The physiological role of endoglin in the cardiovascular system

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Abstract

Endoglin (CD105) is an integral membrane glycoprotein that serves as co-receptor for members of the transforming growth factor beta (TGF-β) superfamily of proteins. A major role for endoglin in regulating TGF-β-dependent vascular remodeling and angiogenesis has been postulated based on: i) endoglin is the gene mutated in Hereditary Hemorrhagic Telangiectasia (HHT) type 1, a disease characterized by vascular malformations; ii) endoglin knockout mice die at mid-gestation due to defective angiogenesis; iii) endoglin is overexpressed in neoangiogenic vessels, during inflammation, and in solid tumors; and iv) endoglin regulates the expression and activity of endothelial nitric oxide synthase (eNOS), which is involved in angiogenesis and vascular tone. Besides the predominant form of the endoglin receptor (L-endoglin), two additional forms of endoglin have been recently reported to play a role in vascular pathology and homeostasis: the alternatively spliced short isoform (S-endoglin) and a soluble endoglin form (sEng) that is proteolytically cleaved from membrane bound endoglin. The purpose of this review is to underline the role that the different forms of endoglin play in regulating angiogenesis, vascular remodeling and vascular tone, as well as to analyze the molecular and cellular mechanisms supporting these effects.
Introduction

Endoglin (also known as CD105) is a type I integral membrane glycoprotein that belongs to the Zona Pellucida family of proteins (64, 105). It is highly expressed on proliferating vascular ECs (25, 119) and has been identified as an accessory receptor for transforming growth factor beta (TGF-β) (30). The human *ENDOGLIN* gene has been localized to chromosome 9q34ter (53) and it is mutated in hereditary hemorrhagic telangiectasia (HHT) type 1 (118). Mice lacking endoglin die during embryonic development due to defective angiogenesis (6, 23, 103) and endoglin plays a major role in tumoral and non-tumoral adult angiogenesis (13, 79). A soluble form of endoglin plays also a central role in pre-eclampsia, a disease characterized by hypertension and severe alterations in placental circulation (172). Overall, these results support the view that endoglin has a pivotal function in vascular development and disease (13, 50, 93, 158). The purpose of this review is to critically assess the role of endoglin in vascular function as well as the mechanisms involved.

Structure of endoglin

Human endoglin is a type I integral membrane protein with a large extracellular domain (561 amino acids), a single hydrophobic transmembrane domain, and a short cytosolic domain (64) (Figure 1A). The expression of two different alternatively spliced isoforms, long (L)-endoglin and short (S)-endoglin, has been demonstrated in human and mouse tissues (12, 64, 130). Human S-endoglin and L-endoglin proteins vary from each other in their cytoplasmic tails that contain 14 and 47 amino acids, respectively, with a sequence of only 7 residues being specific for S-endoglin (Figure 1B). Because L-endoglin is the predominantly expressed isoform, unless stated otherwise, the functional studies in this review will be referred to this isoform. Endoglin is a
The primary structure of endoglin suggests that there are five N-linked glycosylation sites in the N-terminal domain and a probable O-glycan domain, which are rich in Ser and Thr residues proximal to the membrane-spanning domain. Human endoglin also contains an Arg-Gly-Asp (RGD) peptide sequence that is known as a cell recognition site for numerous adhesive proteins present in the extracellular matrix, but this motif is absent from mouse, porcine, rat, and canine endoglin proteins. Structurally, endoglin belongs to the Zona Pellucida (ZP) family of proteins that share a ZP domain of ~260 amino acid residues in their extracellular region. The three-dimensional structure of the extracellular domain of endoglin at 25Å resolution, using single-particle electron microscopy (EM) has been elucidated. Endoglin arranges as a dome made of antiparallel oriented monomers enclosing a cavity at one end. Each subunit comprises one ZP domain in the juxtamembrane region. The N-terminal domain does not show any significant homology to other protein family/domain and thereby has been named “orphan” domain.

The cytosolic domain of endoglin is constitutively phosphorylated and it can be targeted by serine and threonine kinases, including the TGF-β type I and II receptors. It has been shown that the endoglin phosphorylation status can influence its subcellular localization and cellular migration. Endoglin cytoplasmic domain contains a consensus PDZ binding motif that mediates endoglin interaction with several PDZ domain containing proteins and endoglin phosphorylation of distal threonine residues.

**Regulation of endoglin expression**
The cellular and tissue distribution of endoglin suggests its role in vascular development, angiogenesis, and vascular homeostasis. Endoglin is expressed at low levels in resting endothelial cells (ECs), but it is highly expressed in vascular ECs at sites of active angiogenesis, during embryogenesis (80, 131), in inflamed tissues and healing wounds (163), psoriatic skin (145), inflamed synovial arthritis (155), upon vascular injury (21) and in tumor vessels (13, 25, 55, 119). Endoglin is also overexpressed after ischemia and reperfusion in the kidney (47), hind-limbs (79) and heart (168). Endoglin is expressed not only in ECs, but also in several other cell types involved in the cardiovascular system. For example, while endoglin expression is low in normal smooth muscle cells (2), its expression is up regulated in vascular smooth muscle cells of human atherosclerotic plaques (36). Endoglin is expressed in cardiac fibroblasts and modulates the profibrogenic actions of angiotensin II (31). Endoglin is also expressed in other tissues undergoing fibrosis such as the kidney (143) and liver (34). Endoglin is present on monocytes and it is upregulated during the monocyte-macrophage transition (87). During the development of the cardiovascular system, endoglin is found on the vascular endothelium of human embryos during all developmental stages from 4 weeks onwards and it is transiently up-regulated on cushion tissue mesenchyme during heart septation (135). Furthermore, an altered expression of endoglin was observed in human fetuses with cardiac defects (9).

The mechanisms responsible for the increased endoglin expression in activated vessels are probably multifactorial, hypoxia, vascular injury and related cytokines being the most likely stimuli. In fact, endoglin expression is upregulated after ischemia in the heart (168), kidney (47) and hind-limbs (79), as well as upon arterial injury (21, 110). Also, in murine cerebral microvascular ECs, hypoxia induces the expression of endoglin at both the mRNA and protein levels and this induction is regulated by p38 and probably JNK pathways (179). Furthermore, a hypoxia-responsive element (HRE) downstream of the main transcription start site of the
endoglin gene has been characterized. Thus, under hypoxic conditions, the hypoxia inducible
factor-1 (HIF-1) complex binds a functional consensus HRE in the endoglin gene promoter (146). TGF-β signaling, via Smad transcription factors, also potently stimulates endoglin expression
(20, 88, 142). By contrast, tumor necrosis factor-alpha (TNF-α) decreases endoglin protein levels
in ECs (98). Whereas hypoxia alone moderately stimulates endoglin transcription, addition of
TGF-β1 under hypoxic conditions results in a transcriptional cooperation between both signaling
pathways, leading to marked stimulation of endoglin expression. This synergic stimulation
involves the formation of a transcriptional multicomplex containing Smad3/Smad4, Sp1, and
HIF-1, leading to a cooperative effect of these factors on endoglin transcription (146). Also, upon
vascular injury, a transcriptional activation of endoglin mediated by the cooperative interaction
between Sp1 and KLF6 transcription factors has been reported (21).

