Structural and Functional Characteristics of a Soluble Form of Endoglin in the Context of Preeclampsia

by

Allison Gregory

A thesis submitted in conformity with the requirements for the degree of Master of Science
Graduate Department of Immunology
University of Toronto

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Abstract

Endoglin is an auxiliary receptor for ligands of TGF-β receptor superfamily. It is an integral membrane protein highly expressed in the vascular endothelium. It is also present in the placental syncytiotrophoblast layer, and up-regulated during pregnancy. The expression of membrane endoglin (mEng) is further increased in the placenta during preeclampsia, a pregnancy-specific hypertensive syndrome. We hypothesize that the soluble form of endoglin (sEng) released from the placenta leads to endothelial dysfunction and hypertension by disrupting normal BMP-9 signaling. We first analyze the ligand specificity of mEng by comparing endoglin-deficient and normal endothelial cells. We show that the presence of mEng at the cell surface inhibits TGF-β1, BMP-2, and BMP-7 induced Smad1,5,8 phosphorylation while potentiating BMP-9 induced signaling. sEng has been shown to be elevated in the sera of preeclamptic women several weeks before the onset of clinical signs of preeclampsia and is postulated to interfere with endothelial cell function. We engineered a soluble form of sEng for both structural and functional studies. We show that sEng binds to BMP-9 with a 2 nM affinity, much higher than observed for other ligands. We also show that sEng can compete for BMP-9 binding to endothelial cells, resulting in inhibition of downstream Smad1,5,8 phosphorylation. Our results suggest that sEng is contributing to endothelial dysfunction and hypertension by dysregulating BMP-9 signaling in the maternal vasculature.
Dedication

To my family and friends for their love,

faith in me and constant support during the completion of my Master’s Degree.

&

Pete Mason for all his help, confidence, support and enthusiasm

he provided while I wrote my thesis.
Acknowledgments

I would like to thank my supervisor, Dr. Michelle Letarte, for giving me the opportunity to work in her lab, and for all her guidance and support. Without her push and belief in me, I would have never realized my potential in research as well as life in general. I would like to thank Dr. Luca Jovine, from the Karolinska Institutet in Stockholm, Sweden, for allowing me to work in his lab for 6 months. He gave me such an amazing opportunity and experience at the same time as renewing my passion for protein biochemistry. I am also grateful to the members of my graduate supervisory committee Drs. James Rini and Stuart Berger for all their help on this project.

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Ling Han in Dr. Luca Jovine’s Research Team

Supervised and assisted in the generation of stable cell lines, large scale protein expression and protein purification. This data was used to generate Figures 9 and 10.

Dr. Guoxiong Xu

Performed TGF-β1, BMP-2 and BMP-9 stimulation experiments. The data was used to generate Figures 11A, 11B and 11D.

Zhijie Li in Dr. James Rini’s Research Team

Supervised and assisted with all surface plasmon resonance experiments. The data was used to generate Figures 14 and 15, as well as Table 3.

Valentin Sotov

Assisted with all the surface Plasmon resonance experiments. The data was used to generate Figures 14 and 15, as well as Table 3.
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Figure 16 Model of the mechanism through which sEng contributes to endothelial cell dysfunction in the context of PE
Abbreviations

ActRII : Activin receptor type II

ACVRL1 : ALK1 gene (human)

ALK : Activin receptor-like kinase

AT1 : Angiotensin II type I receptor

AT1-AA : Angiotensin II type I receptor agonistic autoantibodies

AVM : Arteriovenous malformation

BMP : bone morphogenic protein

BMPRII : bone morphogenic protein receptor type II

CHO : Chinese hamster ovary

EndoH : endoglycosidase H

ENG : ENDOGLIN gene (human)

Eng : endoglin protein

Eng^{+/+} : Endoglin wild-type

Eng^{+-} : Endoglin heterozygous

Eng^{-/-} : Endoglin homozygous null

eNOS : endothelial nitric oxide synthase

ET1 : endothelin-1

GDF : growth differentiation factor

GlcNAc : glucosamine
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<td>HEK 293T</td>
<td>human embryonic kidney 293T cell</td>
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<tr>
<td>HHT</td>
<td>Hereditary Hemorrhagic Telangiectasia</td>
</tr>
<tr>
<td>HHT1</td>
<td>Hereditary Hemorrhagic Telangiectasia type 1</td>
</tr>
<tr>
<td>HHT2</td>
<td>Hereditary Hemorrhagic Telangiectasia type 2</td>
</tr>
<tr>
<td>HELLP</td>
<td>hemolysis, elevated liver enzymes, low platelets</td>
</tr>
<tr>
<td>HRE</td>
<td>hypoxia-responsive element</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human umbilical vein endothelial cells</td>
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<tr>
<td>I-Smads</td>
<td>Inhibitory Smads</td>
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<td>Id-1</td>
<td>Inhibitors of differentiation-1</td>
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<tr>
<td>IMAC</td>
<td>Ni$^{2+}$ immobilized metal affinity chromatography</td>
</tr>
<tr>
<td>KD</td>
<td>affinity constant</td>
</tr>
<tr>
<td>mEng</td>
<td>membrane endoglin</td>
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<tr>
<td>MISRII</td>
<td>Mullerian inhibitory substance type II receptor</td>
</tr>
<tr>
<td>MT1-MMP</td>
<td>membrane-type 1 matrix metalloproteinase</td>
</tr>
<tr>
<td>PAI-1</td>
<td>Plasminogen activator inhibitor-1</td>
</tr>
<tr>
<td>PE</td>
<td>Preeclampsia</td>
</tr>
<tr>
<td>R-Smads</td>
<td>Receptor activated Smads</td>
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<tr>
<td>sEng</td>
<td>soluble endoglin</td>
</tr>
<tr>
<td>sFlt1</td>
<td>soluble fms-like kinase (soluble VEGF receptor type 1)</td>
</tr>
<tr>
<td>SPR</td>
<td>surface plasmon resonance</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Explanation</td>
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<tr>
<td>-------------</td>
<td>-----------------------------------</td>
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<tr>
<td>TBRII</td>
<td>Transforming growth factor β receptor II</td>
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<td>TGF-β</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor α</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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<td>ZP</td>
<td>Zona pellucida</td>
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Chapter 1

Introduction

1 Introduction

The transforming growth factor β (TGF-β) superfamily of cytokines is one of the most ubiquitous regulators of embryonic development and of physiological and cellular processes. The superfamily is composed of multiple ligands and receptors that lead to a complex signaling network. Endoglin, an accessory receptor, has been shown to be a critical member of this family. This transmembrane glycoprotein is predominately expressed on proliferating endothelial cells. Through modulating the cellular response to multiple ligands from the TGF-β superfamily, endoglin has a critical role in embryonic development and angiogenesis. Endoglin has also been implicated in the pathogenesis of multiple diseases. Mutations in endoglin have been linked to hereditary hemorrhagic telangiectasia (HHT), an autosomal dominant vascular disorder. Endoglin has also been implicated in tumor angiogenesis and a soluble form of the membrane protein has been shown to be released in cancer. A similar form of soluble endoglin has been implicated in the pathogenesis of preeclampsia, a pregnancy-related condition resulting in maternal hypertension. The introduction will summarize briefly our knowledge of the TGF-β superfamily with a particular emphasis on endoglin in terms of structure and function and its soluble form in the context of preeclampsia.

1.1 TGF-β superfamily

The TGF-β superfamily is a large family of evolutionarily conserved cytokines that include transforming growth factor β (TGF-β), bone morphogenetic proteins (BMPs) and activins to name a few (1-3). Also included in this family are all the corresponding membrane receptors (1-3).

1.1.1 Ligands and receptors

The TGF-β superfamily of cytokines contain more than 30 structurally related polypeptide growth factors, which include: TGF-β1, -β2, -β3; activins A and B; inhibins A and B; BMP-1 to BMP-10; nodal and growth differentiation factors (GDFs) (1-3). These proteins all share a conserved cystine knot structure (4). All are initially translated as a precursor protein, composed
of a N-terminal pro-region and a C-terminal ligand (4). These precursors form covalently-linked dimeric structures in the endoplasmic reticulum, and then the pro-region is cleaved (5). In most cases, such as for BMPs, the pro-domain dissociates and the active ligand is released from the cell (5). However, in the case of the TGF-βs, the pro-region remains non-covalently attached to the ligand after secretion, requiring further steps for generation of an active ligand (6).

Briefly, ligands of the TGF-β superfamily initiate their cellular effects by interacting with a heteromeric receptor complex at the cell surface (7). All individual receptors exist as disulphide linked homodimers (7). The TGF-β superfamily of receptors can be divided into three broad categories: type I, type II and type III (7). The subsequent information is summarized in Table 1. The type I family, also known as Activin-like kinases (ALK), has seven members (ALK1 to ALK7), which are serine/threonine kinases (8). The type I receptors can be separated into 3 main groups based on sequence similarity: the ALK5 group which, includes the TGF-β type I receptor, ALK5, the Activin receptor, ALK4, and the Nodal receptor ALK7; the ALK3 group, consisting of the BMP type I receptors, ALK3 and ALK6; and the ALK1 group, with ALK1 and ALK2 interacting with different BMP/TGF-β/Activin family members (8, 9).

The kinase function of these receptors only becomes activated when phosphorylated by a type II receptor (8, 9). The type II sub-family of receptors contains 5 members, which are all constitutively active serine/threonine kinases (3). Type II receptors include the TGF-β type II receptor, TBRII, the Activin and BMP/GDF type II receptors, ActRII and ActRIIB and BMPRII, and the Mullerian inhibitory substance type II receptor, MISRII. The third category of receptors (type III) is commonly referred to as accessory or co-receptors (10). The two main members of this group are betaglycan and endoglin. These receptors function by modulating the signaling of multiple members of the TGF-β superfamily.

1.1.2 Signalling pathways and downstream effects

TGF-β superfamily ligands interact with a heteromeric receptor complex on the cell surface consisting of one of each of the type I and II receptors (11). The ligands have different affinities for different type I and II receptor combinations, that can be modulated by the presence of a type III receptor (3, 10). The ligand initially binds the ligand-binding receptor, type II for TGF-βs as opposed to type I for BMPs, and initiates a conformational change (3, 12-14). This results in the recruitment and binding of the appropriate second receptor, in turn resulting in receptor complex
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<th>Ligands, binding partners</th>
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<td>Activin A, BMP-6, BMP-7, MIS, TGF-β1, TGF-β2, TGF-β3</td>
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formation (7, 15). The type II receptor then phosphorylates the type I receptor, causing activation of the serine/threonine kinase activity (13, 16, 17).

The activated type I receptors mediate their cellular effects through phosphorylation of the Smad proteins, a family of conserved dimeric transcriptional factors (18, 19). Phosphorylation of the receptor Smads (R-Smads: Smad1, Smad2, Smad3, Smad5, Smad8) by the type I receptor results in R-Smad activation (19). Phosphorylated R-Smads complex with the common Smad4, translocate to the nucleus, and regulate gene expression (20, 21). Negative feedback in these pathways is mediated by the inhibitory Smads (I-Smads): Smad6 and Smad7 (22, 23). While it might seem like an infinite possibility of signalling pathways, commonly each type I receptor only activates a defined subset of R-Smads. ALK4, ALK5 and ALK7 are specific for induction of Smad2,3 phosphorylation, while ALK1, ALK2, ALK3 and ALK6 interact and activate Smad1,5,8 (10) (Table 1).

In most cell types, TGF-β1 and -β3 signal via ALK5 and TBRII resulting in Smad2,3 phosphorylation (Fig. 1) (24). This pathway is known to inhibit cellular proliferation, induce apoptosis, facilitate cellular adhesion, as well as promote homotypic cell aggregation. The Smad2,3 pathway induces expression of plasminogen activator inhibitor-1 (PAI-1), a negative regulator of cell migration and angiogenesis. TGF-β-induced Smad2,3 activation has also been shown to increase expression of extracellular matrix components such as collagen and fibronectin. However, in endothelial cells, TGF-β1,3 can also signal through ALK1, another type I receptor only expressed in this cell type (Fig. 1). Although TGF-β/ALK5/Smad2,3 signalling antagonizes TGF-β/Alk1/Smad1,5,8 signaling, ALK5 is essential for recruitment and activation of ALK1 (25). Just as the receptors are antagonistic, so are the pathways, ALK1 and ALK5 have opposite effects on endothelial cell migration and proliferation. The BMPs signal via multiple type I receptors (ALK1, ALK2, ALK3, ALK6), resulting in Smad1,5,8 phosphorylation (Fig. 2) (24, 26). While each BMP elicits its own unique cellular effect, in general, activation of the Smad1,5,8 pathway induces expression of Id-1, an inhibitor of basic helix-loop-helix proteins that promotes cell proliferation and migration (27). The complexity of these signaling pathways is further complicated by the modulating effects of TGF-β superfamily type III accessory receptors.
Both ALK1 and ALK5 are functional TGF-β type I receptors in endothelial cells. The constitutively active kinase, TβRII, binds TGF-β1 or TGF-β3 ligand, recruits ALK1 or ALK5 to the complex and phosphorylates the type I receptor cytoplasmic domain. This signalling complex may be modulated by endoglin, which can bind the ligand in association with TβRII. When ALK5 is phosphorylated, it transmits signals through Smad2/3, while activated ALK1 signals though Smad1/5/8. These receptor-activated Smads then bind the common partner Smad (Smad4) and translocate to the nucleus, where they regulate gene transcription. It has been proposed that TGF-β regulates the state of the endothelium via a fine balance between ALK1 and ALK5 pathways. Activation of the ALK5 pathway could be involved in vessel homeostasis through expression of proteinase inhibitors (eg. PAI-1), and the production of extracellular matrix proteins. On the other hand, ALK1 activation may be involved in new vessel formation, supported by expression of inhibitors of differentiation (Id-1) and genes involved in proliferation and migration.
Figure 2. BMP signalling pathways in endothelial cells.

