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ENDOGLIN Is Dispensable for Vasculogenesis, but Required for Vascular Endothelial Growth Factor-Induced Angiogenesis

Zhen Liu¹ 3, Franck Lebrin² 4 5, Janita A. Maring¹ 3, Sander van den Driesche² 3 6, Stieneke van der Brink², Maarten van Dinther¹, Midory Thorikay¹, Sabrina Martin⁴, Kazuki Kobayashi¹, Lukas J. A. C. Hawinkels¹, Laurens A. van Meeteren¹, Evangelia Pardali¹ 7 8, Jeroen Korving², Michelle Letarte⁵, Helen M. Arthur⁶, Charles Theuer⁷, Marie-José Goumans¹ 8, Peter ten Dijke¹ 9

¹ Department of Molecular Cell Biology, Cancer Genomics Centre, Centre for Biomedical Genetics, Leiden University Medical Center, Leiden, The Netherlands, ² Hubrecht Institute, Utrecht, The Netherlands, ³ Department of Anatomy and Embryology, Leiden University Medical Center, Leiden, The Netherlands, ⁴ Center for Interdisciplinary Research in Biology (CIRB), CNRS UMR 7241/INSERM U1050, Collège de France, Paris, France, ⁵ Molecular Structure and Function Program, The Hospital of Sick Children, Department of Immunology and Heart and Stroke Richard Lewar Center of Excellence, University of Toronto, Toronto, Ontario, Canada, ⁶ Institute of Genetic Medicine, Newcastle University, International Centre for Life, Newcastle upon Tyne, United Kingdom, ⁷ Tracfon Pharmaceuticals, San Diego, California, United States of America

Abstract

ENDOGLIN (ENG) is a co-receptor for transforming growth factor-β (TGF-β) family members that is highly expressed in endothelial cells and has a critical function in the development of the vascular system. Mutations in Eng are associated with the vascular disease known as hereditary hemorrhagic telangiectasia type I. Using mouse embryonic stem cells we observed that angiogenic factors, including vascular endothelial growth factor (VEGF), induce vasculogenesis in embryoid bodies even when Eng deficient cells or cells depleted of Eng using shRNA are used. However, ENG is required for the stem cell-derived endothelial cells to organize effectively into tubular structures. Consistent with this finding, fetal metatarsals isolated from E17.5 Eng heterozygous mouse embryos showed reduced VEGF-induced vascular network formation. Moreover, shRNA-mediated depletion and pharmacological inhibition of ENG in human umbilical vein cells mitigated VEGF-induced angiogenesis. In summary, we demonstrate that ENG is required for efficient VEGF-induced angiogenesis.

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* E-mail: M.J.T.H.Goumans@lumc.nl (MJG); C.L.Mummyer@lumc.nl (CM); p.ten_dijke@lumc.nl (PtD)

† These authors contributed equally to this work.

¤ Current address: MRC Centre for Reproductive Health, Edinburgh University, Edinburgh, Scotland, United Kingdom

¶ Current address: Department of Cardiology and Angiology, University Hospital Münster, Münster, Germany

Introduction

During development of the embryo, blood vessels evolve de novo from hemangioblasts that differentiate into endothelial cells and form a primary vascular plexus. This process is defined as vasculogenesis [1]. Angiogenesis refers to the remodeling and maturation of this primitive vascular network into a branched vascular network [2]. Angiogenesis is a dynamic and carefully balanced process involving an activation phase associated with increased vascular permeability, basement membrane degradation, endothelial proliferation and migration, and a resolution phase accompanied by inhibition of endothelial cell proliferation and migration, in parallel with basement membrane reconstitution [3]. In the maturation phase the recruitment of pericytes and vascular smooth muscle cells is needed to maintain vessel stability and protect endothelial cells from apoptosis [4,5].

Vascular endothelial growth factor (VEGF) plays a very prominent role in vasculogenesis and angiogenesis. VEGF represents a family of related cytokines, of which the VEGF-A isoform is a potent endothelial mitogen strongly induced by hypoxia [6]. Mice lacking one Vegfa allele die at embryonic day E8.5 as a result of vascular malformations [2,7]. VEGF-A signaling occurs via the high affinity tyrosine kinase receptors VEGFR1 (FLT-1), and VEGFR2 (FLK-1) [8,9]; VEGFR2 is the important endothelial VEGF receptor during angiogenesis. Vegfr2 knockout mice die at E8.5 from impaired development of hematopoietic and endothelial cells [10] and closely resemble VEGF-A deficient embryos.

Endoglin (ENG or CD105) is a transmembrane glycoprotein essential for angiogenesis and vascular development, which is predominantly expressed in vascular endothelial cells [11]. Mice lacking Eng die at E10.5-E11.5 from angiogenic and cardiovascular
defects. The early steps of vasculogenesis appear to be normal but the primary endothelial network fails to remodel into a mature circulatory system [12–14]. ENG functions as a co-receptor for transforming growth factor-β (TGF-β) family members, and interacts with their signaling serine/threonine kinase receptors [15,16]. TGF-β relays its signal via Type I receptors (TβRI), also termed as activin receptor-like kinases (ALKs). TβRI acts downstream of type II receptors (TβRII) [17] and mediates the activation of intracellular SMAD effector transcription factors [18]. In endothelial cells, TGF-β can signal via two different TβRIs, ALK1 and ALK5 [3,19]. Activation of ALK1 induces SMAD1 or −5 phosphorylation and mediates endothelial cell proliferation and migration, whereas ALK5 induces SMAD2 and −3 activation leading to vascular quiescence [3,20]. ENG promotes ALK1/Smad1/5 signaling and inhibits ALK5/SMAD2/3 signaling [21–23]. ENG and ALK1 have also been shown to bind other TGF-β family members. Bone morphogenetic protein (BMP) 9, in particular, can bind directly and with high affinity to ENG and ALK1 [24,25].

