Crosstalk between VEGF and BMP9 signalling in the context of preeclampsia

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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Abstract

Preeclampsia is a pregnancy related disorder, characterized by proteinuria and hypertension. The pathogenesis of preeclampsia is poorly understood; however, two proteins, called sFlt-1 and sEng, were found to be highly elevated in the maternal plasma weeks prior to the onset of clinical symptoms. sFlt-1 and sEng are thought to inhibit VEGF and TGF-β receptor signalling respectively. In order to elucidate how these proteins may contribute to preeclampsia, we looked at their ability to affect the secretion of endothelin-1 (ET-1), a powerful vasoconstrictor, shown to be dysregulated in preeclampsia. We found that both TGF-β1 and BMP9, a recently described ligand for sEng, induce ET-1 secretion through Smad1/5/8 and p38 pathways. Moreover, we report that sEng and VEGF can efficiently block ET-1 secretion, induced by BMP9. We propose that the balance between VEGF and BMP9 signalling is disturbed during preeclampsia, leading to excessive release of ET-1, which in turn may cause hypertension.
Dedication

I dedicate this thesis to my father, Alexander Sotov. He was the person who encouraged me to pursue science in the first place, so none of this will be possible without him. His love, faith and constant support helped me immensely during the completion of my Master’s degree.
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Acknowledgements of Contributions

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List of abbreviations

ActRII : Activin receptor type II
ALK : Activin receptor-like kinase
AT\textsubscript{1} : Angiotensin II type I receptor
AT\textsubscript{2} : Angiotensin II type II receptor
AT\textsubscript{1}-AA : Angiotensin II type I receptor agonistic autoantibodies
BMP : bone morphogenic protein
BMPRII : bone morphogenic protein receptor type II
Eng : endoglin protein
eNOS : endothelial nitric oxide synthase
ET-1 : endothelin-1
GDF : growth differentiation factor
HHT : Hereditary Hemorrhagic Telangiectasia
HHT1 : Hereditary Hemorrhagic Telangiectasia type 1
HHT2 : Hereditary Hemorrhagic Telangiectasia type 2
HELLP : hemolysis, elevated liver enzymes, low platelets
HUVEC : Human umbilical vein endothelial cells
I-Smads : Inhibitory Smads
Id-1 : Inhibitors of differentiation-1
K_{D} : affinity constant

mEng : membrane endoglin

MMP : matrix metalloproteinase

PAI-1 : Plasminogen activator inhibitor-1

PE : Preeclampsia

PlGF : Placental growth factor

R-Smads : Receptor activated Smads

sEng : soluble endoglin

sFlt1 : soluble fms-like kinase (soluble VEGF receptor type 1)

SPR : surface plasmon resonance

TβRII : Transforming growth factor β receptor II

TGF-β : Transforming growth factor β

TNF-α : Tumor necrosis factor α

VEGF : vascular endothelial growth factor
Chapter 1
Introduction

1 Introduction

1.1 Overview

Preeclampsia (PE) affects 5-8% of all pregnant women worldwide and is one of the leading causes of maternal death in childbirth. Hallmark symptoms of PE include hypertension and proteinuria after 20 weeks of gestation (1). To date, there is no definite treatment of PE and the only available therapy is to deliver the baby. Pathogenesis of PE is not clearly understood, but it is evident that the placenta plays a crucial role in the disease (2). Specifically, during PE, the placenta produces a number of anti-angiogenic factors, such as sFlt-1 (soluble VEGF receptor 1) and soluble endoglin (sEng), which have been shown to cause systemic endothelial dysfunction (3, 4). It has been postulated that an increase in sFlt-1 and sEng, which block VEGF and TGF-β signalling pathways respectively, is a major cause of hypertension and proteinuria, associated with PE. However, recent studies also implicate increased ET-1 secretion as one of the key events in PE associated hypertension (5). ET-1 is a very potent vasoconstrictor, elevated in preeclamptic patients, though the exact mechanism responsible for the increase in ET-1 secretion during preeclampsia remains unknown. Therefore, we examined the effects of VEGF and TGF-β superfamily ligands on ET-1 secretion. This thesis will focus on signalling pathways employed by VEGF and TGF-β superfamily ligands with respect to ET-1 secretion. To better understand the context of the experiments performed in this thesis, the introduction will give a quick overview of the pathogenesis of preeclampsia, followed by a discussion of TGF-β superfamily receptor signalling and the role of ET-1 in the context of PE.

1.2 Preeclampsia

1.2.1 Overview of the disease

Preeclampsia continues to be the most common cause of maternal and neonatal morbidity and mortality in the world (6). Hallmark symptoms of PE are maternal systemic hypertension and proteinuria after 20 weeks of pregnancy. Two subtypes are recognized: early and late onset PE. Early onset PE occurs before 34 weeks of gestation and is thought to be a fetal disorder, often associated with fetal growth restriction and incomplete trophoblast invasion. In contrast, late
onset PE occurs after 34 weeks of pregnancy and is considered a maternal disorder usually associated with normal uterine resistance and low rate of fetal involvement with no signs of growth restriction. Though both types of PE share a number of features, this thesis will focus on early-onset PE (7, 8).

1.2.1.1 Clinical Presentation and Diagnosis

The hallmark symptoms of PE are proteinuria and hypertension. Classic PE is diagnosed by a systolic blood pressure \( \geq 140 \text{ mm Hg} \) or a diastolic pressure \( \geq 90 \text{ mm Hg} \) on two or more occasions and at least 4 hours apart, after week 20 of gestation. However, PE is a highly heterogeneous disease; the extent of hypertension varies between patients and around 20% of cases show minimal to no signs of proteinuria, making the disease much harder to diagnose (9). PE is categorized into mild and severe forms. About 5% of PE cases will progress to severe disease, associated with headaches, liver dysfunction, pulmonary edema, renal failure, eclampsia or thrombocytopenia (1, 10). By definition, preeclampsia should be a precursor for eclamptic seizures; however, 20% of women progress to eclampsia without proteinuria and in some cases without hypertension (11, 12). There are no reliable tests for eclamptic seizures. Magnesium sulfate, which is often given during preeclampsia to prevent seizures, has a number of known side effects, including areflexia and respiratory distress (13).

Another complication of severe PE is hemolysis, elevated liver enzymes, low platelet (HELLP) syndrome, which occurs in 2% of women with PE. HELLP syndrome can be life threatening for both the mother and fetus, though most deaths result from complications of premature birth before 28 weeks of pregnancy (14, 15). The mild form of PE is rarely dangerous for the fetus or the mother, while the severe form can be fatal and accounts for 10%–15% of maternal deaths worldwide (16).

There is no test to predict preeclampsia and in many cases diagnosis is made too late to keep the symptoms in check. Recent findings, however, indicate that an increase in sFlt-1 and sEng concentrations in the maternal circulation precedes the clinical symptoms and could be used to predict preeclampsia several weeks in advance. In fact, an increase in sEng levels occurs as early as 2-3 months before the onset of maternal symptoms and has a strong association with disease severity (17). Several studies measuring the levels of sFlt-1, VEGF and PIGF (placental growth factor), in the maternal serum, confirmed that sFlt-1 rises shortly after sEng (18, 19). Moreover,
the rise in sFlt-1 is often accompanied by a decrease in VEGF and PI GF levels. Though neither sFlt-1 nor sEng alone can accurately predict preeclampsia, studies have indicated that a rise in the sFlt-1 to PI GF ratio accompanied by an increase in sEng, provides clinicians with an opportunity to accurately predict preeclampsia as early as one month before the onset of clinical symptoms (20). These biomarkers can help in the early detection of PE.

1.2.1.2 Treatments, prognosis

Once diagnosis of PE is established, patients start undergoing frequent blood pressure measurements and blood tests to check kidney and liver function. The fetus is monitored through heart rate measurements and ultrasound. Preeclampsia rarely progresses to the severe stage, and the mild stage can usually be managed expectantly until 37 weeks of pregnancy (21). Women who progress to severe preeclampsia are monitored and are expected to deliver the baby on time unless: a) hypertension can no longer be managed through medication; b) the patient develops either eclampsia or HELLP syndrome; or c) the fetus shows signs of distress. By the 34th week of gestation, risks associated with the birth of the baby are minimal, and delivery is suggested (1). Patients with preeclampsia are usually given a solution of magnesium sulfate, shown to significantly decrease incidents of eclampsia (22).

Interestingly, while delivery of the placenta cures all symptoms associated with preeclampsia, recent studies have suggested that there are long-term effects associated with disease. Specifically, 20% of women with PE develop hypertension within 7 years of the pregnancy, compared with 2% among women with normal pregnancies. Additionally, preeclamptic women were 3 times more likely to develop cardiovascular disorders later in life (23). These numbers are shared with other non-pregnancy related cardiovascular and especially hypertensive disorders, suggesting a common etiology (24).

1.2.2 Pathogenesis of preeclampsia

A number of factors have been shown to contribute to the pathogenesis of preeclampsia. Figure 1 illustrates some of the known molecules and pathways, thought to play an important role in preeclampsia.
Figure 1. A summary of key factors involved in PE pathogenesis.

A number of factors, including excessive production of AT1-AA (Angiotensin 1 auto-antibodies), genetic predisposition, oxidative stress and decreased heme oxygenase activity have been linked to placental dysfunction, which in turn results in the production of anti-angiogenic factors, such as sFlt-1 and sEng, to induce endothelial dysfunction and subsequent hypertension (HTN), proteinuria and other complications.

*Figure reproduced with permission from The American Physiology Society.*

1.2.2.1 Regulation of Trophoblast Invasion

Preeclampsia has been described as a disease of theories, highlighting the fact that, despite 100 years of active research, its pathogenesis is yet to be understood. However, most researchers agree that failure of the cytotrophoblast to invade the spiral arteries occurs in most PE cases. During the first half of gestation, cytotrophoblast start migrating from the fetal compartment into the uterine wall and associated vasculature, thus attaching the fetus to the mother. By doing so, trophoblast cells invade and modify spiral arteries into low-resistance high blood-flow vessels, capable of supplying the growing fetus with enough nutrients (25, 26). To accomplish this remarkable transformation, trophoblast cells undergo a process called molecular mimicry, where they lose molecular markers associated with epithelial cells, but upregulate endothelial markers, which allow them to displace native endothelial cells and modify spiral arteries. However, during preeclampsia, this process is incomplete and spiral arteries remain high-resistance low blood-flow vessels, that may lead to hypoxia (Figure 2). The exact mechanism of trophoblast invasion has not been elucidated; however, a number of molecules have been shown to affect this process.

To invade the spiral artery, the trophoblast must digest the extracellular matrix (ECM). To do this, the trophoblast upregulates metalloproteinases, like MMP9, which are very efficient in degrading ECM (27, 28). Therefore, failure of ECM digestion may ultimately result in a poor trophoblast invasion. Interestingly, Lin et al, showed that trophoblast cells from preeclamptic patients failed to upregulate expression of MMP9 at the mRNA level, and showed reduced invasion (29). Other studies have shown that TGF-β3 and TGF-β1 signalling inhibit trophoblast proliferation and invasion, probably by stimulating tissue inhibitors of metalloproteinases (30). Interestingly, TGF-β3 expression is induced by hypoxia inducing factor 1 (HIF-1α), which is itself upregulated during hypoxia (31). Therefore, hypoxia could be responsible for poor trophoblast invasion. Not surprisingly, the plasma concentration of TGF-β3 was found to be elevated in preeclamptic women (32).

Additionally, endoglin has been shown to play a role in trophoblast invasion. Specifically, antibodies raised against endoglin were shown to enhance trophoblast differentiation and invasion, presumably by interfering with TGF-β1 and TGF-β3 signalling (33). Later reports
In normal pregnancy, the invasive trophoblast invades the spiral artery and transforms it into a low resistance high volume artery. This allows the fetus to receive all required nutrients for successful growth. During preeclampsia, however, the trophoblast fails to invade, resulting in a small caliber high resistance spiral artery.

*Figure reproduced with permission from The American Physiology Society.*

confirmed these findings, showing that the loss of endoglin promotes the invasion of extravillous trophoblast (34). Recent studies have indicated that the plasma of preeclamptic women can inhibit trophoblast invasion, suggesting that certain circulating soluble factors can actively block trophoblast invasion. As of now these factors have yet to be identified (35). Overall, it is likely that both endoglin and TGF-β play a crucial role in remodeling of spiral arteries.

To complete the invasion process, trophoblast cells need to change their adhesion molecule profile from epithelial to endothelial, thus insuring a proper placentation process. However, during preeclampsia trophoblast cells continue to express α_6β_4, α_5β_5 and E-cadherin molecules, which are usually present on epithelial cells and are associated with a non-invasive phenotype. In fact, antibodies against E-cadherin were shown to significantly enhance the invasion process, implying that E-cadherin normally blocks invasion. Alternatively, blockade of VE-cadherin function, using antibodies, resulted in much reduced invasion, suggesting that the role of VE-cadherin is to enhance trophoblast invasion (36). Experiments confirmed that preeclamptic trophoblast cells do not show expression of α_Vβ_3, α_1β_1 integrins as well as VE-cadherin, which were found to be essential for successful invasion (37). Overall, it is clear that upregulation of specific integrin molecules greatly potentiates the invasive capabilities of the trophoblast; however, in PE, trophoblast cells fail to acquire endothelial cells markers, leading to shallow invasion and reduced blood flow (37, 38). It is thought that hypoxia, induced by the failure of trophoblast to remodel spiral arteries, acts on placenta to induce several anti-angiogenic molecules, such as sFlt-1. sFlt-1 will in turn block both VEGF and PlGF signalling in an attempt to increase blood circulation and bring more nutrients to the fetus.

