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Endoglin Plays Distinct Roles in Vascular Smooth Muscle Cell Recruitment and Regulation of Arteriovenous Identity During Angiogenesis

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Abstract

Blood vessel formation is a multi-step process. Endoglin is a TGFβ coreceptor required for angiogenesis. Endoglin null embryos exhibit a loss of arteriovenous identity and defective vascular smooth muscle cell (vSMC) recruitment. Haploinsufficiency of endoglin results in Hereditary Hemorrhagic Telangiectasia (HHT), characterized by a loss of arteriovenous identity and aberrant vSMC incorporation in fragile vessels. We explored a cell autonomous role for endoglin in endothelial and vSMCs during angiogenesis by conditionally activating endoglin expression in wild type or endoglin null embryos using either smooth muscle (SM22αcre) or endothelial cell (Tie2cre) promoters to partially rescue vSMC recruitment to the dorsal aorta. Examination of endoglin null embryos revealed ectopic arterial expression of the venous specific marker COUPTFII. Endoglin re-expression in endothelial cells restored normal COUPTFII expression. These results suggested that endoglin plays distinct and cell autonomous roles in vSMC recruitment and arteriovenous specification via COUPTFII in angiogenesis and may contribute to HHT.

Keywords

Endoglin; Endothelial cell; Smooth muscle; Transforming growth factor beta; COUPTFII; Vascular development; Hereditary hemorrhagic telangiectasia

INTRODUCTION

The embryonic vasculature is the earliest organ system to develop and its function is essential for all subsequent developmental processes (Cleaver and Melton, 2003; Lebrin et al., 2005). The vasculature of the developing embryo forms through distinct but interdependent processes of vasculogenesis and angiogenesis (Carmeliet, 2000; Jain, 2003). Endothelial cells differentiate from mesodermally derived angioblasts in response to signals provided by surrounding tissues (Kappel et al., 1999) and the extracellular matrix (Eliceiri and Cheresh, 2001; Provenzano et al., 2006). The differentiating angioblasts aggregate into a primitive vascular plexus (vasculogenesis), which subsequently undergoes growth, migration, and sprouting (angiogenesis), resulting in the development of a functional circulatory system (Cleaver and Melton, 2003; Coultas et al., 2005).

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During the earliest stages of vasculogenesis, endothelial cells adopt either an arterial or venous identity (Lawson and Weinstein, 2002; Carmeliet, 2005; Swift and Weinstein, 2009). Arterial identity is determined by a cascade that involves VEGF/Neuropilin-1 (NP1) signaling, and results in the expression of Notch signaling components, which, upon activation, upregulate expression of ephrin-b2 (You et al., 2005). Venous identity was previously considered a default pathway. However, COUPTFII (chicken ovalbumin upstream promoter transcription factor II) was demonstrated to be a regulator of venous identity. COUPTFII is specifically expressed in the venous endothelium, although low levels of expression are detected in vascular smooth muscle cells (vSMCs) of arteries. The mechanism through which COUPTFII regulates venous identity appears to involve inhibition of NP1 expression with subsequent inhibition of expression of Notch, ephrin-b2, and arterial specific genes, thereby allowing vein-specific genes, including the ephrin receptor B4 (EphB4), to be expressed (You et al., 2005; Swift and Weinstein, 2009).

Endothelial cells cannot complete mature vessel formation independently. As angiogenesis progresses, vessels become surrounded by layers of mesenchyme-derived pericytes in smaller vessels and vSMCs in larger vessels, which produce signals that are essential for the generation and stabilization of mature blood vessels (Carmeliet, 2000; Armulik et al., 2005). The proper assembly of the vessel wall depends upon reciprocal communication between endothelial cells and vSMCs (Hirschi and D'Amore, 1997; Chakravarthy and Gardiner, 1999; Conway et al., 2001). This communication involves multiple signaling pathways, the best characterized of which are the PDGF, Ang-1/Tie2, and TGFβ pathways (Carmeliet, 2000; Cleaver and Melton, 2003; Coultas et al., 2005). According to the current model, PDGF secreted by endothelial cells recruits pericytes to the developing vessels. Pericytes/vSMC secrete Ang-1, a ligand for the endothelial specific receptor, Tie2, which induces endothelial cell stabilization. TGFβ signaling is required for recruitment of vSMC, though the mechanisms through which it acts are unclear.

TGFβ signaling ensues through ligand binding to heteromeric complexes of type I activin-like kinase (ALK) and type II serine/threonine kinase receptors. The type I TGFβ receptors phosphorylate downstream effectors, the best characterized of which are the Smad family of transcription factors (reviewed in (Feng and Derynck, 2005)). The TGFβ type I and II receptors interact with type III coreceptors, including endoglin, though the function of endoglin within (Blanco et al., 2005; Koleva et al., 2006) or outside (Conley et al., 2004) the receptor complex remains poorly understood.

The importance of TGFβ superfamily signaling in vascular development and homeostasis is revealed by mutational analysis. In humans, hereditary hemorrhagic telangiectasia (HHT) is an autosomal dominant disorder characterized by arteriovenous malformations (AVM) in the brain, lung, liver, and gastrointestinal tract. Specifically, vascular lesions in HHT include loss of capillary beds with direct shunting of arterial blood into postcapillary venules, which become arterialized with increased vSMC in the vessel wall. HHT is caused by mutations in either endoglin (McAllister et al., 1994) or ALK-1 (Johnson et al., 1996). AVMs in HHT likely arise from dysregulation of arteriovenous identity. Analyses of mice carrying mutations of either endoglin or ALK-1 have provided insight into the molecular mechanisms through which they act. Similar to HHT, mouse embryos lacking either endoglin or ALK-1 develop AVMs resulting from anomalous fusions between the dorsal aorta and cardinal vein. In addition, ectopic hematopoietic clusters are observed in the cardinal vein of both endoglin −/− and ALK-1−/− embryos, which is consistent with loss of venous identity. Arterial ephrin-B2 expression is lost in ALK-1−/− embryos, consistent with loss of arterial identity. Intriguingly, arterial ephrin-B2 expression is maintained in endoglin−/− embryos. Notch signaling, which is required for arterial identity, appears unaffected in both endoglin−/− and ALK-1−/− embryos, indicating that the Notch pathway is not downstream of ALK-1/

endoglin signaling during development (Sorensen et al., 2003). Taken together, these results suggest that ALK-1/endoglin signaling involves pathways required for the development and maintenance of arteriovenous identity.

ALK-1 and endoglin also appear to orchestrate smooth muscle cell recruitment to developing vessels, potentially via regulation of arterial versus venous identity. Mice lacking ALK-1 (Oh et al., 2000; Urness et al., 2000) or endoglin (Li et al., 1999; Arthur et al., 2000; Bourdeau et al., 2000) have similar embryonic angiogenic defects, including an overall reduction of vSMC number and recruitment to the vasculature plexus in the yolk sac and embryo. Thus, endoglin and ALK-1 are necessary for recruitment of vSMC or the differentiation of vSMC, or both, in the developing vasculature. However, endoglin's role in vSMC-autonomous versus paracrine signaling from endothelial cells is largely uncharacterized.

Because an endoglin conditional null mouse has not been available, it has not been possible to investigate the cell autonomous requirement for endoglin in vSMC versus endothelial cells. To study this intriguing question in vivo, we employed a transgenic complementation strategy in which endoglin was expressed in either endothelial or vSMCs of endoglin null mice. Interestingly, endoglin expression in either endothelial cells or vSMCs in an endoglin null background partially rescued vSMC recruitment to the dorsal aorta, supporting the hypothesis that endoglin plays a cell-autonomous role in both endothelial cells and vSMCs. Furthermore, we provide evidence that endoglin regulates the expression of COUPTFII, thereby identifying a mechanism by which endoglin contributes to the establishment of arterial versus venous identity. In endoglin null embryos, COUPTFII was misexpressed in arterial endothelial cells, and the reconstitution of endoglin expression in endothelial cells, but not vSMCs, restored normal regulation of COUPTFII expression. These results provide novel insights into the function of endoglin, which is critical to understanding the mechanisms of angiogenesis and vascular homeostasis, and the development of HHT.

RESULTS

To distinguish between primary and secondary effects of endoglin in endothelial cells and vSMCs during vascular development, we used a genetic gain-of-function strategy to express endoglin specifically in endothelial cells and vSMCs by using the $TgEng^{LoxP}$ transgenic strain (Mancini et al., 2007) with two well-characterized cre-driver systems: (i) Tie2cre, for conditional activation in endothelial cells (Kisanuki et al., 2001), and (ii) SM22αcre for conditional activation of endoglin in vSMCs (Holtwick et al., 2002). In vivo cre-recombination and subsequent expression of the L-isoform (Bellon et al., 1993) of endoglin were confirmed by detection of the conditionally expressed endoglin protein from whole Tie2cre;$TgEng^{LoxP}$ and SM22cre;$TgEng^{LoxP}$ embryos. Western blotting of individual E10.5 Tie2cre;$TgEng^{LoxP}$ and SM22cre;$TgEng^{LoxP}$ embryos followed by western blotting (Mancini et al., 2007) confirmed that endoglin transgene expression was evident as a band with electrophoretic mobility just slightly greater than the pair of constant bands comprising endogenous endoglin, and that protein levels were comparable between embryos (Figure 1A). To determine that conditional transgene expression resulted in efficient production of the mature, glycosylated form of endoglin (Lux et al., 2000), immunoprecipitation of total endoglin from a pool of three double-transgenic Tie2cre;$TgEng^{LoxP}$ embryos confirmed that the conditionally expressed protein was predominantly a single polypeptide with the mobility expected for the mature glycosylated form of endoglin (Figure 1B).