ALK1, ALK2, ALK3 and ALK6 are all functional TGF-β type I receptors found on endothelial cells. The ligand-binding receptors (ALK1, -2, -3, -6) bind the BMP ligand, and then recruit the appropriate constitutively active kinase (BMPRII or ActRII). The type II receptor (constitutively active kinase) phosphorylates the type I receptor cytoplasmic domain. This signaling complex may be modulated by endoglin, which can only bind ligand in association with the ligand binding receptors. Endoglin has been shown to interact with BMP-2, BMP-7 and BMP-9. When the type I receptor is phosphorylated, it transmits signals through Smad1/5/8. These receptor-activated Smads then bind the common partner Smad (Smad4) and translocate to the nucleus and regulate gene transcription. Activation of the Smad1/5/8 pathway is associated with expression of inhibitors of differentiation (Id-1) and genes involved in proliferation and migration.
1.2 Endoglin distribution, structure and function

1.2.1 Endoglin expression profile

Endoglin, a type III accessory receptor, is a membrane protein that was first identified, cloned and sequenced in our laboratory. It was initially discovered on a lymphoblastic cell line derived from a pre-B acute lymphoblastic leukaemia, the HOON pre-B cell line, using the monoclonal antibody 44G4 (28). Use of the same antibody showed strong reactivity with endothelial cells from capillaries, arterioles and venules in a variety of tissues ranging from lymph nodes, tonsils, thymus, spleen, kidney, liver and lungs (28-30). This predominant expression in the vascular endothelium led to endoglin being classified as an endothelial cell marker (CD105) at the Vth International Leukocyte Workshop (30). Endoglin expression appears to be regulated by the activation status of the endothelium and is increased during the angiogenic process (31). In fact, endoglin is now widely used as an immunohistochemical marker of tumor angiogenesis (32).

Endoglin expression is not only increased on blood vessels undergoing angiogenesis, but also during an inflammatory state, in association with infiltrating cells (33). Endoglin is also highly expressed by the syncytiotrophoblast layer, which is the outer-most layer of the placenta, bathed in maternal blood (34). This expression is further increased if the mother is suffering from preeclampsia, a pregnancy related condition associated with hypertension (35). Endoglin expression is also highly upregulated on human endocardial cushion tissue mesenchyme during heart septation and valve formation, but returns to baseline as the valves mature (36).

Endoglin is also expressed by erythroid precursors (37), leukemic cells of the lymphoid and myeloid lineages (37, 38) and mesenchymal stem cells (39). Endoglin is induced upon activation of peripheral blood monocytes into macrophages (40). It is also expressed by vascular smooth muscle cells, as well as fibroblasts (41, 42).

1.2.2 Structural features of endoglin

The human CD105 gene was located to chromosome 9q34, identified using fluorescence in situ hybridization; the coding region contains 14 exons (43, 44). This sequence codes for a type I integral membrane protein (45). Endoglin is composed of a large extracellular domain with a short cytoplasmic domain (45). Between species, endoglin exhibits a fair degree of similarity, with the most highly conserved regions being the transmembrane and cytoplasmic domains. The
human and murine proteins share overall 74% identity, with 95% sequence identity in the transmembrane and cytoplasmic domains (42, 46). This could indicate that the cytoplasmic domain, while having no known enzymatic activity, may be critical for endoglin function within the cell. The closest known relative of endoglin is betaglycan, another TGF-β superfamily type III receptor (47). Overall, they share a significant sequence homology, particularly in the transmembrane and cytoplasmic domains (47).

Endoglin is composed of an N-terminal orphan domain followed by a zona pellucida (ZP) domain, juxtamembrane region, transmembrane region and short cytoplasmic domain (48, 49) (illustrated in Figure 3). The orphan domain covers amino acids 1-357 and is termed orphan since it has no known structural or functional homologue. The ZP domain consists of approximately 260 amino acids with 8 conserved cysteine residues (48, 50). This domain can be divided into two ZP sub-domains (ZP-N and ZP-C). The ZP-N sub-domain is highly conserved throughout most ZP proteins, while more variation can be observed in the ZP-C (48). Endoglin has a very similar ZP-N region to other ZP proteins, such as betaglycan, but with a divergent ZP-C sub-domain (48). The ZP domain can be involved in receptor oligomerization, as in the case for the ZP proteins surrounding the oocyte; however there is no evidence showing that endoglin forms oligomeric chains at the cell surface through the use of its ZP domain (48). As mentioned previously, while the entire protein shares roughly 70% homology between different species, the transmembrane and cytoplasmic domains of endoglin are highly conserved (27). The cytoplasmic domain, while having no enzymatic activity, can be phosphorylated by serine/threonine kinases, and likely contributes to the function of endoglin in the cell (51).

At the cell surface, endoglin exists as a 180 kD disulphide-linked homodimeric protein referred to in this thesis as membrane endoglin (mEng) (28, 29). The exact site of the interchain disulphide linkage remains unknown. Some believe that it is located at Cys582, however this residue is not conserved in all species such as mice, which still express a disulphide-linked homodimer (51). A study that utilized a chimaeric construct encoding the N-terminal sequence of monomeric PECAM-1 (CD31) antigen fused to residues Ile281-Ala658 of endoglin was expressed as a dimer, suggesting that cysteine residues contained within fragment Cys330-Cys412 are involved in the intrachain disulphide bond formation (52). More work needs to be done to elucidate the exact residues involved.
Figure 3. Schematic diagram of the endoglin protein.

Endoglin is an integral membrane protein. It is composed on an N-terminal signal peptide (1-26), an orphan domain (27-357), zona pellucida domain (358-586), juxtamembrane region, transmembrane region and short cytoplasmic domain. Within the cell it exists as a disulphide linked homodimer. The polypeptide structure is also illustrated with cysteine residues (●) numbered according to their position in the endoglin sequence.
Endoglin is a highly glycosylated protein with five potential sites for N-linked glycosylation (Asn88, Asn102, Asn121, Asn134, Asn307) within the orphan domain (45). Purified endoglin showed a reduction of 20 kD when treated with N-glycosidase or Endoglycosidase (29), suggesting that at least some of these sites are glycosylated. It has yet to be determined if these sites confer structural or functional importance. There is also evidence for the presence of O-linked glycosylation (45). The extracellular region extending from amino acids 311 to 551 is rich in serine and threonine residues, a characteristic of an area rich in O-glycans. This was confirmed by an observed 15 kD reduction following treatment of endoglin with O-glycanase and neuraminidase (29).

Currently little is known of the actual three-dimensional structure of endoglin (49). Using single particle electron microscopy, the structure of the extracellular domain was determined to a resolution of 25 Å (49). The molecular reconstruction suggested that endoglin might exist as a dome formed of two anti-parallel oriented monomers. The use of data fitting also suggested that each endoglin monomer itself was made up of three well defined regions, one orphan domain and two ZP domains (ZP-N and ZP-C). In contrast, recent experiments using small angle X-ray scattering revealed that endoglin might exist in a more elongated dimeric conformation (53). The information obtained from these structural studies has been very useful; however, a higher resolution structure is needed to further our understanding of endoglin. The generation of a high-resolution three-dimensional image of endoglin using X-ray crystallography is the next step in the quest to better understand not only the biochemistry of this highly important protein but its functional role as well.

**1.2.3 Association of endoglin with multiple members of the TGF-β superfamily**

Endoglin bound to ligand can be isolated as a complex with type I and type II receptors, using cross linkers with isolated cells and radioactive ligand (54). Initially, it was determined that endoglin binds TGF-β1 and TGF-β3 but not TGF-β2, in contrast to betaglycan, which can bind all three isoforms (54-56). Binding of TGF-β was originally demonstrated in human umbilical vein endothelial cells (HUVEC) (54), then in human bone marrow stromal cells (57), and haematopoietic cells (58). In view of the much lower number of receptors type I and II, relative to the amount of endoglin present at the cell surface, it was concluded that only a small fraction of endoglin was part of a TGF-β receptor complex (59). *In vitro* co-immunoprecipitation studies
of endoglin with type I and type II receptors indicated that endoglin can also interact with activin-A, BMP-7, and BMP-2 (54, 59, 60). Each of these interactions required co-expression of the ligand-binding kinase receptor (59, 60). Accordingly, endoglin binds TGF-β1 and TGF-β3 by interacting with TBRII. It can interact with activin-A and BMP-7 via their respective ActRII and ActRIIB receptors, and with BMP-2 via ALK3 and ALK6. More recently, it was shown in transfection experiments that endoglin could bind with high affinity to BMP-9 and BMP-10 in the absence of the ligand-binding kinase receptor, ALK1 (61). Over-expression of endoglin also increased BMP-9 responses (62).

Protein-protein interaction, co-immunoprecipitation, cross-linking, phosphorylation and functional experiments have been used to determine the interactions of endoglin with other receptors of the TGF-β superfamily. Endoglin has been found to interact with ALK5 and TBRII through both its extracellular and cytoplasmic domains (51). Endoglin has only been shown to interact with one other receptor via its cytoplasmic domain, ALK1 (63). The extracellular region is much more likely to interact with other receptors (32).

1.2.4 Functional role of endoglin within the cell and the organism

The evidence to date suggests TGF-β superfamily auxiliary receptors do not have an intrinsic signaling function but regulate ligand access to the signalling receptors. Betaglycan has been characterized as facilitating TGF-β binding to its kinase receptors (64). The exact mechanism through which endoglin modulates signalling remains to be determined. Several studies suggest endoglin functions as an ancillary receptor that contributes to the TGF-β superfamily system of regulation (27). A significant portion of the endoglin research thus far has focused on its involvement in TGF-β signalling. Human U937 myelomonocytic cells over-expressing endoglin were shown to block the following normal TGF-β1 responses: inhibition of cellular proliferation, down-regulation of c-myc mRNA, stimulation of fibronectin synthesis, cellular adhesion, and phosphorylation of PECAM-1 (65). Rat myoblasts transfected to over-express human endoglin showed similar effects (60). In HUVEC cells, which naturally express significant amounts of endoglin, an antisense approach was used to reduce expression, resulting in an increase of TGF-β1 inhibitory effects on cell proliferation and migration (66). It has also been shown that ALK1/Smad1,5,8 activation is enhanced in endothelial cells lacking endoglin, suggesting that endoglin may also inhibit this pathway (67). However other in vitro studies have shown that
endoglin promotes the effects of ALK1 in endothelial cells (63). Relatively little is known regarding endoglin function in BMP signalling. It has been shown that expression of endoglin can enhance BMP-7 and BMP-9-induced Smad1,5 activation and responses, although the mechanism by which this occurs is largely unknown (62, 68).

Endoglin is important in development since endoglin null (Eng<sup>-/-</sup>) mice are embryonic lethal at E10.5 with cardiac and vascular defects (69-71). There is vessel rupturing and a lack of endothelial-mesenchymal transition. Failed endocardial cushion formation, essential for valve development and heart septation, and pericardial edema were also detected (69). However the vasculature appears normal before E8.5. In vitro angioblast differentiation in Eng<sup>-/-</sup> embryonic stem cells was found to be unaffected by the loss of endoglin expression. The endothelial cell network in Eng<sup>-/-</sup> embryos also appeared to be normal. Endoglin has been implicated in early hematopoietic development. In vitro differentiation assays showed that Eng<sup>-/-</sup> mouse embryonic stem cells have reduced myelopoiesis and erythropoiesis, whereas generation of lymphoid and vascular precursors was unaffected (72).

1.3 Endoglin and disease

The importance of endoglin in normal function is highlighted by the fact that alterations in normal endoglin expression have been associated with multiple disease states (73). Mutations in the ENG gene lead to a vascular disease called hereditary hemorrhagic telangiectasia 1 (HHT1), also known as Rendu-Osler-Weber syndrome (43). The observation that endoglin expression is strongly up-regulated in the endothelium of various tumor tissues led to the discovery that endoglin is important, and even critical, in tumor angiogenesis (32). A soluble form of endoglin (sEng) has also been seen to be shed in cancer (32). A circulating form of sEng has also been implicated in preeclampsia, contributing to hypertension, one of the trademark symptoms (35). This section will explore the role of endoglin in these disease states.