### 1.2.2.2 Dysregulation of VEGF/PlGF Signalling Pathway

VEGF and PlGF proteins are structurally and functionally similar. The difference lies in the ability of VEGF to bind to two distinct receptors: VEGFR-1 and VEGFR-2, while PlGF can only bind to VEGFR-1. It is thought that PlGF enhances VEGF function by competing for VEGFR-1 and forcing VEGF to bind to the more active receptor, VEGFR-1 (Figure 3) (39). In response to ischemia, the placenta starts producing a variety of anti-angiogenic proteins (20). One of the most widely recognized proteins is sFlt-1 (or sVEGFR-1), which is primarily secreted from the syncytiotrophoblast into the maternal circulation. sFlt-1 is a splice variant of VEGFR-1 (Flt-1), which lacks the intracellular and membrane regions. VEGFR-1 can bind VEGF and
Figure 3. VEGF signalling in normal and preeclamptic pregnancies.

During normal pregnancy, VEGF can bind to either VEGFR-1 or VEGFR-2 to phosphorylate and activate eNOS to produce the very potent vasodilator NO. In PE, however, high levels of sFlt-1 bind circulating free VEGF and PIGF, blocking their signalling and subsequent NO production, leading to hypertension.

PlGF, therefore sFlt-1 is extremely efficient at scavenging both molecules. It should be noted, that by binding to VEGF, sFlt-1 also blocks signalling of VEGFR-2, as VEGF can signal through both receptors (40). Studies have shown that preeclamptic women have a 6-fold increase in serum concentration of sFlt-1 compared to normal pregnancies. Not surprisingly, elevated sFlt-1 leads to a low concentration of free VEGF and PlGF in the serum (20). Interestingly, inhibition of VEGF alone was not sufficient to induce PE like symptoms in pregnant rats. Both PlGF and VEGF pathways needed to be blocked to induce PE, suggesting that PlGF plays a key role in pathogenesis of PE (3).

The important role of sFlt-1 in PE is underlined by experiments showing that mice injected with sFlt-1, developed several of the symptoms occurring in PE patients, including proteinuria, hypertension and renal failure (4). Moreover, recent studies have indicated the development of hypertension and proteinuria in patients undergoing anti-VEGF therapy as part of their cancer treatment (41). Therefore, it appears that sFlt-1 by itself can mimic the majority of PE symptoms.

VEGF is a known angiogenic molecule that stimulates production of NO and PGI\(_2\), both of which are extremely potent vasodilators (Figure 3). VEGF can induce Akt phosphorylation, leading to phosphorylation and activation of eNOS, which is a major NO-producing enzyme (42). VEGF can also upregulate eNOS expression through ERK phosphorylation (43). Therefore VEGF can induce vasodilatation by upregulating or activating eNOS. Overall, a hallmark symptom of PE, systemic hypertension, could be partially attributed to the lack of free VEGF and PlGF molecules in circulation (44). Additionally, both VEGF and VEGF receptors are highly expressed by the glomerular endothelium and VEGF was shown to have a stabilizing effect on glomerular endothelial cells. A podocyte specific VEGF-knockout mice experienced proteinuria and glomerular endotheliosis, highlighting the importance of locally produced VEGF for glomerular endothelium function (45). It is likely that the lack of VEGF signalling in the glomerular endothelium is a primary cause of proteinuria in PE patients.

1.2.2.3 Heme-oxygenase 1 and PI3K/Akt Pathways

Heme-oxygenase 1 (HO-1) is an inducible enzyme that catalyzes the degradation of heme into biliverdin and carbon monoxide. Therefore, not only can HO-1 protect cells against oxidative damage induced by heme, but it also releases CO, which acts as a blood vessel relaxant.
Therefore, low levels of HO-1 could lead to endothelial dysfunction and hypertension, observed in PE. Indeed, chorionic villous samples taken at 11 weeks of gestation showed a much lower expression of HO-1 (46). Multiple reports have shown that women who smoke during pregnancy are less likely to develop preeclampsia suggesting that inhaling exogenous CO can be protective during PE (47). In fact, HO-1 is one of the few molecules capable of blocking release of sEng and sFlt-1. Zhou et al. showed that HO-1 acts downstream of TNF-α to regulate sEng and sFlt-1 production (Figure 4) (48). It appears that HO-1 exerts its protective function by activating the PI3K/Akt pathway (49, 50).

Recently, the PI3K/Akt pathway has emerged as a key signalling cascade in preeclampsia. Firstly, activation of PI3K/Akt stimulates the expression of both VEGF and VEGF receptors through HIF-1α transcription factor. Therefore, downregulation of PI3K/Akt activity can potentially exacerbate decreased VEGF signalling, whereas lack of VEGF leads to reduction in VEGF and VEGFR-1 expression, which in turn results in even more complete blockade of VEGF signalling (51, 52). Activation of the PI3K/Akt pathway is required for endothelial cell homeostasis and survival after vascular injury. In fact, several molecules including VEGF promote endothelial cell survival through the PI3K/Akt pathway (53). Moreover, inhibition of the PI3K-Akt pathway was shown to directly suppress sFlt-1 secretion from human placenta hypoxia models (54).

1.2.2.4 The Angiotensin II type I Agonistic Autoantibodies (AT₁-AA)

The circulating renin-angiotensin system (RAS) is a signalling cascade, which regulates blood pressure and electrolyte balance. It starts with secretion of renin, which cleaves angiotensinogen into angiotensin I (ANG I). While ANG I is not biologically active, it can be cleaved into ANG-II by angiotensin-converting enzyme (ACE). ANG-II then can bind one of two angiotensin receptors: AT₁ and AT₂, expressed on a number of different cell types, including vascular smooth muscle cells and placental vasculature. Once bound, ANG-II initiates a signalling cascade to induce vasoconstriction by inducing adolsterone production. Adolsterone increases reabsorption of water and ions in the kidney, leading to an increase in blood pressure (55). As ANG-II can readily increase blood pressure during preeclampsia, several studies compared its concentration between normal and preeclamptic pregnancies. Paradoxically, ANG-II was present in much lower quantities in preeclamptic women compared to healthy controls (56).
However, in a pioneering study done by Wallukat et al., researchers found increased levels of AT₁-AA in individuals with PE compared to healthy pregnancies. These autoantibodies were shown to bind and activate the AT₁ receptor (57). Subsequent studies were done to elucidate the function of these antagonistic autoantibodies in preeclampsia. AT₁-AA are present between 18-22 weeks of pregnancy, before the onset of the clinical symptoms. The mechanism of action of these antibodies is complex. While the direct mechanism of activating AT₁ receptor certainly plays a major role in inducing hypertension, recent studies have shown that AT₁-AA also induce expression of pre-pro-endothelin, a very potent vasoconstrictor (58). Moreover, the serum of rats injected with AT₁-AA, contained higher levels of both sFlt-1 and sEng, suggesting that AT₁-AA themselves could potentially initiate the release of these factors into the circulation (Figure 4) (59). Additionally, these antibodies were shown to interfere with the invasive ability of human trophoblast in vitro, suggesting that AT₁-AA could be responsible for the initial ischemia as well. In fact, administration of AT₁-AA, isolated from preeclamptic patients, was shown to induce preeclampsia in pregnant rats (60). The important role of AT₁-AA was further demonstrated by interfering with auto-antibody production in several animal models of preeclampsia, resulting in a significant reduction in blood pressure (60).

Though AT₁-AA are not specific for preeclampsia and occur in many other hypertensive disorders, the mechanism involved in the production of these antibodies remains unclear. Rats exposed to the chronic reduced uterine perfusion pressure (RUPP) model of preeclampsia tested positive for AT₁-AA. Therefore, ischemia itself appears to promote the generation of these antibodies (61).

### 1.2.2.5 Generation and Function of Soluble Endoglin

Soluble endoglin is also increased in the serum of preeclamptic women. In fact, during PE there is a 5-fold increase in sEng levels. sEng starts to rise around 8 to 10 weeks before the onset of preeclampsia and correlates better with disease severity than sFlt-1 (17). However, unlike sFlt-1, for which alternate splice variants have been described, none have thus far been reported for sEng. Based on work from tumor cells, it was proposed that sEng is cleaved from plasma membrane endoglin by the metalloprotease MMP14 (or MT1-MMP) and released into the serum (62). Multiple mechanisms have been proposed to explain sEng generation from the placenta. AT₁-AA are thought to induce production of sEng by upregulating TNF-α expression.
Figure 4. Model of sEng and sFlt-1 generation by AT1-AA.

AT1-AA bind to the AT1 receptor and induce expression of TNF-α. TNF-α can then trigger secretion of sEng and sFlt-1, which cause endothelial dysfunction and impaired angiogenesis. Interestingly, HO-1 can block TNF-α-induced sEng and sFlt-1 generation.

Indeed, several studies have showed that TNF-α upregulates a number of metalloproteases, which in turn could be responsible for cleaving sEng from the cell surface (48). Specifically, MMP14 has been shown to be expressed in preeclamptic placentae and capable of generating sEng by cleaving proteins from the membrane (63). Interestingly, Hawinkels et al. used site-directed mutagenesis to determine that endoglin is cleaved at the G-L bond at positions 586-587, releasing the complete extracellular domain into the circulation (62). However, soluble endoglin that has been isolated from the serum of preeclamptic women is resolved by SDS-PAGE as a 65kDa band, as opposed to the 80kDa mass of the recombinant Δ586 protein (4) (Figure 5). It is therefore unlikely that the circulating soluble form of endoglin (sEng) observed in PE, is produced by MMP14 cleavage at position 586. Mass spectrometry analysis of purified endoglin from sera of PE patients, could only identify peptides up to Arginine 406 (4), missing the C-terminal portion (Figure 5). The exact structure of soluble endoglin associated with PE, remains to be established and current findings with recombinant (Δ586) will have to be reexamined when the true nature of sEng produced by the placenta during PE is identified.

The functional characteristics of sEng were described in a study by Walshe et al. where mice were infected with an adenovirus, engineered to induce the expression of recombinant sEng (Δ586). An increase in sEng was shown to contribute to endothelial dysfunction by impairing perfusion, vasodilatation and barrier function (64). Additionally, sEng was shown to inhibit TGF-β1 –induced phosphorylation of eNOS and to enhance lung and liver microvascular permeability (4). In that same study, injection of sEng (Δ586) alone resulted in a small increase in arterial pressure, however, combination of sFlt-1 and sEng exacerbated hypertension and proteinuria induced by sFlt-1 alone. The authors concluded that sEng interferes with TGF-β1 and TGF-β3 binding to the receptor, thus blocking eNOS phosphorylation and subsequent vasodilatation (4).

Though some papers have reported the ability of sEng to interfere with TGF-β1 signalling (4), recent studies have shown that recombinant sEng (Δ586) has little to no affinity for TGF-β molecules, but rather has high affinity for another TGF-β superfamily ligand, namely bone morphogenic protein BMP9 (and related BMP10) (65). Overall, recombinant soluble endoglin appears to have a destabilizing effect on the vascular endothelium, which probably contributes to the pathogenesis of preeclampsia.
Membrane endoglin (A) is a homodimeric glycoprotein of 180kDa. Each polypeptide chain consists of 658 amino acids and the glycosylated chain has a molecular mass of approximately 90kDa. It is composed of an orphan domain (with unknown structural features) followed by a zona pellucida (ZP) region (generally involved in protein interactions), a juxtamembrane and transmembrane region and a short cytoplasmic region. A recombinant soluble form (B) used by many investigators corresponds to the 586 amino acids of the extracellular domain, and lacks the transmembrane and cytoplasmic regions. Its molecular mass is around 80kDa. The physiological form of sEng (C), identified in the sera of preeclamptic patients was shown to contain at least 406 amino but is likely a bit larger to correspond to its estimated molecular mass of 65kDa.

Figure 5. Structure of different forms of endoglin.
1.3 Overview of TGF-β Receptors and Signal Transduction

1.3.1 TGF-β superfamily

The TGF-β superfamily, including TGF-βs, BMPs, activins and other related proteins, plays a key role in cell proliferation, migration, differentiation and survival. More than 30 proteins have been identified, all of which share the same cysteine knot structure (66). All ligands are synthesized as pre-proproteins and require the pro-domain to form dimers. Therefore, dimerization occurs intracellularly, before pro-domains are cleaved; proteins are then secreted from the cell (67). All ligands signal through a heteromeric complex of Type I and Type II serine/threonine kinase receptors. Five type II receptors and seven type I receptors (also known as Activin receptor like kinases or ALK), have been identified in human. Though most of the ligands were shown to have affinity for either type I (BMP-2, 4, 7, 9) or type II (TGF-β1, TGF-β3) receptors, some can only bind to an already formed type I – type II receptor complex, suggesting that some receptors can associate with each other on the cell surface in the absence of ligand.