In humans, mutations in Eng lead to hereditary hemorrhagic telangiectasia type I (HHT1, also known as Rendu-Osler-Weber syndrome), while HHT2 is associated with mutations in the type I receptor, ALK1 [26], [27]. HHT is an inherited autosomal-dominant vascular disorder that affects the blood vessels of many organs. Characteristic symptoms include epistaxis (nosebleeds), skin and mucosal telangiectases associated with hemorrhage, as well as pulmonary, cerebral and hepatic arteriovenous malformations [28,29].

During the differentiation of mouse embryonic stem cells (ESCs) in vitro, hematopoietic commitment within Vegf2+/− precursor populations are characterized by Eng expression [30]. In particular, Eng is expressed during the progression from the Vegf2+/− Cd45+/− to Vegf2+/− Cd45− stage, marking the hemangioblast [31]. In Eng deficient ESCs, the number of hemangioblast precursors were reduced and myelopoiesis and definitive erythropoiesis were severely impaired, suggesting that the regulated expression of ENG functions to support lineage-specific hematopoietic development from VEGFR2+ expressing precursors [30,31]. Additional studies with forced expression of ENG in ESCs and transcriptional profiling studies on ENG+ and VEGFR2+ expressing cells from E7.5 embryos further supported an important role for ENG in hematopoietic development [32,33].

In the present study, we examined the role of ENG in vasculogenesis and angiogenesis using aggregates of ESCs known as embryoid bodies (EBs). We found that endothelial cell differentiation was not affected by a lack of ENG, but that VEGF-induced angiogenesis was severely impaired. The effects were dependent on the level of Eng heterozygotes exhibited an intermediate phenotype, reminiscent of features in HHT1 patients. These results were validated and consolidated by shRNA-mediated Eng depletion and pharmacological ENG inhibition studies in endothelial cells. The impaired VEGF-induced endothelial cell sprouting in the absence of ENG might provide a suitable cell model to screen for drugs that can rescue this phenotype, which might lead to novel treatment modalities.

**Results**

**Absence of Eng impairs organization of vascular structures in 15-day-old embryoid bodies**

To elucidate the role of ENG in blood vessel morphogenesis we examined the effect of Eng gene dosage using the established assay of differentiation of ESCs into EBs [34]. When induced to differentiate, Eng+/− or Eng−/− ESC lines [13] were found to form EBs of similar size and compactness to those of wild type EBs (Fig. 1A). Next, the assembly of vascular structures was analyzed by platelet endothelial cell adhesion molecule (PECAM)-1 staining of sections of ESC-derived EBs with different Eng gene dosage (Eng+/+, Eng+/− or Eng−/−) obtained after 15 days of differentiation embedded in plastic and sectioned (Fig. 1B). Morphology of the vasculature formed in wild type ESC-derived EBs was very similar to that of the yolk sac in wild type mouse embryos (Fig. 1B). Multiple blood islands, lined with a single layer of thin elongated endothelial cells, were found between the outer endoderm and the inner ectoderm layers (Fig. 1B), as reported previously by Wang et al. [35]. The number of blood islands in Eng−/− ESC-derived EBs appeared less numerous than in the wild type ESC-derived EBs and endothelial cells were found in clusters rather than in elongated single cell layers, confirming the defective formation of vessel-like structures in Eng−/− ESC-derived EBs (Fig. 1B). Vascular structures also developed in Eng+/− ESC-derived EBs, but their frequency and organization were markedly reduced compared to those in wild type ESC-derived EBs, indicating a dose dependent effect of Eng on vascular organization (Fig. 1B).

**ENG does not affect endothelial cell differentiation**

Two processes are responsible for the formation of blood vessels during embryonic development: (i) vasculogenesis, the primary in situ differentiation of endothelial precursors from mesoderm, and their organization into a primary capillary plexus and (ii) angiogenesis, the formation of new vessels by a process of sprouting from pre-existing vessels [1], [36]. RT-PCR analysis of endothelial cell specific markers on ESC-derived EBs collected from days 0 to 20 were used to define the role of ENG during endothelial cell differentiation. Distinct gene expression patterns were induced as differentiation proceeded. Vegf1 was rapidly up-regulated at day 3 and Vegfr2, Tie-1 and Tie-2 more prominently at day 5 (Fig. 2A). The expression patterns of the different EC markers were similar in Eng−/− ESC-derived EBs. In addition, we determined the number of PECAM-1 positive cells in dissociated 11-day-old EBs by FACS analysis and found no differences between wild type, Eng−/− or Eng+/− ESC-derived EBs (Fig. 2B).