Modulation by endoglin of TGF-β-dependent cell responses
Endoglin is an auxiliary TGF-β receptor that modulates TGF-β1- and TGF-β3-, but not TGF-β2-
dependent responses in several cell types. In human monocytic cells, TGF-β1, but not TGF-β2
responses are abrogated in the presence of endoglin (88). In a variety of cell types, including
myoblasts and fibroblasts, endoglin opposes TGF-β1-dependent responses such as the inhibition
of cellular proliferation (88), the expression of the extracellular matrix proteoglycan lumican
(22), as well as the increased expression of extracellular matrix components, including PAI-1,
collagen or fibronectin (46, 72, 88, 96, 125). Moreover, neutralizing anti-endoglin antibodies or
antisense oligonucleotides for endoglin enhance the inhibitory effect of TGF-β on proliferation
and migration (100, 152), whereas endoglin overexpression counteracts the anti-proliferative
effect of TGF-β1 in ECs (100). Inhibition of endoglin expression on ECs increases the anti-
proliferative effect of TGF-β1, and enhances EC apoptosis induced by hypoxia and TGF-β1 (100, 101). These findings are compatible with the fact that endoglin is markedly up-regulated in the proliferating endothelium of tissues undergoing angiogenesis (13, 18, 25, 56, 85). While it is widely accepted that endoglin is expressed at high levels in proliferating ECs, the direct role of endoglin in mediating EC proliferation and migration is controversial. Several experimental evidences support the hypothesis that endoglin promotes endothelial cell proliferation and migration (92). However, other authors have reported that an Eng−/− EC line proliferates faster than Eng+/+ control cells (128) and that Eng−/− progenitors can be expanded and differentiated in culture (33). Using a mouse with a conditional mutation in the Eng gene, it was shown that subcutaneous Matrigel implants in adult mice were populated by reduced numbers of new blood vessels compared with controls, whereas their endoglin-deficient retinas exhibited increased proliferation of ECs (112). These variable results suggest that the endoglin role in cell proliferation might be context dependent and they should be interpreted with caution. Endoglin cytoplasmic and extracellular domains specifically interact with those of the activin like kinase 1 (ALK1), a TGF-β type I receptor (15). Also, colocalization of endoglin and ALK1 has been demonstrated in vascular endothelia (113). Moreover, studies using Eng−/− and Eng+/− embryonic ECs indicate that endoglin promotes endothelial cell proliferation via the TGF-β/ALK1 pathway (92), suggesting the involvement of endoglin and ALK1 in a common signaling pathway (15, 93, 157). Analyses of the downstream target genes regulated by endoglin and ALK1 have been carried out by gene expression fingerprinting of endoglin deficient human ECs from HHT patients with pathogenic mutations in either endoglin or ALK1 genes. These studies allowed the identification of hundreds of down-regulated and up-regulated genes, including those
involved in angiogenesis, cytoskeleton organization, cell guidance, intercellular connections, cell migration and proliferation, or nitric oxide (NO) synthesis (51, 159).

Role of endoglin in vascular pathology

The importance of endoglin in vascular biology is reflected by the fact that mutations in the ENDOGLIN gene (ENG) lead to a vascular disease called the Rendu-Osler-Weber syndrome, or Hereditary Hemorrhagic Telangiectasia type 1 (HHT1) (118). HHT is an inherited autosomal-dominant and highly penetrant disorder characterized by vascular dysplasias, frequent episodes of epistaxis, mucocutaneous telangiectases, and arteriovenous malformations of the lung, brain, liver and gastrointestinal tract (69). A second form of HHT (HHT2) is caused by mutations in the gene coding for the TGF-β type I receptor known as activin receptor-like kinase-1 (ACVRL1 or ALK1). Either of the two genes, ENG or ACVRL1, is mutated in more than 90% of patients with HHT (1, 19, 52, 57, 94, 95). Two additional loci for HHT have been mapped to chromosomes 5 and 7, but the corresponding mutant genes have not been identified yet (10, 35). Moreover, a combined syndrome of Juvenile Polyposis (JP) and HHT was described that is caused by mutations in SMAD4 which encodes a transcription factor of the TGF-β signaling pathway (59). This combined syndrome (JP-HHT) occurs only in 1%-2% of persons clinically diagnosed with HHT, as evidenced by detected mutations in SMAD4.

A common feature in HHT patients is the presence of vascular lesions (telangiectases and arteriovenous malformations) that lead to a loss of the intervening capillary network that connects the arteriole with the venule (24, 74). Interestingly, the frequency of pulmonary arteriovenous malformations in HHT2 (8%) is far less than in HHT1 (45%) patients. Expression studies in lung vessels showed that endoglin and ALK1 have distinct expression profiles in the pulmonary
vasculature and are only co-expressed in the distal (pre-capillary) arteries, distal veins and capillaries, consistent with the tendency for pulmonary arteriovenous malformations to form in the distal pulmonary vessels in HHT (113). In spite of the important pathological implications of these lesions, the mechanisms by which they are generated have not been fully elucidated. Because of the predominant expression of endoglin in ECs, it is tempting to speculate that endoglin loss of function of the mutant allele in this cell type is the cause of the lesion. One of the functions described for endoglin is the protective role against apoptosis in ECs subjected to hypoxia and TGF-β1 stimuli (101). Thus, endoglin haploinsufficiency in HHT may lead to a massive apoptosis in those capillary ECs where endoglin function is required for survival (Figure 2). As a consequence of the EC apoptosis, the capillary network gradually disappears and only a preferential vessel remains that eventually becomes the arterio-venous shunt (Figure 3). An interesting question that arises in HHT patients is why the vascular lesions appear only at distinct sites within certain organs, rather than being present throughout the body and in all organs/tissues. To explain this finding one can postulate the need for an external trigger, or second hit, such as inflammation, infection, vascular injury, ischemia or trauma that synergizes with endoglin haploinsufficiency to generate the lesion. Of note, these potential hits can upregulate endoglin expression (21, 34, 47, 90, 110, 146, 163, 167, 168), suggesting that endoglin function is required under those stressing conditions. Experimental support for the second hit hypothesis has been recently reported using a mouse with a conditional mutation in Eng, demonstrating that arteriovenous malformations develop when an angiogenic stimulus is combined with endoglin depletion (112). Thus, in the HHT setting endoglin protein levels may not reach the minimum threshold to achieve the optimal function and be critical to generate the vascular lesion (Figure 3).
**Endoglin and regulation of angiogenesis**

Angiogenesis is a complex and highly regulated physiological homeostatic process by which the body maintains the supply of oxygen and metabolites depending on the requirements of a given organ or tissue (3, 141). It involves the formation of new vessels with two separate, but coordinated phases: activation and maturation. The whole process consists of a series of endothelial cell responses to angiogenic stimulation; including degradation of extracellular matrix (ECM), budding, proliferation, migration, tube formation, maturation and maintenance of quiescent endothelium. It should be noted that ECs in normal quiescent endothelium have a very low turnover rate, with a doubling time of more than 1,000 days. Angiogenic endothelium, in contrast, has a rapid turnover and it has been termed “activated” endothelium (85). In the activation phase, new sprouts form at distinct locations in the preexisting vessel. Sprout formation is initiated by endothelial cell activation, degradation of the ECM by ECs followed by development of a new bud from the endothelial cell layer. This bud will elongate by EC proliferation and migration towards the source of the angiogenic stimuli. The process culminates with tube formation and maturation. Maturation phase consists of a progressive decrease in EC proliferation and the recruitment of mesenchymal cells to form mural cells, which can be pericytes or vascular smooth muscle cells. Pericytes are thought to stabilize capillaries, whereas vascular smooth muscle cells (VSMC) are critical for arterial structure and function (3, 28, 76, 141).

