases in proximity to the embryo. The parietal endoderm layer and Reichert's membrane are specialized placental structures found only in rodents and bats. Human embryos have a different placentation than rodents and do not

elaborate a functional extraembryonic membrane comparable to parietal endoderm. Fetal and maternal tissues in humans are separated by the syncytiotrophoblast layer (Steven, 1975; Hamilton and Hamilton, 1977). Indeed, TM is produced by these syncytial trophoblast cells (Salem et al., 1984; Maruyama et al., 1985), and there it could exert a function analogous to that in the parietal yolk sac of mice. However, parietal endoderm and trophoblast are not evolutionary homologous structures, but are derived from different embryonic cell lineages, the trophectoderm and primitive endoderm, respectively (Hogan et al., 1986).

The significance and underlying mechanism of the expression patterns of TM in the human and rodent placenta are not clear. Recent insights into the structure of the murine TM locus have raised the possibility that the species-specific expression of TM in the early murine placenta could be controlled by a repetitive intracisternal A particle (IAP)-like element, which was found integrated in the TM promoter of some mouse strains, but not others (Ford and Kennel, 1993). *Cis*-acting regulatory DNA regions in the long terminal repeat of similar IAP-like elements have been reported to promote the placental-specific expression of nearby cellular genes (Chang-Yeh et al., 1991, 1993). TM expression in the parietal yolk sac could therefore be confined to certain mouse strains harboring this mutation. Such a mechanism would imply that the absence of the receptor from this placental tissue has no physiological consequences.

Here, we examined the expression pattern of TM during a critical time window between day 7 and 9.5 of development. We have recently described the generation of a mouse strain in which the bacterial β-galactosidase gene (*lacZ*) was placed under the regulatory control of the endogenous TM promoter by homologous recombination in embryonic stem cells (Weiler-Guettler et al., 1996). This approach allowed us to conduct a rapid and sensitive survey of *lacZ* expression to delineate the embryonic tissues where TM exerts its developmental function. We have identified the murine trophoblast as a previously unrecognized source of TM. The conserved expression of the receptor in the trophoblast of humans and mice further suggests a critical involvment in early maternal-fetal interactions. TM expression in both parietal endoderm and trophoblast was independent of IAP-like elements in the promoter and is shown to be controlled by authentic TM gene sequences. Employing both an in vitro embryonic stem (ES) cell differentiation model and in vivo transgene analysis, we have delineated specific promoter regions which regulate the expression of the TM gene in the parietal yolk sac endoderm of the early murine placenta.

MATERIALS AND METHODS

TM*lacZ*-targeted mice

The generation of mice carrying a mutated TM allele in which the bacterial β-galactosidase gene is placed under the regulatory control of the endogenous TM promoter has been described earlier (Weiler-Guettler et al., 1996). Briefly, the β-galactosidase gene was inserted into the murine TM locus via homologous recombination in embryonic stem cells. The mutation results in the transcription of a hybrid mRNA, consisting of the entire TM 5′ untranslated mRNA region fused to the bacterial reporter gene; and is terminated by a SV40-derived polyadenylation signal. The insertion functionally disrupts the TM gene by separating the TM protein-encoding region from its promoter (see Fig. 5). Heterozygous and homozygous carriers of the mutation phenotypically resemble TM-deficient mice described earlier (Healy et al., 1995). The expression of the β-galactosidase gene in adult transgenic mice recapitulated the widespread expression of the thrombomodulin gene in vascular endothelium and was identical to the distribution of the endogenous gene product (Weiler-Guettler et al., 1996).

DNA constructions and generation of transgenic mice

The isolation of a genomic thrombomodulin clone from 129/Sv mice has been described previously (Healy et al., 1995). TM promoter fragments of varying length were fused to a bacterial β-galactosidase gene to conserve the translational and transcriptional start signals of the TM gene. To this end, an *Sph*I site was introduced in the sequence spanning the translation initiation codon of the β-galactosidase gene of the plasmid pSDKlacZpA (gift from Dr J. Rossant, Mount Sinai Hospital Research Institute, Toronto). TM promoter/reporter gene constructs A, B and C (see Fig. 5) were derived by inserting TM promoter fragments extending from the *Sph*I site spanning the translation start of TM to variable restriction sites in the 5′ promoter region. Construct A, containing 2.4 kb 5′ region, was defined at the 5′ end by an *Apa*I site; construct B (1.1 kb) by a 5′ *Hin*dIII site; construct C (0.85 kb) was defined by a 5′ *Sma*I site. These fragments were inserted into the *Sph*I site of pSDKlacZ. A selectable neomycin-resistance gene expression cassette was ligated into unique restriction sites located downstream of the *lacZ* gene. All plasmids were constructed to have unique restriction sites flanking the TM promoter/*neo* constructs to allow removal of vector sequences prior to transfection experiments. The configuration of the TM promoter/*lacZ* fusion and the neomycin resistance gene was essentially identical to the structure of the *lacZ* targeted TM allele.

For the generation of transgenic mice, constructs A, B, and C were cut with appropriate restriction enzymes to remove vector sequences and the neomycin resistance gene prior to injection. The TM promoter/*lacZ* fragments were recovered from preparative agarose gels and then injected at a concentration of approximately 3 μg/ml into the pronucleus of fertilized eggs from superovulated female mice (FVB/NTacfBr; Taconic Farms). Injected embryos were transferred into pseudopregnant females (CD-1; Charles River Breeding laboratories). Progeny were screened for the presence of the transgene by Southern blot analysis of tail DNA samples with a probe directed against the β-galactosidase gene. Male F_1 transgenic offspring were mated to wild-type FVB/N females to derive F_2 embryos for analysis of transgene expression. The morning of the day when a vaginal plug was found was defined as embryonic day 0.5 post coitum.

