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The Effects of Bone Morphogenic Proteins and Transforming Growth Factor ß on *In-Vitro* Endothelin-1 Production by Human Pulmonary Microvascular Endothelial Cells

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Preface

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The following people and groups contributed either as co authors or as financiers to the research found in the manuscript in this thesis.

Dr. David Langleben - guided with the design of the experiments, collection of data, analysis of results interpretation, and writing of the manuscripts

Mr. Michele Giovinazzo – aided in the design of the experiments, the processing of the samples as well as interpretation of data.

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Abstract

Introduction:

Idiopathic Pulmonary arteriole hypertension (IPAH) is a rare but severely debilitating disease that strikes women to men at a ratio of 3:1. Endothelial cell (EC) dysfunction is a hallmark of the disease. This includes rapid growth of the ECs until the occlusion of the vasculature as well as decreased blood levels of vasodilators. Markedly increased levels of endothelin-1, a potent vasoconstrictor and smooth muscle mitogen, have been noted in IPAH patients.

Recently mutations in the bone morphogenic protein receptor type II (BMPRII) have been linked to the disease. Interestingly mutations in activin-like kinase-1 (ALK-1) and endoglin have been linked to hereditary haemorrhagic telangiectasia (HHT), a disease that results in PAH clinically indistinguishable from IPAH. All of these proteins are either receptors or co-receptors to members of the TGFß superfamily. The connection of these mutations to the disease still remains largely a mystery to researchers and the effects of either bone morphogenic proteins 2, 4, 7 or TGFß levels on endothelin-1(ET-1) production in human microvascular endothelial cells cultured from normal lungs (HMVEC-LBI) are unknown.

Methods:

HMVEC-LBI cells were cultured in the presence of various concentrations of BMP 2, 4, 7 and TGFß, in complete media or serum starved conditions. After allotted time points the media was collected and assayed by ELISA, meanwhile the cells were lysed and protein content assayed for normalization purposes. Small Mothers against Decapentaplegic (SMAD) 1/5 phosphorylation was also measured.

Results and Conclusions:

Despite evidence that all BMPs used were biologically active, namely through SMAD phosphorylation studies, only BMP7 at very high dosages increased ET-1 production levels. TGFß had a more pronounced effect at earlier time points with lower concentrations. The results provide insights on the effects of an important group of proteins, the BMPs and TGFß, on lung microvascular ECs and which are likely the key cellular player In IPAH development. These findings may have clinical relevance in terms of control of the disease and understanding the normal response of these cells BMPs and TGFß.

RÉSUMÉ

Introduction :

L'hypertension pulmonaire artériolaire idiopathique (HPAI) est une maladie rare mais extrêmement débilitante. Elle affecte 3 femmes pour 1 homme. La caractéristique de cette maladie est une dysfonction des cellules endothéliales (CEs) qui implique une croissance rapide des cellules endothéliales jusqu'à occlusion, ainsi qu'une réduction des taux de vasodilatateurs produits. Chez les patients souffrant d'hypertension pulmonaire artérielle (HPA), on a observé une augmentation marquée des taux d'endothéline-1 (ET-1), un vasoconstricteur puissant ainsi qu'un agent mitogen de muscle lisse.

Des mutations du << bone morphogenic protein receptor type II >> (BMPR11) ont été récemment reliées à la maladie. Il est intéressant de noter qu'une mutation de l'« activin-like kinase-1 » (ALK-1) ainsi que de << l'endoglin >> ont été associées à la télangiectasie hémorragique héréditaire (THH) , une maladie qui s'accompagne d'une HPA cliniquement identique à l'HPAI. Toutes ces protéines sont soit des récepteurs ou des co-récepteurs des membres de la « super » famille des << transforming growth factors beta >> (TGFß). La relation entre ces mutations et la maladie demeure une énigme pour les chercheurs. Nous ne connaissons pas les effets des BMP 2, 4, 7, et de TGFß sur le taux de production d'ET-1 par une culture de CEs provenant de réseau micro-vasculaire pulmonaire normal (HMVEC-LBI).

Méthodologie :

L'HMVEC-LBI a été cultivé en présence de concentrations variées de BMP 2, 4, 7 et de TGFß, dans des conditions de media enrichis et appauvris. À des

temps donnés d'une échelle de temps déterminée, les média ont été recueillis et

soumis à des essais par ELISA, alors que les CEs ont été lysées et leur contenu en protéines analysé pour fin de normalisation. La phosphorylation du << Small Mothers against Decapentaplegic >> SMAD 1/5 a aussi été mesurée.

Conclusions et Résultats:

Bien que les études de phosphorylation du SMAD aient démontré que toutes les BMPs utilisées étaient biologiquement actives, seulement la BMP 7 à très haute dose a induit une augmentation de la production d'ET-1. Le TGFß a eu un effet plus prononcé plus tôt, à de plus faibles concentrations. Ces études nous renseignent sur les effets d'un groupe clairement important de protéines, soit les BMPs et le TGFß, sur les CEs de réseau micro-vasculaire pulmonaire, un jour clé dans le développement de l'HPAI. Ces observations non seulement apportent une meilleure compréhension de la réponse normale des BMPs et TGFß, mais aussi peuvent être pertinentes sure le clinique pour la traitement de la maladie.