These processes are driven by a complex interaction of different growth factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and TGF-β and their specific receptors. This complexity is eventually combined with other types of angiogenic stimuli such as hypoxia. The directionality of new vessel growth is driven by leading endothelial tip cells in
response to guidance molecules. Recent studies have shown how endothelial tip, stalk and phalanx cells form sprouts and how ECs arrange in tubular structures in a highly organized way (28). Platelet-derived growth factor (PDGF) signaling is important for the initial recruitment of mesenchymal cells that differentiate to VSMCs in response to TGF-β signaling. Both stimulatory and inhibitory effects of TGF-β on angiogenesis have been reported (129, 157). Whether TGF-β stimulates or inhibits these processes depends on the experimental conditions. For example, low doses of TGF-β in vitro (0.25-0.50 ng/ml) stimulate EC proliferation and migration, whereas higher doses show an inhibitory effect, leading to EC quiescence (17, 68, 91).

A major evidence for the pivotal role of endoglin in angiogenesis is that mice lacking endoglin (Eng−/−) die from cardiovascular defects at mid gestation (E10.5-11.5) with major defects in yolk sac vasculature (6, 23, 103). In these embryos, the first stages of differentiation from hemangioblasts to ECs and the formation of the primitive vascular structures, a process called vasculogenesis, occurs normally, but this primitive system does not develop to a mature vascular network (angiogenesis), indicating a critical role for endoglin in angiogenesis rather than in vasculogenesis (80, 131).

Eng−/− embryos also show a defective development of VSMC (96) and several explanations have been proposed to account for this deficiency. First, ectopic expression of endoglin in neural crest stem cells causes pericardial hemorrhage associated with altered smooth muscle cell investment in the walls of major vessels, suggesting a direct role for endoglin in myogenic differentiation (115). Alternatively, in the yolk sac, the absence of endoglin in ECs of Eng−/− mice results in reduced availability of active TGF-β protein to promote the recruitment and the differentiation of mesenchymal cells into VSMCs, thus leading to weak vessel walls (29). More recently, it has been shown that endoglin, via the venous-specific marker COUPTFII, plays distinct and cell-
autonomous roles in VSMC recruitment and arteriovenous specification in angiogenesis that may contribute to HHT (114).

Endoglin haploinsufficient (Eng$^{+/-}$) mice have been used to investigate the function of endoglin in adult neovascularisation because these adult mice have endoglin protein levels reduced by 50%, as in HHT1 patients (23, 77, 162). Eng$^{+/-}$ mice have a delayed reperfusion following hindlimb ischemia induced by femoral ligation (79). Also, endoglin heterozygous mice implanted with matrigel plugs, to measure EC outgrowth and invasion into the ECM in vivo, showed significantly less vascular structures compared to wild type mice (79). Moreover, Eng$^{+/-}$ mice with myocardial infarction induced by coronary artery ligation showed a defective angiogenesis (168) in agreement with data from a femoral artery-ligated hindlimb model (79).

**Endoglin and tumor angiogenesis**

Most of the events occurring during “physiological” angiogenesis also occur during the process of tumor angiogenesis. Tumor vessels can exist either in an immature state (lack of mural cells) or in association with pericytes/VSMCs. Angiogenesis is a crucial process in tumor growth, as continuous supply of oxygen and nutrients is necessary to support anabolic cell metabolism involved in cell proliferation. Increased tumor size is accompanied by new vessel formation stimulated by hypoxia and by the increased metabolism necessary for tumoral cell growth. If angiogenesis is fully blocked, tumor size can not reach more that a few mm$^3$. Thus, angiogenesis is considered as a major target for the treatment of tumors.

Endoglin is overexpressed in tumor vessels (13, 25, 49, 56, 119) but also in tumor cells (4, 13). Endoglin is a better marker of tumor capillary density than other classical markers of ECs and the assessment of microvascular density with endoglin staining is a good prognostic indicator as it has been demonstrated that the density of endoglin-positive blood vessels negatively correlates
with overall survival, disease–free survival and metastasis in a great variety of solid tumors (reviewed in 13). Moreover, $Eng^{+/−}$ mice bearing subcutaneous lung carcinomas show decreased tumor growth rates and lower capillary densities, as compared to wild-type mice (50), suggesting endoglin involvement in tumor growth (Figure 4). Given the high density of endoglin in tumor ECs, endoglin can be also used to target these vessels with anti-endoglin antibodies, bound or not to toxic substances, in order to destroy the tumoral vessels and limit the tumor growth. In this regard, there are several reported approaches using experimental animal models (55, 117, 156, 164, 165). Among these, the approach of using naked anti-endoglin monoclonal antibodies as a tumor vascular targeting agent is clinically relevant. In fact, a multicenter phase I clinical trial using an anti-endoglin antibody (TRC105) has been recently approved by the United States Food and Drug Administration (FDA) and it is now in progress in patients with advanced and/or metastatic cancer (13).

**Endoglin and regulation of vascular repair**

Mononuclear cells (MNCs) contribute to the formation of new blood vessels (7, 106, 140) and activated MNC express upregulated levels of endoglin (87). These findings prompted van Laake et al. (168) to investigate whether endoglin expression in MNCs might regulate vascular repairing in an infarcted myocardium by injecting MNCs from healthy human donors or from HHT1 patients, which express half levels of endoglin. MNCs from healthy human donors significantly improved heart function in $Eng^{+/−}$ mice, whereas MNCs from HHT1 patients were without significant effect (168). Because MNCs from HHT1 patients showed a reduced homing ability to infarcted mouse myocardium, the possible involvement of stromal-cell derived factor-1 alpha (SDF-1$α$) and its corresponding chemokine receptor CXCR4, that are crucial for homing and are
negatively influenced by CD26, were explored (132). Thus, it was shown that a decreased homing of HHT1-MNCs is caused by an impaired ability of the cells to respond to SDF-1α. Interestingly, modulating the dipeptidyl peptidase IV CD26 levels using inhibitors like Diprotin-
A restored homing in cases where increased expression of CD26 contributes to the underlying pathological mechanism (132).

