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To my Love

Nils Visser

Content

Scope of the investigation

Endothelial cell sprouting is a multi-step process, tightly regulated by diverse signalling pathways. Vascular endothelial growth factor (VEGF) is an essential inducer for angiogenesis as evidenced by *in vivo* and *in vitro* studies. Transforming growth factor (TGF)-β remodels the vascular morphogenesis *in vivo* and regulates the expression of VEGF and VEGF receptors *in vitro*. However, little is known about how these two factors orchestrate the modulation of endothelial cell function. The scope of the research presented in this thesis is to study the interplay between TGF-β and VEGF signalling on endothelial cell function, with the focus on the effect of TGF-β signalling on VEGF- induced endothelial cell function.

TGF-β transduces its effect by binding to two distinct TGF-β type I receptors, ALK1 and ALK5 on endothelial cells. In addition, the concentration of TGF-β affects the degree of activation of these two receptors. In Part I, to investigate the influence of ALK5 activity in response to VEGF stimulation, a selective inhibitor of ALK5 (SB-431542) was applied to investigate the role of ALK5 in VEGF-induced vascular network formation; to address the role of ALK1 in VEGF-induced endothelial cell behavior, BMP9 as a potent ALK1 ligand was used in the presence of VEGF.

Endoglin is a co-receptor for TGF-β and functions as a modulator for ALK1 and ALK5 in endothelial cells. The involvement of endoglin in VEGF-induced endothelial cell function remains unclear. In Part II, the studies were designed to elaborate the role of endoglin and its soluble form on VEGF-stimulated endothelial cell sprouting. Of note, Chapter 6 discusses the pathological contribution of elevated soluble endoglin levels to pre-eclampsia.

In conclusion, TGF-β and VEGF signalling intimately intertwine and crosstalk with each other to affect endothelial cell function accordingly. Since the aim of the thesis was to elucidate the underlying mechanism of vascular pathologies that are associated with perturbed TGF-β and VEGF signalling, the work of this thesis may open new opportunities for future development of new treatment modalities.

Thesis Outline

The studies presented in this thesis have focused on the crosstalk between the TGF-β, BMP9 and VEGF signalling pathways and their roles on EC function, and how endoglin, a co-receptor of TGF-β signalling influences VEGF-induced endothelial sprouting.

Chapter 1 provides a general overview of the role of TGF-β family signalling in vascular development concerning its role in endothelial cell and mural cells as well as the impact of TGF-β signalling in pathological conditions.

Part I: The effect TGF-β signalling in endothelial cell function in response to VEGF

Chapter 2 demonstrates that inhibition of the TGF-β signalling pathway using the TGF-β receptor I inhibitor SB-431542 enhances VEGF-induced endothelial cell function. Sub-optimal doses of VEGF and SB-431542 synergistically induce endothelial cell migration and sprouting.

Chapter 3 reports the new ligand BMP9 for ALK1 signalling and its inhibitory role in endothelial cell function.

Part II: The role of endoglin in endothelial cell function in response to VEGF

Chapter 4 describes the new observation that endoglin is required for efficient VEGF-induced endothelial cell sprouting.

Chapter 5 demonstrates that endoglin is cleaved by MMP14, and that the derived soluble form of endoglin exerts inhibitory effect on VEGF-induced endothelial cell sprouting

Chapter 6 gives an overview of the role of soluble endoglin and soluble Flt1 in pre-eclampsia.

Part III: General Discussion

Chapter 7 discusses the perspectives of the main focuses of this thesis reflected towards the current literature.

Chapter 1

TGF-β signalling in vascular biology and dysfunction

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Abstract

Transforming growth factor (TGF)-β family members are multifunctional cytokines that elicit their effects on cells, including endothelial and mural cells, via specific type I and type II serine/threonine kinase receptors and intracellular Smad transcription factors. Knock-out mouse models for TGF-β family signalling pathway components have revealed their critical importance in proper yolk sac angiogenesis. Genetic studies in humans have linked mutations in these signalling components to specific cardiovascular syndromes such as hereditary hemorrhagic telangiectasia, primary pulmonary hypertension and Marfan syndrome. In this review, we present recent advances in our understanding of the role of TGF-β receptor signalling in vascular biology and disease, and discuss how this may be applied for therapy.

Keywords**:** angiogenesis, BMP, Marfan syndrome, Smad, pre-eclampsia, pulmonary hypertension, TGF-β

Introduction

Transforming growth factor (TGF)-β is the prototypic member of a large family of evolutionarily conserved pleiotropic cytokines. Thirty three members are present in mammals, including three TGF-β isoforms, activins, and bone morphogenetic proteins (BMPs) [1-3]. TGF-β family members have critical and specific roles during embryogenesis and in maintaining the homeostasis of adult tissues. Perturbations in their signalling pathways have been linked to a diverse set of developmental disorders and diseases, including cancer, fibrosis, auto-immune and cardiovascular diseases.

All TGF-β family ligands are generated as dimeric precursor proteins and subsequently cleaved by proteases and secreted [4]. Members of the TGF-β family elicit their cellular effects by binding to a complex of type II and type I serine/threonine kinase transmembrane receptors (Figure 1). Five type II receptors and seven type I receptors, also termed activin receptor-like kinases (ALKs) are present in mammals [1-3]. Within the ligand-induced heteromeric receptor complex, the constitutively active type II receptor phosphorylates the type I receptor on specific serine and threonine residues in the intracellular juxtamembrane region. TGF-β signals in most cells via TGF-β type II receptor (TβRII) and ALK5, activins via activin receptor type IIA (ActRIIA) and IIB and ALK4, and BMPs via BMP type II receptor (BMPRII), ActRIIs and ALK1, 2, 3 and 6. In endothelial cells (ECs) TGF-β can, in addition to ALK5, also signal via ALK1 [5, 6]. Upon type I receptor activation, receptor-regulated Smads (R-Smads) are recruited to, and phosphorylated by the type I receptor at the two serine residues in their extreme carboxyl termini. ALK4 and 5 (and 7) induce R-Smad2 and 3 phosphorylation, while ALK1, 2, 3 and 6 mediate phosphorylation of R-Smad1, 5 and 8. Activated R-Smads form complexes with the common mediator Smad4, and translocate into the nucleus, where they can regulate, together with other partner proteins, the transcription of specific target genes. Inhibitory (I)-Smads, i.e. Smad6 and -7, can inhibit the activation of R-Smads by competing with R-Smads for type I receptor interaction and by recruiting specific ubiquitin ligases or phosphatases to the activated receptor complex thereby targeting it for proteosomal degradation or de-phosphorylation, respectively [1-3].

Access of ligands to the signalling type I and type II receptors is regulated by soluble ligand binding proteins and by accessory type III receptors. Examples of the latter class that have been most intensively studied are endoglin and betaglycan [4, 7]. Both receptors are structurally related trans-membrane proteins with long extracellular and short intracellular domains and without an enzymatic motif. Compared with the type I and type II receptors, betaglycan is present in higher abundance and binds TGF-β1 and 3 with lower affinity [8]. When TGF-β encounters a target cell expressing betaglycan, it is likely to interact first with betaglycan, which then presents it to TβRII. Betaglycan facilitates TGF-β signalling, in particular for TGF-β2 that binds with very weak affinity to TβRII alone [9, 10]. Endoglin (CD105) is a homodimer that interacts with TGF-β1 and TGF-β3, but only when it is associated with TβRII [11, 12]. Both the endoglin extracellular and intracellular domains interact with TβRII and ALK5 [7]. Endoglin is able to bind directly to BMPs [13-15].

In contrast to factors such as vascular endothelial growth factors (VEGFs) and angiopoietins that have prominent effects on EC behavior [16], TGF-β was initially discovered through its effects on fibroblasts [17] and subsequently shown to affect among other cell types, epithelial-, immune-, stem-, endothelial- and mural cells. This together with its highly cellular context dependent properties, frequently having opposite effects depending on the cellular differentiation state or the presence of other specific extracellular cues, has left the elucidation of the complex role of TGF-β family members in the cardiovascular system somewhat under-investigated. However, phenotypic and molecular characterizations of knock-out mice for TGF-β signalling components have demonstrated their critical role in angiogenesis, and importantly several cardiovascular syndromes were directly linked to mutations in their genes [4]. TGF-β family members have now gained a prominent spot among other key cytokines that control vascular function. Our understanding of their complex role in cardiovascular biology and interplay with VEGF and other angiogenesis regulators is proceeding at a rapid pace. In this review we focus on recent insights into the function of TGF-β family members in the cardiovascular system and discuss how dys-regulation of their signalling pathways contributes to vascular pathologies.

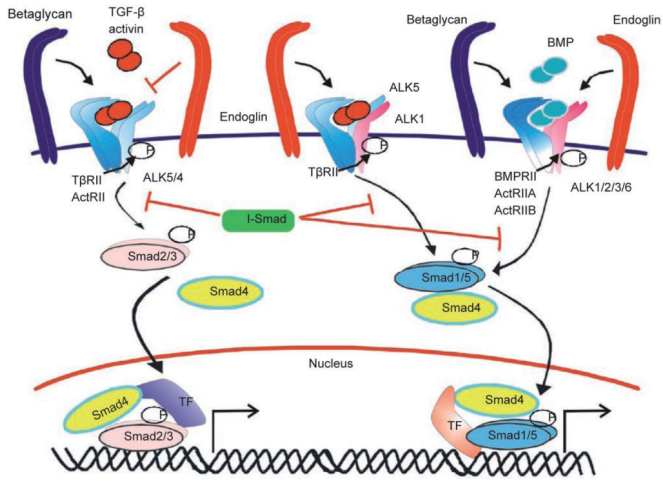

Fig.1 *Signal transduction by TGF-β family members is mediated via specific heteromeric complexes of type I and type II serine/threonine kinase receptors. In most cells TGF-β interacts with TβRII and ALK5, but in endothelial cells it can also signal via ALK1. BMPs signal via BMPRII, ActRIIA and ActRIIB, and type I receptors ALK1, 2, 3 and 6. Co-receptors betaglycan and endoglin can facilitate TβRII/ALK5 and TβRII/ALK1 signalling. Soluble versions of the co-receptors betaglycan and endo- glin have been shown to sequester TGF-β and BMP9, respectively. Intracellular signalling can be divided into two main Smad signalling pathways: ALK5 induces phosphorylation of Smad2 and Smad3, and ALK1, 2, 3 and 6 mediate phosphorylation of Smad1, 5 and 8. Activated R-Smads form heteromeric complexes with common mediator Smad4, which accumulate in the nucleus, where they can act as transcription factor complexes and regulate the expression of specific target genes*

Vascular morphogenesis

Neovascularization, the formation of new functional microvascular networks, is a tightly controlled process regulated by several converging signalling pathways that are tightly coordinated in time and space. The formation of new blood vessels can occur via two processes, vasculogenesis and angiogenesis, both of which result in the formation of endothelial-lined tubes [16]. During vasculogenesis new vessels arise de novo from a mass of proliferating cells, classically forming an inner core of hematopoietic precursor cells and an outer layer of ECs termed a blood island, followed by their subsequent migration, fusion and organization into a primary capillary plexus [18]. Angiogenesis refers to the formation of new capillary networks by sprouting from pre-existing vessels [18, 19]. Neovascularization is involved in growth and development, wound healing and several pathological situations such as tumor growth and metastasis, and cardiovascular disorders [20]. While angiogenesis occurs during embryogenesis and in adult life, vasculogenesis was initially thought to occur only in the embryo, but some studies have suggested that circulating endothelial progenitor cells may also contribute to vessel formation in the adult [21]. The formation of new capillaries involves EC activation, migration, alignment, proliferation, tube formation, branching and maturation of intercellular junctions and the surrounding basement membrane. All new blood vessels begin as simple EC-lined capillaries, but during vessel maturation, some vessels remain as capillaries covered by pericytes, and others develop into large vessels with support from a layer of smooth muscle cells (SMCs), forming a strong vessel wall.

Angiogenesis is a carefully balanced process, under the control of and fine tuned by a multitude of factors, including stimulators like VEGF and inhibitors such as thrombospondin [20]. Considering the context-dependent effects of TGF-β in other cell systems, it is not surprising that its effect on blood vessel formation is biphasic [4]. It can act as both a promoting and an inhibitory factor of angiogenesis for which the underlying mechanisms are starting to be uncovered.

Insights from knock-out mice

In vivo studies show that loss of TGF-β signalling components leads to abnormal formation of the primitive vascular plexus and decreased vessel wall integrity caused by irregular capillary vessel formation or impaired differentiation and recruitment of vascular SMCs. Targeted deletions of ALK1, ALK5, TβRII and endoglin in mice result in remarkably similar phenotypes (Figure 2). Embryos lacking any one of these components die during mid-gestation due to impaired vascular development, exhibiting hyper-dilated, leaky vessels [22], highlighting the importance of TGF-β signalling in the vascular sys- tem. The primary target cell for TGF-β is the EC since mice deficient in endothelial TβRII or ALK5 expression showed vascular defects in the yolk sac and embryonic lethality at embryonic day (E)10.5, which pheno-copied mice lacking receptors in all cells [23]. TGF-β

not only affects the ECs, but is also important for proper differentiation and function of SMCs and pericytes. Mice lacking TβRII specifically in vascular SMCs also showed vascular defects in the yolk sac but at later stages of development, allowing the embryo to survive to E12.5 [23].

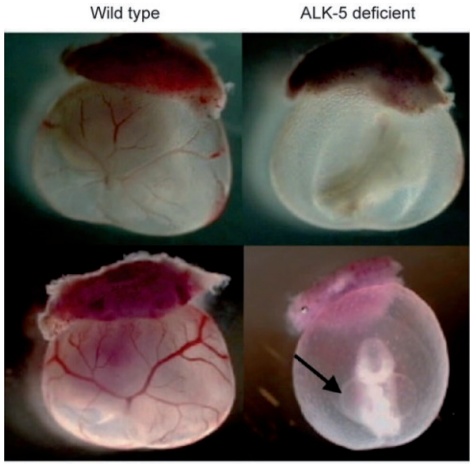

Fig.2 *TβRI/ALK5-deficient embryos exhibit severe defects in vascular development. Gross morphology of whole-mount yolk sacs in mutant embryos is compared with wild type littermates. Arrow indicates the pericardial effusion in the mutant embryo probably caused by a circulation defect [116].*

At the intracellular level Smad1-, Smad2- or Smad4- deficient mice demonstrate pre-angiogenesis lethality in embryos [22]. In Smad1-deficient embryos this is accompanied with defects in chorion-allantoic circulation [24, 25]. Smad5 knock-out and endothelial specific Smad4 knock-out have phenotypes reminiscent of TGF-β receptor knock-out mice [26, 27]. Smad3-lacking mice are viable but die of impaired immunity and colon cancer [22].

The I-Smads, Smad6 and 7 were initially identified as EC shear stress response genes [28] and are highly expressed in specific regions of the heart [29, 30]. Both I-Smads are strongly induced by TGF-β family members and participate in a negative feedback loop [1-3]. Studies of mice deficient in Smad6 or Smad7 demonstrated their critical role in development and heart homeostasis [30, 31]. Mice lacking Smad6 are born, but develop multiple heart abnormalities, including septation defects, ossification of the aorta, and have high blood pressure. While some of the Smad7-deficient mice die shortly after birth because of heart complications and abnormal heart rate, the majority die in utero due to cardiovascular defects, including outflow tract malformation and abnormality in ventricular septum and non-compaction.

Role of TGF-β signalling in ECs

TGF-β has been proposed to regulate the activation state of ECs by differentially activating two type I receptors, ALK5 and ALK1 [5, 6]. ALK5 is widely expressed in almost all tissues, but the expression of ALK1 is restricted to ECs and during embryogenesis at sites of angiogenesis [32].

TGF-β/ALK5 signalling induces Smad2/3 phosphorylation and blocks angiogenesis by inhibiting EC proliferation, tube formation and migration [6, 33]. ALK5 induces the expression of fibronectin and plasminogen activator inhibitor type 1 (PAI-1), a negative regulator of EC migration [34]. Interestingly, in mouse embryonic stem cell-derived ECs, the ALK5 kinase inhibitor SB-431542 enhances EC growth and integrity via up-regulation of the tight junction component Claudin-5, suggesting a role for ALK5 signalling in regulating vascular permeability [35]. ALK5 indeed has been reported to increase TGF-β-induced EC permeability and actin cytoskeleton remodeling [36]. By enhancing TβRII/ALK5 assembly clustered VE-cadherin promotes persistent and elevated TGF-β-induced Smad2/3 activation, indicating a positive role for VE-cadherin in TGF-β/ALK5-induced vessel stabilization [37]. Taken together, TGF-β/ALK5 signalling plays an important role in keeping the endothelium quiescent.

In contrast to TGF-β/ALK5, TGF-β/ALK1 signalling induces Smad1/5 activation and has been shown to stimulate EC migration, proliferation and tube formation [33]. Caveolin1 was shown to associate with ALK1 and to promote TGF-β/ALK1-induced responses [38]. An important intracellular effector of ALK1 is Id1; its upregulation was shown to be required for TGF-β/ALK1-induced EC migration and tube formation [6]. However, an inhibitory effect of ALK1 signalling on EC proliferation, migration and spouting has also been reported [39, 40]. BMP9, identified as a ligand for the ALK1 and BMPR-II complex in ECs, was shown to inhibit EC migration and VEGF-induced angiogenesis [13, 14]. These observations suggest that the effect of ALK1 signalling on angiogenesis is dependent on the context and specific ligand by which it is activated.

ALK1 and ALK5 signalling not only elicit opposite responses, but also physically interact with each other in ECs. ALK5-deficient ECs are not only defective in TGF-β/ALK5 signalling but also exhibit impaired TGF-β/ ALK1 responses; ALK5 was found to be necessary for recruitment of ALK1 into a TGF-β receptor complex, and the kinase activity of ALK5 is essential for maximal ALK1 activation [33]. Furthermore, ALK1 can directly antagonize ALK5/Smad2/3 signalling at the level of Smads [5, 6]. The cross-talk between ALK1 and ALK5 signalling provides ECs with a TGF-β-dependent switch to fine tune EC function.

BMPs also play an important role in EC function. BMP6 promotes EC migration and tube formation via Smad1/5/8 phosphorylation [41]. BMP4 was shown to induce proliferation and migration of ECs via stimulation of VEGF-A and Tie-2 signalling [42]. *In vivo* BMPs were also found

to induce tumor angiogenesis [43]. BMP endothelial cell precursor-derived regulator (BMPER) interacts with BMP4, and regulates BMP4-mediated angiogenesis [44]. Interestingly, inhibition of BMPRII using specific siRNA in human pulmonary arterial ECs was found to induce EC apoptosis via an increase in activated caspase-3 [45]. Upon exposure to hypoxia, BMPRII, phosphorylated Smad1/5/8 and Id1 expression were strongly reduced in these ECs, which may be of relevance to the pathogenesis of hypoxia-induced pulmonary hypertension [46]. BMP9, which interacts with ALK1, is reported to be a circulating vascular quiescence factor [15].

Interestingly, there are ECs that express betaglycan and those that express endoglin. ECs expressing betaglycan respond to all three isoforms of TGF-β, whereas ECs that express endoglin (and not betaglycan) respond with high potency to TGF-β1 and -β3, but not -β2 [47]. In ECs that co-express betaglycan and endoglin, both proteins were shown to be part of a common TGF-β receptor complex [48]. The co-receptor endoglin is predominantly expressed in highly proliferating vascular ECs, but is also reported to be detected on hematopoietic cells, syncytiotrophoblasts of term placenta, stromal cells and mesenchymal cells. Ectopic expression of endoglin inhibits TGF-β-induced growth inhibition in ECs, monocytes and myoblasts [12, 49] and extracellular matrix deposition [50]. Endoglin regulates the fine-tuning between the ALK1 and ALK5 signalling pathways. It is required for TGF-β/ALK1 signalling and indirectly inhibits TGF-β/ ALK5 signalling [49, 51]. Suppression of endoglin gene expression using siRNA resulted in impaired TGF-β/ ALK1 signalling responses [49]. ECs derived from *Eng–/–* embryos were unable to proliferate in culture, displayed reduced migration, VEGF secretion and eNOS expression [49, 52]. Interestingly, up-regulation of endoglin protects ECs from the apoptotic action of TGF-β1 [53]. Endoglin was recently shown to interact via its C- terminal PDZ binding motif with the scaffolding protein GIPC, which promotes endoglin cell surface retention [54]. Elevated endoglin expression levels are used as a marker of tumor angiogenesis and endoglin antibodies coupled with toxin or radioactivity have been successfully used to target ECs in anti-angiogenic therapy [55, 56]. Two endoglin splice forms have been reported, termed long (L) and short (S)-endoglin with pro- and anti-angiogenic activity, respectively [57]. While the L-form is most abundantly expressed and promotes TGF-β/ALK1 signalling, S-endoglin expression was recently shown to be specifically high in senescent ECs and it preferentially promotes TGF-β/ALK5 signalling [58,59]. Transgenic mice overexpressing S-endoglin in ECs showed hypertension. Taken together, whereas L-endoglin contributes to the activation phase of angiogenesis and contributes to pathological neovascularization, S-endoglin is induced during EC senescence and may lead to age-associated pathologies such as hypertension [59].

Role of TGF-β signalling in vascular SMCs

Vascular SMCs express multiple type I and type II receptors for TGF-β family members [60]. TGF-β is a potent stimulator of vascular SMC differentiation by activating the genetic program that

includes a large set of SMC differentiation marker genes [61]. Myocardin, an important coactivator of serum response factor, was shown to potently enhance TGF-β/Smad3-mediated activation of SM22α actin transcription [62]. The zinc finger E-box binding transcription factor DeltaEF1 was also shown to have an important effector role in this respect by forming a complex with serum response factor and Smad3 [63]. TGF-β-induced growth inhibition of vascular SMCs was found to be ALK5-mediated via both Smad3-dependent and p38 MAP kinase-dependent signalling pathways [64]. TGF-β was also shown to inhibit SMC migration in a non-Smad3-dependent pathway via up-regulation of cysteine rich protein 2 expression [65, 66]. Interestingly, Smad3-deficient vascular SMCs demonstrated reduced growth inhibition by TGF-β, but did not show any attenuated TGF-β-mediated migratory response [64, 67].

Endoglin is also important for the formation of vascular SMCs. Ectopic endoglin expression in neural crest stem cells causes pericardial hemorrhage associated with altered vascular SMC investment in the walls of major vessels, suggesting a direct role of endoglin in myogenic differentiation [68].

Vascular SMC differentiation and function are also influenced by BMPs. The effect of BMPs depends on the source of SMCs studied as well as the local environment. While BMP2 stimulates vascular SMC migration, it prevents platelet-derived growth factor induced vascular SMC proliferation via induction of the PPARγ/apoE axis [69, 70]. BMP7 also inhibits the growth of vascular SMCs and helps maintain the vascular SMC phenotype in culture [71]. In pulmonary SMCs both BMP7 and BMP4 induce apoptosis via a caspase 8- and 9-dependent mechanism [72]. Interestingly, BMP4 induces micoRNA- 21, leading to the repression of PDCD4, an inhibitor of smooth muscle cell gene expression; SMC differentiation is thereby stimulated [73].

Interaction between ECs and vascular SMCs

Tight regulation and close coordination between ECs and vascular SMCs are needed to form a mature vascular network. Vascular SMCs form abundant gap junctions with ECs and upon receiving signals from SMCs, ECs line up and recruit more SMCs [74].

TGF-β is an important regulator of the EC-SMC interaction. ECs produce latent TGF-β that upon EC-SMC interaction can be activated and induce SMC differentiation and function [75]. Targeted deletions of several TGF-β signalling components have revealed the importance of TGF-β signalling in vSMC-EC contact. ALK1- and Endoglin-deficient mice display defective remodeling of the primary capillary plexus of the yolk sac and aberrant development of SMCs [4, 5, 22, 76]. Mice lacking TβRII, Smad5, Smad1 and TGF-β1 show defects in vasculature structure or blood vessel organization [22] indicative of a defect in EC lining and impaired SMC development.

TGF-β can also stimulate SMCs to produce VEGF partly in a p38 MAP kinase [77], and a reactive oxygen species generation-dependent manner [78]. The produced VEGF can influence both the ECs as well as SMCs, by inducing their growth and differentiation. Interestingly, VEGF was found to inhibit TGF-β release by ECs [79].

Besides influencing their growth, TGF-β and BMP are also potent SMC differentiation factors and crucial regulators to switch SMCs from an undifferentiated phenotype to a contractile phenotype [61]. Furthermore, TGF-β can also induce trans-differentiation of ECs (Endo-MT) into smooth muscle-like cells. TGF-β induces the expression of α-smooth muscle actin and smooth muscle myosin in ECs in a Snail-dependent manner [80] and blocking TGF-β signalling with neutralizing antibodies abrogates the induction of smooth muscle markers in ECs [75].

Vascular disorders and TGF-β signalling

In recent years multiple cardiovascular disorders have been linked to alterations in TGF-β/BMP signalling pathways, several of which will be discussed below. Increased understanding of the molecular mechanisms has been achieved by studying mouse models that mimic these human diseases. Importantly, these mouse models also allow for testing of therapeutic strategies that aim to normalize the perturbed signalling balance.

Hereditary hemorrhagic telangiectasia (HHT)

Hereditary hemorrhagic telangiectasia (HHT), or Osler-Rendu-Weber syndrome, is an autosomal dominant vascular dysplasia, characterized by the development of mucocutaneous telangiectasias and arteriovenous malformations in the brain, lungs, liver, and gastrointestinal tract (Figure 3). There is variability in the organs affected, even between individuals within families [81]. Three genes are causally related to HHT, i.e. endoglin mutated in HHT type 1 (HHT-1) [82]; ALK1 mutated in HHT-2 [83] and mutation in Smad4 causing a syndrome consist- ing of both juvenile polyposis and an HHT phenotype [84]. *In vivo*, EC-specific deletion of ALK1 caused vascular malformations mimicking all pathologic features of HHT-2 vascular lesions [85], while endoglin heterozygous mice have dilated and fragile blood vessels which resemble clinical manifestations of HHT-1 patients [76, 86].

While circulating levels of TGF-β were reduced in HHT-1 patients compared to control, this was not found in HHT-2 patients, suggesting that reduced endoglin levels on HHT-1 ECs might lead to reduced TGF-β1 secretion [7]. Since TGF-β is important for SMC differentiation, and recruitment of SMCs to new vessels as well as their growth requires TGF-β, this reduced TGF-β secretion might explain the formation of fragile and leaky vessels in HHT-1 patients. Multiple clinical trials in HHT patients are ongoing with anti-angiogenesis agents, including bevacizumab, a neutralizing antibody

against VEGF, thalidomide and interferon α-2b to inhibit bleedings and other vascular malformations associated with HHT (http://www.hht.org/).

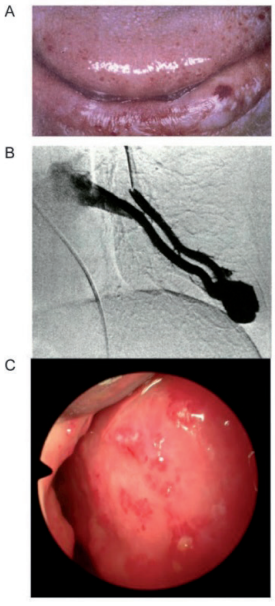

Fig. 3 *Clinical symptoms of hereditary hemorrhagic telangi- ectasia (HHT) include (A) bleedings in tongue and lower lip, (B) arteriovenous malformations (pulmonary angiogram is shown), and (C) nasal telangiectases (courtesy of Dr U Geisthoff). Panels A and B were reproduced from [117] with permission.*

Marfan syndrome and Loeys-Dietz syndrome (MFS and LDS)

Marfan syndrome (MFS) is a genetic connective tissue disorder caused by mutations in the fibrillin gene [87]. Typical MFS can affect the skeletal system, ocular system, cardiovascular system, pulmonary system as well as other organs, with defects in aorta and heart valves giving the most severe complications. Fibrillin contains several motifs homologous to latent TGF-β binding protein (LTBP), and has been shown to interact with LTBP and control TGF-β bioavailability [4]. TGF-β is bound and kept inactive by the LTBP-fibrillin complex. In fibrillin-1-deficent mice, a model of MFS, fibrillin-1 deficiency was found to diminish the sequestration of latent TGF-β to the extracellular matrix, thereby leading to increased TGF-β activation [88]. Marfan syndrome type 2 is caused by mutation in the TβRII gene locus. Three prominent features of Marfan's syndrome in humans, i.e. dilatation of the aortic root, air-space widening and skeletal myopathy, can be prevented and even reversed in the mouse model by treatment with losartan, which blocks the angiotensin II type 1 receptor and antagonizes TGF-β signalling [89, 90].

The Loeys-Dietz syndrome is a recently described autosomal dominant aortic-aneurysm syndrome presenting with cardiovascular and skeletal manifestations consistent with those seen in MFS, along with other features not present in MFS. Typical Loeys-Dietz syndrome is characterized by a mutation in either TβRI or TβRII [91]. The molecular mechanism of Loeys-Dietz syndrome is complex and poorly understood; although mutations in TβR are inactivating its function, they lead paradoxically to overactive TGF-β signalling, as measured by accumulation of phosphorylated

Smad2 in the nucleus and expression levels of connective-tissue growth factor [92]. Thus TGF-β antagonists may also alleviate manifestations of Loeys-Dietz syndrome. In a recent clinical study with 18 MFS patients, losartan decreased the rate of progressive aortic-root dilation [93] (http://www.marfan.org/ nmf/index.jsp).

Pre-eclampsia

Pre-eclampsia, which involves a raise in blood pressure, is a major cause of maternal, fetal, and neonatal mortality. The clinical manifestations of pre-eclampsia reflect widespread EC dysfunction, resulting in vasoconstriction, endo-organ ischemia and increased vascular permeability [94]. Soluble endoglin (solEng), a placenta- derived 65 kD soluble form of endoglin and soluble fms-like tyrosine kinase 1 (sFlt1, an inhibitor of VEGF) are both considered to be clinical indicators for pre-eclampsia [95]. Just before the onset of pre-eclampsia circulating levels of solEng are found to be markedly el- evated and it cooperates with sFlt in the pathogenesis of pre-eclampsia in rats [96]. SolEng was found to inhibit TGF-β-mediated activation of eNOS, thereby affecting vascular tone. SolEng disrupts EC function, inhibiting EC tube formation and enhancing capillary permeability. SolEng is formed by proteolytic cleavage, but the protease involved remains to be identified. Upon identification of this protease involved in endoglin shedding, it will be interesting to explore whether its targeting could be beneficial for treatment of pre-eclampsia. Circulating solEng levels may be a useful diagnostic marker in pre-eclampsia [4].

Pulmonary arterial hypertension

Pulmonary arterial hypertension (PAH) is a disease caused by constriction of small pulmonary arteries leading to an increase in blood pressure (Figure 4). Aberrant proliferation and dysfunction of ECs and SMCs contribute to vascular remodeling [97, 98]. Heterozygous germ line mutations in the BMP receptor II gene (BMPRII) have been found in more than 80% of familial PAH patients. In addition, about 20% of patients with idiopathic PAH were reported to have mutations in BMPRII [99, 100]. A recent study has implicated mutations of ALK1 in children with PAH [101]. Over-expression of a mutant form of BMPRII in SMCs of transgenic mice causes an increase in pulmonary arterial pressure and pulmonary arterial muscularization, resembling some manifestations of PAH patients [102]. Mutations in BMPRII also can deregulate Id gene expression [103]. BMPRII signalling was found to be essential for BMP-mediated regulation of vascular SMC growth and differentiation [104]. Clinical trials with prostanoids, endothelin antagonists and phosphodiesterase inhibitors have shown promising results in the treatment of PAH. It will be interesting to examine the interplay between the targets of these therapeutics and deregulated BMPRII signalling in PAH [105].

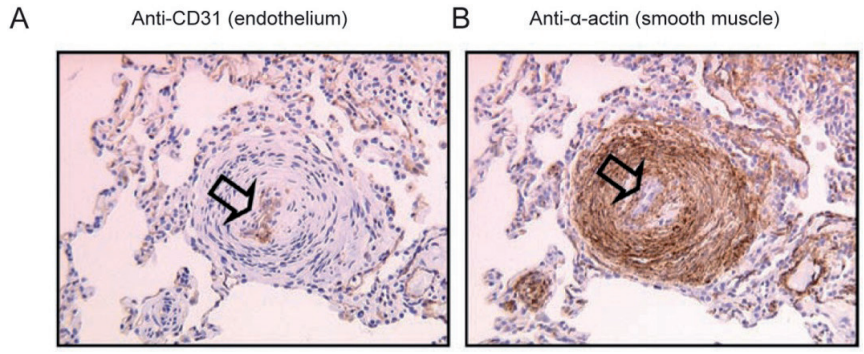

Fig.4 *Photomicrographs of lung sections from patients with primary pulmonary hypertension (PPH). The occlusion of small pulmonary arteries that is typical of PPH is shown. Stainings of lung sections from PPH patients are shown using anti- bodies against (A) CD31 (also known as platelet endothelial cell adhesion molecule-1), an endothelium-specific marker (arrow is pointing to single layer of ECs); and (B) SMC α-actin (arrow is pointing to concentric layers of SMCs). Figure was repro- duced from [97], with permission from Dr N Morrell and Lippincott, Williams and Wilkins.*

Cardiac remodeling and hypertrophy

Cardiac remodeling describes the alteration in size, shape and function of the left ventricle in response to changes in hemodynamic loading conditions, neurohormonal activation, or induction of local mediators that alter the structural characteristics of the myocardium. TGF-β is a crucial regulator of cardiac remodeling through its direct and potent actions in mediating cardiomyocyte growth, fibroblast activation and extracellular matrix deposition [106].

TGF-β levels are markedly induced in the hypertrophic myocardium. Angiotensin II via the angiotensin type 1 receptor promotes cardiomyocyte growth and stimulates fibroblast proliferation and expression of extracellular matrix components [107, 108]. Angiotensin, a potent vasoconstrictor, stimulates TGF-β1 mRNA and protein expression and may through its induction of thrombospondin lead to TGF-β activation [109], indicating that TGF-β1 acts downstream of angiotensin signalling [107]. In addition, angiotensin has been shown to directly lead to Smad3 activation via a mechanism that is not well understood [110]. The renin-angiotensin system (RAS) plays an important role in cardiac remodeling and clinical trials have documented the beneficial effects of angiotensin II inhibition in patients with myocardial infarction and heart failure [111]. Indeed, losartan has been approved for the reduction of cardiovascular events in patients with hypertension and left ventricular hypertrophy [112]. Furthermore it has been reported that TGF-β stimulation alters the program of differentiation-related gene expression in isolated cardiac myocytes, promoting the synthesis of fetal contractile proteins, characteristic of pressure-overload hypertrophy [113]. Over-expression of TGF-β in transgenic mice results in cardiac hypertrophy which is characterized by both interstitial fibrosis and hypertrophic growth of cardiac

myocytes [114]. The local production of TGF-β in hypertrophic myocardium and the link between the RAS system and TGF-β strongly implicate the role of TGF-β in hypertrophic response. All these results demonstrate the importance of TGF-β in mediating hypertrophic cardiac remodeling, however, limited knowledge is available on the signalling pathways responsible for TGF-β action in hypertrophy.

Concluding remarks

The pivotal importance of TGF-β family members in angiogenesis is underscored by the observations that nearly all knock-out mice for specific TGF-β family signalling components die during midgestation due to a yolk sac angiogenesis defect and that mutations in the genes encoding TGF-β pathway components are linked to an increasing number of human pathologies with vascular dysfunction. In concordance with these findings *in vitro* studies with different TGF-β family members have revealed potent effects on the function of ECs and vascular SMCs, affecting e.g., proliferation, differentiation, migration and extracellular matrix production. However, results obtained by different laboratories are sometimes in apparent conflict, but this can largely be attributed to context-dependent functions of TGF-β family members. Ligand concentration, cell density, cell type, media and matrix coating may determine specific cellular responses to TGF-β family members. TGF-β also plays an important role in the interplay between EdCs and vascular SMCs. Recent data indicate that TGF-β is capable of mediating EndoMT, a transdifferentiation of ECs into SMC- like cells [115].

With misregulation of TGF-β signalling at the heart of cardiovascular disorders, the cognate signalling components represent interesting targets for therapy. The first large-scale clinical trials with losartan to treat MFS are underway and results are eagerly awaited. Results from one vascular disorder may open opportunities for development of therapies in other vascular pathologies. Whereas high levels of circulating solEng may lead to pre-eclampsia by causing an EC dysfunction in multiple organs, solEng may be explored for anti-angiogenic activity in curbing tumor angiogenesis.