List of Abbreviations

ActRII: Activin type II receptor

ALK5: Activin Like Kinase 5

BAMBI: BMP and Activin membrane bound inhibitor

BMP: Bone Morphogenic Protein

BMPRI: Bone Morphogenic Protein Receptor type I

BMPRII: Bone Morphogenic Receptor Type II

DAG: Diacylglycerol

EC: Endothelial cell

ECE-1: Endothelin Converting Enzyme 1

ECE-2: Endothelin Converting Enzyme 2

ERK: Extracellular Signal Regulated Kinases

ET-1: Endothelin-1

ET-2: Endothelin-2

ET-3: Endothelin-3

ET-A: Endothelin Receptor A

ET-B: Endothelin Receptor B

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

HHT: Hereditary Hemorrhagic Telanglectasia

HMVEC-LBI: Human Microvascular Endothelial Cell of the Lung

IP3: Inositol Triphosphate

IPAH: Ideopathic pulmonary areteriole hypertension

LIMK1: LIM Kinase 1

MAPK: Mitogen Activated Protein Kinase

MH1: Mad Homology 1

MH2: Mad Homology 2

NO: Nitric Oxide

PKC: Protein Kinase C

PLA2: Phospholipase A2

PLC: Phospholipase C

PXSP: Phospho-MAPK/CDK Substrates

RACK1: Receptor for Activated C Kinase 1

SMAD: Small Mothers against Decapentaplegic

Smurf1: SMAD ubiquitin regulatory factor 1

TGFß: Transforming Growth Factor Beta

TβRI: TGFß receptor Type I

TβRII: TGFß receptor Type II

Chapter 1:

Introduction

1.1 History of the disease

The pathology of an illness consistent with pulmonary arterial hypertension (PAH) was first described in 1891 by Romberg (1). In 1951, David Dresdale coined the term "primary pulmonary hypertension", now termed idiopathic PAH, for cases of pulmonary hypertension without evident aetiology (2;3). Aside from the vasodilator studies of Paul Wood in the 1950's, little progress was made in studying the disease until a World Health Organization meeting was organized in 1973, following an outbreak of seemingly idiopathic PAH related to exposure to the diet pill aminorex (4;5). Another anorexigen-related outbreak of PAH occurred in the early 1990's after exposure to fenfluramine-like agents (6). Anorexigen exposure for longer than 3 months resulted in a 23.1-fold increased risk of developing PAH, as compared to controls (6). In the last twenty years many advances in understanding of the disease have occurred in the areas of genetics, physiology, pathogenesis, and treatment.

1.2 Description of the disease

Pulmonary Hypertension simply refers to elevation of pulmonary arterial pressure, most commonly related to an increased pulmonary vascular resistance. When pulmonary hypertension is severe, patients generally succumb to right heart failure. (7)

Table 1:

Revised Clinical Classification of Pulmonary Hypertension (Venice 2003)

1. Pulmonary arterial hypertension (PAH)
   - 1.1. **Idiopathic** (IPAH)
   - 1.2. Familial (FPAH)
   - 1.3. Associated with (APAH):
     - 1.3.1. Collagen vascular disease
     - 1.3.2. Congenital systemic-to-pulmonary shunts*†
     - 1.3.3. Portal hypertension
     - 1.3.4. HIV infection
     - 1.3.5. Drugs and toxins
     - 1.3.6. **Other** (thyroid disorders, glycogen storage disease, Gaucher disease, hereditary hemorrhagic telangiectasia, hemoglobinopathies, myeloproliferative disorders, splenectomy)
   - 1.4. **Associated with significant venous or capillary involvement**
     - 1.4.1. Pulmonary veno-occlusive disease (PVOD)
     - 1.4.2. Pulmonary capillary hemangiomatosis (PCH)
   - 1.5. Persistent pulmonary hypertension of the newborn
2. Pulmonary hypertension with left heart disease
   - 2.1. Left-sided atrial or ventricular heart disease
   - 2.2. Left-sided valvular heart disease
3. Pulmonary hypertension associated with lung diseases and/or hypoxemia
   - 3.1. Chronic obstructive pulmonary disease
   - 3.2. Interstitial lung disease
   - 3.3. Sleep-disordered breathing
   - 3.4. Alveolar hypoventilation disorders
   - 3.5. Chronic exposure to high altitude
   - 3.6. Developmental abnormalities
4. Pulmonary hypertension due to chronic thrombotic and/or embolic disease
   - 4.1. Thromboembolic obstruction of proximal pulmonary arteries
   - 4.2. Thromboembolic obstruction of distal pulmonary arteries
   - 4.3. Non-thrombotic pulmonary embolism (tumor, parasites, foreign material)
5. **Miscellaneous**
   Sarcoidosis, histiocytosis X, lymphangiomatosis, compression of pulmonary vessels (adenopathy, tumor, fibrosing mediastinitis)

The 2003 World Symposium on PH Classifications of pulmonary hypertension table from Simonneau et al. 2004 (8). Reprinted by permission of Elsevier.

The studies in this thesis will be most relevant to idiopathic and hereditary PAH group 1 (table 1). Idiopathic PAH is a very rare disease with an incidence of 1 – 2 cases per million. For unexplained reasons the idiopathic form of the disease

affects women at a higher rate than men at a ratio of 3:1 *(9)*. Pulmonary arterial hypertension is defined as an elevation of mean pulmonary arterial pressure to more than 25 mm Hg at rest or to more than 30 mm Hg with exercise, in the presence of an elevated pulmonary vascular resistance (> 3 Wood units) and a pulmonary artery wedge pressure of < 15 mm Hg. Idiopathic PAH may only be diagnosed in the absence of heart disease, pulmonary disorders or any other secondary cause.