The low angiogenic activity shown by Eng+/− mice and the abnormal behavior of MNCs from HHT1 patients in the heart infarction experiments suggest a defective homing of endoglin expressing circulating cells. VEGF, in addition to directly stimulating EC proliferation and migration, also induces the expression of SDF1α, a chemokine that regulates MNCs adherence to the vessel walls, where they can act in a paracrine fashion to enhance proliferation of resident ECs (70). It should be noted that VEGF production has been observed to be decreased in ECs derived from Eng+/− mice (79), and this finding could explain the defects in MNC recruitment observed in these animals. MNCs adherence to the vessel wall is regulated by a family of membrane molecules called cell adhesion molecules (CAM), including ICAM-1, VCAM-1, PECAM-1 and P-selectin. After renal ischemia/reperfusion, both CAM expression and MNCs infiltration were reduced in Eng+/− compared with wild type mice (47). Thus, a defective CAM expression in endoglin deficient cells (either in endothelial or circulating cells) could also explain the low angiogenesis rate associated with endoglin deficiency. TGF-β also regulates the formation of podosomes that are highly dynamic structures involved in adhesion, migration, invasion and circulating cell recruitment (104, 120). As reduced endoglin expression affects TGF-β signalling in monocytes (88), this may lead to a defective podosome formation and impaired recruitment to inflamed tissues.
Endoglin and regulation of vascular tone

Mice deficient in endoglin (Eng\(^{+/−}\)) show a defective vasodilator response to endothelium-dependent vasodilator substances such as acetylcholine or bradykinin (77). In agreement with these data, Eng\(^{+/−}\) mice show a decreased NO synthesis and/or a decreased eNOS expression (77, 78, 160). However, opposite results have been reported, in terms of vasodilation, using Eng\(^{+/−}\) mice. Thus, Jerkic et al (77) observed a defective vasodilator response to acetylcholine or bradykinin in the perfused hindlimb. By contrast, a decreased myogenic vasoconstrictory response and an increased endothelium-dependent vasodilatation in phenylephrine-precontracted mesenteric arteries have been reported (160). In addition, the same group has reported that in pulmonary arteries the endothelium-dependent relaxation in response to acetylcholine of adult Eng\(^{+/−}\) mice was higher than that of Eng\(^{+/+}\) control vessels (11). Accordingly, the interpretations provided to explain these opposite results are also different. On one hand, Jerkic et al postulate that the decreased vasodilatation in Eng\(^{+/−}\) mice is due to a decreased NO production as a consequence of decreased levels of eNOS (77). On the other hand, while agreeing with a reduced NO production in these animals, Toporsian et al hypothesize that endoglin haploinsufficiency in ECs leads to the uncoupling of eNOS that is associated with a defective eNOS/Hsp90 association and a subsequent decrease in NO release and increase in \(\text{O}_2^−\) production (160). Due to the increased levels of \(\text{O}_2^−\), these authors explain the increased vasodilatory response in Eng\(^{+/−}\) mice based on the fact that \(\text{O}_2^−\) has been reported to directly inhibit smooth muscle contraction \textit{in vitro} (82) and that the \(\text{H}_2\text{O}_2\), generated from \(\text{O}_2^−\) by dismutation, is a vasorelaxant in mouse mesenteric arteries. However, a wide number of studies have shown that \(\text{O}_2^−\) decreases endothelium-dependent vasodilatation as it removes NO upon chemical reaction, producing peroxinitrites (137). In addition, endogenous \(\text{H}_2\text{O}_2\) may act as a vasoconstrictor in murine resistance vessels
Although other studies have shown that H$_2$O$_2$ is a major endothelium-dependent relaxing factor in mice aorta (27) or cerebral arteries (48).

Because some of these studies were performed in perfused hindlimbs (77) and others in isolated mesenteric arteries (160), a possible explanation for these contradictory results is that the responses and mechanisms involved in endothelium-dependent vascular relaxation may vary with the different vascular beds and the specific experimental approach. For instance, in mouse mesenteric arteries, a chronic rise in blood flow induces a diameter enlargement involving NO and superoxide (37), a finding compatible with the myogenic response data of Toporsian et al (160). Moreover, infusion of mice with angiopoietin II, characterized by hyperproduction of O$_2^-$ and ONOO$^-$, causes an impairment in the NO-mediated component of endothelium-dependent relaxation in response to acetylcholine. This inhibitory effect is mediated by increased O$_2^-$ and ONOO$^-$ in the vascular smooth muscle cells of mesenteric arteries (174), at variance with the increased endothelium-dependent vasodilatation of mesenteric arteries reported in Eng$^{+/−}$ mice (160).

Regarding muscular arteries, that are the ones involved in the hind-limb perfusion experiments of Eng$^{+/−}$ animals (77), it has been reported that mice fed a high salt diet show an increased generation of O$_2^-$ in the skeletal muscle microcirculation, and an impaired endothelium-dependent dilation through reduced NO bioavailability. Specifically, arteriolar dilation in response to acetylcholine was ~50% smaller in high salt mice than in normal salt mice, whereas inhibition of NOS with N(G) monomethyl L-arginine (L-NMMA) significantly reduced resting diameters and responses to acetylcholine in normal salt mice, but not in high salt mice (124). These data are in agreement with the defective vasodilator response to acetylcholine in the perfused hindlimb of Eng$^{+/−}$ mice (77).
With respect to pulmonary arteries and the pathogenesis of pulmonary hypertension, increased production of NO in eNOS transgenic mice prevented the increase in right ventricular systolic pressure (RVSP), lung vascular remodeling, and right-ventricular hypertrophy induced by chronic hypoxia, thus suggesting that a decreased eNOS production contributes to the pathogenesis of pulmonary hypertension (127). Furthermore, decreased eNOS-Hsp90 interaction has been suggested to play a role in the pathogenesis of hypoxia-induced pulmonary hypertension on the basis of decreased eNOS activity and NO bioavailability (84, 122), a result that is in agreement with the pulmonary hypertension observed in Eng+/− mice (161). However, a recent study in lungs of Caveolin KO mice showed that increased eNOS-Hsp90 interaction was involved in the mechanism of pulmonary hypertension due to persistent activation of eNOS and the resultant increased formation of peroxynitrite (178). Thus, high levels of O2- and peroxinitrite, similarly to those reported in Eng+/− mice (160), decreased NO availability and led to nitration of PKG, a critical mediator of the NO-dependent vasodilatation. Consequently, an impaired endothelium-dependent relaxation was observed (178), as opposed to the increased endothelium-dependent vasodilatation found in Eng+/− mice (160), although both animal models share the pulmonary hypertension phenotype (161, 178).

Taken together, these data provide evidence that there is a dysregulated vascular tone in endoglin deficient animals, while underlining the complexity in the regulation of the vascular tone by NO, O2- and their derivatives and in the pathways present in different vascular beds, which may explain the apparently discrepant results observed in the Eng+/− mice by different groups.

TGF-β1 leads to an increased vasodilatation in control mice that is severely impaired in Eng+/− mice, suggesting the involvement of endoglin in the TGF-β regulated vascular homeostasis (147). The decreased vasodilatation shown by Eng+/− mice is not associated with increased arterial
pressure because these animals also show increased COX-2 expression and activity with the 

390 corresponding increase in the production of COX-2 derived vasodilator eicosanoids (78). 