Cre-mediated expression of endoglin in endothelial cells of wild type mice (Tie2cre;$TgEng^{LoxP}$) resulted in hemorrhaging at E9.5 in a subset of the mutants, which appeared embryonic lethal (Figure 1C versus E). The growth of the Tie2cre;$TgEng^{LoxP}$

embryos was not retarded compared to wild type. Histological analysis of the major vessels revealed normal expression of the endothelial marker PECAM in Tie2cre;TgEng^{LoxP} embryos, which was similar to the wild type and the endoglin null embryos (Figure 1F–H) (Bourdeau et al., 1999; Li et al., 1999). Examination of the expression of the vSMC differentiation marker αSMA revealed sparse vSMC coverage of the paired dorsal aorta in contrast to wild type (Figure 1K). However, the overall level of αSMA expression in the Tie2cre-induced transgenic dorsal aorta based on a quantitative analysis appeared to be somewhat diminished compared to wild type (Figure 7A). The finding that conditional expression of endoglin in endothelial cells caused mild hemorrhaging and a reduction in αSMA staining suggested a defect in vascular integrity, a phenotype resembling that observed by activating endoglin in neural crest-derived vSMCs by using Wnt1cre (Mancini et al., 2007). This phenotype may result from alterations in vSMC or precursor investment or differentiation or both.

Using the SM22αcre driver, we conditionally expressed endoglin in vSMCs (SM22cre;TgEng^{LoxP}). SM22cre;TgEng^{LoxP} embryos exhibited hemorrhaging at E9.5 that was more severe than that observed in the Tie2cre;TgEng^{LoxP} embryos (Figures 2A–C and refer to Figure 1C). In contrast to Tie2cre;TgEng^{LoxP} embryos, in which hemorrhaging appeared to be restricted to the dorsal aorta (Figure 1E), hemorrhaging in the SM22cre;TgEng^{LoxP} embryos was observed in the abdomen as well as the aorta (Figure 2C). Histological analysis of the major vessels and the hearts of E9.5 SM22cre;TgEng^{LoxP} embryos revealed normal expression of PECAM in the vessels compared to wild type and endoglin null sections (Figure 2D–F). Interestingly, a substantial increase in αSMA staining was found in the area surrounding the paired dorsal aorta of SM22cre;TgEng^{LoxP} embryos (Figure 2I), which was confirmed by image analysis of αSMA staining (Figure 7B). These results are similar to those observed in transgenic embryos in which endoglin conditional expression was driven by Wnt1cre (Mancini et al., 2007). Taken together with the analysis of the Tie2cre;TgEng^{LoxP} embryos, these data indicate that conditional expression of endoglin in either endothelial cells or vSMCs in vivo results in aberrant vSMC investment in the walls of major vessels, potentially by altering the differentiation of vSMC or their precursor cells.

To examine the potential for distinct cell-specific requirements for endoglin in endothelial cells and vSMCs during development, we used genetic complementation to restore endoglin expression in a cell type-specific manner in endoglin null embryos. By crossing heterozygous endoglin null mice bearing the Cre-inducible endoglin transgene (TgEng/EngHet) with Tie2cre and SM22αcre driver mice on an Eng heterozygous background, we obtained Tie2cre;TgEng/Null and SM22cre;TgEng/Null embryos. We first assayed for rescue of embryonic lethality of the endoglin null phenotype by using the Tie2cre driver. At E11.5, there were no viable Tie2cre;TgEng/Null embryos (data not shown). Confirming our previous results, evidence of hemorrhage was observed in Tie2cre;TgEng^{LoxP} embryos (Figure 3B). In contrast, Tie2cre;TgEng/Null embryos lacked evidence of hemorrhaging seen for conditional endoglin expression on the wild type background, and were grossly indistinguishable from endoglin null embryos at E9.5 (Figure 3C,D). Histological analysis of the Tie2cre;TgEng/Null embryos at E9.5 was performed. Expression of the transgene on the null background was confirmed by immunohistochemistry using an α-endoglin antibody that preferentially recognizes the human form of endoglin (Rodriguez-Pena et al., 2002) (Figure 4A–C). PECAM staining revealed no alterations in location or gross morphology of endothelial cells (Figure 4D–F). Interestingly, αSMA staining in the Tie2cre;TgEng/Null embryos demonstrated partial rescue of vSMC αSMA staining and recruitment to the dorsal aorta and cardinal vein (Figure 4I), which achieved nearly 60% of the wild type αSMA staining (Figure 7C), demonstrating partial rescue of αSMA expression and differentiation in vSMCs.

To analyze the contribution of endoglin to vSMC function, we examined the consequences of restoring endoglin expression specifically in the vSMCs by using the SM22αcre driver (SM22cre;TgEng/Null). No viable embryos of the SM22cre;TgEng/Null genotype could be found at E11.5. SM22cre;TgEng embryos showed hemorrhaging (Figure 5B), but similar to the Tie2cre;TgEng/Null, SM22cre;TgEng/Null embryos lacked evidence of hemorrhaging and were grossly indistinguishable from endoglin null embryos at E9.5 (Figure 5C,D).

Recombination and expression of the transgene on the endoglin null background were verified through immunohistochemical analysis on sections through the heart and major vessels (Figure 6A–C). PECAM staining highlighted a normal pattern of endothelial cells in the SM22cre;TgEng/Null embryos, similar to wild type and endoglin null embryos (Figure 6D–F). Remarkably, examination of αSMA expression in the SM22cre;TgEng/Null embryos revealed the presence of vSMCs around the dorsal aorta at E9.5. The partial rescue of vSMC recruitment was less profound than that seen in the Tie2cre;TgEng/Null embryos because there were no αSMA-positive cells surrounding the cardinal vein (Figure 6G–I). However, there were αSMA-positive cells present in both vessels comprising the paired dorsal aorta, indicating that the rescue occurred to an extent similar to that seen for Tie2cre;TgEng/Null embryos, as confirmed by image analysis (Figure 7D), and the presence of αSMA-positive cells was bilateral.

Smooth muscle cell functional rescue in Tie2cre;TgEng/Null and SM22cre;TgEng/Null embryos was next studied by immunodetection of SM22α. SM22α is an early vSMC marker expressed by E8.0 in precursors surrounding the paired dorsal aorta (Figure 8 row A) (Zhang et al., 2001). In endoglin null embryos, there is a lack of association of SM22α-expressing cells with endothelium of the dorsal aorta (Figure 8 rows B and C), suggesting that vSMC precursors require endoglin for recruitment to vessels.

Consistent with αSMA staining, robust SM22α expression was observed in endothelium-associated cells of Tie2cre;TgEng/Null and SM22cre;TgEng/Null embryos (Figure 8 row C). Quantitation of SM22α immunostaining confirmed that complementation of endoglin expression in either the endothelial or smooth muscle compartment rescued differentiation and recruitment of vSMCs associated with the dorsal aortae (Figure 8D). The disorganized SM22α stained cells can clearly be seen in the endoglin null embryos, and contrasts with the vessel associated SM22α positive cells in the conditional endoglin-expressing null embryos, suggesting that null cells were be blocked in their capacity for vascular recruitment and differentiation.

Endoglin null embryos exhibit apparent defects in arteriovenous identity during vascular development due to the presence of ectopic hematopoietic clusters in the cardinal vein (Sorensen et al., 2003). Similar ectopic hematopoietic clusters are also present in the cardinal vein of COUPTFII null embryos (You et al., 2005). Because COUPTFII was described as a novel regulator of venous identity, we examined expression of COUPTFII in the endoglin null embryos to ascertain whether endoglin might play a role in the arteriovenous specification process. Consistent with previous reports (You et al., 2005), in wild type embryos COUPTFII was expressed in the endothelial cells of the cardinal vein, and its expression was absent from the endothelium of the dorsal aorta (Figure 9A). Interestingly, there was ectopic expression of COUPTFII in the endothelial cells of the paired dorsal aorta in E9.5 endoglin null embryos (Figure 9B). Normal venous expression of COUPTFII was observed in the endoglin null embryos (Figure 9A',B'). Arterial endothelial cells ectopically expressing COUPTFII also coexpressed endoglin, as indicated by Eng-driven LacZ expression (Figure 9C). The expression of COUPTFII in endoglin null embryos was further evaluated using immunofluorescence microscopy. Ectopic COUPTFII expression was confirmed in the null versus wild type sections by immunofluorescence

staining, as was COUPTFII expression in endothelial cells lining the aorta of endoglin null sections, as visualized by double immunofluorescence colocalization using anti- von Willebrand factor (vWF) antibody (Gang et al., 2006; Hasegawa et al., 2007) and anti-COUPTFII antibody (Figure 9D). Aortic vSMCs visualized using anti-SM22α antibody revealed sporadic COUPTFII that was not different between wild type and endoglin null sections (Figure 9E).

To further explore the role of endoglin in the regulation of COUPTFII expression in the arterial endothelium, we examined the expression of COUPTFII in the Tie2cre;TgEng^{LoxP} embryos at E9.5 by immunohistochemistry. Conditional expression of endoglin in the endothelium did not alter expression of COUPTFII relative to wild type (Figure 9F,G) and ectopic expression in the dorsal aorta was not observed (Figure 9G).