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Chapter 2

VEGF and inhibitors of TGF-β type-I receptor kinase synergistically promote blood-vessel formation by inducing α5-integrin expression

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Abstract

Vascular endothelial growth factor (VEGF) and transforming growth factor-β (TGF-β) are potent regulators of angiogenesis. How VEGF and TGF-β signalling pathways crosstalk is not well understood. Therefore, we analyzed the effects of the TGF-β type-I-receptor inhibitors (SB-431542 and LY-2157299) and VEGF on endothelial cell (EC) function and angiogenesis. We show that SB-431542 dramatically enhances VEGF-induced formation of EC sheets from fetal mouse metatarsals. Sub-optimal doses of VEGF and SB-431542 synergistically induced EC migration and sprouting of EC spheroids, whereas overexpression of a constitutively active form of TGF-β type-I receptor had opposite effects. Using quantitative PCR, we demonstrated that VEGF and SB-431542 synergistically upregulated the mRNA expression of genes involved in angiogenesis, including the integrins α5 and β3. Specific downregulation of α5-integrin expression or functional blocking of α5 integrin with a specific neutralizing antibody inhibited the cooperative effect of VEGF and SB-431542 on EC sprouting. *In vivo*, LY-2157299 induced angiogenesis and enhanced VEGF- and basic-fibroblast-growth-factor-induced angiogenesis in a Matrigel-plug assay, whereas adding an α5-integrin neutralizing antibody to the Matrigel selectively inhibited this enhanced response. Thus, induction of α5-integrin expression is a key determinant by which inhibitors of TGF-β type-I receptor kinase and VEGF synergistically promote angiogenesis.

Keywords**:** Angiogenesis, Endothelial cell, Integrin, Signalling, SB- 431542, TGF-β, VEGF

Introduction

During embryogenesis, the formation of new blood vessels depends on vasculogenesis and angiogenesis. Angiogenesis refers to the formation of new blood vessels from pre-existing ones (1), and consists of an activation phase and a resolution phase (2). The activation phase is associated with vessel destabilization and increased permeability, degradation of the extracellular matrix (ECM), and endothelial cell (EC) proliferation and migration. During the resolution phase, ECs become quiescent and pericytes and vascular smooth muscle cells (VSMCs) are recruited to ensure stabilization and maturation of the newly formed vessels (1).

Angiogenesis is tightly regulated by pro- and anti-angiogenic signals, and plays an important role in pathophysiological and physiological processes such as wound healing, tissue remodeling, the female reproductive cycle, autoimmune diseases and cancer (3). Vascular endothelial growth factor (VEGF) is a key regulator of vasculogenesis and angiogenesis. Heterozygous mice lacking a single *VEGF* allele die at embryonic day (E)8.5 with severe vascular defects (4; 5). Overexpression of VEGF also results in embryonic lethality (6). VEGF signals through two distinct tyrosine- kinase receptors, VEGFR1 (also known as Flt-1) and VEGFR2 (also known as KDR and Flk-1) (7), and exerts multiple effects on ECs, including proliferation, survival (8), migration and the formation of capillary-like tubules (9).

Genetic studies in mice and humans have suggested that perturbation of TGF-β signalling results in vascular abnormalities (10). TGF-β exerts its biological effects by binding to and activating type-I and type-II transmembrane serine/threonine-kinase receptors. Binding of TGF-β to the TGF-β type II receptor (TβRII) leads to recruitment and phosphorylation of type I receptor [TβRI, or activin receptor like kinase 5 (ALK5)]. Activin and Nodal, which are structurally related to TGF-β, signal via ALK4 and ALK7, respectively. Activated ALK5, ALK4 and ALK7 propagate the signal into the cells by phosphorylating the downstream effector proteins Smad2 and Smad3 (10). In ECs, TGF-β can also activate ALK1, an alternate type-I receptor, which mediates phosphorylation of Smad1 and Smad5 (11, 12). The TGF-β-ALK5 pathway results in inhibition of EC proliferation and migration, whereas the TGF-β-ALK1 pathway results in the activation of proliferation and the migration of ECs. Balance between the TGF-β-ALK1 and TGF-β-ALK5 signalling pathways play an important role in angiogenesis (11, 12). In line with those results, the effects of TGF-β on angiogenesis are highly context dependent, e.g. at low concentrations, TGF-β promotes angiogenesis, whereas, at high concentrations, it inhibits it (13). In addition to its direct effects, TGF-β can exert effects on angiogenesis by regulating the expression of angiogenic factors such as VEGF and components of the ECM such as matrix metalloproteases (MMPs) and integrins (14; 15; 16).

Integrins are heterodimeric transmembrane proteins consisting of α- and β-subunits mediating cell-ECM interactions. Up to date, nine integrin heterodimers have been implicated in blood-vessel formation, namely α1β1, α2β1, α4β1, α5β1, αvβ1, αvβ3, αvβ5, αvβ8 and α6β4 (17). Despite the

fact that integrins lack intrinsic enzymatic activity, upon ligand-induced integrin clustering specific intracellular signals are initiated by the activation of intracellular associated kinases and adaptor proteins in focal adhesion complexes. Integrins regulate divergent biological events including cell adhesion, migration, proliferation, differentiation, survival and angiogenesis (17).

Several studies have provided evidence suggesting that the interplay of VEGF and TGF-β signalling pathways plays an important role in angiogenesis. TGF-β can induce the expression of VEGF by various cells in the tumor microenvironment, such as tumor cells, stromal fibroblasts and cells of the immune system (18; 19). SB-431542, an ALK5/4/7 kinase inhibitor (20) was shown to exert an inhibitory effect on VEGF secretion in human cancer cell lines (21; 22; 23). Moreover, it was shown that SB-431542 stimulated the formation of FLK1- positive embryonic stem cell (ESC)-derived EC sheets induced by VEGF (24).

However, the molecular mechanisms that regulate the cross-talk between the VEGF and TGF-β signalling pathways in angiogenesis have not been determined. To elucidate the interplay between VEGF and ALK5, we analyzed the effects of VEGF and SB-431542 alone or in combination using different angiogenesis assays. We show that VEGF and SB-431542 synergistically induce angiogenesis both *in vitro* and *in vivo*. Gene expression profiling and functional validation revealed that the upregulation of α5-integrin expression plays a crucial role by which VEGF and TGF-β-type-I-receptor- kinase inhibitor achieve their synergistic angiogenic response.

Results

VEGF and ALK5-kinase inhibitor synergistically enhance angiogenesis in fetal mouse metatarsal assay

To investigate the interplay between VEGF and TGF-β-ALK5 signalling in angiogenesis, we made use of an *ex vivo* fetal mouse metatarsal assay. This model provides a quantitative *ex vivo* assay with the complexity of an *in vivo* assay to study the formation of capillary-like structures (25; 26). Metatarsals of 17-day-old mouse embryos were isolated and cultured in 24-well plates for 72 hours to allow adherence, followed by stimulation with VEGF, TGF-β3, SB-431542 or combinations thereof. Capillary-like structures were visualized by staining the cultures using an EC-specific anti-CD31 antibody (Fig. 1A). Similar to what was previously reported for VEGF and TGF-β2 (25), quantitative image analysis showed that VEGF strongly stimulated the formation of vessel-like structures, which was inhibited by TGF-β3 (Fig. 1). Treatment of bone explants with the ALK4/5/7 inhibitor SB-431542 had no significant effects on basal outgrowth of tube-like structures. However, the combination of VEGF with SB-431542 (VEGF+SB-431542) significantly stimulated EC network formation in a synergistic manner. Interestingly, treatment of metatarsals with LY-2157299, another ALK5-

kinase inhibitor that is structurally divergent from SB-431542 (27), slightly inhibited endothelial sheet formation. However, similar to SB-431542, LY-2157299 strongly promoted VEGF-induced angiogenesis (Fig. 2A,C). Our results indicate that activation of VEGF and inhibition of TGF-β (and/or Activin and/or Nodal) signalling by the SB-431542 or LY-2157299 inhibitors synergistically stimulates angiogenesis *in vitro*.

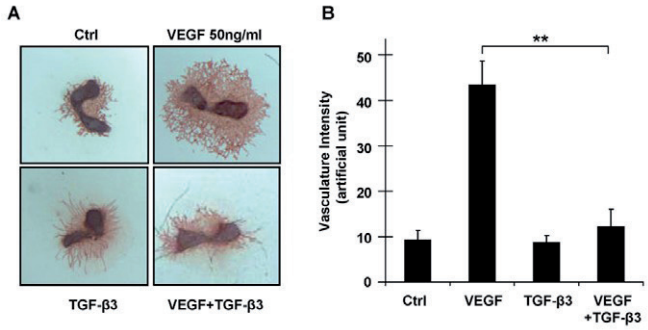

Fig. 1. *TGF-β inhibits VEGF-induced formation of the endothelial network in mouse metatarsal assays. Metatarsals of 17-day-old mouse fetuses were prepared, transferred to cell-culture plates and allowed to adhere, and were then stimulated with VEGF (50 ng/ml), TGF-β3 (5 ng/ml) or both. (A) Cultures were fixed and vessel-like structures were visualized by anti-CD31 staining. Six bones were stimulated per experimental group and one representative picture of each group is shown. Ctrl,control. (B) TGF-β3 did not significantly affect baseline formation of the endothelial network. Incubation with VEGF strongly stimulated the formation of vessel-like structures, which was dramatically decreased by addition of TGF-β3. **P≤0.01.*

ALK5 inhibitor and VEGF synergize in inducing EC sprouts in 3D spheroid culture

To study the effect of combined VEGF and ALK5 inhibitor on EC function, we performed a three-dimensional (3D)-culture collagen EC spheroid assay, which is a suitable model for the analysis of the early regulation of angiogenesis (28). ECs originating from the embedded spheroids invade the gel to form capillary-like structures. Spheroids, generated from human umbilical vein ECs (HUVECs), were embedded into type I collagen gels and stimulated with VEGF, SB-431542 or their combination for 24 hours (Fig. 3). In the absence of stimulation, hardly any EC sprouting was observed. Stimulation with different amounts of VEGF (10-50 ng/ml) dramatically increased the length and the number of sprouts (Fig. 3A). The addition of SB-431542 (1-10 μM) induced the formation of sprouting in a dose-dependent manner, but to a lesser extent compared with VEGF (Fig. 3B).

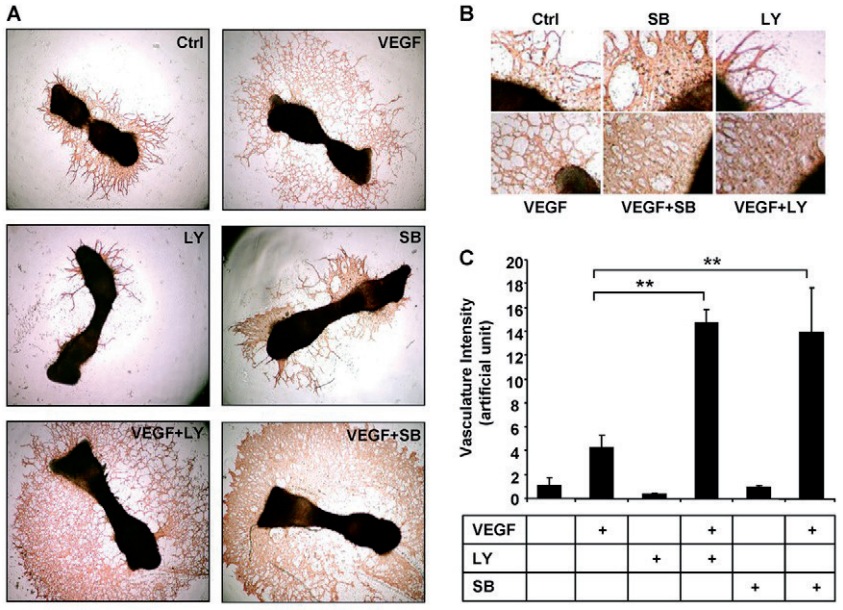

Fig. 2. *Effects of VEGF and SB-431542 on endothelial network formation in mouse metatarsal assays. (A) Metatarsals of 17-day-old mouse fetuses were prepared, transferred to cell-culture plates and allowed to adhere for 4 days. Medium was refreshed and bones were stimulated for 7 days with VEGF (50 ng/ml), SB-431542 [SB; or LY-2157299 (LY)] (10 μM) or both. Cultures were fixed and vessel-like structures were visualized by anti-CD31 staining. Six bones were stimulated per experimental group and one representative picture of each group is shown. Ctrl control. (B) Enlargements of the EC-sheet formation of the images in A. (C) SB-431542 did not significantly affect baseline formation of the endothelial network. LY-2157299 slightly inhibited formation of the endothelial network. Incubation with VEGF strongly stimulated the formation of vessel-like structures, which was dramatically promoted by co-stimulation with SB- 431542 or LY-2157299. **P≤0.01.*

Thus, both VEGF and SB-431542 promote EC sprouting. Interestingly, the addition of a VEGFR2-kinase inhibitor (PTK787) (29) blocked both VEGF- and SB-431542-induced sprouting (Fig. 3D). Because PTK787 can inhibit the stimulatory effect of SB-431542 on EC sprouting, we wondered whether SB-431542 enhances VEGFR2 phosphorylation. We observed no effect of SB-431542 on basal and VEGF-induced VEGFR2 phosphorylation levels or on the VEGF-induced activation of downstream pathways such as ERK and p38 MAP kinases (data not shown). Treatment of HUVECs with SB-431542 decreased basal phosphorylated Smad2, indicating the presence of TGF-β-like factors in media supplements or active TGFβ secreted by HUVECs. VEGF had no effect on the levels of Smad2 phosphorylation (data not shown). Taken together, our results indicate that SB-43152 and VEGF do not influence immediate VEGFR2- or ALK5-induced responses, respectively.

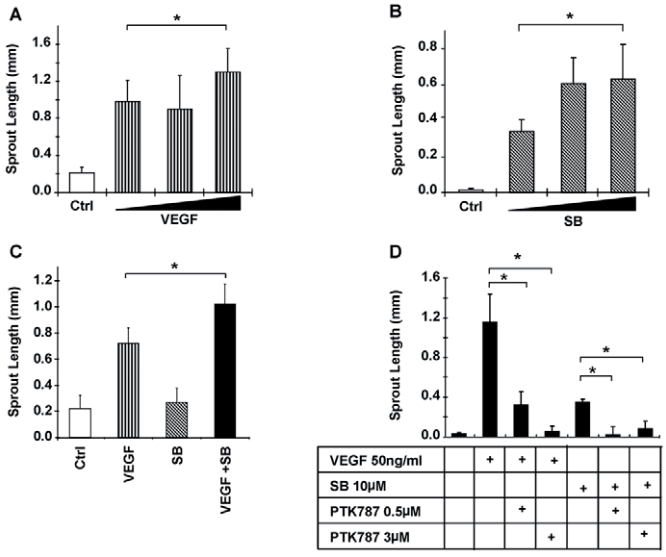

Fig. 3. *Synergistic effect of SB-421542 or TGF-β neutralizing antibody with VEGF on EC sprouting. (A) HUVEC spheroids embedded in collagen were stimulated with increasing amounts of VEGF (10, 25 or 50 ng/ml). (A)HUVEC spheroids embedded in collagen were stimulated with increasing amounts of SB-431542 (SB; 1, 5 or 10 μM). (C) HUVEC spheroids embedded in collagen were stimulated with VEGF (50 ng/ml), SB-431542 (10 μM) or both for 24 hours. (D) EC spheroids were stimulated with VEGF or SB-431542 in the presence or absence of the VEGF-receptor-kinase inhibitor PTK787. (A-D) Quantitative analysis of the mean total sprout length was performed on at least ten spheroids per experimental group. *P≤0.05.*

Although stimulation of the spheroids with VEGF (50 ng/ml) and SB-431542 (10 μM) further increased sprouting (Fig. 3C), this effect was additive and not synergistic. Therefore, we hypothesized that the concentrations of VEGF and SB-431542 are near-to-plateau levels, and we analyzed the effect of sub-optimal concentrations of both VEGF and the ALK5 kinase inhibitor. Stimulation of EC spheroids with 1 ng/ml VEGF or 0.2 μM SB-431542 alone resulted in a small induction of sprouting compared with control (Fig. 4A,B). Interestingly, the combination of low levels of VEGF and SB-431542 synergistically enhanced EC sprouting (Fig. 4A,B). Similar results were obtained when the TGF-β type I receptor kinase inhibitor LY-2157299 was used. The combination of sub-optimal concentrations of LY-2157299 and VEGF resulted in enhanced sprouting compared with VEGF or the inhibitor alone (Fig. 4C,D).

Because SB-431542 interferes with signalling not only of the TGFβ type I receptor ALK5, but also with the activity of the Actvin and Nodal type I receptors ALK4 and ALK7, we investigated whether the effects seen are due to inhibition of TGF-β or antagonism of Actvin-Nodal signalling. We therefore used the 1D11 TGF-β neutralizing antibody (29 30., 1989). EC spheroids were stimulated with a low concentration of VEGF in the presence or absence of the TGF-β neutralizing antibody or an isotype-

matched control antibody (Fig. 4E). The isotype-matched control antibody had no effect on basal or VEGF-induced sprouting. Addition of the TGF-β neutralizing antibody did not induce EC sprouting. However, the combination of TGF-β neutralizing antibody with VEGF significantly induced EC sprouting compared with VEGF or TGF-β blocking antibody alone (Fig. 4E). As expected, ectopic expression of a constitutively active form of ALK5 receptor (caALK5) exerted the opposite response of SB-431542 treatment and inhibited VEGF-induced HUVEC sprouting in the 3D spheroid system (supplementary material Fig. S1A). Moreover, caALK5 overexpression inhibited basal cord formation of mouse embryonic endothelial cells (MEECs) (supplementary material Fig. S1B). Taken together, our results suggest that inhibition of the TGF-β signalling pathway significantly enhances VEGF-induced EC sprouting *in vitro*.

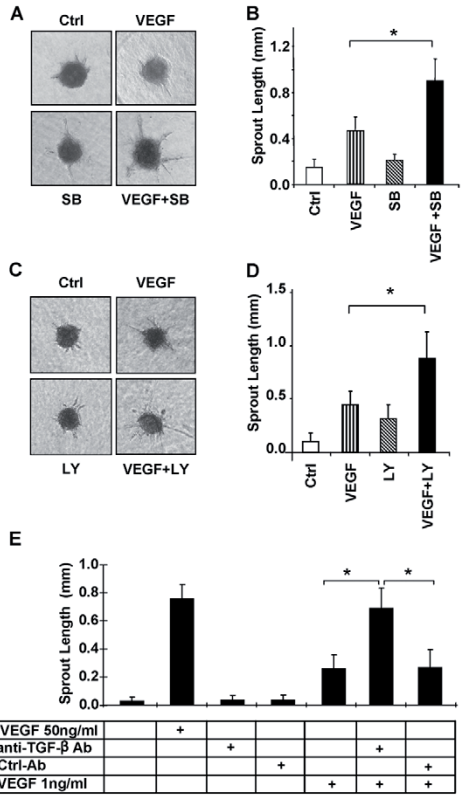

Fig. 4. *Synergistic effect of SB-421542 or TGF-β neutralizing antibody with VEGF on EC sprouting. (A,B) HUVEC spheroids embedded in collagen were stimulated with VEGF (1 ng/ml), SB-431542 (SB; 0.2 μM) or both for 24 hours. One representative picture of each group is shown. Quantitative analysis of the mean total sprout length was performed on at least ten spheroids per experimental group. (C,D) Spheroids were stimulated with VEGF (1 ng/ml), LY-2157299 (LY; 0.2 μM) or both for 24 hours. One representative picture of each group is shown. Quantitative analysis of the mean total sprout length per experimental group is shown. (E) EC spheroids were stimulated with VEGF (1 ng/ml), TGFβ-neutralizing antibody (30 μg/ml), isotype control antibody (10 μg/ml) or a combination for 24 hours. Quantitative analysis of the mean total sprout length per experimental group is shown. *P≤0.05.*

VEGF and ALK5 inhibitor promote EC migration

To further investigate the mechanisms by which VEGF and SB-431542 exert their synergistic effect on angiogenesis, we analyzed their effects on EC migration. To study the effect of VEGF and SB-431542 on migration, serum-starved monolayers of HUVECs were wounded by scratching and were stimulated for 6 hours. There was no effect on migration when cells were stimulated by VEGF or the ALK5-kinase inhibitor alone. However, the combination of VEGF and SB-431542 significantly induced EC migration (Fig. 5A,B). Consistent with these results, over-expression of caALK5 in MEECs resulted in reduced migration, using the transwell migration assay, and invasion, using a Transwell invasion assay with Matrigel coating (11) (supplementary material Fig. S2A,B).

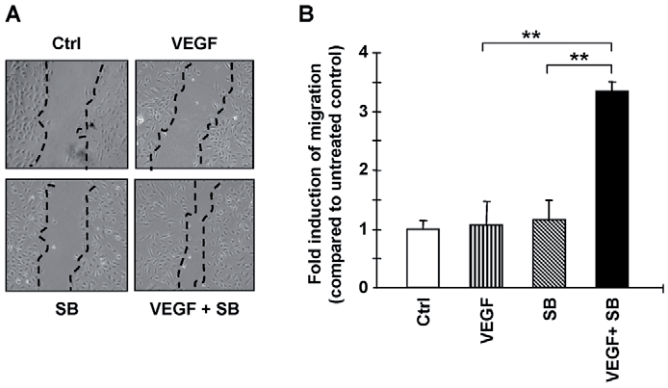

Fig. *5. Effects of SB-431542 and VEGF on EC migration. VEGF and SB- 431542 (SB) synergistically stimulate HUVEC migration. HUVECs were allowed to grow to confluence and serum-starved for 5 hours. Monolayers were wounded and stimulated with VEGF (1 ng/ml), SB-431542 (0.2 μM) or both for 6 hours. (A) One representative picture of each group is shown. Dashed lines indicate the wound edge. (B) Wound closure was measured after 6 hours using ImageJ software. **P≤0.01.*

Transcriptional profiling of the VEGF+SB-431542-induced EC response

Trying to unravel the mechanistic basis of the VEGF and SB-431542 synergistic effects on angiogenesis (EC sprouting), we analyzed expression of a number of genes essential to EC function by quantitative real-time PCR. We used a highly sensitive commercial PCR-based array system containing up to 84 genes related to EC function. From the genes analyzed, the expression of 20 of them was significantly upregulated when cells were stimulated by the combination of VEGF and SB-431542, but not by VEGF or SB- 431542 alone (Table 1). In addition, VEGF+SB-431542 stimulation resulted in the downregulation of 30 genes. Interestingly pro- and anti-apoptotic genes were differentially regulated following VEGF+SB-431542 stimulation. There was an upregulation in expression of the anti-apoptotic gene *Bcl2* and a significant downregulation of pro-apoptotic genes, such as annexin A5, *Bax* and caspase 6 (Table 1), suggesting that VEGF and SB-431542 induce

pathways that promote EC survival. VEGF and SB-431542 synergistically induced the expression of several other genes implicated in angiogenesis, such as angiotensin receptor I, *ACE*, *CCL5*, *IL3* and *IL7* (Table 1). Integrins have been shown to play an important role in EC migration and survival, as well as in capillary sprouting during angiogenesis. In line with this notion, VEGF+SB-431542 stimulation synergistically induced α5 integrin and β3 integrin mRNA expression. To verify these results, we performed quantitative PCR analysis using PCR primer sets different from those used in the array (supplementary material Fig. S3A). Similar results were obtained when mRNA was isolated from EC spheroids embedded in collagen after VEGF and/or SB-431542 stimulation (supplementary material Fig. S3B).

***Table** 1. Summary of genes identified as significantly altered by VEGF/SB-431542 stimulation of ECs, using an EC function PCR-based array system.*

Fold difference compared to control				
Gene	**VEGF**	**SB**	**VEGF/SB**	**p value***
Apoptosis/survival				
Bcl2	2.8	4.0	1×10^{3}	<0.01
Bax	1.1	1.8	0.2×10^{-3}	<0.01
Annexin A5	1.1	2.2	8×10^{-3}	<0.01
CASP6	1.0	1.6	0.1×10^{-3}	<0.01
Angiotensin system				
AGT	0.5	0.8	0.5	0.2
AGTR1	0.5	0.8	0.5×10^{3}	<0.01
ACE	1.6	2.7	0.6×10^{3}	<0.01
Chemokines				
CCL5 (RANTES)	0.93	2.1	5×10^{3}	<0.01
Interleukins				
IL1b	1.0	2.6	21	0.5
IL3	1.0	1.0	2×10^{2}	<0.01
IL6	0.5	0.87	7	<0.05
IL7	0.7	0.99	3×10^{3}	<0.01
IL11	0.9	1.8	45	0.08
Integrins				
ITGA5	1.0	1.31	1×10^{3}	<0.01
ITGB3	7.6	14.7	51	<0.01

**p value of VEGF/SB stimulated versus untreated control cells.*

Crucial role of α5 integrin in VEGF+SB-431542-induced angiogenesis

Stimulation of VEGF and inhibition of the TGFβ type I receptor kinase significantly induces integrins α5 and β3 mRNA expression. Interestingly, pretreatment of HUVECs with cyclohexamide (inhibitor of protein synthesis) diminished the synergistic effect of VEGF+SB-431542 on α5-integrin expression (supplementary material Fig. S4). Those results indicate that the synergistic effect of VEGF and SB-431542 on integrin expression is not direct, but it requires de novo protein synthesis.

To investigate whether integrins α5 and β3 have a functional role in the synergistic effects of VEGF and SB-431542 on capillary sprouting, we studied the loss-of-function effects using an RNA-interference approach. HUVECs were transfected with siRNA oligonucleotides for α5 integrin and β3 integrin or with control siRNA and subsequently seeded to form spheroids, which were then embedded in collagen. In α5 integrin- and β3 integrin- siRNA-transfected cells, the levels of α5-integrin and β3 integrin RNA expression decreased on average 50% (data not shown). Quantification of sprouting demonstrated a significant decrease in VEGF+SB-431542-induced sprout length in the α5 and β3 integrin siRNA transfected spheroids compared with control siRNA (Fig. 6A). It is well recognized that integrin-mediated signalling plays a crucial role in angiogenesis. To exclude the possibility that downregulation of α5 integrin or β3 integrin results in a general block in angiogenesis, we tested the effect of α5 and β3 integrin siRNA on basic fibroblast growth factor (bFGF)- and VEGF (high concentration)-induced sprouting in the spheroid assay. Whereas α5-integrin downregulation diminished the VEGF+SB-431542-induced sprouting, it had no effect on bFGF- or high dose VEGF-induced capillary formation (Fig. 6A). Interestingly, downregulation of β3 integrin by siRNA affected VEGF+SB-431542 as well as bFGF- or high dose VEGF-induced EC sprouting (Fig. 5A). Moreover, α5-integrin downregulation had no effect on VEGF- (1 ng/ml) or SB-431542- (0.2 μM) induced EC sprouting (Fig. 6B).

To further substantiate the role of α5 integrin in the synergistic effect of VEGF and SB-431542 on angiogenesis, we made use of an α5 integrin neutralizing antibody. Addition of α5 integrin function blocking antibody inhibited the synergistic effect of VEGF+SB-431542 on EC sprouting (Fig. 6C). However, α5 integrin neutralizing antibody had no effect on high dose VEGF-induced capillary formation (Fig. 6D). Our results suggest that α5 integrin is necessary for VEGF+SB-431542-mediated synergistic induction of EC sprouting in the 3D EC spheroid assay.

VEGF and ALK5-kinase inhibitor synergistically enhance in vivo angiogenesis in a Matrigel-plug assay in mice

In order to characterize the effect of inhibition of the ALK5 receptor in VEGF-induced angiogenesis, we analyzed the *in vivo* angiogenesis response by placing Matrigel plugs without and with supplements (LY-2157299 and/or VEGF and bFGF) under the skin of mice. Because VEGF

alone did not induce angiogenesis in the *in vivo* Matrigel-plug assay (data not shown), we used a combination of VEGF and bFGF. Vascularization was assayed by CD31 staining of sections from the recovered plugs (Fig. 7B,C). Matrigel plugs without any stimulus showed no vascularization. Addition of LY- 2157299 or VEGF+bFGF combination significantly induced angiogenesis (Fig. 7 A,B).

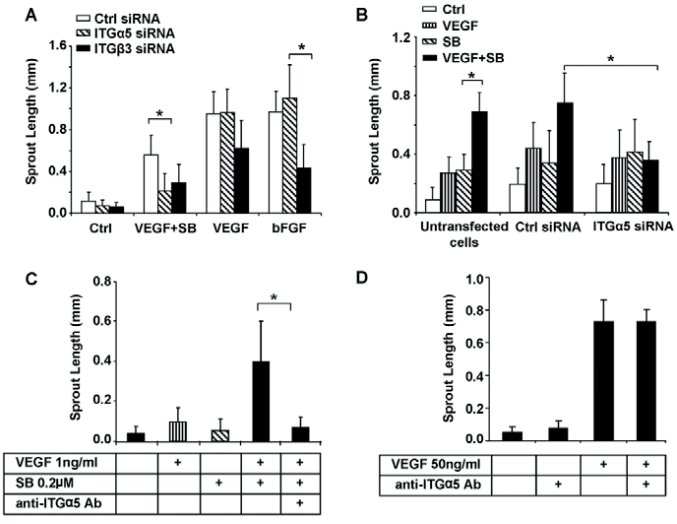

Fig. 6. *Integrin α5 and β3 mediate the synergistic effects of SB and VEGF induced angiogenesis. (A) Downregulation of integrin α5 and β3 inhibits VEGF/SB-431542- induced EC sprouting. HUVECs transiently transfected with control, integrin α5 (ITGα5) or β3 (ITGβ3) siRNA were allowed to form spheroids. Spheroids were embedded in collagen and stimulated with VEGF (1 ng/ml) and SB-431542 (0.2 μM), VEGF (50 ng/ml) or bFGF (100 ng/ml) for 24hr. Quantitative analysis of the mean total sprout length per experimental group is shown. (B) HUVECs were transiently transfected with control or ITGα5 siRNA were plated to form spheroids. Spheroids were embedded in collagen and stimulated with VEGF (1 ng/ml), SB-431542 (0.2 μM) or their combination for 24hr. Quantitative analysis of the mean total sprout length per experimental group is shown. (C) Functional blocking of integrin α5 suppresses VEGF/SB-431542-induced angiogenesis. Spheroids were embedded in collagen and stimulated with VEGF (1 ng/ml), SB-431542 (0.2 μM), VEGF (1 ng/ml) and SB-431542 (0.2 μM), VEGF/SB-431542 or VEGF/SB-431542 and integrin α5 neutralizing antibody (10 μg/ml). Quantitative analysis of the mean total sprout length per experimental group is shown. (D) Functional blocking of α5 integrin does not affect VEGF (high dose)-inducedangiogenesis. Spheroids were embedded in collagen and stimulated with VEGF (50 ng/ml) with or without integrin α5 neutralizing antibody (10 μg/ml). Quantitative analysis of the mean total sprout length was performed on at least 10 spheroids per experimental group using the Olympus Analysis software.*

Addition of LY-2157299 with VEGF+bFGF (VEGF+bFGF+LY) further significantly enhanced the blood-vessel formation (Fig. 7A). To corroborate the role of α5 integrin in VEGF+bFGF+LY synergistic effect on *in vivo* angiogenesis we examined the effect of the α5 integrin neutralizing antibody in the Matrigel plug assay. Whereas addition of α5 integrin neutralizing antibody but not a control antibody inhibited VEGF+bFGF+LY-induced angiogenesis, it had no significant effect on VEGF+bFGF-induced angiogenesis. These results illustrate that inhibition of TGFβ-ALK5 signalling *in vivo* can potentiate the pro-angiogenic effect of VEGF and bFGF, which is critically dependent on the induction of α5 integrin signalling.

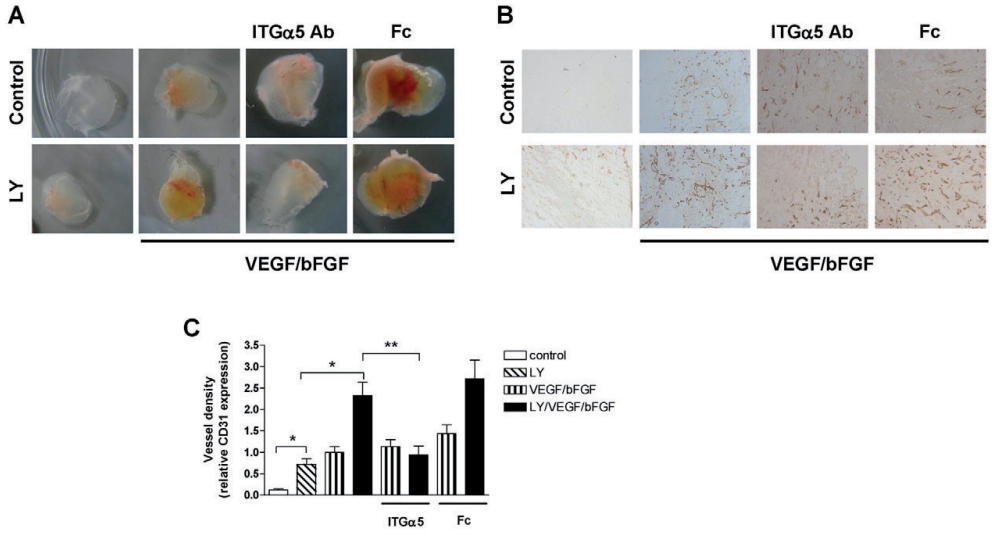

Fig. 7. *LY-2157299 induces angiogenesis in vivo in a Matrigel-plug assay and promotes VEGF+bFGF-induced angiogenesis. Matrigel plugs combined with either PBS (control), VEGF (300 ng/ml) and bFGF (700 ng/ml), and/or LY-2157299 (LY; 0.1 μM), in the presence or absence of α5-integrin-neutralizing antibody (20 μg/ml) or Fc control protein (20 μg/ml), were subcutaneously injected into mice. LY-2157299 induces angiogenesis and enhances VEGF+bFGF-induced angiogenesis. The synergistic effect of LY+VEGF+bFGF on angiogenesis is inhibited by an anti-α5-integrin antibody but not by Fc. (B) Histological analysis of CD31-stained sections recovered from Matrigel plugs with different stimuli. (C) Quantification of the vascular density by CD31 immunostaining as a percentage of the lesional area. *P≤0.05; **P≤0.01.*

Discussion

Here we show that inhibition of TGFβ type-I receptor (ALK5) and VEGF synergistically promotes angiogenesis. Treatment with the ALK5 inhibitors SB-43152 or LY-2157299 strongly enhanced VEGF-induced EC capillary formation and EC sprouting. Similar results were obtained using a TGFβ-neutralizing antibody (30), indicating that inhibition of TGFβ, and not of Activin- Nodal signalling, is likely to cooperate with VEGF in inducing angiogenesis. Addition of the VEGFR2-kinase inhibitor PTK787 (29) blocked not only the VEGF- but also the SB- 431542-induced EC sprouting, suggesting that inhibition of TGFβ signalling potentiates autocrine VEGF signalling in ECs. Importantly, the TGFβ receptor kinase inhibitor promoted basal and VEGF+bFGF-induced angiogenesis *in vivo* in a Matrigel-plug assay. Conversely, ectopic expression of caALK5 potently inhibited all VEGF-induced pro-angiogenic responses of ECs.

Recently it was reported that inhibition of TGFβ antagonized the pro-angiogenic response on human normal dermal microvascular ECs (MVECs) *in vitro* (31). There are several explanations for the discrepancy with our results. First, the pro-angiogenic effects of TGFβ were inhibited using TGFβ antagonistic peptides derived from the TGFβ type III receptor sequence. By contrast, we used pharmacological inhibitors of the TGFβ and/or Activin receptors and a pan TGFβ-neutralizing antibody in combination with VEGF. TGFβ type III receptor facilitates TGFβ signalling by enhancing the binding of ligand to TβRII (32). It was recently shown that the TGFβ type III receptor can bind multiple members of the BMP subfamily, including BMP2, BMP4 and BMP7, and thereby potentiate BMP signalling. BMP2, BMP4 and BMP7 have been shown to induce angiogenesis (33; 25; 34; 35; 36). Consequently, it is possible that the peptide antagonists used by Serrati and co-workers inhibit endogenous BMP signalling (30). Second, ECs of different origin were used. Seratti used MVECs, whereas we used HUVECs. These ECs might respond differently to TGFβ signalling owing to, e.g. expression of different receptors or different levels of TGFβ production. We show that treatment of HUVEC spheroids with the TGFβ-receptor-kinase inhibitor (or TGFβ neutralizing antibody) resulted in decreased basal Smad2 phosphorylation and increased sprouting, suggesting that endogenous TGFβ signalling or TGFβ present in media supplements or produced by the cells inhibits VEGF signalling in HUVECs.