Accordingly, the simultaneous inhibition of COX-2 and NOS markedly increases arterial 

392 pressure in Eng \(^{+/−}\) mice (78).

393 The altered vasodilator response in endoglin-deficient animals suggests a potential mechanism in 

394 the genesis of arteriovenous malformations present in HHT1 patients. Under normal conditions 

395 NO regulates dilatation of precapillary sphincters. However, if this mechanism is impaired, 

396 precapillary sphincters remain closed, whereas the blood circulates only through the preferential 

397 ways, lacking pre-capillary sphincters, existing in the capillary beds. This could lead to capillary 

398 EC apoptosis induced by hypoxia and may be reinforced by endoglin haploinsufficiency as in 

399 HHT1. At the same time, the preferential ways may react to the increased flow by widening their 

diameter and recruiting smooth muscle cells (arterialization), thus leading to the typical vascular 

401 malformations. This hypothesis is supported by the observation that adult Eng \(^{+/−}\) mice display 

402 pulmonary arterial hypertension accompanied by rarefaction of peripheral vessels and dilatation 

403 of central large vessels (161). It should be noted that the lung is a frequent place for arteriovenous 

404 shunts in HHT1 patients (69, 74). Further support for a dysregulated vascular tone in HHT has 

405 been recently reported (11). Adult, but not newborn, Eng \(^{+/−}\) mice, show pulmonary vascular 

eNOS uncoupling. In agreement with this finding, pulmonary arteries from adult Eng \(^{+/−}\) mice are 

407 more dilated and have an enhanced endothelium-dependent smooth muscle relaxation potential. 

408 This increased vasorelaxation may play a role in the formation of pulmonary arteriovenous 

409 malformations later in life and could explain the generally late onset of pulmonary clinical 

410 manifestations in HHT (11). In summary, these results further support a role for endoglin in the 

411 regulation of vascular tone.
Nitric oxide mediates endoglin involvement in angiogenesis and vascular homeostasis.

Nitric oxide synthase (NOS)-derived NO is a major regulator of vascular tone and angiogenesis following arterial occlusion. The ischemic tissue shows an increase in endothelial NOS (eNOS) mRNA, protein expression and NO synthesis (77, 109). The deficiency of the NO pathway either by pharmacological inhibition or by gene disruption of eNOS, diminishes ischemia-induced angiogenesis. Conversely, supplementation of NO by the use of exogenous sources restores ischemia-induced angiogenesis. (109).

Transcription of eNOS is regulated by endothelial shear stress, hypoxia, several hormones, and various mediators and growth factors, including TGF-β1. Endoglin plays a major role in regulating eNOS abundance and NO synthesis. The endoglin-dependent regulation of eNOS abundance seems to be based on two different mechanisms. First, endoglin regulates e-NOS mRNA expression (77, 78, 147). Thus, Eng+/− mice and ECs derived from these mice show reduced levels of both basal and TGF-β1-induced eNOS mRNA and protein levels, without changes in inducible NOS (iNOS) or neuronal NOS (nNOS) (77, 78, 160). Furthermore, ectopic expression of endoglin in ECs in vitro results in increased levels of Smad2 protein leading to an enhanced TGF-β receptor-dependent induction of eNOS mRNA expression (147). The second mechanism involved in the regulation by endoglin of eNOS abundance is the regulation by endoglin of the half-life of eNOS protein and eNOS activity (160). In this regard, functional ECs lacking endoglin loose the capacity to generate NO in response to Ca²⁺-dependent eNOS activation. Thus, endoglin associates with eNOS and Hsp90 and stabilizes the activation complex, resulting in NO production (Figure 5A). In addition, eNOS activity was reported to be uncoupled in Eng-deficient murine ECs, as evidenced by severely reduced eNOS/Hsp90 association and increased eNOS derived O₂⁻, H₂O₂ and presumably, peroxinitrite production.
Accordingly, it has been suggested that endoglin modulates the coupling of eNOS activity by acting as a scaffolding protein and bringing cytoplasmic Hsp90 into close proximity with caveolar eNOS (160). Furthermore, it has been reported that in $Eng^{+/c}$ mouse tissues and in $Eng^{-/-}$ cells, eNOS is uncoupled, leading to a decreased NO availability and increased superoxide and $H_2O_2$ production (11, 161). These findings suggest that endoglin expression and nitric oxide regulation are intimately related. Consequently, a major role for eNOS in endoglin-dependent angiogenesis and vascular tone has been postulated. A demonstration of the importance of NO in mediating the stimulation of angiogenesis by endoglin comes from the fact that blockade of NO synthesis with L-nitroarginine methyl-ester (L-NAME) decreases angiogenesis after hind limb ischemia in normal mice but not in endoglin-deficient mice (79). Similar results were observed in a model of tumor-induced angiogenesis in $Eng^{+/c}$ mice. In these mice, tumor growth and vessel density was lower than in control mice (50). Also, NO synthesis blockade with L-NAME induced a marked inhibition of angiogenesis in control mice that was much lower in $Eng^{+/c}$ mice (Duwell, Eleno, Bernabeu and Lopez-Novoa; unpublished data). Taken together, these data support the hypothesis that e-NOS derived NO plays a major role in the endoglin-dependent regulation of angiogenesis and vascular tone.

**Endoglin, the TGF-β signalling pathway and vascular homeostasis**

TGF-β superfamily members, including bone morphogenetic protein (BMP), activin and TGF-β subfamilies, critically regulate many different processes within the cardiovascular system, including cardiac development and angiogenesis. The importance of TGF-β signalling in the cardiovascular system is underlined by the observation that genetic deletion of several TGF-β family members, their receptors, or downstream signaling proteins in mice results in the death of
most of the mutants due to severe defects in yolk sac vasculature formation (67). Also, alterations in this pathway, including either germ-line mutations or alterations in the expression of members of these signaling pathways may lead to cardiovascular pathology (40, 62, 66, 157).

Members of the TGF-β superfamily signal through specific cell surface receptor complexes containing a heterodimeric association between signaling receptors type I and II and non-signalling type III receptors or co-receptors. The receptors types I and II are serine/threonine kinases and are involved in the downstream signaling, whereas the co-receptors, including endoglin and betaglycan, are proteins without known signaling motifs. The core TGF-β signaling pathway comprises at least seven type I (also known as activin like kinase -ALK- receptors) and five type II receptors, where type I acts downstream of type II, and whose combinatorial heterodimeric association determines the specificity of the ligand signaling. The type I receptors include BMP (ALK1, ALK2, ALK3, ALK6), activin (ALK1, ALK2 and ALK4) and TGF-β (ALK1, ALK2, ALK5) receptors. Upon ligand binding, the type II receptor transphosphorylates the type I receptor, which subsequently propagates the signal by phosphorylating the receptor-regulated Smad (R-Smad; Smad1,2,3,5,8) family of proteins. Once phosphorylated, R-Smads form heteromeric complexes with a cooperating homologue named Co-Smad (Smad4), and translocate into the nucleus where they regulate the transcriptional activity of target genes. (62, 116, 157).