We next examined expression of COUPTFII in the Tie2cre;TgEng/Null and SM22cre;TgEng/Null embryos to determine whether ectopic expression of COUPTFII in the endoglin null could be eliminated by reconstituting endoglin expression in the endothelium or the vSMCs. In the Tie2cre;TgEng/Null embryos, but not in the SM22cre;TgEng/Null embryos, we observed a reduction in ectopic expression of COUPTFII in the dorsal aorta, thereby indicating that replacing endoglin expression in these cells restored normal expression of COUPTFII (Figure 9H,I). Because ectopic expression of COUPTFII in arterial endothelium persisted in the SM22cre;TgEng/Null embryos (Figure 9I', versus 9H'), we suggest that endoglin's regulation of arteriovenous identity occurs in an endothelial cell-autonomous manner in the developing vasculature. Taken together, these results support the notion that lack of endoglin expression in arterial endothelial cells is sufficient to induce ectopic expression of COUPTFII. This finding places endoglin upstream of COUPTFII and suggests a novel mechanism by which endoglin regulates arteriovenous specification.

In embryos in which COUPTFII was conditionally inactivated within the endothelium, the arterial markers Notch1, Jagged1, Ephrin-B2, NP1, and Hey1 were ectopically expressed in the endothelium of mutant veins (You et al., 2005). Previous studies in endoglin null embryos examined the expression of signaling components involved in arterial specification, including Notch1, 3, and 4, Delta-like 4, Jagged 1 and 2, HRT1, and ephrin-B2, and no differences were found compared to wild type controls (Sorensen et al., 2003). Therefore, we examined the expression of the remaining known COUPTFII target genes, NP1 (Figure 10A–F) and EphB4 (Figure 10G–L) in endoglin mutant embryos and found no differences in expression as compared with wild type embryos. Collectively, the data from endoglin null embryos suggest inconsistencies within the current model of determination of arterial versus venous identity (Lawson and Weinstein, 2002; Adams, 2003; You et al., 2005). Specifically, endoglin appears be upstream of COUPTFII, but expression of COUPTFII's downstream targets, including Notch1, Jagged1, ephrin-b2, EphB4, and NP1, are not altered in the endoglin mutant embryos.

DISCUSSION

The present study is the first to examine a dual role for endoglin in angiogenesis through cell autonomous signaling in both vSMC recruitment and arteriovenous specification of endothelial cells. Defects in arteriovenous specification and aberrant vSMCs are hallmark traits associated with HHT. However, the full contribution of endoglin haploinsufficiency to the vascular phenotype of this disorder has not been fully defined.

Here, we approached the problem of assessing the distinct roles of endoglin expression in endothelial and vascular smooth muscle cells using conditional endoglin expression on the systemic endoglin null background. This versatile strategy enables analysis of cell type-

specific effects of endoglin in a well-characterized null mouse model. Well recognized limitations of this approach are that it may lead to levels of protein that may not precisely match the levels or timing of endogenous protein expression. The issue of timing is addressed by using well characterized Cre driver mouse lines. However, the comparison of the wild type mouse endogenous versus conditionally expressed endoglin levels remains challenging, due to the technical limitations of quantitative immunoblot measurements. Despite these limitations, we suggest that this is an informative complementary approach to conditional gene inactivation, which is subject to a distinct set of similarly problematic limitations.

Using both endothelial and vSMC-specific cre driver mice, we observed that conditional expression of endoglin on the wild type background resulted in hemorrhaging due to apparent defects in vascular integrity. The defects in vascular integrity correlated with aberrant vSMC investment in the dorsal aorta and was consistent with the apparently normal endothelial cell morphology characteristic of the endoglin null embryonic phenotype and in HHT (Bourdeau et al., 1999; Li et al., 1999). Reconstitution of endoglin expression in either endothelial cells or vSMCs in the null background was not sufficient to rescue embryonic lethality; however, the vSMC recruitment defect observed in endoglin null embryos was partially restored by reconstitution of endoglin expression either endothelial or vSMC.

These data suggest opposing effects of endoglin conditional expression in endothelial cells on the wild type versus endoglin null backgrounds; that is, hemorrhage versus increased vSMC recruitment, respectively. It is probable that conditional expression of endoglin in wild type embryos produces excessive levels of endoglin, leading to a disruptive effect on endothelial-smooth muscle interactions. However, the partial rescue and the lack of hemorrhage seen upon transgene expression on the null background support the view that a more physiological level of expression is achieved in the null context, leading to a course of development more closely resembling wild type.

Though immunoblotting suggests that conditionally expressed endoglin levels may greatly exceed endogenous levels of endoglin expression, this effect can largely be explained by the greater affinity of the anti-endoglin antibody for human endoglin expressed from the transgene versus mouse endoglin (Rodriguez-Pena et al., 2002). The CAGCAT transgene promoter was chosen over previous CMV-derived versions due to its moderate expression levels (Yamauchi et al., 1999). Indeed, pooled anti-endoglin antibody-immunoprecipitated protein lysates from embryos conditionally expressing endoglin showed no evidence of immature non-glycosylated endoglin, demonstrating that the transgenic endoglin was properly processed. Moreover, primary cultures of both human aortic endothelial cells and vSMCs express very high endogenous levels of endoglin (Conley et al., 2000), suggesting that conditional expression of endoglin on the null background was not excessive.

The intriguing observation that restoration of endoglin expression in vSMCs alone partially rescued their recruitment strongly suggests that endoglin performs a distinct function in vSMC, and that endoglin expression by vSMC or vSMC precursors is required for normal angiogenesis. Furthermore, based on these observations, it is unlikely the lack of vSMCs observed in the null phenotype is solely a secondary effect resulting from a loss of endoglin expression in endothelial cells. Most importantly, we observed a novel function of endoglin in the specification of arteriovenous identity in endothelial cells by regulating COUPTFII expression. The re-expression of endoglin in endothelial cells, and not vSMCs was sufficient to rescue ectopic expression of COUPTFII in arterial endothelial cells. Taken together, our data demonstrate that endoglin plays novel and distinct roles orchestrating vSMC recruitment in both endothelial cells and vSMCs, and regulates arteriovenous identity through a mechanism that involves regulation of COUPTFII.

Conditional expression of endoglin driven by either Tie2cre- or SM22αcre-driven Cre recombination resulted in a vSMC phenotype, however the effects appeared to be distinct and opposing. As compared to wild type embryos, Tie2cre;TgEng^{LoxP} embryos had fewer vSMCs or less vSMC investment in the dorsal aorta at E9.5, and SM22cre;TgEng^{LoxP} embryos appeared to have an increase in the thickness of the vSMC layers in this vessel. Because impaired vSMC investment resulting from conditional expression of endoglin in endothelial cells appeared to act in a similar manner to loss of endoglin expression, it raises the possibility that a critical level of endoglin expression is required for its normal function. In this scenario, altered expression levels of endoglin would not send the appropriate signals to recruit vSMC precursor cells, leading to the malformation of the vSMC layer, and loss of vascular integrity. Similar likenesses between null and conditional expression phenotypes have been found for Notch pathway components (Gridley, 2001). The observation that endoglin conditional expression in vSMC results in increased thickness of the vSMC layer is consistent with the phenotype observed following conditional expression of endoglin in the neural crest, in which vSMC layers were thicker due to increased vSMC specification of neural crest stem cell progenitors (Mancini et al., 2007). These results support the view that endoglin plays a role in the specification of a more general population of vSMC precursors in addition to neural crest-derived vSMC.

Cell type-specific reconstitution of endoglin expression in endothelial cells and vSMCs, on the endoglin null background, both partially rescued the vSMC recruitment defect. This view is consistent with distinct requirements for endoglin expression in endothelial cells and vSMCs, for proper communication with or recruitment of vSMCs. As expected based on this hypothesis, we did not rescue embryonic lethality with individual cre drivers. However, additional factors may also have contributed to this observation, including the timing of expression of the individual promoters. Furthermore, a wider cell type expression of endoglin exists beyond endothelial and vSMCs, including hematopoietic progenitors, in which endoglin expression was not restored and may be required for embryonic survival.

To elaborate on this point, an alternative hypothesis is that endoglin is required in progenitor cells, earlier than the timing of expression of the SM22αcre or Tie2cre transgenes. Indeed, High et al. reported that SM22αcre-driven expression of their transgene did not occur until after induction of vSMC differentiation markers (High et al., 2007). It would be interesting to employ a conditional null endoglin mouse when available in conjunction with an earlier cre expression system to examine vascular development (Allinson et al., 2007). Possibilities include Hoxb6-cre (Lowe et al., 2000) and Meox1cre (Jukkola et al., 2005). Hox6b-cre expression is activated in lateral plate mesoderm cells, which have been considered to be the major source of aortic smooth muscle cells. Recent lineage tracing studies using Hox6b–cre (lateral plate mesoderm) and Meox1cre (paraxial mesoderm) suggest that vSMC on the ventral side of the aorta at E9.0-E9.5 are derived from lateral plate mesoderm, but these cells are replaced by somite-derived smooth muscle cells at E11 (Wasteson et al., 2008). Results fom this study also suggest that aortic endothelial cells arise from the lateral plate mesoderm. Because cellular context and timing of endoglin expression are likely to be critical for elucidating its function, the origins of endoglin-expressing progenitor cell populations must be carefully delineated.