Analysis of β-galactosidase gene expression and immunohistochemistry

For detection of reporter gene expression in whole-mount preparations, embryos were removed from the uteri of pregnant females, dissected free of maternal decidual tissue, rinsed twice with phosphate-buffered saline (PBS) and fixed for 20 minutes at 4°C in PBS containing 1% formaldehyde/0.2% glutaraldehyde. After several washes in PBS, the embryos were incubated in PBS containing 5 mM $K_4Fe(CN)_6.3H_2O$, 5 mM $K_3Fe(CN)_6$, 2 mM $MgCl_2$, 0.02% Nonidet-P40, 0.01% SDS and 1 mg/ml 4-chloro-5-bromo-3-indolyl-β-galactoside at 30°C until color developed. Subsequently, they were washed in PBS and cleared for whole-mount photography in 30% glycerol. To prepare paraffin-embedded histological sections, stained embryos were postfixed in formalin, dehydrated through graded alcohol and embedded in paraffin. To prepare cryosections, the entire conceptus was fixed in Pipes-buffered paraformaldehyde (0.1 M Pipes, pH 6.9; 2 mM $MgCl_2$; 2% paraformaldehyde; 1.25 mM EGTA) for 30 minutes at 4°C, equilibrated in 30% sucrose, embedded in OCT compound and then frozen on dry ice. Cryosections of 8-10 μm thickness were mounted on surface-treated glass slides, fixed briefly with 1%

formaldehyde in PBS, incubated with the chromogenic β-galactosidase substrate as described above and counterstained with eosin. Immunohistochemical detection of TM in acetone-fixed (1 minute at 4°C) cryosections was performed with the TM-specific monoclonal antibodies 34A and 201B (Kennel et al., 1988; antibodies were kindly provided by S. Kennel, Oak Ridge National Laboratories, TE) and secondary antibody/horseradish peroxidase conjugates according to the manufacturer's instructions (Vectastain ABC, Vector Labs).

RNA and DNA analysis

RNA from cultured ES cells and from mouse tissues was prepared by extraction with acidic phenol/chloroform/guanidine isothiocyanate. mRNA levels were quantitated by RNase protection assays with a commercially available kit (Ambion; Austin, TX). cRNA probes for TM, the neomycin resistance gene, and β-galactosidase were obtained by subcloning appropriate restriction fragments of the corresponding genes into the pBluescriptII vector (Stratagene). ES cell RNA (30-50 μg) was hybridized simultaneously with β-actin and one of the TM-, β-galactosidase-, or neo-cRNA probes labeled with [α-^{32}P]rUTP. Samples were digested with RNaseA/T1 and protected fragments were resolved on a 5% urea/polyacrylamide gel. Radioactivity associated with specific fragments was quantitated with a blot analyzer. Gel electrophoresis, capillary blotting and Southern blot hybridization analysis of genomic DNA has been described earlier (Healy et al., 1995).

Tissue culture and transfection of embryonic stem cells

The murine embryonic stem cell line ESD3 (Doetschman et al., 1985) was obtained from Dr R. O. Hynes (MIT, Cambridge, MA). The ESD3 cells were routinely propagated as undifferentiated stem cells on a feeder layer of mitotically inactivated, primary mouse embryonic fibroblasts in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/l glucose, 10 mM Hepes, 0.1 mM β-mercaptoethanol, 2 mM glutamine, 0.1 mM MEM-non-essential amino acids, 15% heat-inactivated fetal calf serum (Intergen), and 10^3 U/ml recombinant murine leukemia inhibitory factor (LIF; ESGRO, Gibco BRL). For differentiation experiments, ES cells were removed from feeder cell layers and maintained for at least two passages on plastic culture dishes coated with 0.3% gelatin (Sigma) in the same culture medium. To induce differentiation, cells were cultured in medium without LIF, containing 0.5 mM dibutyryl-cyclic AMP and 0.5 mM theophylline (referred to as CT), or 1 μM all-*trans* retinoic acid (Sigma) in com-