1.3.1 Hereditary hemorrhagic telangiectasia

HHT is an autosomal dominant disorder with variable penetrance that is characterized by aberrant vascular development (74). HHT is not constrained by race or ethnicity, having a wide geographic distribution. Epidemiological studies have revealed an incidence of one in 5000 people (75, 76). Generally, clinical symptoms present during puberty. Classical symptoms
include spontaneous epistaxis (nosebleeds) and mucocutaneous telangiectases, which are generally the first disease manifestations (74). In addition, arteriovenous malformations (AVMs) in lung, brain, and liver and gastrointestinal telangiectases are the most severe consequences of this vascular disease. AVMs, like telangiectases, are direct connections between arteries and veins, bypassing the capillary bed (77). The shunting of blood through these lesions can lead to serious complications such as gastrointestinal bleeding, high cardiac output heart failure, hypoxemia, stroke, brain abscess and fatal haemorrhage (77, 78). Pulmonary AVMs are generally the first to become apparent, starting at puberty, with other characteristics of HHT developing with age (79).

Genetic linkage studies of families with HHT identified two disease associated loci, the *ENG* gene coding for the co-receptor endoglin (HHT1), and the *ACVRL1* gene coding for the ALK1 receptor (HHT2) (43, 80). In HHT1, 311 unique mutations in ENG, all contained within the extracellular domain, have been identified (74). These mutations are generally unique to a particular family, and include deletions, insertions, missense, and nonsense mutations (77, 81). *ENG* mutations result in unstable mRNA and protein, such that mutant proteins are rarely detectable (81-85). Our laboratory has characterized HHT1 as a haploinsufficiency-related condition, as opposed to a dominant negative genetic disease, since we demonstrated that endothelial cells from HHT1 patients express half the normal amount of endoglin. Further support for the haploinsufficiency model is obtained from the fact that multiple unique mutations of different types result in the same HHT phenotype (86). In the case of HHT2, 249 unique mutations in *ACVRL1* have been identified (74). In contrast to *ENG* mutations, *ACVRL1* mutations, largely missense, can be located in any of the extracellular, transmembrane or cytoplasmic domains. The majority of mutations affect protein folding and stability (74). Studies from our laboratory have demonstrated that HHT2 is also characterized by haploinsufficiency. There were reduced ALK1 protein levels in HUVEC samples of newborns with an ALK1 mutation (87, 88).

HHT is therefore due to a loss of function of endoglin and ALK1 in endothelial cells, where both genes are expressed. However, the mechanisms through which these receptors normally function are still being explored. This pursuit is impeded by the level of complexity associated with the TGF-β superfamily signalling in endothelial cells. Firstly, TGF-β can signal through both the ALK5/Smad2,3 pathway as well as the ALK1/Smad1,5,8 pathway, having opposing effects on
endothelial cell proliferation and migration (25, 89). The presence of these two pathways may allow TGF-β to function in a dose-dependent manner in endothelial cells, promoting proliferation and migration at low concentrations, while inhibiting these responses at higher ligand concentrations (25). The intricacies of this potentially functional balance are still to be determined.

It has been proposed that endoglin may have a protective effect against hypoxia and/or TGF-β induced apoptosis (90). The reduced endoglin expression in HHT may lead to massive apoptosis in capillary ECs, resulting in the loss of capillaries and ultimately development of an arteriovenous shunt. These malformations may appear only at distinct locations because of the need for an external trigger such as inflammation or injury, which are known to cause an upregulation in endoglin expression in known disease states (27, 91). This two hit model stresses the importance of the protective effects of endoglin. However, this model does not highlight the importance of ALK1, which is also implicated in HHT. ALK1 has been identified as the true type I receptor for BMP-9 (61). This ligand has also been shown to interact with endoglin in the absence of the ligand-binding kinase receptor (61). This suggests that the HHT phenotype is associated with decreased ALK1/Smad1,5,8 signalling. The potential role of BMP-9 in HHT must be considered. Clearly, further investigation is required to understand the role of endoglin and ALK1 in the pathobiology of HHT.

1.3.2 Cancer

It has been established that endoglin is expressed at low levels in resting endothelial cells but is upregulated in the vascular endothelium at sites of active angiogenesis (32). While angiogenesis is a normal physiological event, it is also critical for tumour growth, which is dependent on a continuous supply of oxygen and nutrients (27). As in the case of normal angiogenesis, the role of endoglin in tumor angiogenesis is shrouded by the complexity of the TGF-β superfamily. In tumors, enhancement of endoglin expression in endothelial cells is likely due to hypoxic microenvironments (32). In support of this view, a hypoxia-responsive element (HRE) has been characterized downstream of the main transcription start site in the ENG gene. Under low-oxygen conditions, the HIF-1 transcription factor complex binds the HRE and effectively increases endoglin promoter activity (92). This elevated endoglin expression is correlated with the proliferation of tumor endothelial cells (93). This has been shown to have a physiological
effect with $Eng^{+/−}$ mice having reduced angiogenic responses and consequently reduced tumor angiogenesis (94).

As well as being functionally important for tumor angiogenesis, this increased endoglin expression on tumor vessels has a multifaceted clinical value (32). Endoglin staining in microvessels has important prognostic value (32). Monoclonal antibodies to endoglin show greater specificity for tumor microvasculature, a prognostic indicator for multiple human tumors, than other endothelial markers such as CD31, CD34 or vWF (31, 95-98). This has been confirmed in multiple types of solid tumors such as breast carcinoma, prostate cancer and non-small cell lung cancer to name a few (97-99). As well as having prognostic value, endoglin expression has diagnostic value (32). Labelled antibodies to endoglin were shown to effectively accumulate in tumor specific tissue and were detectable with a high signal to noise ratio, suggesting that endoglin is a good target for tumor imaging (100-102). Using a similar mechanism, endoglin was found to be an exceptional target for anti-angiogenic therapy, aimed at preventing the development of new blood vessels within the tumor (32). In vivo studies revealed that injecting naked antibodies to endoglin in mice with human breast or colon cancer xenographs was associated with significantly smaller tumors and greater survival rates as compared to controls (103, 104). Taken together, these findings indicate that while endoglin promotes tumor angiogenesis, it also has potentially great prognostic, diagnostic and therapeutic value as a targeted marker.

Endoglin expression has also been detected on tumor cells of human sarcoma, melanoma, carcinoma and leukemia origin (105, 106). Endoglin expression is differentially regulated in a variety of tumors and correlates differently with tumor progression and metastasis, depending on cancer type. Endoglin expression is downregulated on malignant or metastatic prostate cancer cell lines as compared to the non-tumorigenic counterparts (107). Similarly, downregulation of endoglin expression in esophageal cancer cells is correlated with disease progression (108). Endoglin’s ability to antagonize TGF-β-mediated cell migration has been suggested as a possible mechanism. However, recently, endoglin was shown to have a pro-invasive role in metastatic breast cancer cells (109). Ectopic overexpression of endoglin enhanced migration and invasiveness in breast cancer cells. Endoglin clearly can play an important role in sporadic cancer, however, it may be context-dependent and the mechanism still needs to be determined.
A number of laboratories have also reported the detection of increased levels of a soluble, circulating form of endoglin (sEng) in serum, plasma or other fluids from cancer patients (110). Recently, it was shown that an 80 kD form of sEng was released from the surface of HUVEC cells after proteolytic cleavage by membrane-type 1 matrix metalloproteinase (MT1-MMP) (111). Using cleavage site mutants, it was shown that MT1-MMP cleaved endoglin between amino acids 586 and 587. Cleavage at this site results in the release of a nearly full-length endoglin extracellular domain (~80 kD) into circulation. There is still a significant amount of information to be determined regarding the cleaved protein. Its exact origin is unclear, being either the neovasculature or the cancer cells themselves, or both (110, 112, 113). Its influence on tumor development remains to be elucidated, but sEng has been identified as a marker of poor prognosis in cancer patients (110, 112). The measured serum levels of sEng were significantly higher in individuals with metastatic solid malignancies, mainly breast and colorectal, as compared to controls (112, 113). Increased levels of sEng in serum and urine have also been detected in patients with prostate cancer, making sEng a marker for the potential diagnosis of prostate cancer (114).

1.3.3 Preeclampsia

Both membrane (mEng) and soluble (sEng) forms of endoglin have been implicated in another disease termed preeclampsia. Preeclampsia is a major obstetric problem leading to substantial maternal, fetal and neonatal morbidity (and mortality) in 5-7% of pregnancies worldwide, especially in developing countries (115). This disease can manifest in two non-mutually exclusive ways, as fetal and maternal syndromes (116). The fetal syndrome can be characterized by fetal growth restriction, reduced amniotic fluid and abnormal oxygenation (117-119). This placental dysfunction is thought to give rise to the maternal syndrome that can be further divided into severe, early-onset (that develops before 34 weeks) and mild, late-onset (that appears after week 34) (115, 118). Mild, late-onset is characterized by hypertension and proteinurea. Severe preeclampsia can lead to the appearance of the HELLP (hemolysis, elevated liver enzymes, low platelets) syndrome and seizures (eclampsia) (115, 120). There are many theories concerning the pathogenesis of preeclampsia, but very little is understood about the patho-mechanism of this pregnancy-related condition.
The importance of the fetus and placenta in the development of PE can be seen by the fact that symptoms of disease rapidly disappear upon delivery (115). Currently, this is the only known treatment resulting in total relief of symptoms. It is presently believed that PE is induced by placental ischemia that results in fetal distress and dysfunction (121, 122). This ultimately results in the placenta releasing soluble factors into the maternal blood stream and causing the symptoms associated with PE (35, 123). In normal pregnancy, the extravillous trophoblasts of the placenta penetrate the maternal decidua and invade the maternal spiral arteries (124). The spiral arteries then become extensively remodelled by the invasive trophoblasts. The once high-resistance vessels become dilated, high-capacitance vascular channels that allow for fetal perfusion. In preeclampsia, it has been shown that the trophoblasts do not invade the spiral arteries to the same extent (124). This results in failure to convert the high-resistance vessels to high-capacitance vessels (125). This causes decreased fetal circulation and ultimately leads to placental and fetal ischemia. The exact events that lead to this improper vascular remodelling remain to be determined.

This placental ischemia is thought to lead to fetal and placental distress, resulting in multiple physiological changes. One such change is the increased expression of mEng. Expression of this protein is naturally upregulated on the syncitiotrophoblast during pregnancy; however, women with PE experience on average a fourfold increase in both mRNA and protein levels of mEng in the placenta (35). This change is thought to be brought on by the hypoxic environment created by the placental ischemia. As previously stated, endoglin expression can be upregulated by hypoxia in endothelial cells (92). The HIF-1 transcription factor, known to be induced by hypoxic conditions, has been shown to enhance endoglin promoter activity (92). It has also recently been shown that levels of endoglin are up-regulated by low oxygen both in vitro, using villous explants, and in vivo, using models of placental hypoxia pathology (126). The effect of this increased expression, however, remains to be determined.

Placental ischemia has also been linked to the release of multiple factors into the maternal circulation (121). These factors then target maternal endothelial cells resulting in endothelial cell dysfunction, which in turn leads to hypertension and other clinical manifestations of the maternal syndrome. It has been shown that two of these factors are sFlt-1 (soluble fms-like kinase, also known as soluble VEGF receptor type I) and sEng, which can both be detected in the circulation
6-8 weeks before the onset of clinical symptoms (35, 123, 127-130). Elevated levels of these factors have been shown to correlate with disease severity (35, 131).

Adenoviral expression of sEng in pregnant rats indicated that sEng can induce a significant increase in arterial pressure, further increased by the presence of sFlt1 (35). Low levels of proteinurea were also detected in sEng-treated rats, but nephritic damage was much more severe in sFlt1-treated rats (35). sEng and sFlt1 in combination lead to proteinurea, severe hypertension and the HELLP syndrome (35). While it is obvious that sEng plays an important role in disease pathogenesis, the means through which it is released and the mechanism leading to endothelial dysfunction are still largely unknown.

While sFlt1 is a splice variant, the 65 kD sEng is believed to be generated by cleavage of mEng by a metalloproteinase (35). The exact form of sEng released into circulation is unknown, since insufficient amounts of this soluble protein could be purified from maternal serum to perform C-terminal sequencing (35). MT1-MMP has been implicated in mEng cleavage from endothelial cells in the context of cancer; however, the product predicted to be Eng1-586 is larger (80kD) than the sEng detected in PE (111).

