Essential Role for Thymosin β4 in Regulating Vascular Smooth Muscle Cell Development and Vessel Wall Stability

Alex Rossdeutsch,* Nicola Smart,* Karina N. Dubé, Martin Turner, Paul R. Riley

Rationale: Compromised development of blood vessel walls leads to vascular instability that may predispose to aneurysm with risk of rupture and lethal hemorrhage. There is currently a lack of insight into developmental insults that may define the molecular and cellular characteristics of initiating and perpetrating factors in adult aneurysmal disease.

Objective: To investigate a role for the actin-binding protein thymosin β4 (Tβ4), previously shown to be proangiogenic, in mural cell development and vascular wall stability.

Methods and Results: Phenotypic analyses of both global and endothelial-specific loss-of-function Tβ4 mouse models revealed a proportion of Tβ4-null embryos with vascular hemorrhage coincident with a reduction in smooth muscle cell coverage of their developing vessels. Mechanistic studies revealed that extracellular Tβ4 can stimulate differentiation of mesodermal progenitor cells to a mature mural cell phenotype through activation of the transforming growth factor-beta (TGFβ) pathway and that reduced TGFβ signaling correlates with the severity of hemorrhagic phenotype in Tβ4-null vasculature.

Conclusions: Tβ4 is a novel endothelial secreted trophic factor that functions synergistically with TGFβ to regulate mural cell development and vascular wall stability. These findings have important implications for understanding congenital anomalies that may be causative for adult-onset vascular instability. (Circ Res. 2012; 111:e89-e102.)

Key Words: thymosin ■ vasculature ■ mural cell ■ aorta ■ mouse ■ mouse mutants ■ smooth muscle differentiation ■ vascular biology ■ vascular smooth muscle

The development of a functional vasculature is an essential process during embryogenesis, perturbations in which result in fetal lethality or vascular disease after birth. The formation of systemic blood vessels occurs in a stereotypical fashion—endothelial tubes form through a number of mechanisms (angiogenesis, vasculogenesis, or intussusception). Endothelial cells then recruit mural cells comprising the subsets of vascular smooth muscle cells (VSMCs) and pericytes to the external wall of the vessel. These mural cells are required to provide structural support for the blood vessel and probably play a role in maintaining endothelial health and integrity. The establishment of a vessel wall is accomplished either through the differentiation of de novo mural cells from precursor populations or recruitment from a proliferating pool of mature cells. The former is thought to occur chiefly through the actions of endothelial secreted transforming growth factor-beta (TGFβ) and the latter through paracrine platelet-derived growth factor-B (PDGF-B). Typically, in the embryo, mural cells originate from the in situ differentiation of mesodermal tissues, which surround endothelial tubes. The exception to this is in the central nervous system, where blood vessels recruit to their outer layer via the migration of neurectodermal-derived mature mural cells, as typified by the development of the postnatal retinal vasculature. Consequences of failed mural cell recruitment range widely, depending on the degree of mural cell coverage. Midgestation lethality is seen in Alk5 knockout mice coinci-
dent with a failure to differentiate mural cells. However, mutants with a partial loss of mural cells can survive to later stages. The PDGF receptor-β–null mouse dies perinatally, probably due to hemorrhage and edema as a result of lack of structural support to blood vessels. In contrast, the endothelial-specific PDGF-BB knockout mouse survives into adulthood with reduced mural cell coverage but has deficiencies in renal and cardiac function. Nevertheless, in all of these described mutants, some degree of mural cell contribution to blood vessels is still observed, indicating that additional signals are required for mural cell differentiation from progenitor cells.

Thymosin β4 (Tβ4) is a 43–amino acid peptide encoded by the gene Tmsb4x on the X chromosome in mouse. It was initially identified as a G-actin–binding protein with the ability to regulate the cellular availability of actin monomers for the formation of polymeric F-actin. In recent times, however, novel functions have been ascribed to Tβ4, based on its ability to affect cell behavior when applied extracellularly or in a paracrine fashion. Notably, Tβ4 has been shown to improve cardiac function after ischemic injury. These cardioprotective effects may be due in part to the ability of Tβ4 to stimulate the differentiation of new coronary vascular cells, including coronary VSMCs, and consequently facilitate the process of neovascularization within the infarcted myocardium.

Although several groups have shown that exogenous Tβ4 can promote angiogenesis both in vitro and ex vivo, the role Tβ4 plays in the systemic vasculature in vivo is unknown. We describe a requirement for endothelial Tβ4 in the

Figure 1. Tβ4 is expressed in the endothelium of the developing aorta. Whole-mount in situ hybridization at E9.5 reveals Tβ4 staining in the dorsal aorta (A, white arrowheads). Sagittal (B) and coronal (C) sections at E9.5 confirm the aortic expression of Tβ4 (black arrowheads highlight wall of dorsal aorta) (B and C). From E10.5, Tβ4 begins to be expressed in the microvasculature (D), notably highlighted by expression in the dermal vascular plexus of the limb bud (E) and the intersomitic vessels (F, white arrowheads). Expression of Tβ4 in the developing aorta colocalizes with the endothelial marker endomucin (G) at E10.5, whereas at E12.5 expression was also observed at a relatively lower level in the SmaA+ compartment (H). DA indicates dorsal aorta; ISV, intersomitic vessel; LB, limb bud. Scale bars: A, 500 μm; B and C, 50 μm; D, 400 μm; E and F, 200 μm; G, 20 μm; H, 100 μm.
differentiation of mesoderm-derived mural cells to contribute to the stability of the developing vasculature: a function mediated through synergy with the TGFβ pathway.

Methods
An expanded Methods section is available in the Online Data Supplement.

Mouse Lines
Mice were housed and maintained in a controlled environment, and all procedures were performed in accordance with the Animals (Scientific Procedures) Act 1986, (Home Office, United Kingdom). Global Tβ4 KO mice were generated by deleting exon 2 of the Tmsb4x locus and maintained on a C57Bl6/J background for more than 20 generations. Endothelial-specific Tβ4 knockdown mice were generated by crossing female mice, containing a previously described Tβ4 short hairpin RNA (shRNA)9 targeted to the hypoxanthine phosphoribosyltransferase locus with Tie2-Cre male mice.13 A cardiac-specific Tβ4 shRNA knockdown strain has been previously described,10 using a Nkx2-5-Cre knock-in14 crossed with the Tβ4 shRNA knockdown strain.

Histology, Immunofluorescence, and In Situ Hybridization
Standard histological, immunohistochemical, immunofluorescence, and in situ hybridization were performed on frozen or paraffin embryo sections or on whole-mount fixed embryos or retinas harvested at postnatal day 6, as described in full in the Online Supplement.

Real-Time PCR
Real-time quantitative PCR (qRT-PCR) was performed according to a standard ΔΔCT protocol using SYBR green (Applied Biosystems). Primer sequences are given in the Online Supplement.

Cell Culture
10T1/2 cells and A404 cells were maintained under standard conditions. For stimulation experiments, cells were treated for 4 days with PBS vehicle control, 1 μg/mL TGFβ, 2 ng/mL TGFβ, or 1 μg/mL TGFβ plus 2 ng/mL TGFβ before RNA extraction using Trizol reagent (Invitrogen). For Smad 2 phosphorylation assays, A404 cells were serum-starved (0.5% fetal calf serum) overnight before stimulation, as above, for 20 minutes before protein extraction and standard Western blotting. For coculture experiments, early-passage human umbilical vein endothelial cells (HUVECs) were cultured in 50:50 A404:HUVEC medium (endothelial cell growth medium, PromoCell) for 24 hours; cells were counted, and an equal number of A404 cells were plated in each well. Thereafter, cells were cocultured in A404 medium for 4 days, with or without the addition of a neutralizing rabbit polyclonal anti-Tβ4 antibody (1:250; Immunodiagnostik). 10T1/2 cells were transfected with Smad activity luciferase reporter plasmids (SA Biosciences) using Effectene transfection reagent according to the manufacturer’s instructions. After 16 hours, cells were serum-starved overnight (0.5% FCS in DMEM+Glutamax) before stimulation for 6 hours in the presence of 100 ng/mL TGFβ, 2 ng/mL TGFβ, 100 ng/mL TGFβ plus 2 ng/mL TGFβ, or a PBS vehicle control. Smad activity-dependent firefly luciferase activity was measured by dual luciferase reporter assay (Promega).
Loss of Tβ4 Causes Hemorrhage Due to a Defect in Mural Cell Recruitment to Developing Vessels