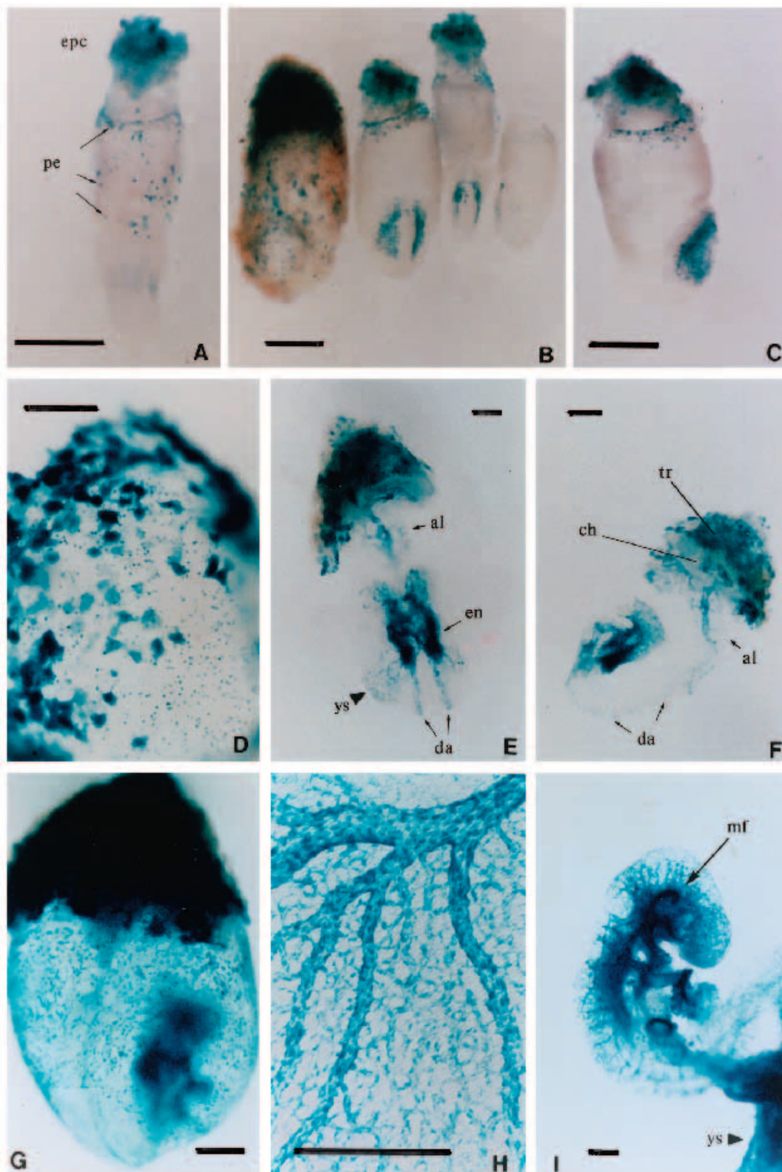

Fig. 1. Whole-mount β-gal staining of E7-E9.5 TM*lacZ*-targeted embryos. (A) Approx. E7 embryos; Reichert's membrane with attached parietal endoderm (pe) was partially removed from the lower half of the embryo to expose the embryo proper. Frontal view towards the prospective head region of the embryo. β-gal staining is seen in the ectoplacental cone (epc), parietal endoderm, mesoderm underlying the neural folds in the anterior, median part of the embryo and in posterior mesoderm lateral to the caudal primitive streak region. (B) approx. E7.5 embryos of one litter, slightly varying in their developmental stage. The intact embryo on the left shows β-gal staining in the trophoblast and smaller PE cells in the underlying parietal yolk sac. The trophoblast and parietal endoderm were removed from the remaining embryos. *lacZ* expression is seen in the laterally expanding mesoderm of the head region. (C) Lateral aspect of the embryo second from left in B. (D) Parietal yolk sac and trophoblast, removed from the embryo shown in E-G, viewed into the interior, embryonic aspect of parietal endoderm. Punctuate blue staining, represents *lacZ* expression by small, round parietal endoderm cells, on the inner surface of Reichert's membrane. Intense staining of giant trophoblast cells attached to the exterior maternal aspect of Reichert's membrane is evident. Frontal (E) and lateral (F) view of the embryo shown in G, with yolk sac removed. Blue staining is associated with angiogenic head mesenchyme, endocardial tubes (en) and endothelial cells in the allantois (al) which has fused with the chorionic plate (ch). The paired dorsal aorta (da) is labeled over its entire length. In the foregut region, a small patch of yolk sac (ys) was left attached to the embryo to show the connection between extraembryonic yolk sac vasculature and intraembryonic *lacZ*-positive cells. tr, trophectoderm. (G) Yolk sac vasculature at E8.5. *lacZ*-positive cells lining the wall of the forming capillary plexus. Trophectoderm and Reichert's membrane with parietal endoderm, removed from the lower part of the embryo, are shown in D. (H) *lacZ* expression in the endothelium of yolk sac blood vessels at E9.5. (I) Approx. E9.5, whole embryo, connected to the yolk sac (ys) through umbilical vessels. Expression of TM*lacZ* in the mesencephalic flexure (mf) is visible as a hook-shaped structure (arrow). Scale bars represent 0.2 mm.

bination with 0.5 mM dibutyryl cyclic AMP and 0.5 mM theophylline (referred to as RACT) for 5 days. Embryoid body formation in stationary suspension culture was initiated by seeding a single cell suspension of feeder-free ES stem cells onto bacterial grade plastic culture dishes at a density of 10^5 cells/ml. The culture medium (DMEM containing 4.5 g/l glucose, 10 mM Hepes, 0.1 mM β-mercaptoethanol, 2 mM glutamine, 0.1 mM MEM-non-essential amino acids, 20% heat-inactivated FBS) was changed every other day by aspiration. For transfection with DNA expression constructs, a single cell suspension of ES cells was prepared by trypsinization, the cells were washed twice in Hepes-buffered saline (25 mM Hepes, 134 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, pH 7.1), suspended at 2×10^7 cells/ml in the same buffer containing 20 μg/ml of linearized DNA and were electroporated at 600 V/cm and 500 μF (gene-pulser, Bio-Rad). The cells were replated on neomycin-resistant feeder-layers and stable transfected cells were selected in medium containing 200 μg/ml of (active) G418 (GibcoBRL). Drug-resistant colonies were isolated after 7-9 days and individually expanded.

RESULTS

We have recently described the generation of mice, in which the bacterial β-galactosidase gene has been inserted into the TM locus via homologous recombination in D3 embryonic stem cells (Weiler-Guettler et al., 1996). This 'knock-in' approach created a loss of TM function and placed the *lacZ* gene under the regulatory control of the endogenous thrombomodulin promoter. The expression of the transgene in adult transgenic mice was identical to the distribution of the endogenous gene product and recapitulated the widespread expression of the thrombomodulin gene in vascular endothelium. In the current report, *lacZ* expression was surveyed in embryos carrying the *lacZ*-targeted allele to characterize the TM expression pattern during the critical time window between day 7 and 9.5 p.c. Wild-type C57Bl/6 females were mated to heterozygous TM-*lacZ* males to collect embryos for the analysis of reporter gene expression. Therefore, the maternal tissue surrounding the embryo had a wild-type genome and did not express β-galactosidase. The presence of both the targeted and the wild-type allele in heterozygous embryos allowed the simultaneous detection of TM antigen and *lacZ* activity.

lacZ expression in postimplantation embryonic placental tissue and the developing cardiovascular system

The histochemical detection of β-galactosidase activity revealed expression of the targeted reporter gene in two cell lineages forming the early placenta: the parietal endoderm and the trophoblast. β-gal-positive staining was present in a subpopulation of parietal endoderm cells of late streak-stage embryos isolated at day 7 p.c. (Fig. 1A). By day 8.5 and 9.5, *lacZ* expression was detected in the majority of parietal endoderm cells covering the embryonic aspect of Reichert's membrane (Fig. 1D).

Between day 7 and 7.5, strong staining was also observed in the trophoblast cells covering the surface of the ectoplacental cone (Fig. 1A-C). Around day 8.5, β-galactosidase expression was associated with trophoblast cells overlaying the mesometrial pole of the embryo, and with individual secondary giant trophoblast cells attached to the maternal surface of Reichert's membrane (Fig. 1D,G). At 9.5 days, the trophoblast formed a coherent layer of TM*lacZ*-positive cells surrounding the entire embryo and has begun to establish the spongiotrophoblastic and labyrinthine layers at the mesometrial pole of the embryo. The expression of reporter gene activity in trophoblast correlated with the expression of the endogenous gene product as detected by immunohistochemical staining with thrombomodulin-specific antibodies (Fig. 2A-C).

In the embryo proper, the first β-gal-positive staining was seen by day 7 in two small streaks in the anterior head-region mesoderm, and in mesoderm lateral to the primitive streak in the posterior part of the embryo (Fig. 1A). Staining of the anterior mesoderm apposed to the prospective neural fold ectoderm intensified thereafter and became more widespread as this cell population expanded laterally (Fig. 1B,C). Weak, speckled staining was also seen in mesoderm of the visceral yolk sac. In 8-8.5 day embryos, the reporter gene was expressed in the cardiogenic mesoderm and throughout the angioblastic head mesenchyme, including sprouting blood vessels establishing the perineural capillary plexus (Fig. 1E,F). The paired dorsal aorta was *lacZ* positive throughout its entire length. The allantoic stalk contained a cluster of β-gal-positive cells that were connected with intraembryonic blood vessels in slightly older embryos. β-gal staining in the vascular endothelium of the yolk sac became markedly stronger as individual blood islands merged to form a capillary plexus. (Fig. 1G). During the subsequent developmental stages, characterized by a significant expansion of the vascular system, the endothelium of all intraembryonic vessels and the yolk sac vasculature stained positive homogeneously (Fig. 1H,I). Within the cardiovascular tree of E9-9.5 embryos, the most rapidly detectable staining was associated with the developing atrioventricular valve tissue of the embryonic heart (Fig. 3A). The reporter gene was expressed in the endocardium, but was absent from the myocardium. The pattern of X-Gal staining in the yolk sac and embryo proper of homozygous *lacZ*-targeted embryos at E8.5 was identical to that observed in wild-type or heterozygous littermates. For the latter analysis, the genotype of individual embryos was ascertained by Southern blot hybridization analysis of DNA prepared from the yolk sac or the embryo proper, while the remaining tissue was analyzed for *lacZ* expression by histochemical staining.