Moreover, TGF-β receptors are often found in a complex with type III receptors also referred to as co-receptors (betaglycan and endoglin). Betaglycan and endoglin are two known co-receptors with some structural and functional homology (68). These co-receptors have been shown to modulate cell signalling depending on the ligand and cell type in question (69). For example, betaglycan facilitates interaction of TGF-β2 to its receptor TβRII (70). Endoglin on the other hand, was shown to generally enhance signalling of TGF-β1/β3 and BMP9 (71).

1.3.2 TGF-β receptor signalling

1.3.2.1 Initial binding to the receptor

TGF-β1 has a high affinity for its type II receptor, TβRII. All type II receptors are constitutively active and can undergo autophosphorylation. Phosphorylation of Ser213 and Ser409 has been identified as essential for TβRII kinase activity, while phosphorylation of Ser416 may have an inhibitory effect (72). For a long time, it was though that in the absence of ligand, each receptor existed on the cell surface as a homodimer. However, recent studies on TβRI and TβRII, using single molecule imaging and total internal reflection fluorescence microscopy technology, clearly showed that receptors are monomers, and dimerize upon ligand binding (73, 74). Once
ligand is bound, TβRII associates with and phosphorylates type I receptor in its GS domain (TTSGSGSG). Wieser et al, have shown that mutations of this sequence strongly inhibit smad-dependent signalling initiated by TGF-β, therefore this phosphorylation is central to TGF-β receptor activity (75). Interestingly, a number of phosphatases have been shown to modulate TGF-β receptor signalling. Protein Phosphatase 1 (PP1) was found to inhibit TβRI activity and be upregulated by TGF-β signalling, thus serving as a negative feedback mechanism. PP2A was found to have dual effects on TBRI activity, depending on which regulatory subunit of the phosphatase was activated (76). As of yet, there are no reports of phosphatases that affect TβRII activity (76). Additionally, ALK5 expression was also shown to be regulated through the ubiquitination pathway (77).

Though TGF-β1 binds primarily to TβRII and recruits the ALK5 receptor, it was also shown to associate with another type I receptor – ALK1. While ALK5 is present almost universally on most cell types, ALK1 is mainly present in endothelial cells; therefore co-activation of ALK1 and ALK5 receptors by TGF-β occurs primarily in the endothelium. Mutation studies have shown that TGF-β itself does not bind to ALK1, but rather that ALK5 activation, by TGF-β binding, allows association between two type I receptors. The kinase activity of ALK5 was required for efficient ALK1 activation (78). Therefore, in endothelial cells, TGF-β1 can activate both ALK5 and ALK1 type I receptors; however, ALK5 activation is necessary for ALK1 function (Figure 6).

Other members of the TGF-β superfamily, capable of activating ALK1 receptors are BMP9 and the structurally related BMP10. Unlike TGF-β, BMP9 and BMP10 have a strong affinity towards the type I receptor ALK1 (79). ALK1 associates with a type II receptor, BMPRII or Activin type II receptor (ActRII) to mediate BMP9 and BMP10 effects (79). Phosphorylation of the type I receptor leads to a conformational change and recruitment and phosphorylation of Smad1/5/8 proteins.

Type III receptors, or co-receptors, have been shown to mediate type I receptor signalling; specifically, endoglin was shown to modulate TGF-β1 and TGF-β3 responses; however, the exact mechanism of this process is uncertain. Both, intracellular and extracellular domains of endoglin physically associate with TβRII and ALK5 receptors (80). In fact, studies have shown that endoglin can bind TGF-β1 and TGF-β3 with high affinity in the presence of TβRII (81).
Figure 6. Function of endoglin with respect to TGF-β1 signalling in endothelial cells.

Endothelial cells contain both ALK1 and ALK5 receptors, meaning that TGF-β1 can induce both Smad2/3 and Smad1/5/8 phosphorylation. The Type III receptor, endoglin, was shown to modulate TGF-β1 signalling. Specifically, endoglin can inhibit ALK5 activation and subsequent Smad2/3 phosphorylation. ALK5 signalling is thought to have quiescent effects in ECs. ALK1 signalling, on the other hand, appears to be enhanced by the presence of endoglin on the cell surface. Therefore, endoglin potentiates ALK1 and Smad1/5/8 activation.

However, it appears that endoglin function is very complex, as it was shown to enhance TGF-β1–induced Smad1/5/8 phosphorylation, but to interfere with TGF-β1–induced Smad2/3 phosphorylation (82). Moreover, endoglin is extremely important for BMP9 signalling, as endoglin deficient endothelial cells were unable to induce Smad1/5/8 phosphorylation in response to BMP9 (83). Since both BMP9 and TGF-β1 induce Smad1/5/8 phosphorylation through ALK1 receptor, it appears that endoglin facilitates ALK1 and blocks ALK5 signalling (Figure 5). However, other studies have reported that endoglin has no effect on Smad2/3 phosphorylation (84), while Santibanez et al. demonstrated that endoglin enhances ALK5 signalling (85). Therefore, the function of endoglin is extremely cell type and context dependent.

1.3.2.2 Smad Pathways

Smads are intracellular proteins, which relay signalling from TGF-β ligands to the nucleus. There are three types of Smads: R-Smad (receptor regulated), Co-Smad (common mediator) and I-Smad (Inhibitory). Smads1, 2, 3, 5, 8 are R-Smads and can be phosphorylated by type I receptors. Binding of Smad proteins to the type I receptors is aided by the presence of Smad anchor for receptor activation (SARA) protein. Once phosphorylated, Smads dissociate from the receptors and form heterotrimeric or dimeric complexes with Smad4. All R-Smads have two conserved domains, termed mad homology (MH) domains 1 and 2 (86). In all R-Smads, with the exception of Smad2, MH1 domain is responsible for DNA binding, while MH2 domain mediates protein-protein interactions. Specifically, the MH2 domain is required for interaction with TβRI receptor. Interestingly, the same region of Smad has been implicated in other protein-protein interactions, suggesting that TGF-β signalling is achieved through a sequential process of competitive interactions (87).

Upon phosphorylation, Smad complexes translocate to the nucleus, where they are able to interact with transcription factors and be recruited to specific promoter elements. Usually, each type I receptor is associated with only a subset of Smads. For example, ALK4, ALK5, ALK7 all phosphorylate Smad2 and Smad3, while ALK1, ALK2, ALK3 and ALK6 induce Smad1, 5, 8 phosphorylation (88). All heteromeric complexes of Smads contain Smad4, implying that Smad4 is central to TGF-β signalling. Yet, Smad4 null mice and a number of Smad4 lacking tumors show TGF-β-induced transcription. It is not known whether this is mediated by other Smads, or represents Smad-independent signalling (89). While R-Smads and Co-Smads are present
universally in almost all if not all cell types, I (inhibitory)-Smads expression is highly regulated. In fact, TGF-β receptor signalling is a known activator of Smad6 and Smad7 expression, suggesting that I-Smads act as a negative feedback loop to control TGF-β signalling (90-92).

TGF-β1 activates ALK5 signalling cascade, meaning that its primary pathway goes through phosphorylation and dimerization of Smad2/3. The dimer then associates with Smad4 and translocates to the nucleus to initiate gene activation. In endothelial cells, however, TGF-β1 was shown to also signal through ALK1, activating the Smad1/5/8 signalling cascade (78, 93). Extensive research showed that though ALK1 is the primary receptor, which initiates Smad1/5/8 phosphorylation, the presence of active ALK5 receptor is an absolute requirement for this process. It appears that binding of TGF-β1 to ALK5 receptor initiates the association of ALK5 to ALK1, which in turn induces Smad1/5/8 signal transduction (78). BMP9, on the other hand, was shown to bind mostly the type I receptor ALK1 and occasionally ALK2. ALK1 activation leads to Smad1/5/8 phosphorylation; therefore, both TGF-β1 and BMP9 are capable of activating the ALK1 receptor and induce Smad1/5/8 phosphorylation (83).

1.3.2.3 Non-canonical pathways

P38 pathway

The TGF-β receptor family not only induces signalling through Smad proteins, but is also capable of activating other signalling molecules such as MAPKs, ERK, p38 and others (Figure 7). Perhaps the most recognized non-canonical pathways initiated by TGF-β are p38 MAPK and JNK pathways. Both p38 and JNK exist at the 3rd level of MEK phosphorylation, meaning that there are least two sequential phosphorylation events before p38 or JNK can become phosphorylated. It is thought that JNK is activated through MKK4 and p38 MAPK through MKK3/6 (94). Further upstream, both MKK4 and MKK3/6 are activated through a TGF-β activated kinase (TAK1). Multiple studies have shown that TAK1 deficient cells are unable to signal through p38 or JNK pathways (95, 96). Though Watkins et al showed that TAK1 can directly interact with TβRII, most studies agree that TRAF6 is needed for efficient recruitment of TAK1 to the receptor (97). Interestingly, JNK and p38 pathways are completely independent from Smad signalling, as Smad2/3 or Smad4 deficient cells are perfectly capable of activating these pathways (98). Recent studies revealed that TGF-β receptors cannot only be phosphorylated on their serine/threonine resides, but can also be activated through
Figure 7. Canonical and Non-Canonical signalling pathways of TGF-β.

Upon binding to its receptor, TGF-β1 induces phosphorylation of TβRII and TβRI, which leads to recruitment, phosphorylation and dimerization of Smad2/3 molecules. This dimer then interacts with Smad4 and the whole complex translocates to the nucleus to activate gene transcription. This pathway is referred to as the canonical TGF-β1 pathway. Additionally, TβRI can also activate PI3K/Akt pathways as well as TAK, which would in turn phosphorylate JNK and p38 proteins. Moreover, activation of TβRII can also lead to RAS/RAF activation and subsequent ERK phosphorylation. These non-Smad pathways are usually called non-canonical pathways.

phosphorylation of tyrosine residues. TβRII cytoplasmic domain contains three tyrosine residues, which upon phosphorylation can recruit scaffold proteins, which in turn initiate non-canonical signalling pathways (99).

**ERK pathway**

Several studies have shown that TGF-β can induce ERK activation. Interestingly, in some cells ERK phosphorylation occurs rapidly within minutes of TGF-β binding, suggesting a direct method of recruitment (100). In contrast, other cells show a delayed response, happening hours after stimulation, implying that protein synthesis is required for signalling to occur (101). As in the case of p38 signalling, phosphorylation of tyrosine residues on TβRII plays a crucial role in ERK signalling. Moreover, tyrosine residues on TβRI can also be phosphorylated by TβRII, which then serve as docking sites for Grb2 and Shc proteins. Grb2 protein is usually in a complex with Sos protein and therefore brings Sos to the TβRI – TβRII complex. The ShcA/Grb2/Sos complex is then capable of activating RAS at the plasma membrane, resulting in activation of c-RAF, MEK and ERK (102). Mutations in ShcA have been shown to interfere with ShcA /Grb2 interactions, causing decreases in ERK phosphorylation. Therefore, it appears that ShcA is essential for efficient ERK signalling and actively holds the ShcA/Grb2/Sos complex together (102). Moreover, as in the case with p38, Smad involvement is not needed to induce ERK phosphorylation (103).

**Akt pathway**

Several findings suggest that TGF-β can also activate PI3K/Akt pathway in a Smad independent manner (104). The exact mechanism of this activation is not yet clear; however, recent studies have shown that TβRI can indirectly associate with one of the p85 subunit of PI3K (105). It is thought that PI3K/Akt pathway signals downstream to activate mammalian target of rapamycin (mTOR), which is a key regulator of protein synthesis. Inhibition of either PI3K or Akt phosphorylation showed a significant decrease in mTOR activation level, suggesting a direct link between PI3K/Akt and mTOR pathways (106). Interestingly, TGF-β-induced apoptosis and growth inhibition, which are Smad-dependent processes, can be attenuated by PI3K/Akt activation, implying that two pathways are in direct conflict with one another. Later studies have
shown that phosphorylated Akt can directly bind to Smad3 and prevent its translocation to the nucleus, thus inhibiting Smad3 signalling (107).

**BMP9 signalling**

BMP9 can also activate non-Smad pathways; however, no extensive research has been done to elucidate the exact mechanisms of Smad-independent pathways. It is likely that BMP9 initiates non-canonical signalling similarly to TGF-β, by phosphorylating tyrosine residues allowing for protein docking (108). Several papers have shown that BMPs, including BMP9, can induce MAPK pathways, through TAK1, leading to p38 MAPK or JNK activation. Additionally, BMPs were also shown to induce ERK phosphorylation (109). Interestingly, BMP9 was also found to have an inhibiting effect on PI3K/Akt pathway, which is activated by TGF-β signalling, implying that ALK1 and ALK5 pathways could function in opposition to each other (110).