To determine whether Tβ4 plays an essential role during the development of the systemic vasculature, a global loss of function model of Tβ4 was created by replacing exon 2 of the Tmsb4x gene with a neomycin resistance cassette resulting in a functionally null allele (Online Figure I). Hemizygous null male (Tβ4 −/Y) mice reach adulthood, but in reduced numbers, indicating a degree of embryonic lethality (Table 1). The loss of Tβ4 −/Y embryos was progressive from insignificant lethality before and up to E10.5 (21% versus 25% expected; \( P = 0.482 \)) through to significantly reduced recovery of mutants at E14.5 (16% versus 25% expected; \( P = 3.01 \times 10^{-3} \)) and up to birth (17% versus 25% expected; \( P = 1.01 \times 10^{-3} \)), indicating incomplete penetrance (Table 1). When E10.5 Tβ4 −/Y embryos were examined, it was apparent that approximately 10% of Tβ4 −/Y embryos displayed abnormal accumulation of blood in their hearts (Figure 2A and 2B). Mutant embryos also revealed extensive cranial bleeding in the midbrain region compared with +/Y controls (Figure 2C and 2D) and in axial sections there was clear evidence of both pericardial and coelomic cavity hemorrhage (Figure 2E through 2H). Severe vascular hemorrhaging at E10.5 was incompatible with continued survival accounting for the significant loss of Tβ4 −/Y mutant between E10.5 and E14.5 (Table 1). Over the course of the studies, we observed a reduction in embryonic lethality, reflecting an overall increase from approximately 60% to 80% survival of Tβ4 −/Y mutant mice to postnatal stages. This was coincident with a reduced incidence of the more severe hemorrhagic phenotype identified at E10.5, down from 44% to 20%, and reflected in an increased recovery of viable embryos at E14.5. The data in Table 1 represent current embryonic lethality versus survival rates against expected mendelian ratios.

Such hemorrhagic phenotypes can arise through defective mural cell investiture of developing blood vessels.15,16 Thus, Tβ4 −/Y embryos were examined for mural cell defects. Immunofluorescence studies for NG2, a marker that we initially confirmed as specific for mural cells and excluded from the developing endothelium (Figure 3A and 3B), showed that by E10.5 wild-type embryos displayed a dorsal aorta invested with NG2-positive mural cells (Figure 3C). Immunofluorescence analysis revealed that Tβ4 was predominantly expressed in the developing endothelium at E10.5, which was retained at E12.5. Relatively weaker expression was observed in the mural cell population of the dorsal aorta at E12.5, consistent with the onset of a more differentiated smooth muscle cell layer (Figure 1G and 1H).

Results

Tβ4 Is Expressed in the Endothelium of the Developing Embryo

To investigate vascular expression of Tβ4 during development, whole-mount and section in situ hybridization was performed on midgestation embryos. Tβ4 was expressed in the dorsal aorta of the developing embryo from E9.5 onward (Figure 1A, 1B, and 1C). At E10.5, Tβ4 expression appeared relatively ubiquitous at low resolution (Figure 1D) but was detected in all developing blood vessels in the embryo (Figure 1D through 1F), including those within the developing limb bud (Figure 1E) and the intersomitic vessels (Figure 1F). Immunofluorescence analysis revealed that Tβ4 was predominantly expressed in the developing endothelium at E10.5, which was retained at E12.5. Relatively weaker expression was observed in the mural cell population of the dorsal aorta at E12.5, consistent with the onset of a more differentiated smooth muscle cell layer (Figure 1G and 1H).

Exon Array Analysis

Micro-arrays were performed on Affymetrix Mouse Exon 1.0ST arrays. Raw data were processed with Affymetrix expression console software before being analyzed for gene expression changes in Partek. Statistics were performed in R and pathway analysis performed using the Metacore software (Genego).

Statistics

Statistical analysis was performed with Graphpad Prism software. Contingency tables were analyzed by \( \chi^2 \) test. Two-tailed, unpaired, nonparametric \( t \) tests were used for all other statistical tests.

Figure 2. Embryos lacking Tβ4 reveal a gross hemorrhagic phenotype. A proportion of E10.5 Tβ4 −/Y embryos displayed overt pericardial hemorrhage (A and B, red arrowheads) and cranial hemorrhage in the midbrain region (C and D, red arrowheads). Bleeding into the pericardium was confirmed by sagittal section through Tβ4 +/Y and −/Y hearts at E10.5 (E and F, black arrowheads highlight presence of blood in pericardial cavity). Coelomic cavity hemorrhage was also seen in the Tβ4 −/Y embryos (G and H; black asterisks indicate coelomic bleeding). CC indicates coelomic cavity; DA, dorsal aorta; LV, left ventricle; MB, midbrain. Scale bars: A as applies to B, 500 μm; C as applies to D, 50 μm; E as applies to F, 50 μm; G as applies to H, 100 μm.

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Figure 3. Impaired mural cell coverage in Tβ4-null vasculature. Immunofluorescence staining for the mural cell-specific marker NG2, which is excluded from the PECAM positive endothelium (A and B), revealed that by E10.5 Tβ4+/Y embryos which exhibited a mild phenotype with intact vasculature (mild) and Tβ4−/Y mutants with hemorrhaging (severe) display reduced coverage of mural cells in comparison to control Tβ4+/Y embryos (C through E). This defect in mural cell investiture is quantifiable and statistically significant (F; n=6 embryos per genotype; *P<0.05). Reduced mural cell coverage was confirmed by immunohistochemistry for smooth muscle myosin heavy chain (SM-MHC) in the wall of the aorta in Tβ4−/Y (G and J) and −/− mild (H and K) and severely affected (I and L) littermates, which revealed reduced and irregular staining of the mutant vessel wall in both transverse and sagittal sections through the aorta. qRT-PCR studies reveal significant global defects in the expression of a large panel of mural cell marker genes between somite matched pairs of E10.5 Tβ4+/+Y and Tβ4−/−Y embryos implying a reduction in mural cells across mutant embryos (n=5 embryo pairs run in triplicate per qRT-PCR run) (M). Ang1 indicates angiopoietin 1; DA, dorsal aorta. Scale bars: A, 30 μm; B, 20 μm; E as applies to C and D, 20 μm; L as applies to G through L, 30 μm. Statistics: *P≤0.05, **P≤0.01, ***P≤0.001; 2-tailed Mann-Whitney U test.

Tβ4−/Y embryos, such that investiture was less significantly reduced in mildly affected embryos, in which the vascular wall appeared intact, as compared with severely affected mutants with evident hemorrhaging (Figure 3F). A reduced mural cell pool, impaired support of the mutant −/−Y dorsal aorta, and correlation with severity of phenotype was further illustrated by immunohistochemistry for smooth muscle myosin heavy chain (SM-MHC) in transverse and sagittal sections Figure 3G through 3L). Globally impaired mural cell development in E10.5 Tβ4−/−Y embryos was confirmed by downregulation of a wide panel of mural cell markers in somite matched pairs of E10.5 Tβ4+/+Y and Tβ4−/−Y embryos, quantified by qRT-PCR. Significant changes in gene expression were observed for markers of VSMCs such as smooth muscle α-actin (SMαA) and smooth muscle 22α (SM22α) as well as pericyte markers such as endosialin, NG2, CD13, and angiopoietin 1 (Figure 3M). Consistent with the ubiquitous expression of Tβ4 in developing blood vessels, Tβ4−/−Y embryos at E14.5, displayed statistically significant levels of dermal hemorrhage compared with littermate controls (Online Figure IID through C). Immunostaining of the skin from E14.5 Tβ4−/−Y embryos for SMαA demonstrated a qualitative lack of mural cell-derivatives in comparison to specimens from control Tβ4+/+Y littermates (Online Figure IID and E). We next examined the integrity of the developing endothelium in Tβ4+/+Y versus Tβ4−/−Y aortas (Figure 4A and 4B). Even in selected areas of the walls of the dorsal aortas which appeared to have a disrupted endothelium by gross histological staining (Figure 4A and 4B; black inset boxes), immunofluorescence for the endothelial-specific adhesion molecule, VE-cadherin (Figure 4C and 4D), suggested that the endothelial cell contacts were intact across all genotypes, including both in mildly and severely affected mutants relative to Tβ4+/+Y controls (Figure 4E through 4G). This was supported by immunostaining for zona occludens 1 (ZO-1) (Figure 4H through 4N), a marker of
endothelial tight junctions, which revealed an integral endothelium within Tβ4 −/Y aortas (Figure 4J through 4N) comparable to that of littermate controls (Figure 4H and 4I).