Multiple mechanisms have been proposed to explain the release of sEng. Similar to mEng, the levels of sEng are also positively correlated with hypoxic conditions. Low oxygen levels have also been shown to upregulate multiple proteases, such as MT1-MMP; combined with increased mEng expression, this may ultimately lead to increased release of sEng. A significant amount of work has also linked this increased expression to a more immunological pathogenesis model (115) (Fig. 4). Walluket et al. showed that women with PE have autoantibodies that stimulate the angiotensin II type I receptor (AT1), which is an important component of the renin-angiotensin system (132). Through activation of the AT1 receptor, angiotensin II type I receptor agonistic autoantibodies (AT1-AA) have been shown to produce preeclamptic symptoms in pregnant women (133-135). It is suggested that placental ischemia leads to production of AT1-AA. Pregnant rats exposed to chronic reduced uterine perfusion pressure (RUPP) showed the presence of AT1-AA (136). These antibodies were also detectable between 18-22 weeks of pregnancy in women with abnormal uterine perfusion determined by Doppler ultrasound (137). AT1-AA from preeclamptic patients were shown to effectively interact with the AT1 receptors in pregnant mice and induce symptoms of disease, hypertension and proteinurea (138). The
Figure 4. Model of the angiotensin II type I receptor agonistic antibodies (AT₁-AA) mediated release of placental factors.

This diagram represents a possible signaling cascade by which placental ischemia leads to the generation and release of AT₁-AA. These antibodies are free to activate the angiotensin II type I receptor (AT₁R) which results in an increased expression of tumor necrosis factor-α (TNF-α). This leads to the increased release of soluble factors into the maternal circulation. Soluble endoglin (sEng), soluble VEGF receptor 1 (sFlt-1) and endothelin-1 have all be shown to contribute to maternal hypertension in preeclampsia.
hypertension is thought to be partially mediated by the ability of AT$_1$-AA to induce endothelin-1 (ET$_1$) expression, a well-known vasoconstrictor. Injection of IgG from women with PE into pregnant mice also showed that AT$_1$-AA lead to increased placental sEng expression by AT$_1$ activation (139). The mechanism is mediated by increased TNF-α expression in the placenta. Both membrane and soluble endoglin expression were increased in human extravillous explants treated with IgG from preeclamptic women, as compared to controls, and this was prevented by the presence of a TNF-α inhibitor. Increased shedding of endoglin could also be mediated by the ability of TNF-α to upregulate multiple proteases. Both hypoxia and agonistic antibodies represent viable means of inducing the expression of both forms of endoglin, and are likely not mutually exclusive (115).

While a significant amount of progress has been made in understanding the release of sEng, these is still a large amount of work to be done in regards to the role of sEng in endothelial dysfunction and ultimately hypertension. It has been shown that sEng inhibits TGF-β-mediated eNOS-dependent vasodilatation (35). Both TGF-β1 and TGF-β3 induced vasodilatation in isolated rat renal microvessels (35). sEng was able to attenuate these effects. TGF-β mediates this effect via eNOS dephosphorylation at Thr495, which precedes activation of eNOS via Ser1177 phosphorylation. TGF-β could act synergistically with VEGF, since VEGF induces eNOS Ser1177 phosphorylation (140, 141). sFlt1 leads to hypertension by binding VEGF and no longer allowing for eNOS activation or its downstream vasodilation (131). It is logical to suggest sEng works in a similar manner. With the use of a radio-labelled binding assay, it was found sEng significantly reduced the ability of TGF-β1 to bind TBRII (35). This would effectively limit the ability of TGF-β to allow for eNOS activation. sEng also inhibited TGF-β1-induced activation of the Smad2,3 pathway (35). Taken together, these data suggested that sEng competes for TGF-β binding to its receptor; however, there is no direct evidence indicating that sEng binds TGF-β in the absence of the ligand-binding receptor, TBRII.

1.4 Thesis objectives

1.4.1 Rationale

For a long time, it has been known that endoglin modulates the signalling of multiple members of the TGF-β superfamily. Endoglin effects differ based on cell type and environmental milieu, and are therefore context-dependent. The significant amount of research conducted thus far has
allowed us a basic understanding of endoglin biochemistry and function. Structural studies have
provided a globular image of endoglin; however, a higher resolution structure is needed to
further our understanding in both physiological and pathological settings.

Within the context of preeclampsia, a soluble form of endoglin is released from the
syncytiotrophoblast into the maternal blood stream. This soluble protein has been shown to
contribute to the development of high blood pressure, a characteristic symptom of preeclampsia;
however, the exact mode of action has yet to be understood. The current dogma is that soluble
endoglin sequesters TGF-β1,3, resulting in endothelial dysfunction and ultimately leading to
hypertension. However, it was shown that endoglin can only bind TGF-β1,3 in the presence of
TBRII. Interestingly, preliminary results have shown that endoglin may have the capacity to bind
BMP-9 in the absence of its ligand-binding receptor and this specificity may be more relevant to
the role of endoglin in preeclampsia.

1.4.2 Hypothesis
Soluble endoglin contributes to endothelial dysfunction within the context of preeclampsia by
binding and effectively competing for BMP-9.

1.4.3 Specific objectives
Our main objectives were:

I) To generate soluble and stable endoglin proteins that could be produced in large-scale
quantities for the generation of protein crystals for X-ray crystallography and for
functional studies.

II) To determine if soluble endoglin can interact with various ligands of the TGF-
β superfamily, in order to better understand how sEng leads to preeclampsia.
Chapter 2
Materials and Methods

2 Materials and Methods

2.1 DNA Constructs

Constructs containing the full-length endoglin coding sequence (Eng1-658) and the extracellular domain (Eng1-586) had been previously generated in the laboratory. They were inserted between Kpn1 and Xba1 restriction sites in a modified version of the mammalian pCMV5 vector (Invitrogen), with a C-terminal Flag-tag. All subsequent endoglin constructs (Eng1-357, Eng358-581, Eng358-586) were generated by PCR amplifying segments of the full length human endoglin sequence (accession number: NM_001114753) to contain Kpn1 and Xba1 restriction sites to facilitate their subcloning into the pCMV5 vector with the C-terminal Flag-tag. The forward (F) and reverse (R) primers used for cloning are shown in Table 2.

Glycosylation mutants were obtained by sequential site-directed mutagenesis of glycosylation sites from asparagine (Asn;N) to glutamine (Gln;Q) residues. Site-directed mutagenesis was performed using the GeneTailor Site-Directed Mutagenesis kit (Invitrogen) along with KOD XL DNA polymerase (Novagen). The protocol was optimized from the manufacturer’s specifications. The primers are shown in Table 2.

Wild-type and glycosylation mutant forms of Eng1-586 and Eng1-357 were sub-cloned into Chinese Hamster Elongation Factor 1 (CHEF1) plasmids pDEF28 and pNEF38 (CMC Biologics) with a C-terminal 6xHis tag. The desired endoglin sequences were inserted between Sal1 and Xba1, with the cut sites added to the inserts during PCR amplification. The primers are shown in Table 2.

All constructs were confirmed by DNA sequencing and aligned to control sequence using Vector NTI.
Table 2: Primers used for cloning, site-directed mutagenesis and sub-cloning

<table>
<thead>
<tr>
<th>Purpose</th>
<th>Construct</th>
<th>Direction</th>
<th>Cut Site</th>
<th>Primer</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Eng1-357</td>
<td>F</td>
<td>Kpn1</td>
<td>5'-CCGAGTACATGGAGCCGC GGCACGGTCTCGT-3'</td>
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<td>Xba1</td>
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<td>Kpn1</td>
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2.2 Cell Culture

Human renal epithelial (HEK) 293T cells (ATCC: CRL-1573) were cultured in DMEM medium (GIBCO) and supplemented with 10% fetal bovine serum (GIBCO) and 5% antibiotics (Penicillin/Streptomycin solution, GIBCO).

Immortalized mouse embryonic endothelial cells expressing either wild-type levels of membrane endoglin \((\text{Eng}^{+/+})\) or no membrane endoglin \((\text{Eng}^{-/-})\) were originally developed in Letarte laboratory from murine embryo and yolk sacs harvested at E9.0 of gestation (67). These cells are cultured in MCDB 131 medium (GIBCO) supplemented with 15% fetal bovine serum (GIBCO), 5% glutamine solution (GIBCO), 5% antibiotics (Penicillin/Streptomycin solution, GIBCO) and 25 \(\mu\)g/ml endothelial mitogen (Biomedical Technologies Inc.).

2.3 Transient transfection and small-scale protein expression

HEK 293T cells were transiently transfected with 1 \(\mu\)g of the indicated plasmids using FUGENE6 (Roche) as described by the manufacturer. 24 h after transfection, cells were washed with PBS (Invitrogen), and cultured for 24 h in DMEM without supplements. Culture media was then collected, debris removed by centrifugation (4˚C, 15800xg, 5 min) and the media concentrated 10-fold using an Ultracel\textsuperscript{TM} 10kD centrifugal filter unit (Millipore). Lysate fractions were prepared by scraping cells and solubilizing in lysis buffer (50mM Tris-HCl pH 7.4, 100mM NaCl, 1mM EDTA, 1% Triton X-100, 10mM sodium pyrophosphate, 25mM NaF, 1mM Na\textsubscript{3}VO\textsubscript{4}, 1% ProteoBlock\textsuperscript{TM} protease inhibitor cocktail (Fermentas)). Protein concentration was determined by the Dc protein assay, a modified Lowry assay (Bio-rad), and samples solubilised in SDS sample buffer (60 mM Tris-HCl pH 6.8, 2% Sodium dodecyl sulfate, 5% glycerol, 10mM Dithiothreitol, 0.005% Bromophenol blue). Samples intended for non-reducing gels were solubilised in the same SDS sample buffer only lacking the reducing agent, Dithiothreitol.

2.4 Generation of protein and subsequent deglycosylation

HEK 293T cells were transiently transfected with the indicated plasmids using FUGENE6 (Roche) as described by the manufacturer. Cells were grown in complete medium (lacking antibiotics) supplemented with 5 \(\mu\)M kifunensine (Toronto Research Chemicals). After 24 h, cells were washed with PBS and cultured for 24 h in DMEM supplemented with 5 \(\mu\)M
kifunensine. Tissue culture supernatant containing the secreted proteins was harvested, debris removed by centrifugation (4°C, 15800xg, 5 min) and then concentrated 10-fold using an Ultracel™ 10kD centrifugal filter unit. Proteins contained in the media were deglycosylated with EndoH (New England Biolabs). The EndoH was initially prepared by serial dilutions in 0.1 M sodium acetate pH 5.2. Samples of concentrated media (20μl) were exposed to a range of EndoH (100U, 200U, 400U) concentrations, and incubated for 3h at 4°C (control) or 37°C. The extent of deglycosylation was monitored by immunoblot.

2.5 Cell-based stimulation and inhibition assays

In stimulation studies, equivalent numbers of endothelial cells were seeded in complete medium. After 48 h, cells were starved for 3 h in serum-free medium and treated with increasing concentrations of ligand (TGF-β1, BMP-2,7,9; R&D) for 30 min. Lysates were prepared by scraping the cells and solubilizing in lysis buffer as described above. Protein concentration was estimated and samples were further solubilized in SDS sample buffer (60 mM Tris-HCl pH 6.8, 2% Sodium dodecyl sulfate, 5% glycerol, 10mM Dithiothreitol, 0.005% Bromophenol blue). Samples intended for non-reducing gels were solubilised in the same SDS sample buffer only lacking the reducing agent, Dithiothreitol.

In inhibition studies, 5.0x10^5 endothelial cells were seeded in complete medium. After 48 h, cells were starved for 2 h in serum-free medium, exposed to increasing concentrations of soluble receptors (sAlk1, sEng, sTBRII ; R&D) for 1 hour and then treated with a defined concentration of each ligand (TGF-β1, BMP-2,7,9; R&D). Lysates were collected as previously described. Protein concentration was determined by the Dc protein assay, a modified Lowry assay (Bio-rad), and samples were solubilized in SDS sample buffer (60 mM Tris-HCl pH 6.8, 2% Sodium dodecyl sulfate, 5% glycerol, 10mM Dithiothreitol, 0.005% Bromophenol blue). Samples intended for non-reducing gels were solubilised in the same SDS sample buffer only lacking the reducing agent, Dithiothreitol.

2.6 Generation of stable cell lines and large-scale protein expression

For stable expression, dihydrofolate reductase-deficient CHO DG44 cells (Invitrogen), grown in α-MEM medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), were co-
transfected with the pDEF38 and pNEF38 CHEF1 vector pairs containing wild-type and triple glycosylation mutant forms of Eng1-586 and Eng1-357 using the Lipofectamine™ 2000 system. Stably transfected clones were grown in α-MEM medium lacking hypoxanthine/thymidine, and supplemented with 8% dialyzed fetal bovine serum (Invitrogen) and 0.8mg/ml G418 (Invitrogen). 96 single cell colonies per cell line were selected and plated at constant density. Clones expressing high levels of the C-terminally histidine-tagged endoglin constructs were identified by immunoblot. The 8 clones (12 for Eng1-357-N-121,134,307-Q-His) expressing the highest levels of C-terminally histidine-tagged endoglin constructs were selected, plated at constant density, media analyzed via immunoblot and cells counted at time of harvest. The 3 best clones for each cell line, selected based on endoglin production and low cell number at time of harvest, were amplified and stored.