Transcriptional profiling revealed that the VEGF+SB-431542 stimulation of HUVECs synergistically regulates a number of genes involved in angiogenesis. Our results showed that VEGF+SB- 431542 stimulation of ECs resulted in a dramatic decrease in the expression of pro-apoptotic genes and an increase in that of anti-apoptotic genes. TGFβ was shown to induce apoptosis of ECs via autocrine and/or paracrine stimulation of VEGF expression and signalling by VEGFR2 via downstream activation of p38 (37). We observed no effects of SB-431542 on VEGF-induced ERK and p38 phosphorylation (data not shown). Thus, on the basis of our results, we suggest that stimulation of ECs with VEGF and SB-431542 protects ECs from apoptosis and enhances their survival and proliferation. This might explain the dramatic induction of EC-sheet formation we observed upon VEGF+SB- 431542 challenge in the ex vivo metatarsal assay.

α5 integrin, a cell-surface receptor for fibronectin, is one of the genes that is synergistically upregulated by VEGF+SB-431542 co-stimulation. We confirm the importance of this upregulation in VEGF+SB-431542-induced EC sprouting because genetic ablation and function neutralizing antibodies of α5 integrin inhibit VEGF+SB-431542-induced EC sprouting *in vitro* and *in vivo*. Our results underline the important role of α5 integrin demonstrated before in blood vessel development; α5 integrin null embryos exhibit abnormal blood vessel formation and a lower complexity vascular network (38). Furthermore, α5β1 integrin function blocking antibody was shown to inhibit HUVEC tube formation *in vitro* (39), and to inhibit tumor neovascularization and growth in animal models (40).

VEGF and TGFβ signalling play a central role in tumor angiogenesis and thus in tumor development and metastasis. Several therapeutic strategies targeting TGFβ signalling have been shown to prevent the growth and metastasis of certain cancers (41). Interestingly, a recent study using low doses of the TβRI kinase inhibitor (LY364947) in experimental tumors decreased VSMC coverage of the tumor endothelium and promoted the accumulation of anticancer drugs in the tumor tissue (42). Our results suggest that inhibition of TGFβ signalling renders the ECs more sensitive to VEGF-induced sprouting *in vitro* and *in vivo*. Therefore, anti-TGFβ-based therapeutic strategies must be carefully considered before administration of TGFβ antagonists because there might be adverse effects, including the induction of tumor angiogenesis. A better treatment modality, as suggested by this study, is combining VEGF and TGFβ pathway inhibitors to inhibit tumor cell metastasis and angiogenesis.

In conclusion, we investigated the effect of TGFβ type I receptor inhibitor(s) or TGF-β neutralizing antibody on VEGF-induced EC sprouting. Our results suggest that combining VEGF and inhibition of TGFβ-ALK5 signalling synergistically promotes angiogenesis *in vitro* and *in vivo* by inducing a cascade of expression of genes that play important roles in EC survival and in angiogenesis. Functional studies revealed that the induction and function of α5 integrin is a key determinant in VEGF+SB-431542-induced angiogenesis.

Materials and Methods

Recombinant proteins, inhibitors and antibodies

The VEGF165 isoform was purchased from R&D Systems and bFGF from Peprotech. SB-431542 was purchased from Tocris Biotrend, LY-2157299 from Calbiochem and PTK787 from Novartis. α5-integrin neutralizing antibody was purchased from BD Biosciences, the TGFβ-neutralizing antibody and isotype control obtained from Genzyme, and the Fc domain of IgG1 (MOPC-21) from Bio Express, West Lebanon, NH.

Ex vivo metatarsal angiogenic assay

All animal experiments were approved by the local animal ethics committee. Metatarsals from 17-day-old mouse fetuses were dissected as described earlier (26). Six bones per experimental group were transferred to 24-well 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 the stimuli. Cultures were fixed 7 days after stimulation and vessel formation was visualized by anti-CD31 staining (Deckers 25, 2001).

Cell culture

HUVECs cells were cultured in Medium 199 with Earle's salt and L-glutamine (Gibco), 10% FCS, heparin (LEO pharma), bovine pituitary extract (Gibco) and PS on plates coated with 1% gelatin, at 37°C, 5% CO2. HUVECs were used up to passage 4. Experiments were confirmed with HUVECs from different donors. MEECs were cultured in DMEM (Gibco) supplemented with 10% FCS and PS on 0.1% gelatin- coated plates, at 37°C, 5% CO2.

3D-culture spheroid assay

HUVECs (400 cells per spheroid) were suspended in Medium M199 containing Earle's salt and L-glutamine, 10% FBS, 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. EC sprouts were measured by Olympus Analysis software.

Migration scratch assay

HUVECs were seeded in six-well plates coated with 1% gelatin and allowed to grow to confluence. After serum starvation, monolayers were wounded with three scratches and medium

was replaced by fresh medium containing stimuli. Cell migration was measured in five areas per well directly after wounding and 6 hours later by automated image analysis using ImageJ software.

Matrigel plugs

Male 7- to 8-week-old C57BL/6 mice (Charles River Laboratories, Sulzfeld, Germany) were injected subcutaneously near their abdominal midline with 0.4 ml of Matrigel basement membrane, high concentration (BD Biosciences, San Jose, CA) combined with either PBS, VEGF (300 ng/ml) and bFGF (700 ng/ml), and/or LY- 2157299 (0.1 μM), in the presence or absence of α5-integrin-neutralizing antibody (20 μg/ml) or Fc control protein (20 μg/ml). Groups of four plugs were injected for each treatment and the experiment was repeated twice. Seven days later Matrigel plugs were removed, fixed in formalin and embedded in paraffin. Sections were subjected to histological analysis with eosin. Sections were deparaffinized. Quenching of endogenous peroxidase activity was done using 0.3% H2O2 in methanol for 20 minutes at room temperature (RT), followed by antigen retrieval using citrate buffer and blocking with 1% BSA in PBS for 1 hour at RT. The primary antibody against CD31 (1:1000, Santa Cruz Biotechnologies, Santa Cruz, CA) was incubated in 1% BSA in PBS overnight at RT. Biotin-conjugated secondary antibodies were applied followed by amplification using the strep-AB-complex/HRP (DAKO, Hamburg, Germany). Finally, diaminobenzidine substrate (Sigma) was added to visualize peroxidase activity. The area covered by CD31-positive staining was quantified with image analysis.

Cell transfection and RNA interference

Cells were seeded in six-well plates and the following day were transiently transfected with control siRNA or siRNA against α5 integrin and β3 integrin, purchased from Dharmacon, according to the manufacturer's instructions. One day after transfection, cells were trypsinized and seeded for spheroid formation or used for RNA isolation.

RNA isolation and EC PCR array

Total DNA-free cellular RNA was extracted with RNeasy kit (Macherery-Nagel) according to the manufacturer's instruction. RNA from HUVECs grown in monolayers that were either non-treated or treated with VEGF, SB-431542 or VEGF+SB-431542 for 24 hours was isolated from three independent biological experiments and separately analyzed on the array. A commercial EC PCR-array (purchased from Superarray) was used to investigate gene expression profiling of selected angiogenesis- related genes involved in EC function (http://www.sabiosciences.com/rt_pcr) product/HTML/PAHS-015A.html). The experiment was performed in triplicate. The manufacturer's instructions were strictly followed (http://www.sabiosciences.com/). Gene expression levels were determined by using data analyzer template provided by Superarray (http://www.sabiosciences.com/pcrarraydataanalysis.php), using GAPDH, β-actin and ribosomal protein L13a as reference. The non-stimulated condition was set to 1.

Adenoviral infection

HUVECs or MEECs growing in 70-80% confluent monolayers were infected with an adenovirus expressing either caALK5 or lacZ with an MOI of 500. At 48 hours after infection, cells were trypsinized and re-seeded to be used in different assays.

EC cord formation

Matrigel Basement Membrane Matrix Growth Factor Reduced (Becton Dickinson) was added at 50 µl to each well of a 96-well plate and allowed to polymerize for 1 hour at 37°C. Cells were removed from culture by trypsinization and resuspended at 30,000 cells/ml. 100 µl cell suspension were plated in each well in triplicates and plates incubated for 48 hours. Pictures were acquired with a phase-contrast microscope in four different fields. The length of branches was quantified using automated image analysis using the Olympus Analysis software.

Transwell migration and invasion assays

Transwell migration was performed in 24-well plates with filter inserts of a pore size of 0.8 µm (Costar). For the Transwell invasion assay, filters were coated with Matrigel. 30,000 cells were seeded in the upper chamber. Experiments were done in triplicates. Cells were fixed after 20 hours with 4% paraformaldehyde and stained with 0.1% crystal violet. Pictures of the filters were acquired with phase-contrast microscopy at 10x magnification. Three fixed positions were imaged of each membrane and the number of cells was counted.

RNA isolation and quantitative PCR analysis

RNA from HUVECs growing in monolayers or EC spheroids embedded in collagen [non-treated, or VEGF- (1 ng/ml), SB-431542- (0.2 µM) or VEGF+SB-431542-treated for 24 hours) was isolated from three independent biological experiments with RNeasy kit (Macherery-Nagel) and subjected to cDNA synthesis with RevertAid H Minus first strand cDNA synthesis kit (Fermentas) according to the manufacturer's instruction. Expression of α5 integrin (ITGα5) and house-keeping gene acidic ribosomal phosphoprotein (ARP) were analyzed using the following primers:

ITGα5 forward: 5-ATACTCTGTGGCTGTTGGTGAATTC-3;
ITGα5 reverse: 5-ATTAAGGATGGTGACATAGCCGTAA-3;
ARP forward: 5-CACCATTGAAATCCTGAGTGATGT-3;
ARP reverse: 5-TGACCAGCCGAAAGGAGAAG-3.

Taqman PCR reactions were performed using the ABI prism HT7900 sequence-detection system (Applied Biosystem). All samples were plated in duplicates. Gene expression levels were determined with the comparative ΔCt method using ARP as reference and the non-stimulated condition was set to 1.

Cyclohexamide experiments

HUVECs growing in monolayers were either untreated or pretreated with 5 µg/ml cyclohexamide for 1 hour, and subsequently unstimulated or treated with 1 ng/ml VEGF, 0.2 µM SB-431542 or a combination of both VEGF and SB-431542 for 24 hours. RNA was isolated and subjected to cDNA synthesis followed by quantitative real-time PCR analysis.

Statistical analysis

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

Acknowledgments

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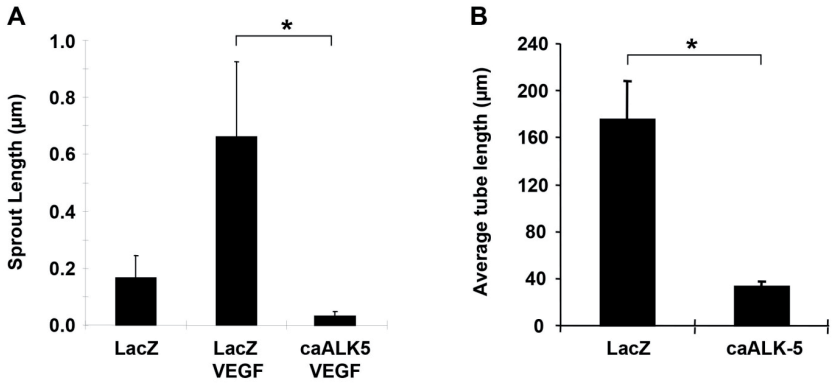
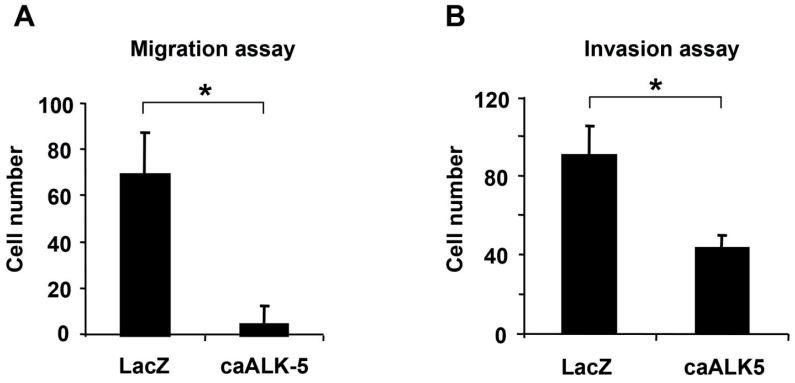
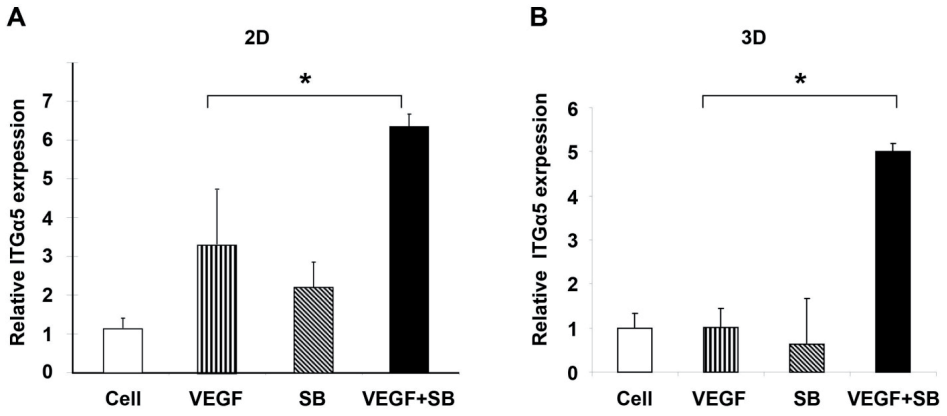
Supplementary Information



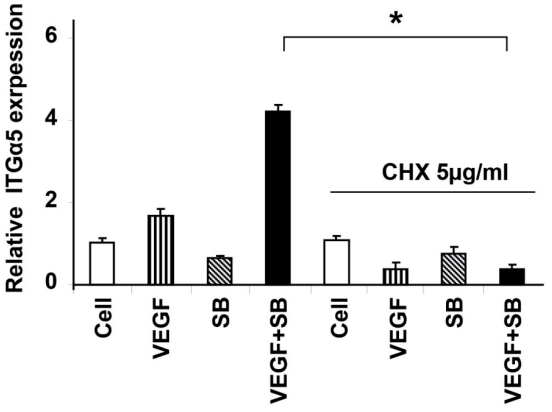
Supplemental Fig. 1 *Overexpression of constitutively active (ca)ALK5 inhibits EC sprouting and cord formation. (A) HUVECs infected with adenoviruses expressing caALK5 or lacZ were plated to form spheroids and embedded in collagen were stimulated with VEGF (50 ng/ml) for 24 hours. One representative picture of each group is shown. Quantitative analysis of the mean total sprout length was performed on at least ten spheroids per experimental group. (B) MEECs infected with adenoviruses expressing caALK5 or lacZ were plated on Matrigel-coated plates for cord formation for 48 hours. One representative picture of each group is shown. Quantitative analysis of the mean total cord length per experimental group is shown.*



Supplemental Fig. 2 *Overexpression of caALK5 inhibits EC Transwell migration and invasion. MEECs infected with adenoviruses expressing caALK5 or lacZwere plated on the upper chamber of 24-well plates with filter inserts. (A) Cells were fixed after 20 hours with 4% paraformaldehyde, stained with 0.1% crystal violet and the number of migrating cells was counted in three different fields. (B) For the Transwell invasion assay filters were coated with Matrigel. After 20 hours, cells were fixed, stained and the number of invaded cells was counted in three different fields*



Supplemental Fig 3 *SB-431542 and VEGF synergistically induce expression of integrin α5 in 2D and 3D EC cultures. HUVECs growing in monolayers (A) or in EC spheroids treated (B) with VEGF (1 ng/ml), SB-431542 (0.2 μM) or their combination for 24 hours. Extracted mRNA was reverse transcribed and used in a quantitative PCR array to determine relative integrin α5 mRNA expression patterns. The mRNA levels of integrin α5 in each sample was first normalized to the expression of ARP in that sample and then normalized to the expression of that gene in untreated HUVECs.*



Supplemental Fig 4 *VEGF+SB-431542-induced α5-integrin expression depends on new protein synthesis. HUVECs growing in monolayers were pretreated with 5 μg/ml cyclohexamide (CHX), followed by VEGF (1 ng/ml), SB-431542 (0.2 μM) stimulation or their combination for 24 hours. Extracted mRNA was reverse transcribed and used in a quantitative PCR array to determine relative α5-integrin mRNA expression patterns.*

Chapter 3

BMP-9 signals via ALK1 and inhibits bFGF-induced endothelial cell proliferation and VEGF-stimulated angiogenesis

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Abstract

Genetic studies in mice and humans have shown that the transforming growth factor-β (TGF- β) type-I receptor activin receptor like kinase 1 (ALK1) and its co-receptor endoglin play an important role in vascular development and angiogenesis. Here, we demonstrate that ALK1 is a signalling receptor for bone morphogenetic protein-9 (BMP-9) in endothelial cells (ECs). BMP-9 bound with high affinity to ALK1 and endoglin, and weakly to the type-I receptor ALK2 and to the BMP type II receptor (BMPRII) and activin type II receptor (ActRII) in transfected COS cells. Binding of BMP-9 to ALK2 was greatly facilitated when BMPRII or ActRII were co-expressed. Whereas BMP-9 predominantly bound to ALK1 and BMPRII in ECs, it bounds to ALK2 and BMPRII in myoblasts. In addition, we observed binding of BMP-9 to ALK1 and endoglin in glioblastoma cells. BMP-9 activated Smad1 and/or Smad5, and induced ID1 protein and endoglin mRNA expression in ECs. Furthermore, BMP-9 was found to inhibit basic fibroblast growth factor (bFGF) stimulated proliferation and migration of bovine aortic ECs (BAECs) and to block vascular endothelial growth factor (VEGF)-induced angiogenesis. Taken together, these results suggest that BMP-9 is a physiological ALK1 ligand that plays an important role in the regulation of angiogenesis.

***Keywords*:** ALK1, Angiogenesis, bFGF, BMP, Endoglin, Signalling, Smad, VEGF

Introduction

Bone morphogenetic proteins (BMPs) belong to the TGF-β_superfamily and were originally identified for their ability to induce ectopic bone growth and cartilage formation (1, 2). Since then, substantial knowledge has been obtained about the multiple functions of these growth factors. BMPs regulate cell growth, differentiation andapoptosis of various cell types, and they are critically involved in the morphogenesis and differentiation of tissues and organs. In addition, they play an important role in adult tissue homeostasis (3, 4). BMP-9 is a secreted protein (5) that is expressed in the liver (6, 7). It has been associated with the regulation of genes involved in glucose and fatty acid metabolism (6), and it induces expression of hepcidin, which plays a key role in iron homeostasis (8). BMP-9 stimulates proliferation of non-endothelial cells (non-ECs) such as liver tumour cells, pre-adipocytes or myoblasts (6, 7). In addition, BMP-9 induces ectopic bone growth and hypertrophic chondrocyte formation and supports the differentiation of mesenchymal cells into cartilage (9). BMP-9 is also expressed in the septum and spinal cord of mouse embryos, and it is a differentiation factor for cholinergic neurons of the central nervous system (10).

In a recent study, BMP-9 was shown to bind with high affinity to the immobilised extracellular domain of activin receptor like kinase 1 (ACVL1, also known as, and hereafter referred to, as ALK1) in a BIAcore assay (5). The ALK1 receptor is mainly expressed in ECs (11), regulating EC proliferation and migration *in vitro* (12), and angiogenesis *in vivo* (13, 14). ALK1-deficient mice display impaired vessel remodelling, dilated blood vessels and defective recruitment of smooth muscle cells (13, 14). *In vitro* studies have shown that the co-receptor endoglin (ENG) is able to form complexes with ALK1 and to promote the effects of ALK1 on ECs (15, 12, 16). The phenotype of endoglin deficient mice is highly reminiscent of ALK1-deficient mice, thereby suggesting that endoglin also plays a role in ALK1 signalling in angiogenesis (17). Further evidence for the importance of ALK1 and endoglin in vessel formation and maintenance is derived from the vascular disorder hereditary haemorrhagic telangiectasia (HHT or Osler-Weber-Rendu disease). HHT is an autosomal-dominant vascular dysplasia that is characterised by recurrent epistaxis, telangiectases in mucosa and skin, gastrointestinal haemorrhage and arteriovenous malformations in brain, lung and liver. Mutations in the genes encoding ALK1 (responsible for HHT2) or endoglin (responsible for HHT1) have been identified to be the cause for the observed phenotype in most of the cases (18,19).

So far, TGF-β1 is the only described functional ALK1 ligand (20, 12, 13). Yet, ALK1 alone is not sufficient to transduce the TGF-β signal across the plasma membrane. TGF-β and also BMP signalling require a specific heteromeric complex of type I and type II serine/threonine kinase receptors (21, 22). Whereas TGF-β1 first binds to the type II receptor and subsequently recruits the type-I receptor, this order is reversed for some members of the BMP family (23, 24). Ligand-

induced receptor complex formation results in phosphorylation of the type-I receptor by the type II receptor kinase and in subsequent phosphorylation of downstream receptor-regulated Smads (R-Smads, i.e. Smad1, Smad2, Smad3, Smad5 and Smad8). Activated R-Smads then form heteromeric complexes with the common mediator (Co)-Smad4, which then translocate into the nucleus where they regulate specific gene transcriptional responses (21 - 23). In mammals, three BMP type II receptors (BMPRII, ActRIIA, ActRIIB) and three BMP type I receptors (ALK2, ALK3, ALK6) have been identified. Activation of BMP receptors leads to phosphorylation of Smad1, Smad5 and Smad8. In most cell types, TGF-β signals via the broadly expressed receptor type I receptor ALK5 thereby inducing phosphorylation of Smad2 and/or Smad3. The type I receptor ALK1 transduces TGF- β signals in ECs, which then leads to activation of Smad1, Smad5 and Smad8 (12, 13, 20).

The reported interaction of BMP-9 with ALK1 tempted us to investigate whether ALK1 is also a receptor for BMP-9 in ECs and how BMP-9 signalling influences EC function. Our results demonstrate that BMP-9 binds with high affinity to ALK1 and endoglin in ECs. In non-ECs, BMP-9 strongly bound to ALK2, thereby providing an explanation for the potent effects of BMP-9 in these cell types. BMP-9 was found to induce phosphorylation of Smad1 and/or Smad5 and to stimulate the expression of ALK1 target genes. Moreover, BMP-9 stimulated activation of a Smad1- and/or Smad5-responsive transcriptional reporter, which was blocked using small interference RNA (siRNA) targeting ALK1. The importance of BMP-9 signalling in ECs was shown by the inhibiting effect of BMP-9 on basic fibroblast growth factor (bFGF)-induced EC proliferation and by the abrogation of vascular endothelial growth factor (VEGF)-induced angiogenesis. Taken together these results show that BMP-9 signals via ALK1 and functions as a potent regulator of angiogenesis.

Results

BMP-9 binds with high affinity to ALK1 and endoglin in transfected COS-7 cells

To study BMP-9 receptor binding, we transfected COS-7 cells with cDNAs for ALK1 or ALK2 in combination with the type II receptors BMPRII or ActRIIB that have been shown to bind ALK1 in the cell-free BIAcore assay (5). Transfected cells were first affinity-labelled with [125I] BMP-9 and then ligand-receptor complexes were chemically crosslinked and subsequently immunoprecipitated with specific type I or type II antisera. Expression of ALK1, ALK2, BMPR-II and ActR-IIB after transfection was checked in parallel by western blotting (data not shown). Resolution of immunoprecipitated complexes by SDS-PAGE and detection of the radioactive signal on a phosphorimager screen showed that BMP-9 strongly binds to ALK1, but not to ALK2 when expressed without a type II receptor (Fig. 1A; lanes 1, 2). The two bands seen after

immunoprecipitation of ALK1 are probably caused by monomeric or dimeric BMP-9 crosslinked to the receptor. BMP-9 binding to the type II receptors BMPRII and ActRIIB alone was weak (Fig. 1A; lanes 3, 4). Binding of BMP-9 to ALK1 was slightly increased when ALK1 and BMPRII or ActRIIB were co-expressed (Fig. 1A; lanes 5, 6). By contrast, BMP-9 binding to ALK2 was greatly enhanced when the receptor was co-expressed with either BMPRII or ActRIIB (Fig. 1A; lanes 7, 8). Thus, whereas BMP-9 can bind to ALK1 alone, it can only interact with ALK2 in combination with BMPRII or ActRII.

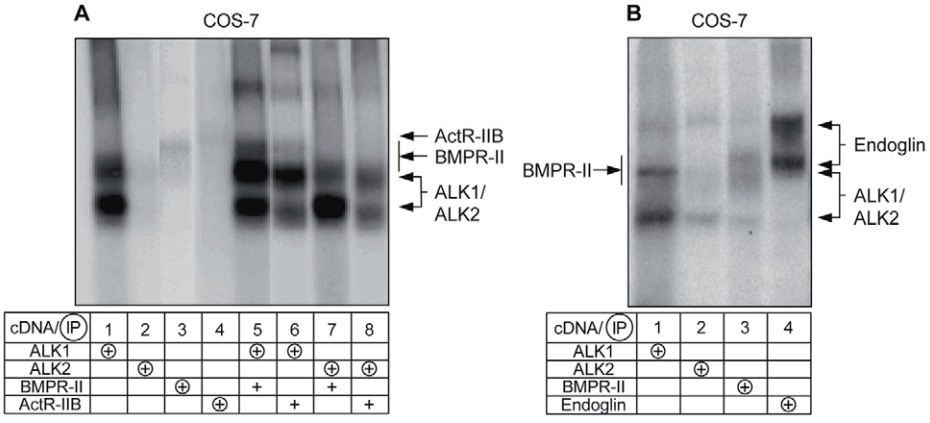

Fig. 1. *BMP-9 binds to ALK1 and endoglin in the absence of type II receptors. COS-7 cells were transiently transfected with cDNAs for ALK1, ALK2, BMPRII, ActRIIB or endoglin and affinity-labelled with [125I]BMP-9. Crosslinked complexes were immunoprecipitated with specific antisera (marked with a circle) and subjected to SDS-PAGE and autoradiography. (A) [125I]BMP-9 binds with high affinity to ALK1, but not to ALK2 in the absence of type II receptors. BMP-9 binding to ALK2 is strongly enhanced when BMPRII or ActRIIB are co-expressed. (B) [125I]BMP-9 binds to endoglin in the absence of type I or type II receptors.*

The accessory receptor endoglin has been described to form complexes with ALK1 and to promote ALK1 signalling (15, 16). To investigate whether BMP-9 binds to endoglin, we transiently transfected COS-7 cells with cDNAs encoding ALK1, ALK2, BMPRII or endoglin and incubated them with [125I]BMP-9. Fig. 1B shows that BMP-9 binds endoglin and ALK1 in the absence of type I or type II receptors.

BMP-9 binds to ALK1, ALK2, BMPRII, ActRII and endoglin in ECs

We then analysed the ability of BMP-9 to bind to endogenous endoglin, type I and type II receptors in primary ECs. Bovine aortic ECs (BAECs), human umbilical vein ECs (HUVECs) and human dermal microvascular ECs (HDMVECs) were incubated with [125I]BMP-9, and crosslinked receptor-ligand complexes were immunoprecipitated with antisera against endoglin, type II receptors and type-I receptors known to be involved in BMP signalling. BMP-9 strongly bound to ALK1 in all EC types investigated and it weakly bound to ALK2 in BAECs and HDMVECs (Fig. 2A, B and data not shown). In addition, in all EC types, BMP-9 bound to BMPR-II, ActR-IIA and endoglin. In HUVECs, BMP-9 was also found to bind ActRIIB. The observed co-immunoprecipitation of type I with type-II receptors (and vice versa) and of type I and type II receptors with endoglin (and vice versa) indicates that these receptors form heteromeric complexes with each other on the cell surface.

To verify that BMP-9 binding to ALK1 is specific, we competed [125I]BMP-9 binding to ALK1 in HUVECs with excess of unlabelled 'cold' BMP-9, activin, BMP-7 or TGF-β and immunoprecipitated receptor-ligand complexes with ALK1 antiserum (Fig. 2C). Only excess of cold BMP-9 competed with [125I]BMP-9-receptor binding, demonstrating the specificity of the BMP-9-receptor interactions. Moreover, incubation of BAECs with [125I]BMP-6 showed that BMP-6 does not bind to ALK1, but only to ALK2 and BMPRII (data not shown).

BMP-9 can bind ALK1 and ALK2 in non-ECs

We subsequently investigated BMP-9 binding to non-ECs. In C2C12 myoblasts, BMP-9 was found to bind to ALK2 and the type II receptors BMPRII and ActRIIA, but not to ALK1 (Fig. 3A). In XTH-1 breast cancer cells, BMP-9 bound to ALK2 and also to endoglin (Fig. 3B). By contrast, BMP-9 strongly bound to ALK1 and endoglin, and only weakly to ALK2 in T98G glioblastoma cells (Fig. 3C). These results suggest that BMP-9 signalling in non-ECs occurs via ALK1 and/or ALK2 and, thus, provides an explanation for the potent effects of BMP-9 on non-ECs described in the literature.

BMP-9 induces phosphorylation of Smad1 and/or Smad5, and stimulates expression of ID1 protein and endoglin mRNA in ECs

We then went on to investigate whether Smad1 and Smad5, which are downstream in the ALK1 signalling pathway, are activated by BMP-9. BAECs, HUVECs, and HDMVECs were stimulated for 45 minutes with different doses of BMP-9, lysed and subjected to SDS-PAGE and subsequent western blotting.

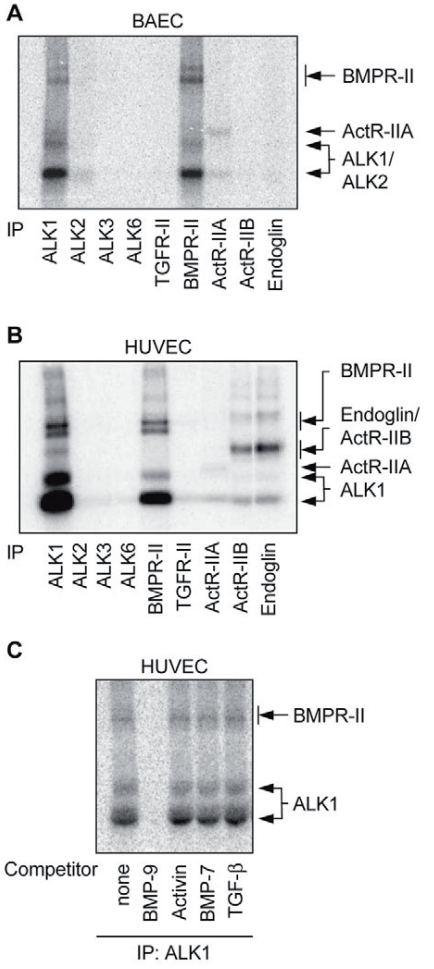

Fig. 2. *BMP-9 binds to endogenous receptors in endothelial cells. (A,B) BAEC (A) or HUVECs (B) were affinity-labelled with [125I]BMP-9 and crosslinked ligand-receptor complexes were immunoprecipitated with specific antisera as indicated. BMP-9 predominantly binds to ALK1 and BMPR-II, but also to ALK2, ActR-IIA, ActR-IIB and endoglin. (C) Competition of [125I]BMP-9 binding to ALK1 in HUVECs with either excess unlabelled (cold) BMP-9, activin, BMP-7 or TGF-β. Only cold BMP-9 can compete with [125I]BMP-9 binding to ALK1.*

Probing of membranes with an antibody that detects phosphorylated Smad1 and Smad5 demonstrated that BMP-9 dose-dependently induces phosphorylation of Smad1 and Smad5 in all cell types investigated (Fig. 4A-C). BMP-6, which is known to activate Smad1 and Smad5, was used as positive control. Equal loading was demonstrated by probing membranes with an anti-β-actin antibody. We also stimulated cells with BMP-6 and BMP-9, and analysed the expression

of *ID1*, described to be a direct target gene of Smad1 and/or Smad5 signalling (25). In HUVECs and HDMVECs, ID1 protein levels increased at the lowest BMP-9 concentration tested (Fig. 4B,C). In BAECs, upregulation of ID1 protein levels was seen at a concentration of 1 ng/ml BMP-9 (Fig. 4A).

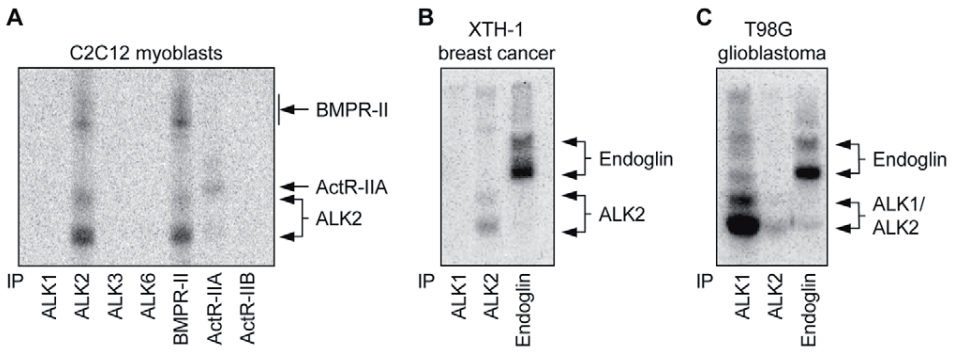

Fig. 3. *BMP-9 binds to endogenous receptors in non-endothelial cells. (A-C) Autoradiography of crosslinked complexes of [125I]BMP-9 with cell surface receptors in C2C12 myoblasts (A), XTH-1 breast cancer cells (B), and T98G glioblastoma cells (C). Ligand-receptor complexes were immunoprecipitated with specific antisera as indicated in the figure and subjected to SDS-PAGE. BMP-9 binds to ALK2 in all cell lines, to ALK1 in glioblastoma cells and to endoglin in breast cancer and glioblastoma cells.*

Endoglin has been shown to be induced by TGF-β (26) and by overexpression of constitutively active ALK1 (27, 28). Using quantitative PCR, we found that, similar to TGF-β, BMP-9 induces endoglin mRNA expression in BAECs (Fig. 4D). This suggests that BMP-9 regulates its own signalling pathway by inducing the expression of its co-receptor endoglin.

BMP-9 activates the Smad1- and Smad5-responsive BRE reporter via ALK1

The ID1-promoter-derived BMP reporter element (BRE)-luciferase reporter construct is a readout system transcriptional responses induced by Smad1 and/or Smad5. BAECs were transiently transfected with the BRE reporter and stimulated with TGF-β, BMP-6 or BMP-9 overnight. BMP-9 and BMP-6 but not TGF-β induced BRE activation (Fig. 5A). We then investigated whether addition of the Fc-ccoupled extracellular domain of ALK1 inhibits BMP-9-induced BRE activation. Incubation of BAECs with ALK1-Fc efficiently blocked BMP-9-induced BRE activity (Fig. 5B).