### 1.3.3 Effects of TGF-β superfamily signalling

#### 1.3.3.1 Functional role of TGF-β1

Members of TGF-β superfamily have multiple and diverse functions, including but not limited to proliferation, differentiation, migration, wound repair and inflammation (86). There are three isoforms of TGF-β: -β1, -β2 and -β3. They have very similar properties *in vitro*, but appear to have different functions *in vivo* (111). TGF-β1 is secreted as a proprotein, the prodomain being required for dimerization. Upon release, the prodomain is usually cleaved from the protein, but remains non-covalently attached to TGF-β1, inhibiting the ability of TGF-β1 to bind to its receptor. Dimeric TGF-β attached to the prodomain, is called latency-associated protein (LAP). Early in the assembly of TGF-β1, LAP is in complex with latent TGF-β binding protein (LTBP) through disulfide bonds. LTBP was shown to have covalent interactions with the ECM, tethering TGF-β1 in the extracellular matrix and preventing it from entering the bloodstream. It is therefore thought that the major pool of TGF-β1 exists in the extracellular matrix, while only a fraction of TGF-β1 is in circulation (112). Indeed, the concentration of TGF-β1 in plasma is only around 1ng/ml (113).

One of the most acknowledged functions of TGF-β proteins is in wound repair. Upon injury, TGF-β1 is rapidly secreted by macrophages, platelets and keratinocytes (114). TGF-β1 is thought to be important for cell migration during wound repair, as well as production of ECM
proteins, which provide structural integrity for the wound (115). Many animal models with impaired wound repair show reduced expression of TGF-β, and usually benefit greatly from exogenous administration of TGF-β (116). Paradoxically, recent studies found that Smad3 null mice experience accelerated wound healing (117). Subsequently, multiple studies have reported that blocking TGF-β signalling leads to a significantly faster wound repair (118-120). Therefore, the intricacies of how wound repair is regulated by TGF-β signalling remain to be determined.

Additionally, TGF-β plays an important role in immunity. TGF-β was shown to inhibit IL-2 production in T cells, thus significantly inhibiting T cell proliferation (121). Moreover, TGF-β is a known inducer of FoxP3 during naïve T cell activation, forcing T cells into regulatory T cell (Treg) lineage (122). Overall, the majority of the studies agree that TGF-β has strong immunosuppressive properties (123).

The importance of TGF-β in angiogenesis is highlighted by the fact that mice deficient in TβRII, ALK1, ALK5 or endoglin, experience a very similar phenotype, showing lethal abnormalities in cardiovascular development (124). ALK5 activation usually blocks angiogenesis by up-regulating fibronectin and plasminogen activator inhibitor type 1 (PAI-1), which has been shown to inhibit EC migration (125). Surprisingly, TGF-β-induced ALK1 activation stimulates EC migration, proliferation and tube formation (78). In fact, multiple studies have reported that ALK1 and ALK5 pathways act in opposition to each other in terms of ECM production (126) and endothelial cell proliferation and migration (78). The mysterious nature of TGF-β is further outlined in cancer pathogenesis, where TGF-β was shown to be highly beneficial at the early stages by inducing cell cycle arrest and apoptosis. However, during the late metastatic stages, cells become resistant to quiescent effect of TGF-β1 and start benefiting from its angiogenic properties (127).

1.3.3.2 Functional role of BMP9

BMP9 is also known as GDF2 (Growth and differentiation factor 2) and has been identified as a ligand for the ALK1 receptor (83). Like all other TGF-β1 ligands, BMP9 is synthesized as a large pre-pro-protein. Once dimerization occurred, the pro-domain is cleaved from the active BMP9; however, it still continues to be attached to BMP9 through non-covalent interactions (128). Bidart et al. looked at the expression of BMP9 in different organs and found that hepatocytes are by far the best producers of BMP9. Moreover, they found that similarly to TGF-
β, BMP9 circulates in active and non-active forms. However, unlike TGF-β, the pro-domain of BMP9 does not seem to bind to ECM, meaning that BMP9 is not inhibited to enter the circulation, resulting in a much higher concentration of BMP9 in the serum. The estimated level of BMP9 in the blood is around 5-10ng/ml, which is much higher than the EC$_{50}$ for ALK1 activation. Indeed, aortic endothelial cells showed endogenously phosphorylated Smad1/5/8 proteins, presumably due to sustained activation by circulating BMP9 molecules (129).

The functional properties of BMP9 are poorly understood. In fact, BMP9 knockout mice did not show defects in angiogenesis (130), even though multiple studies identified BMP9 as an important factor for vasculature maintenance (79). Further studies revealed that another protein, BMP10, is present at high levels in the circulation and can also bind to the ALK1 receptor and can function as a substitute for BMP9 (130). Knockout of the BMP9/10 receptor, ALK1, is embryonically lethal, while haploinsufficiency in ALK1 leads to a genetic disorder called hereditary hemorrhagic telangiectasia type 2 (HHT2), which is characterized by abnormal vessel formation (131). Knockout of endoglin, which also interacts directly with BMP9/BMP10, also leads to a very similar disease, HHT1.

Early studies described BMP9 as a vascular quiescence factor (132). Subsequent papers used constitutively active ALK1 to confirm that BMP9 inhibits VEGF-induced angiogenesis and endothelial cell proliferation (79). Recent studies, however, reported the ability of BMP9 to induce proliferation of multiple types of endothelial cells both in vitro and in vivo, most likely by up-regulating VEGF receptors and Angiopoetin-1/Tie2 expression (133). These findings are not surprising, as ALK1 signalling up-regulates ID-1 and ID-3 protein expression (134), which are strong promoters of angiogenesis (135). Interestingly, the soluble form of ALK1 was recently reported to inhibit tumor growth in mouse models (136). As with other TGF-β superfamily ligands, the functional role of BMP9 remains controversial and is likely extremely cell and context dependent.

Apart from angiogenesis, BMP9 was also found to have many other effects in vitro and in vivo. As most other BMPs, BMP9 can function as an osteogenic and chondrogenic factor (137). Additionally, BMP9 can also regulate metabolism, by inhibiting glucose production and upregulating key enzymes of lipid homeostasis (138). Moreover, two recent papers reported that BMP9 can induce secretion of Endothelin-1 (ET-1), a very potent vasoconstrictor (139, 140).
1.4 Endothelin-1

1.4.1 Structure and Function of ET-1

1.4.1.1 Biosynthesis of ET-1

Endothelin was first described in 1985 as an endothelium-derived constrictor factor (141). Since then, 3 different isotypes of ET-1 have been found, termed ET-1, ET-2 and ET-3. ET-1 is by far the most prominent form, and responsible for the majority of functions exerted by ETs (142). Endothelin isoforms are highly homologous; in fact ET-2 and ET-3 differ from ET-1 only by 3 and 6 amino acids respectively. All three are synthesized as pre-pro-proteins, with a 17 amino acid leader sequence that targets pre-pro-ET-1 to the endoplasmic reticulum(143). In the cell, the pre-domain (165 amino acids) is cleaved from pro-ET-1 by furin-like proteases, leaving 38 amino acids, termed Big-ET1. Big-ET1 is then further cleaved into active ET-1 (21 amino acids) by endothelin-converting enzymes (ECE) (144). It is thought that activation of ET-1 by ECE is a rate-limiting step in ET-1 production (145). ET-1 is mainly produced by endothelial cells in relatively small quantities, but also by other cell types, such as cardiomyocytes and vascular smooth muscle cells. The reported concentration of ET-1 in the serum was much below the EC\textsubscript{50} of the ET-1 receptors, suggesting that ET-1 acts as a local agent (146).

1.4.1.2 The receptors of ET-1

There are four known receptors for endothelins called ET\textsubscript{A}, ET\textsubscript{B1}, ET\textsubscript{B2} and ET\textsubscript{C}, all of which are G-protein coupled receptors. ET\textsubscript{A} binds ET-1 and ET-2 with greater affinity than ET-3, while ET\textsubscript{B} appears to bind all isoforms with the same strength. These receptors are expressed in different levels in various cell types (147). Studies revealed that ET\textsubscript{A} receptors are mostly present on vascular smooth muscle cells (VSMC) and upon activation initiate calcium ion influx and subsequent vasoconstriction. ET\textsubscript{B} receptors, on the other hand, are mostly present in endothelial cells, and induce release of NO and prostacyclin, causing vasodilatation (148). Typically upon ET-1 injection, arterial pressure briefly goes down due to the extremely fast secretion of NO, induced via ET\textsubscript{B} receptors, but then quickly rises due to the constriction effect via ET\textsubscript{A} receptors (149). Interestingly, an isotype of ET\textsubscript{B} receptors has been recently found to be present in VSMC. It was named ET\textsubscript{B2} receptor, as opposed to ET\textsubscript{B1} found on endothelial cells; upon activation, ET\textsubscript{B2} initiates contraction of the smooth muscle cells (150). Constriction by ET\textsubscript{A} receptor can usually last for up to one hour. Studies have shown that vasoconstriction is initiated by prolonged
influx of Ca\(^{2+}\) from the extracellular space. Removal of ions from the extracellular space resulted in the absence of sustained vasoconstriction (149). Interestingly, NO donors were found to interfere with channels responsible for ET-1-induced calcium ion influx, implying that ET\(_A\) and ET\(_B\) receptors might work in opposite ways (151). Moreover, several studies have reported that NO decreases ET-1 production from endothelial cells, once again suggesting a delicate balance between ET\(_A\) and ET\(_B\) receptors (152). Though it would seem logical to inhibit ET\(_A\) receptor, rather than inhibiting both types of receptors, most studies agree that inhibiting both ET\(_{A/B}\) receptors produces a much more effective vasodilatation (146). It is likely, that exclusive inhibition of the ET\(_A\) receptor, allows the ET\(_B\) receptor on VSMC to mediate ET-1-induced vasoconstriction.

1.4.1.3 Physiological role of ET-1

Endothelin-1 is one of the most potent vasoconstrictors known, second only to urotensin II (153). In humans, ET-1 increases mean arterial blood pressure, reduces heart rate and causes sustained vasoconstriction in the pulmonary vasculature (154). Since its discovery in 1988, multiple studies have shown a link between ET-1 and various cardiovascular disorders, such as heart failure, hypertension, renal failure and many others. Blockade of ET\(_A\) receptor was shown to result in an increase in forearm blood flow in healthy humans (155), while inhibition of ET\(_B\) signalling yielded local vasoconstriction. Therefore, it appears that endothelial cells are constantly stimulated to induce production of ET-1, which plays an important role in maintenance of vascular tone. The importance of ET-1 is highlighted by the fact that the ET-1 homozygous knockout phenotype is lethal with morphological abnormalities of craniofacial and thoracic blood vessels (156). Mice deficient in receptors for endothelin-1 show a similar phenotype (157). ET-1 heterozygous mice, paradoxically suffer from increased systemic blood pressure, even though serum ET-1 concentration was found to be much lower than in wild type mice. The most probable explanation is the fact that the ET\(_B\) receptor is no longer activated, leading to decreased in NO production and subsequent hypertension (156, 158). This is further strengthened by the fact that mice deficient in ET\(_B\) receptor also experience hypertension (158).

Apart from its cardiovascular effect, ET-1 is also associated with inflammation processes. In fact, ET-1 was shown to activate macrophages, leading to release of multiple pro-inflammatory cytokines, including IL-1, IL-6 and TNF-\(\alpha\). In turn, both TNF-\(\alpha\) and IL-6 were able to induce
ET-1 secretion from endothelial cells (159). It appears that ET-1 works in concert with TNF-α to up-regulate adhesion molecules on ECs. Blockade of ET-1 receptors has been shown to significantly decrease the accumulation of neutrophils and myeloperoxidase activity in the ischemic myocardium (160).

1.4.2 Role of ET-1 in Preeclampsia

1.4.2.1 ET-1 levels in PE

Several studies have looked into ET-1 as being a potential contributor to preeclampsia-associated hypertension. Most studies have found a 2 to 3 fold increase in ET-1 concentration in the serum of preeclamptic women and at least one study found a correlation between ET-1 levels and severity of the disease (161, 162). Additionally, Nishikawa et al. looked at serum nitrate levels in preeclamptic patients and showed a negative correlation between ET-1 levels and those of vasodilators NO and cGMP (162). Another group compared the expression levels of ECE and found it was up-regulated in preeclamptic women compared to healthy controls, implying that increased activity of ECE might be contributing to the increase in ET-1 production (163). Moreover, ET-1 was shown to induce oxidative stress and inhibit proliferation of JEG-3 cells (a trophoblast line), suggesting that increased levels of ET-1 may contribute to extravillous trophoblast differentiation in vivo (164).

1.4.2.2 ET-1 in preeclampsia models

There are several animal models, which were developed to study preeclampsia. One of the most widely used models is RUPP (Reduced uterine perfusion pressure), to partially block blood flow to the uterus at the later stages of gestation (165). As a result, the placenta experiences acute hypoxia and ischemia, an approximation of what happens when the trophoblast fails to invade the spiral artery. This model was applied to a wide variety of animals, including rodents, dogs and non-human primates. Most studies reported that animals were experiencing all hallmark symptoms of preeclampsia, such as elevated sFlt-1, renal injury and endothelial dysfunction (165-167). Other studies looked at the expression of ET-1 in RUPP animals and concluded that pre-pro-endothelin mRNA was significantly elevated compared to healthy pregnant controls (168, 169). Moreover, endothelial cells incubated with serum from RUPP rats secreted significantly more ET-1 when compared to cells incubated with healthy control serum (170). The
most interesting finding, however, was that hypertension induced by this model can be completely abolished using an \( \text{ET}_A \) specific antagonist (171).