For large scale protein expression, selected stable cell lines were grown in 3L spinner flasks at initial concentrations of 0.3x10⁶ cells/ml in serum-free medium HyQ SFM4CHO-utility medium (HyClone). Cells were grown until they reached 60% viability (roughly 2-3 weeks), then the media was harvested.

2.7 Protein Purification

Secreted sEng proteins were captured from conditioned medium by batch binding with Ni-NTA Superflow beads (QIAGEN) overnight. The IMAC column was gravity packed and then equilibrated with Buffer A (20 mM NaHEPES [pH 7.8], 150 mM NaCl, 20 mM Imidazole). Bound protein was eluted using a 20 mM to 500 mM imidazole gradient for 100 min at 1ml/min. Subsequently, the eluted peak was concentrated and then dialyzed for 24 h against gel filtration buffer (10mM Tris-HCl pH 7.2, 50mM NaCl). Samples were subjected to size exclusion chromatography using a 10/300 Superdex-200 column (GE Healthcare) equilibrated against gel filtration buffer and fractionated at 0.3 ml/min in 10mM Tris-HCl pH 7.2, 50 mM NaCl.

2.8 SDS-PAGE and Western blotting

All protein samples were run on NuPAGE Bis-Tris 4-12% SDS-PAGE gels (Invitrogen) for approximately 60 min at 170V, followed by electrophoretic transfer to nitrocellulose (Millipore) for 90 min at 200 mA. Blocking proceeded for 90 min at room temperature in blocking solution [5% milk powder (Bio-Rad) in Tris-buffered saline/0.1% Tween-20 (TBS-T)]. The blots were
stripped using a 1M glycine solution (pH 2.5) and washed with TBS-T. Western blot (or immunoblots) were used to detect expression of endoglin constructs using a primary antibody, rabbit polyclonal H300 (Santa Cruz; 1:1000), that recognizes both reduced and non-reduced endoglin. Dot blot and Western blot experiments, for analysis of stably transfected clones, used the primary mouse monoclonal antibody to the 5His tag (QIAGEN; 1:1000). For functional studies, rabbit polyclonal anti-phospho-Smad1/5/8 (Cell Signalling; 1:1000), and mouse monoclonal anti-Smad1(Santa Cruz; 1:1000). Secondary antibodies were horseradish peroxidase-conjugated AffiniPure sheep anti-mouse and donkey anti-rabbit (GE Healthcare, 1:10,000). Bands were visualized using Western Lighting™ Chemiluminescent Reagent Plus (PerkinElmer) followed by exposure to Hyperfilm™ ECL™ (Amersham Biosciences). All immunoblots were performed in duplicate.

2.9 BIAcore Biosensor Analysis

All analyses were carried out on a BIAcore X (BIAcore, Uppsala, Sweden) at 25°C. Research grade CM5 sensor chips, N-hydroxysuccinimide (NHS), N-ethyl-N’-(3-diethylaminopropyl) carboxydiimide (EDC), and ethanol-amine hydrochloride were purchased from the manufacturer (BIAcore Inc.). sALK1-Fc and sTBRII-Fc were purchased from R&D. sEng was generated and purified as previously described. All ligand/analytes (TGF-β1, BMP-2,7,9) were purchased from R&D and prepared according to the manufacturers specifications. All buffers were filtered and degassed.

For amine coupling, carboxymethylated dextran surfaces on CM5 chips were activated using the standard NHS/EDC procedure. Soluble receptors were diluted to 20 nM in 10 nM sodium acetate buffer, pH 6. The receptor solutions were injected over the activated surface until the desired surface densities were achieved. Activated coupled surfaces were then quenched of reactive sites with 1 M ethanolamine, pH 8.5, for 7 min. Reference surfaces were activated as described above and then quenched with 1M ethanolamine, pH 8.5, for 7 min.

All data was collected at 10 Hz using at least 5 injections for each receptor/ligand pair. Flow rates during each injection were maintained, and ranged from 2-20 μL/min based on usable sample volume. Regeneration of sALK1 chip was carried out with a 5 μL injection of 3M MgCl₂. Regeneration of all other active surfaces (when necessary) was carried out with a 5 μL
injection of regeneration buffer (0.4M urea, 0.9M MgCl₂, 25mM EDTA, 0.9M guanidine-HCl, 10mM glycine pH 9.5).

2.10 Analysis of Biosensor Data
BIAevaluation 4.1 software was used to measure and plot the \( k_{on} \) and \( k_{off} \) values directly, which were then used to calculate the affinity (\( K_D \)). We confirmed the BIAevaluation 4.1 software affinities using the plateau method. In this method we plotted RU difference vs. analyte concentration. Hyperbolic curve fitting was used to determine the hyperbolic equation describing the relationship between RU difference and analyte concentration. The concentration that generated the half-maximal RU difference represents the affinity of the interaction (\( K_D \)). The highest RU difference observed was assumed to be the maximal RU difference.

2.11 Statistical Analysis
Data was evaluated by one-way analysis of variance (ANOVA). Paired t-test was used to compare two sets of data determine significant difference. Statistical significance was accepted at P<0.05.
3 Results: Generation of Materials for Structural and Functional Studies

Despite two reports of the low resolution structure of endoglin (49, 53), its biochemical nature remains mostly unresolved. We therefore generated stable and soluble forms of the extracellular domain of endoglin for the purpose of X-ray crystallography and functional studies.

3.1 The complete extracellular and orphan domains of endoglin are readily expressed and secreted as soluble proteins

The membrane form of endoglin consists of a N-terminal orphan domain followed by the zona pellucida (ZP) domain, juxtamembrane region, transmembrane region and a short cytoplasmic domain (48, 49). Full-length endoglin was previously cloned into the mammalian pCMV5 vector with a C-terminal Flag-tag (Fig. 5A), and used as a template to generate multiple constructs of endoglin delineating different domains. pCMV5-Eng1-586-Flag codes for the complete extracellular domain, encompassing both the orphan and ZP domains (Fig. 5A). pCMV5-Eng1-357-Flag codes for the endoglin N-terminal orphan domain (Fig. 5A). Two different constructs coding for the ZP domain were generated, one terminating at residue 581 (pCMV5-Eng358-581-Flag) and the other at residue 586 (pCMV5-Eng358-586-Flag) (Fig. 5A). These two forms were generated to test the role of Cys-582 in the structure and stability of endoglin ZP domain.

Expression levels and solubility of each construct were evaluated using transfected HEK 293T cells. Twenty-four hours after transfection, cells were washed and then cultured in serum-free medium, after which the cell lysate and medium were collected and analyzed by SDS-PAGE and immunoblotting (Fig. 5B). Within the lysates, Eng1-586 (80 kD) and Eng1-357 (55kD) were readily identified, whereas both ZP domain constructs (27 kD) were barely detectable, despite loading five times more material, relative to the other constructs (Fig. 5B). This implies that both ZP domain constructs are expressed poorly relative to the orphan or complete extracellular domain. Within the media, Eng1-586 (95 kD) and Eng1-357 (75 kD) were easily detectable,
**Figure 5. Cloning and expression of endoglin fragments.**

(A) Schematic representation of the full length endoglin cDNA and the engineered extracellular domain constructs containing a FLAG tag. Human endoglin cDNA codes for full length endoglin (L-Eng) (Met1-Ala658). Select endoglin fragments were cloned into the pCMV5 vector. The five glycosylation sites (Asn88, 102, 121, 134, 307) are marked (•). (B) Immunoblotting of endoglin constructs expressed in HEK 293T Cell Lysate and Media using a polyclonal antibody to endoglin (H300) that reacts with both monomers and dimers. 5 µg of cell lysate proteins were loaded for the empty vector control (1), L-Eng (2), Eng1-586 (3) and Eng1-357 (4) while 25 µg protein was used for Eng358-581 (5) and Eng358-586 (6). Culture media, concentrated 10-fold, was loaded with three times the volume loaded for the equivalent cell lysate samples. Samples were run under reducing (R) and non-reducing (NR) conditions.
demonstrating that both constructs are expressed and secreted in a soluble form (Fig. 5B). Neither ZP domain was detectable in the media.

Western blot analysis also provided useful information regarding the oligomeric structure of the endoglin constructs. Full-length endoglin is a transmembrane disulfide-linked dimer of 180,000 Da as previously reported (45), and resolved as monomers under reducing conditions (Fig. 5B). In cell lysates, Eng1-586 was found in both dimeric and monomeric forms under non-reducing conditions while Eng1-357 was mostly monomeric. Since the disulphide bond is likely within the 1-357 region (52), the decreased presence of dimeric protein can likely be attributed to improper orientation due to no longer being tethered to the membrane. Both forms of the ZP domain exist only in monomeric form under both reducing and non-reducing conditions. In the culture media, Eng1-586 was also present as dimer and monomer under non-reducing conditions, whereas Eng1-357 was mostly monomeric. Taken together these results indicated that both Eng1-586 and Eng1-357 were expressed and secreted as stable forms of the extracellular domain of endoglin (142) and could be used for subsequent studies aimed at determining endoglin structure and function.

3.2 Generation of deglycosylated forms of soluble endoglin

Since these soluble proteins were intended for X-ray crystallography we needed to ensure that they would be suitable. Some of the most difficult proteins to crystallize are glycoproteins, owing to the fact that crystallization is usually inhibited by the chemical and conformational heterogeneity of carbohydrate moieties (142). In an attempt to facilitate protein crystallization, it was decided that glycosylation mutants would be generated using site-directed mutagenesis. Human endoglin contains 5 putative N-linked glycosylation sites (Asn 88, 102, 121, 134, 307) based on sequence analysis (45). These sites were altered sequentially by site-directed mutagenesis and converted to Gln residues. The corresponding constructs were analyzed for expression and secretion in HEK 293T cells (Fig. 6A). Progressively mutating glycosylation sites resulted in a progressive downward shift in molecular weight. This observation gives the first evidence that each of these sites are in fact glycosylated.

While removal of glycosylation sites is beneficial in the context of crystallography it may come at the cost of protein expression (142). When one or two of the five sites (N-134-Q and N-
Figure 6. Generation of deglycosylated endoglin fragments by site-directed mutagenesis.

(A) Expression of Eng1-586 glycosylation mutants. cDNA (1 μg) corresponding to Eng1-586 (WT) and its glycosylation mutants were transfected into HEK 293T cells and equivalent amounts of culture media samples analyzed by SDS-PAGE and anti-FLAG Western blot. This is a representative image of 7 experiments. Eng1-586-N134Q (88kD) and Eng1-586-N121,134Q (82kD) showed 80% expression relative to WT (95kD) while Eng1-586-N121,134,307Q (72kD) and Eng1-586-N88,121,134,307Q (67kD) showed 50% expression. Eng1-586-N102,121,134,307Q (66kD) was only expressed at 10% and the completely deglycosylated (45kD) form was not detectable, indicating that Asn102, and to a lesser extent Asn307, glycosylation sites are important for protein folding/stability. 

(B) Generation of Eng1-357 triple (N121,134,307Q) and quadruple (N88,121,134,307Q) mutants. Eng1-357-N121,134,307Q and Eng1-357-N88,121,134,307Q mutants were generated by sub-cloning from the equivalent Eng1-586 constructs. cDNA (1 μg) was transfected into HEK 293T cells and equivalent amounts of culture media samples were run under reducing conditions and immublotted with anti-endoglin (H300).
121,134-Q, respectively) of Eng1-586 were mutated, protein expression was reduced to 80% relative to wild-type (WT) (Fig. 6A). On average, the triple (N-121,134,307-Q) and quadruple (N-88,121,134,307-Q) mutant forms of Eng1-586 showed 50% expression relative to WT. An alternate form of the quadruple mutant (N-102,121,134,307-Q) had drastically reduced expression, 10% relative to WT, while a completely deglycosylated mutant was not detectable in the media. The large loss of protein stability and subsequent expression experienced by mutating residue 102 suggests that glycosylation at this site is required for endoglin stability. We decided that the triple (N-121,134,307-Q) and quadruple (N-88,121,134,307-Q) mutant forms of Eng1-586 were the most viable options to pursue since they were partially deglycosylated, expressed and secreted, and may yield enough protein for functional and structural studies.

These mutants were then used as templates for the generation of the deglycosylated forms of the orphan domain, Eng1-357 (Fig. 6B). On average, the triple mutant form of Eng1-357 (N-121,134,307-Q) had approximately 70% expression relative to WT, while the quadruple mutant (N-88,121,134,307-Q) was not detectable in the media. This suggests that the ZP domain may offer some shielding near residue 88, thereby allowing the quadruple (N-88,121,134,307-Q) mutant forms of Eng1-586 to be expressed.

Due to decreased protein expression with mutation of glycosylation sites, an alternate means of deglycosylation was explored. Within mammalian cells, once a glycoprotein reaches the Golgi, all glycosylation sites contain a high-mannose chain (Fig. 7A) (142). This chain is then trimmed by mannosidase I, forming the substrate needed for the generation of complex glycosylation chains. However, growing cells in the presence of kifunensine, a potent inhibitor of mannosidase I, blocks its action, leaving only high-mannose chains. These high-mannose chains, as opposed to complex chains, can be cleaved by endoglycosidase (Endo) H leaving a single glucosamine (GlcNAc) residue.