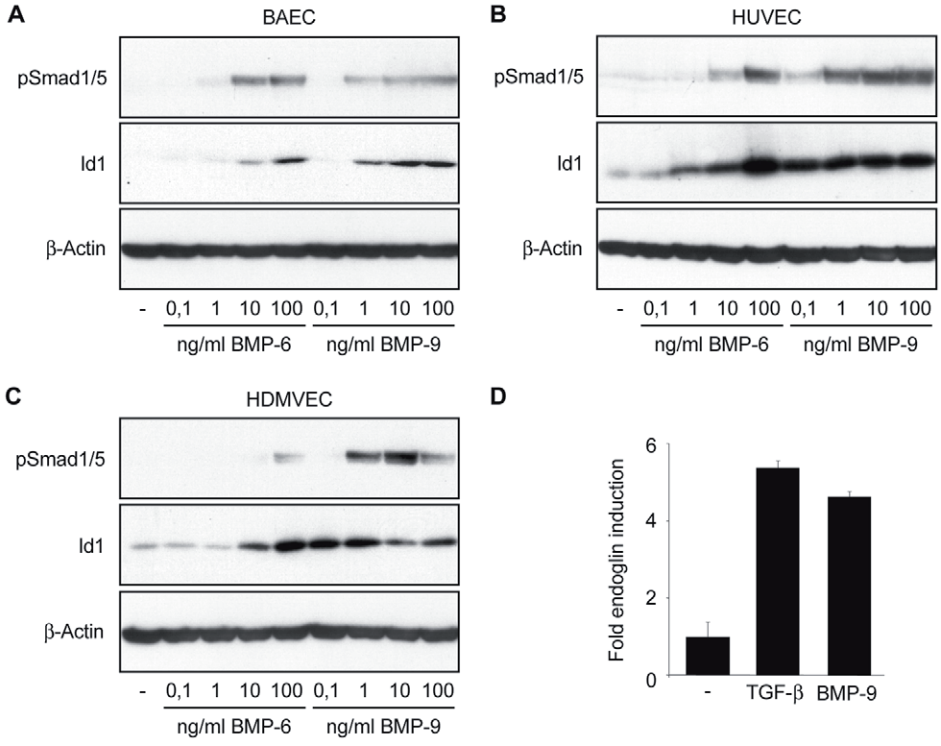

Fig. 4. *BMP-9 activates Smad1 and Smad5, and upregulates ID1 and endoglin in endothelial cells. (A-C) BAECs (A), HUVECs (B) or HDMVECs (C) were incubated with increasing doses of BMP-9 and BMP-6 as indicated in the figure. After 45 minutes, cells were lysed and samples subjected to SDS-PAGE and subsequent western blotting. Membranes were either probed with an antibody that specifically recognises phosphorylated Smad1 and Smad5 or with an antibody directed against ID1. An anti-β-actin antibody was used to confirm equal loading. (D) Effect of BMP-9 and TGF-β on endoglin mRNA expression in BAECs as measured by quantitative real-time PCR.*

To analyse whether BMP-9-mediated BRE activation occurs via ALK1 or ALK2, we designed short hairpin RNA (shRNA) expression constructs targeting ALK1 or ALK2. Transient transfection with ALK1 shRNA but not ALK2 shRNA inhibited BMP-9-induced reporter activity (Fig. 5C). By contrast, BMP-6-stimulated reporter activation was only affected by downregulation of ALK2 but not of ALK1 (Fig. 5D). To control the specificity of the shRNA constructs, we cotransfected BAECs with the ALK1 shRNA construct and with expression vectors for human ALK1 or ALK2. Inhibition of BMP-9-induced BRE activity by ALK1 shRNA was rescued by the ectopic expression of ALK1 but not of ALK2 (Fig. 5E). These experiments show that BMP-9 specifically activates the Smad1- and Smad5-responsive BRE reporter via ALK1 inBAECs.

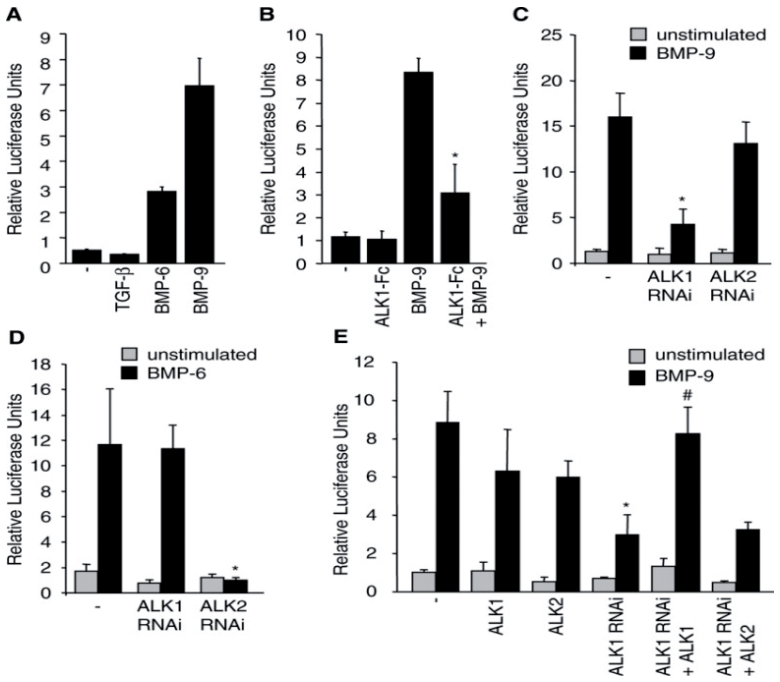

Fig. 5. *BMP-9 activates the Smad1- and Smad5-responsive BRE luciferase reporter through ALK1. (A-B) BAECs were transiently transfected with an ID1-promoter-derived luciferase reporter construct (BRE) and a construct for β-galactosidase as internal expression control. Before stimulation, cells were serum-starved for 8 hours and then incubated with the respective ligands overnight. (A) BRE activity is induced by BMP-9 (100 ng/ml) and BMP-6 (100 ng/ml) but not by TGF-β (5 ng/ml). (B) BMP-9 (5 ng/ml) stimulated BRE-induction can be inhibited by a 15-fold molar excess of ALK1-Fc. (C,D) shRNA constructs against ALK1 or ALK2 were cloned into the pSuper vector and co-transfected into BAECs. ALK1 shRNA blocks BMP-9 stimulated (5 ng/ml) (C), but not BMP-6-induced (25 ng/ml) BRE activation (D). (E) ALK1 shRNA-mediated reduction of BRE activity is rescued by overexpression of human ALK1, but not by human ALK2. *P<0.01 compared with BMP-9 stimulated control; #P<0.01 compared with ALK1 RNAi after BMP-9 stimulation.*

BMP-9 inhibits migration and proliferation of ECs

We next investigated the effect of BMP-9 on EC function. To study the effect of BMP-9 on migration, serum-starved monolayers of BAECs were wounded by scratching and stimulated with the ligands for 24 hours. BMP-9 weakly, albeit significantly, inhibited EC migration at a dose of 10 ng/ml (Fig. 6A; $P<0.01$).

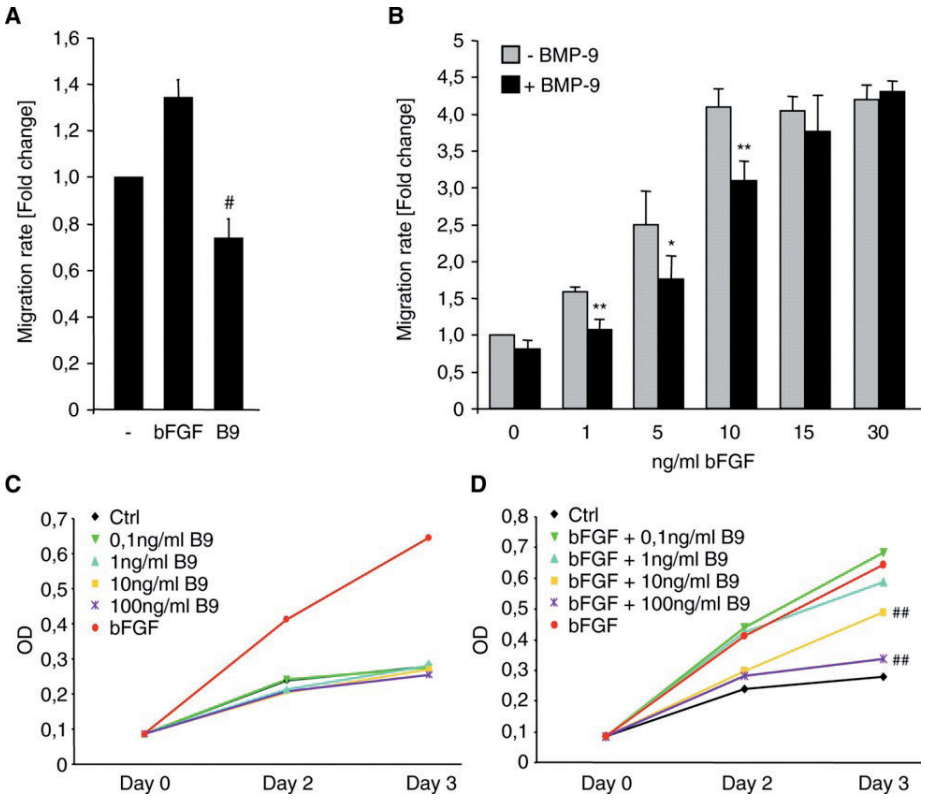

Fig. 6. *BMP-9 attenuates migration and inhibits proliferation of ECs. (A) BAECs were allowed to grow to confluence and serum-starved overnight. Monolayers were wounded and stimulated with either 30 ng/ml bFGF or 10 ng/ml BMP-9. Wound closure was measured after 24 hours using the Olympus Analysis software. (B) Scratched BAEC monolayers were incubated with 10 ng/ml BMP-9 and different concentrations of bFGF as indicated in the figure and cell migration was measured after 24 hours. (C, D) 3000 BAECs were seeded in 96-well plates and stimulated with different concentrations of BMP-9, 30 ng/ml bFGF or combinations of bFGF and BMP-9. Cell proliferation was determined after 2 and 3 days by adding the MTS reagent and measuring the absorbance at 490 nm. B9, BMP-9. ##P<0.001 compared with bFGF treatment; #P<0.01 compared with control; **P< 0.005 and *P<0.05 compared with the respective bFGF treatment without BMP-9*

We also studied the influence of BMP-9 on migration induced by bFGF and found that, in the presence of BMP-9, inhibition of bFGF-stimulated migration decreased with increasing concentrations (0-30 ng/ml) of bFGF (Fig. 6B). Furthermore, we analysed the effect of BMP-9 on cell growth and found that it potently inhibited bFGF-stimulated cell growth when applied at 10 and 100 ng/ml (Fig. 6D). However, BMP-9 alone had no effect on proliferation of BAECs (Fig. 6C).

BMP-9 inhibits vessel formation in vitro

Considering the data obtained from our functional studies and the established role for ALK1 in angiogenesis, we sought to elucidate the effect of BMP-9 on angiogenesis. Metatarsals of 17-day-old mouse embryos were used to study the effect of BMP-9 on EC outgrowth and vessel formation. Culturing of explanted bones leads to formation of a feeder layer of fibroblast-like cells, on which a tubular network of ECs is formed (29). Staining of the EC network using an anti-CD31 antibody showed that BMP-9 inhibits baseline sprouting of ECs and completely abolishes VEGF- induced formation of tube-like structures (Fig. 7). Of note, BMP-9 increased the size of the bone ends, which may reflect the reported stimulatory effect of BMP-9 on chondrogenesis (9). These results show that BMP-9 is a powerful inhibitor of angiogenesis *in vitro*.

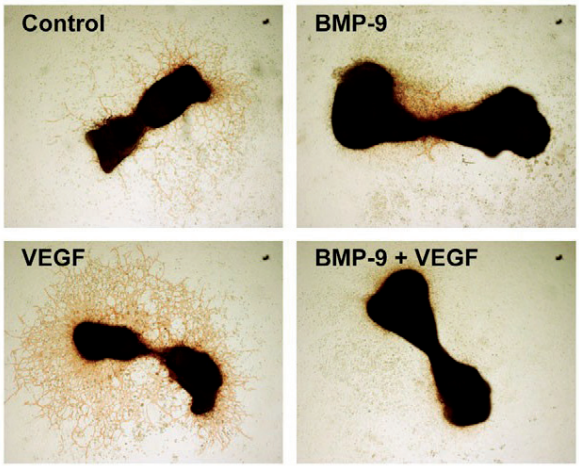

Fig. 7. *BMP-9 blocks endothelial network formation. Metatarsals of 17-day-old mouse foetuses were prepared, transferred to cell culture plates and allowed to adhere for 4 days. Medium was refreshed and bones were stimulated for 7 days with BMP-9 (100 ng/ml), VEGF (50 ng/ml) or both. Cultures were fixed and vessel-like structures were visualised by anti-CD31 staining. BMP-9 inhibits baseline formation of the endothelial network. Incubation with VEGF strongly stimulated the formation of vessel-like structures, which was completely abrogated by co-stimulation with BMP-9. Six bones were stimulated per experimental group and one representative picture of each group is shown.*

Discussion

The functional consequences of *Alk1* gene disruption in mice and loss-of-function mutations in humans have clearly demonstrated that ALK1 is crucial for angiogenesis. (13, 14, 18, 19,). The reminiscent phenotypes of TGF-β1-deficient and ALK1 deficient mice, together with the *in vitro* finding that TGF-β1 signals via ALK1, have suggested that TGF- β1 is the ALK1 ligand *in vivo* (30). In the present study, we demonstrate that BMP-9 is also a functional ligand of ALK1 in ECs. Moreover,

we show that BMP-9 inhibits bFGF-induced proliferation and VEGF- induced angiogenesis *in vitro*, suggesting that BMP-9 also plays a role in ALK1 signalling and vascular morphogenesis *in vivo*.

To study the binding pattern of BMP-9 in ECs, we performed crosslinking studies with 125I-labelled BMP-9 and found that BMP-9 binds to the type I receptors ALK1 and ALK2, and to the type-II receptors BMPRII, ActRIIA and ActRIIB. Compared with ALK2 binding, BMP-9 binding to ALK1 was strong even in the absence of a type II receptor. This finding is consistent with a study, in which BMP-9 was shown to bind with high affinity to immobilised Fc-coupled ALK1 in the cell-free BIAcore assay (5). However, BMP- 9 binding to ALK2 was enhanced when co- expressed with either BMPRII or ActRIIB. The affinity of TGF-β superfamily members for the type I receptor is low or absent, but is strongly increased after heteromeric complex formation with the type II receptor. BMP-9 binding to ALK2 resembles the binding pattern of BMP-6 and BMP-7 that bind only efficiently when both ALK2 and ActRII are present (24, 31). The high affinity of BMP-9 for ALK1 resembles more the binding pattern of BMP-2 and BMP-4 (24, 31). BMP-2 and BMP-4 strongly bind to the type I receptors ALK3 and ALK6, respectively, and show only low affinity for their type II receptor BMPRII.

In primary ECs, we identified ALK1 and BMPRII as the predominant BMP-9 receptors. Moreover, BMP-9 also bound to endoglin even in the absence of type II and type I receptors. So far, BMP-2, BMP-7, activin A, TGF-β1 and TGF-β3 have been described to bind endoglin. However, these molecules can only bind endoglin in the presence of either type I or type II receptors (33, 34). Together with our finding that BMP-9 potently induces endoglin mRNA expression; these results suggest an important role for endoglin in BMP-9 signalling.

BMP-9 has been described to have strong effects on non-ECs (6, 9, 35). We identified the type-I receptor ALK2 and the type II receptor BMPR-II as predominant BMP-9 receptors in myoblasts. In addition, BMP-9 bound to ALK2 and endoglin in XTH-1 breast cancer cells and to ALK1 and endoglin in T98G glioblastoma cells. These results indicate that BMP-9 elicits its broad effects on non-ECs by signalling via ALK2 and/or ALK1. In a recent study, BMP-9 has been shown to signal via ALK1 and the type II receptors BMPRII and ActRIIA in HDMVECs (36). We further extend these findings by showing that BMP-9 binds to these receptors in other EC types as well, that BMP-9 also binds to endoglin, ALK2 and ActRII-B in ECs, and that ALK1 and ALK2 are both receptors for BMP-9 in non-ECs.

We then sought to elucidate whether BMP-9 activates the signalling pathway downstream of ALK1. BMP-9 strongly induced phosphorylation of Smad1 and/or Smad5, and activated the Smad1- and Smad5-responsive BRE luciferase reporter in ECs. BMP-9-induced BRE activation was inhibited by ALK1-Fc. However, we were unable to inhibit BMP-9- stimulated BRE activity with a 500-fold higher concentration of the monomeric extracellular domain of ALK1 (data not shown). As proteins with Fc-fusion are artificially dimerised this suggests that ALK1 dimers are needed for high-affinity binding of BMP-9. Conclusive evidence for a role of ALK1 in BMP-9 signalling was

obtained by shRNA-mediated down-regulation of ALK1 or ALK2 in ECs. Decreased ALK1, but not ALK2 expression abrogated the BMP-9-induced BRE signal. By contrast, BMP-6 stimulated reporter activity was completely blocked by ALK2 shRNA but not by ALK1 shRNA, thereby proving the functionality of the shRNA constructs. Future studies will reveal whether ALK2 plays a role in BMP-9 signalling in ECs. Consistent with the BMP-9- induced activation of the *ID1*-promoter-derived BRE-luciferase reporter, we found an upregulation of ID1 protein after BMP-9 stimulation in all EC types investigated. *ID1* is one of the most important BMP target genes and has also been characterised as ALK1 target gene (27, 28). BMP-9 was also found to activate a Smad3- and Smad4-responsive transcriptional reporter, which could be blocked by ALK1 shRNA (P.t.D., unpublished observation). We are currently analysing the significance of this finding.

We further investigated the effect of BMP-9 on EC function. BMP-9 inhibited EC migration and significantly delayed bFGF-induced migration. Moreover, BMP-9 was found to inhibit proliferation of ECs stimulated with bFGF. Importantly, BMP-9 completely abrogated VEGF-induced formation of tubular EC structures in a bone-explant angiogenesis assay. The mechanism by which BMP-9 negatively interferes with bFGF- and/or VEGF-induced cellular responses remains to be elucidated. It is probable that BMP-9 exerts its anti-angiogenic activities by decreasing the expression of angiogenic factors and/or their receptors.

The observed negative effects of BMP-9 on angiogenesis are in contrast with other studies showing that several other BMP family members promote angiogenesis (37 - 41). BMP-9, however, is the first BMP family member described to signal through ALK1 and this may explain the discrepancy. As mentioned earlier, ALK1 and its accessory receptor endoglin are established as crucial regulators of angiogenesis *in vivo*. However, the question whether ALK1 plays a role in the activation or resolution phase of angiogenesis remains open. *In vitro* data show that expression of constitutive active ALK1 in ECs leads to positive as well as negative effects on EC migration and proliferation (21, 41). The lack of vessel sprouting in ALK1- or endoglin-deficient mice suggests that these receptors play a role in the activation phase of angiogenesis. However, these mice also have dilated, unstable vessels that lack smooth muscle cell coverage, which would point to a role in the resolution phase of angiogenesis. An explanation for all these contradictory findings could be that ALK1 and endoglin are involved in both the activation and in the resolution phase, and that a fine balance of receptor levels, receptor types (TβRII or BMPRII) and ligands (TGF-β or BMP-9) determines the effect on angiogenesis in the respective vascular bed.

Materials and Methods

Cell culture

BAECs were grown in low glucose Dulbecco's modified Eagle's medium (DMEM, Gibco) with 10% foetal calf serum (FCS) and penicillin-streptomycin (PS). HUVECs were cultured in Medium 199 with Earle's salts and L-glutamine (Gibco), 10% FCS, heparin (LEO pharma), bovine pituitary extract (Gibco), and PS. HDMVECs were grown in EGM-2 MV (Clonetics). C2C12 mouse myoblast cells were grown in DMEM, 10% FCS, L-glutamine and PS. XTH-1 breast cancer cells (kind gift from Hartmut Beug, IMP, Vienna, Austria) were cultured in RPMI medium supplemented with 15% FCS, PS and 2.5 ng/ml TGF-β. T98G glioblastoma cells (42) were grown in DMEM with 10% FCS. All cells were cultured at 5% CO2, apart from BAECs which were cultured at 10% CO2.

RNA isolation and quantitative real-time PCR

Total DNA-free cellular RNA was extracted with the RNeasy kit (Macherery-Nagel). The oligonucleotide primers for PCR were designed using the Primer Express Software (Applied Biosystems). Expression of bovine endoglin and bovine hypoxanthine phosphoribosyltransferase (HPRT) were analysed using the following primers: endoglin forward, 5'-tcctcaactgggcgaatacg-3'; endoglin reverse, 5' gatgctttgcggcttgct-3'; HPRT forward, 5'-acgacagcactttgaggcatt-3'; HPRT reverse, 5'-agttaatactaccgaaacctactgaaacac-3'. Taqman PCR reactions were performed using the ABI prism HT7900 sequence-detection system (Applied Biosystems). All samples were plated in triplicate. Gene expression levels were determined with the comparative ΔCt method using *HPRT* as reference and the non-stimulated condition was set to 1.

Expression plasmids and shRNA constructs

Expression constructs for full-length human ALK1 and ALK2 have been cloned into the pcDNA3 vector as described previously (43). Vectors expressing short hairpin RNA (shRNA) targeting ALK1 and ALK2 were made by cloning bovine ALK1- and ALK2-derived constructs and complementary oligonucleotides into the pSuper vector (44). Experiments were performed with different shRNA constructs:

bovineALK1(5'-3') construct1,
gatccccGTGAGAGCGTAGCCGTCAAttcaagagaTTGACGGCTACGCTCTCACttttg gaaa;
bovine ALK1 (5'-3') construct 2
gatccccGACTTATTGTGAC- ATGAAAttcaagagaTTTCATGTCACAATAAGTCttttggaaa;
bovine ALK2 (5'-3') construct 1,
gatccccGATGAGAAGTCGTGGTTTAttcaagagaTAAACCACG- ACTTCTCATCttttggaaa.

The shRNA sequence (uppercase letters) was linked to its reverse complementary antisense shRNA sequence by a short spacer (lowercase letters). The shRNA sequence is flanked at the 3' end by a string of T residues that serve as a transcription termination site. Representative results of bovine ALK1 construct 1 and bovine ALK2 construct 1 were shown.

Recombinant proteins

Recombinant BMP-9 was obtained from Human Genome Sciences. The purity and specific activity of the preparation was accessed as previously described (5). Recombinant BMP-6 and BMP-7 were obtained from K. Sampath (Creative Biomolecules, Inc., Hoptinton, MA) and recombinant activin from Y. Eto (Ajinomoto Co, Kawasaki, Japan). Recombinant TGF-β3 was obtained from K. Iwata (OSI Pharmaceuticals, Inc., Boulder, CO). The VEGF165 isoform and ALK1- Fc were purchased from R&D systems and bFGF from Peprotech.

[125I]BMP-9 binding assay

Iodination of BMP-9 was performed according to the chloramine T method and cells were subsequently affinity-labelled with the radioactive ligand as described before (31, 45). In brief, cells were incubated on ice for 3 hours with the radioactive ligand. After incubation, cells were washed and crosslinking was performed using 54 mM disuccinimidyl suberate (DSS) and 3 mM bis(sulfosuccinimidyl)suberate (BS3, Pierce) for 15 minutes. Cells were washed, scraped and lysed. Lysates were incubated with the respective antisera overnight and immune complexes were precipitated by adding proteinA Sepharose (Amersham). Samples were washed, boiled in SDS sample buffer and subjected to SDS-PAGE. Gels were dried and scanned with the STORM imaging system (Amersham).

Transcriptional reporter assay

BAECs were seeded in 24-well plates and transiently transfected for 4 hours with the BRE-luciferase reporter construct (25) using Lipofectamine reagent (Invitrogen) according to the manufacturer's protocol. Expression vectors for human ALK1 or human ALK2 and shRNA expression constructs for bovine ALK1 or bovine ALK2 were co-transfected where indicated. An expression plasmid for β-galactosidase was co-transfected and used to correct for transfection efficiency. Cells were serum-starved the following the day (or two days after transfection for RNAi experiments) and stimulated overnight with the respective ligands. Cells were washed, lysed and luciferase and β-galactosidase activity were determined. Each transfection was carried out in triplicate and representative experiments are shown.

Western blot analysis

Cells were seeded in six-well plates and allowed to grow to 90% confluence. Cells were washed with PBS and serum-starved for 6 hours (HUVECs, HDMVECs) or overnight (BAECs). Cells were stimulated with different concentrations of BMP-6 and BMP-9 for 45 minutes, washed with PBS and lysed in SDS sample buffer. Samples were boiled for 10 minutes and subjected to SDS-PAGE and western blotting. Smad phosphorylation was detected with an antibody specifically recognising phosphorylated Smad1 and Smad5, which has been described previously (46). The anti-ID1 antibody was from Santa Cruz. Equal loading was shown with an anti-β-actin antibody (Sigma).

Cell growth and migration assay

BAECs were seeded at a density of 3000 cells/well in 96-well plates. The next day, medium was aspirated and replaced by fresh medium containing the respective ligands. Proliferation of cells was determined after 2 and 3 days by adding MTS solution (Promega), and by measuring the absorbance at 490 nm. To determine cell migration, BAECs were seeded in six-well plates and allowed to grow to confluence. After serum starvation, monolayers were wounded with three scratches and medium was replaced by fresh medium containing the ligands. Cell migration was measured in four areas per well directly after wounding and 24 hours later by automated image analysis using the Olympus Analysis software.

Metatarsal angiogenesis assay

Metatarsals from 17-day-old mouse foetuses were dissected as described earlier (47). Six bones per experimental group were transferred to 24-well tissue culture plates containing a.-MEM (Gibco), 10% FCS and PS and allowed to adhere for 4 days. Then, medium was replaced by fresh medium containing the ligands. Cultures were fixed 7 days after stimulation and vessel formation was visualised by anti-CD31 staining (29).

Statistical analysis

All results are expressed as the mean ± s.d. Statistical differences were examined by one-way ANOVA followed by Bonferroni's multiple comparison test. For statistical comparison of two samples, a two-tailed Student's *t*-test was used where applicable. $P<0.05$ was considered as statistically significant.

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Chapter 4

Endoglin is dispensable for vasculogenesis, but required for vascular endothelial growth factor-induced angiogenesis

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Abstract

Endoglin 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 endoglin are associated with the vascular disease known as hereditary hemorrhagic telangiectasia type l (HHTl). Using mouse embryonic stem cells we observed that angiogenic factors, including vascular endothelial growth factor (VEGF), induce vasculogenesis in embryoid bodies even when endoglin deficient cells or cells depleted of endoglin using shRNA are used. However, endoglin 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 endoglin heterozygous mouse embryos showed reduced VEGF-induced vascular network formation. Moreover, shRNA-mediated depletion and pharmacological inhibition of endoglin in human umbilical vein cells (HUVECs) mitigated VEGF-induced angiogenesis. In summary, we demonstrate that endoglin is required for efficient VEGF-induced angiogenesis.

Key words: embryonic stem cell; embryoid body; endoglin; TRC105; angiogenesis; VEGF

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 (E)8.5 as a result of vascular malformations [2], [7]. VEGF-A signalling 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 (CD105) is a transmembrane glycoprotein essential for angiogenesis and vascular development, which is predominantly expressed in vascular endothelial cells [11]. Mice lacking *Endoglin* die at El0.5-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]. Endoglin functions as a co-receptor for transforming growth factor-β (TGF-β) family members, and interacts with their signalling 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]. Endoglin promotes ALK1/Smad1/5 signalling and inhibits ALK5/Smad2/3 signalling [21] - [23]. Endoglin 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 endoglin and ALK1 [24], [25].

In humans, mutations in *endoglin* lead to hereditary haemorrhagic 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 *Vegfr2*$^{+}$ precursor populations are characterized by endoglin expression [30]. In particular, endoglin is expressed during the progression from the *Vegfr2*$^{+}$*Cd45*$^{-}$ to *Vegfr2*$^{-}$*Cd45*$^{+}$ stage, marking the hemangioblast [31]. In endoglin deficient ESCs, the number of hemangioblast precursors were reduced and myelopoiesis and definitive erythropoiesis were severely impaired, suggesting that the regulated expression of endoglin functions to support lineage-specific hematopoietic development from *Vegfr*2^{+} precursors [30], [31]. Additional studies with forced expression of endoglin in ESCs and transcriptional profiling studies on *Eng*$^{+}$*Vegfr2*$^{+}$ from E7.5 embryos further supported an important role for endoglin in hematopoietic development [32], [33].

In the present study, we examined the role of endoglin 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 endoglin, but that VEGF-induced angiogenesis was severely impaired. The effects were dependent on the level of endoglin: heterozygotes exhibited an intermediate phenotype, reminiscent of features in HHT1 patients. These results were validated and consolidated by shRNA-mediated endoglin depletion and pharmacological endoglin inhibition studies in endothelial cells. The impaired VEGF-induced endothelial cell sprouting in the absence of endoglin 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 endoglin impairs organization of vascular structures in 15-day-old embryoid bodies

To elucidate the role of endoglin in blood vessel morphogenesis we examined the effect of endoglin 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 endoglin gene dosage (*Eng*$^{+/+}$, *Eng*$^{+/-}$ or *Eng*$^{-/-}$) obtained after 15 days of differentiation embedded in plastic and sectioned (Fig. lB). 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 endoglin on vascular organization (Fig. 1B).

Endoglin 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 endoglin during endothelial cell differentiation. Distinct gene expression patterns were induced as differentiation proceeded. VEGFR1 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 endoglin gene dosage (Fig. 2C). Taken together, our results show that endoglin is not required for endothelial and mural cell differentiation.

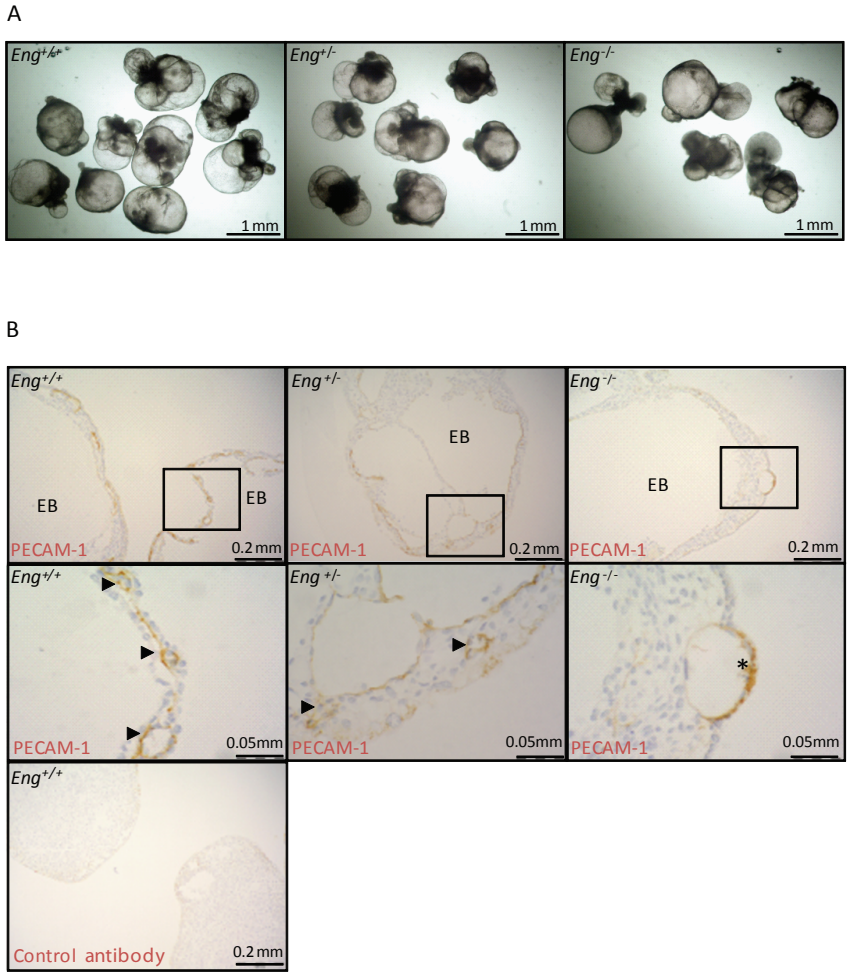

Fig 1. *Impaired vasculature in Eng null mutation ESC-derived 11-day-***old EBs.** *(A) Eng$^{+/-}$ or Eng$^{-/-}$ ESC lines form EBs with no difference when compared to EBs derived from wild type ESCs (B) PECAM-1 whole mount immunohistochemistry of representative wild type, Eng$^{+/-}$, and Eng$^{-/-}$ 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.*

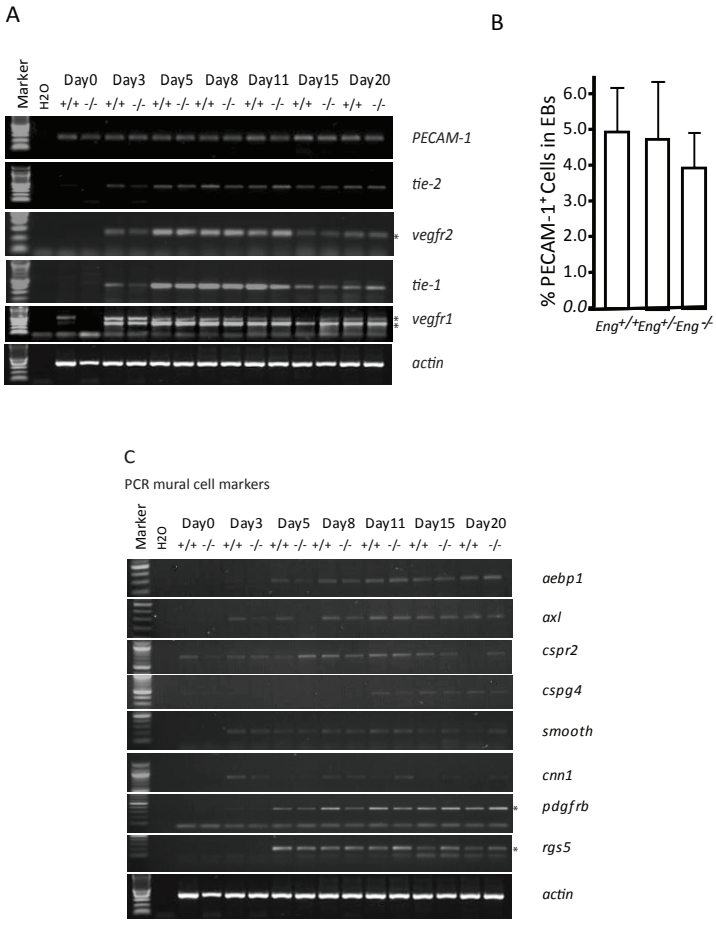

Fig 2. *Expression of endothelial-specific markers during vascular development in EBs. (A) RT-PCR analysis of endothelial cell markers was performed on ESC and EBs cultured for the indicated number of days. Abbreviations: PECAM, platelet endothelial cell adhesion molecule; tie, tyrosine kinase with immunoglobulin-like loop and epidermal growth factor homology domain; VEGF, vascular endothelial growth factor receptor (B) The number of PECAM-1 positive cells was quantified by FACS analysis of the cell suspension of wild type, $Eng^{+/-}$, and $Eng^{-/-}$ ESC-derived 11-day-old EBs. (C) RT-PCR analysis of pericyte-smooth muscle cell markers was performed on ESC and EBs cultured for the indicated number of days. Abbreviations: aebp, adipocyte enhancer binding protein; axl, a receptor tyrosine kinase; cspr, cysteine- and glycine-rich protein; cspg, chondroitin sulfate proteoglycan; cnn, calpoin; pdgf, platelet-derived growth factor, rgs, regulator of G protein signalling.*

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 endoglin 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 observed a reduction in both number of sprouts and sprout length in the *Eng*$^{-/-}$ EBs (Supplementary Fig. 1).

To validate the data obtained with the *Eng*$^{-/-}$ ES cell line we depleted endoglin by shRNAs targeting endoglin in ESCs. Essentially we obtained the same results as for ESCs in which gene dosage was reduced (Fig. 4). Partial knock down of endoglin in ESC in differentiated EBs (Fig. 4B) interfered with efficient VEGF-induced sprouting (Fig. 4D), whereas expression of endothelial markers PECAM-1 and VE-Cadherin was not significantly affected (Fig. 4A and 4C).