The data presented identified a novel role for endoglin in arteriovenous identity through regulating COUPTFII expression. COUPTFII was ectopically expressed in aortic endothelial cells in endoglin null embryos, suggesting that endoglin represses COUPTFII expression in arterial endothelial cells. Our data indicated that ectopic COUPTFII expression did not result in altered expression of EphB4 or NP1 (You et al., 2005). The lack of altered expression of COUPTFII target genes may reflect reduced levels of the ligand for COUPTFII (Lin et al., 2000) or a COUPTFII cofactor (Pipaon et al., 1999; Kang et al.,

2003), such that COUPTFII signaling is blocked in the endoglin null embryo. In addition, endoglin may be participating in a feedback loop in arterial endothelial cells preventing the expression of COUPTFII. Our data suggest that endoglin expression in arterial endothelial cells is necessary and sufficient to prevent ectopic expression of COUPTFII, though the mechanisms through which it exerts it effects remain obscure. Examination of this mechanism in vitro poses a challenge. To date, there are no in vitro based assays available because many, if not all, arterial endothelial cell lines express COUPTFII. Therefore, cell culture-based systems do not accurately recapitulate the function of COUPTFII in vivo and future mechanistic studies will have to be conducted using genetic models in animal systems.

In the absence of two functional copies of endoglin, HHT lesions may originate, remodel, and regress early in human development (Morgan et al., 2002; Mei-Zahav et al., 2006). The data presented here provide novel insights into distinct roles for endoglin in endothelial cells and vSMCs contributing to the knowledge of the multifaceted nature of HHT. HHT is comprised of both aberrant vSMC investment, accompanied by fragile vessels, and loss of arteriovenous identity. Taken together, our data suggest that defects in endoglin expression in humans may contribute to both of these HHT phenotypes. We have provided further evidence that endoglin is involved with specification and possibly recruitment of vSMCs from progenitors in a cell autonomous manner. This view is consistent with previous work in which we demonstrated that endoglin specified vSMCs from neural crest stem cell populations (Mancini et al., 2007). It will be important to determine these independent mechanisms of endoglin function to further understand the complex processes of angiogenesis and design treatments for vascular diseases such as HHT.

EXPERIMENTAL PROCEDURES

Endoglin null and transgenic mouse studies

Embryos were harvested at either E9.5 or E10.5, as indicated in the text. Endoglin null C57Bl6 mice developed by Dr. H. Arthur (Arthur et al., 2000) were kindly provided by Dr. D. Marchuk. Transgenic mouse embryos used in this study were maintained on an FVB background (Mancini et al., 2007). Mice were maintained according to the NIH standards established in the “Guidelines for the Care and Use of Experimental Animals.” Protocols and procedures were approved by the Institutional Animal Care and Use Committee at the Maine Medical Center Research Institute. Briefly, full-length human endoglin cDNA was ligated to an EGFP construct containing an intervening internal ribosomal entry site, as previously described (Mancini et al., 2007). This construct was blunt end-ligated into the CAGCAT vector (Yamauchi et al., 1999). Microinjection into fertilized FVB oocytes and other surgical procedures were performed as described (Kisanuki et al., 2001). Chloramphenicol acetyl transferase (CAT) enzyme-linked immunosorbent assay was used to normalize the transgene locus CAT activity levels before establishment of these mouse lines.

The Tie2cre (Constien et al., 2001) and SM22αcre (Miano et al., 2004) drivers were used to achieve conditional LoxP excision, recombination, and expression of the endoglin transgene in endothelial cells and vSMCs, respectively. Kisanuki et al. determined that Tie2cre expressed with the CAGCAT vector system recombined in the E8.5 yolk sac, and was expressed in most endothelial cells by E9.5 (Kisanuki et al., 2001). Similarly, Miano et al. determined that SM22αcre-mediated excision through embryonic day E9.5 produced phenotypic effects by E10.5 in vSMC recruitment to the dorsal aorta (Miano et al., 2004). Therefore, the timing of excision by these cre LoxP systems is advantageous because it precedes endoglin-null lethality occurring at E10.5. Mating of Tie2cre and SM22αcre mice with $TgEng^{LoxP}$ generated cell type-specific $TgEng^{LoxP}$ embryos that were identified by genotyping using PCR primers, as previously described (Rodriguez-Pena et al., 2002).

Briefly, yolk sac genomic DNA samples were amplified using Hot Master Mix (Eppendorf), according to the manufacturer's instructions. The primers used for genotyping are listed in Table 1, and all PCR products were confirmed by sequence analysis.

Mouse strains used in the genetic complementation assay were generated as follows: Endoglin heterozygous mice (Arthur et al., 2000) were bred individually to the Tie2cre and SM22αcre lines to generate Tie2cre;EngHet and SM22cre;EngHet mice. In parallel, endoglin heterozygous mice were bred with $TgEng^{LoxP}$ to generate EngHet;$TgEng^{LoxP}$ mice. Breeding between Tie2cre;EngHet and SM22cre;EngHet males with EngHet;$TgEng^{LoxP}$ females produced cre;$TgEng^{LoxP}$/Null embryos in a ratio of ~1/16.

Immunoprecipitation and western blot analysis of embryos

Embryos were harvested at E9.5. Whole embryos were dissociated by passing through a 1 mL syringe into RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 300 mM sucrose, 1.0% Triton-X100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and 10 mM β-glycerol phosphate) supplemented with a protease inhibitor cocktail (Roche), and processed as previously described. Total protein from each lysate was determined by Bradford assay (BioRad) prior to immunoprecipitation. Immunoprecipitation of endoglin was conducted using the P4A4 anti-endoglin antibody (Mancini et al., 2007). For western blotting, the anti-human endoglin antibody Clone 35 at 1:2000 (BD Transduction), was used to demonstrate cre-recombination and conditional expression of the human endoglin transgene in the $TgEng^{LoxP}$ embryos.

Histological analysis

X-gal staining was performed according to standard procedures (Hogan et al., 1994). For hematoxylin and eosin staining and immunohistochemistry, embryos were fixed in 4% paraformaldehyde overnight, subsequently dehydrated through a graded ethanol series, and paraffin embedded. Five mm sections were stained with hematoxylin and eosin for morphological analysis, and immunohistochemistry (IHC) was performed using: anti-PECAM antibody (BD Pharmingen) 1:3000, α-smooth muscle actin antibody (αSMA, Sigma) 1:500, α-COUPTFII antibody (R&D Systems), 1:500, α-human endoglin antibody (SN6h) 1:500 (Dako), goat α-NP1 antibody 1:300 (R&D Systems), and α-EphB4 antibody 1:300 (R&D Systems). Anti-goat horseradish peroxidase-conjugated antibody was from Vector Laboratories. Immunohistochemistry for PECAM was performed using the TSA Biotin kit (PerkinElmer) with trypsin antigen retrieval per manufacturer's instructions. IHC for endoglin was performed by using citrate retrieval and the TSA Biotin kit. αSMA IHC was performed by using the Avidin/Biotin kit (Vector Laboratories) with citrate buffer antigen retrieval (Dako) per manufacturer's instructions. IHC for NP1 and EphB4 were performed on embryos fixed in 4% paraformaldehyde for 15 minutes, and frozen in OCT (Tissue Tek), followed by citrate buffer antigen retrieval (Dako) and Avidin/Biotin development of the alkaline phosphatase detection system (ABC, Vector Labs).

For vWF and COUPTFII immunofluorescence, deparaffinized and hydrated sections were subjected to antigen retrieval by steaming for twenty-two minutes in antigen retrieval solution (Dako Cytomation, 1:10 dilution). Sections were incubated at 4°C over night in the presence of anti-vWF (Dako, 1:200) or COUPTFII (Perseus Proteomics Inc. 1:100). Anti-Mouse TRITC-conjugated secondary antibody (Sigma, 1:200 dilution), Alexa fluor 488 anti-Goat secondary antibody (Invitrogen, 1:200 dilution), and Alexa fluor 488 anti-Rabbit secondary antibody (Invitrogen, 1:200 dilution) were applied for one hour at 37 °C. DAPI nuclear staining (Invitrogen, 1:40,000 dilution) was conducted for 10 minutes at room temperature. The stained sections were visualized using an Axiovert 200 (ZEISS, Göttingen, Germany) microscope.