β-gal-staining was also prominent in intraembryonic, non-vascular structures of E9-10 embryos, such as the leptomeninges, the ventral midline of the mesencephalic flexure neuroepithelium (Fig. 3B), and the developing lung. In the latter, β-gal-staining was seen in submucosal tissue surrounding the already differentiated trachea and bronchi (Fig. 3A,C);. In more caudal and less mature regions of the lung, the airway epithelium itself was strongly β-gal positive (Fig. 3D).

Expression in the fetal placenta is independent of a IAP-like element in the TM promoter

Previous investigations into the structure of the TM gene have revealed a polymorphism within the receptor locus in different strains of laboratory mice that resulted from the insertion of an intracisternal A-particle (IAP)-like element into the TM promoter region (Ford and Kennel, 1993). IAP-associated mRNA transcripts have been detected specifically in rodent placenta and have been shown to originate from promoter sites within the long terminal repeat of IAP-like elements (Chang-Yeh, 1991). We therefore investigated whether TM expression

in the fetal placenta correlated with the presence of a IAP-element in the TM promoter.

The insertion of the defective retrovirus into the TM promoter of TM*lacZ* mice occurred 2462 bp upstream of the transcriptional start site and generated a direct repeat of 6 bp of TM sequence at the insertional breakpoints (Fig. 5). The presence or absence of the mutation in different mouse strains and cell lines was then ascertained by Southern blot hybridization analysis of genomic DNA to detect a discriminating restriction fragment length polymorphism (data not shown). The IAP-element was detected in both TM alleles of the ESD3 cell line used to generate *lacZ*-targeted mice, in the mouse strain 129, and in the F9 embryo carcinoma cell line, which has been employed in previous analyses of TM gene expression (Weiler-Guettler et al., 1992; Niforas et al., 1993). The hybridization analysis of genomic DNA from FVB/N-mice was consistent with the absence of the insertion (data not shown). Northern blot analysis of RNA isolated from the trophoblast and parietal yolk sac of day 9.5 TM*lacZ* embryos and from FVB/N mice revealed the presence of TM mRNA transcripts of identical size (approximately 3600 nucleotides) in both mouse lines (data not shown). The synthesis of TM in the trophoblast of both TM*lacZ* and FVB/N mice was further confirmed by immunohistochemical staining with TM-specific antibodies (Fig. 2). The lack of correlation between the presence of an IAP-like element in the TM promoter and receptor expression in the trophoblast and parietal endoderm showed that TM gene activity in the early placenta is controlled by authentic TM promoter sequences. This finding prompted us to search the 5′ TM promoter region between the transcriptional start site and the IAP insertion point for the presence of regulatory domains governing placental TM expression.

Functional characterization of the TM promoter in differentiating ES cells

TM promoter fragments of varying length were fused to the reporter gene bacterial β-galactosidase and a selectable neomycin expression cassette (see Fig. 5). The DNA constructs were electroporated into ES cells and at least four independently derived, stable transfected cell clones were established for each construct. Reporter gene expression in transfected cell lines was then compared to the distribution of β-galactosidase activity in TM*lacZ*-targeted ES cells in which reporter gene expression is controlled by the TM promoter in its intact chromosomal context.

In undifferentiated TM*lacZ* targeted stem cells, no TM or β-galactosidase transcripts were found and *lacZ* staining was spurious. Addition of cAMP/theophylline (CT) or retinoic acid in combination with CT (RACT) induced both TM and β-galactosidase mRNA. Exposure to CT alone activated *lacZ* gene expression in the entire cell population without inducing any obvious morphological changes. Reporter gene expression in TM*lacZ*-targeted cells exposed to RACT was confined to a characteristic subpopulation of cells (Fig. 4A) previously described as resembling parietal endoderm (van de Stolpe et al., 1993). The distribution of *lacZ*-positive cells in embryoid bodies (Fig. 4B) was similar to the morphology of presumptive endothelial cells organized into vascular chord-like structures, that have been characterized earlier (Doetschman et al., 1985, 1993; Risau et al., 1988; Wang et al., 1992).

Constructs A and B, including 2.4 and 1.1 kb of 5′ TM promoter, respectively, displayed an identical pattern of expression during the in vitro differentiation of ES cells. In contrast to the targeted β-galactosidase gene, constructs A and B directed a low level (relative to CT or RACT treated cells) of reporter gene expression in stem cells indicating a derepression of the reporter gene constructs as compared to the endogenous TM promoter. Intense β-gal staining occurred in RACT-treated cells and was confined to the same subpopulation of cells in which the targeted *lacZ* gene was active. (compare Fig. 4A and C). Both promoter constructs were also responsive to transcriptional activation by CT. Further truncation of the promoter to 0.85 kb upstream of the transcriptional start site (construct C) resulted in the loss of expression in response to CT or RACT, and in undifferentiated stem cells. ES cell lines containing genomically integrated copies of the constructs A, B and C were then cultured as suspension aggregates to induce differentiation into embryoid bodies in the absence of exogenously added hormones. Expression driven by a 2.4 kb or 1.1 kb TM promoter fragment (construct A and B) was seen in the cell mass at the apical pole of EBs (Fig. 4D), thus partially matching the distribution of the targeted *lacZ* gene product, but was absent from vascular-like chords in the large, cystic portion of the embryoid bodies. Construct C was again silent throughout embryoid body development over a time span of 14 days.

These data indicated, (1) that a 1.1 kb TM promoter fragment was sufficient to confer RACT-mediated upregulation of a reporter gene in a specific subpopulation of differentiated ES cells most likely resembling parietal endoderm; (2) that DNA regions necessary for gene activation by cAMP and expression in parietal endoderm-like cells mapped to a 250 bp interval located between position −1100 and −850 in the TM promoter; (3) that the 2.4 kb TM promoter fragment did not contain the necessary information to reproduce the reporter gene expression of the targeted *lacZ* gene in a cell population reminiscent of endothelial-like cells.

2.4 kb of the TM promoter directs reporter gene expression selectively to the parietal endoderm of transgenic animals

Transgenic animals were generated by pronuclear injection of reporter constructs into oocytes in order to identify unambiguously the embryonic structures, in which the TM promoter constructs were active. This route to generate transgenic animals – as opposed to the generation of ES-cell chimeras – was chosen because the limited contribution of ES cells to extraembryonic membranes of chimeric embryos could complicate the analysis of reporter gene activity in parietal endoderm and trophoblast (Beddington and Robertson, 1989).