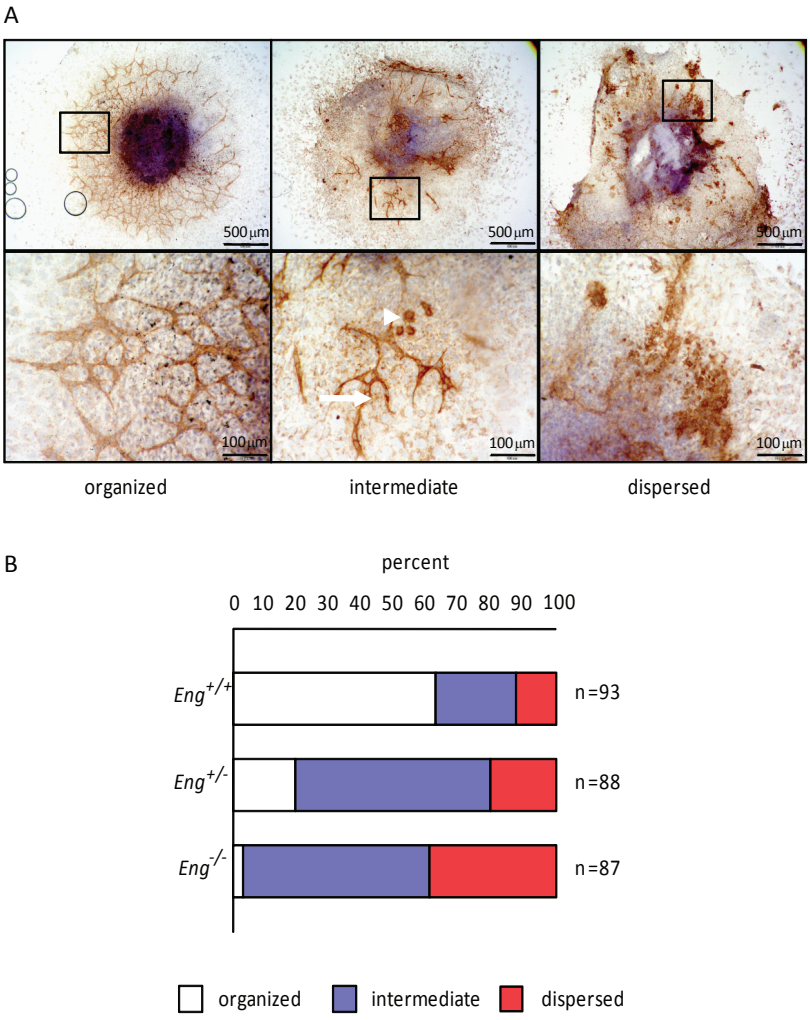

Fig 3. *Eng$^{-/-}$ ESC-derived EBs plated on gelatin coated plates lack organized vessel structures. (A) PECAM-1 immunohistochemical staining of ESC-derived 11-day-old EBs plated on gelatin for 4 days displayed "organized", "intermediate" or "dispersed" phenotype. (B) Quantification of wild type, Eng$^{+/-}$, and Eng$_{-/-}$ ESC-derived 15-day-old EBs vascular phenotypes as they were defined in A.*

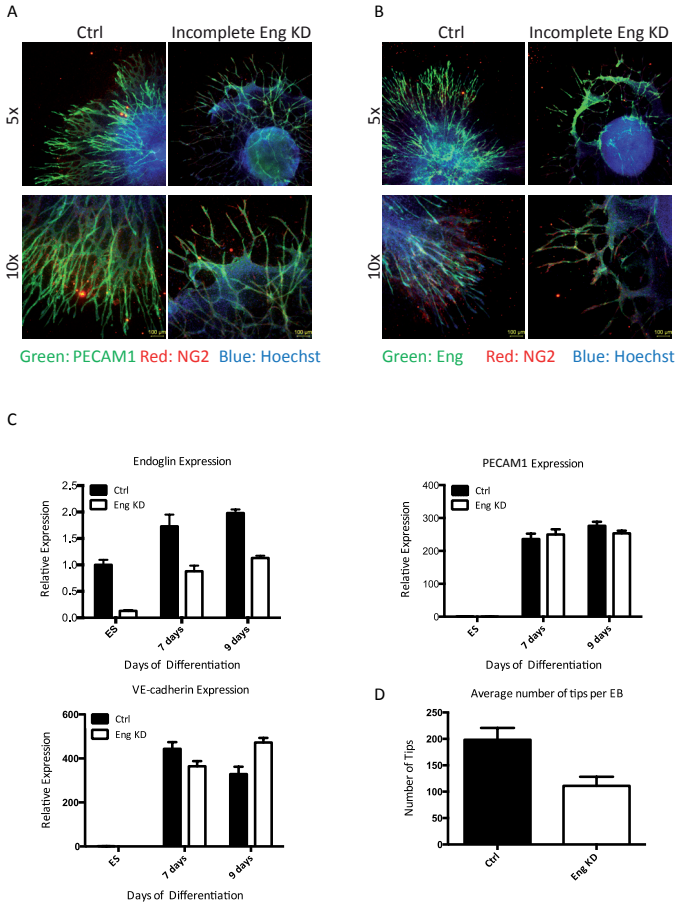

Fig 4. *shRNA-mediated knock down of endoglin inhibits VEGF-induced endothelial cell sprouting of EBs. ES cells were transduced with either scrambled or endoglin targeting shRNA. ES cells formed EBs during 4 days of hanging drop culture before embedding in collagen and stimulation with VEGF (30ng/ml). 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 endoglin 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 endoglin knockdown stained for endoglin (green) and DAPI (blue). Endoglin 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 endoglin mainly in the tips of the outgrowing sprouts. The cellular sheets hardly had endoglin expression. C) qPCR analysis of endoglin, PECAM-1 and VE-cadherin expression during differentiation of the control and endoglin knockdown ES cells. Endoglin expression is reduced by approximately 85% in the ES cells, but during differentiation, at day 7 and 9, expression is restored to half of the normal levels. Expression of PECAM-1 and VE-cadherin did not differ between control and endoglin knockdown EBs at the ES cell state or at day 7 and 9 of differentiation. D) Analysis of number of tips per EB. Endoglin knockdown EBs exhibit significantly less sprouts than the control EBs.*

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 endoglin expression has been associated with activated endothelial cells in tumor stroma [38]. To investigate a possible interplay between VEGF and endoglin 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*$^{+/-}$ metatarsals (Fig. 5). This result suggests that endoglin is required for efficient VEGF-induced angiogenesis.

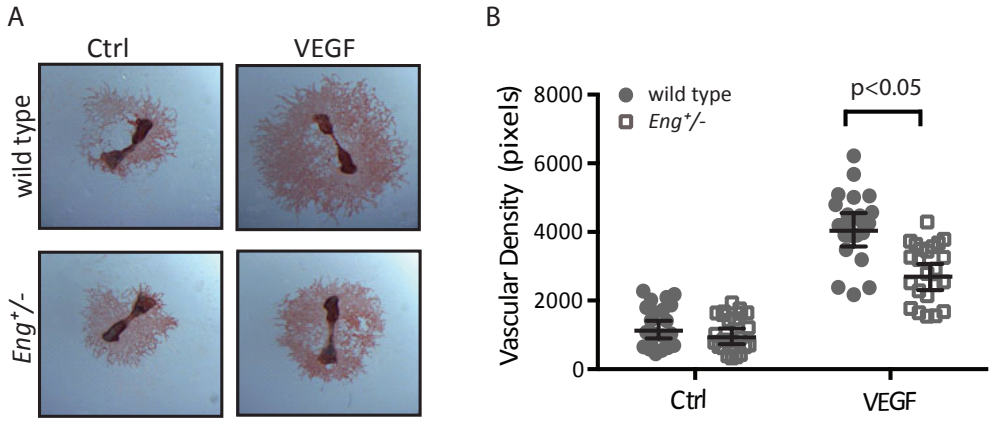

Fig 5. *VEGF-induced angiogenesis is impaired in Eng+/- fetal metatarsal bones. Metatarsals of 17-day-old mouse fetuses were prepared from wild type and Eng+/- mice, transferred to cell-culture plates, allowed to adhere, and then stimulated with VEGF (50 ng/ml). (A) Cultures were fixed and vessel-like 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.*

Inhibition of endoglin expression or function mitigates VEGF-induced sprouting of HUVECs

To elucidate the role of endoglin in VEGF-induced endothelial sprouting, we used a 3D-endothelial cell spheroid-sprouting assay, an established model for studying early *in vitro* angiogenic responses [40]. Non-stimulated spheroids of human umbilical vein endothelial cells (HUVECs) in collagen remain quiescent, and mimic the quiescent endothelial cells in the vessel wall. When stimulated with VEGF, tube-like protrusions emerge from the HUVEC spheroid within one day. We observed that shRNA-mediated depletion of endoglin in HUVECs significantly reduced the VEGF-induced response in this assay (Fig. 6A, B). In addition, treatment of HUVECs with the endoglin neutralizing antibody TRC105 also mitigated this response (Fig. 6C), confirming that inhibition of endoglin function attenuates the VEGF-induced angiogenic response.

Direct VEGF-induced signalling is not affected in endoglin-deficient cells

The data above suggest an involvement of endoglin in the VEGF signalling pathway or crosstalk between endoglin and the VEGF pathway. We therefore further investigated the effect of endoglin on VEGF signalling. The first event in the VEGF signalling cascade is binding of VEGF to its receptor, VEGFR2. However, specific depletion of endoglin in HUVECs using shRNA did not affect VEGF binding to VEGFR2 as measured by affinity crosslinking with radiolabeled VEGF (data not shown). After VEGF-VEGFR2 interaction, VEGFR2 autophosphorylates itself at amino acid 1175, and thereafter initiates activation of the ERK kinase pathways. However, analysis of VEGF-induced VEGFR2, phospho-ERK pathways did not reveal any significant changes upon endoglin knock down (Fig. 6D) These data indicate that endoglin deficiency does not affect VEGF-induced ERK signalling directly.

Discussion

In the present study, we examined the role of endoglin in vasculogenesis and angiogenesis using aggregates of mouse ESCs known as EBs that were challenged with angiogenic supporting 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 endoglin (*Eng*$^{+/-}$ and *Eng*$^{-/-}$, respectively). We found that the endothelial cell differentiation program in ESC-derived EBs is not affected by homozygous deletion of endoglin. 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 endoglin 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].

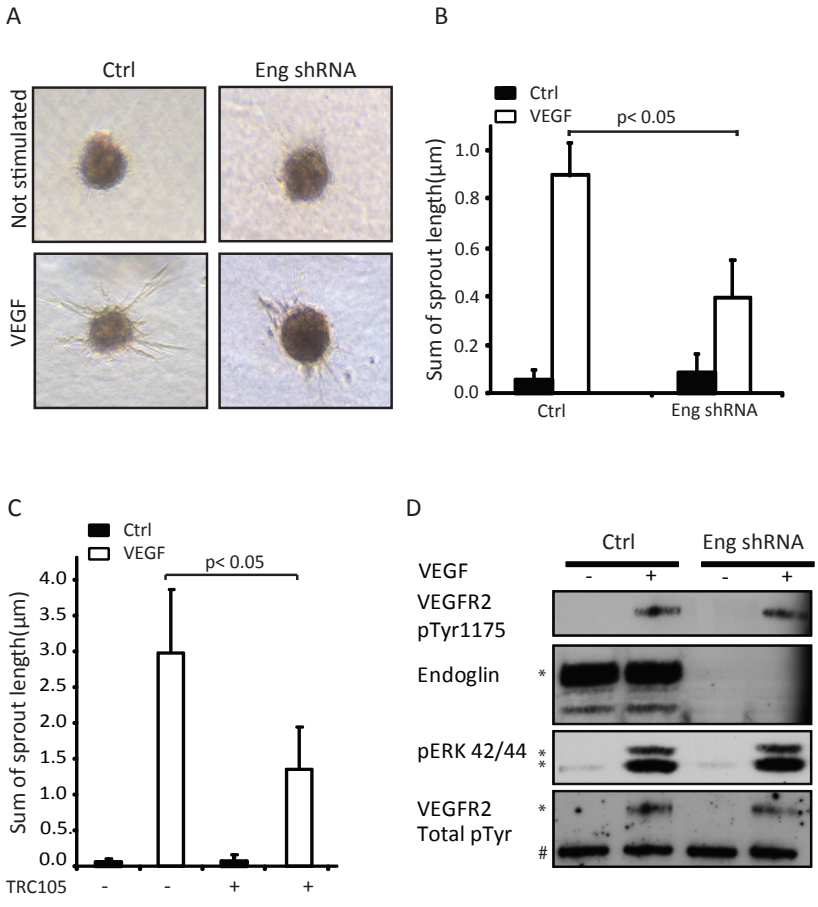

Fig 6 *Endoglin deficiency inhibits VEGF-induced sprouting of HUVEC spheroids. (A) Effect of shRNA-mediated depletion of endoglin on VEGF-induced endothelial cell sprouting. HUVECs were transduced with lentivirus expressing endoglin shRNA overnight. HUVEC spheroids with deficient endoglin expression were embedded in collagen and stimulated with VEGF (50ng/ml). (B) Quantitation of effects seen in (A). A representative experiment is shown. (C) Effect of TRC105 endoglin antibody on VEGF-induced endothelial cell sprouting. HUVEC spheroids were embedded in collagen and stimulated with VEGF (50ng/ml), TRC105 (10µg/ml), or both overnight. As control antibody for experiments using TRC105, the Fc domain (MOPC-21) from Bio Express, West Lebanon, NH, was used. Pictures were taken by phase-contrast microscopy. Quantitative analysis of the mean total sprout length was performed on 10 spheroids per experimental group. P≤0.05. (D) VEGF-induced VEGFR2 phosphorylation at site 1175 and extracellular regulated kinase (ERK) mitogen activated protein (MAP) kinase phosphorylation was examined in shRNA-mediated endoglin knockdown cells. Two bands were detected with the phosphor ERK MAPK antibodies with a molecular weight of 44 and 42 kDa; they represent ERK1 and ERK2 isoforms, respectively. Number sign (#) represents a background band, indicating the even loading for the experiment. Asterisks indicate the protein bands with expected size.*

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 endoglin 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 endoglin depletion at an early stage, before any long term adaptation responses occur. Thus, we conclude that endoglin is responsible for the lack of VEGF-induced endothelial vascular organization.

We observed interdependence for endoglin in VEGF-induced angiogenic responses. Genetic depletion of endoglin from endothelial cells and pharmacological inhibition using TRC105 endoglin antibody severely affected VEGF-induced endothelial cell sprouting. These results are in line with previous studies, which demonstrated that endoglin 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 endoglin has been shown to inhibit tumor angiogenesis [51], [52], and elevated placental expression of endoglin results in high serum levels of soluble endoglin that contribute to vascular dysfunction in pre-eclampsia [53].

Remarkably, mouse embryonic endothelial cells (MEECs) isolated from *Eng*$^{-/-}$ 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 endoglin expression *in vivo* [21], [54-56].

Analysis of yolk sac vasculature in endoglin 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-β l was reduced in the smooth muscle cells although TGF-β l mRNA levels in the adjacent endothelial cells were unaffected [55]. 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 endoglin 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 signalling by interfering with endoglin function.

Materials and Methods

Cell culture

HUVECs

Human umbilical vein endothelial cells (HUVECs) cells 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% CO_2. HUVECs were used up to passage 4. Experiments were confirmed with HUVECs from different donors.

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 endoglin 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 MEFI and MERl amplify normal Exon 1 (300 bp) and primers MEFR1 and MEZR 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-Mercaptoethanol, lx non-essential amino acids and 1000 U/ml recombinant Leukemia Inhibitory Factor (LIF).

Lentiviral transduction

HUVECs were infected with lentivirus encoding an shRNA sequence against human endoglin (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 endoglin (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 endoglin 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]. Briefly, 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 20µl 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 50ng/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-o1d 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% CO_2 atmosphere [30], [42]. For further analysis of sprouting vessels, the 35 mm gel dish was inverted over a 50 mm x 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. The 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 MEC13.3, BD Biosciences).

Method 2: Collagen solution was made as following: Purecol (Advanced Biomatrix, 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 350µl 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 30ng/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 endoglin, FLK-1 and VE-cadherin. 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.

Target	Forward primer	Reverse primer
Endoglin	GGTCATGACTCTGGCACTCA	AGGCGCTACTCAGGACAAGA
PECAM-1	ACCAAGGCGACTATGTTTGC	GGGCAAGTCACTTCAATGGT
VE-cadherin	ATTGAGACAGACCCCAAACG	TGTTTTTGCCTGAAGTGCTG
GAPDH	AACTTTGGCATTGTGGAAG	ACACATTGGGGGTAGGAACA

Immunofluorescence staining

For cryosections, ESC-derived l5-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 l hour. The slides were then incubated with rat anti-mouse PECAM-1 (Clone MEC14.7, 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) at RT. The slides were then washed four times in PBS and mounted in Mowiol before confocal laser microscope analysis. Slides containing EBs cultured in 3D-collagen gel and zinc fixed were permeabilized for 15 minutes with 0.2% Triton X-100 in PBS, followed by blocking with TNB blocking solution for 1 hour at RT, as described above. EBs were stained with rat anti-mouse PECAM-1 (Clone MEC13.3, BD Biosciences) and mouse anti-smooth muscle cell actin (Clone 1A4, Sigma) simultaneously o/n at 4°C. The slides were then washed in TBS and incubated 1 hour with donkey anti-rat FITC (Jackson ImmunoResearch Laboratories) and goat anti-mouse (Jackson IrnmunoResearch Laboratories) secondary antibodies diluted in TNB.

The slides were then washed four times in TBS and mounted in Mowiol before confocal laser microscope analysis. EBs matured in 3D according to *method 2* were excised from collagen. The EBs were blocked in blocking solution (Tris-Buffered Saline Tween (TBST) with 3% BSA) for 2 hours or overnight at room temperature. Staining with primary and secondary antibodies was done overnight. The EBs were stained with Hoechst to visualize the nuclei. The following antibodies were used: rat anti-mouse PECAM-1 (BD Pharmingen) and rat anti-mouse endoglin (CD105 MJ7/18, BD Pharmingen). Secondary antibodies: donkey anti-rat Alexa 488 (Invitrogen) and goat anti-rabbit Alexa 594 (Invitrogen). EBs were stored at 4^{o}C in PBS until analysis with fluorescence microscopy. Endothelial cell sprouting from the EBs was quantified by measuring the length of the tubes.

Flow Cytometric analysis

To obtain single cell suspensions for FACS analysis, 15-day-old EBs were collected from agar coated-dishes and washed twice with PBS before being incubated for 30 minutes in a dissociation solution containing 0.2% collagenase B (Roche Diagnostics). EBs were gently flushed every 5 minutes using one ml tip. After centrifugation, the cell pellet was washed twice with 2% FBS in PBS and then incubated for one hour at 4°C with a FITC conjugated anti-mouse PECAM-1 before FACS analysis.

Western blot analysis

Cells 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-VEGFRII, phospho-ERK antibodies were purchased from Cell signalling Technology. Endoglin was analyzed with an antiserum recognizing human endoglin [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 endoglin 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. All animal experiments were approved by the local animal ethics committee

Statistics

All results are expressed as the mean ± s.d. Statistical differences were examined by two-tailed Student's t-test and $P \leq 0.05$ was considered to be statistically significant (in the figures, $^{*}P \leq 0.05$ and $^{**}P \leq 0.01$).

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Chapter 5

Matrix Metalloproteinase-14 (MT1-MMP)-mediated endoglin shedding inhibits tumour angiogenesis

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Abstract

Endoglin is a transforming growth factor-β coreceptor with a crucial role in angiogenesis. A soluble form of endoglin is present in the circulation, but the role of soluble endoglin (sEndoglin) is poorly understood. In addition, the endoglin shedding mechanism is not known. Therefore, we examined the role of sEndoglin in tumor angiogenesis and the mechanism by which the extracellular domain of endoglin is released from the membrane. In colorectal cancer specimens, we observed high endothelial endoglin protein expression, accompanied with slightly lower sEndoglin levels in the circulation, compared with healthy controls. *In vitro* analysis using endothelial sprouting assays revealed that sEndoglin reduced spontaneous and vascular endothelial growth factor–induced endothelial sprouting. Human umbilical vascular endothelial cells were found to secrete high levels of sEndoglin. Endoglin shedding was inhibited by matrix metalloproteinase (MMP) inhibitors and MMP-14 short hairpin RNA, indicating MMP-14 as the major endoglin shedding protease. Coexpression of endoglin and membrane-bound MMP-14 led to a strong increase in sEndoglin levels. Endoglin shedding required a direct interaction between endoglin and membrane-localized MMP-14. Using cleavage site mutants, we determined that MMP-14 cleaved endoglin at a site in close proximity to the transmembrane domain. Taken together, this study shows that MMP-14 mediates endoglin shedding, which may regulate the angiogenic potential of endothelial cells in the (colorectal) tumor microenvironment.

***Keywords*:** MMP14, Angiogenesis, sEndoglin, MMP inhibitors, VEGF

Introduction

Endoglin (CD105) is a 180-kDa integral membrane-bound glycoprotein, which serves as a high-affinity coreceptor for transforming growth factor (TGF)-β1 and TGF-β3, in the presence of the TGF-β type II receptor (1, 2). Mutations in the gene encoding endoglin lead to hereditary haemorrhagic telangiectasia type I, a multisystemic vascular disease characterized by bleeding from small vascular lesions in the mucocutaneous tissues and the presence of arteriovenous malformations (3, 4). Endoglin is highly expressed by activated endothelial cells, in which it has a crucial role in angiogenesis, shown by the fact that endoglin knockout animals die in utero because of defects in the vascular system (5). Expression of endoglin can be induced by hypoxia (3, 6), TGF-β1 (1, 7, 8), and/or TGF-β3 (9). Endoglin expression was shown to be upregulated in various cancers (1) and correlated with the development of metastatic disease in colorectal cancer (CRC; ref. 6). Together, these data indicate a crucial role for endoglin in tumor angiogenesis. Besides membrane-bound endoglin, in the circulation, a soluble form (sEndoglin) exists (3). Elevated levels of sEndoglin have been reported in pregnant women suffering from preeclampsia (10, 11) and colorectal and breast cancer patients (12–14). However, the studies on sEndoglin in cancer are few and not conclusive.

Receptor shedding is important in regulating cellular homeostasis by influencing cytokine and growth factor signalling. Ectodomain shedding of receptors is mainly regulated through the proteolytic cleavage of the extracellular part of the receptor. The TGF-β type I receptor (TβRI) is released through tumor necrosis converting enzyme (TACE)–mediated cleavage, which decreases cell surface localized receptors and therefore inhibits TGF-β signalling (15). The TGF-β coreceptor betaglycan is released through proteolytic cleavage by membrane-type 1 matrix metalloproteinase (MMP-14). Soluble betaglycan can compete with membrane-bound betaglycan for TGF-β binding and thereby inhibit TGF-β effects on cells (16). sEndoglin has been shown to contribute to endothelial cell dysfunction (11, 17), but the mechanism of shedding and the consecutive effects of sEndoglin on tumor angiogenesis remain to be identified.

The aim of this study was to analyze the role of sEndoglin in regulating tumor angiogenesis and to identify the mechanism by which the extracellular part of endoglin is cleaved from cells. First, endoglin protein expression was determined in CRC tissue and sEndoglin levels in the circulation of CRC patients. Next, we evaluated the effect of sEndoglin on angiogenesis and the endoglin shedding mechanism in endothelial cells. The data revealed that MMP-14 sheds endoglin at a cleavage site close to the transmembrane domain.

Results

Endoglin expression and sEndoglin levels in CRC patients

We determined the expression and cellular localization of endoglin in CRC specimens, corresponding normal mucosa, and premalignant adenomatous polyps using immunohistochemistry. In normal colonic tissue, endoglin protein expression was low and only present in submucosal endothelial cells (n = 7; Fig. 1, left). In contrast, in tumors, strongly increased endoglin protein expression in angiogenic endothelial cells was observed, confirmed by staining for the endothelial marker CD31 and vimentin, and absence of pan-cytokeratin staining (n = 7; Fig. 1, right). ELISA analysis (Supplementary Fig. S1A) revealed that endoglin levels were significantly increased in carcinoma as compared with normal mucosa, (5.4 ng/mg protein versus 2.7 ng/mg protein, respectively; n = 191; $P < 0.0001$) or premalignant adenomas (2.5 ng/mg protein; n = 82).

Next, we determined plasma sEndoglin levels in healthy volunteers and CRC patients pre-operation and 3 months post-operation. Mean preoperative sEndoglin levels in CRC patients were slightly but significantly lower (mean, 4.9 ng/ mL; n = 48) compared with healthy controls (mean, 7.6 ng/ mL; n = 96) and increased postoperatively (mean, 5.2 ng/mL; n = 25; Supplementary Fig. S1B). In this cohort of CRC patients, sEndoglin levels were not related to the Dukes stage of the tumor (Supplementary Fig. S1C). Together, these data indicate that endoglin expression is not increased in benign adenomas, but strongly increased in CRC with slightly lower sEndoglin levels in the circulation of these patients.

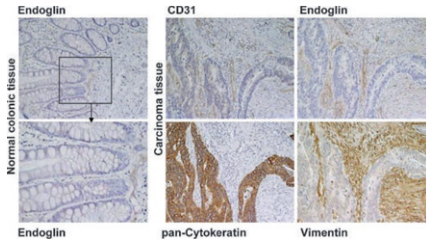

Fig. *1. Endoglin expression in CRC. Localization of endoglin in normal colonic tissue (left) and in CRC (right). Staining was performed for endoglin, the pan-endothelial marker CD31, pan-cytokeratin (epithelial marker), and vimentin (mesenchymal marker) on sequential sections. Magnification ×200; bottom left, ×400.*

sEndoglin inhibits angiogenesis

Endoglin plays an important role in endothelial cell function and angiogenesis. Therefore, we evaluated the role of sEndoglin in angiogenesis. HUVEC cells were subjected to cord formation assays on Matrigel and three-dimensional endothelial sprouting assays. HUVEC cells form tubes on Matrigel, which was efficiently inhibited (~65%) by the addition of 1 µg/mL recombinant endoglin-Fc chimera (Eng-Fc), but not by control Fc protein (Fig. 2A). In addition, endoglin-Fc also inhibited VEGF-induced endothelial sprouts in three-dimensional collagen matrices (~55% inhibition; Supplementary Fig. S2). To further investigate the inhibiting role of sEndoglin in angiogenesis, we used ECRF (immortalized HUVEC endothelial cells) and HUVEC cells, which were transduced with lentiviral vectors expressing endoglin-Fc or Fc only as a control. Expression of the Fc constructs was confirmed by Western blot analysis (anti-Fc and anti-endoglin antibodies; data not shown) and did not influence the proliferation of the cells (Supplementary Fig. S3A and B). Spheroids of ECRF cells formed spontaneous sprouts once embedded in collagen, which were left unaffected by the lentiviral-induced expression of the Fc protein. In contrast, sprouting was reduced ~50% when cells were transduced with an endoglin-Fc construct (Fig. 2B).

Furthermore, endoglin-Fc also efficiently inhibited VEGF-induced and basal sprouting of HUVEC spheroids (Fig. 2C). Together, these data indicate that sEndoglin reduces spontaneous sprout formation in ECRF cells, cord formation in HUVECs, and VEGF-induced sprouting of HUVEC cells. To confirm these data, we used a metatarsal assay. This model provides a combination of a quantitative *in vitro* assay combined with the complexity of a multicellular system. Metatarsals from mice were transduced with Fc- or endoglin-Fc expressing adenoviruses and capillary outgrowth was determined. The data revealed that endoglin-Fc significantly reduced VEGF- induced capillary outgrowth from fetal bones compared with Fc or non-infected cells (Fig. 2D).

Identification of MMP-14 as the endothelial endoglin shedding protease

MMPs have been identified as important regulators of tumor invasion and metastasis, by influencing extracellular matrix remodeling, growth factor activation, and receptor shedding. To analyze the potential role of MMPs in regulating endoglin shedding, we used HUVEC endothelial cells that secrete high levels of sEndoglin into the medium (~1 ng/mL, ELISA). Western blot analysis revealed that the sEndoglin cleavage product has a similar size as the recombinant endoglin extracellular domain (Fig. 3A), which implies a cleavage site close to the transmembrane domain. To investigate the role of proteases in mediating endoglin shedding, HUVEC cells were treated with various protease inhibitors and sEndoglin levels were determined by ELISA.

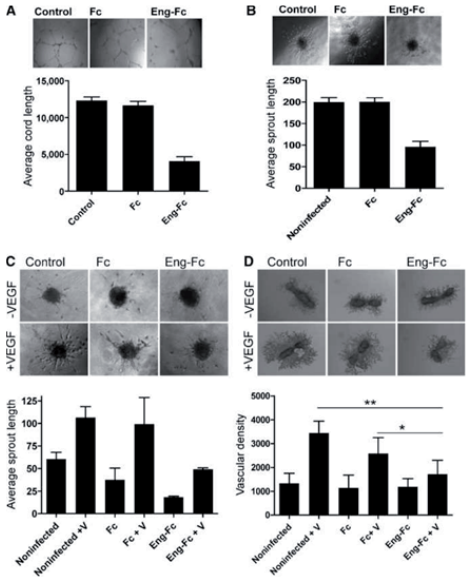

Fig.2 *Effect of sEndoglin on the angiogenic potential of endothelial cells. A, HUVEC cord formation assay. The average cord length in the presence of endoglin-Fc (Eng-Fc) or a control Fc protein (both 1 µg/mL) was analyzed by phase-contrast microscopy. Columns, mean from a representative experiment; bars, SD. B and C, ECRF (B) and HUVEC (C) endothelial sprouting assay with endoglin-Fc or Fc-expressing cells (+V, VEGF stimulated). Data represent mean of at least three independent experiment performed in quadruplicate. D, metatarsal assay. Endoglin-Fc expression reduces vascular density compared with Fc (*, P = 0.047) and control (**, P = 0.0005).*

The broad-spectrum MMP inhibitors GM6001 and Marima-stat strongly inhibited sEndoglin levels in contrast to inhibitors of cystein proteases (E64) and serine proteases (Aprotinin), including cathepsins and plasmin, which had no inhibitory effect. Inhibition of sEndoglin release was 50% by both GM6001 and Marimastat (Fig. 3B). Specific inhibitors of gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3), had only a mild effect on sEndoglin release, whereas a collagenase inhibitor (MMP-13) did not affect sEndoglin levels at all. This indicated that these classes of MMPs were probably not involved in the shedding process and suggested membrane-type MMPs to be the primary endoglin shedding candidates because they are inhibited by these broad-spectrum inhibitors (29).

Therefore, we analyzed expression of MT-MMPs in HUVEC and ECRF cells by real-time PCR, which revealed that MMP- 14 is the most abundantly expressed MT-MMP in endothelial cells (Fig. 3C). MMP-15, MMP-16, MMP-17, and MMP-24 are also expressed but to a lower extent, whereas MMP-25 was undetectable. MMP-14 plays an important role in cancer progression by influencing angiogenesis, invasion, and receptor shedding (30). Therefore, we analyzed the tissue distribution of MMP-14 in relation to endoglin in CRC cancer (n = 7). MMP-14 expression was observed in malignant epithelial cells and endothelial cells. Interestingly, staining of sequential sections for MMP-14 and endoglin revealed that strong MMP-14 staining was accompanied by lower or absent endoglin staining (Fig. 3D, top). Accordingly, we observed that strong endoglin staining was accompanied by low or absent MMP-14 staining (Fig. 3D, bottom). These data indicate MMP-14 as the primary candidate to mediate endothelial endoglin shedding and was therefore further analyzed.

MMP-14 knockdown strongly reduces endoglin shedding

To confirm the role of MMP-14 on endoglin shedding, we performed shRNA-mediated knockdown experiments. MMP-14 shRNA efficiently reduced MMP-14 expression in HUVEC and ECRF cells (Fig. 4A). Levels of sEndoglin were reduced up to 75% in the conditioned media of HUVEC cells (constructs #2, #3, and #4; Fig. 4B). A milder MMP-14 knockdown (construct #1) resulted in a minor decrease of sEndoglin, indicating that MMP-14–mediated endoglin shedding is very efficient and low levels of MMP-14 are already sufficient to mediate endoglin shedding. In ECRF cells, sEndoglin levels were reduced by 60% to 75% (Fig. 4C). Interestingly, a reduction of MMP-14 and sEndoglin was accompanied by a higher membrane–bound endoglin expression (Fig. 4D). These data Indicate that in endothelial cells, MMP-14 is the main endoglin shedding protease.

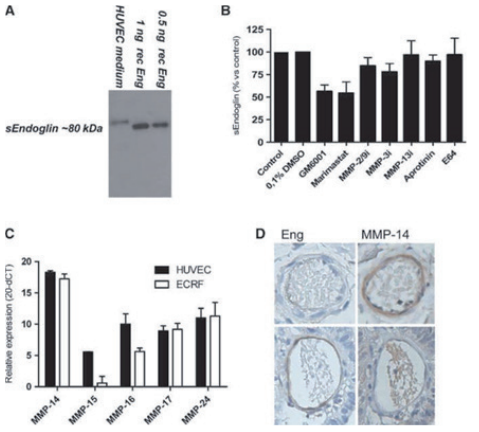

Fig.3 *MMP-14–mediated endoglin shedding in endothelial cells. A, Western blot analysis HUVEC conditioned medium. A band at similar height as recombinant human endoglin extracellular domain (Rec Eng) was observed. B, sEndoglin levels in HUVEC conditioned medium after additional protease inhibitors. GM6001 and Marimastat: broad-spectrum MMP inhibitors, Aprotinin and E64, and serine and cystein protease inhibitors, respectively. % versus appropriate control, n = 4 to 6 independent experiments performed in triplicate; columns, mean; bars, SEM. C, real-time quantitative PCR for membrane-type MMPs in HUVEC and ECRF cells. Columns, mean from representative experiment performed in duplicate; bars, SD. E, Endoglin (Eng; left) and MMP-14 (right) staining on sequential colorectal carcinoma sections. Magnification, ×400.*

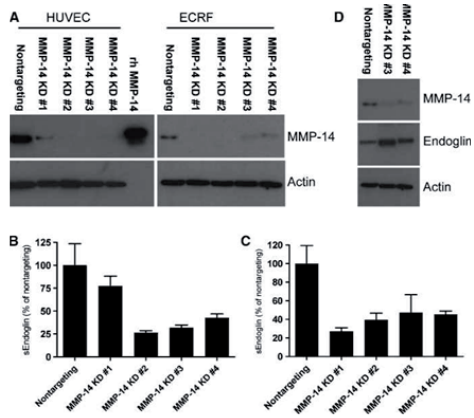

Fig. *4 MMP-14 knockdown in endothelial cells. A, MMP-14 knockdown efficiency in HUVEC and ECRF cells analyzed by Western blot. Rh MMP-14, recombinant human MMP-14. sEndoglin levels in HUVEC (B) or ECRF (C) conditioned medium after MMP-14 knockdown. D, Western blot analysis of ECRF cells revealing increased membrane localized endoglin upon MMP-14 knockdown. All experiments were performed at least thrice in triplicate. Data shown are from a representative experiment.*

Membrane localization of MMP-14 is required for efficient endoglin shedding

To examine the endoglin shedding mechanism, we used COS-7 cells transiently transfected with endoglin and MMP-14 expression plasmids. After O/N starvation, sEndoglin levels were determined in the conditioned media. Co-expression of endoglin with mem brane localized MMP-14 led to increased sEndoglin levels in the medium. This increase was not observed with the MMP-14 mutant lacking the transmembrane domain MMP-14ΔTM (Fig. 5A). Accordingly, treatment with recombinant soluble MMP-14 did not increase sEndoglin levels (data not shown). The increase in endoglin shedding could be inhibited by the addition of the MMP inhibitor GM6001, but not by the addition of the serine protease inhibitor aprotinin. Finally, experiments showed that co-transfection with MMP-13, which is activated by MMP-14, did not increase endoglin shedding (data not shown).

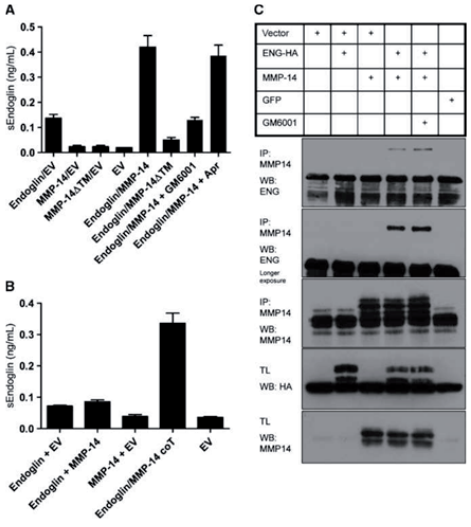

Fig .5 *Endoglin and MMP-14 transfection in COS cells. A, COS cells were transfected with endoglin, and MMP-14 expression plasmids and sEndoglin levels were determined in the medium. EV, empty vector (pcDNA3.1); MMP-14 ΔTM, MMP-14 lacking the transmembrane domain; GM6001, broad-spectrum MMP inhibitor; Apr, aprotinin. B, COS cells were transfected with endoglin, MMP-14, or cotransfected (coT) expression plasmids and were reseeded. sEndoglin levels were determined. Columns, mean of three independent experiments performed in triplicate; bars, SEM. C, COS cells were cotransfected with endoglin and MMP-14. Next, immunoprecipitation of MMP-14 followed by endoglin Western blotting (WB) was performed. IP, immunoprecipitate; TL, total lysates before immunoprecipitation; HA, hemaglutinin tag. Green fluorescent protein was used as a transfection control.*

Next, we studied if the expression of endoglin and MMP-14 on the same cell is required for efficient shedding. Cells were transfected with endoglin or MMP-14 plasmids or co-transfected with both. After reseeding, the cells in a 1:1 ratio sEndoglin levels were determined in the medium. Figure 5B shows that only cotransfection of endoglin and MMP-14 leads to endoglin shedding, indicating that expression of endoglin and MMP-14 on the same cell is required. To further evaluate if endoglin directly interacts with MMP-14, we performed coimmunoprecipitation experiments. Lysates from endoglin and MMP-14 co-transfected cells were immunoprecipitated with MMP-14 antibodies and subjected to Western blot analysis for endoglin. Figure 5C shows that endoglin coprecipitates with MMP-14 and that addition of GM6001 increased interaction between these proteins. Together, these data revealed that membrane localization of MMP-14 is required for efficient endoglin shedding and that the shedding process involves a direct interaction between endoglin and MMP-14.