To test the suitability of removing glycosylated side-chains by kifunensine treatment and EndoH digestion on endoglin expression, HEK 293T cells were transfected with either wild-type Eng1-586 or Eng1-357 and grown in the absence or presence of 50 μM kifunensine for 24 h. The cell media was then concentrated 10-fold and incubated with increasing concentrations of EndoH. Growing cells in the presence of kifunensine did not affect protein expression and secretion (Fig.
Figure 7. Generation of deglycosylated endoglin fragments by kifunensine and EndoH treatment.

(A) Pictorial representation of complex glycosylation pathways and their inhibition by kifunensine and cleavage of high-mannose glycosyl groups by EndoH. Immunoblotting of kifunensine and EndoH treated Eng1-586 (B) and Eng1-357 (C). Transfected HEK 293T cells were grown in either the absence (lanes 1-5) or presence (lanes 6-10) of 50 µM kifunensine. The concentrated culture media were then treated with varying concentrations of EndoH for 3 h at 37°C (lanes 3-5, 7-10). Protein stability was assessed in relation to temperature, 4°C (lanes 1 and 6) and 37°C (lanes 2 and 7). EndoH dependent deglycosylation was only apparent when cells were grown in the presence of kifunensine.
As expected, incubation of proteins obtained from kifunensine untreated but EndoH treated cells did not alter the degree of glycosylation. However, proteins obtained from kifunensine treated cells allowed for nearly complete deglycosylation by EndoH for both Eng1-586 and Eng1-357. Therefore, treatment with kifunensine and EndoH represents a viable alternate means of generating deglycosylated endoglin for structural studies.

This method is very useful since it allows for proper folding due to glycosylated side-chains, which can be removed later. Also, the single GlcNAc residue left behind at each glycosylation sites allows for partial shielding of the potentially hydrophobic regions of the protein surface. This method is now being implemented in the laboratory of our collaborator in order to produce deglycosylated Eng1-586 and ultimately to perform X-ray crystallography.

### 3.3 Sub-cloning endoglin constructs into high-expression CHO vectors

In order to generate stable CHO cell lines expressing endoglin for structural and functional studies, the constructs of interest must be sub-cloned into specialized vectors for stable transformation. The vectors chosen were the CHEF1 vectors (pDEF38 and pNEF38) since they were designed for high-level protein expression in CHO cells (Fig. 8A) (143). These vectors contain the 5’ and 3’ UTR regions of a highly expressed CHO gene, EF1-α, and the dihydrofolate reductase gene, as well as the AmpR selection marker. It was decided that the wild-type, triple (N-121,134,307-Q) and quadruple (N-88,121,134,307-Q) mutant forms of both Eng1-586 and Eng1-357 would be inserted into the high-expression vectors, pDEF38 and pNEF38. All of the desired endoglin fragments were successfully sub-cloned into the proper vectors with a C-terminal His-tag. The expression and secretion of the pDEF38 vector constructs were compared to that of the pCMV5 constructs (Fig. 8B, C). For both Eng1-586 and Eng1-357, each high-expression construct was readily expressed and secreted in HEK 293T cells; however, protein expression was slightly lower relative to expression of the same protein fragment in the pCMV5 vector. This decreased expression can be explained due to the fact that the CHEF1 vectors are designed for use in CHO cell lines, and we were testing them in HEK 293T cells to simply verify expression of the constructs. Increased protein expression in CHO cells of these vectors is due to desired sequences being flanked by the 5’ and 3’ UTR regions of a highly expressed CHO gene.
Figure 8. Expression of endoglin fragments in vectors designed for large scale protein generation.

(A) Schematic of high-expression vectors (pDEF38/pNEF38). These vectors are engineered for high expression in CHO cells by containing the 5’ and 3’ flanking sequences of a highly expressed CHO gene (EF-1α). The wild-type and triple mutant (N-121,134,307-Q) forms of both Eng1-586-His and 1-357-His were sub-cloned into both pDEF38 and pNEF38 and then used in the generation of stable CHO cell lines. The WT, triple (GS3, N121,134,307Q) and quadruple (GS4, N88,121,134,307Q) mutant forms of both Eng1-586 (B) and Eng1-357 (C) in either pCMV5 (P) or pDEF38 (D) were transfected into HEK 293T cells. Equivalent amounts of culture media samples were run under reducing conditions and immunoblotted with anti-endoglin (H300).
3.4 Large-scale expression and purification of Eng1-586-His and Eng1-357-His proteins

With the ultimate goal being the production of sufficient quantities of protein for structural and functional studies, four stable cell lines were generated, each expressing one of the following constructs: Eng1-586-His, Eng1-586-N-121,134,307-Q-His, Eng1-357-His and Eng1-357-N-121,134,307-Q-His. Due to resource and space limitations, only the cells expressing Eng1-586 and Eng1-357 were used for large scale protein expression since they are the most physiologically relevant forms for functional studies. Each cell line was grown in a spinner flask for 2-3 weeks. Secreted proteins were purified via Ni²⁺ immobilized metal affinity chromatography (IMAC).

The initial Eng1-586-His preparation yielded ~2mg of protein from a 1.5 L culture volume. Purity of the endoglin preparations was evaluated by SDS-PAGE followed by Coomassie blue staining. The Eng1-586-His samples had very high purity after IMAC with little visible protein contaminates (Fig. 9A). This protein was subsequently dialyzed against the desired buffer for functional studies.

Unfortunately the cells expressing Eng1-357-His responded poorly to the serum-free medium and failed to produce detectable levels of protein. With adjustments to the production protocol, 3.1L of Eng1-357-His culture yielded ~2.2mg of protein. The Eng1-357-His samples had very high purity after IMAC, with some visible protein contaminates based on the Coomassie blue stained SDS-PAGE gel (Fig. 9B). This protein was subsequently dialyzed against the desired buffer for future functional studies.

Based on the Coomassie blue stained SDS-PAGE gels, Eng1-586-His was found in both monomeric and dimeric forms under non-reducing conditions (Fig. 9A). There was a greater proportion of dimeric protein (~80%), as compared to Eng1-586-Flag obtained from transient transfection in HEK 293T cells (~50%) (Fig. 5B vs. Fig. 1B). The proportion of dimeric form of purified Eng1-357 (~50%) was also higher than observed for the transiently expressed Eng1-357-Flag (~2%) (Fig. 5B vs. Fig. 1B). Evidence from other research has shown that the presence of a His-tag may actually increase the likelihood of dimerization, giving a possible explanation for the differences observed in this research (144).
Eng1-586-His and Eng1-357-His in the high expression vectors (pDEF38/pNEF38) were stably expressed in CHO cell lines. Secreted proteins were purified from the media by Ni\(^{2+}\)-IMAC purification and resolved by SDS-PAGE and analyzed by coomassie blue staining. (A) Under reducing (R) conditions, Eng1-586-His exists as a monomer; under non-reducing (NR) conditions it exists in both monomeric and dimeric forms. (B) Under reducing conditions, Eng1-357-His is resolved as a monomer; under non-reducing conditions it exists as a mixture of monomers and dimers. Both samples are of high purity.
Once we had obtained protein samples for functional studies, we then optimized the procedures to increase production of Eng1-586-His for the generation of protein crystals for X-ray crystallography. With adjustments to the growing protocol such as addition of more cells, 2.8L of Eng1-586-His culture yielded ~15mg of protein following IMAC fractionation. The IMAC fractions containing Eng1-586 were further subjected to gel filtration chromatography using a Hi-Load Superdex-200 gel filtration column, separated into monomer and dimer samples and then used for crystallography screens.

Another important criterion for the generation of protein crystals suitable for crystallography is a pure homogenous protein solution. Based on Coomassie-stained SDS-PAGE gels (Fig. 9), the proteins eluted from the IMAC column were very pure, with few visible contaminants. Another possible form of contamination is the heterogeneity of monomeric and dimeric proteins in solution. In order to analyze the relative stability of purified Eng1-586-His, IMAC eluted samples from the second preparation, were run on a small 10/300 Superdex-200 column. It was resolved mostly as a dimer (eluting at 10.07 mL) and with an additional peak corresponding to the monomer (eluting at 11.93 mL) (Fig. 10A). Monomeric and dimeric Eng1-586-His initially separated using the Hi-Load Superdex-200 column were subsequently run on a 10/300 Superdex-200 column. Monomeric Eng1-586-His remained in monomeric form, eluting at 11.94mL, with only slight dimer contamination, eluting at 10.05mL (Fig. 10B). Conversely, Eng1-586-His dimer remained stable eluting at 10.08mL (Fig. 10C). This successfully demonstrates that the two oligomeric forms of Eng1-586-His are stable and do not readily interconvert. Taken together, these results indicate that we have generated and purified a sufficient amount of structurally stable and highly pure proteins to be used in structural and functional studies.
Eng1-586-His was expressed in CHO cell lines using high-expression CHEF1 vectors (pDEF38/pNEF38) and purified by Ni$^{2+}$-IMAC. Fractions containing Eng1-586 were pooled and 100 μg subjected to gel filtration chromatography using a 10/300 Superdex-200 column. (A) UV absorbance of eluted fractions of Eng1-586-His displaying a mixture of oligomeric states. The eluted fractions were subjected to a second round of gel filtration chromatography to assess their stability. UV absorbance chromatograph of purified monomers (B) and dimers (C) of Eng1-586-His.
4 Results: Functional Studies of Soluble Endoglin

4.1 Endoglin modulates the signaling of multiple TGF-β superfamily ligands

While a significant amount of research has been focused on the role of endoglin, much remains to be understood about its mechanisms of action. It is generally accepted that endoglin does not bind ligand on its own, but is able to modulate the downstream signaling of multiple members of the TGF-β superfamily, in a context-dependent manner. Our interests lie in better understanding the role of endoglin in endothelial cells. We first determined the effect of membrane endoglin, mEng, on signaling for multiple members of the TGF-β superfamily. We then tested if a soluble form of endoglin, sEng, could disrupt ligand-induced Smad activation as a first step towards elucidating how sEng leads to endothelial cell dysfunction and ultimately hypertension in the context of preeclampsia.

To better define the role of mEng in response to specific ligands acting via the BMP pathways, we measured Smad1,5,8 phosphorylation as a surrogate for downstream signaling. Smad 1,5,8 phosphorylation was examined in the presence or absence of membrane endoglin, in immortalized E\textsuperscript{ng}+/+ and E\textsuperscript{ng}--/ mouse embryonic endothelial cells respectively (Fig. 11). We first tested Smad1 phosphorylation in response to increasing doses of TGF-β1 using a phospho-specific antibody that recognizes activated Smad1. Levels of Smad1 phosphorylation were higher in E\textsuperscript{ng}--/ cells compared to their wild-type counterpart, E\textsuperscript{ng}+/+ cells with increasing concentrations of TGF-β1 (Fig. 11A). This is in agreement with previous studies from this laboratory (67) and suggests that membrane endoglin inhibits Smad- mediated TGF-β1 responses in endothelial cells.

Next we examined Smad1 activation in response to BMP-2 stimulation and observed higher phosphorylation with increasing ligand concentration, being close to saturation at 400 pM, the highest dose tested (Fig. 11B). Similar to the case of TGF-β1, the level of Smad1
Figure 11. Smad1 phosphorylation in response to stimulation by TGF-β superfamily ligands.

Eng\(^{+/+}\) and Eng\(^{-/-}\) cells were serum-starved for 30 min, and stimulated for 30 min with varying concentrations of TGF-β1 (A), BMP-2 (B), BMP-7 (C) and BMP-9 (D). Cell lysates were resolved by SDS-PAGE and analyzed by Western blot with an antibody to phosphorylated Smad1,5,8 (PSmad1) and to total Smad1,5,8 (Smad1). The amount of PSmad1 was quantified relative to total Smad1 levels. The graphs represent the mean ± s.e.m. of 3 experiments, except for panel A which is a representative experiment of a previously published observation (67). Panels (A), (B) and (D) were generated by Dr. Guoxiong Xu. Based on ANOVA analysis both the Eng\(^{+/+}\) and Eng\(^{-/-}\) curves were statistically different for Panels B and D. Time points showing statistically different values between Eng\(^{+/+}\) and Eng\(^{-/-}\) samples are illustrated by *; \(P < 0.05\).
phosphorylation was consistently higher in \( \text{Eng}^{-/-} \) than control cells, suggesting that endoglin also inhibited BMP-2 induced signaling. We next investigated the response of endothelial cells to BMP-7 and found that higher concentrations were needed to activate Smad 1 phosphorylation, reaching near saturation at 1200 pM, the highest dose tested (Fig. 11C). Membrane endoglin gave a small but not statistically significant inhibition of the BMP-7 response. However, membrane endoglin significantly potentiated the BMP-9 induced Smad1 phosphorylation (Fig. 11D). Near maximal activation of Smad1 was obtained at 25 pM BMP-9 for \( \text{Eng}^{+/+} \) cells as compared to 400 pM for \( \text{Eng}^{-/-} \) cells.