Six independent transgenic founder animals were generated carrying 2 to 6 copies per haploid genome of a reporter construct containing 2.4 kb of TM promoter fused to the bacterial β-galactosidase gene (see Fig. 5). Four of the founders transmitted the transgene through their germline to produce F_2 transgenic progeny for analysis. Two transgenic lines did not express the reporter gene at any stage during embryonic development or in adult animals. In the remaining two lines, A36 and A27, the transgene was selectively active in the parietal endoderm layer of the yolk sac of E9.5 embryos (Fig. 6B). Expression in parietal endoderm cells occurred in all parts of the parietal yolk sac resembling the expression pattern

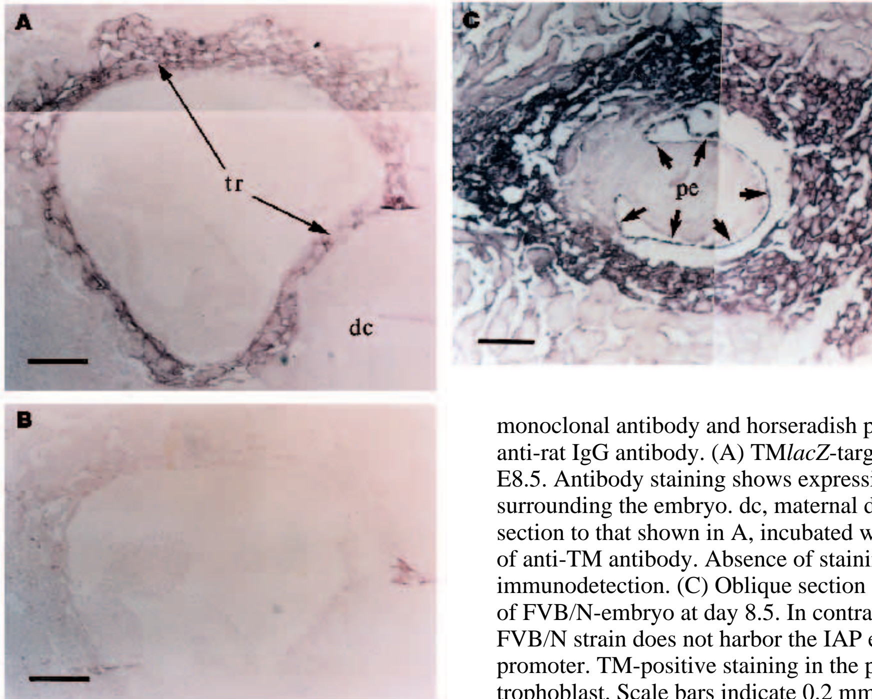

Fig. 2. Immunohistological detection of TM in fetal extraembryonic tissues. Deciduas were dissected from pregnant mice, fixed and embedded in paraffin. Histological sections were incubated with an anti-murine TM rat monoclonal antibody and horseradish peroxidase-conjugated secondary anti-rat IgG antibody. (A) TM*lacZ*-targeted embryo, sagittal section, E8.5. Antibody staining shows expression of TM in the trophoblast (tr) surrounding the embryo. dc, maternal decidual cells. (B) Adjacent section to that shown in A, incubated with non-immune serum instead of anti-TM antibody. Absence of staining shows specificity of TM immunodetection. (C) Oblique section through ectoplacental cone area of FVB/N-embryo at day 8.5. In contrast to TM*lacZ*-targeted mice, the FVB/N strain does not harbor the IAP element insertion in the TM promoter. TM-positive staining in the parietal endoderm (pe) and trophoblast. Scale bars indicate 0.2 mm.

observed in TM*lacZ* targeted embryos. *lacZ* activity was absent from all other cell types making up the extraembryonic membranes, such as trophoblast cells, visceral endoderm, or the yolk sac vasculature, and from intraembryonic vascular endothelium. The only structure within the embryo proper to exhibit detectable reporter gene expression in both transgenic lines at this stage was the developing heart (Fig. 6A). In line A27, *lacZ* reaction product appeared within the prospective ventricular chamber. In line A36, staining was localized in the outflow tract. β-galactosidase expression was also detected in the ventral midline of the developing midbrain in line A27, but not in adjacent head mesenchyme; thus partially matching the expression pattern of the endogenous TM gene in the neuronal epithelium. Spurious ectopic expression was associated with small clusters of cells at the base of the mandibular arch and in the otic vesicle in the mouse line A27. Reporter gene activity was absent from the corresponding structures in line A36. In adult animals of both lines, no detectable reporter expression was present in any tissue analyzed, including kidney, liver, spleen, lung, brain, skeletal muscle and heart.

Out of four transgenic founder animals carrying a transgene containing a 1.1 kb TM promoter fused to the *lacZ* gene, two

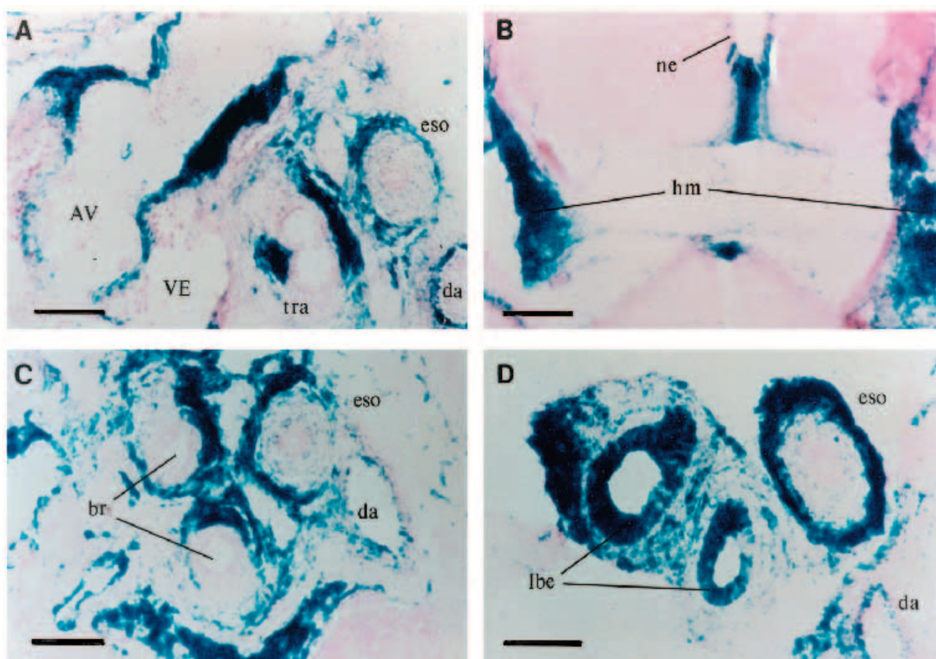

Fig. 3. Histological cross sections of paraffin-embedded TM*lacZ*-targeted embryos. (A) Cross section through the heart at the level of the atrioventricular valve and tracheal bifurcation, at E9.5. There is intense staining of the tissue forming the atrioventricular valve (AV) and in the mesenchyme surrounding esophagus (eso) and trachea (tra). Note staining of the endothelium of the dorsal aorta and absence of staining from airway epithelium of the tracheal bifurcation. VE, ventricle. (B) Brain, cross section through the mesencephalic flexure at E9.5. Rostral portion of mesencephalon on top. hm, head mesenchyme. ne, neuroepithelium. (C,D) Bronchi and lung buds at E10. Serial cross sections, spacing between section shown in A, C and D approx. 3.5 mm, from rostral (A) to caudal (D). The epithelium of the bronchi (br) is negative for β-gal staining in C, lung bud epithelium (lbe) is positive (D). Scale bars represent 0.2 mm.