To confirm that the mural cell defects observed in Tβ4 −/Y embryos were due to a deficiency of Tβ4 in the endothelium, as suggested by the expression data (Figure 1), rather than a lack of Tβ4 acting cell autonomously in mural cells, we made use of a mouse strain that enables transcription crossing this mouse with a Tie2-Cre strain,13 knockdown of Tβ4 under the control of Cre recombinase with gfaTβ4 shRNA (Figure 5).4 Initially, we observed a gross hemorrhagic phenotype (including cranial, pericardial, and coelomic cavity bleeding) in 30% of the endothelial-specific Tβ4-knockdown embryos at E10.5 (n=24 embryos analyzed), which was equivalent to the one-third documented for the global Tβ4 −/Y mutants.4,9 In addition, we recorded an overall incidence of embryonic lethality in Tie2-Cre Tβ4 shRNA animals of 12% (animals lost in utero between E10.5 and E14.5 and up to P1 according to expected mendelian ratios at birth) compared with 17% for the −/Y knockout animals (Table 1).4 The reduced incidence of loss in utero in the EC specific Tβ4-shRNA model may reflect both inefficient knockdown via the Cre/shRNA as demonstrated by qRT-PCR (Figure 5) and additional developmental roles for Tβ4 highlighted by global loss of function, such as an essential requirement in epicardial-derived coronary vasculogenesis.4 The latter was confirmed as a contributory factor to the differential embryonic lethality between E14.5 and P1 by analyzing the coronary vasculature of Tβ4 −/Y embryos at E14.5 (Online Figure III). We observed a significant incidence of VSMCs abnormally residing within the epicardial layer and reduced presence in the underlying myocardium, compared with Tβ4 +/Y littermate controls (Online Figure IIIA and B), a phenotype consistent with that described after myocardial shRNA knockdown of Tβ4.4,9

Serial sections through the developing dorsal aorta of Tie2-Cre Tβ4 shRNA embryos revealed defective NG2-positive mural cell recruitment to the vessel wall in comparison to Cre-only littermate controls (Figure 5A and 5B). The defective recruitment was equivalent to that observed after global knockdown of Tβ4 (Figure 3C through 3E). Knockdown of Tβ4 expression in Tie2-Cre Tβ4 shRNA embryos was confirmed by qRT-PCR on isolated dorsal aortas (Figure 5C) and protein knockdown via immunostaining for Tβ4 with that of a mild (F) and severe (G) mutant. The integrity of the endothelium after loss of Tβ4 was confirmed by positive immunostaining for the tight junction marker ZO-1, between +/Y (H and I), mildly affected (J through L), and severely affected (M and N) mutant littermates. Scale bars: A as applies to B, 100 μm; D as applies to C, 40 μm; G as applies to E through G, 20 μm; L as applies to I through L, 20 μm; N as applies to M, 20 μm.
developing vascular plexus. Whereas T
migration of phenotypically mature mural cells along the
retinal vasculature, consistent with its status as a central
Mesodermal Cells to a Mature Mural
VC through E), suggesting that impaired migration of mural
defects independently of the P19 embryonal carcinoma cell
line known as A404, clonally selected for a propensity to
differentiate into mural cells. Treatment of A404 mural cell
progenitors with synthetic Tβ4 resulted in an increased
number of SM22α- and SMαA-positive cells in culture
compared with vehicle-treated controls (Figure 6A, 6B, 6E,
and 6F). TGFβ was used as a positive control in these
experiments and resulted in an equivalent increase in the
incidence of SM22α- and SMαA-positive cells that was
further augmented by the combined addition of both Tβ4 and
TGFβ (Figure 6C, 6D, 6G, and 6H). The phenotypic changes
in the A404 cells and coincident expression of the smooth
muscle differentiation markers in culture were accompanied
by a significant upregulation of mural gene expression, as
quantified by qRT-PCR. Markers of both VSMCs such as
SMαA and SM22α and pericytes, such as endosialin and
desmin, were all significantly upregulated (Figure 6I). These
data indicate that Tβ4 can act in a paracrine fashion to
stimulate mural cell differentiation from mesoderm.

Blood vessels in mesoderm-derived tissues are thought to
recruit mural cells via the in situ differentiation of mesoderm
progenitors into mature mural cells.5 Given that mural cell
defects in Tβ4−/Y embryos are present only in mesodermal
tissues, such as the aorta and subdermal vessels and not in
tissues that derive their mural cells via migration, such as the
postnatal retina, we investigated whether Tβ4 acts to induce
mesoderm differentiation into a mature mural cell lineage.
Initially, we assessed the ability of exogenous Tβ4 to stim-
ulate the differentiation of a P19 embryonal carcinoma cell
line known as A404, clonally selected for a propensity to
differentiate into mural cells. Treatment of A404 mural cell
progenitors with synthetic Tβ4 resulted in an increased
number of SM22α- and SMαA-positive cells in culture
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SMαA and SM22α and pericytes, such as endosialin and
desmin, were all significantly upregulated (Figure 6I). These
data indicate that Tβ4 can act in a paracrine fashion to
stimulate mural cell differentiation from mesoderm.

Endothelial Tβ4 Stimulates Differentiation of
Mesodermal Cells to a Mature Mural
Cell Phenotype
Mural cell defects in developmental mutants have previously
been attributed to either aberrant migration, survival, prolif-
eration, or differentiation of mural cells.6 The postnatal
retinal vasculature, consistent with its status as a central
nervous system tissue, derives a mural cell layer via the
migration of phenotypically mature mural cells along the
developing vascular plexus. Whereas Tβ4 is expressed in
the primary plexus vasculature of the early postnatal (P6)
retina (Online Figure VA and B), P6 retinas from Tβ4−/Y
mice did not show any defect in mural cell coverage when
compared with Tβ4 +/Y littermate controls (Online Figure
VC through E), suggesting that impaired migration of mural
cells is unlikely to be responsible for the defective mural cell
coverage of Tβ4−/Y embryos. In addition, mural cells
displayed no overtly abnormal levels of proliferation or
apoptosis, as measured by phospho-histone H3 or cleaved
caspase 3 immunofluorescence staining, respectively, in either
Tβ4 +/Y or Tβ4−/Y embryos (Online Figure VIA through F),
thus ruling out the possibility that the mural cell
defects observed were due to defective mural cell hyperplasia
or survival.

Dorsal aorta after endothelial-specific knockdown of T
VE-cadherin confirmed the endothelium was intact in the mutant
embryos (Figure 5B, white arrowheads highlight endothelial cell contacts). Scale
bars: B as applies to A, 20 μm; F as applies to E, 30 μm; Statistics: **P<0.01, ***P<0.001; 2-tailed Mann-Whitney U test.
Error bars represent standard error of the mean.

Tβ4 Stimulates Mural Cell Differentiation by
Enhancing the Activity of TGFβ Signaling
To gain greater insight into the molecular mechanisms
underlying the role of Tβ4 in mural cell differentiation, gene
expression arrays were carried out on E12.5 Tβ4−/Y and
+/Y embryos and compared with array data from Tβ4−/Y and
−/Y adult hearts to further facilitate the identification of
Tβ4-dependent gene expression. Metacore software from
GeneGo was used to highlight the expression changes of
signaling factors in the embryo and adult heart datasets and
identify the most likely underlying pathways. Four of the top
highlighted pathways included TGFβ (Table 2), previously
highlighted as a key molecule involved in mural cell differ-
entiation.17,18 Thus, we hypothesized that Tβ4 may exert its
effects on mural cell differentiation by interaction with and/or
modulation of the TGFβ pathway.

Activation of the canonical TGFβ pathway in mural cells
leads to the transcription of stereotypical TGFβ-responsive
genes such as plasminogen activator inhibitor-1 (PAI-1),
Id-1, and c-myc.19,20 Levels of the mRNAs encoding these
proteins can be used as a read-out of TGFβ pathway activity.

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Figure 6. Tβ4 induces in vitro mural cell differentiation via the TFGβ signaling pathway. Culture of A404 cells in the presence of exogenous Tβ4 led to an upregulation in the smooth muscle cell markers, SM22α and SMαA, and corresponding change in A404 progenitor morphology (A, B, E, and F; white arrowheads in E highlight background differentiation in control cultures). Ectopic administration of TGFβ served as a positive control for mural cell differentiation (C and G; white arrowheads in G highlight TGFβ-induced differentiation), which in combination with Tβ4 augmented a further significant effect on cell morphology and smooth muscle cell marker expression (D and H; white arrowheads in D highlight differentiated cells). The addition of Tβ4 alone or in combination with TGFβ resulted in a significant upregulation of a wide panel of mural cell markers as measured by qRT-PCR (I; n=8 per treatment group) and also significantly enhanced the expression of the TGFβ target genes PAI-1, Id-1, and c-myc, an effect that was blocked by the addition of Tβ4-neutralizing antibody (J). Coculture of A404 cells with HUVECs (PECAM−) induced differentiation of the progenitors as indicated by a change in cell morphology and expression of SMαA (K and L). A404 differentiation was inhibited by the addition of Tβ4-neutralizing antibody (M and N), suggesting that Tβ4 is a key paracrine factor secreted from HUVECs to regulate mural cell differentiation. HUVEC
Treatment of A404 cells with Tβ4 led to significant upregulation of these TGFβ target genes, comparable to that induced by TGFβ alone, as measured by qRT-PCR, and, moreover, combined Tβ4 and TGFβ acted to further significantly increase expression of all 3 target genes, indicating that Tβ4 can enhance TGFβ signaling in these cells (Figure 6). We next examined cocultures of A404 progenitors withHUVECs, plus or minus Tβ4 neutralizing antibody. Importantly, Tβ4 antibody alone had no effect on A404 differentiation per se as confirmed by addition to A404 cultures followed by qRT-PCR (Figure 6f and 6j; black hatched bars). HUVECs express Tβ4 at high levels, and in the control setting, HUVEC coculture brought about differentiation of the A404 cells, as indicated by changes in cell morphology (adoption of a more elongated and differentiated phenotype) and expression of SMαA (Figure 6K and 6L). However, the addition of Tβ4 antibody significantly blocked the visible differentiation of A404 cells in culture (Figure 6M and 6N) and significantly reduced the expression of both mural cell markers (Figure 6O) and the TGFβ targets PAI-1, Id-1 and c-myc (Figure 6P).