Analysis of the expression of multiple pericyte-vascular smooth muscle markers by RT-PCR also did not reveal striking differences between ESC-derived EBs with different Eng gene dosage (Fig. 2C). Taken together, our results show that ENG is not required for endothelial and mural cell differentiation.

**Endothelial cell organization is disrupted in Eng−/− ESC-derived EBs plated on gelatin**

EBs plated on a gelatin-coated substrate can develop branching vascular structures indicative of vascular morphogenesis [37]. Endothelial cells are initially aggregated in dense clusters but when plated, rapidly form thin branching tubes, in a process resembling angiogenesis. To determine the role of ENG in this process, we plated 11-day-old EBs derived from Eng+/+, Eng+/− and Eng−/− ESCs and maintained them in culture for four additional days before staining them with an antibody to PECAM-1 and hematoxylin to reveal the vascular network. Three different phenotypes could be identified in the EBs: (i) those with an extensively branched vascular network without endothelial cell clusters categorized as “organized”, (ii) those forming some vessels and still containing endothelial cell clusters referred to as “intermediate”, (iii) those with endothelial cells clusters only; these were designated as “dispersed” (Fig. 3A). Of around 90 EBs scored in each case in two independent experiments, on average
about 63% of Eng+/− EBs showed an organized phenotype, ∼26% an intermediate phenotype and only ∼11% a dispersed phenotype (Fig. 3B). By contrast, in the Eng−/− EBs, ∼39% lacked cord-like structures entirely and were classified as dispersed, whereas ∼59% had an intermediate phenotype. Furthermore, the length of the vessel sprouts that did form was greatly reduced compared to those of the Eng+/− EBs and vessels appeared often wider. Quantitative analysis also showed that an intermediate vascular phenotype predominated in the Eng+/− EBs with ∼20% dispersed and ∼60% intermediate phenotypes (Fig. 3B). When EBs were embedded into a collagen gel and allowed to form vascular sprouts in 3D, we

Figure 1. Impaired vasculature in Eng null-mutation ESC-derived 11-day-old EBs. (A) Eng+/− or Eng−/− ESC lines form EBs with no difference when compared to EBs derived from wild type ESCs (B) PECAM-1 whole mount immunohistochemistry of representative wild type, Eng+/−, and Eng−/− ESC-derived 11-day-old EBs. Wild type ESC-derived EBs form a primitive vascular plexus. In contrast, Eng−/− ESC-derived EBs form irregular vascular structures with endothelial cell clusters. Light microscopy of serial plastic sections of wild type, Eng+/−; and Eng−/− ESC-derived 11-day-old EBs stained as whole mount for PECAM-1. Black arrowhead indicates vessel like structures. Asterisk indicates endothelial cell clusters. doi:10.1371/journal.pone.0086273.g001
observed a reduction in both number of sprouts and sprout length in the Eng−/− EBs (Fig. S1).

To validate the data obtained with the Eng−/− ES cell line, we depleted Eng by shRNAs targeting Eng in ESCs. Essentially we obtained the same results as for ESCs in which gene dosage was
Reduced (Fig. 4). Partial knock-down of Eng in ESC in differentiated EBs (Fig. 4B, 4C) interfered with efficient VEGF-induced sprouting (Fig. 4D), whereas expression of endothelial markers Vegfr2 and VE-Cadherin mRNA was not significantly affected (Fig. 4C).

VEGF-induced angiogenesis is reduced in fetal metatarsals from Eng^{−/−} mice

In the studies above, VEGF was provided as the angiogenic stimulus. VEGF is a potent mitogen for endothelial cells and elevated ENG expression has been associated with activated endothelial cells in tumor stroma [38]. To investigate a possible interplay between VEGF and ENG in angiogenesis, we compared the VEGF-induced angiogenic response in fetal mouse metatarsals derived from wild type and Eng^{+/−} mice. After adherence of the fetal bones to the culture dish, fibroblast-like cells migrate from the bones to form a monolayer, on which a tubular network of endothelial cells is formed [38,39]. Staining of this endothelial cell network with an antibody to PECAM-1 showed that the VEGF-induced angiogenic responses, as measured by the number and the length of capillary sprouts were significantly reduced in the Eng^{−/−}
Figure 4. shRNA-mediated knock down of Eng inhibits VEGF-induced endothelial cell sprouting of EBs. ES cells were transduced with either scrambled or endoglin targeting shRNA. ES cells formed EBs during 4 days of hanging drop culture before embedding in collagen and stimulation with VEGF (30 ng/ml). Sprouting EBs were analyzed after 8 days of VEGF stimulation. A) Control EBs and EBs with incomplete endoglin knockdown stained for endothelial marker PECAM-1 (green) and DAPI (blue). Control EBs have large and many outgrowing sprouts of endothelial cells, which form extensive networks. EBs with incomplete Eng knockdown show less sprouts, which do not seem to form as extensive networks as control EBs. Sheets of cells that are mostly PECAM-1 negative have formed between the sprouts. B) Control EBs and EBs with incomplete Eng knockdown stained for ENG (green) and nuclear marker hoechst (blue). ENG is present in the entire sprout in the control EBs, with the highest expression towards the tip of the sprout. In the EBs with incomplete knockdown showed expression of ENG mainly in the tips of the outgrowing sprouts. The cellular sheets hardly had ENG expression. C) qPCR analysis of Eng, PECAM-1 and VE-cadherin expression during differentiation of the
control and Eng knockdown ESCs. Endoglin expression is reduced by approximately 85% in the ESC cells, but during differentiation, at day 7 and 9, expression is restored to half of the normal levels. Expression of Vegf2 and Vc-cadherin did not significantly differ between control and endoglin knockdown EBs at the ES cell state or at day 7 and 9 of differentiation. D) Analysis of numbers of tips per EB. Endoglin knockdown EBs exhibit significantly less sprouts than the control EBs (p<0.01).