Determination of the MMP-14 cleavage site in endoglin

Because we observed that MMP-14 cleaves endoglin, releasing a fragment close to the size of the complete extracellular domain, we evaluated potential cleavage sites in proximity to the transmembrane domain. Analysis of 79 MMP-14 substrates with known cleavage site revealed that the majority of the cleavage sites have a flanking G-L amino acid sequence (Supplementary Fig. S4A and B). The endoglin sequence contains four G-L sequences located in different domains of the protein (Fig. 6A). One of these cleavage sites at position 586 to 587 is in close proximity to the transmembrane domain and would therefore lead to a cleavage product consisting of the complete extracellular domain. Therefore, we hypothesized that MMP-14 cleaves endoglin at position 586-587.

To investigate this possibility, we mutated amino acids 586 and 587 using site-directed mutagenesis. Four mutants were created, based on non-occurring amino acids in the natural MMP-14 substrates and keeping as much as possible the amino acid characteristics intact. The wild-type (WT) endoglin G-L sequence was mutated to V-P, V-H, H-P, and H-H. WT and mutant plasmids were cotransfected with MMP-14 in COS cells and sEndoglin levels in the medium were determined after O/N starvation. Mutation of these amino acids did not change the membrane localization of endoglin, as confirmed by FACS analysis (data not shown). Decreased sEndoglin levels were observed especially for the V-H, H-P, and H-H mutants and to a lesser extent with the V-P mutant (Fig. 6B). Western blot analysis confirmed the decrease in 80 kDa sEndoglin in the medium (data not shown). Taken together, these data indicate that MMP-14 cleaves endoglin at the G-L bond at position 586 to 587, releasing nearly full-length endoglin extracellular domain into the circulation.

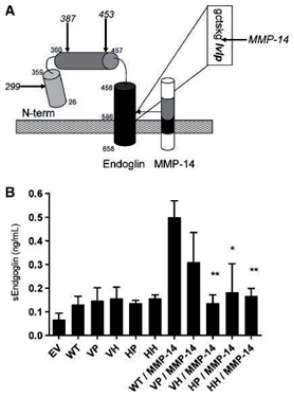

Fig. *.6 Mutation of MMP-14 cleavage site in endoglin. A, endoglin contains four potential cleavage site located in the extracellular domain at positions 299, 387, 453, and 586. B, the proposed MMP-14 cleavage site was mutated using site-directed mutagenesis and sEndoglin levels were determined in the conditioned media. EV, empty vector transfected cells; WT, WT endoglin. Columns, mean of representative experiment from three independent experiments; bars, SD. *, P = 0.02 versus WT; **, P < 0.002 versus WT.*

Discussion

In this study, we identify MMP-14 as the main endoglin shedding protease. We reveal that coexpression of endoglin with MMP-14 on the cell membrane leads to the cleavage of endoglin at the G-L bond at position 586, releasing the nearly complete endoglin extracellular domain. In addition we show that sEndoglin has antiangiogenic properties; it is capable of reducing spontaneous and VEGF- induced angiogenesis.

Our work shows that MMP-14 is the most abundantly expressed MT-MMP in endothelial cells and that knockdown of MMP-14 strongly reduces sEndoglin levels in the conditioned media of these cells. This process is very efficient be- cause milder shRNA-mediated knockdown of MMP-14 still results in high sEndoglin levels. MMP-14 belongs to the sub- class of the membrane-type MMPs and is highly expressed by (malignant) epithelial cells, endothelial cells, and several other cell types. MMP-14 can directly degrade extracellular matrix components, but also cleaves cell surface molecules such as EMMPRIN (CD147, an inducer of MMP expression), LRP, CD44, and cadherins (30–33). In addition, MMP-14 is the major activator of MMP-2 and MMP-13 (34) and it has an important role in tube formation during angiogenesis (35). This study shows that the coexpression of membrane-localized MMP-14 and endoglin results in increased endoglin shedding and this effect depends on a direct interaction between MMP-14 and endoglin. Cotransfection with MMP-13, which is downstream of MMP-14 in the MMP activation cascade, did not show this increase. Moreover, we identified the cleavage sequence used by MMP-14 to shed endoglin. Analysis of natural MMP-14 substrates revealed broad substrate specificity, but in the majority (40%) of the cases, the cleavage site was flanked by a glycine and leucine residue. Endoglin contains four G-L sequences in the extracellular domain, of which one is very close to the transmembrane domain. Mutation of the G-L sequence at position 586 to 587 resulted in a dramatic decrease in sEndoglin levels and therefore indicates this site to be the natural cleavage site.

In this study, we observed strongly increased tissue endoglin levels in colorectal carcinoma, but not in premalignant adenomas, corresponding to what has previously been shown for benign gastric lesions (36). High endoglin expression on tumor-associated endothelial cells has been associated with poor survival in various types of cancers (1, 6, 13), even being prognostically superior to other angiogenic markers such as CD31 or CD34 (13, 36, 37). Disturbance in the balance between membrane-localized and soluble receptors can result in pathologic conditions. For example, in pregnant women suffering from preeclampsia (a severe complication during pregnancies characterized by hypertension and proteinuria), soluble VEGF receptor (sFlt-1) and sEndoglin levels are strongly increased (10). Interestingly, gene expression analysis recently showed that in preeclampsia patients, both endoglin and MMP-14 gene expression is upregulated (38). Soluble receptors are capable of scavenging circulating ligands. VEGF can be bound to sFlt,

whereas it has been described that endoglin can bind different TGF-β superfamily members (11, 39), which could influence angiogenesis. Our data show that sEndoglin inhibits spontaneous cord formation in HUVECs, sprout formation in ECRF cells, and VEGF- induced HUVEC sprouting. In addition, we show that sEndoglin inhibits capillary outgrowth from fetal mouse metatarsals. Our data are consistent with previous findings showing that adenoviral overexpression of sEndoglin interferes with vascular function by decreasing perfusion of the vessels and increasing their permeability (17). Preliminary data from our group show that endoglin-Fc strongly reduces microvessel density in a mouse model of invasive ductal breast carcinoma[4]. The exact role of sEndoglin in malignancies remains unclear. Several studies revealed increased levels in cancer patients compared with controls (12, 14, 40), whereas other showed no increased levels (41–44). On the other hand, our results from CRC patients support an anti-angiogenic role of sEndoglin because we observed lower circulating sEndoglin levels in these patients and would therefore suggest higher angiogenic activity in these patients. In line with our results, changes of sEndoglin levels in the circulation reported previously are generally low. This indicates that sEndoglin in the circulation cannot be used as a powerful marker of angiogenic activity in cancer patients. However, local regulation of endoglin shedding could result in dramatic changes in sEndoglin levels in the tumour microenvironment and therefore have stronger effects on the angiogenic potential of tumor-associated endothelial cells. Local up-regulation of endothelial MMP-14 expression will increase sEndoglin, decrease membrane localized endoglin, and transform the endothelium to a quiescent state. This notion is supported by our immune-staining results, showing the absence of endoglin expression with high MMP-14 staining on endothelial cells and vice versa.

Recent data showed a cross-talk between the TGF-β signalling pathway and the VEGF-induced angiogenesis (45). Furthermore, we have shown that combined TGF-β and BMP-9 treatment synergistically induces angiogenesis (46). Although the exact mechanism is subject of ongoing research, we speculate that scavenging of ligands such as BMP-9 and/or TGF-β by sEndoglin can disturb this delicate balance required for efficient angiogenesis and the normalization of blood vessels. Together, these data indicate an important role for sEndoglin in regulating (tumour) angiogenesis and highlights the importance of the identification of the endoglin shedding protease.

Materials and Methods

Patient material

Preoperative and 3 months postoperative citrate plasma samples (n = 48 and n = 25, respectively) and tissue specimens (n = 191) from patients undergoing resection for primary CRC at the Department of Oncologic Surgery, Leiden University Medical Centre, were collected as described before (18, 19). In addition, plasma samples were collected from 22 healthy volunteers and an additional group of 74 healthy controls previously described (20). Colorectal adenomas (n = 82) were removed endoscopically at the Department of Gastroenterology-Hepatology. Tissue was homogenized and protein concentrations were determined as previously described (18). For immunohistochemistry, tumour tissue and adjacent normal mucosa were collected, fixated, dehydrated, and embedded in paraffin. Human samples were used according to guidelines of the Medical Ethics Committee of the Leiden University Medical Centre.

Cell culture

COS cells were purchased from the American Type Culture Collection and maintained in DMEM, supplemented with 10% fetal bovine serum (FBS) and penicillin/ streptomycin. Human umbilican vein endothelial (HUVEC) cells were isolated from umbilical cords as described before (21). Cells were maintained in M199 medium supplemented with 20% FBS, penicillin/streptomycin, heparin (Leo Pharma), and bovine putitary extract. ECRF cells (an immortalized HUVEC cell line; ref. 22) were a gift from Dr. R. Fontijn, Amsterdam Medical Center (Amsterdam, the Netherlands) and were cultured in HUVEC medium in fibronectin-coated cell culture flasks.

Endoglin and sEndoglin ELISA

Endoglin levels in tissue homogenates (5 µL) and cell culture media (50 µL) were determined essentially according to the DY1097 human endoglin ELISA (R&D Systems). For sEndoglin determination in plasma samples, the capture antibody was replaced by mouse monoclonal anti-endoglin (clone E9, Cell Sciences). Detailed information can be found in Supplementary Materials and Methods.

Immunohistochemistry

To determine tissue localization of endoglin, immunohistochemistry was performed as previously described (23). Representative photomicrographs were taken with a Nikon Eclipse E900 microscope equipped with a Nikon DXM1200 digital camera. Detailed information can be found in Supplementary Materials and Methods.

Cord formation assay

HUVEC cells (3 × 103 cells/well) were resuspended in Medium 199 containing 4% FBS, 1 µg/mL endoglin extracellular domain/Fc chimera (endoglin-Fc), or 1 µg/mL Fc protein and seeded on growth factor reduced Matrigel coated (BD Biosciences) 96-well plates. After 16 hours, photographs were acquired with a phase contrast microscope in four different fields. The length of branches was quantified by automated image analysis using the Olympus analysis software.

Endothelial sprouting assay

Endothelial sprouting assays were performed in the presence or absence of endoglin-Fc (1 µg/mL; R&D Systems) or Fc protein (1 µg/mL) as described before (24, 25). In addition, ECRF and HUVEC cells were infected with lentiviral vectors encoding endoglin-Fc or Fc only. Expression of the proteins was examined by Western blot using anti-Fc antibodies (dilution, 1:1,000; R&D Systems). Angiogenic activity was analyzed as described above. ECRF spheroids form spontaneous sprouts in collagen and were therefore not vascular endothelial growth factor (VEGF) stimulated.

Metatarsal assay

Metatarsals were dissected from 17-day-old albino fetal mice and the assay was performed as described before (26). Bones were transduced with endoglin-Fc or endoglin-Fc adenovirus at 1 × 10^6 plaque-forming unit at day 4. After stimulation with 50 ng/mL VEGF for 7 days, vessel formation was visualized by anti-CD31 staining. Vascular density was quantified by automated image analysis with Image J. Animal experiments were approved by the Dutch animal ethics committee.

Protease inhibitor experiments

HUVEC cells were seeded in 24-well plates and treated with 0 to 160 ng/mL recombinant human MMP-14 (Chemicon) or with proteinase inhibitors: 20 µmol/L E64 (cystein protease inhibitor), 10 µg/mL Aprotinin (serine protease inhibitor, both Sigma- Aldrich), 1 µmol/L GM6001 (broad-spectrum MMP inhibitor), 10 µmol/L Marimastat (kindly provided by British Biotech Pharmaceuticals), 100 nmol/L MMP-2/MMP-9 inhibitor, 100 nm MMP-13 inhibitor, or 1 µmol/L specific MMP-3 inhibitor (all Calbiochem). Serum-free M199 medium and 0.1% DMSO were included as controls. The percentage inhibition versus the appropriate control was calculated.

Quantitative real-time PCR

To analyze the expression of membrane-type MMPs, total RNA was isolated from HUVEC and ECRF cells using Nucleospin RNA columns (Macherey-Nagel) followed by cDNA synthesis using Revertaid first strand cDNA synthesis kit (Fermentas). Quantitative PCR was performed as

previously described (27), according to the manufacturers' protocol, using MMP-14, MMP-15, MMP-16, MMP-17, MMP-24, and MMP-25 primers ordered at the supplier (Applied Biosystems).

Knockdown experiments

Lentiviral constructs expressing short hairpin RNAs (shRNA) targeting human MMP-14 were obtained from the Sigma Mission shRNA library. HUVEC and ECRF cells were infected with the lentiviral knockdown vectors, and 1 week after infection, the cells were plated in 24-well plates. Knockdown was confirmed by MMP-14 Western blot (Abcam; dilution, 1:2,000). After O/N starvation, serum-free medium was collected and subjected to sEndoglin ELISA.

Transfections

COS cells were transfected with plasmids encoding HA-tagged endoglin (28), full-length MMP-14, MMP-14 lacking the transmembrane domain (MMP- 14ΔTM, both kindly provided by Dr. R. Hanemaaijer, TNO Quality of life BioSciences, Leiden, the Netherlands), or empty vector in triplicate. DNA (0.4 µg) was transfected using Lipofectamin, according to the manufacturers' protocol (Invitrogen). Twenty-four hours after transfection, cells were serum starved for 16 hours and sEndoglin levels were determined by ELISA. To examine the interaction between endoglin and MMP-14, COS cells were transfected with endoglin and MMP-14 plasmids as described above in six-well plates. After 48 hours, cells were lysed and incubated with rabbit monoclonal MMP-14 antibodies (Abcam; 1:100) for 3 hours at 4°C, followed by protein-A Sepharose beads for another 30 minutes at 4°C. After extensive washing, samples were subjected to SDS-PAGE and Western blot detection with MMP-14 (Abcam; dilution, 1:2,000) and endoglin (R&D; dilution, 1:500) antibodies.

Mutation of MMP-14 cleavage sequence in endoglin

The predicted MMP-14 cleavage site in endoglin [glycine-leucine (G-L) amino acid 586-7] was mutated into V-P, V-H, H-P, and H-H variants. PCR-mediated overlap extension method was used for site-directed mutagenesis using the pDisplay-endoglin plasmid as a template (28). Detailed information can be found in Supplementary Materials and Methods. The obtained constructs were sequenced and transfected into COS cells. Transmembrane localization of endoglin mutants was confirmed by fluorescence-activated cell sorting (FACS). After O/N starvation, medium was collected and subjected to sEndoglin ELISA.

Statistical analysis

Differences were calculated using the t test, Mann-Whitney U test, or the Wilcoxon signed-rank test using the SPSS 14.0 statistical package. P values of <0.05 were considered statistically significant.

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Supplementary Information

Supplementary materials and methods

Endoglin and sEndoglin ELISA

96 well plates (MaxiSorp, Nunc, Glostrup, Denmark) were coated with mouse monoclonal ant human endoglin antibodies (R&D Systems, 2 µg/ml in PBS) overnight at 4°C. Plates were washed with PBS containing 0.05% Tween-20 (Merck, Darmstadt, Germany) after each step. Nonspecific binding was blocked with PBS containing 5% Tween-20, 0.05% NaN3 for 2 h at room temperature (RT). Samples (5 µl tissue homogenates or 50 µl cell culture media) or standard (0-4 ng/ml recombinant human endoglin, R&D Systems) diluted in PBS with 1% bovine serum albumin (BSA, Sigma, Germany) were incubated for 2h at RT. Immunodetection was performed with biotinylated goat anti-human endoglin antibodies (2 µg/ml in PBS/1% BSA, R&D systems) for 2h at RT, and a substrate reagent pack according to the manufacturers' protocol (R&D Systems). Values were calculated in ng/ml for cell-culture media and in ng/mg total protein for tissue samples. The ELISA was validated by western blot analysis and spiking with a fixed amount of recombinant human endoglin. For determination of endoglin levels in citrate plasma samples the capture antibody was replaced by mouse monoclonal anti-endoglin (clone E9, Cell Sciences, Canton, MA, USA). The standard curve was diluted in PBS containing 40 mg/ml human serum albumin (Sigma) to correct for background absorption. The ELISA was validated using a serially diluted plasma samples and spiking of these samples with a fixed amount recombinant human endoglin, which accordingly increased the ELISA signal (data not shown). The ELISA was performed and validated as described above.

Immunohistochemistry

For immunohistochemical staining slides were deparaffinized, rehydrated and antigen retrieval was performed by boiling in 0.01M sodium citrate buffer pH 6.0. Slides were incubated overnight (O/N) at RT with unlabeled primary antibodies: mouse monoclonal anti-pan-cytokeratin (1:1000), mouse monoclonal anti-vimentin (1:400, both Santa Cruz Biotechnologies, Santa Cruz, USA), mouse monoclonal anti-CD31 (1:400, Dako, Glostrup, Denmark), mouse monoclonal anti-MMP-14 (1:1600), or biotinylated goat-anti human endoglin (1:200, both R&D systems). Immunodetection was performed with goat anti-mouse antibodies and streptavidin-biotin complex (all Dako). Staining was visualized with diaminobenzidine and H2O2.

Endothelial sprouting assays

To analyze the angiogenic potential, endothelial sprouting assays were performed. In short, endothelial spheroids were prepared (750 cells/spheroid) and after overnight incubation embedded

in a collagen type-1 matrix. HUVEC spheroids were stimulated with 50 ng/ml vascular endothelial growth factor (VEGF, R&D systems), which stimulates endothelial sprouting, in the presence or absence of endoglin-Fc (1 µg/ml, R&D systems) or Fc protein (1 µg/ml) as a control. After overnight incubation sprout length was determined as described before (1, 2). In addition ECRF and HUVEC cells were infected with lentiviral vectors encoding Endoglin-Fc or Fc only. Expression of the constructs was examined by western blot using anti-Fc antibodies (dilution 1:1000, R&D systems). Angiogenic activity was analyzed as described above. ECRF spheroids form spontaneous sprouts once embedded in collagen, and were therefore not VEGF stimulated.

Mutation of of MMP-14 cleavage sequence in endoglin

The predicted MMP-14 cleavage site in endoglin sequence G-L (aa 586-7) was mutated into V-P, V-H, H-P and H-H variants. PCR-mediated overlap extension method was used for site-directed-mutagenesis.

In brief, two flanking primers: for 5'-cccaaggacacttgtagcccg-3', rev 5'-acgtggcttcttctgccaaagc-3' and two mutagenic primers per construct:
V-P: for 5'-aagcaaagtccccgtcctgccc-3', rev 5'-caggacggggactttgcttgtg-3';
V-H: for 5'-aagcaaagtccacgtcctgccc-3', rev 5'-caggacgtggactttgcttgtg-3';
H-P: for 5'-aagcaaacaccccgtcctgcccg-3', rev 5'-caggacggggtgtttgcttgtgc-3';
H-H: for 5'-aagcaaacaccacgtcctgcccgc-3', rev 5'-gcaggacgtggtgtttgcttgtgc-3' were used for generation of intermediate PCR products (two per construct) with overlapping regions containing the desired mutations (mutations introduced into nucleotide sequence are underlined). pDisplay-endoglin plasmid was used as a template (3). The two PCR fragments per construct were mixed and used for a second round of PCR with flanking primers. Subsequently the obtained products were digested and cloned into SacI/SalI site in pDisplay-endoglin vector leading to an exchange of wildtype G-L site with the desired mutations. All PCR reactions were performed using Phusion High-Fidelity PCR Master Mix (Finnzymes, Espoo, Finland) and 98°C-30", (98°C-5", 60°C-20", 72°C-15") x 35, 72°C-5' conditions. The obtained constructs were sequenced and used for transfection experiments. Wild type or endoglin mutants were transfected into COS cells in 6 well plates (250.000 cells/well). Transmembrane localization of endoglin mutants was confirmed by flow cytometry (FACS). After O/N starvation medium was collected and sEndoglin levels were determined by ELISA as described above.

References

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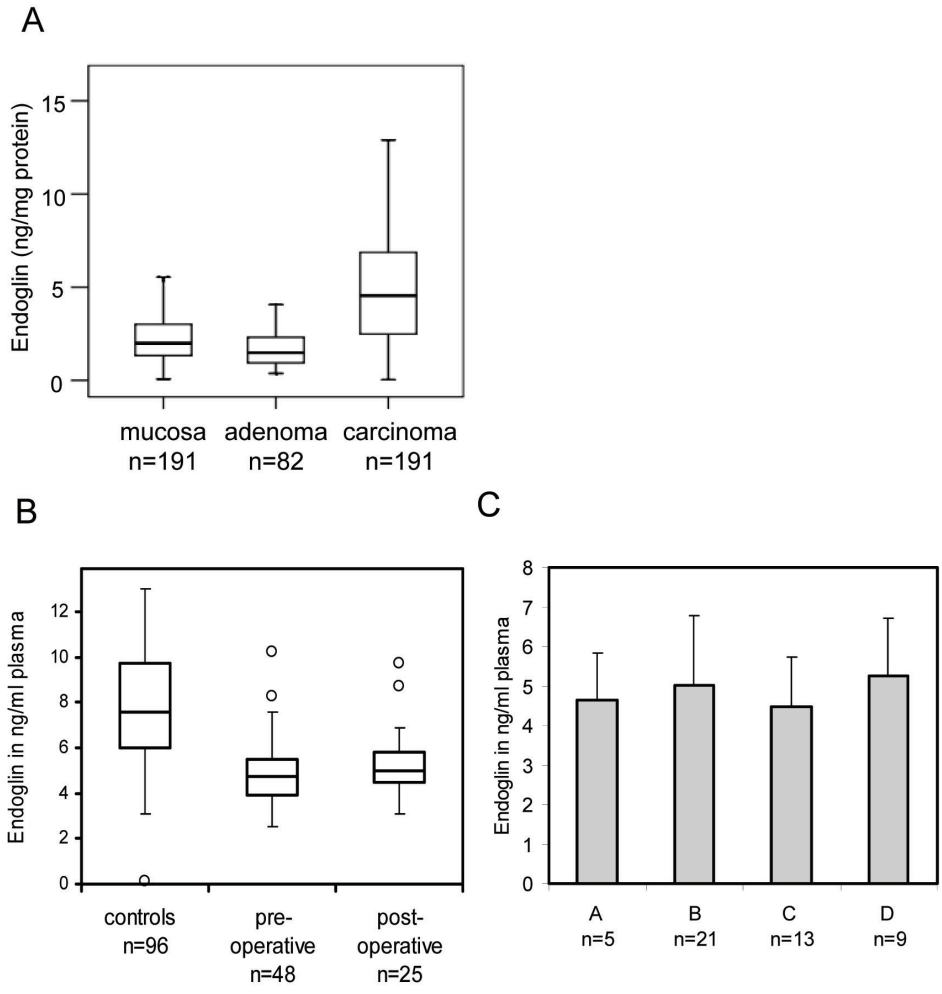
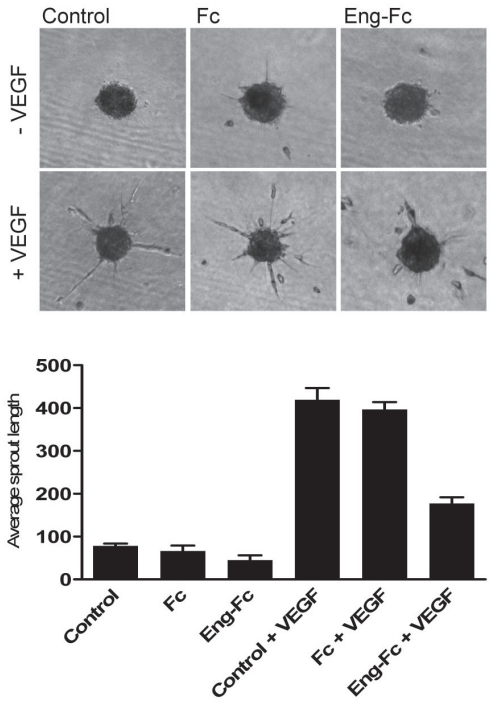
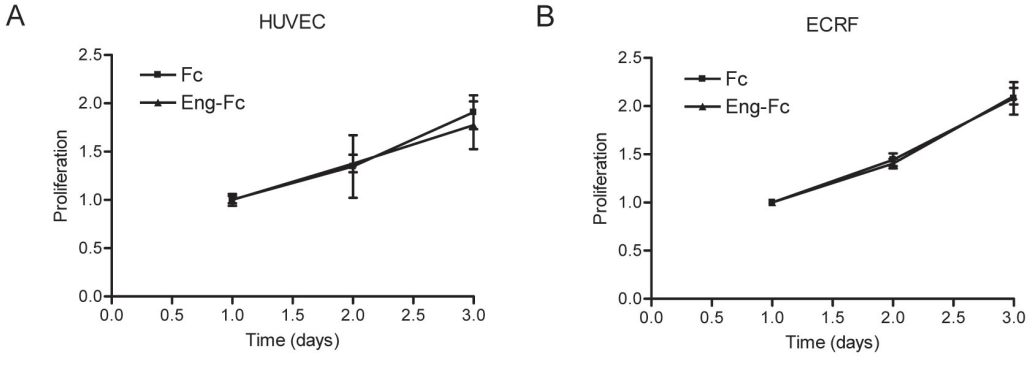


Supplemental Fig 1. *Endoglin levels in colorectal cancer A, Endoglin levels in colon tissue homogenates determined by ELISA. Levels are increased in carcinomas, compared to normal tissue and premalignant adenomas. B, Box-whisker plots showing levels of soluble endoglin in citrate plasma samples in colorectal cancer patients pre- and 3 months post-operation, and healthy controls. Levels were determined by ELISA. Soluble endoglin levels are lower in CRC patients before operation compared to healthy volunteers. (Healthy versus pre-operation, P<0.0001). C, Soluble endoglin levels in CRC patients show no relation with Dukes stage.*



Supplemental Fig 2.*HUVEC endothelial sprouting assay with Endoglin-Fc HUVEC spheroids were prepared, embedded in collagen and stimulated with 50 ng/ml VEGF in the presence of control Fc protein or Endoglin-Fc (both 1 µg/ml). Sprout length was determined from at least 10 spheroids per experimental condition after O/N incubation. Data represent average sprout length ± SD from a representative experiment.*



Supplemental Fig 3. *Influence endoglin-Fc on proliferation endothelial cells***.** *HUVEC and ECRF cells were transduced with Fc- or Endoglin Fc expressing lentivirusses. Proliferation was determined using the MTS assay. Data show no effect of Endoglin-Fc on proliferation of HUVEC (A) or ECRF cells (B).*

A

Supplementary Figure 4

G	12	P	20	A	13	G	26	C	L	39	V	12	T	12	E	14
L	11	L	18	Q	11	A	12	L	I	11	A	9,7	A	11	A	9,7
A	8,6	A	9,7	G	9,7	R	11	E	Y	9,7	E	8,6	L	9,7	S	8,6
D	8,6	S	8,6	E	8,6	N	9,7	A	S	7,5	N	8,6	E	9,7	L	8,6
Q	8,6	V	8,6	S	8,6	D	7,5	V	M	5,4	R	7,5	G	8,6	R	8,6
V	7,5	R	5,4	L	7,5	P	5,4	A	D	4,3	Q	6,5	S	8,6	N	7,5
N	6,5	Q	4,3	V	6,5	S	5,4	G	T	4,3	S	6,5	K	7,5	Q	6,5
T	6,5	T	3,2	P	6,5	K	4,3	E	Q	3,2	G	5,4	V	6,5	P	6,5
R	6,5	I	3,2	I	4,3	Q	4,3	-	V	3,2	L	5,4	P	4,3	T	4,3
P	5,4	Y	2,2	R	4,3	T	3,2	S	A	3,2	K	4,3	R	4,3	K	4,3
E	4,3	E	2,2	Y	3,2	L	3,2	I	F	3,2	T	4,3	N	3,2	G	4,3
I	3,2	D	2,2	F	3,2	Y	3,2	T	G	2,2	F	4,3	H	3,2	F	3,2
S	3,2	F	2,2	K	3,2	E	3,2	E	N	1,1	Y	3,2	Q	3,2	H	3,2
K	3,2	G	2,2	N	3,2	H	1,1		W	1,1	M	3,2	F	3,2	D	3,2
H	2,2	C	2,2	T	2,2	W	1,1		R	1,1	P	2,2	D	2,2	V	3,2
C	1,1	N	1,1	D	2,2				E	1,1	D	2,2	C	1,1	Y	2,2
M	1,1	K	1,1	C	1,1						I	2,2	I	1,1	W	1,1
F	1,1	W	1,1	H	1,1						H	2,2	Y	1,1	I	1,1
		H	1,1	M	1,1						C	1,1				
		M	1,1								W	1,1				

B

1 *mdrgtlplav alllascsls ptslae* tvhc dlqpvgperg evtyttsqvs kgcvaqapna
61 ilevhvlfle fptgpsqlel tlqaskqngt wprevllvls vnssvflhlq algiplhlay
121 nsslvtfqep pgvnttelps fpktqilewa aergpitsaa elndpqsill rlgqaqgsls
81 fcmleasqdm grtlewrprt palvrgchle gvaghkeahi lrvlpghsag prtvtvkvel
241 scapgdldav lilqgppyvs wlidanhnmq iwttgeysfk ifpeknirgf klpdtpqgll
301 gearmlnasi vasfvelpla sivsldassc ggrlqtspap iqttppkdtc spellmsliq
361 tkcaddamtl vlkkelvahl kctitgltfw dpscqaedrg dkfvlrsays scgmqvsasm
421 isneavvnil sssspqrkkv hclnmdslsf qlglylsphf lqasntiepg qqsfvqvrvs
481 psvseflql dschldlgpe ggtveliqgr aakgncvsll spspqegdprf sfllhfytvp
541 ipktgtlsct valrpktgsq dqevhrtvfm rlniispdls gctskg ***lvlp avlgitfgaf***
601 ***ligalltaal w***yiyshtrsp skrepvvava apassessst nhsigstqst pcstssma

Supplemental Fig.4 . *Determination MMP-14 cleavage sequence in endoglin A, Analysis of amino acids flanking the MMP-14 cleavage site in natural substrates. 79 natural MMP-14 substrates with known cleavage sites were analyzed. Occurrence of the amino acids flanking the cleavage site is shown in % of total. B, Endoglin amino acid sequence with potential MMP-14 cleavage sites based on the consensus G—L sequence indicated by arrows (G-L sequences). Transmembrane domain is indicated in* **bold**

Chapter 6

Soluble fms-like tyrosine kinase 1 and soluble endoglin are elevated circulating anti-angiogenic factors in pre-eclampsia

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Abstract

Pre-eclampsia, characterized by hypertension and proteinuria, affects approximately 3–5% of all pregnancies worldwide and is a major cause of maternal and fetal morbidity and mortality. Maternal endothelial dysfunction is associated with disease pathogenesis. Recently, reports have shown that elevated levels of circulating soluble fms-like tyrosine kinase 1 [sFlt1] and soluble endoglin [sEng] are associated with pre-eclampsia. Flt1 is a receptor for vascular endothelial growth factor receptor [VEGF], whereas endoglin [Eng] is an auxiliary receptor for transforming growth factor-β [TGF-β] super-family members. Both signalling pathways modulate angiogenesis and are involved in vascular homeostasis. Increased levels of sFlt1 and sEng dysregulate VEGF and TGF-β signalling respectively, resulting in endothelial dysfunction of maternal blood vessels. This review summarizes our current knowledge of Flt1 and endoglin and soluble forms in pre-eclampsia. Furthermore, it highlights the predictive and early-screening value of circulating levels of sFlt1 and sEng for the risk of developing pre-eclampsia

***Keywords*:** Angiogenesis, Endothelial dysfunction, Pre-eclampsia, sFlt1, Soluble endoglin, TGF-β, VEGF

Introduction

Pre-eclampsia is a pregnancy-specific syndrome that develops after mid-gestation and is characterized by de novo hypertension [systolic blood pressure P140 mm Hg or diastolic blood pressure P90 mm Hg] accompanied by new onset proteinuria defined as 300 mg of protein/24 h or more after 20 weeks of gestation [1]. To date, the clinical incidence of pre-eclampsia is 3–5% of pregnancies, which in some instances can lead to complications such as maternal renal failure, hemolysis, elevated liver enzymes and low platelets [HELLP], seizures, liver failure, stroke or death [2–4]. For the fetus, pre-eclampsia can result in intrauterine growth restriction [IUGR], preterm delivery and death [2–4]. Pre-eclampsia resolves after placental delivery; however, women with a history of pre-eclampsia are at higher risk of developing cardiovascular complications later in life [5,6].

The etiology of pre-eclampsia remains incompletely understood. Its origin lies in defective placentation resulting in the release of placenta-derived angiogenic modulators into the maternal circulation. Studies have shown that levels of soluble fms-like tyrosine kinase 1 (sFlt1) and soluble endoglin (sEng) are elevated in the blood of pregnant women suffering from pre-eclampsia [7–9]. Flt1 is a vascular endothelial growth factor [VEGF] receptor and also known as VEGF receptor 1 (VEGFR1), while endoglin is a co-receptor for transforming growth factor (TGF)-β super-family members. Both receptors are highly expressed on endothelial cells and are involved in maintaining vascular homeostasis. The aberrantly large quantities of these placenta-derived soluble receptors in pre-eclamptic women can cause maternal endothelial cell dysfunction and thereby the resulting clinical symptoms of pre-eclampsia.

Besides the contribution of these anti-angiogenic factors, inflammatory responses and metabolic syndromes are also considered to be associated with the etiology of pre-eclampsia [1,10]. In this review we focus on discussing the roles of placenta-derived sFlt1 and sEng in endothelial dysfunction and their potential as biomarkers to assess the risk of developing pre-eclampsia.

The biology of sFlt1 and sEng

The biology of sFlt1

VEGF is a multifunctional molecule that is involved in numerous biological processes during postnatal development and in both physiological and pathological conditions. The mammalian VEGF family is composed of VEGF- A, VEGF-B, VEGF-C VEGF-D and placenta growth factor (PlGF). The predominant form, VEGF-A, is alternatively spliced in different isoforms which have pro- and anti- angiogenic properties [11]. Three types of VEGF receptors are activated upon ligand binding,

namely Flt1, Flk1 (VEG- FR2) and Flt4 (VEGFR3). Co-receptors, such as neuropilins and heparan sulfate proteoglycans modify VEGFR activities in diverse fashions [12–16]. Flt1 binds VEGF-A, -B and PlGF, whereas Flk1 binds to VEGF-A and -C and Flt4 binds VEGF-C and -D [17].

VEGFs and their receptors are crucial for vascular development and lymphatic vessel formation. VEGFRs consist of an extracellular domain, a trans-membrane domain and an intracellular protein tyrosine kinase domain. Ligand binding leads to VEGFR dimerization and activation of the tyrosine kinase activity, upon which diverse intracellular signalling pathways are initiated that modulate vascular biological functions. Flt1 and Flk1 mediated signalling events regulate endothelial cell function, such as endothelial cell proliferation, migration, tube formation and branching [18]. VEGFR3 signalling is mainly important for lymphatic vessel development [19].

The importance of Flk1 and Flt1 in vascular function became apparent from the analysis of knockout mice. Depletion of either Flt1 or Flk1 in mice resulted in embryonic lethality due to vascular malfunction, but with distinct differences in the phenotypes. Flt1 knockout mice showed irregular vascular organization due to endothelial cell overgrowth, whereas Flk1 knockout embryos had an absence of blood vessels and blood islands [20,21]. The extracellular domain of both Flk1 and Flt1 consists of seven extracellular immunoglobulin (Ig) homology domains. The 2nd Ig homology domain is sufficient for VEGF binding to Flt1, while Flk1 requires both the 2nd and 3rd Ig homology domains for adequate VEGF binding [22–25]. VEGF-A has a higher affinity for Flt1 than Flk1, which may be explained by the difference in ligand-binding domains. Yet the kinase activity of Flt1 in response to VEGF-A binding is weak [26–28], possibly because the juxtamembrane domain of the intracellular domain represses kinase activation [29]. Interestingly, mice deficient in the kinase domain of membrane-anchored Flt1, but with an intact ligand binding domain, revealed no defects in vascular development, suggesting the kinase activity of Flt1 is dispensable for its role in vasculature formation [30]. Taken together, these data suggest that Flt1 may function as a decoy receptor for VEGF-A, thereby regulating VEGF-A bio-availability for Flk1 [18]. PlGF binds directly to Flt1 and amplifies VEGF-driven angiogenesis through Flk1 [31]. PlGF can outcompete VEGF-A for the binding of Flt1, indirectly promoting VEGF-A/Flk1 signalling by increasing the bio-availability of VEGF-A for Flk1 [32,33]. Nonetheless, other experimental evidence indicates that Flt1 not only serves to fine-tune the VEGF-Flk1 axis, but is also directly involved in signal transduction. VEGF-A and PlGF induce Flt1 receptor phosphorylation and downstream signalling [31]. Loss of Flt1 kinase activity shows deficient inflammation and angiogenesis in various disease models such as atherosclerosis, choroidal neovascularization, lung metastasis and rheumatoid arthritis [34–37]. In addition, Flt1 can act as a negative regulator for endothelial tip cell sprouting and branching morphogenesis in zebra fish system [38]. Up to date, the apparent function of Flt1 in angiogenesis is evident, but the exact signalling of Flt1 remains to be elucidated.