The pathology of PAH has certain hallmark features, most prominently the cellular occlusion and obliteration of small precapillary arterioles. The plexiform lesion, classic for PAH, was originally thought to be an aberrant attempt of the lung to revascularize the occluded or obliterated segments of the vasculature. However, recent studies which demonstrated that the plexiform lesions are primarily composed of endothelial cells, that the cells may be monoclonal in origin, and that there is a more diffuse upstream endothelial intimal proliferation, have led to the hypothesis that deregulated endothelial cell growth may be the cause of PAH and might represent a localized endothelial neoplasia of the lung. (10). Further research has suggested that the initiating event might be widespread endothelial apoptosis, with emergence of apoptosis resistant endothelial clones that gradually occlude the vascular lumen (11). In support of this hypothesis, many of these abnormal cells express survivin, a tumor-related antiapoptotic peptide (11;12). Abnormal endothelial proliferation is perhaps the most significant endothelial cell

abnormality, but numerous other abnormalities of endothelial function are also found in PAH, including:

- Reduced levels of nitric oxide synthase, the enzyme that produces beneficial nitric oxide.
- reduced levels of prostacyclin synthase, the enzyme that produces beneficial prostacyclin *(13)*
- an unfavourable imbalance in the levels of beneficial prostacyclin and detrimental thromboxane A2, as assessed by their metabolites (14)
- Increased levels of endothelin-1 (ET-1) a potent vasoconstrictor and muscle mitogen (15) *(16)*

Neomuscularization of small vessels also contributes to the increased pulmonary vascular resistance. Alteration of the extracellular matrix may promote further cellular proliferation as well as changing the physical properties (compliance and elastance) of the vessels (17). While it had originally been proposed that PAH was principally related to vasoconstriction, it is now clear that the structural remodeling in vessels is the predominant factor in the development of PAH, with little, if any, vasoconstrictive component being present. The increased after load and vascular resistance causes right heart dilatation and low cardiac output, and the heart eventually is unable to overcome the pressure and the patient succumbs to right heart failure. Recent therapies have extended mean survival rates from 3 to 6 years but are not curative (9)

1.3 Endothelin-1

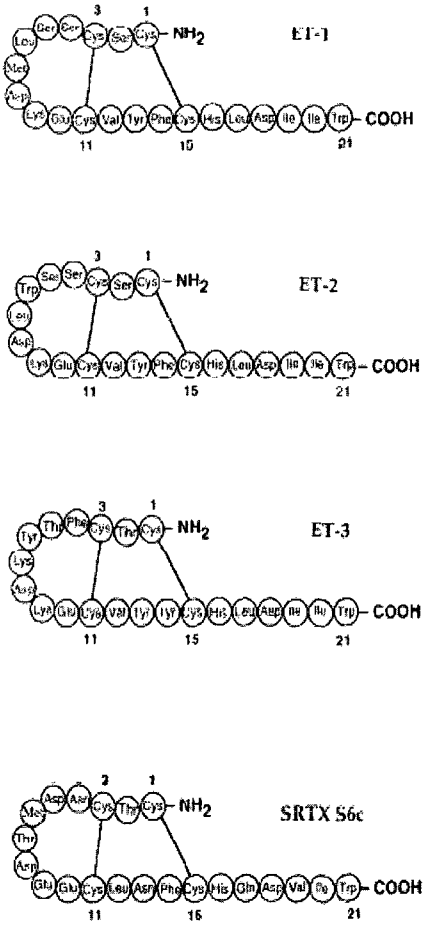

Figure 1: A comparison of the amino acid content and secondary structure of the three Endothelins as well as the venom Sarafotoxin Taken from Gray et al. 1996 (18). Reprinted with permission from Elsevier.

It had long been thought that the endothelial layer was simply a barrier to diffusion through the vessel wall (18). However by the 1980's it was become clear that endothelial cells play a vital role in the maintenance of vascular homeostasis. Studies in the 1960's and 1970's showed that the pulmonary endothelium was

metabolically active, transforming or taking up a variety of molecules from the pulmonary circulation. Furchgott's detection of an endothelial-derived relaxant factor in 1980 led to the identification of nitric oxide as a critical endothelium-derived vasodilator (19;20). In 1985, it was recognized that bovine aortic endothelial cell conditioned media constricted coronary artery sections (21). Yanagisawa (22) isolated a naturally occurring 21 amino acid peptide that was the most potent vasoconstrictor heretofore identified. It was termed endothelin. Endothelin contains two di-sulphide bonds in a short-length polypeptide. This type of structure had previously not been seen in mammals; however it was similar to the structure of a group of toxins, known as sarafotoxins, found in the venom of burrowing asps and other venomous animals.

There are three different endothelins found in human systems (figure 1). All of which have a mature 21 amino acid polypeptide. In their mature form ET-1 differs from ET-2 by 2 amino acids and ET-3 by 6 (18) . ET-1 is found throughout in many tissue types (18); aside from endothelial cells it is produced in many other cell types as well such as smooth muscle cells, neurons and astrocytes (23). ET-2 is mainly produced in the kidneys and intestine, and to a lesser extent the myocardium, placenta and uterus. ET-3 circulates in the plasma like ET-1, it is also found in large amounts in the brain and in lower levels in the gastrointestinal tract, kidneys and lungs (23).

The human endothelin-1 gene is 6.8kb in length and has been localized to chromosome 6. Prepro-ET-1 mRNA is 2117 nucleotides in length. The gene contains 5 exons and 4 introns, all of the exons contribute to prepro-ET-1 mRNA, as well as 5' and 3' flanking regions (figure 3) *(24)*. There are several regulatory sequences in the flanking regions of the gene. These include a binding sequence for the transcription factor NF-1, an Activator Protein-1 (AP-1) binding site, several copies of the hexanucleotide CTGGGA *(24)*. There is also a SMAD binding factor at -193/-171 (25).