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Discussion
In the present study, we examined the role of ENG in vasculogenesis and angiogenesis using aggregates of mouse ESCs known as EBs that were challenged with angiogenic factors, including VEGF. Under appropriate conditions, both vasculogenesis and angiogenesis take place in EBs [41–45]. We compared EBs from wild type mouse ESCs with those from mouse ESCs with heterozygous or homozygous deletions in Eng (Eng\(^{-/-}\) and Eng\(^{-/-}\), respectively). We found that the endothelial cell differentiation program in ESC-derived EBs is not affected by homozygous deletion of Eng. However, homozygous mutant endothelial cells were severely inhibited in their ability to form organized vascular structures either following plating of EBs on gelatin in 2D or in 3D collagen gels, supporting evidence for an essential role of ENG in VEGF-mediated angiogenesis. This is consistent with reports by Bourdeau et al. [13], Li et al. [14] and Arthur et al. [12], and more recently by Park et al. [46]. However, these data are different from earlier reports on the Eng\(^{-/-}\) ESCs claiming no effect on endothelial cell organization in differentiating embryoid bodies. However, different methods were used, which might have contributed to the different outcomes [47]. To validate the defect in sprouting of Eng\(^{-/-}\) ESC lines compared with control Eng\(^{+/+}\) ESC, we depleted Eng by shRNA. Essentially we were able to confirm the results obtained using the knock out cells in that they are also defective in VEGF-induced endothelial cell sprouting, albeit not as dramatically as knock out cells. shRNA-mediated depletion has the advantage of looking at the effects of Eng depletion at an early stage, before any long term adaptation responses occur. Thus, we conclude that ENG is responsible for the lack of VEGF-induced endothelial vascular organization.

Figure 5. VEGF-induced angiogenesis is impaired in Eng\(^{-/-}\) fetal metatarsal bones. Metatarsals of 17-day-old mouse fetuses were prepared from wild type and Eng\(^{-/-}\) mice, transferred to cell-culture plates, allowed to adhere, and then stimulated with VEGF (50 ng/ml). (A) Cultures were fixed and vessel-like structures were visualized by anti-PECAM-1 staining. Six bones were stimulated per experimental group and one representative picture of each group is shown. (B) VEGF addition stimulated the formation of vessel-like structures. No significant difference in the baseline vascular network formation was observed between wild type and Eng\(^{-/-}\) metatarsals. The induction of the vascular network of wild type metatarsals is significantly stronger than the network of Eng\(^{-/-}\) metatarsals. P<0.05.

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We observed interdependence for ENG in VEGF-induced angiogenic responses. Genetic depletion of Eng from endothelial cells and pharmacological inhibition using TRC105 ENG antibody severely affected VEGF-induced endothelial cell sprouting. These results are in line with previous studies, which demonstrated that ENG is essential for normal growth, migration and cord formation of endothelial cells [21,48,49]. In addition, our results are consistent with a recent report that showed that TRC105 inhibited VEGF and FGF-induced HUVEC endothelial tube formation when co-cultured with dermal fibroblasts [50]. Moreover, soluble ENG has been shown to inhibit tumor angiogenesis [51,52], and elevated placental expression of ENG results in high serum levels of soluble ENG that contribute to vascular dysfunction in preeclampsia [53].

Remarkably, mouse embryonic endothelial cells (MEECs) isolated from Eng2/2 embryos have been described as exhibiting...
enhanced proliferation [54]. The basis for the differences between our findings here and these studies is not clear. One explanation may be adaptive mechanisms that take place in endothelial cells in order to compensate for reduced ENG expression in vivo [21,54–56].

Analysis of yolk sac vasculature in Eng mutant mice has shown previously that vascular smooth muscle cells are sparse in the vicinity of vessels lacking endoglin and it was striking that immunodetectable TGF-β1 was reduced in the smooth muscle cells although TGF-β1 mRNA levels in the adjacent endothelial cells were unaffected [53]. The impaired ability of endothelial cells to secrete or activate TGF-β1 was believed to explain the lack of phosphorylated Smad2 in the adjacent mesothelium and the subsequent failure of these cells to differentiate into vascular smooth muscle cells. In the EB vasculogenesis assay used here, vascular smooth muscle cells did form and organize to some extent, albeit abnormally, in the absence of Eng in contrast to the observations in vivo. However, the culture conditions used included the use of fetal bovine serum as a medium supplement, which could provide active TGF-β and facilitate partial rescue. Since the EB vasculogenesis assay closely models aspects of vascular development and includes both the differentiation and organizational aspects of EC and vascular smooth muscle cell components, it is potentially useful in screening anti-or pro-angiogenic drugs as well as in understanding the underlying molecular mechanisms.