The transmembrane Flt1 protein is encoded by 30 exons; sFlt1 is generated through alternative splicing (Fig. 1A) and consists of exons 1 through 13 with an intron 13 derived encoded carboxy tail [39–41]. sFlt1 and Flt1 share the first six Ig-homology domains, which are essential for ligand binding [42,43]. The splicing of sFlt1 is possibly regulated by hypoxia and histone jumonji domain-containing demethylases [44–46]. Recent studies have described additional splice variants of sFlt1, (termed as sFlt1-v2,-v3,-v4). While all share an extracellular domain followed by a short C-terminal tail, they differ in composition [47,48]. Different from the other variants, sFlt1-v2 has been reported to be highly expressed in placental tissues as a non-endothelial cell form of sFlt1 [48,49]. However the relevance of this secreted sFlt1 on placenta function *in vivo* has not yet been investigated. In addition to alternative splicing, a ligand-induced proteolytically cleaved version of soluble Flt1 has been found to be expressed by leukemic cells [50]. Whether proteolytic shedding also contributes to sFlt1 in pre-eclampsia is not known.

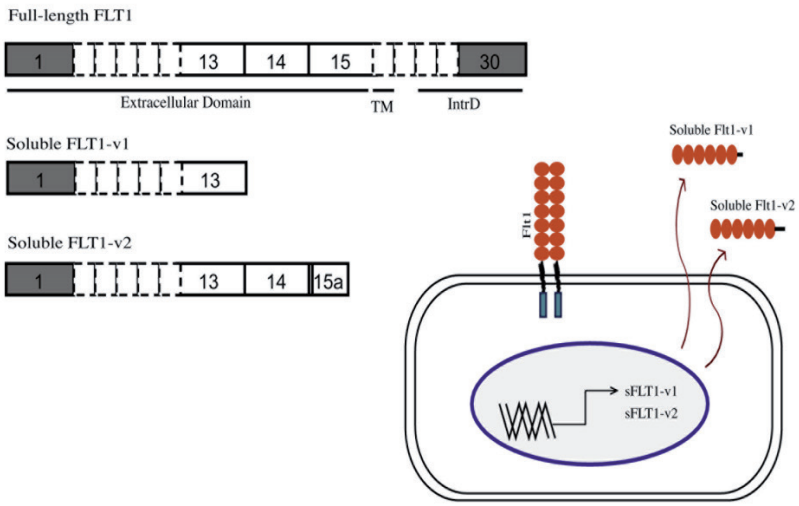

Fig.1A. *Different transcripts of sFlt1 and the generation of sFlt. The transmembrane VEGF receptor, including the extracellular domain, transmembrane domain (TM), and intracellular catalytic domain (IntrD), is encoded by all 30 exons of the FLT1 gene. The sFLT1 proteins are the result of alternative splicing, generating a secreted form of FLT1 with a unique 31 amino acid (sFLT1-v1) or 28 amino acid (sFLT1-v2) c-terminal tail. In human placenta, 80% of FLT1 expression corresponds to transcript sFLT1-v2, 15% to sFLT1-v1, and 5% to the transmembrane version.*

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sFlt1 sequesters circulating VEGF-A, VEGF-B and PlGF [31,42] (Fig.1B). In addition, it can form a stable receptor complex with the extracellular domain of Flk1, thereby interfering with Flk1 dimerization and subsequent intracellular signalling [43]. Due to the neutralizing function of sFlt1 for its natural ligands, sFlt1-based trap has been developed for preclinical purpose, which contains the first three Ig-homology domains of Flt1 [51]. sFlt1 and the sFlt-based trap have been shown to act as negative regulators for endothelial function [52] and angiogenesis in several pre-clinical studies [53–56]. Notably, Flt1 and sFlt1 are highly expressed in placental tissues, particularly in the trophoblast [49,57–59] and can be released into the maternal circulation [60].

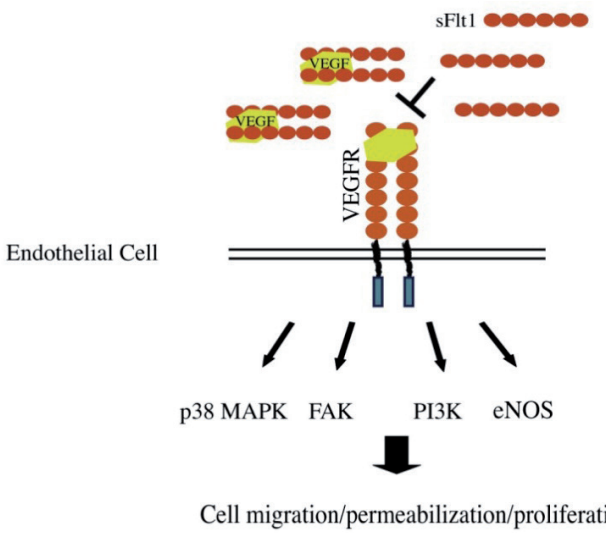

Fig.1B. *VEGF/Flt1 signalling and effect of sFlt1 thereon. VEGF induces Flt1 receptor dimerization. As a result of Flt1 receptor activation, downstream signalling pathways are indirectly or directly activated, such as p38 mitogen-activated protein kinases (p38 MAPK), focal adhesion kinase (FAK), phosphoinositide 3-kinase (PI3K), endothelial nitric oxide synthase (eNOS). The VEGF/Flt1 system regulates endothelial cell migration, proliferation and permeabilization. Increased sFlt1 levels in the circulation can sequester VEGF locally and thereby interfere with VEGF-induced endothelial function.*

The biology of sEng

Endoglin (CD105) is a membrane-bound glycoprotein which functions as an auxiliary/co-receptor for TGF-β super-family members [61]. The TGF-β superfamily includes TGF-βs, activins and bone morphogenetic proteins (BMPs); they affect not only vascular cells, but also many other cell types. The TGF-β superfamily signals via complexes of type I and type II serine/threonine kinase receptors [62].

Endoglin contains an extracellular domain, a single transmembrane domain and a short cytoplasmic domain [63] (Fig. 2A). The extracellular domain of endoglin harbors a signal peptide, an orphan domain and a zona pellucida (ZP) domain. The ZP domain is involved in endoglin oligomerization; the extracellular and intracellular domains mediate heteromeric interactions with TGF-β receptors I and II (TβRI/II) [64]. TGF-β only binds to endoglin in complex with TβRII, whereas BMP-9 directly binds to the orphan domain of endoglin [65,66]. Endoglin is closely related to the TGF-β co-receptor betaglycan. Endoglin binds to TGF-β1 and β3, while betaglycan interacts with all three TGF-β isoforms [67]. In part, the cell type-specific effects of TGF-β are mediated by the auxiliary receptors that demonstrate differential expression patterns and interact with different affinity to TGF-β isoforms. Due to differential mRNA splicing, two membrane-bound forms of endoglin have been described: long-form endoglin (L-endoglin) and short-form endoglin (S-endoglin). L-endoglin is the most abundantly expressed isoform [68,69]. Apart from the membrane-bound forms, endoglin can exist as a soluble form (sEng) (Fig. 2A).

Endoglin is expressed at low levels in quiescent endothelial cells, but is highly expressed in proliferating endothelial cells [70]. In addition to endothelial cells, endoglin is also expressed in syncytiotrophoblasts [70,71], stromal cells [71,72] and hematopoietic cells [73] of full term placentas. Endoglin expression can be induced and regulated by hypoxia, TGF-β1, TGF-β3 and BMP-9 [66,75,76]. Mouse aortic endothelial cells (MAEC) isolated from endoglin null mice display reduced proliferation, migration, VEGF secretion and decreased endothelial nitric oxide synthase (eNOS) expression [77–79]. Lebrin *et al.* also found that endothelial cells derived from endoglin null embryos demonstrated impaired proliferation in culture [61]. Up-regulation of endoglin has been shown to protect endothelial cells from TGF-β1-induced apoptosis [79]. The importance of endoglin in vascular biology was demonstrated by the embryonic lethality of mice deficient in endoglin due to defective angiogenesis *in vivo* [80,81]. Mutations in endoglin cause the autosomal dominant disease hereditary hemorrhagic telangiectasia [HHT] type I. HHT is a vascular dysplasia disease characterized by the development of mucocutaneous telangiectasias and arteriovenous malformations in the brain, lungs, liver and gastrointestinal tract. Endoglin heterozygous mice exhibit vascular lesions due to capillary malformation, resembling HHT type 1 [81–83]. Thus, endoglin has a pivotal role in endothelial cell function [74,84].

Levels of sEng are elevated in the sera of pre-eclamptic patients [7,9] as well as in colorectal and breast cancer patients [85,86], which results in abnormal angiogenic responses. It has been reported that sEng is generated via metalloproteinase (MMP)-14 (MT1-MMP) mediated shed- ding of membrane bound endoglin in colorectal cancer, at the site close to the transmembrane domain of endoglin [86] (Fig.2A). This cleaved sEng contains the entire extracellular domain, retaining the ability of binding to TGF-β and BMP-9 [9,66]. Thus, local shedding of endoglin is a potential source of sEng, which subsequently can affect tumor angiogenesis in the tumor microenvironment. MMP14 was recently shown to be involved in the generation of sEng in pre-eclampsia patients. MMP14 was expressed by syncytiotrophoblasts and interacted with endoglin within the pre-eclamptic placenta. Challenging trophoblasts with MMP14 inhibitors attenuated the production of sEng [87].

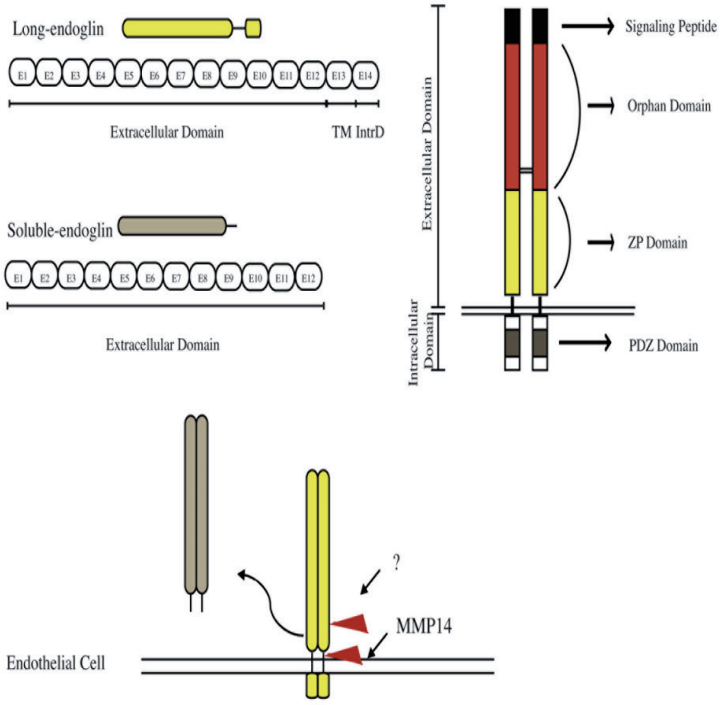

Fig. 2A. *Different forms of endoglin and the generation of sEng. Endoglin is a 90kDa protein that contains a large extracellular domain and short intracellular domain that lacks an enzymatic motif. Endoglin is a dimeric protein and monomers are connected by a disulphide bridge. Several sub-domains have been identified, including orphan domain and TGF-ß/BMP binding domains, receptor interacting domains. The long form (L; yellow) and the soluble form (S; gray) forms differ in the size of their intracellular domain. At its carboxy terminus endoglin has a PDZ interaction motif. sEng consists of part of the extracellular domain of endoglin; it lacks a transmembrane and intracellular domain. sEng can be generated by shedding of membrane bound endoglin possibly via MMP14 activity. It is not excluded that endoglin can be cleaved by any other unknown enzymes because it contains multiple potential proteolytic cleavage site.*

It has been shown that sEng inhibits angiogenesis in ex vivo assays, such as the fetal mouse metatarsal [86] and chick chorioallantoic membrane assay [88]. sEng can exert anti-angiogenic effects on endothelial cells by modulating TGF-β/BMP signalling [9,86,88,89] (Fig. 2B); Studies *in vitro* have shown that sEng interferes with TGF-β signalling and eNOS activity [9]. The explanation for these findings could be that sEng functions as a scavenger for circulating ligands such as TGF-β1/3 and BMP-9 [88], and thereby affects the vascular balance maintained by TGF-β/BMP signalling. It is also possible that by binding to TGF-β receptors, sEng interferes with the availability of TGF-β receptors for membrane-bound endoglin [88].

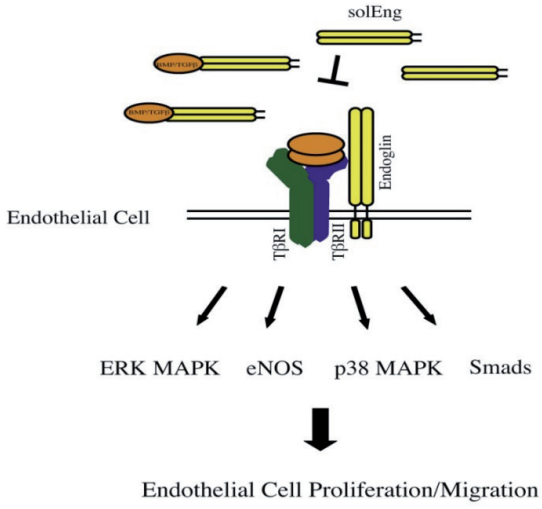

Fig.2B. *TGF-β/BMP/endoglin signalling and the effect of sEng thereon. TGF-β and BMP bind to heteromeric type II/type I serine/threonine kinase receptor complexes. Endoglin is an auxillary receptor and can interact with, and modulate ligand binding to type II/type I receptor complexes. The active receptor complex can activate intracellular Smad pathways and non-Smad pathways: early response kinase (ERK); phosphoinositide 3-kinase (PI3K); p38 mitogen-activated protein kinases (p38 MAPK), endothelial nitric oxide synthase (eNOS). In the endothelial cell, TGF-β/ BMP signalling modulates cell migration and proliferation. sEng functions as a negative regulator for TGF-β/BMP signalling in endothelial cells.*

Endothelial cell dysfunction with increased levels of sFlt1 and sEng in pre-eclampsia

Angiogenesis is tightly controlled by pro- and anti-angiogenic factors. An imbalance in this process can lead to excessive or insufficient angiogenic responses, which has been associated with different diseases. Studies in humans and animal models have demonstrated that TGF-β and VEGF signalling play crucial roles in maintaining physiological vascular homeostasis by modulating endothelial cell function [90–92].

Initial insights from human studies

In 2003, the first studies were published that clearly demonstrated increased sFlt1 levels in the circulation of pre-eclamptic women as to compared normotensive pregnant women [8,92]. Also, free VEGF and PlGF levels were lower in the blood of pre-eclamptic patients compared to

normotensive pregnancies [8,93], confirming the antagonistic effects of sFlt1 on the VEGF system. Subsequently, many other studies have confirmed these findings with the agreement that sFlt1 holds predictive value reviewed by Lapaire [94]. Most importantly, the increase in sFlt1 was detected before the onset of pre-eclampsia. A human study performed by Levine and colleagues has shown the significant predictive value for sFlt1 [7]. Sera were analyzed for sFlt1 by ELISA from a total number of 120 women with pre-eclampsia and matched healthy pregnant women during their pregnancy. Women who developed pre-eclampsia showed higher levels of sFlt1 compared with the control group. An increase in sFlt level was detected 5 weeks before the clinical onset of pre-eclampsia. Meanwhile, PlGF concentration was significantly lower in women who later developed pre-eclampsia compared to controls [95]. Because the increase of sFlt1 is accompanied with a decrease in PlGF levels, the angiogenic balance was most likely shifted toward inhibition in women with a high risk of developing pre-eclampsia. This subverted anti-angiogenic status in pre-eclampsia patients is further supported by a renal biopsy study, which illustrated mild glomerular endotheliosis in normal pregnancy, but severe lesions in pre-eclampsia [96].

A landmark study, performed by Venkatesha et al., showed that the expression of sEng is elevated both in placenta tissues as well as serum of pre-eclamptic patient compared to controls. The high levels of sEng in pre-eclamptic patients were dramatically reduced after placental delivery, indicating that the increase in sEng is pregnancy-associated. Similar to the role of excessive sFlt1 in kidney dysfunction, elevated sEng levels induce glomerular endotheliosis [9,97].

Insights from rodent studies

Many of the clinical symptoms of pre-elampsia can be recapitulated in animal models. As the mouse placenta shares molecular and structural similarities with the human placenta reviewed by Rossant [98], a variety of rodent models have been proposed for pre-eclampsia studies. These models include the sFlt1/sEng induction model, the reduced uterine perfusion pressure (RUPP) model and the renin angiotensin abnormalities (AT-AAs) model.

Rats administered with adenoviral sFlt1 showed a clear increase in blood pressure and proteinuria recapitulating major features observed in human patients [8]. Similar responses were observed after intravenous injection of sFlt1 protein into mice [99]. Introduction of adenoviral sEng into pregnant rats also resulted in a significant increase in mean arterial pressure, mild renal endotheliosis and vascular damage in the placenta [9,100]. Furthermore, the combination of adenoviral induction of sEng and sFlt1 in pregnant rats increased complications of pre-eclampsia, including infarction at the maternal-fetal interface, fetal growth restriction, hepatic ischemia and necrosis [9]. Reversely, neutralization of free sFlt1 in pre-eclamptic mice by the increase of VEGF or PlGF ameliorated the pre-eclampsia syndrome [99,101].

In the RUPP model, the bilateral utero-ovarian arteries are ligated in pregnant animals. This results in the development of pre-eclampsia-like symptoms such as hypertension, proteinuria and glomerular endotheliosis [102]. This technique has been applied in different animals including rabbits [103], dogs [104,105], monkeys [106], baboons [107] and sheep [109] to study hypertension in relation to blood pressure changes during pregnancy. In a recent study, using the RUPP model to induce pre-eclamptic hypertension in rats, it was shown that both the sFlt1 and the sEng levels [99,108] in serum were significantly increased compared to the control group, again confirming the association of elevated sFlt1/sEng levels with pre-eclamptic hypertension.

Another mouse model to induce pre-eclampsia is achieved by the injection of angiotensin receptor activating autoantibodies (AT1-AAs), which are obtained from women with pre-eclampsia, into healthy pregnant mice. When mice were co-treated with an AT1 receptor antagonist [losartan], pre-eclampsia did not develop [9,110] validating the importance of AT1-AAs for pre-eclampsia onset. The injection of AT1-AAs resulted in elevated levels of sFlt1 and sEng in pregnant mice, whereas these levels were not affected in non-pregnant AT1-AA treated control mice [110,111], further suggesting the relevance of sFlt and sEng in pre-eclampsia. Similar to human studies, increased levels of sFlt1 and sEng are associated with pre-eclampsia in rodent models. These models provide researchers with great opportunities to investigate the intervention value of sFlt1 and sEng as potential therapeutic targets.

sFlt1 and sEng as circulating biomarkers for pre- eclampsia

As hypertension and proteinuria are not specific symptoms for pre-eclampsia, a specific diagnostic marker is greatly needed for pre-eclamptic patients for early diagnosis and prevention. For this purpose a number of biomarkers have been under investigation [112]. The expression of sFlt1 and sEng is increased in pre-eclamptic patients prior to the occurrence of the pre-eclamptic syndrome, indicating their predictive value for the onset of pre-eclampsia. Nevertheless, the level of sFlt1 during pregnancy is variable between individuals, so it is difficult to interpret its accuracy as a circulating biomarker. Therefore, recent studies have analyzed the sFlt1/PlGF ratio to enhance the sensitivity of this marker as an indicator for the onset of pre-eclampsia [7]. This ratio increases before the onset of pre-eclampsia, but it does not distinguish between women with subsequent gestational hypertension and the control group [7]. Using an automated sFlt1/PlGF assay [Elecsys® sFlt1 and PlGF assay developed by Roche] the sFlt1/PlGF ratio was shown to be a valuable tool in the assessment of pre-elampsia [112,113].

Besides increased sFlt1 levels, sEng levels are also elevated in pre-eclamptic patients with an increased sFlt1/ PlGF ratio [7]. Increased sEng has been shown to be associated with pre-eclampsia development in a cohort study [114] and supported by another independent study in a Korean cohort [115]. In addition, sEng is reported to be informative to discriminate pre-eclampsia from gestational hypertension and chronic hypertension [116]. Of note, its increase also occurred in normotensive pregnancies with IUGR [117]. However, the association between IUGR and increased sEng levels was challenged by other studies in which no association between IUGR with sEng levels could be demonstrated [118].

The currently available enzyme-linked immunosorbent assay (ELISA) kits for the detection of sFlt1 are comprised of antibodies that do not distinguish the different isoforms of sFlt1. sFlt1 variants are expressed at different levels in the placenta [49]. Use of antibodies which discriminate between the different subtypes could more specifically evaluate the potential roles of different isoforms in the pathogenesis of pre-eclampsia. With regard to sEng detection, different molecular weights of sEng have been reported. A study by Hawinkels *et al.* reported sEng to be around 80 kDa in endothelial cells, whereas Venkatesha *et al.* detected a 65 kDa form of sEng in placental tissue. The size of sEng appears to be variable in different experimental settings. To validate the prediction value of sEng, more studies are needed to understand the source of different sEng forms.

Concluding remarks and future perspectives

Pre-eclampsia is a complex pregnancy-related syndrome. Deficient placental function has been recognized as the major cause, but the pathological mechanism that underlies this disease remains less understood. Concerning the high incidence among pregnant women globally, an effective diagnostic tool for pre-eclampsia is urgently needed. Notably, the predictive potential of sFlt1 and sEng has drawn great attention in the research field of pre-eclampsia in recent years, as substantial evidence implies that aberrant sFlt1 and sEng levels are involved in the angiogenic imbalance in pre-eclampsia patients. Whereas the application of combining sFlt1/PlGF ratio together with the sEng concentration as diagnostic markers may contribute to better prediction and early identification of pre-eclamptic patients, further studies regarding sFlt1 and sEng as predictive biomarkers for pre-eclampsia are required. Furthermore, it would not be surprising if aberrant sEng or sFlt1 levels are associated with other diseases, such as vascular complications in hypertension, diabetes and gestational proteinuria patients [119,120].

To explore potential therapeutic approaches for suppressing of pre-eclampsia progression the perturbed VEGF-sFlt1 balance was restored in mice by the administration of VEGF. This indeed alleviated the pre-eclampsia-like symptoms [101]. Targeting endoglin shedding may be an option to suppress sEng levels in the circulation. Alternatively, pharmalogically targeting of

the signalling upstream of sFlt1 and sEng expression might be more effective. Increasing levels of Heme-oxygenase-1 [HO-1] may be beneficial for ameliorating pre-eclampsia HO-1 is a negative regulator for both sFlt1 and sEng expression in human placenta explants at the transcriptional level [121,122]. This strategy has been pursued n the ongoing trial StAmP [Statin to Ameliorate Early Onset Pre-eclampsia] to validate the use of statins in early-onset pre- eclampsia [123]; statins, a class of anti-oxidant agents, induce HO-1 expression [121,124]. However, an issue which should not be neglected is that all aforementioned angiogenic-based therapeutic approaches may affect the systemic endothelial homeostasis.

Recent findings have provided evidence suggesting that levels of sFlt1 and sEng associate with pathology of pre-eclampsia. Yet understanding the exact function of sFlt1 and sEng in pre-eclampsia remains incomplete. Future advances in understanding the biology of sFlt1 and sEng in vascular biology could possibly reveal the etiology of pre-eclampsia and potentially be beneficial in diagnosing patients at risk for the development of pre-eclampsia.

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Chapter 7

General Discussion

The TGF-β family signalling pathways are highly fine-tuned cellular signalling networks and their biological effects dependent on the local microenvironment and context. Signalling cascades of TGF-β family members have been shown to be important in vascular development and angiogenesis. Mutations in TGF-β family receptors or Smads have been linked to vascular disorders such as HHT, PAH and Marfan Syndrome, as described in the introduction of this thesis. Mice deficient in specific TGF-β family signalling components, i.e. ligands, receptors or Smad transcription factors, frequently develop embryonic lethality due to vascular defects. The exact mechanism by which signalling of TGF-β family members, and perturbations therein, affects vascular function remains largely unknown. Therefore, this discussion will focus on our current perspectives of mechanisms underlying the crosstalk between TGF-β and VEGF, the perspective of BMP9 and endoglin in endothelial cell biology and the emerging role of TGF-β family signalling in angiogenic sprout remodeling.

The interplay between TGF-β and VEGF signalling pathways in endothelial cell function

TGF-β signalling in endothelial cells

In endothelial cells (ECs), after the binding to type II receptor (TβRII), TGF-β signals via two opposing type I receptors (TβRI) and thereby activate distinct Smad signalling pathways: (i) the ALK5/Smad2/3 signalling pathway leading to inhibition of EC migration and proliferation, and (ii) the ALK1/Smad1/5 cascade that resulting in the induction EC migration (1). In addition, endoglin, a co-receptor for TGF-β ligands, promotes ALK1- but inhibits ALK5-induced signalling (2). Although the TGF-β/ALK1 pathway appears to directly antagonize TGF-β/ALK5 signalling, the presence of ALK5 is a requirement for efficient TGF-β/ALK1 signalling (3). Interestingly, when ECs are plated on fibronectin the requirement of ALK5 for TGF-β/ALK1 signalling is bypassed (4). To intervene with the pro-angiogenic effects of the TGF-β superfamily, tools have been generated to specifically inhibit the function of ALK1 and endoglin. Soluble chimeric forms of ALK1 (ALK1-Fc) or endoglin (endoglin-Fc) fused to an antibody Fc domain can attenuate the ALK1 signalling pathway possibly by scavenging pro-angiogenic ligands of the TGF-β superfamily. As a result, the fine-balance between TGF-β family and VEGF signalling is disturbed resulting in impaired VEGF-induced EC sprouting *in vitro* and *in vivo* (5 - 6).

TGF- β regulates VEGF expression

Besides the direct effects that TGF-β has on EC behavior, TGF-β also regulates EC function indirectly by regulating the expression of angiogenic factors in non-endothelial cell types, such as smooth muscle cells, macrophages and tumor cells. Notably, in macrophages TGF-β induces

vascular endothelial growth factor (VEGF) production in a Smad-dependent manner to mediate angiogenic responses (8). In addition, TGF-β induces the expression of VEGF in tumor cells and tumor associated-stromal fibroblasts via the ALK5/TβRII complex (9, 10). SB-431542, an ALK4/5/7 kinase inhibitor (11), has been shown to exert an inhibitory effect on VEGF secretion in human cancer cell lines (12 - 14). Moreover, TGF-β synergizes with hypoxia, a potent stimulator of angiogenesis, in stimulation of VEGF promoter activity in a Smad3-dependent manner (9, 15). In contrast, the activation of the ALK1/BMPRII complex upon BMP9 binding has been shown to suppress VEGF expression both in cells *in vitro* and in mouse aorta, lungs, liver, and intestine (16), indicating distinct regulatory effects of ALK1 and ALK5 on VEGF expression by different TGF-β family members.

Thus the regulation of VEGF production is mediated by different TGF-β superfamily members via distinct signalling transduction routes in diverse cell types. Therefore, the effect of TGF-β family members on local angiogenesis is mediated in part through modulation of VEGF signalling (17 - 19). This cross-talk between VEGF and TGF-β signalling orchestrates in an intricately regulated manner the vascular balance, and interfering with either one will result in vascular malformations

VEGF and TGF-β intracellular crosstalk

VEGF and TGF-β family members play prominent roles in angiogenesis, and the interaction between the two signalling pathways modulates EC behavior. While VEGF controls endothelial cell functions in angiogenesis such as migration, proliferation, sprouting and permeability, TGF-β family members are prerequisite for vascular network modeling via the regulation of EC migration, death and survival (20). *In vitro* TGF-β/ALK5 signalling can induce apoptotic effects and inhibit EC proliferation, migration and sprouting (Chapter 2, 1, 3). Interestingly, treatment with a sub-optimal dose of the ALK5 kinase inhibitors SB-43152 or LY-364947 synergistically enhanced EC capillary formation and EC sprouting in response to VEGF, suggesting that crosstalk between TGF-β and VEGF signalling regulates endothelial cell behavior (Chapter 2, 21). This synergistic effect in EC sprouting is also observed when using TGF-β neutralizing antibodies in combination with VEGF (Chapter 2, 21). Transcriptional analysis revealed that inhibition of ALK5 signalling in combination with VEGF stimulation promotes the expression of anti-apoptotic factors and angiogenesis-associated integrins and down-regulates pro-apoptotic genes (Chapter 2). In the similar research line, Watabe *et al.* reported that the inhibition of TGF-β improves stem cell derived EC survival and endothelial sheet formation (22). Furthermore, TGF-β was shown to be able to convert VEGF activity from being anti-apoptotic to pro-apoptotic in EC by shifting the activation of p38 MAP kinase from the pro-survival p38β to the pro-apoptotic p38α MAP kinase (23, 24,). Remarkably the activation of p38α by TGF-β is transient and the ECs become refractory to TGF-β induced apoptosis due to reduced levels of p38α and increased levels of p38β MAP kinase (24), showing that the crosstalk between TGF-β and VEGF signalling dynamically regulates EC biology.

Since modulation of a signal transduction pathway can occur at different levels, e.g. at the level of the receptor or downstream modulators, we made attempts to identify at what level the crosstalk between VEGF and TGF-β occurred. Unfortunately, we did not observe any significant changes in either Smad signalling, p38 or ERK MAP kinase activity after treatment with ALK5 kinase inhibitor or co-stimulation with TGF-β and VEGF (unpublished data). VEGR2 triggers different and diverse signalling pathways to regulate EC function. Yet we cannot exclude the possibility of involvement of other downstream signalling events such as endothelial cell permeability-related nitric oxide synthase (eNOS/NOS3) activation, cell migration related focal adhesion kinase (FAK) activity or PI3 Kinase activity. In addition, our studies (Chapter 2) have only addressed the role of TGF-β in EC angiogenic property both *in vitro* and *in vivo*. A more extensive investigation on the effect of the other TGF-β super family members such as BMPs and their receptors on EC function will improve the current understanding of the TGF-β/VEGF crosstalk.

In summary, TGF-β and VEGF signalling intimately intertwine and crosstalk with each other to regulate EC function. Inhibition of TGF-β signalling potentiated the VEGF-induced angiogenesis (22, Chapter 2). However the fine-tune of this crosstalk in EC is also dependent on various factors: ligand gradient, time windows and local vascular bed condition. The interpretation of TGF-β/VEGF crosstalk can not only be based on any particular specific biological setting, but requires the local EC status and its interaction with neighboring other cell types in the local microenvironment.

Crosstalk between VEGF and TGF-β at the receptor level

Although our experiments did not provide evidence for direct interaction of the downstream intracellular VEGF and TGF-β signalling cascades, other studies offered alternative clues pointing to possible crosstalk at the receptor levels. Glinka and co-workers showed that in cancer cells the co-receptors of VEGF, neuropillin-1 and 2 have affinity for ALK5 and TβRII, and over-expressing of neuropillin-1 and 2 increased the levels of phosphorylated Smad2/3 (25) indicating neuropillins potentiates ALK5/Smad2/3 pathway. Furthermore, neuropillin-1 and -2 capture and activate latent TGF-β to be accessible by changing the conformation of the bound form of latent TGF-β. Given that neuropillin-1 and -2 are required for efficient VEGF signalling and enhance ALK5/Smad2/3 pathway (22, 26), it is possible that neuropillins may be a modulator for the interplay between TGF-β and VEGF signalling in endothelial function.

VE-cadherin is another interesting potential mediator for TGF-β and VEGF interplay. Clustered VE-cadherin improved the sensitivity of Smad activation upon TGF-β stimulation by enhancement of TβRI/TβRII assembly; disruption of VE-cadherin clustering results in reduction of TGF-β-induced inhibition of EC growth and migration (27). In addition, TGF-β induces assembly of the adherens junction complex by separating VEGF receptor 2 (VEGFR2) from VE-cadherin but increasing β-catenin association with both VEGFR2 and VE-cadherin (28). These studies suggest

that the interplay between TGF-β and VEGF pathways may be regulated by the formation of complexes containing TGF-β receptors and VEGF receptors with VE-cadherin as the core. It is plausible that the affinity of VE-cadherin for VEGFR2 and TGF-β receptor determines the TGF-β and VEGF crosstalk.

BMP9: both inhibit and stimulate angiogenesis

Out of more than 30 TGF-β superfamily ligands, there are over 20 BMP subfamily members. BMPs signal through both BMP receptors and activin type II receptors to relay their signals to the downstream cascades via different BMP type I receptors such as ALK1,-2,-3, and -6, while they are also regulated by intrinsic antagonists (e.g. noggin) (29). BMPs exert broad functions as a result of great complexity between combinations of ligands, type I and II receptors. BMP9 is a secreted circulating protein (30) that is highly expressed in the damaged liver (31) and binds to the extracellular domain of ALK1 and endoglin (30, 32). BMP9 signals via ALK1 to modulate endothelial cell function (Chapter 3; 33). Mutations in ALK1 are associated with the vascular disease HHT2. Mutations found in the extracellular domain of ALK1 interfere with the binding of BMP9, whilst HHT2 mutations in the intracellular domain are shown to reduce ALK1 activation upon BMP9 stimulation (34, 35), suggesting that there might be a role of BMP9 in the vascular disease HHT2.

BMP9 has an important role in angiogenesis (33, 36, 37). However, its effect on EC function and the mechanism of action remain unclear. Chapter 3 demonstrates an inhibitory effect of BMP9 on VEGF-induced angiogenesis. This result is supported by observations by David *et al.*, who reported that BMP9 functions as a circulating vascular quiescence factor (38). A recent *in vivo* study has confirmed that activation of ALK1 by BMP9 inhibits retina angiogenesis and blockade of the BMP9/ALK1 signalling by ALK1-Fc induced hypervascularization of retina (39). Interestingly, BMP9 has also been shown to stimulate EC proliferation and to be a pro-angiogenic factor in Matrigel plug vascularization and tumor angiogenesis in a xenograft model (40, 41). In addition, BMP9 has been shown to stimulate endothelial tube formation *in vitro* (43). Moreover, anti-hALK1 and anti-BMP9 antibodies can inhibit endothelial cell sprouting (37). These different results can in part be ascribed to differences in experimental setup: a low dose of BMP9 was found to have a pro-angiogenic effect, while a high concentration exerts an inhibitory effect on EC function (41; Chapter 3). However other factors can also contribute to the different results obtained. Differential receptor usage and/or different levels of activation of intracellular effectors of BMP9 may underlie its complex effects in different types of ECs.

The role of endoglin in endothelial cell function

Endoglin is expressed at the cell surface as a disulfide-linked homodimer, especially in active ECs (42, 44 45). It exists as long-form (L-endoglin) and short-form (S-endoglin) based on the different length of the cytoplasmic domain (46, Chapter 7). Apart from the membrane-bound forms, endoglin can be shedded by Matrix Metalloproteinase-14 (MMP14) existing as a soluble form (sol-Eng) (Chapter 3), which will be discussed later in detail.