Binding of TGFβ ligands to their cognate receptors leads to phosphorylation of Smad adaptor proteins. Treatment of A404 cells with Tβ4 alone resulted in increased levels of phospho-Smad2, to an equivalent level to TGFβ alone (Figure 7A). Most notably, Tβ4 in combination with TGFβ was able to induce higher levels of Smad2 phosphorylation than TGFβ treatment alone (Figure 7A). To investigate whether Tβ4 might be required for TGFβ signaling via Smad2 in vascular development, we assessed phospho-Smad2 levels in global Tβ4 –+/Y embryos and observed a significant downregulation of phospho-Smad2 in both mildly and severely affected –+/Y mutants compared with littermate (+/Y) controls (Figure 7B).

These data collectively suggest that endothelial Tβ4, acting in a paracrine fashion, regulates mural cell differentiation to VSMCs via synergistic activation of the TGFβ signaling pathway.

Inhibition of the TGFβ Pathway Correlates With the Penetration of Hemorrhage in Tβ4-Null Aortas and Alterations in Downstream Signaling

To further determine whether TGFβ signaling was impaired with loss of Tβ4 in vivo, we examined aortas at E10.5 from Tβ4-null mutants, with or without evidence of vascular

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Table 2. Metacore Analyses of Array Data Revealed Misregulation of the TGFβ Pathway in the Tβ4-Null Background

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The top 200 hundred upregulated and downregulated genes identified by affymetrix exon array between Tβ4 +/Y and Tβ4 –/Y E12.5 embryos and Tβ4 +/Y and Tβ4 –/Y adult hearts were analyzed using Genego Metacore software http://www.genego.com/metacore.php. Reverse pathway analysis based on altered gene expression levels and comparison to a systems biology database identified potential disrupted signaling pathways in the Tβ4 –/Y embryos. Four of the top 5 potentially disrupted pathways involved TGFβ as a major signaling node. Displayed are the nodes present in the top 5 ranked putative pathways alongside levels of differential expression per transcript in the exon arrays.

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Figure 6 (Continued). coculture also upregulated the wide panel of mural cell markers as measured by qRT-PCR, which were downregulated in the presence of Tβ4-blocking antibody (O). An equivalent HUVEC dependent upregulation was observed for the TGFβ target genes in A404 cultures, which was also antagonized by Tβ4 antibody treatment (P). Scale bars: A as applies to A through D, 10 μm; E as applies to E through H, 10 μm; K as applies to M, 10 μm; L as applies to N, 5 μm. Statistics: *P≤0.05, **P≤0.01, ***P≤0.001; 2-tailed Mann-Whitney U test. Error bars represent standard error of the mean.
hemorrhage, compared with wild-type littermates, by immunostaining for SMαA and phospho-Smad2 (pSmad2) with Image J quantification (Figure 8A through 8F). Consistent with the previous NG2 and SM-MHC analyses (Figure 3), the VSMC layer was significantly reduced in the mutants with hemorrhaging as compared with those with a mild, nonhemorrhagic phenotype and wild-type littermates (Figure 8A through 8D). This was accompanied by a significant reduction in both the percentage of mural cells positive for pSmad2 and intensity of pSmad2 staining within the aortic wall (Figure 8E and 8F). Interestingly, in the mildly affected Tβ4-null mutants the percentage of pSmad2-positive cells was significantly increased as compared with the wild-type controls, suggesting some form of overcompensation to maintain the integrity of the aorta wall (Figure 8E). To provide further evidence for the ability of Tβ4 to augment TGFβ vascular signaling, 10T1/2 cells were transfected with a construct encoding firefly luciferase under the control of a Smad responsive element (SRE). Treatment with Tβ4 stimulated SRE-dependent reporter activity to a level significantly higher than that of PBS alone and in combination with TGFβ treatment alone (Figure 8G). Consistent with the in vivo pSmad2 data (Figure 8A through 8F) expression levels of the TGFβ responsive genes, PAI-1, Id-1, and c-myc were found to be significantly downregulated in E10.5 Tβ4 +/Y embryos compared with somite-matched Tβ4 +/+ controls, as additional proof of defective TGFβ downstream signaling in vivo after loss of Tβ4 (Figure 8H).

Discussion

This study reveals that in the absence of endothelial Tβ4, the secreted signals from the developing endothelium are no longer adequate to induce differentiation of mesodermal progenitor cells to a mural cell phenotype. At a molecular level, this is due to a deficiency in TGFβ signaling in the mesodermal progenitor cell population and manifests as impaired mural investiture of the developing systemic vasculature in general and the aorta in particular.

We initially mapped the vascular expression of Tβ4 predominantly to the developing endothelium. Global knockout of the Tβ4 gene in the developing embryo resulted in a proportion of the resulting E10.5 embryos exhibiting pericardial and coelomic cavity hemorrhage. An explanation for this hemorrhagic phenotype was evident through the observation that dorsal aortas in the Tβ4-null mice had reduced mural cell coverage in comparison to control littermates. The phenotype of the global knockout embryos was incompletely penetrant and subject to apparent compensation, such that we observed mutant embryos with both intact vasculature and those with a more severe hemorrhagic phenotype. This was reflected in survival rates across distinct stages in development. Lethality occurred both between E10.5 and E14.5 due to hemorrhaging, and beyond E14.5 up to birth whereby global knockout embryos revealed epicardium-derived coronary vascular de-
ence in images across the 3 categories were recorded at the same filter and light intensity settings and exposure time (D and E). SMuA fluorescence was significantly reduced in the severe KO mutant aortas compared with both wild-type and mild mutants (D). The percentage of pSmad2-positive nuclei within the mural cell layer was significantly reduced in the severe mutants, whereas in the mild mutants, the percentage was significantly increased compared with wild-type indicative of a compensatory response to upregulate TGFβ signaling and maintain the integrity of the aorta wall (E). pSmad2 fluorescence was significantly reduced in the severe KO compared with both wild-type and compensated mutants (F). To confirm a synergistic effect of Tβ4 on TGFβ signaling, treatment of 10T1/2 cells transfected with a luciferase reporter construct under control of a Smad-responsive element (SRE) with 100 ng/mL Tβ4 showed a significant increase in luciferase activity compared with treatment with PBS. Treatment with 2 ng/mL TGFβ activated luciferase expression higher than Tβ4 alone, whereas a combination of both Tβ4 and TGFβ at the same doses revealed a significant additive effect on luciferase activity (G; n=6 per treatment group). TGFβ-responsive transcription factors PAI-1, Id-1, and c-myc were significantly decreased in Tβ4−/−Y embryos in comparison to somite matched Tβ4+/−Y controls as measured by qRT-PCR (H; n=8 embryo pairs run in triplicate per qRT-PCR run). DA indicates dorsal aorta. Scale bar in A applies to A through C, 10 μm. Statistics: *P<0.05, **P<0.01, ***P<0.001; Student t test (D and E); 2-tailed Mann-Whitney U test (G and H). Error bars represent standard error of the mean.