Endoglin forms a protein complex with the TGF-β type I (ALK1 and ALK5) and type II receptors and the ligand (14, 30, 73). Several members of the TGF-β superfamily, including TGF-β1 and TGF-β3 (but not TGF-β2) activin-A, BMP-7, and BMP-2 are able to bind endoglin, and this binding requires the presence of the corresponding signaling receptors (8, 30, 96). By contrast, endoglin is able to bind BMP-9 in the absence of signaling receptors (149), in
agreement with the endoglin-dependent increase of the cellular response to BMP-9 (40). Interestingly, BMP-9 has also been shown to be a specific ligand of ALK1. Among the BMP-9-dependent effects are inhibition of EC proliferation and migration in vitro, as well as the inhibition of neoangiogenesis in vivo (40, 41, 42, 166). These BMP-9 effects appear to be mediated by a receptor complex formed by ALK1, the BMP receptor type II (BMPRII) and endoglin (40). Endoglin modulates ligand binding and signaling by association with ALK1 and ALK5. These type I receptors activate signaling pathways via Smad1,5,8 (ALK1) or Smad2,3 (ALK5) to regulate, among others, the proangiogenic Id1 or plasminogen activator inhibitor-1 (PAI-1) target genes, respectively. The balance between ALK1 and ALK5 signalling pathways in ECs and VSMCs plays a crucial role during vascular remodeling and angiogenesis, although the exact molecular mechanisms remain to be elucidated (41, 93, 126, 151, 157). In the ALK1/ALK5 setting, endoglin inhibits the TGF-β/ALK5/Smad3-mediated cellular responses (15, 71, 92, 96, 150, 169) and enhances ALK5/Smad2-mediated responses (29, 73, 147). In addition, endoglin promotes TGF-β1/ALK1 (15, 92) and BMP-9/ALK1 (40) signalling in ECs. Also, endoglin enhances the BMP-7 signal via Smad1/Smad5 pathway in myoblasts (150). Thus, endoglin appears to be a critical modulator of the balance between ALK1 and ALK5 signaling (92). The mechanism by which endoglin potentiates TGF-β/ALK1 signaling involves the direct association of ALK1 with the cytoplasmic and extracellular domains of endoglin, whereas the extracellular domain mediates the enhancement of ALK1 signaling (15). These studies support the view that endoglin and ALK1 participate in a common signaling pathway that is critical for EC responses to TGF-β family members. This conclusion agrees with the fact that pathogenic mutations in endoglin or ALK1 genes result in HHT (1) and that ALK1 and endoglin null mice have similar vascular phenotypes (6, 23, 103, 126, 153). The extracellular and cytoplasmic domains of
endoglin also interact with ALK5 and the type II receptor, but ALK5 interacts with the endoglin
cytoplasmic domain only when the kinase domain is inactive. Upon association, ALK5 and the
type II receptor phosphorylate the endoglin cytoplasmic domain; then ALK5, but not the type II
receptor, dissociates from the complex (73). These data suggest the hypothesis that the
extracellular and cytoplasmic domains of endoglin play distinct roles in receptor signalling that
are downstream of ligand binding and receptor activation.

Role of other endoglin forms in vascular physiopathology

Short endoglin isoform.

The endoglin data described above is referred to the most abundant form of endoglin, the
membrane bound full length L-endoglin (L, long). However, another isoform of membrane
endoglin has been described, which is generated by alternative splicing of the same gene, giving
rise to a shorter form S-endoglin (S, short) (12, 130). In humans, S-endoglin protein contains a
cytoplasmic domain that is 33 amino acids shorter than that of L-endoglin (Figure 1B). Comparative studies between L-endoglin and S-endoglin have revealed distinct functions for
each isoform. Thus, S-endoglin seems to have an anti-angiogenic effect, in contrast to the pro-
angiogenic role attributed to L-endoglin. Mice transgenic for human S-endoglin (170) exhibit a
deficient angiogenic phenotype which drives to a significant delay in tumor growth (130), similar
to that shown by mice deficient in L-endoglin (50, 136). S-endoglin is also involved in
senescence of ECs. The S/L ratio of endoglin isoforms is increased during senescence of human
ECs in vitro, as well as during aging of mice in vascularized tissues (14). Furthermore, transgenic
mice overexpressing S-endoglin in ECs showed hypertension, decreased hypertensive response to
NO inhibition, decreased vasodilatory response to TGF-β1, and decreased eNOS expression in
lungs and kidneys, supporting the involvement of S-endoglin in the NO-dependent vascular homeostasis (14). These results suggest that S-endoglin is induced during endothelial senescence and may contribute to age-dependent vascular pathology.

Signaling by S-endoglin seems to be also different from that by L-endoglin. In myoblasts and ECs L-endoglin enhanced the ALK1/Id1 pathway, while S-endoglin promoted the ALK5/PAI-1 route (14, 169). These effects on signaling are supported by biological effects on TGF-β1-induced collagen I expression and inhibition of cell proliferation. Thus, while L-endoglin decreased TGF-β1-induced collagen I and CTGF expression and increased TGF-β1-induced proliferation, S-endoglin strongly increased TGF-β1-induced collagen I and CTGF expression, and reduced TGF-β1-induced cell proliferation (169). The mechanism underlying the different behavior of S- and L-endoglin might reside in their different interaction with the signaling TGF-β receptors. In ECs, S-endoglin interacts with both ALK5 and ALK1, although the interaction with ALK5 is stronger than with ALK1 (14), at variance with L-endoglin that shows a higher affinity for ALK1 versus ALK5 (15). Moreover, S-endoglin behaves differently than L-endoglin in relation to several TGF-β-responsive specific reporter constructs with different specificities. Thus, S-endoglin expression increased the ALK5 signaling pathway, whereas L-endoglin inhibited the same pathway. On the other hand, L-endoglin, but not S-endoglin, stimulated the ALK1 signaling pathway (14, 169). These results suggest that the S-Endoglin/L-Endoglin ratio in ECs may contribute to balancing the TGF-β signal through ALK5 or ALK1 and their important roles in vascular pathophysiology (Figure 6).

A role for S-endoglin in the regulation of vascular tone has been postulated. Mice transgenic for human S-endoglin (S-Eng+) show a defective NO synthesis and a decreased eNOS expression in lungs and kidneys (14), in contrast with the positive relationship between levels of L-endoglin
and eNOS previously reported in both mice and cultured ECs (77, 78, 160). However, at variance with \( \text{Eng}^{++/-} \) mice, in which the arterial pressure was normal, \( S-\text{Eng}^+ \) mice were hypertensive (14). Furthermore, in both cultured ECs and endoglin-deficient mice, low levels of L-endoglin are associated with high COX-2 expression (77) and this increased COX-2 expression is also found in tissues from \( S-\text{Eng}^+ \) mice (14). Moreover, \( S-\text{Eng}^+ \) transgenic mice show a reduced hypotensive response to TGF-\( \beta \)1 administration (14). This observation is in agreement with the finding that TGF-\( \beta \)1 regulates eNOS expression (148) and induces vasodilatation in wild type mice, this vasodilatation being severely impaired in \( \text{Eng}^{++/-} \) mice (147). Thus, in vivo overexpression of S-endoglin appears to result in the same phenotype as L-endoglin deficiency, suggesting opposing functional effects of both isoforms on the NO and COX-2 systems.