In Chapter 4 we demonstrate that the lack of endoglin decreases the potential of VEGF signalling and VEGF-induced sprouting, resulting in defective endothelial cell sprouting. Mutations in endoglin are associated with the human vascular disease HHT1 (47). Endoglin heterozygous mice exhibit vascular lesions due to capillary malformation, resembling HHT1 syndromes (48 - 50). EC-specific deletion of endoglin in adult mice caused severe cerebrovascular dysplasia with the injection of adeno associated virus carrying VEGF; while one copy loss of endoglin only develops minimal irregular vascular formation (51). Our *in vitro* observations together with *in vivo* findings in the literature underscore the importance of endoglin in VEGF-induced vascular physiology. Yet the requirement of endoglin for efficient VEGF responses is likely not only restricted to ECs since endoglin is also expressed by fibroblasts, smooth muscle cells, and macrophages. Therefore, it is worthwhile to investigate the role of endoglin in VEGF-induced angiogenesis also by addressing other modulators of EC sprouting and the angiogenic microenvironment in order to achieve more insights.

Different forms of endoglin through differential splicing and membrane shedding

It remains very difficult to clarify the role of endoglin in EC function. Up to now, most of the studies about endoglin have focused on the characterization of the functional role of L-endoglin, which is usually referred to endoglin. A few studies have tried to elucidate the role of S-endoglin in EC function. Surprisingly S-endoglin has binding preference for ALK5 rather than ALK1, which provides a possible explanation for the different effects of L-endoglin on EC function (52, 53). Endoglin has been reported to impose opposing effects on EC. While endoglin was shown to be pro-angiogenic and required for EC function (2, Chapter 4), another study demonstrates that endoglin inhibits EC migration (54). The discrepancies among studies are not only due to differences in the experimental setup, such as the source of ECs model system, cell density, or even the expression ratio of S-endoglin/L-endoglin; but also the engagement of endoglin in distinct TGF-β/BMP receptor complexes in response to diverse TGF-β superfamily ligands. Endoglin is a pivotal modulater for ALK1 and ALK5 signalling in ECs. Yet further studies are required to elucidate the precise role of different forms of endoglin in EC function regulation upon TGF-β treatment in different biological environments.

Endoglin with its interactors other than TGF-β receptors in EC function

As addressed previously endoglin is required for VEGF-induced sprouting, however the exact mechanism remains unknown. Apart from its function as co-receptor for TGF-β/BMP receptor complexes, endoglin interacts with VE-cadherin (27). Our preliminary data suggested that endoglin-deficiency reduced the endogenous interaction between VE-cadherin and VEGFRII in ECs (unpublished data). Moreover VE-cadherin modulates the sensitivity of VEGFR2 signalling to cell density (55). These results suggest the possibility of a potential association between endoglin and VEGFR signalling activity via its interaction with VE-cadherin (Fig.1A). It would be interesting to investigate the effect of endoglin deficiency on VE-cadherin signalling as VE-cadherin fine tunes VEGFR2 and TGF-β signalling (Fig.1B).

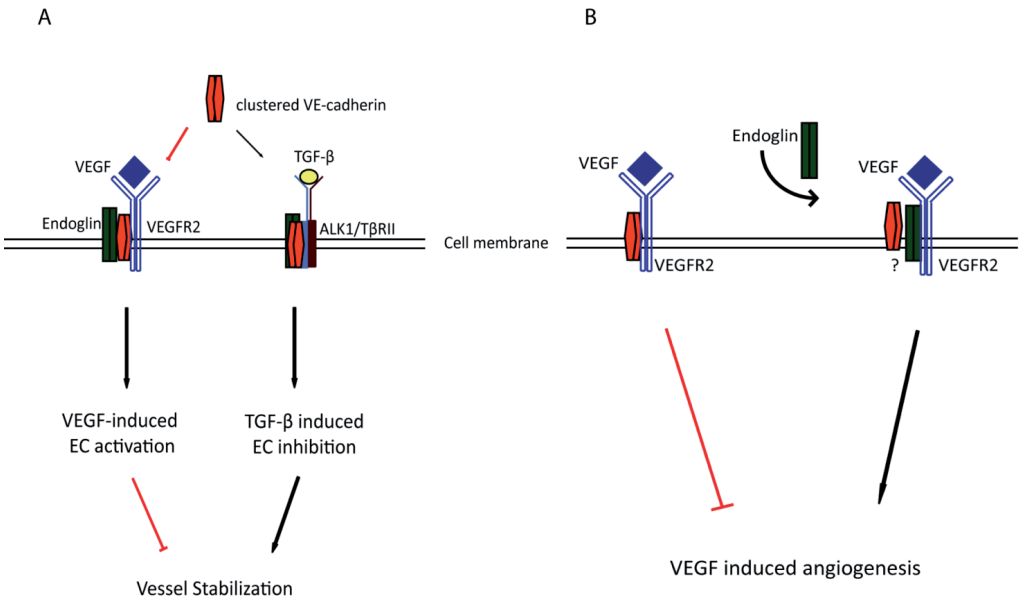

Fig 1 *Schematic possible roles of VE-cadherin and endoglin in controlling the angiogenic balance by regulating VEGF and TGF-β signalling pathways. (A) Clustered VE-cadherins may potentiaate TGF-β signalling and exert inhibitory effect on VEGFR2 signalling in ECs at high cell density, resulting in vessel stabilization. (B) Endoglin is indispensable for VEGF-induced angiogenesis. However, it is not well understood how endoglin deficiency affects VEGF-induced angiogenesis. It is possible that the endoglin may release the inhibitory effect of VE-cadheirn on VEGF signalling by altering the receptor complex composition.*

To further investigate the mechanism of the impact of endoglin on VEGFR2 signalling, we examined the effect of endoglin deficiency on VEGFR2. As tyrosine 1175 of VEGFR2 is an essential phosphorylation site for VEGFR2 activation (56, 57), the phosphorylation level of this site was examined in ECs with endoglin deficiency. Surprisingly, the tyrosine 1175 phosphorylation level was

strongly reduced in endoglin knockdown cells at 5 min of VEGF stimulation (Fig. 2). Subsequently we studied the phosphorylation level of downstream signalling components of VEGFR2 such as ERK and p38 MAP kinases. Unfortunately no changes in ERK and p38 phosphorylation were observed in endoglin deficient ECs upon VEGF stimulation (data not shown). It is possible that the ERK and p38 MAP kinases were not affected in this particular experimental setting due to the high amount of VEGF stimulation (25ng/ml), which may saturate the sensitivity of VEGF-induced MAP Kinase activity. Furthermore, a previous study showed that VEGFR2 preferentially signals to PI3 Kinase in confluent endothelial cells but promotes MAP Kinase activation in the sparse cells or VE-cadherin deficient ECs (55) (Fig. 1A). Thus VEGFR2 signalling may behave differently dependent on the local cell condition. In order to further characterize the influence of endoglin on VEGF signalling future studies should include the other downstream cascades of VEGFR2 such as FAK, AKT, eNOS, Src and PLCγ. Finally, it is not yet known whether lack of endoglin will influence the effect of VE-cadherin on VEGFR2 signalling.

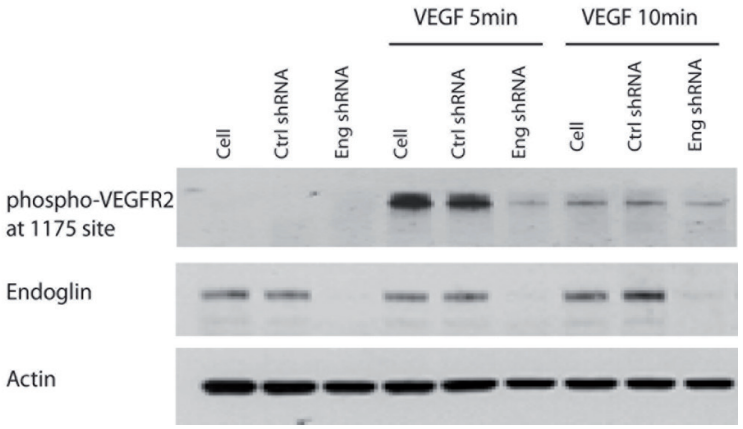

Fig. 2 *Phosphorylation level of VEGFR2 is reduced upon VEGF stimulation in endoglin knockdown ECs. HUVEC cells were stimulated with VEGF (25ng/ml) for 5min and 10 min. Non-infected cells (cell), control shRNA infected cells (Ctrl shRNA) and shRNA-mediated endoglin depleted cells (Eng shRNA) were used. Afterwards cells were lysed and samples subjected to SDS-PAGE and subsequent western blotting. Membranes were either probed with an antibody that specifically recognizes phosphorylated VEGFR2 at the site of 1175 and with an antibody directed against endoglin. An anti-β-actin antibody was used to confirm equal loading.*

The interaction between endoglin and VE-cadherin also provides a hint that endoglin may be involved in cell junctions/contacts. Our preliminary data of electron microscopic analysis showed that endoglin was localized at the cell-cell contacts (data not shown). Tight alignment of endoglin at the cell-cell contact in addition to its interaction with VE-cadherin could further enhance TGF-β signalling in EC stabilization (Fig. 3A). Furthermore, endoglin has been shown to be situated at the cell membrane and to control focal adhesion via its interaction with zyxin (58), and mediate the

activation of PI3 kinase and AKT at the cell membranes, leading to the stabilization of EC sprouting (59). These studies demonstrate the possible function of endoglin in EC junction, which can be important for vessel integrity during angiogenesis.

It is reported that endoglin not only interacts with integrin α5β1 to modulate the crosstalk between TGF-β/ALK1 signalling and fibronection/α5β1signalling, but also co-internalize with α5β1 to propagate integrin α5β1 signalling (4). The fibronection/α5β1signalling suppresses TGF-β induced EC migration in endoglin-depending manner, which could also be in part the explanation of the opposing role of endoglin in EC function in different experiment settings. Of note, the co-localization of endoglin and α5β1 in endocytosis is required for developmental angiogenesis in zebrafish (4), take together with the evidence that VEGF promotes α5β1 internalization to induce EC migration, It is plausible that endoglin could regulate VEGF-induced angiogenesis via its co-endocytotic partner integrin α5β1 leading to enhanced ALK1 signalling (Fig. 3B).

In summary, all these results indicate that endoglin does not function merely as a co-receptor for TGF-β signalling in controlling EC function. Endoglin appears to be involved in EC cell junctions and endocytosis. Current understanding of this possible function is limited. Screening for novel endoglin interactors will provide more insight in endoglin biology. Novel interactors and/or regulators may reveal a new function of endoglin in angiogenesis, in TGF-β/BMP signalling and beyond.

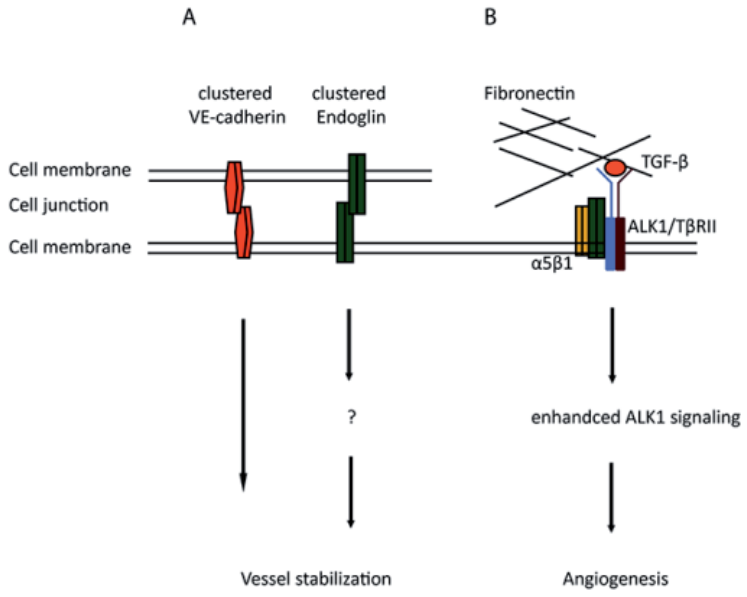

Fig. 3. *Possible role of endoglin in EC functions. (A) Endoglin is situated at the cell-cell contacts. Similar to clustered VE-cadherin, it is possible that the clustered endoglin contribuates to the vessel stabilization. (B) Fibronectin/α5β1 promotes ALK1signalling in a endoglin-dependet fashion, thereby promoting angiogenesis (Tian et al, 2012).*

The emerging therapeutic value of soluble endoglin and its pathological role in pre-eclampsia

Soluble endoglin (sol-Eng) is a circulating factor in serum (60). Elevated levels are observed in patients with pre-eclampsia (60, 61), cancer patients with metastasis (62, 63) and diabetic patients (64). Sol-Eng can be generated via matrix metalloproteinase (MMP)-14 mediated shedding of membrane bound endoglin on endothelial cells in colorectal cancer. The MMP14-mediated cleavage occurs at a site close to the transmembrane domain of endoglin (Chapter 5). Interestingly, it has been shown recently that MMP-14 can induce the release of soluble endoglin in pre-eclamptic placenta (65). The cleaved sol-Eng contains the extracellular domain, retaining the ability of binding to TGF-β and BMP-9 (32, 61). The reported sizes of sol-Eng appear to be variable in different studies. The sol-Eng from pre-eclampsia patients is around 65kDa; but the one cleaved by MMP14 is around 80kDa in tumor cells. This suggests that the endoglin may harbor multiple potential cleavage sites and that the generation of sol-Eng in tumors and pre-eclampsia may occur via different mechanisms. In chondrocytes an increase in the ALK1/ALK5 ratio leads to MMP13 production (66), but it is not known yet whether MMP13 harbors cleavage potential for endoglin. Yet it remains to be examined whether the ALK1/ALK5 ratio regulates MM14 production resulting in modulation of sol-Eng production.

Circulating sol-Eng may have systemic effects on angiogenesis. Sol-Eng contributes to pre-clampsia in concert with soluble VEGFR1 (solFlt1) (61). Elevated sol-Eng levels in the brain causes brain arteriovenous malformation via modulation of MMP activity but without changing the expression of membrane bound forms of endoglin (67). Yet further studies are required to establish a comprehensive understanding of the role of sol-Eng *in vivo*. High levels of circulating sol-Eng might lead to EC dysfunction due to its anti-angiogenic effect, which provides a potential therapeutic window for the endoglin neutralizing antibody to restore EC function. On the other hand, the anti-angiogenic effect of sol-Eng may be interesting for cancer treatment in the future, as an adjuvant therapy to selectively ablate blood vessels and thereby limiting tumor growth.

Future Perspective: TGF-β signalling shapes the sprout tip

A vessel sprout consists of tip and stalk cells. Tip cells are localized at the leading edge of vessel sprouts. The gradient of VEGF guides the direction of tip cells (68). With enriched filopodia-like cell protrusion at their migrating front tip cells are sensitive to local directional cues and thereby orientate the direction of sprouts accordingly (68, 69), while adjacent stalk cells contribute to the extension of the nascent sprouts (Fig. 4). However, the tip and stalk cell phenotypes are subject to a highly dynamic process of phenotype selection along vascular sprouts. As discussed before, in addition to their direct effects on EC behavior, a role for TGF-β family members in other events of vascular modeling is emerging, such as tip-stalk cell phenotype specification.

The TGF-β downstream components Id-1 and its upstream phosphorylated Smad1/5 (p-Smad1/5) exhibit distinct localization patterns in EC sprouts. At embryonic day 9.5 phosphorylated Smad1/5 is ubiquitously expressed in tip and stalk cells, whereas Id-1 is only distributed in the stalk cells (70). In the absence of Smad1/5 signalling, the stalk cells acquire plasticity, becoming tip-cell-like cells (70). This suggests that Smad1/5 signalling is important for stalk/tip cell selection and that Smad1/5 and Id-1 may exert divergent roles in the modulation of tip and stalk cell behavior. Moreover, p-Smad1/5 interacts with the Notch intracellular domain (NICD) to potentiate the expression of the Notch signalling downstream targets Hey1 (Herp2) and JAG1 (71, 72), while Id-1 form heterodimers with Hes-1 for Hes-1 stabilization in ECs (70). It thus appears that the interaction between Smad1/5 and Notch signalling regulates tip and stalk cell morphogenesis. Any impairment of the interplay may affect downstream target gene expression, and thereby result in loss of tip and stalk cell specification. Along with this notion, Larrviée *et al.* reported that blockade of ALK1 in tip cells displayed loss of stalk cell phenotype (39). Hence, TGF-β superfamily signalling cooperates with Notch signalling to specify the tip and stalk cell selection in early vascular sprouting events.

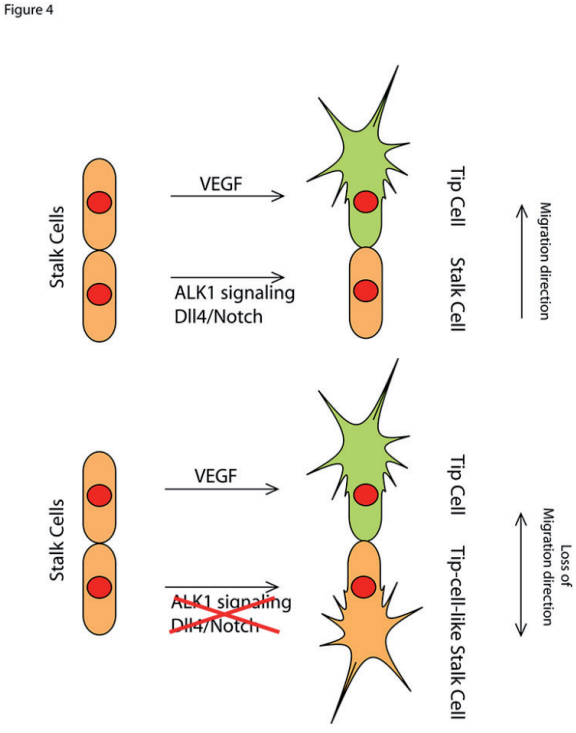

Fig. 4 *Scheme of stalk/tip cell phenotype selection. Stalk cell (orange) and tip cell (green) are under dynamic selection by several signalling pathways. The VEGF pathway drives the transformation of stalk cells into tip cells. ALK1-Smad1/5 signalling integrated with Notch signalling to maintain the adjacent stalk cell phenotype. Impaired ALK1 or Notch signalling turns the adjacent stalk cells into tip-like cells with hypervascularization, resulting in the loss of directed EC migration.*

Chapter 4 demonstrated the requirement of endoglin for VEGF-induced EC sprouting both *in vitro* and *ex vivo. In vivo* endoglin deficiency mice displayed abnormal vascular structure development upon VEGF stimulation. In addition, endoglin is reported to promote EC proliferation and migration (2, 54). These studies open the possibility that endoglin may hold an important function in tip cell development as it is a critical determinant in controlling endothelial behavior.

The crucial role of TGF-β superfamily signalling in angiogenesis has been underlined by numerous studies both *in vivo* and *in vitro.* The exact molecular mechanisms of TGF-β superfamily signalling in vascular development remain complex and require further elucidation. However, the increasing knowledge of TGF-β superfamily signalling and its crosstalk with other signalling pathways rationalize its physiological and pathological contributions to vascular remodeling. Intervention of TGF-β holds great potential as therapeutic agents in anti-angiogenesis therapy of cancer.

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Abbreviations

ACE	Angiotensin converting enzyme
ActR	Activin Receptor
AGT	Angiotensinogen
AGTR	Angiotensinogen receptor
AKT	Protein Kinase B
ALK/ACVL	Activin receptor-Like Kinase
AT-AAs	Angiotensin receptor activating autoantibodies
BAEC	Bovine aortic endothelial cell
Bax	Bcl2 associated X gene
Bcl2	B-Cell CLL/Lymphoma 2
bFGF	Basic Fibroblast Growth Factor
BMP	Bone morphogenetic protein
BMPER	BMP endothelial cell precursor-derived regulator
BMPRII	BMP receptor II
BRE	BMP reporter element
caALK5	Constitutivelt active ALK5
CASP6	Caspase 6
CCL5	Chemokine (C-C Motif) Ligand 5
CRC	Colorectal cancer
E	Embryonic day
EB	Embryonic body
EC	Endothelial cell
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
Endo-MT	Trans-differentiation of endothelial cell
Eng-Fc	recombinant endoglin-Fc chimera
eNOS	Endothelial nitric oxide synthase
ERCF	Immortalized HUVEC
ERK	Early response kinase/mitogen-activated protein kinase 1
ESC	Embryonic stem cell
FACS	Fluoresceent-activated cell sorting
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
Flk-1	Fetal liver kinase 1/ Vascular endothelial growth factor receptor 2
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HELLP	Hemolysis, elevated liver enzymes and low platelets
HHT	Hereditary Hemorrhagic Telangiectasia
HO-1	heme-oxygenase 1
HUVEC	Human umbilical vein endothelial cell
ID1	Inhibitor of DNA binding 1

Ig	Immunoglobullin
IL	interleukin
I-Smad	Inhibitory Smad
ITGA5	Integrin α5
ITGB3	Integrin β3
IUGR	Intrauterine growth restriction
LTBP	Latent TGF-β binding protein
MAEC	Mouse aortic endothelial cell
MEEC	Mouse embryonic endothelial cell
MEF	Mouse embryonic fibroblast
MFS	Marfan Syndrome
MMP	Matrix metallopeptidase
MVEC	Human normal dermal microvascular endothelial cell
PAH	Pulmonary arterial hypertension
PAI-1	Plasminogen activator inhibitor type 1
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PDCD4	Programmed cell death 4
PECAM-1/CD31	Platelet/endothelial cell adhesion molecule 1
PI3 Kinase	Phosphoinositide-3-kinase
PLCγ	Phospholipase C gamma
PlGF	Placental growth factor
qPCR	Real-time quantitative reverse transcription PCR
RAS	The renin-angiotensin system
R-Smad	Regulatory Smad
RT-PCR	Reverse transcription PCR
RUPP	Reduced uterine perfusion pressure
sEng	soluble Endoglin
sFlt-1	Solub;e fms-related tyrosine kinase 1/soluble VEGFR1
shRNA	short hairpin RNA
Smad	Small mothers against decapentaplegic
SMC	Smooth muscle cell
Src	V-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog
StAMP	Statin to ameliorate early onset pre-eclampsia
TBST	Tris-buffered saline and tween 20
TGF-β	Transforming growth factor β
TβRI	Transforming growth factor β receptor I
TβRII	Transforming growth factor β receptor II
VEGF	Vascular growth factor
VEGFR2	Vascular growth factor receptor 2
VSMC	Vascular smooth muscle cell
ZP	Zona pellucida

Summary

New blood vessels arise from pre-existing vessels when the supply of oxygen and nutrients and removal of waste products for the formation of new tissues is limited by diffusion (also termed as angiogenesis). Upon activation and under the guidance of vascular growth factors, endothelial cells (ECs) sprout from mother vessels and branch into nascent vessels. Subsequently pericytes/smooth muscle cells are recruited to the newly formed sprout for vessel stabilization. The primary vascular plexus is remodeled into a highly hierarchical vascular tree. Whereas angiogenesis is beneficial during wound healing, pregnancy, etc. and a prerequisite for development, abnormal angiogenesis is implicated in numerous vascular disorders, such as tumor angiogenesis, atherosclerosis, hypertension and other cardiovascular diseases.

The transforming growth factor (TGF)-β signalling pathway plays a major role in angiogenesis. It has been shown that TGF-β signalling affects both endothelial and mural cell function in genetic studies and *in vitro* cell culture models. Aberration of the TGF-β signalling cascade leads to abnormal remodeling and maturation of the primitive vascular plexus and decreased vessel wall integrity in adults. Targeted deletion of TGF-β signalling receptors in mice, such as ALK1, ALK5, TβRII or endoglin, results in embryonic lethality due to impaired vascular development. In humans, mutations in ALK1, ALK5, TβRII or endoglin are associated with human vascular diseases such as HHT and pulmonary hypertension (PAH). Vascular endothelial growth factor (VEGF) is a multifunctional molecule that is involved in vascular growth and remodeling. Similarly, in genetic mouse models abnormalities in or mis-regulation of the VEGF signalling pathway results also in embryonic death or severe vascular dysfunction. Perturbation in VEGF signalling also contributes to the pathology of tumor angiogenesis and cardiovascular diseases in humans.

This thesis is focused on the characterization of the crosstalk between the TGF-β and VEGF signalling pathways, on EC function, the effect of bone morphogenetic protein (BMP)9 on EC function and the role of endoglin in VEGF-induced angiogenesis. The results of these studies may give us insights into the impacts/effects of these two angiogenic signalling cascades on EC function. This can be beneficial for the understanding of the etiology of certain vascular diseases and the development of new treatment modalities in the future.

The key findings in this thesis are:

- Inhibition of the TGF-β type I receptor by a pharmaceutical kinase inhibitor enhances VEGF-induced angiogenesis both *in vitro* and *in vivo*. The crosstalk between TGF-β and VEGF leads to the induced expression of the pro-angiogenic integrin α5. *[Chapter 2]*
- BMP-9 is a potent stimulator of the ALK1 signalling pathway in endothelial cells and it inhibits VEGF-stimulated angiogenesis at high dosing. *[Chapter 3]*

- BMP-9 directly binds to endoglin and it potently induces endoglin expression at the transcriptional level. *[Chapter 3]*
- Endoglin is required for VEGF-induced angiogenesis in endothelial cells. VEGF-induced EC sprouting is decreased in ECs with endoglin-specific knockdown, but reduced endoglin expression does not affect major VEGF-induced signalling cascades. *[Chapter 4]*
- Absence of endoglin impairs the remodeling of vascular structures embryoid bodies, but doesn't affect endothelial cell differentiation in embryoid bodies. *[Chapter 4]*
- Shedding of endoglin is mediated in part by MMP14. *[Chapter 5]*
- The soluble form of endoglin exerts inhibitory effects on VEGF-induced angiogenesis. *[Chapter5]*

Our studies demonstrate that a crosstalk at the receptor level between VEGF signalling and TGF-β signalling affects EC function and angiogenesis *in vitro* and *in vivo*. In other words, these two signalling pathways work, together with other pathways, in a coordinated manner to maintain the balance in EC function or the optimal angiogenic output via the regulation of gene expression. Once the balance between TGF-β and VEGF signalling is disturbed, downstream gene expression profiles change followed by cell morphology, as well as cell functions such as migration and proliferation. These changes affect EC function and vessel formation. Consequently the development of the vasculature will be modified accordingly. Therefore, the studies described in this thesis further confirm the crucial role of TGF-β in EC function and provide some novel molecular mechanisms. On endothelial cells, TGF-β transduces its effect by binding to two distinct TGF-β type I receptors, ALK1 and ALK5. The two receptors result in distinctive impacts on endothelial cell behavior due to the local environment. Activation of ALK1 by BMP9 leads to inhibition of VEGF-induced angiogenesis, but the inhibition of ALK5 by pharmacological compounds results in enhancement of vascular network stimulated by VEGF.

Endoglin, an auxiliary TGF-β receptor, has been well studied and is considered as an important angiogenic molecule. It is shown in this thesis that endoglin is required for efficient VEGF-induced angiogenesis. The role of endoglin in VEGF signalling further supports the notion for interplay between the TGF-β and VEGF pathways in controlling EC function. Moreover, we show that soluble endoglin (sEng) and an endoglin neutralizing antibody (Tracon105) function as a suppressor/inhibitor of VEGF-induced angiogenesis (chapter 4). sEng negatively influences the angiogenic potential of EC and elevated level of sEng was found to be associated with EC dysfunction in pregnant women with hypertension and pre-eclampsia. Interestingly, aberrant concentrations of soluble fms-like tyrosine kinase 1(sFlt-1), also known as the soluble form of the VEGF receptor, contributes the onset of pre-eclampsia as well. This indicates again the interplay and non-redundancy of these two pathways in maintenance of EC homeostasis. Detection of circulating sEng and sFlt-1 hold promise for the early diagnostic potential for patients with pre-eclampsia.

Nederlandse samenvatting

Nieuwe bloedvaten ontstaan uit reeds bestaande als tijdens de ontwikkeling van nieuwe weefsels de toevoer van zuurstof en voedingsstoffen en de afvoer van afvalstoffen beperkt wordt door diffusie (bekend als angiogenese). Endotheel cellen ontspruiten dan uit de moeder-vaten en vertakken zich in beginnende nieuwe vaten wanneer ze geactiveerd en beïnvloed worden door vasculaire groeifactoren Voor stabilisatie van de nieuw gevormde vertakkingen worden vervolgens pericyten en vasculaire gladde spiercellen (murale cellen) gerecruteerd. De primaire vasculaire plexus wordt gehermodelleerd via een zeer hierarchische vasculaire genealogie. Waar angiogenese normaal gesproken gunstig is tijdens bijvoorbeeld wondheling en zwangerschap, is het zelfs een vereiste voor ontwikkeling. Abnormale angiogenese daarentegen wordt gerelateerd aan talrijke vasculaire afwijkingen zoals tumor-angiogenese, atherosclerose, hypertensie en ander cardiovasculaire aandoeningen.

De transforming growth factor (TGF)-β signaaltransductie route speelt een belangrijke rol in angiogenese. Het is aangetoond in genetische studies en *in vitro* celkweek modellen dat TGF-β signaaltransductie effect heeft op de functie van zowel endotheel als murale cellen. In volwassenen leidt aberratie in de TGF-β sigaaltransductie cascade tot een abnormale hermodellering en maturatie van de primitieve vasculaire plexus en een verlaging van de integriteit van de vaatwand. Gerichte deletie van TGF-β signaleringsreceptoren in muizen, zoals ALK1, ALK5, TβRII of endoglin, resulteert in embryonale lethaliteit door verstoorde vasculaire ontwikkeling. In mensen worden mutaties in ALK1, ALK5, TβRII of endoglin geassocieerd met humane vasculaire ziekten zoals hereditary hemorragic telangiectasia (HHT) en pulmonaire hypertensie (PAH). Vascular endothelial growth factor (VEGF) is een multifunctioneel molecuul betrokken bij de groei en hermodellering van bloedvaten. In genetische muismodellen ontstaat op vergelijkbare wijze embryonale lethaliteit of ernstige vasculaire afwijkingen wanneer er sprake is van misregulatie in VEGF signaaltransductie. Verstoring in VEGF signaaltransductie in mensen draagt tevens bij aan de pathologie van tumor-angiogenese en cardiovasculaire aandoeningen.

In dit proefschrift wordt de interferentie tussen de signaaltransductie routes van TGF-β en VEGF, het effect van bone morphogenic protein (BMP)-9 op endotheelcel functie, en de rol van endoglin in VEGF-geïnduceerde angiogenese gekarakteriseerd. De resultaten van deze studies kunnen ons inzicht geven in de angiogenese-gerelateerde effecten van deze twee signaaltransductie cascades op endotheel cel functie, wat van toegevoegde waarde is voor het begrijpen van de etiologie van bepaalde vasculaire afwijkingen en de ontwikkeling van nieuwe behandelmogelijkheden in de toekomst.

De belangrijkste bevindingen in dit proefschrift zijn:

- Inhibitie van de TGF-β type I receptor door een farmaceutische kinase inhibitor versterkt VEGF-geïnduceerde angiogenese zowel *in vitro* als *in vivo*. Interferentie tussen TGF-β en VEGF leidt tot een inductie van de expressie van pro-angiogeen integrin α5. *[Hoofdstuk 2]*
- BMP-9 is een krachtige stimulator van ALK1 signaaltransductie in endotheelcellen en blokkeert VEGF-gestimuleerde angiogenese in hoge dosering. *[Hoofdstuk 3]*
- BMP-9 gaat een directe binding aan met endoglin en geeft een sterke inductie van endoglin expressie op transcriptioneel niveau. *[Hoofdstuk 3]*
- Endoglin is noodzakelijk voor VEGF-geïnduceerde angiogenese in endotheelcellen. VEGF-geïnduceerde endotheelcel-ontspruiting is gereduceerd in endotheelcellen met een endoglin-specifieke knockdown, maar gereduceerde endoglin expressie heeft geen effect op de belangrijkste VEGF-geïnduceerde signaaltransductie routes. *[Hoofdstuk 4]*
- Het ontbreken van endoglin verhinderd hermodellering van vasculaire structuren in embryoid bodies, maar heeft geen effect op endotheelcel diferentiatie in embryoid bodies. *[Hoofdstuk 4]*
- 'Shedding' (verwijdering van het actieve gedeelte van het molecuul van het celmembraan) van endoglin wordt deels gereguleerd door MMP14. *[Hoofdstuk 5]*
- De oplosbare vorm van endoglin heeft een remmend effect op VEGF-geïnduceerde angiogenese. *[Hoofdstuk 5]*

Ons onderzoek demonstreert dat endotheelcel functie en angiogenese zowel *in vitro* als *in vivo* beïnvloed wordt door interferentie tussen VEGF en TGF-β signaaltransductie op receptor niveau. Met andere woorden, deze twee signaalroutes werken op een gecoördineerde manier samen (ook met andere routes) om zodoende de balans tussen endotheelcel functie en de optimale angiogene *output* te behouden via de regulatie van genexpressie. Zodra deze balans wordt verstoord, worden wijzigingen in het genexpressie profiel geïnduceerd, gevolgd door aanpassingen in de celmorfologie en eveneens in celfuncties zoals proliferatie en migratie. Deze veranderingen hebben invloed op endotheelcel functie en de vorming van vaten. Als gevolg hiervan zal de vasculatuur zich vormen naargelang de veranderingen in signalering.

De studies beschreven in dit proefschrift geven verdere bevestiging van de cruciale rol van TGF-β in de functie van het endotheel en voorziet in enkele nieuwe moleculair-mechanistische inzichten. TGF-β signaaltransductie in endotheelcellen verloopt via binding aan twee verschillende TGF-β type I receptoren, ALK1 en ALK5. Deze twee receptoren hebben een andere uitwerking op het gedrag van de endotheelcel, afhankelijk van het lokale milieu waarin de cel zich bevindt. Activatie van ALK1 door BMP-9 leidt tot remming van VEGF-geïnduceerde angiogenese, echter, inhibitie van ALK5 met farmacologische stoffen resulteert in een versterking van het vasculaire netwerk, gestimuleerd door VEGF.

Endoglin, een TGF-β co-receptor, wordt reeds lang bestudeerd en beschouwd als een belangrijk angiogeen molecuul. In dit proefschrift wordt bewezen dat endoglin noodzakelijk is voor efficiente VEGF-geïnduceerde angiogenese, wat nogmaals de relatie tussen de VEGF en TGF-β signaaltransductie routes in endotheelcel functie onderstreept. Bovendien demonstreren wij dat oplosbaar endoglin (sol-Eng) en een endoglin neutraliserend antilichaam functioneren (TRC105) als een remmer van VEGF-geïnduceerde angiogenese *(hoofdstuk 4)*. Sol-Eng beïnvloedt het angiogene potentieel van endotheel cellen negatief en gevonden is dat een stijging van het niveau van sol-Eng is geässocieerd met dysfunctie van endotheelcellen in zwangere vrouwen met hypertensie en pre-eclampsie. Interessant hierbij is dat afwijkende concentraties van oplosbaar fms-like tyrosine kinase-1 (sFlt-1), ook bekend als de oplosbare vorm van de VEGF receptor, ook bijdragen aan het ontstaan van pre-eclampsie. Dit is opnieuw een duidelijke indicatie van het samenspel tussen deze twee signaaltransductie routes, en van hun noodzakelijkheid in het onderhouden van de endotheelcel homeostase. De mogelijkheid tot het detecteren van circulerend sol-Eng en sFlt-1 beloven dan ook een belangrijke bijdrage te leveren aan het in een vroeg stadium kunnen diagnosticeren van pre-eclampsie in patiënten.

Curriculum Vitae

Zhen Liu was born on January 24th, 1981 in Qingdao, China. She graduated from Qingdao No2 Middle School. From 1999 to 2002, she majored in General biology in the college of life science, HuaZhong Agriculture University, whilst obtained minor in Economy in WuHan University as secondary bachelor. From 2003 to 2005, She attended the master course of molecular biology and bioinformatics in Amsterdam University (UvA). In master program, she performed her first 3-month internship in the department of microbiology at UvA. Thanks to the strong interests in signalling transduction in tumor biology, she was motivated to investigate the LPA signalling as her second internship at NKI under the supervision of Prof. Dr. Wouter Molenaar and Dr. Laurens van Meeteren.

In Sep 2005, Zhen started her PhD study under the supervision of Prof. Dr. Peter ten Dijke at the Leiden University Medical Centre (LUMC). Her research topic was to elucidate the cross talk between TGF-β and VEGF signalling on endothelial cell behavior. From Oct 2009 to Dec 2011, she worked as a post-doc fellow in Dr. Metello Innocenti's group at the department of cell biology at Dutch Cancer Institute NKI, with the focus on the role of actin dynamics in cell motility.

Life is full of surprises. Sometimes it is fulfilled with joy, but other times it offers tough time. Due to family reasons, Zhen resigned the post-doc fellowship in Jan 2012 and went back to China for the most important people in her life --- her family! Since Nov 2012, she has been working in the biotech company ACS. Biomarker B.V.

List of publications

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