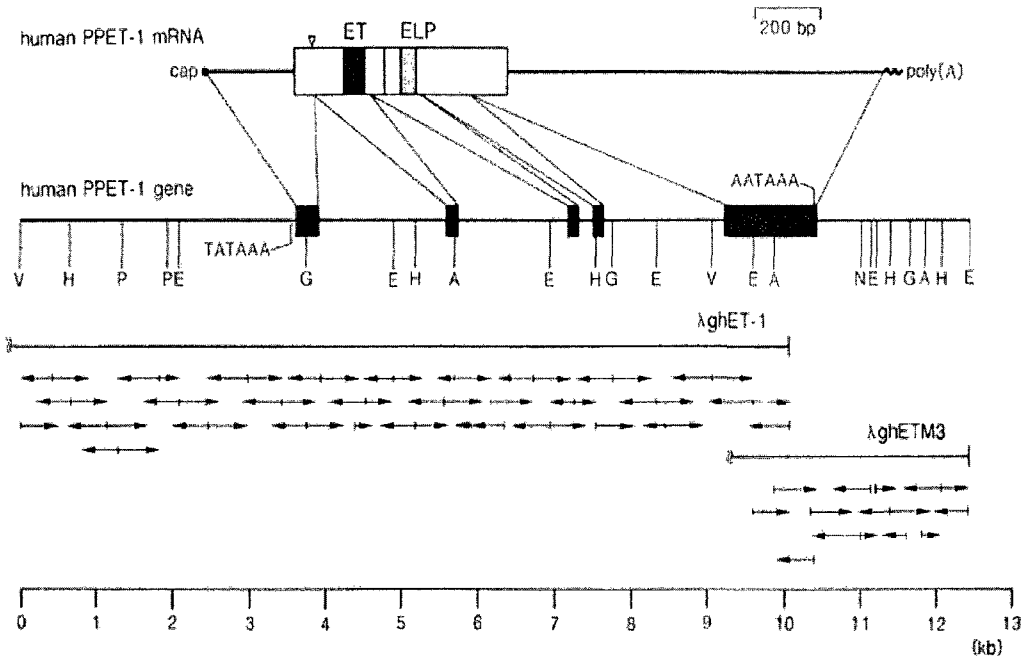

Figure 2: A representation of the complete nucleotide sequence of human ET-1. From Inoue et al. 1994 (24)

1.3.1 Endothelin receptors

To date there are two known receptors for endothelin in mammalian systems, identified as ET-A and ET-B, and localized to chromosomes 4 and 13 respectively (26;27). The receptor proteins are 427 and 442 amino acids in length, and depending on which tissue is studied show a 55% to 64% identity to one another *(18)*. The endothelin receptors are both classical g-protein coupled receptors, with a seven transmembrane form (28;29). They are glycoproteins; this characteristic appears to affect the ligand binding properties of the receptors (28). Exposure to insulin can up regulate ET-A *(30)* and Angiotensin 2 has been shown to increase the expression of ET-B *(31)*. TGFß has been shown to down regulate endothelin binding sites, presumably ET-A, on smooth muscles cells *in vitro (32)*. Expression can be down regulated by prolonged exposure to ET-1 (33). Both of the receptor genes contain several potential poly adenylation sites in their 3' untranslated regions that may lead to unstable mRNA (18)

ET-A has a very high affinity to ET-1 and ET-2 but very low affinity to ET-3. The amino terminal loop structure and the linear structure with Trp^{21} in position 21 are essential for ET-A binding of ET-1 (34). As a result of ET-Bs less stringent requirements, it is capable of binding all three forms of endothelin equally, only requiring a linear C terminus with Trp at the 21'st position.

In the micro-vasculature ET-A is found predominantly in the smooth muscle cells, while ET-B is most abundantly expressed in endothelial cells (18). ET-A is the

main receptor responsible for constriction of muscle cells upon binding of endothelin; however it had been demonstrated that even in the presence of ET-A antagonists the pressor response can not be completely suppressed (35). This led the researchers to suggest the presence of ET-B receptors on the smooth muscle cells which initiate a much less intense pressor response than to ET-A receptors. Indeed ET-B mRNA is expressed in smooth muscle cells. However, the ET-B receptor is predominantly expressed by endothelial cells. In the endothelium, ET-B is the site of ET-1 clearance from the blood circulation and endothelial ET-B activation induces the release of vasorelaxant factors such as nitric oxide and prostacyclin.