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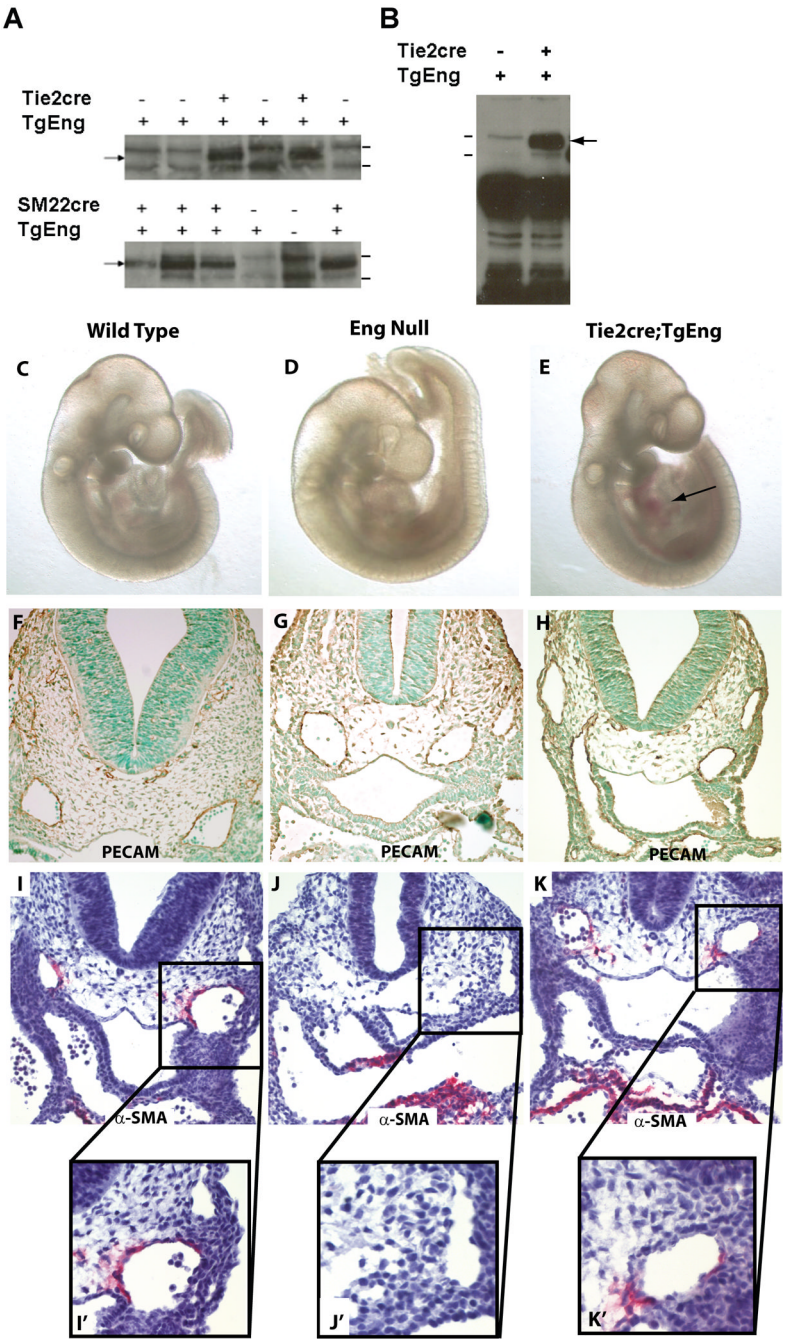


Figure 1. Conditional expression of endoglin in wild type embryonic vascular endothelial cells results in hemorrhaging, correlating with altered vSMC investment in the dorsal aorta

(A, B) Immunoprecipitation and western analysis of endoglin from E10.5 wild type, Tie2cre;TgEng^{LoxP} and SM22cre;TgEng^{LoxP} embryos **(A)**. The same analysis was performed on a pool of E10.5 wild type and Tie2cre;TgEng^{LoxP} embryos showed that the conditional endoglin transgene product migrates as a single polypeptide in the double-positive transgenic embryo pool **(B). (A,B)**: Arrows indicate the position for human endoglin, tic marks indicate mobilities for mature (upper) and immature (lower) endoglin forms. **(C–E)** Whole embryo pictures at E9.5 of **(C)** wild type, **(D)** endoglin null, and **(E)** Tie2cre;TgEng^{LoxP} embryos. Note bleeding in the heart region of the Tie2cre;TgEng^{LoxP}

embryo (Arrow). **(F–H)** Immunohistochemistry on paraffin sections through the heart region of E9.5 mouse embryos for PECAM expression highlighting endothelial cells in **(F)** wild type, **(G)** endoglin null, and **(H)** Tie2cre;TgEng^{LoxP} embryos. Sections were counterstained with methyl green. **(I–K)** Immunohistochemistry on paraffin sections through the heart region of E9.5 mouse embryos for alpha smooth muscle actin (αSMA) expression highlighting vSMCs. **(I'-K')** Higher magnification of the dorsal aorta demonstrating expression of αSMA in **(I')** wild type, (**J'**) endoglin null, and **(K')** Tie2cre;TgEng^{LoxP} embryos. Sections were counterstained with hematoxylin.

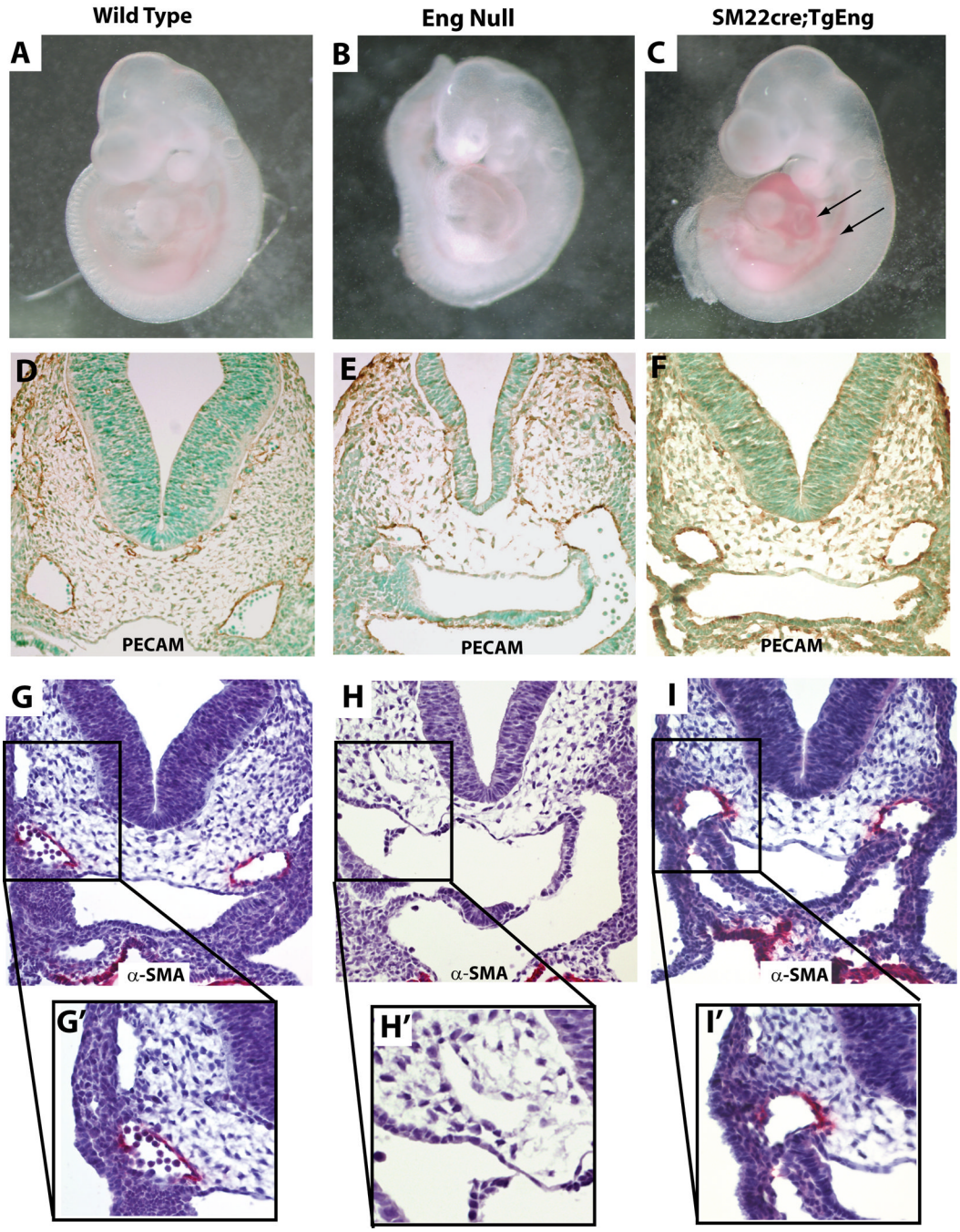


Fig. 2. Conditional expression of endoglin in vSMCs results in hemorrhaging, correlating with an increase in αSMA expression in the dorsal aorta

(A–C) Whole embryo pictures at E9.5 of **(A)** wild type, **(B)** endoglin null, and **(C)** SM22cre;TgEng^{LoxP} embryos. Arrows highlight bleeding in the abdomen and dorsal aorta of the SM22cre;TgEng^{LoxP} embryos. **(D–F)** Immunohistochemistry on paraffin sections through the heart region of E9.5 mouse embryos for PECAM expression highlighting endothelial cells in **(D)** wild type, **(E)** endoglin null, and **(F)** SM22cre;TgEng^{LoxP} embryos. Sections were counterstained with methyl green. **(G–I)** Immunohistochemistry on paraffin sections through the heart region of E9.5 mouse embryos for αSMA expression highlighting vSMCs in the walls of major vessels in **(G)** wild type, **(H)** endoglin null, and **(I)**

SM22cre;TgEng^{LoxP} embryos. **(G'-I')** Higher magnification of the dorsal aorta demonstrating expression of αSMA in **(G')** wild type, **(H')** endoglin null, and **(I')** SM22cre;TgEng^{LoxP} embryos. Sections were counterstained with hematoxylin. These sections are representative of those used for SMA quantitation (Figure 7).