In conclusion, our results provide insights into the molecular mechanisms that underlie vascular defects reminiscent of those in HHT1 patients and opens new avenues for inhibition of VEGF signaling by interfering with ENG function.

Materials and Methods

Cell culture

HUVECs. Human umbilical vein endothelial cells (HUVECs) were cultured in Medium 199 with Earle’s salt and L-glutamine (Gibco), 10% FCS, heparin (LEO pharma), bovine pituitary extract (Gibco) and penicillin/streptomycin (PS) on plates coated with 1% gelatin, at 37°C and 5% CO₂. HUVECs were used up to passage 4. Experiments were confirmed with HUVECs from different donors.

HUVECs were isolated from umbilical cords. The LUMC has the policy that umbilical cords are considered as “rest material” and collection can be performed without permission of the ethical committee, provided that the donor of the umbilical cord has signed a written consent and that collection and processing of the umbilical cord can be performed without permission of the ethical committee, provided that the donor of the umbilical cord has signed a written consent and that collection and processing of the umbilical cord can be performed without permission of the ethical committee.

Embryonic stem cell lines and culture. Two independent R1 ESC lines were used as controls. Eng+/− mouse embryonic stem cells (ESCs) were generated by gene targeting of the parental wild-type 129/Ola-derived E14 ES cell lines, deleting 609 base pairs (bp), including Eng exon 1 and its initiation codon and leaving the Eng promoter intact [13]. Eng−/− ESCs were derived in vitro from Eng+/− ESCs by selection with high concentrations of G418 [30]. Genomic DNA was isolated from ESC lines using standard techniques [57]. Primers MEFL1 and MER1 amplify normal Exon 1 (300 bp) and primers MEFLR and ME2R amplify the recombinant product (476 bp), as previously described [13]. ESC lines were cultured in the presence of mouse embryonic fibroblasts (MEFs) in DMEM, supplemented with 20% heat-inactivated fetal bovine serum (FBS), 0.1 mM 3-Mercaptopento- 

Lentiviral transduction

HUVECs were infected with lentivirus encoding an shRNA sequence against human Eng (TRCN0000003273, TRCN0000003276) selected from the MISSION shRNA library (Sigma) and a third lentivirus encoding shRNA was generated in our lab [58]. R1-ES cells were infected with lentivirus encoding an shRNA targeting mouse Eng (TRCN0000094355, MISSION shRNA library Sigma). As a control, a non-targeting shRNA sequence (SHC002) (Sigma) or empty vector pRRL was used. Virus transduction was performed overnight, and the infected cells were selected using culture medium containing puromycin (1 μg/ml) for 48 h. The efficiency of Eng knockdown was verified by qPCR.

In vitro differentiation of embryonic stem cell clones

Two different methods were used to differentiate ES cells in vitro.

Method 1: ESC lines were cultured in hanging drops to form EBs, as described previously [59]. Brie, 800 cells were cultured in 20 μl of DMEM, supplemented with 20% FBS, 25 ng/ml VEGF, 50 ng/ml bFGF-2, hanging from the lid of the culture dish for 5 days, which allows the formation of cell aggregates (EBs). This makes it possible to control the size of the EBs and circumvents paracrine stimulation between EBs, and therefore allows a very high degree of reproducibility. Subsequently, EBs were either (i) cultured in suspension on bacterial dishes coated with 1% agar for 11 or 15 days. EBs were then washed with PBS and fixed in methanol (MeOH)-dimethyl sulfoxide (DMSO) in a ratio of 4:1, overnight (o/n) at 4°C before staining; or (ii) 11-day-old EBs were plated on gelatin coated coverslips for 4 days and then fixed in Zinc fixative o/n at 4°C before staining.

Method 2: Mixed Feeder-ES cell cultures were trypsinized and subsequently cultured for 45 minutes on gelatin-coated plates before the experiment in order to deplete the MEFs, which adhere faster to the plate. The ES cells were harvested and plated in suspension as hanging drops of 20 μl in complete ES-medium, containing 1200 cells/drop, for four days.

Embryoid body maturation in 2D culture

Four-day-old EBs obtained with method 2 were plated in gelatin-coated 6 well-plates with 15–20 EBs per well. EBs were cultured in ES medium without LIF and supplemented with 50 ng/ml hVEGF-165 (PeproTech, Rocky Hill, USA). After 7 or 9 days, EBs were washed with PBS and RNA was isolated for qPCR analysis.

Embryoid body maturation in 3-D Collagen matrix

Method 1: All the ingredients of the collagen medium (DMEM, 20% FBS, 25 ng/ml VEGF, 50 ng/ml bFGF-2) with the exception of collagen were mixed and stored on ice before harvest of the EBs to avoid prior polymerization of the medium. Prior to use, rat tail type 1 collagen was added and mixed to a final concentration of 1.25 mg/ml. 11-day-old EBs were immediately incorporated into the collagen medium at a final concentration of 50 EBs/ml. 12 ml was poured into a 35 mm bacterial grade Petri dish and cultures incubated for 3 days at 37°C in a 5% CO₂ atmosphere [30,42]. For further analysis of sprouting vessels, the 35 mm gel dish was inverted over a 50 mm × 75 mm glass slide. The collagen gel was gently laid out on the slide and excess liquid around the gel removed by pipetting with a dispenser. The gel was then dehydrated using nylon linen and absorbent filter cards.
slide was air-dried for 12 hours and incubated in zinc fixative o/n at 4°C before staining as previously described [42]. The EBs were stained for PECAM-1 (Clone ME1C13.3, BD Biosciences).