1.3.2 Physiological effects of ET-1

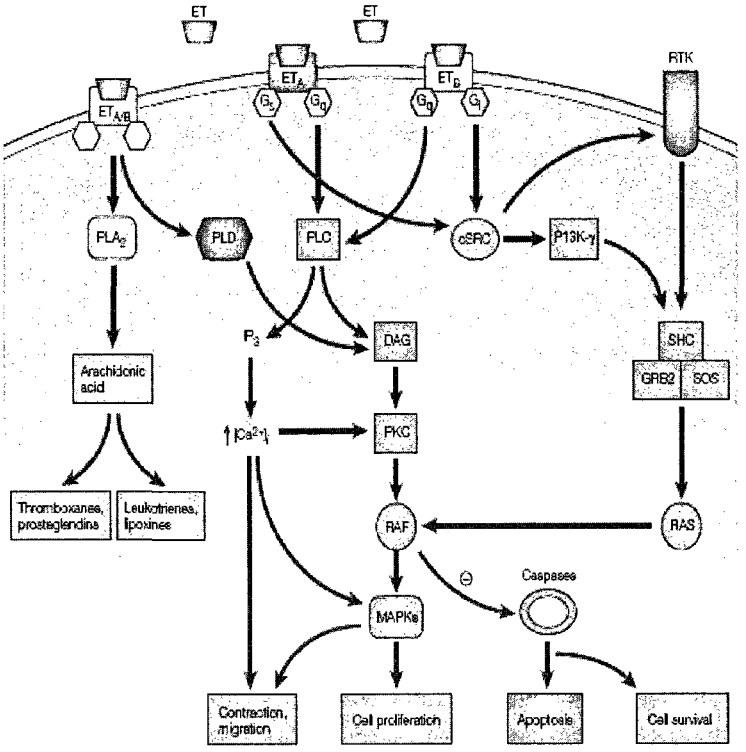

Figure 3: A figure showing all the different signaling cascades after ET-1 binds to either the ET-A or ET-B receptor. From Remuzzi G et al. 2002 (36). Reprint permission granted from Nature Publishing Group.

1.3.3 Cellular effects of ET-1

Binding of ET-1 to the ET-A receptor leads to the activation of proteins G_s and G_q, while ET-B activates G_q and G_i. Gq activates phospholipase C (PLC), which then hydrolyzes phosphotidylinositol to form cytosolic Inositol triphosphate (IP_3) and

diacylglycerol (DAG) (36-38). IP_3 then increases in intracellular Ca^{2+} by release from intracellular stores; at the same time DAG activates Protein Kinase C (PKC), which increases cellular sensitivity to Ca^{2+} (39). DAG also induces a signaling mechanism that promotes long term cellular actions such as migration and proliferation via the MAPK system (36). ET-1 also activates phospholipase A2 (PLA2) which increases production of arachidonic acid and in turn prostaglandins (36). ET-1 also stimulates the release of NO in endothelial cells, which can antagonize some of the effects of ET-1, namely smooth muscle contraction and proliferation (36). However these two agents do not act in parallel as NO synthesis is rapid and has a short half life; ET-1 is controlled at a transcriptional level (36). As such NO effects can be brought on rapidly and decrease almost just as quickly (36). ET-1 takes longer to be expressed and it binds tightly to the receptors and can have a long-lasting effect on its target cells (figure 4).

The circulating levels of ET-1 are very low, in the picomolar level, however at least 75% of the ET-1 is released abluminally towards the smooth muscle of the vasculature where the concentration must be significantly higher and therefore capable of stimulating local receptors suggesting a paracrine/autocrine role of the protein. *(40;41)*

1.3.4 Systemic effects of ET-1

1.3.4.a Role of Endothelin in Organ Development

The endothelin system has been shown to be important in normal physiological development. ET-3, ET-B, and ECE-1 contribute to the development of epidermal melanocytes as well as enteric neurons. ET-3-, ET-B- or ECE-1- knockout mice have normal pigmentation of the eyes but lack melanocytes of the skin and hair follicles need that for white fur. ET-1, ET-A and ECE-1 have been shown to be vital in the normal development of head and cardiac neural crest derived outpouring structures. ET-1 and ET-A knockout mice die at birth from asphyxia due to facial and throat malformation *(42)*

1.3.4.b Endothelin in the Brain

The endothelin system is found throughout the brain suggesting that it has several roles there. ET-1, ET-3, ET-A, and ET-B are expressed by the vascular, neuronal, and glial cells of the brain *(43-45).* ET-1 is the predominant endothelin in the brain with the exception of ET-3 being predominant in the pituitary gland *(46) (47)* along with both ECE-1 and ECE-2. Interventricular injection of ET-1 increases heart rate, renal sympathetic nerve activity, arterial pressure and respiratory rate (42;48). These changes are due to direct glial and neuronal stimulation because they occur prior to the effects that are seen in the local vasculature. The endothelin system in the brain plays a critical role in normal respiration (18).

1.3.4.c The Endothelin System in the Kidneys

The endothelin system plays a vital role in normal renal physiology. It controls renal blood flow, reabsorbtion of water and sodium as well as acid base balance. ET-1 is produced in the blood vessel endothelial cells of the kidneys and ET-1 and ET-3 are made in different cells of the nephron including the epithelial cells of the collecting ducts (49) (50;51). Both receptor types are found in the kidneys, but ET-B is the predominant type in the tubular epithelial cells and is critical to water homeostasis. (42)

1.3.4.d Endothelin and the Lungs

In the lung ET-1 is produced by the endothelial cells, airway epithelial cells (52), and macrophages. ET receptors are found throughout the lungs, but in varying ratios depending on their location. For example in human bronchi ET-B has been found to be tenfold more abundant than ET-A. ET-B is also found on the neuronal processes of the intramural tracheal autonomous nervous system. There is a high density of receptors in the tracheal and bronchial smooth muscle (53).