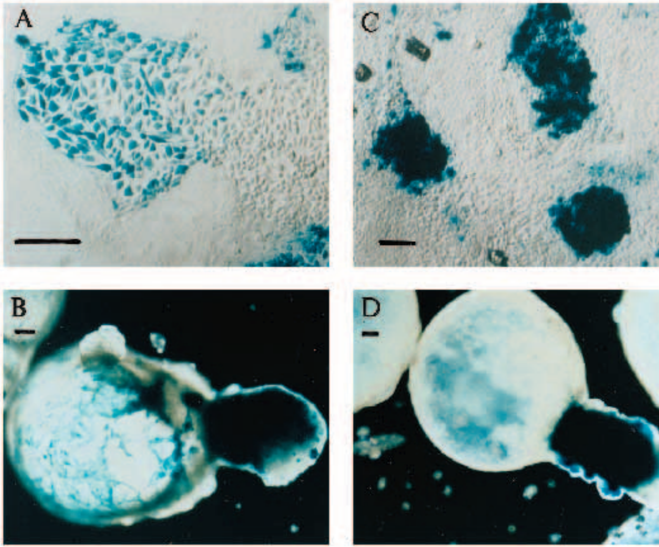

Fig. 4. *lacZ e*xpression during ES cell in vitro differentiation. ES cells carrying various *lacZ* reporter constructs were maintained in the absence of feeder cells as adherent cultures and induced to differentiate by addition of hormones to the culture medium, or cultured in suspension to allow the spontaneous formation of embryoid bodies. Reporter gene activity was detected by histochemical staining with a substrate for β-galactosidase. (A,B) *lacZ*-targeted ES D3 cells treated with retinoic acid and cAMP (A), or cultured as embryoid bodies (B). Note plexus of *lacZ*-positive cells in the cystic part of the embryoid body. (C,D) *lacZ* expression in ES D3 cells (C) and in embryoid bodies (D) stably transfected with a 2.4 kb TM promoter construct (construct A) and treated with both retinoic acid and cAMP (C). Scale bars indicate 0.2 mm.

gave rise to progeny that exhibited reporter gene expression in transgenic embryos. *lacZ* activity was detected in the parietal endoderm of both transgenic lines at E9.5, but was again absent from other cell types of the extraembryonic membranes. In both lines, the smaller promoter directed significant reporter expression to ectopic sites. Similar to the two lines carrying the 2.4 kb TM promoter construct, reporter expression controlled by the 1.1 kb promoter fragment in the embryo proper was transitory; no *lacZ*-positive tissue could be identified in adult animals.

The analysis of TM promoter fragments in transgenic animals thus confirmed and extended the results obtained from in vitro experiments in differentiating ES cells: 2.4 kb of 5′ promoter region fused to the *lacZ* gene were sufficient to reproduce the developmentally regulated expression of the endogenous TM gene selectively in the parietal endoderm. Notably, expression controlled by up to 2.4 kb of 5′ flanking DNA sequences was absent from the vascular endothelium, trophoblast and other cell types in which the endogenous TM gene is active.

DISCUSSION

The present study was undertaken to determine the anatomical sites where TM exerts it’s pivotal function allowing embryonic survival. We have used *lacZ* as a reporter gene under the regulatory control of the endogenous TM promoter to conduct a rapid and sensitive survey of the embryonic pattern of TM expression. This analysis has led to the previously unrecognized localization of TM in the embryonic trophoblast.