**Soluble endoglin**

A soluble form of endoglin (sEng) has been detected by ELISA and western blot analysis in plasma, serum and urine from patients with different pathologies, including preeclampsia and cancer. Circulating sEng has been purified from preeclampsia patients and its partial peptide sequence suggests that it is an N-terminal cleavage product of full-length membrane bound endoglin (172).

Preeclampsia is a systemic syndrome of pregnancy clinically characterized by new onset of proteinuria and hypertension associated with significant morbidity and mortality to both mothers and fetuses. In these patients, sEng plasma levels are upregulated in a pattern similar to that of a soluble VEGF receptor known as sFlt1 (97, 172). Interestingly, levels of cell surface endoglin are significantly increased in preeclamptic placentas (97). Because endoglin is expressed at high levels in the syncytiotrophobast and invading cytотrophoblasts (65) and membrane-type
metalloprotease-1 (MT1-MMP or MMP-14) is expressed in trophoblasts (175), it has been postulated that increased sEng levels in preeclampsia derive from the proteolytic action of MT1-MMP on the full-length membrane-bound endoglin expressed in trophoblasts or in nearby cells (107) (Figure 7). Experimental support for this hypothesis has been recently reported demonstrating that MT1-MMP is in fact a major endoglin shedding protease (75). This is compatible with the fact that betaglycan, another TGF-β co-receptor with partial sequence homology to endoglin, can be shed by MT1-MMP (171). Of note, increased levels of membrane-bound endoglin have also been reported in circulating cells of women with preeclampsia, thus suggesting that this is not only a local, but a systemic endoglin upregulation (134). It has been shown that uterine ischemia and /or hypoxia plays a major role in increased sEng release. In an experimental model with pregnant rats, placental ischemia induced by a reduction of uterine perfusion pressure increases the expression of sEng and provokes hypertension, thus mimicking the pathophysiologic features of preeclampsia (61). Compatible with these findings, women with preeclampsia have alterations in placental HIF-1 and its targets (138) and hypoxia has been shown to upregulate expression and secretion of sFlt1 protein in primary trophoblast cultures from first-trimester placentas (123). Hypoxia has been shown to upregulate endoglin expression in ECs (146, 179), although this upregulation was not observed in cultured villous trophoblasts (121). Preeclampsia has been attributed to increased oxidative stress in the placenta (139), whereas heme oxygenase-1 (HO-1) and its metabolite carbon monoxide (CO) exert protective effects against oxidative stimuli (39). In this regard, it has been reported that overexpression of HO-1 in endothelial cells inhibited VEGF-mediated sFlt-1 release and interferon-gamma- and TNF-α-induced sEng release, whereas HO-1 inhibition potentiated sFlt-1 and sEng production
from ECs and placental villous explants. Furthermore, mice lacking HO-1 produced higher levels of sFlt-1 and sEng compared with wild-type mice (39).

In addition to being a reliable biomarker of the disease, it has been suggested that sEng plays a major role as anti-angiogenic factor in preeclampsia. Thus sEng amplifies the vascular damage mediated by sFlt1 in pregnant rats, inducing a severe preeclampsia-like syndrome with features of the HELLP syndrome (172). Moreover, overexpression of sFlt1 and sEng in rodents was found to induce focal vasospasm, hypertension, and increased vascular permeability that were associated with brain edema, producing images reminiscent of reversible posterior leukoencephalopathy associated with human eclampsia (111). This effect may be mediated by interference of the nitric oxide-mediated vasodilation. In vitro studies demonstrate that sEng impairs EC proliferation and capillary formation (172). Interestingly, the angiogenic process is disturbed in pre-eclamptic placentas, thus suggesting that sEng has anti-angiogenic properties. Compatible with this finding, it has been shown that injection of sEng to rats induced hypertension (172). To explain the mechanism involved, it has been proposed that sEng plays its anti-angiogenic and pro-hypertensive effects through interaction with circulating endoglin-binding molecules, such as the TGF-β protein superfamily thus preventing the binding of these molecules to the cell membrane TGF-β receptor complex (108). In fact, in vitro studies have shown that sEng inhibits TGF-β1 signaling and competes for TGF-β1 binding to its receptors, abolishing ALK5 signaling-dependent responses in ECs, and consequently the pro-angiogenic effects of TGF-β1 in the normal endothelium (172). Of note, the short form of membrane endoglin (S-endoglin), with only 14 amino acids in its cytoplasmic domain, shows opposite effects to the pro-angiogenic L-endoglin isoform (14, 130, 169), supporting a critical role for the extracellular domain in this process. Another possible mechanism involved in the anti-angiogenic effects of sEng is based on
its inhibitory effect on TGF-β1-mediated eNOS activation in ECs (172). Thus, while there is an increased NO synthesis in pregnant rats, evidence for NO deficiency in preeclampsia has been obtained from experimental rat models of preeclampsia. Endothelial dysfunction has been reported in women with preeclampsia. Accordingly, it has been postulated that preeclampsia is a disease with a major endothelial dysfunction component that plays a role in preeclampsia-associated hypertension (43, 173). Endothelial dysfunction in preeclampsia has also been attributed to placental oxidative stress, the excess production of damaging reactive oxygen species, as markers of high oxidative stress are detected in preeclamptic placentas (134). A key animal model of preeclampsia is produced by infusion of the NO synthesis inhibitor of L-NAME in pregnant rats, leading to hypertension, proteinuria and thrombocytopenia (176). As described above, decreased NO synthesis is associated with decreased angiogenesis, as occurs in endoglin-deficient mice (77, 78, 147), suggesting that sEng behaves as an antagonist of L-endoglin in endothelium-dependent responses such as angiogenesis. Supporting this view, sEng inhibits the proangiogenic activity of VEGF, another protein that plays a major role in angiogenesis (54, 75). sEng also seems to be a regulator of vascular tone. It has been reported that administration of sEng to mice induces an increase in arterial pressure by increasing vascular resistance (172). Most probably, this effect could be attributed to the inhibitory effect of sEng on TGF-β1-mediated eNOS activation in ECs and it has been suggested that high levels of circulating sEng could contribute to the hypertension shown by women with preeclampsia (172). Thus, these data suggest that sEng behaves as an antagonist of L-endoglin in endothelium-dependent responses such as vasodilatation and angiogenesis.

Recently, a role for sEng in brain arteriovenous malformations (AVMs) has been postulated. Thus, patients with brain AVMs had higher mean sEng levels compared with controls (32). To
determine whether sEng affects the vasculature of the adult mouse brain, injection of adenovirus expressing human sEng into brain areas previously exposed to adeno-associated virus expressing VEGF was carried out. Indeed, increased number of dysplastic blood vessels associated with increased membrane-type matrix metalloprotease-2 (MMP-2) and MMP-9 activity was revealed in the sEng-treated animals (172). These results suggest that elevated sEng may play a role in the generation of sporadic brain AVMs and may provide new targets for therapeutic intervention in patients with brain AVMs.