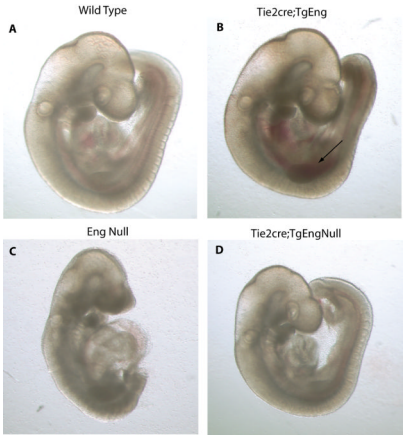


Fig. 3. Replacement of Tie2-driven endoglin expression in the null background does not alter gross embryonic morphology

Images of E9.5 whole embryos from: **(A)** wild type, **(B)** Tie2cre;TgEng^{LoxP}, **(C)** endoglin null, and **(D)** Tie2cre;TgEng/Null. Arrow in panel B highlights a region exhibiting hemorrhage, which is absent in D.

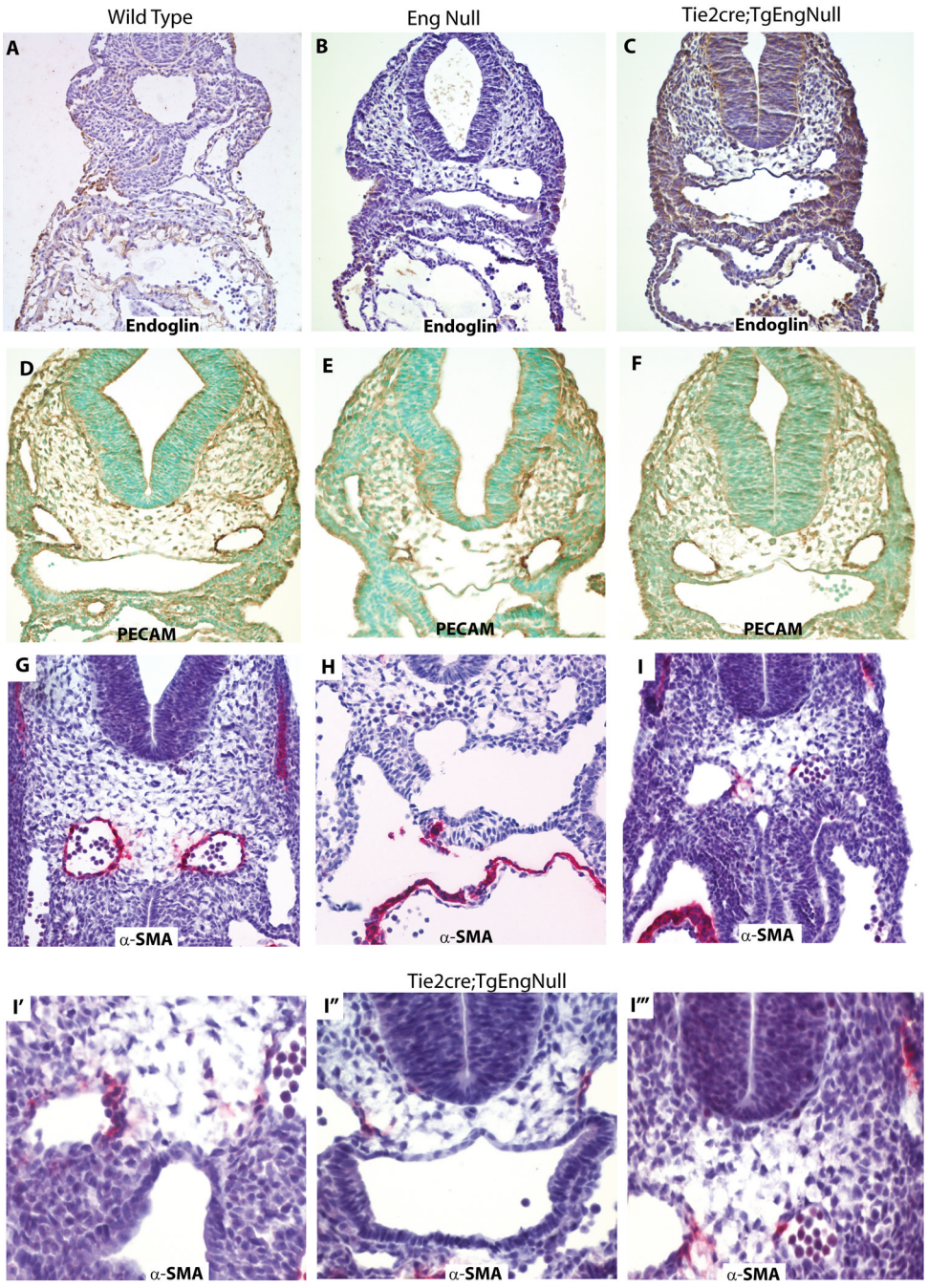


Fig. 4. Replacement of Tie2-driven endoglin expression in the null background partially rescues vSMC recruitment to the dorsal aorta and cardinal vein

(A–C) Immunohistochemistry for human endoglin in paraffin sections through the heart region of E9.5 mouse embryos in **(A)** wild type, **(B)** endoglin null, and **(C)** Tie2cre;TgEng/Null embryos. **(D–F)** Immunohistochemistry for PECAM expression highlighting endothelial cells in **(D)** wild type, **(E)** endoglin null, and **(F)** Tie2cre;TgEng/Null embryos. **(G–I)** Immunohistochemistry for αSMA expression highlighting vSMCs in the walls of major vessels in **(G)** wild type, **(H)** endoglin null, and **(I)** Tie2cre;TgEng/Null. **(I'-I"')** Representative examples of equivalent sections from different E9.5 Tie2cre;TgEng/Null embryos.

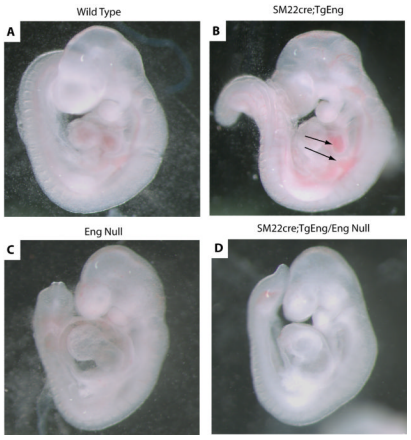


Figure 5. Replacement of SM22α promoter-driven endoglin expression in the null background does not alter gross embryonic morphology

Images of E9.5 whole embryos from: **(A)** wild type, **(B)** SM22cre;TgEng, **(C)** endoglin null, and **(D)** SM22cre;TgEng/Null. Arrows in panel B highlight areas of hemorrhage.

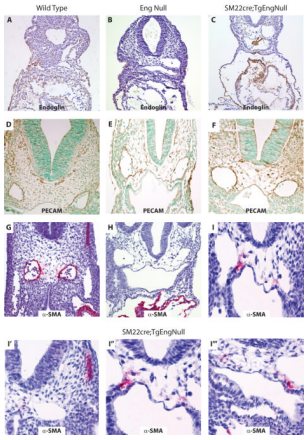


Fig. 6. Replacement of SM22*α* promoter-driven endoglin expression in the null background partially rescues vSMC recruitment to the dorsal aorta and cardinal vein

(A–C) Immunohistochemistry for human endoglin in paraffin sections through the heart region of E9.5 mouse embryos in **(A)** wild type, **(B)** endoglin null, and **(C)** SM22cre;TgEng/Null embryos. **(D–F)** Immunohistochemistry for PECAM expression highlighting endothelial cells in **(D)** wild type, **(E)** endoglin null, and **(F)** SM22cre;TgEng/Null embryos. **(G–I)** Immunohistochemistry for αSMA expression highlighting vSMCs in the walls of major vessels in **(G)** wild type, **(H)** endoglin null, and **(I)** SM22cre;TgEng/Null. **(I'-I''')** Additional representative examples of equivalent sections from different E9.5 SM22cre;TgEng/Null embryos.

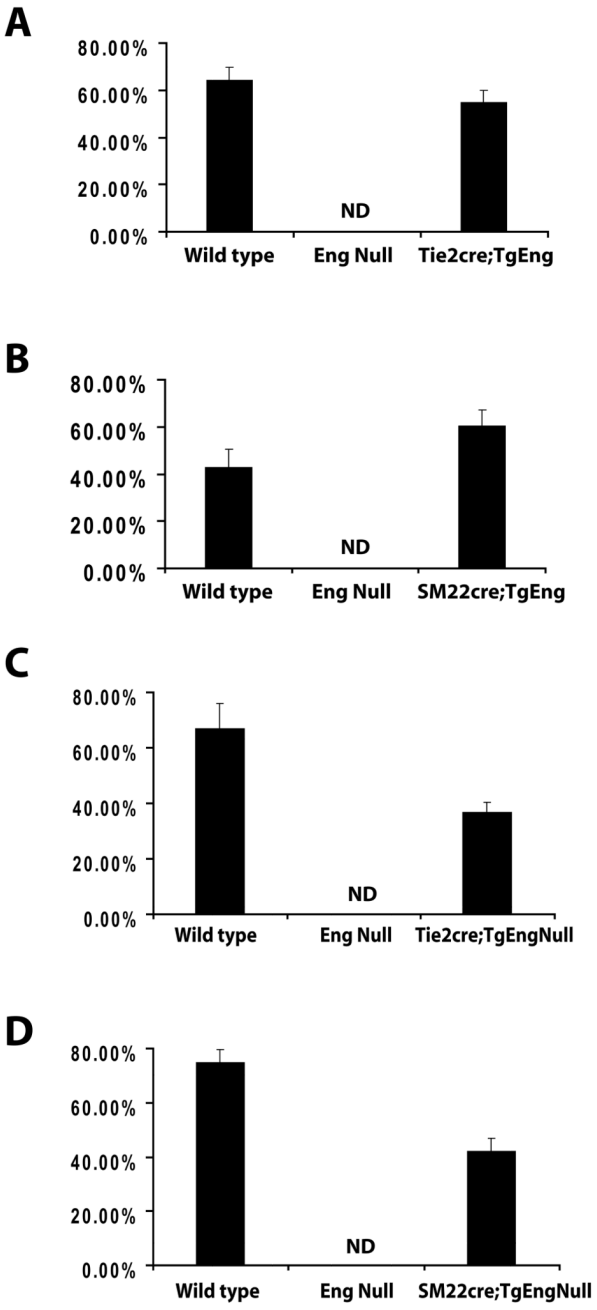


Fig. 7. Quantitative analysis of αSMA expression in dorsal aortae upon conditional endoglin expression in wild type and endoglin null embryos