Another method to induce PE-like disease in animals is to introduce exogenous sFlt-1 molecules. Several groups reported proteinuria, hypertension and renal failure in rats infused with recombinant sFlt-1 or using an adenovirus for delivery of the gene (3). One study looked at ET-1 production in these animals and reported significantly higher levels of ET-1 mRNA in renal cortex. They also looked at the ability of \( \text{ET}_A \) antagonist to relieve sFlt-1-induced hypertension and found that by blocking ET-1 signalling could completely abolish the hypertensive response (18).

In yet another model, \( \text{AT}_1 \)-AA are delivered into pregnant mice, leading to preeclampsia-like symptoms. In the same study, the authors reported an increase in ET-1 mRNA levels, suggesting that induced hypertension was in part due to ET-1 signalling (58). Zhou et al. looked into the mechanism of ET-1 secretion and found that \( \text{AT}_1 \)-AA induced TNF-\( \alpha \) expression, which leads to IL-6 expression, which is necessary for ET-1 production. In fact, \( \text{ET}_A \) antagonists were able to completely abolish the induced hypertension, implying a key role for ET-1 in this model of preeclampsia. Additionally, inhibition of either TNF-\( \alpha \) or IL-6 expression resulted in normal pregnancy with no hypertension and no ET-1 up-regulation (172).

1.4.2.3 Anti-ET-1 therapeutics

It is clear that ET-1 is a key mediator of hypertension in preeclampsia. Multiple studies have shown that administration of ET-1 blocking agents can attenuate, if not completely abolish, PE-associated hypertension (171-173). However, genetic studies with ET-1 or \( \text{ET}_A \) receptor deficient mice showed a number of serious birth defects. In fact, administration of endothelin receptor antagonists is contraindicated during pregnancy (174). Other groups looked into the possibility of administrating ET antagonists during specific times in pregnancy; however, more studies are needed to evaluate the safety of this method (175). In summary, it is evident that ET-1 plays a crucial role in preeclampsia. However, direct inhibition of ET-1 signalling might not be the best way to manage rising blood pressure. Indirect methods of inhibiting ET-1 secretion might prove safer and more beneficial in fighting preeclampsia-associated hypertension.
1.5 Thesis Objectives

1.5.1 Rationale

Preeclampsia is responsible for more than 15% of all premature births in North America, yet pathogenesis of the disease is poorly understood and currently there is no cure but to deliver the baby prematurely. The crucial role of ET-1 in preeclampsia-associated hypertension has been documented by several groups; however, the exact mechanism underlying increased ET-1 generation is not established. Recent reports that some members of TGF-β superfamily can induce ET-1 secretion raise the possibility that they could be responsible for increased levels of ET-1 during preeclamptic pregnancies. Moreover, recent findings have shown that sEng binds BMP9 with high affinity, creating an intriguing possibility that sEng production could be beneficial for preeclampsia patients by blocking BMP9 signalling and subsequent ET-1 generation. Therefore, we investigated the ability of endothelial cells to produce ET-1 in response to TGF-β1 and BMP9. Additionally, we examined signalling pathways responsible for ET-1 secretion and looked at the effects of sEng and VEGF, two known factors involved in PE, on BMP9 – and TGF-β1 –induced ET-1 secretion.

1.5.2 Hypothesis

sEng counteracts sFlt-1-induced hypertension by blocking BMP9 signalling and subsequent secretion of ET-1.

1.5.3 Specific Objectives

(I) To test the effects of sEng and VEGF on BMP9 – and TGF-β1 –induced secretion of ET-1 from mouse endothelial cells.

(II) To examine and compare different signalling pathways responsible for ET-1 secretion from endothelial cells stimulated with either BMP9 or TGF-β1.
2 Materials and Methods

2.1 Cell culture

Mouse embryonic endothelial cells (EC) were a generous gift from Dr E. Dejana (Milan, Italy). They were derived from mouse embryos at E9.0 and immortalized using polyoma middle T antigen (176). Cells were cultured in MCDB131 medium plus 1% glutamine, 1% penicillin/streptomycin, 15% heat-inactivated fetal bovine serum (All GIBCO) and 25 µg/ml endothelial mitogen (Biomedical Technology Inc.), as previously described (177).

2.2 Cell based stimulation and inhibition assays

Endothelial cells were seeded at $5 \times 10^5$ cells per well on 6-well plates and incubated in complete medium at 37°C and 5% CO₂. After 48 hours, cells were washed with phosphate-buffered saline (PBS, GIBCO) and incubated in serum-free media for 3 hours. After another wash, cells were incubated in serum-free MCDB131 media with various inhibitors: increasing concentrations of dorsomorphin (10, 20, 40 µM; Bioscience), SB525334 (1, 2, 4 µM; Selleckchem), or BIRB796 (0.2, 0.5, 1 µM; Selleckchem) for 30 minutes. Cells were then treated with 40 pM of either TGF-β1 or BMP9 for another 30 minutes.

2.3 Western Blotting

Cell lysates were obtained by scraping cells from the 6-well plates and solubilizing in 500 µL of cell lysis buffer (0.05 M Tris-HCl pH 7.4, 0.01 M NaCl, 1% Triton X-100, 0.01 M sodium pyrophosphate, 0.25 M NaF, 0.001 M Na₃VO₄; all from Sigma), supplemented with 1% ProteoBlockTM protease inhibitor cocktail (Fermentas). Samples were then rotated for 30 minutes at 4°C and centrifuged at 10,000g for 30 minutes at 4°C, and the supernatants collected.

Protein concentration measurements were done in duplicate according to the protocol from BioRad Protein Assay II kit. Once concentration was established, a 20 µg protein aliquot was mixed with reducing buffer (Invitrogen), supplemented with 62 µg/mL DL-Dithiothreitol, in 3:1 ratio. Samples were then heated at 95°C for 5 minutes, centrifuged for 1 minute at 10,000g and fractionated on 10 or 12 well 4-12% Bis-Tris Gels (Invitrogen). Samples were fractionated at 150 volts for approximately 1 hour in SDS Running Buffer (NuPAGE MES, Novex, Invitrogen)
and transferred to a PVDF membrane in Transfer Buffer (0.025 M Tris pH 7.4, 0.192 M glycine, 20% methanol; all Sigma) at 4°C for 90 minutes at 200 amps.

Membranes were blocked in 5% milk (Bio-Rad) for 1 hour and incubated with one of the following antibodies: Phospho-Smad1/5/8 (1:1000), Phospho-Smad2/3 (1:1000), Phospho-p38α (1:1000), phospho-p38β (1:1000), purchased from Cell Signalling; total Smad2/3 (1:1000), total Smad1/5/8 (1:1000), phospho-p38 (1:1000), purchased from Santa-Cruz, or β-actin (1:1000), purchased from Sigma. Membranes were incubated overnight at 4°C, washed 3 times in TBS-T buffer (0.02 M Tris base, 0.137 M NaCl, 0.1% Tween 20; Sigma) for 10 minutes and incubated in secondary antibody (anti-mouse or anti-rabbit, purchased from GE Healthcare, 1:10,000 dilution in 5% milk). After 3 washes, membranes were incubated in Western Lighting TM Chemi luminescent Reagent Plus and developed using Konica Minolta SRX-101A developer. Radiographs were scanned and analyzed using FluorChem software. Stripping was done using 1 M glycine solution, pH=2.5 (Sigma).

2.4 Enzyme-Linked Immunosorbent Assay (ELISA) for ET-1
Cells were seeded in 6-well plates at 5·10^5 cells per well and incubated at 37°C for 48 hours. Cells were washed with PBS and starved for 12 hours in MCDB131 media without any additives. Cells were then incubated for 30 minutes with inhibitors (dorsomorphin at 40 µM; Bioscience, BIRB796 at 1 µM; Sellechchem, SB525334 at 8 µM; Sellechchem) and stimulated with various ligands, including TGF-β1 (1 ng/ml, R&D), BMP9 (1 ng/ml, R&D), VEGF (50 ng/ml; R&D), soluble ALK1-Fc (125 ng/ml, R&D) and recombinant sEng [100 nM; this protein was generated by Allison Gregory and contains only the extracellular region, 1-586 amino acids (∆586)]. After 10 hours of stimulation, media was collected, centrifuged for 20 minutes at 10,000g, transferred to a new tube and stored at -80°C. Cell lysates were collected and tested for protein concentration to ensure similar cell numbers in each sample. Protein concentrations estimated in the various samples were within 10% of each other.

The ET-1 ELISA kit was used according to the protocol supplied by EnzoBiosciences. Briefly, 100 µL of the stored media samples were added to the 96-well plate coated with ET-1 antibodies and incubated for 2 hours at 23°C. The plate was then washed 5 times in supplied washing buffer and incubated with antibodies to ET-1 for 1 hour. Once again, the plate was washed 5 times and
visualizing solution was added for 30 minutes. Stop solution was then added to stop the reaction and the plate was read at a wavelength of 450 nm. A standard curve was constructed using the supplied ET-1 protein.

2.5 Biacore Biosensor Analysis

All experiments were done using BIAcore X (BIAcore, Uppsala, Sweden). First, research grade CM5 sensor chips were activated using the standard NHS (N-hydroxysuccinimide)/EDC (N-ethyl-N’-3-diethylaminopropyl) procedure. For coupling, proteins were diluted to 20 nM in 10 nM sodium acetate buffer, pH 6. Soluble receptors were then passed over the activated surface until the desired amount of molecules was covalently attached to the chip. 1 M ethanolamine was then used to quench any remaining active sites. The data were collected at 10 Hz using at least 5 injections of each ligand. Flow rate was maintained at 2-20 µL/min. Regeneration of the chip with attached sALK1-Fc molecules was carried out using 3 M MgCl₂. Regeneration of the chip with sTβRII receptor was done using 0.4 M urea, 0.9 M MgCl₂, 0.025 M EDTA, 0.9 M guanidine-HCl, 0.01 M glycine, pH 9.5.

BIAevaluation 4.1 software was used to measure $K_{on}$ and $K_{off}$ of each interaction, and subsequently the $K_D$ was calculated using these numbers. A hyperbolic curve was then fitted to the graph. Concentration at which RU (relative units) difference was half-maximum represents the affinity of the interaction ($K_D$). The highest observed RU was taken as the maximum.

2.6 Statistical Analysis

Data are reported as mean ± SEM. Comparisons were performed by ANOVA, and significant differences were evaluated post hoc using the Newman-Keuls method. Results are expressed as the mean ± SEM with $P<0.05$ representing significance.
3 Results

3.1 BMP9 and TGF-β1 induce ET-1 secretion in mouse embryonic endothelial cells

Recent studies have reported that BMP9 and TGF-β1 are potent inducers of ET-1 production in human endothelial cells (139, 140). Since ET-1 is a major vasoconstrictor protein (141) implicated in PE pathogenesis (178), I examined the ability of BMP9 and TGF-β1 to stimulate ET-1 secretion in endothelial cells.

To better understand the kinetics of ET-1 release from EC, I exposed cells to increasing concentrations of either BMP9 or TGF-β1 (Figure 8). Unstimulated EC secrete close to 15 pg/ml of ET-1 in 10 hours. This value did not vary significantly throughout the experiments. When treated with low concentrations of BMP9 (0.1, 0.5 ng/ml), EC showed only a slight increase in ET-1 secretion; however, when stimulated with 1 ng/ml of BMP9, EC demonstrated a significant 2-fold increase in ET-1 secretion. EC secreted more ET-1 when treated with higher concentrations of BMP9 (2, 4 ng/ml); however, doubling BMP9 concentration from 2 to 4 ng/ml produced only a modest increase in ET-1 secretion (from 42 to 50 pg/ml), suggesting that near maximum stimulation had been achieved. A software-constructed hyperbolic curve showed a high degree of correlation with the acquired data, resulting in $R^2 = 0.99$.

Treatment of EC with TGF-β1 also induced secretion of ET-1. However, unlike BMP9, low concentrations of TGF-β1 had a pronounced effect on the ability of endothelial cells to secrete ET-1. In fact, 0.4 ng/ml of TGF-β1 induced a significant increase in ET-1 levels. Cells reached their maximum ET-1 secretion of 47 pg/ml when treated with 0.8 ng/ml of TGF-β1. Subsequent increase in TGF-β1 concentration failed to induce a further rise in ET-1 secretion, suggesting that saturation was achieved. A fitted hyperbolic curve showed good correlation with $R^2 = 0.86$. This is most likely due to the large standard errors in the linear portion of the TGF-β1 titration curve, resulting from small changes in ligand concentration causing big changes in ET-1 release, making numbers more variable. Overall, treatment with either BMP9 – or TGF-β1 –induced a strong increase in ET-1 secretion in mouse embryonic endothelial cells.
Figure 8. BMP9 and TGF-β1 induce ET-1 secretion from mouse endothelial cells.