In addition to preeclampsia and brain AVMs, altered sEng levels have been reported in several pathologies such as cancer (13, 26, 99), atherosclerosis and coronary artery disease (16, 102), hepatitis (34), diabetes (5), systemic sclerosis (44, 58), malaria (45), biliary atresia (133) or sickle cell disease (86). Interestingly, a number of laboratories have reported increased levels of sEng in serum, plasma or other fluids from cancer patients as a marker of poor prognosis [reviewed by Fonsatti et al. (56) and Bernabeu et al. (13)]. Also, it has been reported that changes of sEng plasma levels after an acute myocardial infarct are accurate predictors of acute mortality in these patients (38). Although there are numerous reports on the possible participation of sEng in different diseases, the specific molecular mechanism of action of this soluble form of endoglin in these pathologies remains to be elucidated.

Taken together, these results suggest that both the membrane-bound short isoform (S-endoglin) and the soluble endoglin are involved in several pathological conditions and play opposite roles with respect to the predominant membrane-bound L-endoglin isoform.
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Figure 1. Structural representation of endoglin. A. Endoglin is a type I membrane protein with a large extracellular domain that contains a Zona Pelucida (ZP) domain of 260 amino acids in the juxtamembrane region and an N-terminal orphan domain. Endoglin forms dimers and the corresponding monomers are disulphide-linked. Consensus motifs to attach N-linked glycans and O-linked glycans to the extracellular domain have been identified. The cytoplasmic domain of endoglin is phosphorylated at Ser/Thr residues and contains a consensus PDZ-binding motif present at the carboxyl terminus. The cytoplasmic (CYT), transmembrane (TM) and extracellular (EC) domains of the protein are indicated. The scheme is not to scale. B. Amino acid sequences from S-endoglin and L-endoglin cytoplasmic tails. Sequences that differ between L and S isoforms are in blue. The PDZ binding motif in L-endoglin is underlined. C and D. Three-dimensional model of endoglin. C. The atomic model predicted in silico shows the presence of three different subdomains in red, yellow and blue. The orphan domain contains amino acid residues Glu26-Ile359 (red), whereas the ZP domain encompasses the fragment Gln360-Gly586. The ZP-N and ZP-C sub-domains are colored in yellow and blue, respectively. The amino acid numbers corresponding to the border regions of the globular domains are indicated. D. The electron microscopy density map of soluble endoglin (grey volume) allows the fitting of the atomic model of dimeric endoglin. Adapted from Llorca et al. (105).

Figure 2. Hypothetical model for the role of endoglin in EC apoptosis and its relevance in HHT vascular lesions. Under certain stimuli, such as TGF-β and hypoxia, ECs undergo apoptosis that is prevented by the induced expression of endoglin in healthy subjects. In HHT patients, endoglin haploinsufficiency may lead to a massive apoptosis in ECs where endoglin function is required.
for survival leading to capillary regression. A cross-section of an individual vessel is depicted with ECs in red, pericytes in blue and apoptotic ECs in pink.

**Figure 3.** Hypothetical model for the generation of arteriovenous malformations in HHT. The capillary network subjected to the apoptotic stimuli, as shown in Figure 2, is not affected in normal subjects (A). However, in HHT patients (B and C), as a consequence of the EC apoptosis, the capillary network gradually disappears and only a preferential vessel remains that eventually becomes the arterio-venous shunt (C).

**Figure 4.** Role of endoglin in tumor vascular endothelium. In vivo and in vitro studies support the involvement of endoglin in tumor suppression and progression, modulating angiogenesis and tumor proliferation. Increased endoglin expression correlates with increased tumor angiogenesis, probably due to the pro-angiogenic role of endoglin in endothelial cells.

**Figure 5.** Hypothetical model on the role of endoglin in eNOS expression and activation. A. In *Endoglin*^{+/+} cells, a pool of endoglin resides in caveolae, a cholesterol rich plasma membrane domain containing caveolin and eNOS. Endoglin acts as a molecular scaffold facilitating calcium-bound calmodulin and heat shock protein-90 (Hsp90) association to eNOS during endothelial activation (160), thus producing normal amounts of NO and the consequent vasodilatation. B. In endoglin haploinsufficiency (*Endoglin*^{+-}), both the amount of eNOS (77,78) and the eNOS/Hsp90/calmodulin association are reduced, leading to decreased NO as well as increased eNOS uncoupling and formation of eNOS-derived superoxide anion (O_2^-) (160). In turn, O_2^- produces hydrogen peroxide (H_2O_2) by dismutation (11, 161) and presumably reacts with NO to generate peroxynitrite (ONOO^-). Thus, the removal of the vasodilator NO and the presence of oxygen free radicals results in an impaired vasomotor tone.

**Figure 6.** Hypothetical model of S-endoglin functions during endothelial senescence. In the normal state, the TGF-β response is modulated by L-endoglin, but upon senescence of ECs, S-
endoglin is up-regulated, interacting with the TGF-β receptor complex containing ALK1 and ALK5. As a consequence of this interaction, S-endoglin regulates the expression of different target genes including PAI-1, Id1, eNOS and COX-2. Thus, S-endoglin allows a switch that triggers the cardiovascular pathology: i) Up-regulation of PAI-1/extracellular matrix (ECM) synthesis may lead to increased fibrosis; ii) Down regulation of Id1 is associated with decreased angiogenesis; and iii) Down-regulation of eNOS and up-regulation of COX-2 are involved in endothelial dysfunction and impaired vascular relaxation. The involvement of TβRII, TGF-β and S-endoglin/L-endoglin heterodimers has been omitted for simplification. Adapted from Blanco et al. (14).

**Figure 7.** Generation of soluble endoglin by proteolytic processing of membrane-bound endoglin. A recent report suggests that MT1-MMP is a major endoglin shedding protease acting on the juxtamembrane region and leading to the secretion of the large ectodomain of endoglin (75). Several functions reported for soluble endoglin are indicated.
TGF-β / HGF

Hypoxia

HHT1

Non Affected

Endoglin reaches threshold

Repair

Regression

Endothelial cell

Apoptotic endothelial cell

Pericyte

Endoglin does not reach threshold

TGF-β / HGF

Hypoxia
Arteriovenous shunt

Destruction of the capillary network

Apoptotic Stimuli (TGF-β, Hypoxia)

A

Arteries Veins

B

HHT1

C

HHT1

Destruction of the capillary network

Arteriovenous shunt
Vascular endothelium

Endoglin

Tumor suppression

Endoglin ↓
Angiogenesis ↓

Tumor progression

Endoglin ↑
Angiogenesis ↑
Id1

Angiogenesis

Fibrosis

Hypertension

PAI-1/ECM synthesis

eNOS

COX-2

L-Endoglin

Smad3

ALK5

ALK1

Smad1

S-Endoglin

Smad3

ALK5

ALK1

Senescence
Soluble Endoglin

Protease (MT1-MMP)

Endoglin

EC

TM

CYT

Ligand binding (TGF-β superfamily)

Anti-angiogenic activity

Regulation of vascular homeostasis

Endoglin

Soluble Endoglin

Ligand binding (TGF-β superfamily)

Anti-angiogenic activity

Regulation of vascular homeostasis