In summary, mEng inhibits Smad1,5,8 dependent signaling induced by TGF-\( \beta \) 1, BMP-2, and to some degree BMP-7 while it potentiates BMP-9 signaling.

### 4.2 The soluble extracellular domain of endoglin can inhibit BMP-9 induced Smad1 phosphorylation

Soluble forms of endoglin are cleaved from the placental surface in patients with preeclampsia and released into the maternal bloodstream, contributing to endothelial dysfunction and ultimately hypertension. While an increase in circulating soluble endoglin is associated with preeclampsia (35), its mechanism of action remains elusive. One proposed model is that soluble endoglin alters endothelial homeostasis via competitive binding of TGF-\( \beta \)1, thus preventing its binding to membrane-tethered receptors on endothelial cells. However, endoglin has been proposed to bind TGF-\( \beta \)1 in association with TBRII. It therefore remains unclear how sEng elicits its effects within the context of preeclampsia.

Towards this end, we determined if commercially available soluble endoglin (sEng; Eng 1-586) alters downstream Smad1 activation by TGF-\( \beta \)1 or BMP-2, -7 and -9 in wild-type \( \text{Eng}^{+/+} \) endothelial cells. As proof of principle, we tested if Smad1 phosphorylation was inhibited by known soluble receptors, such as TBRII in the case of TGF-\( \beta \)1, or ALK1 in the case of BMP-9. Therefore, we first incubated endothelial cells with increasing concentrations of soluble receptors and after 1 h stimulated the cells with a constant amount of the cognate ligand for 30 min in the presence of the soluble receptors. (Fig. 12). Incubation of TGF-\( \beta \)1-stimulated cells with soluble TBRII reduced Smad1 phosphorylation by 90% at 10 nM, the highest concentration tested (Fig. 12A). Similarly, a soluble form of ALK1 (sALK1), was able to reduce BMP-9-induced Smad1 phosphorylation by approximately 50% at 10 nM (Fig. 12B). Therefore, competitive binding of
Figure 12. Blocking of Smad1 phosphorylation by soluble ligand-binding receptors.

Eng$^{+/+}$ cells were serum-starved for 2h, incubated for 1h with varying concentrations of soluble TGF-β receptor II (sTBRII) (A) or soluble type I receptor ALK1 (B) as indicated and stimulated for 30 min with 100 pM TGF-β1 (A) or 25 pM BMP9 (B) respectively. Cell lysates were resolved by SDS-PAGE and analyzed by Western blot with anti-phospho-Smad1 or anti-Smad1. The amount of phospho-Smad1 was quantified relative to total Smad1 levels. The data represent the average of two experiments.
soluble receptors to either TGF-β1 or BMP-9 leads to inhibition of Smad1 phosphorylation, which can be detected by immunoblot. We then tested if sEng affects Smad1 signaling by TGF-β1, BMP-2, -7 and -9 (Fig. 13). No detectable changes in TGF-β1- or BMP-2-induced Smad1 phosphorylation were observed when endothelial cells were incubated with up to 10nM sEng (Fig. 13A and B, respectively). In contrast, sEng inhibited BMP-7-induced Smad1 signaling by nearly 50% at a 500 nM concentration (Fig. 13C). A more potent inhibition of BMP-9 induced Smad1 phosphorylation was observed when cells were co-incubated with sEng and BMP-9, with nearly 50% inhibition obtained with 10 nM sEng (Fig. 13D). Taken together, these data indicate that sEng can effectively compete with the membrane receptor complex for binding to BMP-9, and to a lesser extent BMP-7, but not to TGF-β1 and BMP-2.

4.3 Relative binding of immobilized purified soluble endoglin to ligands of the TGF-β superfamily

Activation of Smad1 by BMP-7 and BMP-9 can be inhibited by sEng. To determine if this inhibition is mediated by direct binding of sEng to ligands and effective competition with membrane bound receptors, surface plasmon resonance (SPR) was used to determine binding affinities. To ensure that our experimental system was working properly, SPR was used to delineate the $K_D$ for known interactions between TGF-β1/TBRII and BMP-9/ALK1 (145, 146).

Binding of increasing concentrations of TGF-β1 (0.1 nM – 200 nM) to a soluble dimeric TBRII immobilized on a sensor chip was analyzed using a BIAcore X (Fig. 14A). We were unable to remove bound TGF-β1 from the chip, implying a strong interaction. Therefore, kinetic analyses were accomplished using a combination of the plateau method and values generated using the BIAevaluation software. Due to the very tight nature of this interaction resulting in incomplete ligand dissociation, the initial base-line was used to determine the actual RU difference that could be obtained had the TBRII chip been free of bound ligand. The results from kinetic analyses revealed a $K_D$ of approximately 100 pM, weaker than previously described values (5 pM) (Table 3 and Fig. 14A) (145). We next tested the binding of BMP-9 at concentrations ranging from 0.5 nM to 400 nM to immobilized dimeric sALK1-Fc (Fig. 14B). BIAevaluation-based kinetic analysis, verified by the plateau method, gave a $K_D = 1.8$ nM, a value in agreement with a previously reported study (146).
Figure 13. Analysis of the ability of sEng to inhibit ligand induced Smad1 phosphorylation.

Eng<sup>+/−</sup> cells were serum-starved for 2 h, incubated for 1 h with varying concentrations of sEng and stimulated for 30 min with (A) 100 pM TGF-β1, (B) 100 pM BMP-2, (C) 300 pM BMP-7, (D) or 25 pM BMP-9. Cell lysates were resolved by SDS-PAGE and analyzed by Western blot with anti-PSmad1 or anti-Smad1. The amount of PSmad1 was quantified relative to total Smad1 levels. The graphs represent the mean ± s.e.m. of 3 experiments. Inhibition with sEng versus untreated was significant for points denoted by an asterisk; *P < 0.05.
Figure 14. Kinetic analysis of TGF-β1 and BMP-9 binding to their respective TBRII and Alk1 receptors.

Commercially available forms of TBRII-Fc (A) and ALK1-Fc (B) were amine-coupled to a CM5 chip and different concentrations of (A) TGF-β1 (50-200 nM) or (B) BMP-9 (20-400 nM) were injected over the captured receptors. The raw data (coloured lines) are overlaid with a global fit 1:1 model (black lines) obtained by the BIAevaluation software. The difference in resonance signal was plotted against concentration and the data was overlaid with a hyperbolic fit curve (insets).
Table 3. Kinetic and thermodynamic constants for the interaction of multiple ligands of the TGF-β superfamily with the extracellular domain of selected receptors using the BIAevaluation software.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Ligand</th>
<th>( k_\text{a} ) (M(^{-1})s(^{-1}))</th>
<th>( k_\text{d} ) (s(^{-1}))</th>
<th>( K_\text{D} ) (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>sTBRII</td>
<td>TGF-β1</td>
<td>-</td>
<td>-</td>
<td>0.1</td>
</tr>
<tr>
<td>sALK1</td>
<td>BMP-9</td>
<td>2.5x10^5</td>
<td>4.6x10^-4</td>
<td>1.8</td>
</tr>
<tr>
<td>sEng</td>
<td>BMP-9</td>
<td>9.9x10^5</td>
<td>4.9x10^-3</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>BMP-7</td>
<td>4.9x10^4</td>
<td>2.9x10^-3</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>TGF-β1</td>
<td>4.5x10^3</td>
<td>5.6x10^-3</td>
<td>1,230</td>
</tr>
<tr>
<td></td>
<td>BMP-2</td>
<td>26.1</td>
<td>4.9x10^-3</td>
<td>186,000</td>
</tr>
</tbody>
</table>
We then estimated the sEng binding affinities for TGF-β1, BMP-2, -7, and -9 (Fig. 15 and Table 3). The sEng was obtained from stably transfected CHO cell lines, purified and characterized as described in Chapters 3. The dimeric form of sEng, retrieved after size exclusion chromatography was coupled to the chip. Binding of increasing concentrations of ligand (TGF-β1 (1 nM – 800 nM), BMP-7 (1 nM – 300 nM), BMP-9 (0.1 nM – 500 nM) and BMP-2 (1 nM – 300 nM), to sEng immobilized on a sensor chip was analyzed using a BIAcore X instrument. Kinetic and equilibrium analysis were performed using the BIAevaluation software and then confirmed using the plateau method where possible.

Analyses revealed that sEng (Eng1-586) binds TGF-β1 with an affinity of 1.23 μM. The kinetic studies indicated rapid on and off rates (4.5x10³ M⁻¹ s⁻¹ and 5.6x10⁻³ s⁻¹, respectively) (Figure 15A and Table 3). We were unable to reach plateau values using the available ligand concentrations, meaning that we could not confirm the binding affinity using the plateau method. The interaction of BMP-7 with immobilized sEng is considerably stronger (K_D = 60 nM) with a faster on rate (4.9x10⁴ M⁻¹ s⁻¹) but a comparable off rate (2.9x10⁻³ s⁻¹) (Fig 15B and Table 3). sEng interacts most strongly with BMP-9, having an affinity of 5 nM (Fig 15C and Table 3). This higher affinity results from the fast on rate (9.9x10⁵ M⁻¹ s⁻¹) as the off rate (4.9x10⁻³ s⁻¹) is similar to those observed for sEng and TGF-β1 and BMP-7. The interaction between sEng and BMP-2 was much weaker (K_D = 186 μM) with a slow on rate (26.1 M⁻¹ s⁻¹) but an off rate (4.9x10⁻³ s⁻¹) comparable to that of sEng with other ligands (Table 3). Due to resource limitations, only the two highest ligand concentrations tested produced a change in the RU and neither reached a plateau. This means that the determined K_D will likely change with a complete SPR analysis with more concentrated ligand samples.

Together, these data demonstrate that sEng can in fact bind directly to ligands of the TGF-β superfamily but with different affinities. The strongest binding of sEng is with BMP-9 (K_D = 5nM), implying that BMP-9 is functionally the most relevant ligand for endoglin. The interaction of sEng with BMP-7 is one order of magnitude less and likely also of biological relevance. The interaction of TGF-β1 with sEng is in the μM range and 10,000 times less than its binding to TBRII, implying that sEng is unable to compete effectively for binding of TGF-β1 to cells. The weakest interaction observed for sEng was with BMP-2 with an estimated K_D close to 200 μM, leading us to propose that sEng cannot compete with surface receptors for BMP-2 effects.
Figure 15. Kinetic analysis of TGF-β1, BMP-7 and BMP-9 binding to sEng.

The dimeric fraction of purified Eng 1-586 (sEng) produced in CHO cells was amine coupled to a CM5 chip and different concentrations of (A) TGF-β1 (25-800 nM), (B) BMP-7 (3-300 nM) and (C) BMP-9 (1-500 nM) were injected over the captured receptors. The raw data (coloured lines) are overlaid with a global fit 1:1 model (black lines) obtained by the BIAevaluation software. The difference in resonance signal was plotted against concentration and the data was overlaid with a hyperbolic fit curve (insets).
5 Discussion

Both forms of endoglin, mEng and sEng, have been implicated in preeclampsia. There has been shown to be an increased mEng expression in placenta of women with preeclampsia. We have shown in this thesis that the presence of mEng in endothelial cells inhibits TGF-β1, BMP-2 and BMP-7-induced Smad1,5,8 phosphorylation. On the other hand, the presence of mEng potentiates BMP-9 Smad1,5,8 signaling. Research has also shown that there are increased levels of sEng in the sera of women with PE and it correlates with disease severity. Our results show that sEng can bind BMP-9 with high enough affinity to inhibit downstream Smad1,5,8 phosphorylation. However, the affinity between sEng and multiple TGF-β superfamily ligands (TGF-β1, BMP-2, BMP-7) was not sufficient to inhibit Smad1,5,8 signaling within the physiological range.

5.1 Biochemical characterization of sEng

Currently the exact nature of sEng associated with preeclampsia is unknown. Very small amounts could be purified from the serum of women with PE. Mass spectrometry allowed the identification of several peptides and indicated that the N-terminal region extended at least as far as Arg406, however, there was insufficient protein to resolve the C-terminal sequence. This means that we could not generate the exact fragment corresponding to the circulating form of endoglin found in PE. However, we chose to express the Eng1-586 construct, corresponding to the extracellular domain of endoglin.

5.1.1 Expression of sEng

To fulfill our ultimate goal of determining how sEng contributes to endothelial cell dysfunction and hypertension in PE, we first needed to engineer components of the extracellular domain that could be readily expressed and soluble. Previous studies of endoglin truncation mutations have shown that most variants are unstable and would not generate soluble fragments (85). Our construct expressing the entire extracellular domain (Eng1-586) was in fact readily expressed
and secreted into the media. This protein fragment containing the complete extracellular domain had been shown to be expressed and secreted previously (111). This fragment may also have a physiological role. Hawinkels et al. showed that HUVEC cells released a large amount of a soluble form of endoglin into the media (111). Through the use of protease inhibitors and cleavage site mutants, they were able to determine that this 80 kD form of sEng was generated by MT1-MMP cleavage at residues 586-587. This further supports our choice of Eng1-586 as a soluble protein that may permit the study of the role of sEng in a physiological context.