EC were starved for 12 hours in serum-free media and incubated with increasing concentrations of BMP9 (A) or TGF-β1 (B) for 10 hours. Media were collected and tested for the presence of ET-1 by ELISA and results are expressed as pg/ml media. A hyperbolic curve was fitted by the software for each ligand, based on acquired results. Data are the mean ± SEM of 3 experiments, all done in duplicate. * and # denote $P<0.05$ versus control value.
3.2 sEng (Δ586) binds with high affinity to BMP9, but not to TGF-β1

In contrast to sFlt-1, the exact sequence of the physiological form of sEng, how it is released from the placenta and its role in PE remain relatively unknown. As a membrane protein, endoglin was shown to physically associate with a number of TGF-β superfamily receptors and to modulate responses to several TGF-β ligands (82, 177, 179). The function of endoglin as a soluble protein, however, is poorly understood and a recombinant form of sEng (Δ586; 80kDa monomer) is used in most studies, which represents the complete extracellular domain of the membrane protein, which is of larger size than the physiological form of sEng purified from PE sera (65kDa monomer) (see Figure 5) (4). Thus, the complete amino acid sequence of the physiological form of sEng remains to be determined. Several papers have proposed that sEng interferes with TGF-β1 signalling (4, 180); however, recent studies indicate that recombinant sEng (Δ586) has a much higher affinity for BMP9 (65, 181).

To examine the functional properties of sEng, we used BIACore X to evaluate binding affinities of sEng to TGF-β1 and BMP9 ligands (Figure 9). As a proof of principle, the $K_D$ value between two known high affinity receptor/ligand pairs was also measured. Binding of increasing concentration of TGF-β1 (0.1 nM – 200 nM) to immobilized soluble TβRII dimer, yielded a $K_D = 0.1\text{nM}$. That is an extremely tight interaction; in fact, we were unable to strip TGF-β1 from TβRII, which made our calculations less reliable. It is likely that the $K_D$ value was underestimated (Figure 9A). Next, we examined the strength of binding between BMP9 and recombinant sALK1-Fc, which are known to interact with each other from both functional and structural studies (83, 181). My experiments confirmed that BMP9 binds to soluble ALK1 with an extremely high affinity with a $K_D = 1.8\text{nM}$, which though weaker than TGF-β1/TβRII interaction, is still of high affinity.

We then looked into the binding affinities of sEng for TGF-β1 and BMP9. Purified dimeric recombinant sEng (Δ586) was fractionated through size exclusion chromatography and coupled to the CM5 chip. Various concentrations of TGF-β1 and BMP9 were passed over sEng to look at the strength of the interactions. TGF-β1 showed no significant binding to the immobilized sEng, with an estimated $K_D$ value of 1230 nM. In none of the trials were we able to fully saturate the bound sEng, indicating extremely low affinity of interaction. It is evident that TGF-β1 does not
bind to recombinant sEng at lower and more physiological concentrations. Interestingly, sEng was found to have a high affinity towards BMP9. Our experiments showed that sEng/BMP9 interaction has a $K_D = 5$ nM, which is in the same range as the ALK1 – BMP9 interaction. Together these data demonstrate that, despite multiple reports of recombinant sEng being able to block TGF-β1 signalling, it cannot do so by scavenging TGF-β1 directly, but rather that it has a strong affinity towards BMP9 and could scavenge that ligand.
Figure 9. sEng binds with high affinity to BMP9, but not to TGF-β1.

Commercially available recombinant TβRII (A) or ALK1-Fc (B) were amine-coupled to a CM5 chip and increasing concentrations of TGF-β1 or BMP9 were passed over their respected receptors to determine the on and off constants and calculate the affinity constants. sEng (Δ586) was coupled to a different CM5 chip and increasing concentrations of TGF-β1 (C) or BMP9 (D) were injected over the captured protein. Colored lines represent the actual data acquired in the course of the experiment and dotted lines show the expected graph assuming 1:1 binding mode using the calculated $K_D$ values.

*These experiments were done with Allison Lindsay Gregory from our laboratory and Zhijie Li in Dr. James Rini’s laboratory, Department of Biochemistry, University of Toronto.*
3.3 sALK1-Fc and sEng block BMP9 – but not TGF-β1 –induced ET-1 secretion

During preeclampsia, the placenta produces a number of anti-angiogenic factors, including circulating sEng and sFlt-1, which show a strong correlation with PE severity (182, 183). Our own experiments (Figure 8) and recent papers concluded that BMP9 is the most relevant ligand for recombinant sEng. Knowing that BMP9 is a strong inducer of ET-1 secretion, we hypothesized that one of the functions of sEng in the context of PE, could be to scavenge BMP9 and interfere with its ability to block ET-1 secretion.

To examine if sEng is capable of blocking ET-1 secretion induced by BMP9 or TGF-β1 ligands, we treated cells with either BMP9 or TGF-β1 in the presence or absence of 25 nM ALK1-Fc or sEng. Treatment with 25 nM ALK1-Fc alone (in the absence of ligand) induced a small but significant reduction in ET-1 secretion from 16 to 8 ng/ml, implying that endothelial cells themselves secrete a small amount of BMP9, which can be blocked with addition of its soluble receptor. The addition of sEng (∆586) alone decreased ET-1 secretion, but to a lower extent than sALK1-Fc did (Figure 10A).

sALK1-Fc was used as positive control, since this protein is known to have high affinity for BMP9 but not TGF-β1. EC treated with 1 ng/ml of BMP9 showed a significant (2.5-fold) increase in ET-1 secretion. As expected, addition of 25 nM sALK1-Fc abolished BMP9 – induced ET-1 secretion. EC treated with a combination of BMP9 and sEng (∆586), showed significant inhibition in ET-1 secretion compared to BMP9 treated cells (Figure 9B).

Treatment with 1 ng/ml of TGF-β1 produced a significant (2.5-fold) increase in ET-1 secretion. However, neither sALK1-Fc nor sEng could block TGF-β1–induced ET-1 secretion (Figure 9C). Taken together these data indicate that sALK1-Fc and sEng (∆586), can interfere with ET-1 release through direct binding to BMP9.
Figure 10. sALK1-Fc and sEng block BMP9 – but not TGF-β1 – induced ET-1 secretion.

EC were starved for 12 hours in serum-free media and incubated with 25 nM of sALK1-Fc or sEng (Δ586), alone (A) or in combination with 1 ng/ml of BMP9 (B) or TGF-β1 (C) for 10 hours. Media were collected in duplicate and analyzed by ET-1 ELISA. Each column represents data mean ± SEM of 5 experiments. *and # denote significance versus control and stimulated levels respectively, P< 0.05.
3.4 VEGF interferes with BMP9 but not TGF-β1 signalling to block ET-1 secretion

Several studies have implicated altered PlGF/VEGF signalling pathways in PE pathogenesis (4, 182). sFlt-1, shown to be elevated in the serum of preeclamptic women, directly binds to both PlGF and VEGF, blocking their downstream signalling. Several studies have reported lower levels of free PlGF and VEGF proteins in the serum of women with PE (20, 183, 184). Lack of VEGF signalling is thought to be directly responsible for the observed hypertension and proteinuria. In fact, external administration of sFlt-1 has been shown to lead to PE-like symptoms (4). Since both PlGF and VEGF have been implicated in PE, we examined if they could affect TGF-β superfamily receptor signalling. Specifically we looked at the ability of either PlGF or VEGF to alter ET-1 release, which is known to be dysregulated in PE (178).

To test PlGF/VEGF involvement in ET-1 secretion, EC were incubated with 1 ng/ml of BMP9 or TGF-β1 in the presence or absence of PlGF or VEGF (Figure 11). First, we looked at the effect of VEGF and PlGF on basal ET-1 secretion. Neither molecule significantly affected the basal level of ET-1 secreted in the media (Figure 10A). Cells treated with BMP9 showed a robust 2-fold increase in ET-1 secretion. Surprisingly, when cells were treated with a combination of BMP9 and VEGF, we saw a significant decrease in ET-1 secretion, compared to cells treated with BMP9 alone. Treatment with PlGF did not have any effect on ET-1 secretion (Figure 10B). Similar to BMP9, TGF-β1–induced a strong increase in ET-1 secretion. Cells co-incubated with both TGF-β1 and VEGF did not show any difference in ET-1 secretion compared to cells treated with TGF-β1 alone. Similarly, PlGF was not able to influence TGF-β1 stimulated ET-1 secretion (Figure 10C). In summary, PlGF did not have any effect on ET-1 secretion, induced by either BMP9 or TGF-β1. VEGF, on the other hand, strongly inhibited ET-1 secretion induced by BMP9, however failed to do so with cells treated with TGF-β1.
**Figure 11. VEGF inhibits BMP9 – but not TGF-β1 –induced ET-1 secretion.**

EC were seeded in 6-well plates and incubated at 37°C for 48 hours. Cells were then starved for 12 hours in serum-free media and incubated with 50 ng/ml of either VEGF or PlGF (A) in the presence or absence of 1 ng/ml of either BMP9 (B) or TGF-β1 (C). After 10 hours, media were collected for determination of ET-1 by ELISA. Each sample was run in duplicate. Data are shown as mean ± SEM of 5 experiments. * and # denote significance (P< 0.05) compared to control and stimulated levels respectively.
3.5 Additive effects of BMP9 and TGF-β1 on ET-1 secretion

The fact that VEGF can lower ET-1 levels induced by BMP9 but not by TGF-β1 suggests that there could be differences in signalling cascade between these two proteins. If two ligands use different pathways to induce ET-1 secretion, we should see an additive effect when both ligands are present. In fact, a recent study reported that co-incubation of endothelial cells with both TGF-β1 and BMP9 proteins lead to an additional 29% increase in ET-1 secretion (140). To see if mouse embryonic endothelial cells respond similarly, I incubated cells with 1 ng/ml of both TGF-β1 and BMP9 (Figure 12).

Unstimulated cells produced 15.5 pg/ml of ET-1. Treatment with either BMP9 or TGF-β1 produced a similar 3-fold increase in ET-1 secretion. However, their combination showed a 5-fold increase in ET-1 levels, compared to cells treated with either protein individually.

These results suggest that TGF-β1 and BMP9 use different pathways to induce ET-1 secretion. Cells treated with 1 ng/ml of TGF-β1 were fully saturated and an additional increase in TGF-β1 concentration did not result in an increase of ET-1 secretion (Figure 1B). However, treatment with TGF-β1 and BMP9 clearly induced a stronger response than each protein individually and gave an additive effect, suggestive of distinct pathways of ET-1 secretion.
Figure 12. Combination of BMP9 and TGF-β1 has an additive effect on ET-1 secretion.

EC were starved for 12 hours in serum-free media and incubated with BMP9, TGF-β1 or a combination of both ligands for 10 hours. Media were collected and analyzed for ET-1 by ELISA. *P<0.05 compared to control levels, # and & P<0.05 compared to BMP9 and TGF-β1 treated cells respectively. Bars represent mean ± SEM of 5 experiments.
3.6 Analysis of the ability of inhibitors to block their respective phosphorylation pathways

BMP9 and TGF-β1 are known ligands for the TGF-β receptor superfamily. BMP9 was recently shown to bind with high affinity to ALK1 to induce strong Smad1 phosphorylation, while TGF-β1 is a strong inducer of Smad2/3 phosphorylation (79, 83, 185). Furthermore, in endothelial cells, TGF-β1 was also shown to signal through ALK1 receptor to initiate Smad1/5/8 phosphorylation (84). Besides the canonical Smad pathways, both ligands are known to activate other signalling cascades including ERK and p38 (186, 187). Recent studies have implicated Smad1 and p38 in BMP9-induced ET-1 release from human endothelial cells (139, 140). Given that VEGF could interfere with BMP9– but not TGF-β1–induced ET-1 secretion, we hypothesized that these proteins use distinct pathways to stimulate ET-1. In order to examine the pathways responsible for ET-1 release, we treated cells with inhibitors of Smad1/5/8 (dorsomorphin), Smad2/3 (SB525334), and p38 (BIRB796) phosphorylation. Before using inhibitors in ELISA experiments, we looked at their specificity in blocking their respective pathways.

Cells were serum starved for 3 hours and incubated for 30 minutes with either TGF-β1 or BMP9 in the presence or absence of various inhibitors. Preliminary experiments were performed to determine the optimal concentration of the inhibitors. Dorsomorphin was able to significantly reduced Smad1/5/8 phosphorylation at 40 μM; further increase in concentration of the inhibitor did not affect the phosphorylation. SB525334 completely blocked phosphorylation of Smad2/3 at 8 μM; therefore this concentration was chosen for our experiments. BIRB796 at 1 μM resulted in a mild decrease of p38 phosphorylation. Increase in BIRB796 concentration did not show further down-regulation of p38 phosphorylation (data not shown).

TGF-β1 was the only molecule capable of inducing Smad2/3 phosphorylation. BMP9 showed only a small increase in Smad2/3 phosphorylation, which did not reach significance. Since Smad2/3 phosphorylation is mediated by ALK5 receptor, neither BIRB796 nor dorsomorphin could interfere with ALK5 signalling. On the other hand, SB525334, an ALK5 inhibitor completely abolished Smad2/3 phosphorylation induced by TGF-β1 treatment (Figure 13A).

Both BMP9 and to a lesser extent TGF-β1 were able to induce Smad1/5/8 phosphorylation. TGF-β1–induced phosphorylation was blocked by both dorsomorphin and SB525334, implying
that both receptors are required for TGF-β1 activation of the Smad1/5/8 pathway. These results are in agreement with the reports showing that TGF-β1 binds to ALK5 to promote association with ALK1 receptor and can activate both Smad1/5/8 and Smad2/3 pathway simultaneously (78). As expected, BIRB796 could not affect Smad phosphorylation induced by TGF-β1 and BMP9.