The discrepancy between the previously reported distribu-

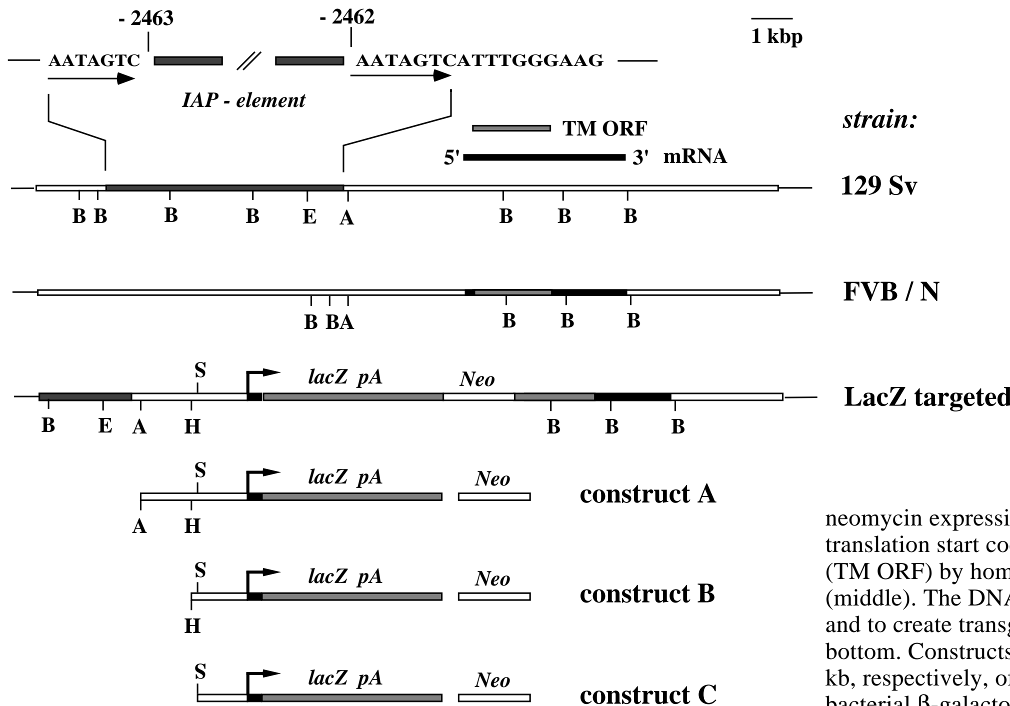

Fig. 5. TM*lacZ* expression constructs. The structure of the endogenous TM locus is shown at the top. In 129Sv mice, but not in FVB/N mice, a defective retroviral intracisternal-A-particle (IAP)-like element of approximately 5 kb is present as an insertion in the TM promoter. In *lacZ*-targeted mice, the β-galactosidase gene and a selectable neomycin expression cassette was inserted into the translation start codon of the TM open reading frame (TM ORF) by homologous recombination in ES cells (middle). The DNA constructs used to transfect ES cells and to create transgenic animals are shown at the bottom. Constructs A, B and C contain 2.4, 1.1 and 0.85 kb, respectively, of TM promoter region coupled to the bacterial β-galactosidase gene. The structure of the fusion between the promoter and reporter gene is identical to that of the targeted allele. Arrows indicate the transcriptional start site of the TM promoter. The neomycin resistance gene was not included in constructs used to create transgenic mice. A, *Apa*I; B, *Bam*HI; E, *Eco*RI; H, *Hind*III; S, *Sma*I; kb, kilobase pairs.

tion of receptor expression during murine development (Imada et al., 1990; Ford and Kennel, 1993) and our finding of TM expression by trophoblast cells prompted us initially to consider the possibility that TM expression in trophoblast occurs only in certain mouse strains, which are characterized by insertion of a repetitive IAP-like element in the TM promoter region. Employing RNA analysis and immunohistochemical detection methods we have shown that the distribution of TM in placental tissues is identical in mouse strains that carry this mutation or contain a normal TM allele. Our results thus showed that the placental expression of TM is not regulated by retroviral DNA elements, but is indeed controlled by authentic TM promoter sequences.

A defect in trophoblast function may be the underlying cause of lethality observed in TM-deficient embryos. Trophoblast giant cells attached to the maternal aspect of Reichert's membrane, the parietal endoderm and the underlying visceral endoderm constitute the earliest placental structure for the exchange of substances between the growing embryo and the maternal blood supply. The parietal and visceral yolk sac are then replaced as the principal route of nutrient exchange by the chorioallantoic placenta, where the fetal endothelium forms a loose network with maternal vascular cells in the labyrinthine layer of the mature placenta (reviewed by Cross et al., 1994). Assuming that the function of TM in development and in the vascular tree of adult mice are related, the receptor must interact with maternally derived thrombin. The hemochorial type of placentation in humans and rodents is indeed characterized by a breakdown of maternal endothelium and thus results in a direct exposure of embryonic tissue to maternal blood constituents (Kirby and Bradbury, 1965; Steven, 1975; Hamilton and Hamilton, 1977). In murine development, this contact occurs first between day 6.5 and 7.5, when blood begins to drain from maternal decidual blood vessels into the lumen surrounding the implantation site. The subsequent development of the chorioallantoic placenta involves expansion and reorganization of the trophoblast into the spongiotrophoblastic and labyrinthine layer and is accompanied by a significant increase in the vascularization of the decidual tissue at the mesometrial pole of the conceptus (Welsh and Enders, 1987, 1991, 1993). Breaching of these expanded maternal blood sinuses produces a surge of blood supply in direct contact with the trophoblastic surface. TM could conceivably be required to counter the thrombin-mediated activation of proteases in proximity to the embryo during these developmental stages. The unusually rapid and complete progression from the occurrence of the first signs of damage to the resorption of virtually all mutant embryos may indicate that between day 6.5 and 8.5 the absence of TM in parietal endoderm and/or the trophoblast causes initially only a comparably mild growth retardation. In contrast, the lack of receptor expression in the trophoblast of the later developing chorioallantoic placenta may then have catastrophic consequences and cause the rapid resorption of mutant embryos. Preliminary results from the analysis of TM null embryos in intermediate stages of resorption indicate indeed that the disorganization and destruction of embryonic structures originates at the mesometrial pole of the implantation site.

A critical developmental function of TM on the surface of the placental trophoblast rather than in the developing vascular system may also be deduced from the observation, that the cardiovascular system developed normally in homozygous TM null mice. This finding supported our earlier conclusion that ablation of the TM gene does not cause a defect in the morphogenetic differentiation of the vascular endothelium (Healy et al., 1995), despite the early onset of TM synthesis in mesodermal progenitors of the endothelial lineage around day 7. The consequences of TM deficiency also differ significantly from the outcome of mutations known to affect the connection of maternal and fetal blood vessels in the chorioallantoic placenta. Embryos with homozygous loss-of-function mutations of the *Mash-2,* α4-integrin, or VCAM-1 genes, which show a defect in placental function, die after day 9.5 and can be retrieved from the implantation site in various states of resorption over a time span of at least two days (Guillemot et al., 1994; Kwee et al., 1995; Yang et al., 1995; Gurtner et al., 1995). In contrast, TM null embryos exhibited a slight, but recognizable retardation in growth and development already at day 8.5, and even remnants of mutant fetuses could rarely be recovered 24 hours after the first phenotypic indications of lethality. The mechanism leading to the resorption of TM mutant mice is therefore likely to differ significantly from previously reported genetic defects interfering with the development of the embryonic vasculature or its fusion with the maternal circulation.

Together, the available information indicates a critical developmental function of TM on the surface of trophoblast cells. The evolutionary conserved expression of TM in the trophoblast of humans and mice suggests that the receptor might play a comparable role in mediating maternal-fetal interactions during placental development in both species. Similar to our observations in mutant mice, homozygous TM deficiency may cause early embryonic lethality in humans, while the heterozygous mutation may be largely asymptomatic. This hypothesis would also predict that heterozygous carriers of a mutated TM allele will be more likely identified in patients affected by recurrent, early failures of pregnancy than in a patient population presenting with thrombotic complications.