Figure 8. TGFβ signaling is reduced in Tβ4-null embryonic aortas and correlates with the severity of hemorrhagic phenotype. Immunostaining for SMuA and pSmad2 revealed a loss of smooth muscle cell coverage and corresponding reduced TGFβ signaling in the aortas of severely affected (KO severe) Tβ4−/−Y embryos at E10.5, which exhibited hemorrhaging compared with both +/Y wild-type and Tβ4−/−Y mutants that had a mild phenotype and intact vasculature (KO mild; A through C). Inset in A highlights punctuate pSmad2 staining in the nuclei of a smooth muscle cell and underlying endothelial cell within the aorta wall; white arrowheads (A through C) highlight pSmad2-positive nuclei. The reduced mural cell coverage and pSmad2 signal was quantified using Image J to record relative fluorescence of SMuA. The percentage of pSmad2-positive nuclei and pSmad2 fluorescence was significantly reduced in the severe KO compared with both wild-type and compensated mutants (D). To confirm a synergistic effect of Tβ4 on TGFβ signaling, treatment of 10T1/2 cells transfected with a luciferase reporter construct under control of a Smad-responsive element (SRE) with 100 ng/mL Tβ4 showed a significant increase in luciferase activity compared with treatment with PBS. Treatment with 2 ng/mL TGFβ activated luciferase expression higher than Tβ4 alone, whereas a combination of both Tβ4 and TGFβ at the same doses revealed a significant additive effect on luciferase activity (G; n=6 per treatment group). TGFβ-responsive transcription factors PAI-1, Id-1, and c-myc were significantly decreased in Tβ4−/−Y embryos in comparison to somite matched Tβ4+/−Y controls as measured by qRT-PCR (H; n=8 embryo pairs run in triplicate per qRT-PCR run). DA indicates dorsal aorta. Scale bar in A applies to A through C, 10 μm. Statistics: *P<0.05, **P<0.01, ***P<0.001; Student t test (D and E); 2-tailed Mann-Whitney U test (G and H). Error bars represent standard error of the mean.

effects, as previously described after shRNA knockdown of Tβ4 in the developing myocardium.9 Interestingly, survival of Tβ4 global knockout mice was in stark contrast to the lethality observed between E14.5 to 16.5 after optimal cardiac-specific knockdown.9 The previous RNAi model revealed a direct correlation between the severity of defective coronary angiogenesis and level of Tβ4 protein, and, moreover, reciprocal Tβ4 gain of function precisely restored the epicardial-derived vascular cell migration and differentiation disrupted by shRNA-induced loss of function. Thus we excluded, to all intents and purposes, the possibility of off-target effects, as can be associated with shRNA gene silencing, increasing the incidence of embryonic lethality. Rather, the differences between our global knockout and knockdown model are probably attributed to the fact that RNAi targeting in vivo, when sufficiently optimal to abrogate expression of the target gene, can result in a more severe phenotype than a corresponding global-null. Genetic ablation via homologous recombination through the germline, leading to complete loss of function from the outset in development, may be partially compensated for by functional orthologs, whereas RNAi-mediated efficient knockdown, occurring rapidly and at a defined developmental stage, may not be permissive for compensation.21 In a recent study, Tβ4 was described as dispensable for murine cardiac development and function after both global and cardiac-specific knockouts.24 This contrasts with our findings describing partially penetrant vascular phenotypes after complete loss of Tβ4; however, we acknowledge that over successive generations the incidence of embryonic lethality decreased from 40% at the outset of the study (60% survival) to a current level of 20% loss in utero (80% survival) due to presumptive modifier effects. That said, there is a clear vascular phenotype in our Tβ4-null background both within the developing systemic and coronary vasculature, and therefore differences between our findings herein and that previously described may reflect allelic variation in the targeting strategy or genetic background-dependent events. It should be noted, however, that in the earlier study, the Tβ4 knockout aortas had an apparent reduction in α-SMA+ cell coverage within the vessel wall relative to wild-type controls at E14.524; while not commented at the time, this finding is consistent with our phenotype as described.
at E10.5, we hypothesized that the process of mural cell embryonic vasculature. Smad2 phosphorylation with TGF
SM increased transcription of canonical mural cell genes such as their target locations. In contrast, the vascular defects in the
exerting its effects by reducing the migration of mural cells to a fully formed media wall. In the absence of T
mural cell differentiation and investiture of the endothelium with migration, or differentiation of mural cells.3 A proliferation
Figure 9. Developmental model for the role of T
 stronger threshold, the vessel loses structural integrity and hemorrhage results.

Embryonic mural cell defects generally arise as a result of perturbation of one or more of the proliferation, survival, migration, or differentiation of mural cells.4 A proliferation defect was ruled out on the basis of examination of E10.5 dorsal aorta mural cells for the presence of the proliferative marker phospho histone H3 and apoptosis excluded by a lack of cleaved caspase 3 expression. The anatomic location of mural cell defects also provided a clue to the role of Tβ4. The mural cell investiture of the postnatal retina is thought to rely on the migration of phenotypically mature mural cells, along a vascular plexus.2,5 As no defects were observed in the postnatal retina of Tβ4 −/Y mice, it is unlikely that Tβ4 is exerting its effects by reducing the migration of mural cells to their target locations. In contrast, the vascular defects in the Tβ4 −/Y mouse tend to occur in vessels which derive their mural cell coverage from the in situ differentiation of overlying mesoderm, notably the E10.5 dorsal aorta and the E14.5 dermal vasculature. Given that the normal induction of a mural cell layer around the dorsal aorta occurs over a narrow time window in murine development, between E9.5 and E10.5, and that Tβ4 −/Y mice first exhibit mural cell defects at E10.5, we hypothesized that the process of mural cell differentiation is aberrant in Tβ4 −/Y mice. Further insight into the cellular basis of Tβ4-induced mural cell differentiation arose from the observation that endothelial cell (EC)-specific Tβ4 knockdown recapitulated the aortic mural cell defects observed in the global knockout. In this instance, despite overlap in the mural cell phenotype, the embryonic lethality was not as significant as in the global knockout at stages between E10.5 and E14.5, reflecting reduced systemic vessel hemorrhaging due to incomplete knockdown of Tβ4. Beyond E14.5, through to birth, the EC knockdown of Tβ4 had no effect on the coronary vasculature, the cause of later onset lethality in the global knockout embryos. Importantly, a reduction in mural cell differentiation after EC-specific knockdown in this study was complemented by mural cell differentiation from mesodermal progenitors on Tβ4 treatment, further supporting the specificity of the particular shRNA used in the EC targeting, identical to that previously described for the myocardial knockdown and defective coronary vasculature.9

Thus, Tβ4, produced by the developing endothelium, acts in a paracrine fashion to stimulate the differentiation of overlying mesoderm into mature mural cells for vascular support. In support of this, exogenous administration of Tβ4 to an in vitro cell model of mural cell differentiation caused the induction of mature mural cell markers and Tβ4-neutralizing antibody in coculture experiments prevented HUVEC-induced differentiation of A404 mural progenitors. Bioinformatic analysis of gene expression data in the Tβ4 −/Y embryos implicated the TGFβ pathway as a molecular mediator of the defects observed in the Tβ4 −/Y mice. During embryogenesis, TGFβ has been identified as one of the central factors in the formation of a normal mural cell component of the vessel wall,10 and in this study, we implicate TGFβ signaling as an important modulator of Tβ4 function in the developing vasculature. Exogenous Tβ4 was observed to upregulate the expression of TGFβ target genes during Tβ4-induced A404 mural cell differentiation. Moreover, Tβ4 was able to stimulate the activity of a Smad-responsive transcriptional element in transfected 10T1/2 cells and induce higher levels of phosphorylation of Smad2 when used in combination with TGFβ than with TGFβ alone. In vivo expression profiling of E10.5 Tβ4 −/Y embryos revealed a global downregulation of the TGFβ pathway in the absence of Tβ4, and importantly, within the mural cell and VSMC layers of E10.5 Tβ4 mutant aortas, we observed correlative changes in TGFβ signaling via immunostaining for phospho-Smad2 with severity and penetrance of the Tβ4-mutant phenotype. In those Tβ4 −/Y embryos whose vasculature appeared phenotypically normal, we observed a compensatory increase in phospho-Smad2 expression, which may underlie the incomplete penetrance of the vascular phenotype after loss of Tβ4, whereas in the mutants with reduced mural cells and, accompanying hemorrhage, phospho-Smad2 was reduced in the VSMC layer. The implication of signaling downstream of TGFβ in this instance is consistent with a recent study which conditionally inactivated TGFβ type II receptor to reveal an essential role in the VSMC differentiation of the descending aorta such that mutant embryos displayed occasional aneurysms.25

Downloaded from http://circres.ahajournals.org/ at Babraham Institute on July 8, 2015
We previously observed that in the developing heart, Tβ4 acts as a secreted myocardin factor on the migration and differentiation of epicardium-derived progenitor cells to form the smooth muscle layer of the coronary vasculature.9 More recently, we have also shown that Tβ4 is a key transcriptional target of the basic helix-loop-helix transcription factor Hand1 and its downregulation is, in part, responsible for the defects seen in the yolk sac vasculature of Hand1-null mutants.28 The present study allows us to expand the role of Tβ4 acting in these highly stereotyped situations to a more generalized function in vascular development. Tβ4 is a significant regulator of VSMC development, functioning as a paracrine signal from myocardial,9 extra-embryonic mesodermal,26 and endothelial lineages (herein), in an analogous manner to components of the Notch pathway.27 Thus, Tβ4 occupies a central role in the formation of a functional circulatory system such that absence or perturbation in Tβ4 function leads to serious deleterious consequences for the growth and stability of blood vessels.