BMP9 induces Smad1/5/8 phosphorylation by directly binding to and activating the ALK1 receptor (79, 83). Therefore, the ALK1 inhibitor, dorsomorphin, was the only molecule tested capable of inhibiting Smad1/5/8 phosphorylation induced by BMP9. Neither SB525334 nor BIRB796 produced any noticeable effect (Figure 13B).

Treatment with BMP9 and TGF-β1 resulted in a slight increase in p38 phosphorylation, which, however, did not reach significance. Phosphorylation of p38 is activated by a number of signalling pathways and by stress signals; it is therefore possible that p38 phosphorylation was already induced in the control state. Among the 3 inhibitors, only dorsomorphin and BIRB796 showed a trend towards lowering control levels of p38 phosphorylation. These results are in agreement with several papers reporting the ability of dorsomorphin to block p38 phosphorylation (188, 189). The ALK5 inhibitor, SB525334, was unable to affect p38 phosphorylation levels (Figure 13C).

In summary, these results indicate that SB525334 is a very potent inhibitor of TGF-β1 effects. It blocks both Smad1/5/8 and Smad2/3 phosphorylation induced by TGF-β1. Dorsomorphin blocked Smad1/5/8 phosphorylation initiated by BMP9 and TGF-β1, but also blocked p38 phosphorylation as an off-target effect. BIRB796 is a specific inhibitor of p38 and did not influence phosphorylation of Smad1/5/8 or Smad2/3 molecules.
Figure 13. Effects of inhibitors on Smad1/5/8, Smad2/3 and p38 phosphorylation.

Cells were seeded on 6-well plates and cultured for 48 hours at 37°C. After 3 hour starvation in serum-free media, cells were incubated for 30 minutes with the following inhibitors: dorsomorphin (D; 40 μM), SB525334 (S; 8 μM) or BIRB796 (B; 1 μM). Afterwards, cells were treated with 1 ng/ml of either BMP9 or TGF-β1. Cell lysates were resolved by SDS-PAGE and analyzed by Western blot with antibodies to phosphorylated Smad1/5/8 (PSmad1/5/8), Smad2/3 (PSmad2/3) and p38 (P-p38) as well as total Smad1/5/8 (Smad1/5/8), Smad2/3 (Smad2/3) and β-actin. Densitometry was performed to quantify each band. The extent of Smad phosphorylation was quantified relative to total Smad levels, while p38 phosphorylation was quantified relative to β-actin levels. The graphs represent the mean ± SEM of 3 experiments, done in duplicate.
3.7 SMAD1 and p38 phosphorylation are involved in BMP9 – and TGF-β1 –induced ET-1 secretion

Recent studies have looked into pathways involved in ET-1 release. Specifically various inhibitors were used to show that p38 and Smad1 pathways are extremely important for BMP9-induced ET-1 secretion (139, 140). Both studies also concluded that ERK phosphorylation was not involved in ET-1 secretion. However, TGF-β family receptor signalling can be extremely cell dependent; BMP9, for example, was able to promote cell growth of multiple endothelial cell lines (133); however BMP9 inhibited proliferation and invasiveness of breast cancer cells (190). It is, therefore highly important to examine signalling pathways involved in ET-1 release in mouse embryonic endothelial cells.

EC cells were serum-starved for 12 hours and incubated with inhibitors of Smad1, Smad2 and p38 pathways for 30 minutes. Cells were then treated with 1ng/ml of either BMP9 or TGF-β1 for 10 hours. Media were collected for ET-1 ELISA measurements (Figure 13). Treatment of EC with dorsomorphin but not SB525334 inhibitor resulted in a decrease of ET-1 secretion, implying that cells produce small amounts of BMP9, which contribute to the background level of ET-1. These results are in agreement with our previous experiments, showing that sALK1-Fc is capable of lowering background levels of ET-1, presumably by binding and neutralizing free BMP9 molecules in the media. Treatment with BIRB796 did not affect baseline ET-1 secretion (Figure 13A).

Dorsomorphin is an inhibitor of ALK1 (the receptor for BMP9) and was extremely efficient at blocking BMP9-induced ET-1 release, completely abolishing it. SB525334, the inhibitor of ALK5, a receptor not used by BMP9, showed no effect on BMP9-induced ET-1 secretion. Treatment with BIRB796 showed a significant reduction in ET-1 levels, though this inhibitor was half as efficient as dorsomorphin, implying both p38 dependent and independent pathways (Figure 13B).

A similar picture was observed with cells treated with TGF-β1. Dorsomorphin, which blocks both Smad1/5/8 and p38 pathways, completely inhibited ET-1 secretion back to baseline levels, implying that the majority of ET-1 is produced via these two pathways. ALK5 catalytic activity is needed for ALK1 activation as well as for p38 phosphorylation. Not surprisingly, SB525334, an ALK5 inhibitor, completely abolished ET-1 secretion in TGF-β1-treated cells. Incubation
with p38 inhibitor showed a significant decrease in ET-1 secretion, suggesting that indeed the p38 pathway is involved in TGF-β1 stimulated ET-1 release (Figure 13C).

Overall, both BMP9 and TGF-β1 use similar pathways to induce ET-1 production, specifically phosphorylation of Smad1 and p38. Most of the ET-1 is getting produced through activation of the Smad1/5/8 signalling cascade, while about half of ET-1 may be produced through the p38 pathway.
**Figure 14. Smad1 and p38 pathways contribute to both BMP9 – and TGF-β1 –induced ET-1 secretion.**

Endothelial cells were serum starved for 12 hours and then incubated with various inhibitors for 30 minutes (A). Subsequently, either 1 ng/ml of BMP9 (B) or TGF-β1 (C) was added to the cells. After a 10 hour incubation, supernatants were collected and analyzed by ET-1 ELISA.

*P<0.05 with respect to controls; # P<0.05 versus ligand stimulation. Bars represent mean ± SEM for at least 3 experiments done in duplicate.
3.8 VEGF may lower BMP9–induced p38β phosphorylation

Even though the literature indicates that both BMP9 and TGF-β1 should induce a significant increase in p38 phosphorylation (186), mouse embryonic endothelial cells showed only a small increase in phospho-p38 levels. Yet, treatment with the p38 inhibitor resulted in a significant decrease in ET-1 levels, implying that p38 is involved in ET-1 secretion. To explore this further we examined two isotypes of p38, which are present in endothelial cells: p38 alpha (p38α) and p38 beta (p38β). In this experiment we looked into the phosphorylation status of these two p38 isotypes in endothelial cells at two different time points.

Cells were serum-starved for 3 hours and then treated with either BMP9 or TGF-β1 in the presence of absence of VEGF. We looked at the phosphorylation state of p38α and p38β after 1 and 4 hours of treatment (Figure 14). Similar to what we saw previously (Figure 12), levels of phospho-p38 regardless of isotypes remained unchanged after 1 hour of incubation with either BMP9 or TGF-β1. After 4 hours, however, we saw an increase in p38β phosphorylation levels in cells treated with either ligand. Surprisingly, VEGF treatment resulted in somewhat lower levels of phospho-p38β. Additionally, cells that were co-incubated with either BMP9 or TGF-β1 along with VEGF, showed a small decrease of p38β phosphorylation compared to the cells treated with just BMP9 or TGF-β1 alone. The phosphorylation state of p38α, on the other hand, remained unchanged throughout the experiment.

In summary, it appears that p38β is being phosphorylated in response to BMP9 or TGF-β1 treatments. However when cells are incubated with BMP9 or TGF-β1 in the presence of VEGF, phospho-p38β levels were reduced, compared to untreated cells, implying that VEGF may block BMP9– and TGF-β1–induced p38β phosphorylation. The increase in phosphorylation only after 4 hours of incubation suggests that protein synthesis is needed to initiate phosphorylation of p38β. It is likely to be an indirect way of p38 activation, potentially through genes activated by the canonical Smad pathway.
Figure 15. VEGF lowers BMP9–induced p38β phosphorylation.

Cells were serum-starved for 3 hours and then treated with 1 ng/ml of BMP9 or TGF-β1 in the presence of absence of 50 ng/ml of VEGF. Cells were then incubated for 1 or 4 hours, lysed and resolved on SDS-PAGE gel and analyzed by Western Blot using antibodies for Phospho-p38α and p38β expression relative to β-actin. The graph shows Phospho-p38α and Phospho-p38β expression relative to β-actin after 4 hours of incubation. Bars represent mean ± SEM for 4 experiments.
4 Discussion

Our studies confirmed previous findings, indicating that sEng (Δ586) binds BMP9 and not TGF-β1 with high affinity. Additionally, I found that BMP9 and TGF-β1 can induce ET-1 secretion from mouse endothelial cells and hypothesized that the function of sEng in preeclampsia could be to block BMP-9 signalling and subsequent ET-1 secretion. Therefore, I examined the ability of sEng to block ET-1 secretion from BMP9 treated ECs, and conclude that sEng can indeed block BMP9 signalling. Furthermore, I discovered that VEGF, but not PlGF, could block BMP9-induced ET-1 secretion, suggesting a new way for VEGF to inhibit vasoconstriction. Finally, I used inhibitors of ALK1, ALK5 and p38 pathways to dissect the molecular mechanism behind BMP9 and TGF-β1 induction of ET-1 secretion. My findings indicate that ALK1 and to a lesser extent p38 pathways are important for ET-1 secretion. Though my data did not show a significant down-regulation by VEGF of p38β phosphorylation induced by BMP9, there was a trend. It is, therefore, possible that VEGF blocks BMP-9–induced ET-1 secretion by down-regulating p38β pathway; however, more experiments are needed to support this claim. Additionally, the fact that VEGF cannot block TGF-β1–induced ET-1 secretion suggests that other signalling pathways are involved in this process.

4.1 BMP9 and TGF-β1 induce ET-1 secretion from mouse embryonic endothelial cells

Recently two papers were published reporting the ability of BMP9 and TGF-β1 to induce ET-1 secretion from human umbilical or bovine aortic endothelial cells (139, 140). Both studies raised the possibility that this mechanism might be important for hypertensive disorders, including preeclampsia(139, 140). We performed similar experiments with mouse embryonic ECs and confirmed that incubation with either BMP9– or TGF-β1–induced release of endothelin-1. Both papers reported a two-fold increase in ET-1 production in treated cells, which is quite comparable with our own findings (Figure 1). Thus, our studies extend the observations to a distinct type of endothelial cells and in different species suggesting that stimulation of ET-1 secretion by BMP9 and TGF-β1 is likely a general mechanism with relevance to systemic hypertension and a condition such as preeclampsia.
4.2 Potential role of sEng in PE

The role of sFlt-1 in preeclampsia is still not fully understood; however, it is clear that it exerts its effects by interfering with VEGF and PlGF signalling. The functional role of sEng, however, has yet to be defined. Several studies looked at the effects of recombinant sEng (Δ586) and concluded that it contributes to endothelial dysfunction (64) and a slight increase in systemic blood pressure (4). It was postulated that sEng is able to scavenge TGF-β1 and interfere with ALK5 signalling. However recent studies (65, 181) and our own experiments (Figure 2) clearly showed that the recombinant sEng (Δ586) only binds with high affinity to BMP9 and not to TGF-β1. Both studies (65, 181) determined that recombinant sEng binds TGF-β1 in the µM range or weaker, which is consistent with our estimated K_D value of 1.2 µM. It is clear that with that kind of binding affinity, recombinant sEng would not be able to scavenge circulating TGF-β1 under physiological concentrations. However, binding of recombinant sEng to BMP9 was strong; our experiments showed a K_D = 4.8nM for sEng/BMP9 interaction. Similar studies were performed by other groups, including Castonguay et al. who determined that recombinant sEng-Fc binds to BMP9 with an affinity of 40 pM (181). This is somewhat surprising, considering that the same group published a previous paper, claiming that the K_D of sALK1-Fc for BMP9 was 2 nM (191). It is unlikely that sEng can bind BMP9 with an affinity 100 times greater than for its natural receptor ALK1, especially considering that the amount of endoglin on the cell surface is at least 100 times more than the amount of ALK1. This would cause BMP9 to preferentially bind to endoglin, ignoring ALK1 receptors. It should be mentioned that the group used proteins expressed as chimeras with the immunoglobulin Fc domain. It is possible that the addition of the Fc domain could alter the protein structure, making a more stable protein with higher affinity for BMP9. Recently, a new study was published showing that K_D value of sEng/BMP9 interaction is around 4 nM, which is similiar to the value obtained in our experiments (65). In summary, I conclude that BMP9 and not TGF-β1 is a binding partner of recombinant sEng (Δ586), and we suggest that the true physiological sEng in circulation would have a similar relative affinity for these ligands. However, this should be demonstrated experimentally.