1.3.5 Endothelins and PAH

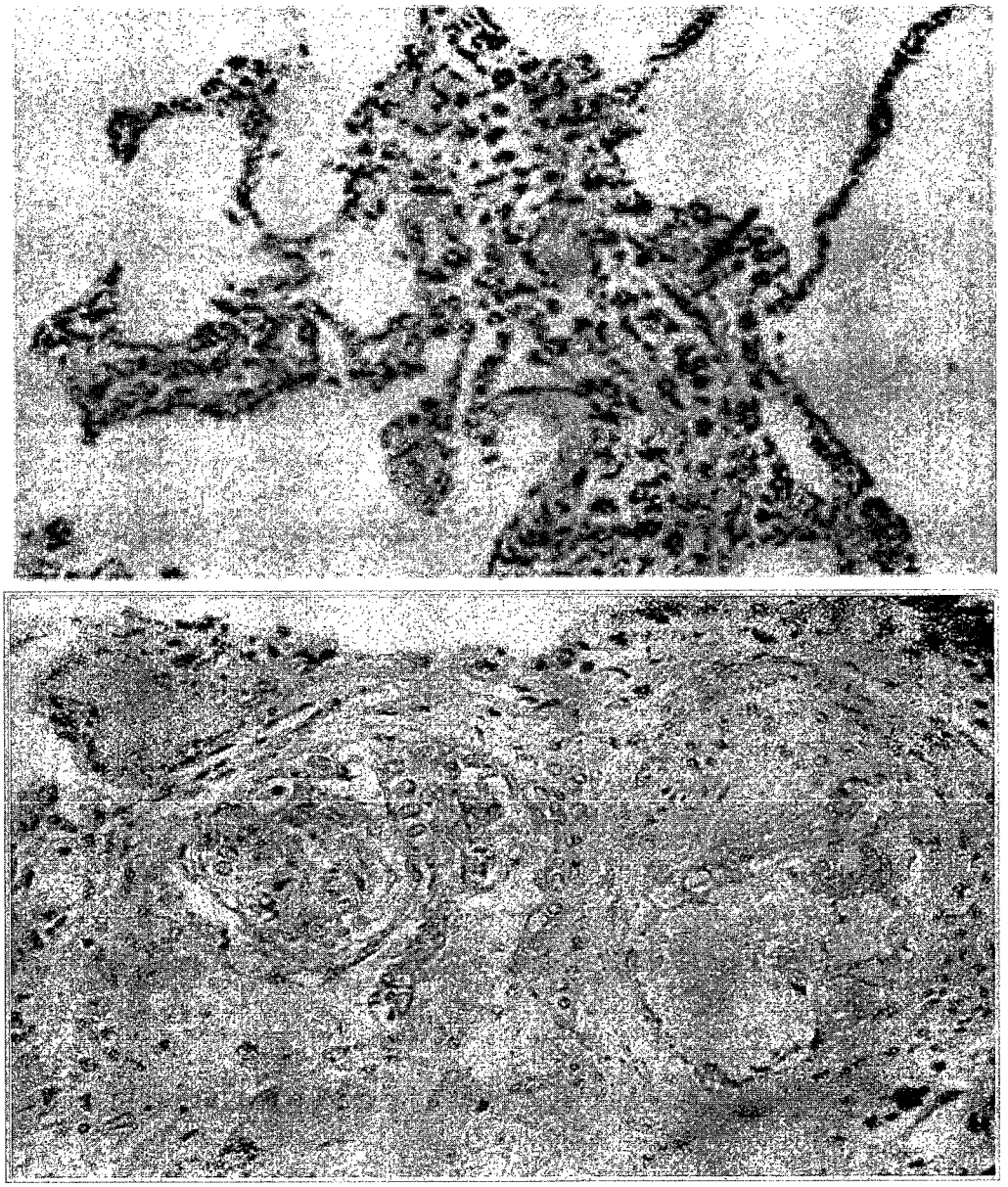

Figure 4: Lung from normal patient (top) and IPAH patient (bottom). There is markedly increased staining in the lungs of IPAH patients. From Giaid A. et al. 1993 (15). Reprinted by permission of the Massachusetts Medical Society.

Increased circulating plasma ET-1 levels have been described in patients with pulmonary hypertension, including PAH (16). Immunohistochemical study of lung tissue from patients with pulmonary hypertension demonstrated greatly increased ET-1 levels in remodelled microvessels and plexiform lesions (figure 5) (15). However, it remained unclear whether the increased levels of circulating ET-1 in the pulmonary vasculature were only a result of overproduction of ET-1 or also reduced pulmonary ET-1 clearance. A recent study of patients with PAH demonstrated that the majority have normal ET-1 extraction. Thus increased ET-1 levels in PAH are mainly due to increased synthesis. The discovery of high ET-1 levels in PAH suggested the therapeutic potential of endothelin receptor antagonists. This hypothesis has proven correct in clinical studies.

1.3.6 Processing of Endothelin

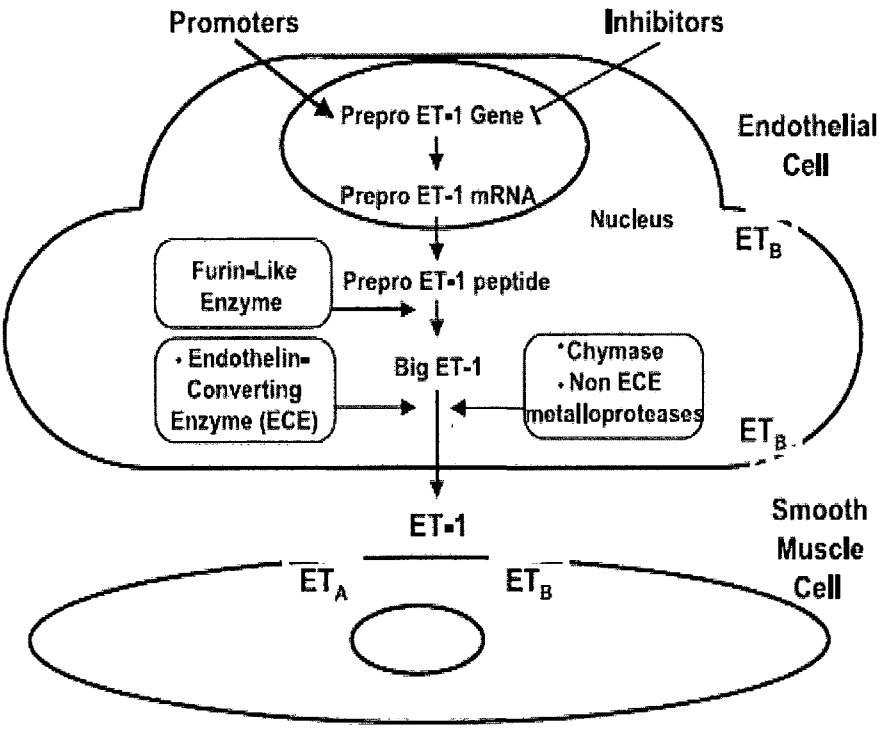

Figure 5: Processing of endothelin from prepro-ET-1 all the way to mature protein. From Galie et al. 2004 (54). Reprinted by permission of Oxford University Press