The significance of Tβ4-dependent abnormalities in vascular development is their potential relevance to adult health and disease. Many pathological processes are caused by, or involve, the subversion of normal mural cell development,28 and an insult that results in depletion of medial VSMCs or a loss of functional VSMCs may be critical for adult aortic stability and vascular function. The model we propose (Figure 9) highlights the importance of secreted paracrine factors acting on mural cells and their progenitor populations, specifically in terms of their functional developmental role. Interest in identifying novel candidate molecules that function in these developmental pathways stems from the ability to agonize or antagonize their effects for therapeutic benefit. Thus, our discovery of Tβ4 as a secreted endothelial factor, which stimulates mesodermal progenitor differentiation into mural cells, can be seen in the context of not only a critical role in vascular development but highlights Tβ4 as a possible mediator of postnatal aortic function.

Acknowledgments
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Disclosures
None.

References
6. Seki T, Hong KH, Oh SP. Nonoverlapping expression patterns of ALK1 and ALK5 reveal distinct roles of each receptor in vascular development. Lab Invest. 2006;86:116–129.
Novelty and Significance

What Is Known?

- The stability of developing blood vessels depends on recruitment and differentiation of mural cells to support developing vascular smooth muscle cells.
- Impaired support of the vessel wall results in vascular instability and can lead to hemorrhage.
- Thymosin β4 (Tβ4) is an actin monomer-binding protein previously implicated in regulating yolk sac and coronary vessel development.

What New Information Does This Article Contribute?

- Tβ4 is required for systemic vascular development.
- An endothelial source of Tβ4 functions to maintain adequate recruitment and differentiation of mural cells/pericytes to maintain the stability of the developing aorta, cranial, and trunk vessels.
- Tβ4 functions with the TGFβ pathway to regulate mural cell development and vascular wall stability.

Vascular instability due to impaired smooth muscle support can lead to aortic aneurysm, with a worldwide prevalence of 5% among the elderly, and, due to rupture and lethal hemorrhage, is associated with a 50% to 80% mortality rate. Until now, most studies have focused on the adult pathology and existing models of the disease have identified only a limited number of causative factors. We reveal a novel congenital mouse model in which either global or endothelial-specific loss of the actin monomer-binding protein thymosin β4 (Tβ4) directly affects TGFβ-induced vascular smooth muscle cell development, predisposing mutant vessels to wall defects ranging from lethal hemorrhage to vascular instability. Although previous studies have shown that Tβ4 plays an important role in smooth muscle contribution to extra-embryonic and coronary vascular beds, our findings reveal a more unifying function of Tβ4 in systemic vessel development per se and suggest the possibility of a new developmental paradigm for adult-onset vascular instability and aneurysm.
Online Supplementary Material

Online Methods

Mouse Lines

Mice were housed and maintained in a controlled environment and all procedures were performed in accordance with the Animals (Scientific Procedures) Act 1986, (Home Office, UK).

Global Tβ4 KO mice. The Tβ4 gene targeting vector was created by ligating a 6.5kb fragment containing a 5’ arm (exon 1; BamHI-EcoRV) and 3’ arm (exon 3; Nhe1-BamH1) flanking a neomycin resistance cassette, thereby deleting exon 2 of the Tmsb4x gene. The construct was linearised and electroporated into 129Sv ES cells which were subject to positive-negative selection and screening by Southern blot analysis for homologous recombination using 5’ flanking and internal probes. Male chimaeras were generated from targeted ES cell lines and F1 mice generated by crossing to wild type C57Bl6/J females Progeny were genotyped using a PCR that amplifies both mutant and wild type alleles (Forward primer (intron I): 5’-GTGCTTTTGGAACTGGGAGA-3’; Reverse primer for WT intron II: 5’-AGCCCGTTCTGAAAAATGG; Reverse primer for mutant, in neomycin cassette: 5’-GGCCTTCTTGACGAGTTCTT). Mice have been maintained on a C57Bl6/J background for more than 20 generations.

Endothelial-specific Tβ4 knockdown mice. The Tβ4 shRNA knockdown strain has been previously described (10). 23-base-pair sense and antisense Tβ4 sequences were inserted downstream of the H1 RNA pol III promoter in a modified pcDNA3 vector. Sequence of the shRNA: CTGAGATCGAGAAA TTCGATAAG; nucleotides 151–173 (Accession: NM 021278.2 Mus musculus thymosin β4, X chromosome (Tmsb4x) mRNA). A 5T stop sequence, flanked by two loxP recombination sequences, was inserted upstream of the hairpin sequences to enable activation of Tβ4 knockdown by Cre recombinase. This construct was
targeted to the HPRT locus and, in order to generate endothelial-specific knockdown of Tβ4, female Tie2-Cre +/+ HPRT Tβ4 shRNA +/- mice were crossed with male Tie2-Cre +/- HPRT Tβ4shRNA mice; the Tie2-Cre strain has been previously described.14

**Antibodies**

The following antibodies were used: rabbit anti-Tβ4 (Immundiagnostik), rat anti-endomucin (eBioscience), rabbit anti-mouse SM-MHC and rabbit anti-mouse SM22α (both from Abcam), rat anti-mouse PECAM and rat anti-VE-Cadherin (both from BD Pharmingen), rabbit anti-human PECAM (ProteinTech Group), rabbit anti-ZO-1 (Invitrogen) Cy3-conjugated mouse anti-α-SMA (Sigma), rabbit anti-NG2 (Chemicon), mouse anti-GAPDH (Chemicon), rabbit anti phospho-Smad2, rabbit anti-Smad 2 (Cell Signalling Technology). Secondary, Alexafluor conjugated antibodies were purchased from Invitrogen.

**Recombinant Protein**

Recombinant Tβ4 was a kind gift from RegeneRx Biopharmaceuticals Inc. Recombinant TGF-β 1 was purchased from R&D systems.

**Immunofluorescence Staining**

E10.5 embryos were fixed in 4% paraformaldehyde in PBS, embedded in OCT and cryosectioned at 10µm. Cryosections were washed for 10 minutes in PBS to remove OCT and then permeabilised in 0.5% Triton-X 100 for 10 minutes. After washing in PBS, sections were blocked at room temperature for 1 hour in blocking buffer (10% BSA, 10% sheep serum or 10% goat serum and 0.1% Triton-X 100 in PBS). Sections were then incubated in the primary antibody at an appropriate dilution (1:300 for Cy3-conjugated mouse anti-SMA; 1:50
for PECAM; 1:100 for all other antibodies) overnight in blocking buffer. Sections were then washed 5 times in 0.1% Triton-X 100 in PBS over the course of 1 hour. Incubation in the secondary antibody took place in blocking buffer for 1 hour at room temperature. Sections were again washed 5 times in 0.1% Triton-X 100 in PBS over the course of an hour, before mounting with coverslips using Vectashield plus DAPI. For immunofluorescence on cultured cells, cells were washed twice in PBS, fixed in 4% paraformaldehyde in PBS for 10 minutes at room temperature and stained according to the above protocol with antibodies at the following dilutions (1:250 for SM-MHC; 1:200 for SM22α; 1:50 for PECAM).

**Immunohistochemical Staining**

E10.5 embryo sections were prepared, as described above, except that sections were treated with 0.6% hydrogen peroxide in 80% methanol to block endogenous peroxidase activity following permeabilization. Blocking and incubation with primary antibody (1:100 anti-SM-MHC) were performed as described above. After three 10 minute washes in PBS (0.1% Triton-X 100), sections were incubated with biotinylated anti-rabbit antibody (Dako) for 1 hour at room temperature in blocking buffer. After three 10 minute washes in PBS (0.1% Triton-X 100), sections were incubated with diluted streptavidin-HRP complex (diluted in blocking buffer), washed 3 times in PBS (0.1% Triton-X 100) and developed using 3,3′-Diaminobenzidine Liquid Substrate System (Sigma). Sections were counterstained with haematoxylin, mounted with coverslips and imaged.