Method 2: Collagen solution was made as following: Purecol (Advanced Bionatrix, San Diego, USA) with 34.65% HAM’s F12 (Gibco), 6.25% NaOH (0.1M), 6.25% 10x F12, 1.25% 4/2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (1M), 0.975% Sodium bicarbonate 7.5%, and 0.625% Glutamax. Four-day old EBs were suspended in 350 µl collagen and transferred to a 24-well collagen pre-coated plate (one EB per well). EBs were cultured in complete ESC-medium without LIF and supplemented with 30 ng/ml hVEGF-165. The EBs were cultured for eight days in collagen and the medium was changed every four days. Afterwards, EBs in collagen were washed with phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde in PBS for 30 min at room temperature.

RNA isolation and quantitative PCR

Total DNA-free cellular RNA was extracted with Trizol reagents, according to manufacturer’s protocol (Invitrogen). Samples were DNase I treated to eliminate genomic DNA and 1 µg RNA was reversed transcribed as described [60]. All PCR analyses of the endothelial cell and SMC specific markers were as previously described [61]. RNA from ESCs and EBs from method 2 was isolated with the NucleoSpin RNA II kit according to manufacturer’s protocol (Bioké, Leiden, Netherlands). qPCR was performed with SYBRGreen reagent (Roche) for Eng, Vegf2 and VE-cadherin. See Table 1 for sequences. The ΔΔCt method was applied for the expression profiling. Gene expression is normalized to house-keeping gene GAPDH and wild type ES cells as the reference sample.

Immunofluorescence staining

For cryosections, ESC-derived 5-day-old EBs were processed as previously described [62] and subsequently sectioned at 7 µm before acetone fixation for 10 minutes at 4°C, followed by 30 minutes air drying at RT. Next, slides were permeabilized for 5 minutes with 0.2% Triton X-100 in PBS, followed by blocking with 2% BSA in PBS at RT for 1 hour. The slides were then incubated with rat anti-mouse PECAM-1 (Clone ME1C14.7, Santa Cruz) o/n at 4°C. The slides were then washed four times in PBS and incubated for 1 hour with goat anti-rat Cy3 (Jackson ImmunoResearch Laboratories) and goat anti-rabbit Alexa 594 (Invitrogen). Secondary antibodies: donkey anti-rat PECAM-1 (BD Pharmingen) and rat anti-mouse ENG (CD105 domain (MOPC-21) from Bio Express, West Lebanon, NH, was used. EC sprouts were measured by Olympus Analysis software. Western blot analysis

HUVECs were seeded in six-well plates and allowed to grow to 90% confluence. Cells were washed with PBS and serum-starved for 5 hours. Cells were stimulated with VEGF 50 ng/ml for 5 minutes, washed with PBS and lysed in SDS sample buffer. Samples were boiled for 10 minutes and subjected to SDS-PAGE and western blotting. Phospho-VEGFR1, phospho-ERK antibodies were purchased from Cell signaling Technology. ENG was analyzed with an antiserum recognizing human ENG [63].

3D-culture spheroid assay

HUVECs (400 cells per spheroid) were suspended in Medium M199 containing Earle’s salt and L-glutamine, 10% FBS, methylcellulose, heparin, bovine pituitary extract, PS and seeded in non-adherent round-bottom 96-well plates. After 24 hours, spheroids were embedded into collagen and stimulated with corresponding stimuli in the presence or absence of inhibitors or neutralizing antibodies for another 24 hours. As control antibody for experiments with ENG neutralizing antibody TRC105, the Fc domain (MOPC-21) from Bio Express, West Lebanon, NH, was used. EC sprouts were measured by Olympus Analysis software.

Ex vivo fetal mouse metatarsal angiogenic assay

Metatarsals from 17-day-old mouse fetuses from Eng+/− and Eng−/− mice [12] were dissected as described previously [64]. Six metatarsals per experimental group were transferred to 24-wells tissue-culture plates containing α-MEM (Gibco), 10% FBS and penicillin/streptomycin (PS), and allowed to adhere for 4 days. Then, medium was replaced by fresh medium containing 50 ng/ml VEGF. Cultures were fixed 7 days after stimulation and vessel formation was visualized by anti-PECAM-1 staining [39]. Vascular density was quantified by automated image analysis with Image J. Mouse Image was performed by the Institutional Committee for Animal Welfare of the Leiden University Medical Center (LUMC) and were performed according to the regulatory guidelines.

| Target       | Forward primer | Reverse primer |
|--------------|----------------|----------------|
| Eng          | GGTCACTGACTGGACACTCA | AGGCGTACTGAGGAAG |
| Vegf2        | ACCAGGGCCGACATGTTGCG | GGGCAAGCTCATTGATG |
| VE-cadherin  | ATTTTAGAGACACCCCAAG | TGGTTTTGCGTGAAGTCG |
| GAPDH        | ACTATGGGGCACCTAGGAG | ACACATGGGGGTAGAAC |

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ENDOGLIN in VEGF-Induced Angiogenesis

Statistics
All results are expressed as the mean ± s.d. Statistical differences were examined by two-tailed Student’s t-test and P≤0.05 was considered to be statistically significant (in the figures, P≤0.05 and **P≤0.01).