Next I examined the function of circulating BMP9. Research on BMP9 effects on endothelial cells reported somewhat controversial results as BMP9 was found to be both a quiescence (132), and an angiogenic factor (133) for endothelial cells. Moreover, recent studies reported that BMP9 can induce ET-1 production. Since ET-1 has been implicated in PE, I hypothesized that
the role of sEng in preeclampsia could be to sequester BMP9 molecules and inhibit ET-1 secretion. I first looked at the ability of soluble ALK1-Fc to inhibit BMP9-induced ET-1 secretion. As expected, ALK1-Fc was able to completely abolish ET-1 stimulation. From my BIAcore studies, I knew that sEng was not as effective at binding BMP9 as ALK1; therefore, it was not surprising that sEng was only half as effective at blocking ET-1 secretion. Neither recombinant sALK1-Fc nor sEng (∆586) could block TGF-β1–induced ET-1 secretion, which correlates well with our BIAcore studies.

Interestingly, cells incubated with sALK1-Fc alone showed a significant decrease in basal levels of ET-1 secretion over a period of 10 hours, indicating that unstimulated endothelial cells can produce a fair amount of BMP9. This hypothesis was further strengthened by the fact that cells treated with an inhibitor of ALK1 signalling, dorsomorphin, also showed a decrease of ET-1 release compared to unstimulated cells. It is likely that BMP9 molecules, present in the cultures, are responsible for the background accumulation of ET-1. Levels of BMP9 have yet to be measured in patients with preeclampsia.

Multiple studies have reported increased levels of ET-1 in preeclampsia; in fact, blockade of ET-1 receptors was able to completely abolish PE-associated hypertension in mouse and rat models (171). It is therefore possible that one of the functions of sEng is to scavenge BMP9 molecules to inhibit ET-1 secretion and subsequent hypertension. Release of sEng could represent a protective mechanism, initiated in attempt to keep hypertension under control (Figure 16). During preeclampsia an increase in circulating sFlt-1 molecules would diminish VEGF signalling, leading to an increase in ET-1 secretion, since BMP9 signalling would remain unopposed. ET-1 would in turn, lead to an increase in blood pressure. We hypothesize that release of sEng might be a response to VEGF blockade aimed at reducing hypertension by inhibiting ET-1 secretion.

Interestingly, elevated sEng is usually detected 1-2 weeks in advance of a detectable increase in sFlt-1. Therefore, it is unlikely that sEng is being released in response to sFlt-1. However, AT1-AA have been shown to be present in PE patients at much earlier time points and are thought to be at least partially responsible for the secretion of sFlt-1 and sEng. Therefore, it is possible that sEng is released in response to AT1-AA, which would eventually initiate sFlt-1-induced hypertension.
Figure 16. Model of Preeclampsia.

In normal pregnancy, BMP9 signalling leading to ET-1 secretion is counteracted by VEGF signalling, which prevents excessive ET-1 secretion and keeps blood pressure at the normal level. However, during preeclampsia, high levels of sFlt-1 block VEGF signalling. ALK1 signalling becomes unopposed leading to increase in ET-1 secretion, which in turn results in hypertension. We hypothesize that sEng gets released in response to hypertension and represents a physiological attempt to control blood pressure. sEng would then bind BMP9 and block ALK1 signalling, therefore down-regulating ET-1 secretion and subsequent hypertension.
4.3 VEGF signalling and hypertension

By far the most interesting finding of my work is the fact that VEGF can block BMP-9-induced ET-1 secretion (Figure 4B). As far as I know, we are the first group to report a negative effect of VEGF on ET-1 secretion in endothelial cells. The role of VEGF in preeclampsia has never been disputed as multiple groups agree that the lack of VEGF signalling due to the increased production of sFlt-1 by the placenta can mimic the majority of preeclampsia symptoms, including hypertension and proteinuria (192). Hypertension, induced by the injection of sFlt-1 was usually attributed to the lack of eNOS activation (193). NO is one of the most common vasodilators in physiology, however quantifying NO production is not an easy task. NO levels in preeclampsia were examined by several groups; somewhat controversial findings were reported. Various studies found NO levels to be either reduced (194), normal (195) or increased (196) in preeclampsia. In theory, reduced NO could explain PE-associated hypertension, while increased NO could represent a physiological response to already induced hypertension.

In this study, I propose a new mechanism by which sFlt-1 can induce hypertension. I report that ET-1 secretion induced by BMP9 can be efficiently blocked by VEGF. It is interesting to note that BMP9 concentration in the serum is 5 to 10 ng/ml, meaning that BMP9 is constantly activating ALK1 signalling in endothelial cells. This was clearly demonstrated by Bidrart et al; they showed phosphorylated Smad1/5/8 molecules in the nucleus of aortic endothelial cells, attributed to BMP9 in circulation (129). Therefore, there should be a constant secretion of ET-1 molecules into the serum. At the same time, other studies report that the concentration of ET-1 in circulation is quite low (197), suggesting additional factors that keep ET-1 production in check. We propose that in healthy individuals VEGF signalling opposes BMP-9-induced secretion of ET-1, thus keeping a low concentration in circulation and preventing hypertension. Upon release of sFlt-1, however, VEGF signalling is blocked. BMP9-induced secretion of ET-1 is no longer inhibited, leading to an increase in ET-1 circulating levels and subsequent systemic hypertension (Figure 14).

Interestingly, PlGF was not able to replicate the effect of VEGF on ET-1 secretion. One possible explanation is the difference in signalling. PlGF signals through VEGFR-1 (Flt-1), while VEGF signals through both VEGFR-1 and VEGFR-2 (198). A number of studies reported that certain functions can be mediated strictly by one receptor. For example, adipose tissue expansion was
induced by VEGF exclusively through VEGFR-2, as an antibody to VEGFR-1 was unable to block the expansion (199). Moreover, Koolwijk et al. examined the VEGF effect on tube formation by human microvascular endothelial cells and found that VEGFR-2 is the only receptor involved in this process (200). Therefore, our data support that VEGF mediates its blocking effect on ET-1 secretion via VEGFR-2.

4.4 Pathways involved in ET-1 production

VEGF was able to inhibit BMP9-induced ET-1 secretion, however failed to block ET-1 secretion induced by TGF-β1. One possible explanation might be that BMP9 and TGF-β1 use different signalling pathways to induce ET-1 release. I therefore incubated cells with both ligands together to see if they have an additive effect. Indeed, cells co-incubated with BMP9 and TGF-β1 secreted significantly more ET-1 than in response to either ligand alone. It should be noted that we used a concentration of 1 ng/ml for both ligands. Our titration experiments (Figure 1) showed that cells treated with TGF-β1 become saturated at a concentration of 0.8 ng/ml and addition of extra TGF-β1 molecules did not result in an increase in ET-1 secretion. Therefore, it is likely that additional ET-1 released by cells co-incubated with the two ligands was initiated by a separate pathway, which is not activated by TGF-β1 signalling alone. To examine the differences between TGF-β1 and BMP9 signalling leading to ET-1 secretion we tested inhibitors of ALK5 activation and of Smad1/5/8 and p38 pathways.

Our experiments showed that BMP9 signals exclusively through ALK1 receptor to induce ET-1 secretion, which is not surprising as ALK1 activity is needed to activate all BMP9-related signalling. These results are in agreement with previous studies showing that BMP9-induced ET-1 release is dependent on Smad1 and p38 phosphorylation (139). Similarly, in our studies inhibition of either pathway led to a significant decrease in ET-1 secretion (Figure 12B). Interestingly, Park et al, showed that though HUVEC cells need Smad1 protein to initiate ET-1 secretion, the presence of Smad4 protein is not required (139).

I also performed similar experiments to elucidate the pathways responsible for TGF-β1–induced ET-1 secretion. I observe that activation of Smad1/5/8 and p38 signalling pathways are also necessary for TGF-β1 to induce ET-1 secretion. In fact, inhibition of ALK1 pathways reduced ET-1 production to background levels. The p38 inhibitor was not as effective as dorsomorphin, but was still capable of significantly decreasing ET-1 secretion. It appears that these two
pathways together are responsible for most of the ET-1 release, suggesting that Smad2/3 phosphorylation is less important in this process. Still, the ALK5 inhibitor, SB525334, completely blocked ET-1 secretion, implying that the kinase activity of ALK5 is necessary. These results are in agreement with our own data (Figure 11) and multiple other studies (82, 201, 202), showing that activation of ALK5 is required for ALK1 signalling.

Interestingly, Castañares et al. demonstrated that TGF-β induces ET-1 secretion through ALK5/Smad3 and not through ALK1 pathways. In fact, knockdown of ALK1 and Smad1 proteins by siRNA had absolutely no effect on ET-1 secretion, suggesting that the ALK1 pathway does not play a role in this process (203). My results, on the other hand, clearly demonstrate that ALK1 pathway is needed for TGF-β-induced ET-1 secretion. This discrepancy could possibly be explained by difference in cell types used in two studies. Castañares et al. used bovine aortic endothelial cells (BAEC), while I used mouse embryonic endothelial cells for our experiments. Additionally, Star et al, clearly showed that the p38 pathway is also involved in TGF-β-induced ET-1 secretion, which was also found in my studies (140).

Though TGF-β1 and BMP9-induced only a slight increase in p38 phosphorylation, BIRB796 could significantly decrease ET-1 secretion, implying that the p38 pathway is involved in this process. One explanation for the phenomenon is that I might not have looked at the p38 phosphorylation at the right time point. Usually, p38 gets activated almost instantly by TβRII associated kinases (97); however several papers raised the possibility that p38 could become phosphorylated hours after the initial stimulation (204, 205). Recent studies have also shown that though there are 4 isoforms of p38 molecules, TGF-β1 does not activate all of them (206). Therefore, it is possible that I did not detect p38 phosphorylation, because only one isoform became phosphorylated. Moreover, a recent study by Ferrari et al, looked at crosstalk between VEGF and TGF-β1 signalling, reporting that co-incubation of cells with both ligands could shift the phosphorylation pattern from p38α to p38β molecules (207). I therefore thought that co-incubation of endothelial cells with both VEGF and BMP9 may affect p38 phosphorylation in a similar manner. Since endothelial cells primarily express p38α and p38β proteins, we tested p38α and p38β phosphorylation following stimulation with either TGF-β1 or BMP9 in the presence or absence of VEGF.
My results showed that neither BMP9 – nor TGF-β –induced p38α phosphorylation, at either 1 or 4 hours after treatment. That was surprising, since it was reported that TGF-β1 specifically up-regulated p38α phosphorylation in multiple cell types (208). P38α is thought to be activated by TGF-β to induce apoptosis, while phosphorylation of p38β is usually associated with survival and is induced by VEGF. However, in my study, both BMP9 – and TGF-β1 –induced phosphorylation of p38β after 4 hours of treatment. Interestingly, although our findings were not statistically significant, co-incubation of VEGF with either ligand suggested that VEGF may interfere with p38β phosphorylation induced by BMP9 and TGF-β1 ligands and could explain how VEGF is able to block BMP9-induced ET-1 secretion. This mechanism, however, would not explain why VEGF is unable to block TGF-β1 –induced ET-1 release from endothelial cells. It is possible that p38 phosphorylation is not as important for TGF-β1 signalling, or alternatively TGF-β1 uses a different isoform of p38 to induce ET-1 secretion. There is also the possibility that TGF-β1 uses a completely different pathway to induce ET-1 secretion, though the fact that p38 inhibitor BIRB76 had some blocking effect suggests that the p38 pathway does play some role in TGF-β1 signalling. Further studies are needed to elucidate the exact signalling pathway, involved in ET-1 secretion by both BMP9 and TGF-β1.

4.5 Conclusion

I report that both recombinant sEng (∆586) and sALK1-Fc can block BMP9 –, but not TGF-β1 induced ET-1 secretion. Also, I found that VEGF and not PIGF can block ET-1 secretion. In terms of molecular pathways implicated, ALK1 and to a lesser extent p38 signalling are important for both TGF-β1 and BMP9-induced ET-1 secretion. My studies on the effects of VEGF on Smad phosphorylation showed that VEGF is not able to induce or block either Smad1/5/8 or Smad2/3 phosphorylation. However, it is known that VEGF can activate the p38 signalling cascade and in certain cases compete with TGF-β1 for p38 activation. Our own experiments suggest that VEGF may block BMP9 –induced ET-1 release at least in part by lowering p38β phosphorylation. It is not clear why VEGF was unable to block TGF-β1 –induced ET-1 secretion. Further studies are needed to dissect the mechanisms accounting for the blocking by VEGF of BMP9 – but not TGF-β1 –induced signalling.
Moreover, in the context of preeclampsia, it appears that sEng and sFlt-1 converge on the same pathway of ET-1 production, but have opposing effects (Figure 14). sEng can directly bind to BMP9 molecules and remove them from circulation, blocking ALK1 signalling and ET-1 secretion. sFlt-1 on the other hand, can sequester VEGF molecules, thus interfering with the ability of VEGF to block BMP9-induced ET-1 secretion. Therefore, induction of sEng can be viewed as a beneficial process, aiming to counteract systemic hypertension induced by sFlt-1. In fact if our hypothesis is correct, sEng or even sALK1-Fc, which has a much higher affinity towards BMP9, could potentially be used as a therapy for PE. Artificial increase in sALK1-Fc would sequester BMP9 molecules and relieve ET-1-induced hypertension. We hope that future research will be able to further investigate role of sEng, sFlt-1 and ET-1 in preeclampsia.
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