Currently, there are three known forms of endothelin, these being endothelin-1, endothelin-2 and endothelin-3. The endothelins show considerable homology to one another and to the sarafotoxins of *Atractaspis engaddensis* a venomous snake. Each is encoded by a different gene and each produces its own respective big-ET. The ET-2 gene is on chromosome 1 (55) while the ET-3 gene is on chromosome 20 (56). The human endothelin 1 gene encodes the prepro-ET-1

mRNA, which is 2117bp (source from NCBI) in length. The mRNA is translated to a 212 amino acid polypeptide (source NCBI). This prepro-ET-1 is then cleaved by a furin convertase in to the 38 amino acid BIG-ET-1. The final step in the process to mature ET-1 involves the endothelin converting enzymes (ECE) which cleave BIG-ET-1 in to mature 21 amino acids ET-1. Big ET-1 and Big ET-2 are both cleaved at the same site Trp^{21}-Val^{22}, whereas Big ET-3 is cleaved Trp^{21}-Ile^{22} *(57)*. All mature endothelins contain two disulphide bonds at position 1-15 and 3-11 *(58)*. There are no storage granules for ET-1 in endothelial cells so stimuli induce transcription (54;59). To date there are several known stimuli for ET- production, including shear stress, insulin and angiotensin 2 (table 2).

Table 2:

Stimuli that can promote or inhibit the expression of pre-pro ET-1 gene

Promoters	Inhibitors
Hypoxia	Nitric oxide
Ischemia	Prostacyclin
Shear stress	Atrial natriuretic peptides
Pulsatile stretch	Estrogens
pH	
Angiotensin II	
Vasopressin	
Cathecolamines	
Insulin	
LDL (oxidized), HDL	
Cytokines	
Growth factors	
Adhesion molecules	
Thrombin	

A table demonstrating the known stimulators or inhibitors of ET-1 synthesis. From Galie N et al.2004 (54). Reprinted by permission of Oxford University Press

Prepro-ET-1 mRNA has a half life of approximately 15-20 minutes and the mature peptide ET-1 has a plasma half life of 4-7 minutes (54). This implies extremely tight control over circulating levels. There are 6 isoenzymes of ECE: ECE-1a, ECE-1b, ECE-1c, ECE-2, ECE-3 *(54;57)*. All ECE-1 isoforms are derived from the same gene; however they differ in their n-terminus structure due to differing promoters (60). Alternate enzyme systems including chymases and non ECE metalloproteases can create mature ET-1. ECE-1-/ECE-1 homozygous knockout mice embryos showed large amount of mature ET-1 *(61),*. Chymases have also been shown to cleave Big ET-1 Tyr^{31}-Gly^{32} to produce ET_{1-31}. This alternative product has vasoconstrictive properties, and competes for binding with ET-1 but its true physiological role remains unclear *(62)*.

1.4 The TGFß/BMP system in heritable PAH

Early descriptions of primary pulmonary hypertension now referred to as idiopathic (IPAH) , by Dresdale in 1954 established that it could be familial (2). Mutations in the gene for the Bone Morphogenic Protein Receptor Type 2 (BMPRII) were described in 2000 in heritable PAH (63;64). BMPRII, a type 2 receptor of TGFß superfamily, and the bone morphogenic proteins it binds had until that point been known only as factors in the osteogenic process. Hereditary hemorrhagic telangiectasia (HHT, Osler-Weber-Rendu syndrome) is also associated with a form of PAH indistinguishable from the idiopathic and familial forms. In 2001, mutations in the gene for the Activin receptor-like kinase 1 (ALK1)

were linked to this disease (65). ALK1 is a type 1 receptor of TGFß, which also binds other members of the TGFß superfamily. Heritable PAH and HHT and their respective mutations provide a compelling link between the TGFß molecule superfamily and PAH. Approximately 25% of all seemingly spontaneous idiopathic cases and 60-70% of heritable PAH have identifiable mutations in BMPR-2 (9). Despite this high connection to the disease only 20% of all people with mutations in the BMPRII gene actually develop clinical PAH. This has led some to believe that these mutations only predispose the patients to PAH, and second stimuli such as a viral infection or exposure to toxins or drugs are required for full development of the disease (figure 7).