**Fluorescence Imaging**

Fluorescent images were captured on an upright Zeiss Z1 fluorescent microscope or an inverted Zeiss LSM 710 confocal microscope.
**Quantification of NG2, α–smooth muscle actin (SMA) and phospho-Smad2 (pSmad2)**

**Immunofluorescence**

In order to quantify the mural cell density around embryonic E10.5, ten axial sections from each embryo providing sections throughout the length of the dorsal aorta were examined. NG2, SMA or pSmad2 immunofluorescence was imaged under constant exposure. Images were thresholded to eliminate background fluorescence. Total channel fluorescence was quantified with ImageJ software. Two perpendicular measurements of the diameter of each aortic section were averaged and used to calculate the vessel circumference. NG2 or SMA total fluorescence was then normalised to vessel circumference to produce a measure of mural cell density. The fluorescence intensity of pSmad2 positive nuclei was measured after using the ImageJ Region of Interest tool to delineate nuclei (24 nuclei per section were selected while blinded to genotype). Lumenal aortic areas were calculated using the Image J freehand selection tool and measure command, after calibrating the scale (pixels/µm), according to the scale bar of the image.

**Histological Methods**

Sections of embryos were stained with hematoxylin and eosin, using a standard protocol, mounted with coverslips and imaged.

**Whole Mount in situ Hybridisation**

Embryos were dissected in diethyl pyrocarbonate (DEPC) treated PBS. Embryos were then fixed overnight in 4% PFA in DEPC PBS and transferred to absolute methanol for storage at -20°C. Embryos were then rehydrated by incubating in a gradient of methanol diluted in PBT (DEPC PBS + 0.1% Tween-20). Embryos were digested in proteinase K (10µg/ml in PBT) at room temperature for 8-25 minutes depending on stage. Post-fixing was conducted
for 20 minutes at room temperature in 4% PFA in PBT + 0.1% gluteraldehyde. After washing with PBT, embryos were pre-hybridized in hybridisation solution (50% formamide, 1.3x SSC, 5mM EDTA, 0.2% Tween-20, 0.5% CHAPS, 100µg/ml heparin in DEPC water) overnight at 68°C. Hybridisation was then carried out at 68°C overnight using a digoxigenin-labelled antisense riboprobe specific for the 3’UTR of Tmsb4x, in hybridisation solution. The following day, embryos were washed several times with hybridisation solution. After washing with TBS-T (0.8% NaCl, 0.02% KCl, 0.1M Tris-Cl pH7.5, 1.1% Tween-20 in DEPC water) embryos were blocked overnight in 10% sheep serum + 1% BSA in TBS-T. Embryos were then incubated with anti-digoxigenin-AP Fab fragments (Roche; 1:2000 in block) overnight at 4°C before washing and developing in NBT/BCIP solution until the desired colour change was achieved. Embryos were then dehydrated in a progressive methanol series before rehydrating and fixing in 4% PFA in PBS overnight, prior to imaging.

**RNA In Situ Hybridisation on Embryo Sections**

RNA *in situ* hybridisation on paraffin sectioned embryos was performed, as previously described (A. F. Moorman, et al., J. Histochem. Cytochem. 49(1) 2001), using a digoxigenin-labelled antisense riboprobe specific for the 3’UTR of Tmsb4x, alongside a sense control. Sections were mounted with coverslips and imaged.

**qRT-PCR**

Real time quantitative PCR (qRT-PCR) was performed according to a standard \(\Delta\Delta CT\) protocol using SYBR green (Applied biosystems).

Primers sequences used were as follows:

Tβ4: 
F-ATGTCTGACAAACCGATATGGC
R-CCAGCTTGCTTCTTTGTCA
SMA: F – GTCCCAGACATCAGGGAGTAA
R – TCGGATACCTCAGCGTCAGGA

SM22α: F – CAACAAGGGTGCCATCCTACGG,
R – ATCTGGGCGGCCTACATCA

NG2: F – GGGCTGTGTGCTGTCTGTTGA
R – TGATTCCCTTCAGGTAAGGCA

Endosialin: F – CAACGGGCTGCTATGGATTG
R – GCAGAGGTAGCCATCGACAG

CD13: F – ATGGAAGGAGGCGTCAAGAAA
R – CGGATAGGGCTTGGACTCTTT

Ang1: F – CACATAGGGTGCGACGAACCA
R – CGTCTGTTTCTGGGAAGAATGA

Desmin: F – GTGGATGCAGCCACTCTAGC
R – TTAGCCGCGATGGTCTCATAC

PAI-1: F – TTCAGCCCTTGCTTGCTCTC
R – ACACCTTTTACTCCGAAGTGGGT

Id-1: F – CCTAGCCTGTTCGCTGAAG
R – CTCCGACAGACCAAGTACCAC

c-myc: F – ATGCCCTCTAACGTAAGCTTC
R – CGCAACATAGGATGGAAGAGCA

Quantification of Haemorrhage at E14.5

E14.5 embryos were harvested from female Tβ4 +/- mice crossed with Tβ4 -/Y males. Immediately after dissection, the amount of surface haemorrhage visible under a dissection stereomicroscope was quantified according to the following scheme. Score 0 – no visible
haemorrhage, 1 – some small spots of dermal haemorrhage observed in a single location, 2 – some small spots of dermal haemorrhage observed in more than one location, 3 – a large area of haemorrhage observed in one location, 4 – a large area of dermal haemorrhage (usually flank or head) observed in one location with small foci of dermal haemorrhage observed in at least one other location, 5 – more than one large area of dermal haemorrhage observed.

**Cell Culture**

10T1/2 cells were maintained in Dulbecco’s modified Eagles’ medium plus Glutamax (DMEM-Gibco) supplemented with penicillin/streptomycin and 10% heat inactivated fetal calf serum. A404 cells were maintained in α-modified Eagles’ medium (α-MEM) supplemented with 7.5% fetal bovine serum, 200mg/µl L-glutamine and penicillin/streptomycin. For stimulation experiments, cells were plated at 50,000 per well in a 6 well plate and maintained in complete medium. They were treated for 4 days with either a control volume of PBS, 1µg/ml Tβ4, 2ng/ml TGF-β, or 1µg/ml Tβ4 plus 2ng/ml TGF-β or a neutralising rabbit polyclonal anti-Tβ4 antibody (1:250; Immundiagnostik). Each day, the medium was changed and the cells re-stimulated with the appropriate factors. On the fourth day, medium was aspirated, cells washed briefly with PBS and RNA extracted using Trizol reagent (Invitrogen). For Smad 2 phosphorylation assays, A404 cells were serum starved in 0.5% fetal calf serum in α-MEM containing 200mg/µl L-glutamine and penicillin/streptomycin (Gibco) overnight. The following day, cells were stimulated with 1µg/ml Tβ4, 2ng/ml TGF-β, or 1µg/ml Tβ4 plus 2ng/ml TGF-β or a control volume of PBS. Cells were stimulated for 20 minutes. Following stimulation, medium was aspirated and cells briefly washed in PBS prior to protein extraction.

**A404: HUVEC Co-culture**
Early passage human umbilical vein endothelial cells (HUVECs) were cultured in Endothelial Cell Growth Medium (PromoCell). For co-culture HUVECs were seeded at 20,000 cells per well in 6 well plates. After 24 hours, medium was replaced with 50:50 A404:HUVEC culture medium. After a further 48 hours, attached HUVECs were counted and an equal number of A404 cells were plated in each well. Thereafter, cells were co-cultured in A404 medium for four days, with or without the addition of a neutralising rabbit polyclonal anti-Tβ4 antibody (1:250; Immundiagnostik).

**Cell Transfections**

10T1/2 cells were seeded in 24 well plates at 50,000 cells per well. The following day 1µg of the relevant plasmid was transfected into 10T1/2 cells with Effectene transfection reagent (Qiagen) according to the manufacturer’s instructions. Smad activity luciferase reporter plasmids and appropriate positive and negative controls were purchased from SA Biosciences. Smad responsive constructs contained the Smad2/3/4 binding element AGCCAGACA. Following 16 hours of transfection, cells were serum starved by replacing the transfection medium with 0.5% FCS in DMEM + Glutamax. Cells were left overnight and the following day cells were stimulated for 6 hours in the presence of 100ng/ml Tβ4, 2ng/ml TGF-β, 100ng/ml Tβ4 plus 2ng/ml TGF-β or a control volume of PBS. Smad activity dependent firefly luciferase activity was then measured by means of a dual luciferase reporter assay (Promega) used according to the manufacturer’s instructions. Renilla luciferase activity was also measured as a transfection efficiency control, and firefly luciferase activity expressed as a proportion on renilla luciferase activity.

**Western Blotting**
Following stimulation, A404 cells were washed twice in PBS and protein extracted immediately by addition of hot (~90°C) Laemmli buffer (250mM Tris-Cl pH 6.8, 4% SDS, 25% glycerol, 0.1% bromophenol blue, 5% β-mercaptoethanol). Samples were separated on a 10% SDS-polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane which was blocked for 2 hours in blocking buffer (5% milk in Tris buffered saline, pH7.5). Membranes were then incubated overnight at 4°C in primary antibody (1: 500 in blocking buffer for Smad antibodies; 1:1000 for GAPDH). Membranes were washed 3 times over the course of 40 minutes in TBS plus 0.05% Tween-20. Membranes were then incubated in the secondary antibody at concentration 1 in 1,000 in blocking buffer at room temperature for 1 hour. After washing 3 times over the course of 40 minutes in TBS plus 0.05% Tween-20, protein bands were visualised by application of ECL western detection reagents (GE Healthcare). Bands were quantified using densitometry with ImageJ software.