We found that the isolated orphan domain (Eng1-357) was readily expressed, stable, and secreted from HEK 293T cells. The stability could be due to the domain being self-contained. Thus Eng1-357 represents an alternate soluble fragment for structural and functional studies. The isolated ZP domains were only weakly expressed and not released from the cell, suggesting that the orphan domain confers some stability to the ZP domain. While we were unable to readily express the isolated ZP domain, although another group was able to transiently express Eng360-586 in HEK 293T cells (147). The increased expression and stability of this particular protein fragment may be due to the use of the pAID-4 vector or the presence of a C-terminal Fc, fragment which may confer some degree of stability (147).

5.1.2 Potential multiple forms of sEng

The physiologically relevant form of sEng associated with PE remains to be determined. While MT1-MMP was shown to cleave mEng, resulting in the release of a soluble form, there are discrepancies to suggest that sEng fragments of different size may also exist. The protein released by MT1-MMP in HUVEC cells was approximately 80 kD based upon Western blot analysis. However, the sEng purified from the sera of women with PE was 65 kD, based upon Western blot analysis. Thus, there might be different forms of sEng likely generated through cleavage by different proteases. The true form of sEng in PE needs to be identified to obtain a better understanding of its nature and function. With the development of more sensitive C-terminal sequencing techniques, it should be possible to identify the placenta-derived sEng and its mechanism of release, and to generate the appropriate constructs for structural and functional studies.
5.1.3 Disulphide linkages

Another critical piece of information that is yet to be resolved is the location of the interchain disulphide bond(s) that maintain the dimeric structure of endoglin. Our work and that of others have shown that Eng1-586 can exist as a disulphide linked homodimer. As previously stated, work done by Raab et al. (52) has suggested that the location of the cysteine residues involved are likely to reside between residues 330 and 412. Within this region there are six cysteine residues (Cys330, Cys350, Cys363, Cys382, Cys394 and Cys412) that could potentially be involved. The possibilities could be narrowed down based on our observation that Eng1-357, generated and expressed in our laboratory, can exist in dimeric form. Taken together, these data suggest that the cysteine residues involved in maintaining the dimeric structure of endoglin are either Cys330 or Cys350. Based on sequence analysis from the sEng found in PE (to Arg406), both these cysteine residues were within the cleaved section of the extracellular domain, suggesting that the physiological sEng in PE is dimeric.

5.2 Functional understanding of sEng

The mechanisms involved in the contribution of sEng to endothelial dysfunction and hypertension remain to be determined. Our research was aimed at increasing our understanding of the physiological and pathological role of endoglin.

5.2.1 mEng role in modulating Smad1,5,8 signaling

It is well known that ligands of the TGF-β superfamily induce intracellular Smad signaling by interacting with a heteromeric receptor complex, composed of both a type I and type II receptor, at the cell surface. While mEng is not an essential component of this receptor complex it has been shown to modulate the signaling of multiple members of this family. In this study, we demonstrate that mEng inhibits the ability of TGF-β1 to stimulate Smad1,5,8 phosphorylation. This inhibition is not surprising, as similar results have been reported using the same endothelial cell lines (67). In addition, L6E9 myoblast cells, lacking endoglin expression, also showed decreased Smad signaling when transfected with a mEng-expressing construct (60). The exact mechanism involved in this inhibition is unknown, since endoglin has not been shown to bind and effectively sequester ligand. It is possible that the presence of endoglin changes the conformation of the ligand-binding receptor present in the receptor complex. The cytoplasmic
domain of endoglin could also be interfering with signal transduction. The mechanism involved in the ability of mEng to modulate signal remains to be determined.

We also tested other TGF-β superfamily ligands that have been shown to interact with endoglin, namely BMP-2, BMP-7, and BMP-9. We show for the first time that mEng inhibits BMP-2-induced Smad1,5,8 signalling. We also see that mEng slightly inhibits, however not significantly, BMP-7-induced Smad1,5,8 activation. This is contrary to what is seen in mEng overexpression studies in a rat myoblast cell line, where the presence of mEng actually strengthens BMP-7 signaling (68). This difference could be due to the different cell type, as the mode of action of endoglin has been shown to be very context dependent.

Interestingly, we found that mEng significantly potentiates BMP-9-induced Smad1,5,8 signaling. Similarly, overexpression of mEng in NIH-3T3 fibroblast cells was shown to lead to increased BMP-9-induced B recognition element (BRE) promoter activity (62). This mEng-mediated modulation may occur through a different mechanism as compared to those stated above, since mEng has been shown to interact with BMP-9 in the absence of its ligand-binding receptor ALK1 (61). This suggests that mEng may be binding ligand and facilitating its interaction with its cell surface receptor complex, ALK1, similar to the means through which betaglycan has been proposed to potentiate TGF-β2 signaling (32).

5.2.2 sEng role in binding ligand and modulating Smad1,5,8 signaling

Next, we wanted to better understand how sEng contributes to endothelial cell dysfunction in PE. sEng has been proposed to interfere with TGF-β1,3 signaling by effectively competing for ligand, even though it is known that sEng can only bind TGF-β1,3 in the presence of the ligand-binding receptor, TBRII. Contrary to what was seen previously using a luciferase assay (35), the presence of sEng did not affect TGF-β1-induced Smad1,5,8 signaling. While these results are contrary to the current accepted model of the role of sEng in PE, they agree with reports indicating that endoglin cannot bind TGF-β1 (59). However, incubation with a soluble extracellular domain of TBRII resulted in a significant and almost complete inhibition of TGF-β1-induced Smad1,5,8 phosphorylation. This is consistent with the kinetic data we obtained.

For the first time we show that sEng can in fact bind TGF-β1 with a 1.23 μM affinity; however this would be too weak to inhibit Smad1,5,8 phosphorylation in our inhibition assay or produce a
physiological change. During the writing of this manuscript, another group published a study looking at sEng binding ligand, also using BIAcore (147). They reported that the sEng/TGF-β1 interaction was in the μM range or weaker, in agreement with our observations. Consistent with the cell-based assay, we observed that the interaction between TBRII and TGF-β1 (100 pM) was much stronger than for sEng. This interaction has been extensively studied and our value is similar, although weaker than published data (~5 pM) (145). Together, this data infers that TBRII has a significantly higher affinity for TGF-β1 than sEng. This demonstrates that sEng could not effectively compete with TBRII at the cell surface for TGF-β1 binding. Given our results, it is unlikely that sEng is leading to endothelial dysfunction in the context of PE by effectively competing for TGF-β1 binding and interrupting downstream signaling.

We also found that sEng was unable to inhibit BMP-2-induced Smad1,5,8 phosphorylation and subsequent downstream signaling. This is consistent with the data suggesting that BMP-2 can only bind endoglin in the presence of ALK3 or ALK6 (59). Our BIAcore data showed that sEng bound BMP-2 with an estimated affinity of 186 μM. This is lower than what was seen for TGF-β1. It can reasonably be concluded that BMP-2 is not the ligand of interest in the context of PE.

sEng was only able to inhibit BMP-7-induced Smad1,5,8 phosphorylation at high concentrations. These sEng concentrations are 10-20 fold higher than what would be seen in PE (35). We found that the interaction between sEng and BMP-7 has an affinity of 60 nM. This interaction is stronger than what has recently been reported. Castonguay et al. reported that if there was any interaction it would be in the μM range or weaker; however, they did not determine the exact affinity (147). While we see that sEng can in fact bind BMP-7, the affinity is likely too weak to cause a significant physiological change and the serum level of sEng in PE is approximately 1.5 nM, which is far less than what was needed to inhibit Smad signaling.

On the other hand, we showed that sEng can interfere with BMP-9-induced Smad1,5,8 phosphorylation. Roughly 50% inhibition of Smad1,5,8 signaling was obtained at 10 nM sEng. This is consistent with studies showing that endoglin can actively interact with BMP-9 without the ligand-binding receptor (61). These observations are supported by the binding affinities. We determined that sEng binds BMP-9 with 5 nM affinity. This is a strong interaction; however, Castonguay et al. reported an observed kD of 26 pM. This large discrepancy could be due to experimental differences, such as linking sEng to the sensor chip using a C-terminal Fc, as
opposed to our protein with a His-tag and attached to the chip in a random orientation. Our determined affinity for the sALK1 and BMP-9 interaction (2 nM) is similar to what we found for sEng/BMP-9. This is consistent with a previously reported affinity of 2 nM (146). This kinetic data is consistent with our cell-based assays. sEng and sALK1, which have been shown to have a similar kD, both show roughly 50% inhibition of Smad1,5,8 phosphorylation at a 10 nM soluble receptor concentration. Since sEng and ALK1 have similar affinities, it is reasonable to suggest that sEng could effectively compete with membrane ALK1 for BMP-9 binding.

Based on this work and other binding studies that were recently done, it looks as if sEng can effectively compete for BMP-9 binding and actually affect downstream signaling. This gives credence to the theory that sEng may lead to endothelial cell dysfunction by interfering with BMP-9 signalling. These results are contrary to the widely held view that sEng interferes with TGF-β1,3 signalling. We were unable to detect a significant affinity between TGF-β1 and sEng to allow it to effectively compete for ligand binding and subsequently disrupt signaling. We were also unable to detect sEng interfering with mEng in the context of Smad signaling. However, we cannot rule out the possibility that sEng may be interfering with TGF-β1 mediated Smad independent pathways by interfering with mEng.

5.2.3 sEng in the context of PE

This new information could completely change the concept of how sEng leads to endothelial cell dysfunction. A lot of work must now be done to elucidate how inhibition of the Smad1,5,8 pathway could lead to the symptoms associated with PE. Recent work within the field of cancer may provide some potential mechanisms. BMP-9 stimulation causes Smad1,5,8 phosphorylation, which can then interact with Smad4, translocate to the nucleus and increase expression of Id-1 (Fig. 16). When this pathway was disrupted by the presence of sALK1, which could mimic the effects of sEng based on our results, this lead to decreased Id-1 expression and ultimately decreased expression of VEGF. sFlt-1 is known to sequester VEGF in the context of PE. These results suggest that sEng and sFlt-1 are ultimately disrupting the same pathway, namely VEGF signalling. This explains previous results that show that sEng and sFlt-1 individually cause mild symptoms, but together have a synergistic effect and contribute to severe PE. VEGF signaling has been shown to increase eNOS expression as well as activation. sEng and sFlt-1 may be inhibiting
Figure 16. Model of the mechanism through which sEng contributes to endothelial cell dysfunction in the context of PE.

This diagram represents a possible mechanism by which placentally derived sEng leads to maternal endothelial cell dysfunction in PE. sEng is released from the placenta into the maternal circulation. sEng effectively competes for BMP-9 binding thereby decreasing the amount of ligand available to induce downstream Smad1,5,8 phosphorylation. This would result in decreased Id-1 expression directly resulting in decreased VEGF production. This decreased production, along with sFlt-1 sequestering VEGF in the circulation, results in decreased VEGF induced expression and activation of eNOS. This means that the pregnant women are no longer able to vasodilate in response to the increased blood volume associated with normal pregnancy resulting in hypertension.
production and signaling of VEGF, respectively. This could lead to less eNOS expression and activation. This would not cause vasoconstriction, but would no longer allow for the maternal vasculature to adjust to the increased blood volume associated with pregnancy, since the vessels are no longer able to produce sufficient NO to allow for vasodilatation. This proposed mechanism gives a plausible means through which sEng, in conjunction with sFlt-1, contributes to hypertension in the context of PE. Such a model will guide future research.

5.3 Concluding remarks

Our results have afforded us a better understanding of endoglin biochemistry. This protein is highly stable as a dimer with the cystine residues involved in the interchain disulphide bond being either Cys330 or Cys350. The sEng found in PE is likely to exist as a disulphide-linked homodimer. While mechanisms of endoglin cleavage have been reported, the exact mechanism and form of sEng in the context of PE remains to be determined. Based on our functional studies we have determined that mEng can potentiate the endothelial cell response to BMP-9, while inhibiting responsiveness to TGF-β1, BMP-2 and BMP-7. More importantly, we demonstrated that sEng can actually bind BMP-9 with a high enough affinity to effectively compete with the membrane receptor for ligand binding. We propose that sEng inhibits BMP-9-induced Smad1,5,8 phosphorylation, ultimately resulting in reduced VEGF expression, thereby contributing to the maternal symptoms of PE. Reduced VEGF could lead to decreased eNOS expression and activation, resulting in maternal vasculature that can no longer vasodilate in response to the increased blood volume associated with pregnancy. The challenge of future research in this field will be to investigate the merits of this proposed mechanism.
References


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