As a first step towards a tissue- and stage-restricted complementation of TM deficiency in knock-out embryos, we attempted to define the regulatory DNA domains directing TM gene expression to placental tissues. In vitro experiments in differentiating ES cells revealed that 1.1 kb of 5′ upstream TM promoter contained the genetic information required to direct reporter gene expression to parietal endoderm-like cells. Analysis of a further truncated promoter allowed us to delineate a 250 bp fragment located between 850 and 1100 bp upstream of the transcriptional start site, that was instrumental for parietal endoderm expression in vitro and also conferred responsiveness to cAMP. This region colocalized with a previously mapped TM promoter fragment shown to mediate the hormone-dependent augmentation of TM gene activity in the F9 embryo carcinoma cell line and other cell types (Shirayoshi et al., 1993). These in vitro results were confirmed and extended by the analysis of the same promoter constructs in transgenic animals. 2.4 kb of 5′ TM promoter sequences directed placental expression specifically to the parietal endoderm. The genetic information for expression in this cell type was contained within 1.1 kb of 5′ promoter sequences. In three transgenic mouse lines where the reporter gene is driven by a further truncated TM promoter devoid of the cAMP responsive region between −850 and −1100, we were unable to document any *lacZ* expression

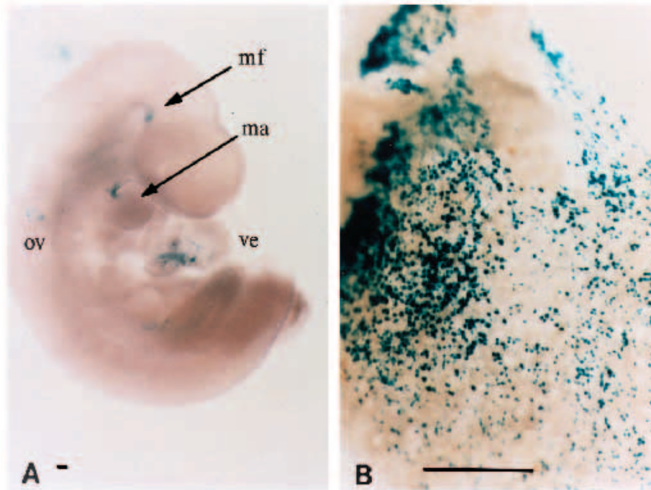

Fig. 6. *lacZ* expression in transgenic embryos. (A) Whole-mount β-gal staining of a transgenic E10.5 embryo, line A27; posterior part of the embryo and tail removed to expose heart region. ov, otic vesicle; ma, mandibular arch; mf, mesencephalic flexure; ve, ventricle. (B) Whole-mount stained embryo showing the trophoblast and parietal endoderm; E10. β-gal staining is restricted to small parietal endoderm cells attached to Reichert's membrane and absent from the underlying trophoblast. Scale bars indicate 0.2 mm.

in adult or embryonic mice (unpublished data). This suggests, that the latter 250 bp DNA fragment is indeed required for in vivo TM expression in parietal endoderm. In contrast to the reproducible pattern in the parietal yolk sac, the transgene activity within the embryo proper varied between individual mouse lines. Reporter gene expression in the mesencephalic flexure neuroepithelium and heart of the transgenic line A27 partially matched the distribution of the endogenous gene product, indicating that promoter elements contributing to a transient transcriptional activation in these sites might be present in the transgene, but are sensitive to integration site-specific effects. However, expression in other tissues than parietal endoderm was only transitory and was not detected in adult animals. Notably, in transgenic animals carrying either the 2.4 kb promoter or the 1.1 kb construct, *lacZ* activity was consistently absent from embryonic and adult vascular endothelium, as well as from trophoblast. The in vitro and in vivo analysis of TM promoter constructs thus provides experimental evidence for a disparate and cell-type specific mechanism controlling receptor production by parietal endoderm. Despite extended similarities between the human and murine gene sequences, the human TM promoter does not support expression in murine parietal endoderm-like cells (Weiler-Guettler et al., 1992). This observation provides further circumstantial evidence, that the regulatory pathway for TM gene transcription in murine parietal endoderm is functionally not related or interconnected with the mechanism underlying the conserved expression in trophoblast and endothelial cells. The cAMP responsive region described above could nevertheless be involved in counteracting receptor downregulation in endothelial cells in response to cytokines or hypoxia (Dufourcq et al., 1995; Koga et al., 1995). This possibility could be examined in future experiments by deleting this DNA fragment from the TM promoter by homologous recombination in ES cells. The functional significance of the cAMP-responsive region could then be evaluated in the otherwise intact genomic context of the chromosomal TM locus.

The simultaneous expression in trophoblast and the endothelial cell lineage is not unique to TM, but has also been reported for other early endothelial gene products. The TM expression pattern in postimplantation embryos overlaps remarkably with the distribution of endothelial cell precursors characterized by expression of the receptor tyrosine kinases, flk-1, flt-1, tek and tie-1, and the more recently described murine vascular endothelial (VE) cadherin gene (Dumont et al., 1992; Oelrichs et al., 1993; Millauer et al., 1993; Yamaguchi et al., 1993; Korhonen et al., 1994; Dumont et al., 1995; Breier et al., 1995, 1996). The onset of TM gene activity matches the early appearance of detectable *flk-1* expression in prevascular mesoderm of late primitive streak-stage embryos and precedes the appearance of the other receptors. In slightly older embryos, all of the above markers are, like TM, present in the angioblastic mesenchyme of the head, the vasculogenic yolk sac mesoderm, developing heart tissue, and the dorsal aorta. However, only flk-1, flt-1 and VE-cadherin are also produced by derivatives of the trophectoderm lineage (Dumont et al., 1995; Breier et al., 1995, 1996). This group of genes is therefore distinguished from other early endothelial differentiation markers not only by expression in both vascular endothelium and trophoblast, but also by their early induction in the endothelial lineage. The concerted activation of this gene battery in trophoblast and in developing blood vessels may indeed indicate a related transcriptional mechanism governing expression in these cell types.

At the present time, we can only speculate about the relative contributions of trophoblast- and parietal endoderm-derived TM or the detailed mechanism through which TM expression in these tissues ensures proper development. The early lethality of TM null embryos also precludes the analysis of the coagulation system in adult mice lacking TM and could in addition conceal secondary developmental abnormalities revealed only at later stages of organogenesis. Our analysis of the TM promoter defines a strategy to manipulate, in vivo, the function of the early murine placenta by targeting the expression of foreign gene products to parietal yolk sac endoderm of transgenic mice. TM promoter fragments as delineated in the present study will help to restore receptor expression of TM knockout mice in a tissue-restricted pattern and to examine the effect of a parietal endoderm-specific complementation on embryonic survival. The contribution of trophoblast-derived TM to the successful outcome of pregnancies could in turn be documented by directing receptor expression with trophoblast-specific promoters derived from the mouse placental lactogen II- or *4311*-gene (Shida et al., 1992; Calzonetti et al., 1995). The recently reported cell type restricted activity of the pre-proendothelin-1-, von Willebrand Factor-, *tie*-, or *tek*-promoter in embryonic endothelial cells could similarly be exploited to ablate the TM gene specifically in vascular endothelium (Harats et al., 1995; Aird et al., 1995; Korhonen et al., 1995;. Dumont et al., 1994, Schlaeger et al., 1995). Such a tissue-restricted complementation of TM deficiency, or ablation of the receptor gene should provide further insight into the role of TM in embryonic development and aid in elucidating the function of the TM-protein C anticoagulant pathway in the cardiovascular system of mice.

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