Supporting Information
Figure S1 Eng+/− ESC derived EBs have impaired endothelial cell-derived vessel structures. A) Bright field image and PECAM-1 staining of EBs from Eng+/− and Eng−/− ESCs. Both bright field image and the PECAM-1 staining show that the Eng−/− EB has less endothelial sprouts than the Eng+/− EB. B) Quantification of the number of sprouts per EB and length of the sprouts. (TIF)

Author Information
Conceived and designed the experiments: ZL FL JM Swid MJG CM PfD. Performed the experiments: ZL FL JM Swid MnD MT Jk SM. Analyzed the data: ZL FL JM Swid MJG CM PfD Ljach Lm Ep. Contributed reagents/materials/analysis tools: Kk ML Hma Ct. Wrote the paper: ZL FL JM Swid MJG CM PfD.

References
1. Risau W (1997) Mechanisms of angiogenesis. Nature 386: 671–674. 2. Carapuzo P, Ferreira V, Bereza G, Bullejos S, Kieckens L, et al. (1996) Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature 380: 435–439. 3. Gougos A., Letarte M. (1988) Identification of a human endothelial cell antigen with monoclonal antibody 4H4 produced against a pre-B leukemia protein. J Biol Chem 263: 6417–6423. 4. Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L, et al. (1996) Vascular endothelial growth factor gene. Nature 380: 439–442. 5. Bouck N, Stellmach V, Hsu SC. (1996) How tumors become angiogenic. Adv Cancer Res 69: 135–174. 6. Levy NS, Chung S, Furneaux H, Levy AP. (1998) Hypoxia stimulation of vascular endothelial growth factor mRNA by the RNA-binding protein HuR. J Biol Chem 273: 6417–6423. 7. Bourdeau A, Dumont DJ, Letarte M. (1999) A murine model of hereditary hemorrhagic telangiectasia. J Clin Invest 104: 1343–1351. 8. Mustonen T, Altitalo K. (1995) Endothelial receptor tyrosine kinases involved in angiogenesis. J Biol Chem 270: 895–898. 9. Landgren E, Schiller P, Gao Y, Claesson-Welsh L. (1998) Placenta growth factor stimulates MAP kinase and mitogenesis but not phospholipase C-gamma and migration of endothelial cells expressing Flt-1. Oncogene 16: 359–367. 10. Shahaly F, Rossant J, Yamaguchi TP, Gertsenstein M, Xu XF, et al. (1995) Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. EMBO J 14: 2199–2208. 11. Gougos A., Letarte M. (1988) Identification of a human endothelial cell antigen with monoclonal antibody 4H4 produced against a pre-B leukemia protein. J Biol Chem 263: 6417–6423. 12. Bade C, Mallet C, Feige JJ, Bally S. (2007) Identification of BMP and BMP9 as functional activators of the orphan activin receptor-like kinase 1 (ALK1) in endothelial cells. Blood 109: 1953–1961. 13. Schaper W, van Dunber M, Liu Z, van Bezoijen RL, Zhao Q, et al. (2007) BMP-9 signals via ALK1 and inhibits bFGF-induced endothelial cell proliferation and VEGF-stimulated angiogenesis. J Cell Sci 120: 964–972. 14. Bouck N, Stellmach V, Hsu SC. (1996) How tumors become angiogenic. Adv Cancer Res 69: 135–174. 15. Risau W (1997) Mechanisms of angiogenesis. Nature 386: 671–674.

Endoglin promotes endothelial cell proliferation and TGF-β signaling in extraembryonic mesoderm. J Cell Sci 129: 1525–1533. 16. Li DY, Sorensen LK, Brooke BS, Urness LD, Davis EC, et al. (1999) Defective hemorrhagic telangiectasia. J Clin Invest 104: 1343–1351. 17. Johnson DW, Berg JN, Balinon MA, Gallione CJ, Maroudel L, et al. (1994) Endoglin, a TGF-β binding protein of endothelial cells, is the gene for hereditary haemorrhagic telangiectasia type 1. Nat Genet 8: 345–351. 18. Johnson DW, Berg JN, Balinon MA, Gallione CJ, Maroudel L, et al. (1994) Endoglin, a TGF-β binding protein of endothelial cells, is the gene for hereditary haemorrhagic telangiectasia type 1. Nat Genet 8: 345–351. 19. Li X, Claesson-Welsh L. (2009) Embryonic stem cell models in vascular biology. J Thromb Haemost. 7 Suppl 1: S3–S16. 20. Wang R, Clark R, Bautch VL. (1992) Embryonic stem cell-derived cystic endothelial bodies form vascular channels: an in vitro model of blood vessel development. Development 114: 303–316. 21. Deckers M, van der Pluijm G, Dooijewaard S, Kroon M, van, Hinsbergh V, et al. (2001) Effect of angiogenic and antiangiogenic compounds on the outgrowth of capillary structures from fetal mouse bone explants. Lab Invest 81: 5–15. 22. Deckers M, van der Pluijm G, Dooijewaard S, Kroon M, van, Hinsbergh V, et al. (2001) Effect of angiogenic and antiangiogenic compounds on the outgrowth of capillary structures from fetal mouse bone explants. Lab Invest 81: 5–15.
47. Nomura-Kitabayashi A, Anderson GA, Sleep G, Mena J, Karabegovic A, et al. (2009) Endoglin is dispensable for angiogenesis, but required for endocardial cushion formation in the midgestation mouse embryo. Dev Biol 335: 66–77.