Retinal Immunostaining

P6 mouse pups were culled by cervical dislocation and the globes enucleated. Retinas were dissected from globes in 2x PBS and subsequently stored in methanol at -20°C. For staining, methanol was aspirated and the retinas were fixed for 2 minutes in 4% formaldehyde. Formaldehyde was then aspirated and retinas left to block for 1 hour in retinal blocking buffer (2x PBS, 0.1% azide, 1% BSA, 3% Triton X-100, 0.5% Tween-20). Retinas were then incubated in primary antibodies at a concentration of 1 in 200 in retinal blocking buffer overnight at 4°C. The following day, retinas were washed five times in retinal blocking buffer over the course of an hour. Retinas were incubated in secondary antibody at a concentration of 1 in 200 in retina blocking buffer for 1 hour at room temperature. Retinas were again washed 5 times in retinal blocking buffer over the course of an hour in the dark at
room temperature. Retinas were post-fixed for 2 minutes in 4% formaldehyde before mounting and imaging.

**Exon Array Analysis**

Micro-Arrays were performed on Affymetrix Mouse Exon 1.0ST arrays on both E12.5 embryos (Tβ4 +/Y and Tβ4 -/Y) and on 10-week old adult hearts (Tβ4 +/-Y and Tβ4 -/-Y). Raw data were processed with Affymetrix expression console software before being analysed for gene expression changes in Partek. Statistics were performed in R. The top 200 up- and downregulated genes were fed into Metacore software from Genego and the reverse pathway analysis tool used to determine the most statistically perturbed signalling pathways.

**Statistics**

Statistical analysis was performed with Graphpad Prism software. Contingency tables were analysed by the chi squared test. Two tailed, unpaired, non-parametric T tests were used for all other statistical tests.
Online Figure Legends

Online Figure I. Generating a Thymosin β4 knockout mouse line.

Tβ4 was targeted by replacing exon 2 of the tmsb4x gene with a neomycin resistance cassette. A restriction map (A) of the wild type tmsb4x locus on the X chromosome, the targeting construct and the homologous recombinant allele. RV, EcoRV; H, HindIII; B, BamHI, S, SalI. Southern blot analysis to illustrate appropriate homologous recombination in an ES cell clone (B). Two separate probes were used: one against a 5’ flanking sequence (Tβ4 probe) and another within the neomycin selection cassette (neo probe), as shown in (A). Correct targeting was verified by the detection of genomic DNA fragments of the predicted sizes, with two separate restriction digests, EcoRV and HindIII, compared with a non-targeted WT 129/Sv ES cell line. Correctly targeted ES cells do not express Tβ4 (C), as determined by northern blotting of mutant and WT ES cells, using a cDNA probe against the Tβ4 coding region; Tβ4 mRNA levels were quantified by phosphorimaging and normalised against γ-actin levels. A mouse line was established from the correctly targeted ES clone and progeny were genotyped using a PCR that amplifies both mutant and wild type alleles (D, upper gel panel). A PCR to amplify a fragment of the SRY gene is used to distinguish male and female embryos (D, lower gel panel). Tβ4-null embryos do not express Tβ4, as confirmed by qRT-PCR analysis on whole E10.5 embryos (E) and immunofluorescence (F, G) on sagittal sections of whole E10.5 embryos (F) and frontal sections of E14.5 hearts (G). Scale bars: (F), 500 μm; (G), 200 μm.

Online Figure II. Dermal haemorrhage and reduced mural cell coverage in Tβ4-null embryos at E14.5
At E14.5, a proportion of Tβ4 -/Y embryos show dermal vascular haemorrhage (A, B) (black arrowheads). Whilst blinded to genotype, embryos were assigned a score 0 (none) to 5 (severe global bleeding) to quantify the extent of dermal haemorrhage observed. A significant difference was seen between the haemorrhage scores of Tβ4 +/Y and Tβ4 -/Y embryos (C) (n=21 per genotype). The dermal vasculature was investigated by immunostaining of endothelium (endomucin) and mural cells (NG2). Vessels in Tβ4 +/-Y specimens displayed a robust coverage with NG2 positive mural cells (D) whereas in Tβ4 -/Y specimens there was a reduction in resident mural cells with a complete absence in some sections of endothelium (E; white arrowheads).

Scale bars: (A, B) 2mm, (E) 10µm. Statistics: * p < 0.05; Two-tailed Mann–Whitney U test. Error bar represents standard error of the mean (SEM).

**Online Figure III.** Tβ4 global knock-out embryos which survive to E14.5 have epicardial-derived coronary vascular defects.

Sections through E14.5 hearts from Tβ4 +/-Y (A) and -/Y (B) embryos immunostained for PECAM and SMαA to detect coronary endothelial and smooth muscle cells (SMCs), respectively, revealed vascular cells in the epicardial and sub-epicardial layer in the Tβ4 -/Y mutant hearts which had failed to migrate into the underlying myocardium (B; highlighted by white arrowheads) and a lack of defined coronary arterioles as compared to Tβ4 +/-Y hearts (A; highlighted by white asterisks). Note also the sparse and disrupted myocardial layer in the Tβ4 -/Y mutants (B), arising from defective coronary vessel development, consistent with that previously demonstrated following shRNA knock down of Tβ4 (Smart et al., 2007). Ep, epicardium; my, myocardium. Scale bar: (A as applies to B) 50µm. The defective migration of coronary vascular precursors from the epicardium into the myocardium
was both quantifiable and highly significant (C: density of PECAM+ cells within the epicardium/subepicardium vs. myocardium; inset: ratio of myocardial:epicardial PECAM+ cell density; n=7 +/Y; n=6 −/Y; Statistics ***p≤0.001 (0.0004); Student’s t-test. Error bars represent standard error of the mean).

Online Figure IV. TB4 is knocked down in the endothelium of Tie2-Cre Tβ4 shRNA embryos.

Immunostaining for Tβ4 and PECAM confirmed knockdown of TB4 protein in the endothelium of the developing aorta in mutant Tie2-Cre Tβ4 shRNA embryos at E10.5 versus littermate controls (A-D). Two representative sections are shown for the control aorta (A, C) versus equivalent plane of sections of the mutant vessel (B, D). Tβ4 immunostaining alone highlighted reduced protein level in the mutant aortas (E-H) which was significant, as determined using Image J analysis across n=6 matched pairs of embryo sections. Statistics ***p≤0.001 (5.64995E-05). Two-tailed Mann–Whitney U test. Error bars represent standard error of the mean (SEM). DA, dorsal aorta; Scale bars: (A-H) 25μm.

Online Figure V. Mural cell coverage of vessels is normal in Tβ4-null postnatal retinas

Tβ4 is expressed in the primary vascular plexus of the post-natal (P6) retina (A, B; red arrowheads in A highlight expression, and dashed line in B demarcates primary plexus boundary). NG2 immunostaining for isolectin b4 (Ilb4) and NG2 of P6 retinas revealed no difference in the mural cell coverage of the retinal vascular primary plexus between Tβ4 −/Y animals and Tβ4 +/Y controls (C-E). Scale bars: (A)
100µm, (B) 50 µm, (C, D) 50µm. Error bars represent the standard error of the mean (SEM).

**Online Figure VI. Normal levels of apoptosis and cell proliferation in the mural cell layer of Tβ4 null dorsal aortas**

Co-immunostaining for the apoptotic marker cleaved caspase 3 (CC3) and the mural cell marker SMA revealed an absence of apoptosis in the mural cell layer of dorsal aortas in E10.5 Tβ4 -/Y embryos and Tβ4 +/+Y controls (A, C). Punctate nuclear CC3 staining in the overlying surface epithelium of the embryo in Tβ4 +/+Y and Tβ4 -/-Y animals serve as a positive control for this staining (B, D). Similarly, co-immunofluorescence for SMA and the proliferative marker phospho-histone H3 (PHH3) demonstrated a lack of proliferation in the dorsal aorta mural cells of both Tβ4 +/+Y and Tβ4 -/-Y E10.5 embryos. PHH3 staining, in cells in the embryonic mesoderm serve as positive controls for this staining (white arrowheads) (E, F). Scale bars: (A-F) 25µm.
Essential Role for Thymosin β4 in Regulating Vascular Smooth Muscle Cell Development and Vessel Wall Stability
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