αSMA IHC was performed using the Avidin/Biotin method with citrate buffer antigen retrieval as described in Experimental Procedures. The proportion of αSMA-positive cell staining in the immediate area of the aorta was estimated using an established morphometric approach (Kumar et al., 1997) modified as follows: Briefly, the area of the vessel lumen was obtained by delineating the lumenal space, in triplicate, using Photoshop CS2 (Adobe Systems). Next, the total area of the vessel to be considered, comprising the lumen plus the area encompassing one cell layer thickness surrounding the lumen was determined as above; subtraction of these two measurements provided the area of the one cell layer surrounding

the lumen. Finally, the area of αSMA-positive cell staining was obtained, and the proportion of αSMA-positive cell staining was expressed as a percent of the total cell area. **(A–B)** Histograms showing the proportion of αSMA positive vSMC in wild type, endoglin null, and the $TgEng^{LoxP}$ expressed under the control of **(A)** Tie2cre or **(B)** SM22αcre recombination. **(C–D)** Histograms showing the proportion of αSMA-positive vSMCs in wild type, endoglin null, and TgEng/Null embryos under the control of **(C)** Tie2cre or **(D)** SM22αcre. Error bars represent the standard error of the mean for a minimum of three independent measurements. ND, no SMA staining detected.

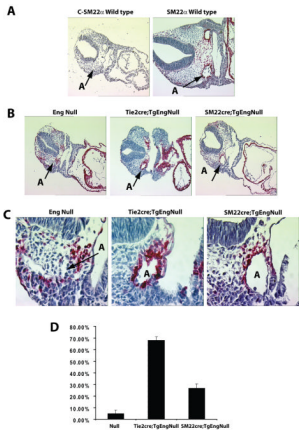
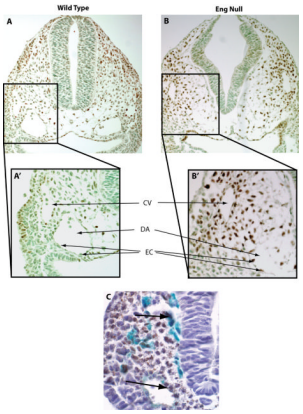
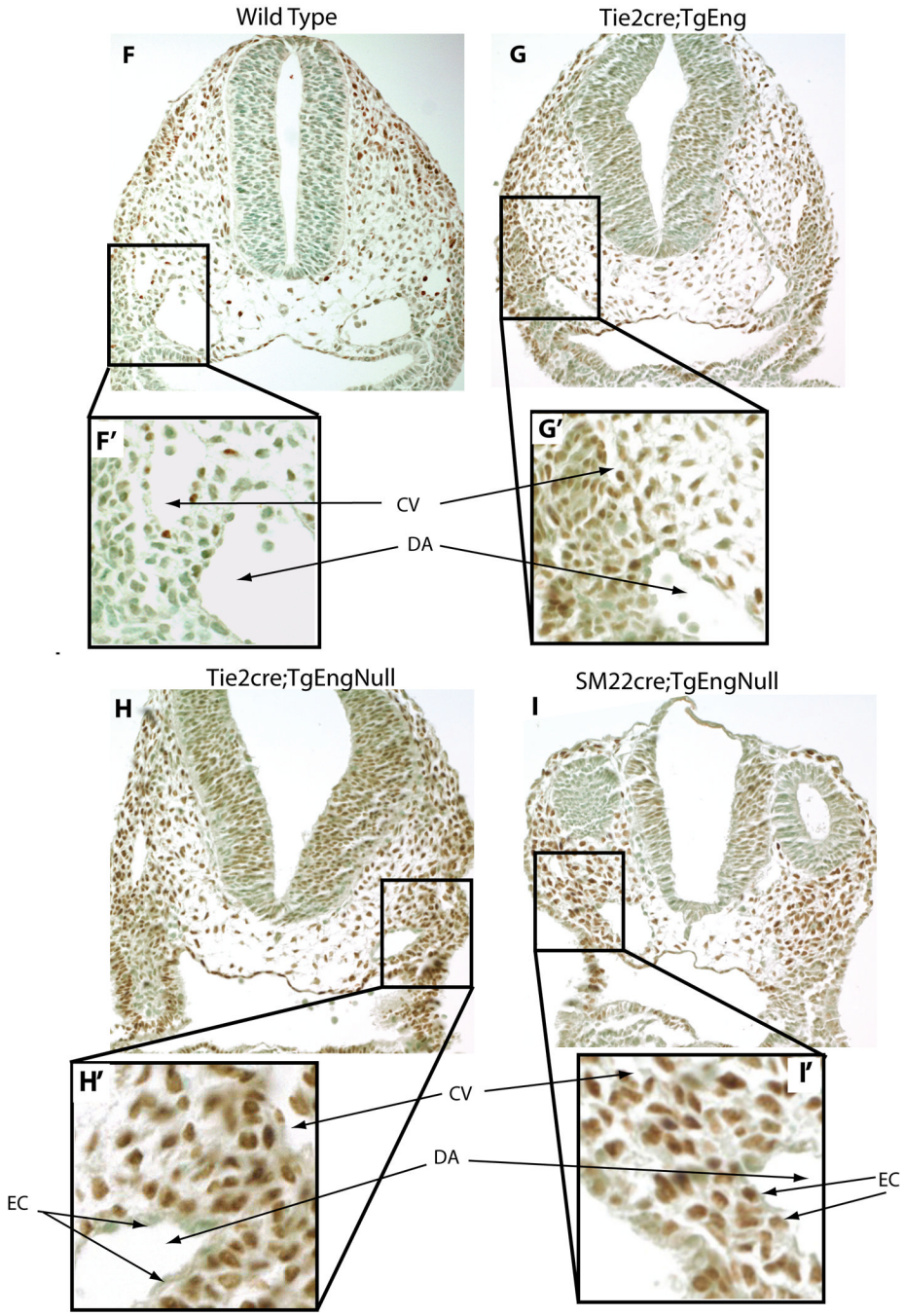


Fig. 8. Endoglin expression rescues vSMC SM22α expression

Anti-SM22α antibody staining was analyzed within equivalent anterior portions of E9.5 wild type, null, and transgenic embryos on the endoglin null background. **(A)** Immunostaining of a representative wild type embryo section using secondary antibody in the absence (C-SM22α Wild type, 10×) or presence (SM22α Wild type, 20×) of primary anti-SM22α antibody. **(B,C)** 10× **(B)**, and 40× **(C)** thoracic sections of E9.5 null, and endoglin transgene-expressing endoglin-null embryos. Aortae (A) are indicated by arrows. **(D)** Quantitative analysis of anti-SM22α staining in dorsal aortae upon conditional cell type-specific endoglin expression in endoglin null embryos. Estimation of SM22α staining was conducted as described in the legend to Figure 7. Error bars represent the standard error of the mean for a minimum of three independent measurements.