48. Li C, Hampson IN, Hampson L, Kumar P, Bernabeu C, et al. (2000) CD105 antagonizes the inhibitory signaling of transforming growth factor-β1 on human vascular endothelial cells. FASEB J 14: 55–64.

49. She X, Matsuno F, Harada N, Tsai H, Seon BK. (2004) Synergy between anti-endoglin (CD105) monoclonal antibodies and TGF-β in suppression of growth of human endothelial cells. Int J Cancer 108: 251–257.

50. Nolan-Stevaux O, Zhong W, Culp S, Shaffer K, Hoover J, et al. (2012) Endoglin requirement for BMP9 signaling in endothelial cells reveals new mechanism of action for selective anti-endoglin antibodies. PLoS One. 7: e50920.

51. Castonguay R, Werner ED, Matthews RG, Presman E, Mulivor AW, et al. (2011) Soluble endoglin specifically binds bone morphogenetic proteins 9 and 10 via its orphan domain, inhibits blood vessel formation, and suppresses tumor growth. J Biol Chem 286: 30034–30046.

52. Havinkels J, Kupper P, Wiercinska E, Verspaget HW, Liu Z, et al. (2010) Matrix metalloproteinase-14 (MT1-MMP)-mediated endoglin shedding inhibits tumor angiogenesis. Cancer Res 70: 4141–4150.

53. Liu Z, Aflak G, ten Dijke P. (2012) Soluble fms-like tyrosine kinase 1 and soluble Endoglin are elevated circulating anti-angiogenic factors in preeclampsia. Pregnancy Hypertension 2: 358–367.

54. Pece-Barbara N, Vera S, Kathirkamathambly K, Lücker S, Di Guglielmo GM, et al. (2005) Endoglin null endothelial cells proliferate faster and are more responsive to transforming growth factor-β1 with higher affinity receptors and an activated Alk1 pathway. J Biol Chem 280: 27800–27808.

55. Carvalho RL, Jonker L, Goumans MJ, Larsson J, Bousman P, et al. (2004) Defective paracrine signalling by TGF-β in yolk sac vasculature of endoglin mutant mice: a paradigm for hereditary haemorrhagic telangiectasia. Development 131: 6237–6247.

56. Xu B, Wu YQ, Hsue M, Arthur HM, Marchuk DA, et al. (2004) Vascular endothelial growth factor induces abnormal microvasculature in the endoglin heterozygous mouse brain. J Cereb Blood Flow Metab 24: 237–244.

57. Sambrook J, Frisch EF, Maniatis T. (1989) Molecular Cloning: A Laboratory Manual. 2nd edn. Cold Spring Harbor Laboratory Press, New York.

58. Bot PT, Hoefner IE, Sluiter JP, van Vliet P, Smit AM, et al. (2009) Increased expression of the transforming growth factor-beta signaling pathway, endoglin and early growth response-1 in stable plaques. Stroke 40: 439–447.

59. Slager HG, Freiend E, Biuring AM, Feijen A, Mummery CL. (1993) Secretion of transforming growth factor-β1 isoforms by embryonic stem cells: isoform and latency are dependent on direction of differentiation. J Cell Physiol 156: 247–256.

60. Roelen BA, Lin HY, Knezevic V, Freiend E, Mummery CL. (1994) Expression of TGF-βs and their receptors during implantation and organogenesis of the mouse embryo. Dev Biol 166: 716–728.

61. Sinha S, Hoofnagle MH, Kingston PA, McCanna ME, Owens G. (2004) Transforming growth factor-β1 signaling contributes to development of smooth muscle cells from embryonic stem cells. Am J Physiol Cell Physiol 287: C1560–C1568.

62. Bajanca F, Luz M, Daxson MJ, Thorsteinsson S. (2004) Integrins in the mouse myotome: developmental changes and differences between the epaxial and hypaxial lineage. Dev Dyn 231: 402–415.

63. Pardali E, van der Schaft DW, Wiercinska E, Gorter A, Hogendoorn PC, et al. (2011) Critical role of endoglin in tumor cell plasticity of Ewing sarcoma and melanoma. Oncogene 30: 334–345.

64. van der Pluijm G, Lowik CW, de Groot H, Alblas MJ, van der Wee-Pals IJ, et al. (1991) Modulation of PTH-stimulated osteoclastic resorption by bisphosphonates in fetal mouse bone explants. J Bone Miner Res 6: 1203–1210.