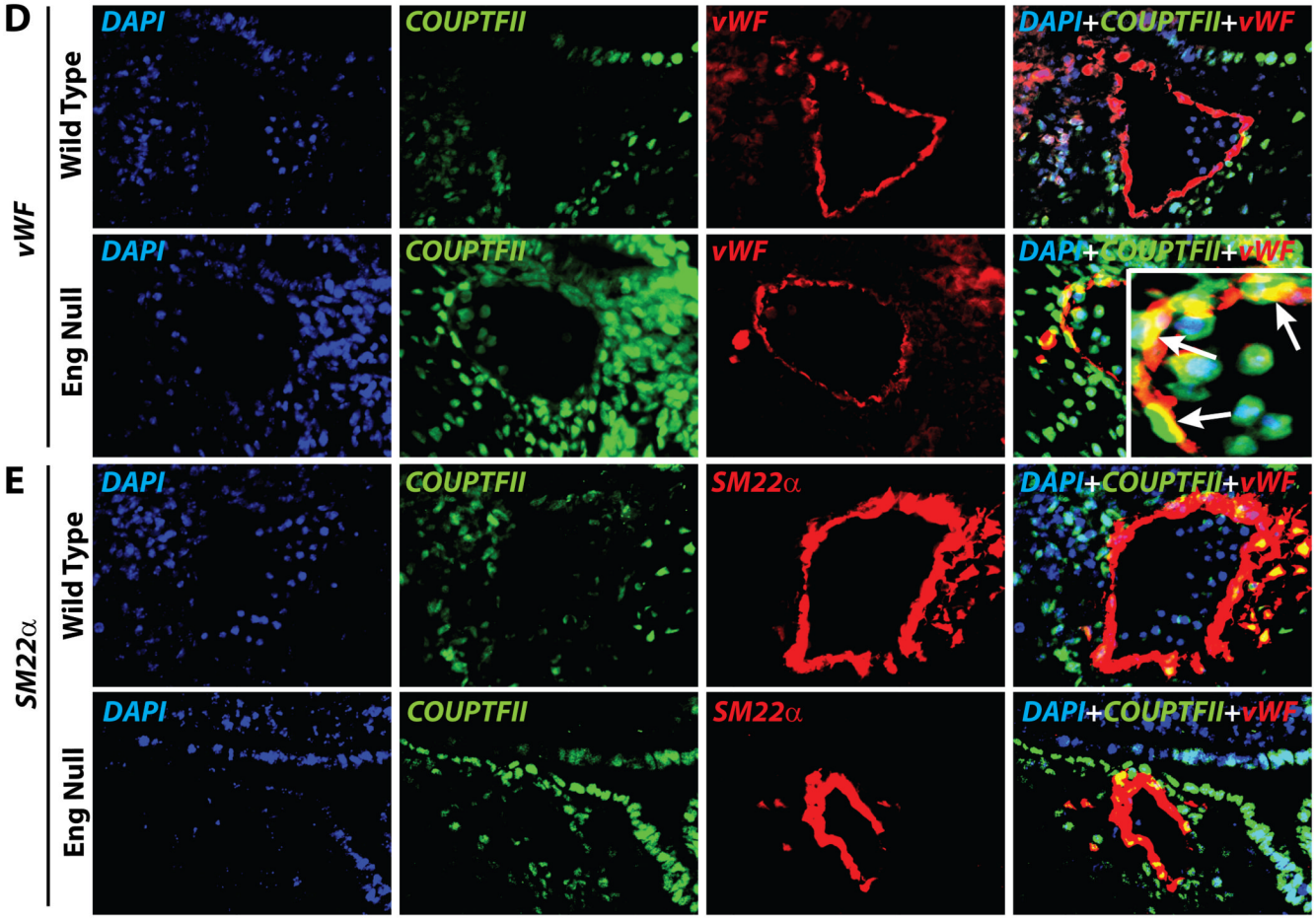


Fig. 9. COUPTFII is ectopically expressed in endothelial cells of the dorsal aorta in endoglin null embryos

(A) Immunohistochemistry for COUPTFII in paraffin sections of an E9.5 wild type mouse embryo through the dorsal aorta (DA) and cardinal vein (CV). **(B)** Immunohistochemistry for COUPTFII in paraffin sections of E9.5 endoglin null mouse embryos in a region similar to **(A). (A', B')** Higher magnification of **(A, B)** showing the cardinal vein and dorsal aorta. Arrows highlight dorsal aortic endothelial cells lacking COUPTFII expression **(A')** and exhibiting ectopic COUPTFII expression **(B')** in the dorsal aorta (DA; cardinal vein, CV; endothelial cell, EC). Sections were counterstained with methyl green. **(C)** Sections of endoglin null embryos in which LacZ was inserted into the endoglin locus were stained for β-galactosidase activity in combination with COUPTFII immunohistochemistry. Arrows indicate arterial endothelial cells ectopically expressing COUPTFII and Eng-driven LacZ. Sections were counterstained with hematoxylin. **(D,E)** Immunofluorescence analysis for nuclear-staining DAPI, and endothelial cell-specific vWF **(D)**, or SM22α **(E)**, and COUPTFII in paraffin sections of wild type and endoglin null embryos. Arrows in the merged vWF and COUPTFII staining Eng null panel inset indicate examples of cells coexpressing vWF and the diffuse nuclear-staining COUPTFII. Panels were obtained using 200× magnification. **(F,G)** Immunohistochemistry for COUPTFII in paraffin sections comparing wild type **(F)** to Tie2cre;TgEng^{LoxP}**(G)** embryos at E9.5. **(F', G')** Higher magnification of the CV and DA. Arrows highlight normal COUPTFII expression in the wild type cardinal vein (CV) that is absent in the dorsal aortae (DA). **(H)** Immunohistochemistry for COUPTFII in Tie2cre;TgEng/Null sections at E9.5. **(H')** Higher

magnification of **(H)**. Arrows indicate rescue of ectopic expression of COUPTFII in endothelial cells of the dorsal aorta of Tie2cre;TgEng/Null embryo. **(I)** Immunohistochemistry for COUPTFII in SM22cre;TgEng/Null sections at E9.5. **(I')** Higher magnification of **(I)**. Arrows indicate that in SM22cre;TgEng/Null embryos, ectopic expression of COUPTFII in endothelial cells of the dorsal aorta is not rescued (DA, dorsal aorta, CV, cardinal vein, EC, endothelial cell).

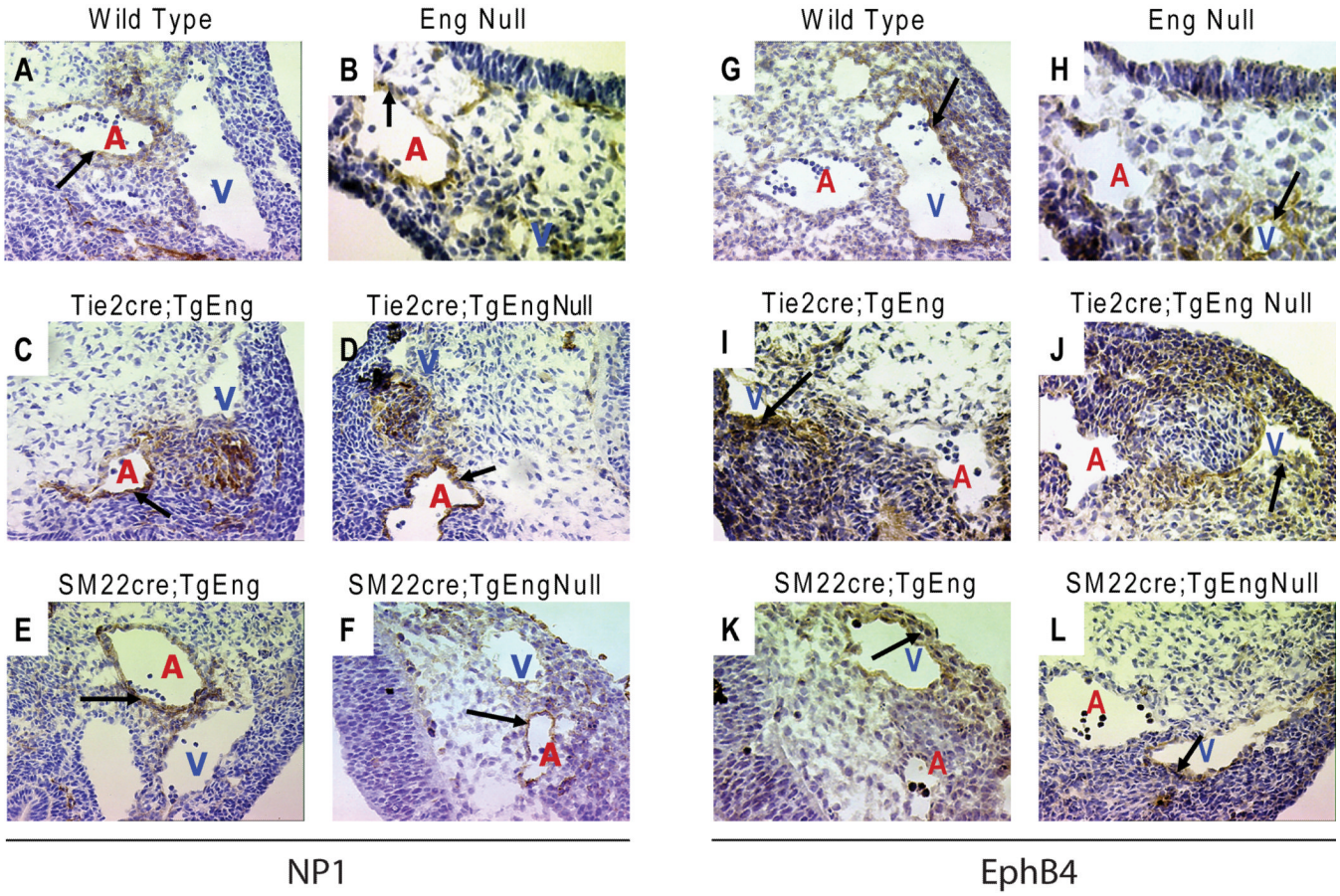


Fig. 10. NP1 and EphB4, targets of COUPTFII, are not altered in endoglin mutant embryos

Panels **(A–F)** IHC for NP1 and panels **(G–L)** EphB4 staining on cryosections of E9.5 mouse embryos. Arrows **(A–F)** indicate that NP1 expression is restricted to the aorta, and for EphB4, arrows **(G–L)**, expression is restricted to the cardinal vein, in wild type and endoglin null embryos.

Table 1

Primer sequences used for genotyping and RT-PCR

Primer	Sequence FW	Sequence RV
Cre	GCTGGTTAGCACCGCAGGTGTAGAG	CGCCATCTTCCAGCAGGCGCACC
Human Eng	CAGAGTGTCCCCATCCGT	TGGGTATGGGTACTGTGTAGAAGT
Neo	GCTCTGATGCCGCCGTGTTCC	CTTCGCCCAATAGCAGCCAGTC
Wild Type Eng	ACCATCTTGTCCTGAGTAGCG	TGAGCCTGACGGGAAACTG