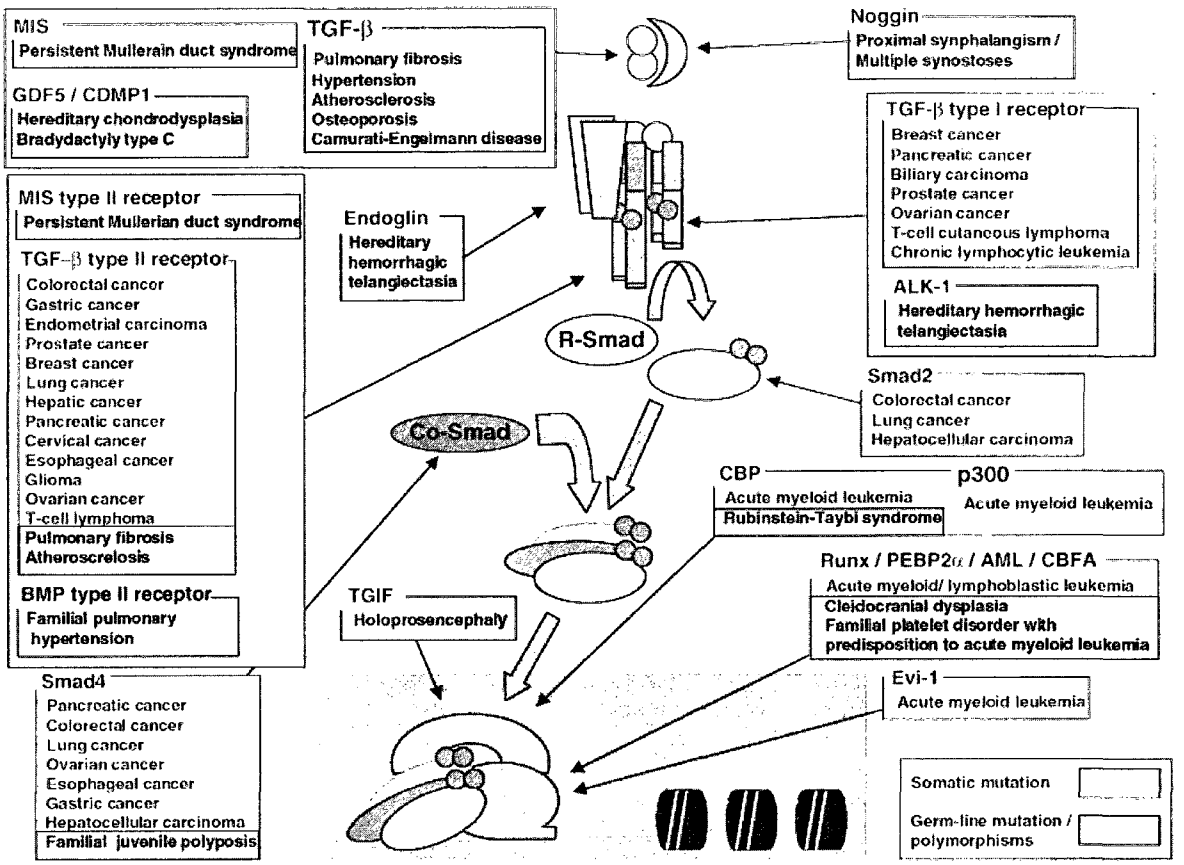

Figure 6: Diagram of TGFß signaling and a list of diseases or defects associated with their respective component. From Miyazono et al. 2001 (66) Reprinted with permission of Wiley-Liss, Inc. a subsidiary of John Wiley & Sons, Inc

1.4.1 BMP System

The BMPs were first discovered as proteins involved in the repair and development of bones and cartilage. Two receptor groups have been described; the type 1 bone morphogenic receptors BMPRIA and BMPRIB, and the type 2 receptors BMPR-II with both a long and short variant (67) The short variant lacks

almost all of exon 12 entirely (68). Although the short form is found ubiquitously, it is not known if it has a different function than the long variant (68). There are several human diseases other than PAH that have been linked to mutations in various components of the BMP signaling cascade: mutations in BMPRIA result in juvenile colonic polyposis (69), and a mutation in BMPRIB causes hereditary brachydactyly (70).

1.4.2. BMPRII

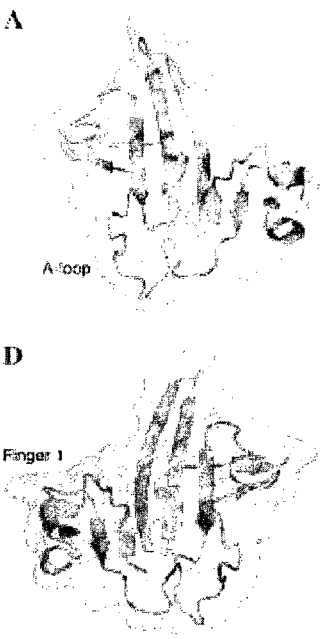

Figure 7: Crystal structure of BMPRII from Mace et al, 2006 (71). Reprinted by permission of Elsevier.

The gene for BMPRII is located on chromosome 2, and the mRNA is 11449bp with 13 exons (source NCBI accession NM_001204). The mature peptide

consists of 1038 amino acids with a shorter splice variant of 530 amino acids *(72)*. BMPRII is activated primarily by BMP 2, 4, 6, and 7 but does not bind TGFß (73). These ligands bind with high affinity to the type I receptors and only low I affinity to BMPRII. BMP-6 is exceptional in that it binds tightly to BMPRII. As with the other type II TGFß family receptors, BMPRII is constitutively active and only forms a heterodimer with either BMPRIA or BMPRIB upon binding of ligands (68). Unlike other type II receptors of TGFß superfamily, BMPRII has a long carboxy terminal sequence following the kinase domain. This domain must be essential to proper receptor functioning because many of the mutations found in PAH patients occur in this domain (74). In cultured smooth muscle cells, the C domain interacts with c-Src tyrosine kinase (74), Receptor for Activated C-Kinase 1 (RACK1) (75), as well as LIM kinase 1 (LIMK1)(76). TCTEX-1 a light chain of the motor complex dynein of the cytoskeleton was also shown to be phosphorylated by the cytoplasmic c terminal in the long isoform but not by the short isoform (77). BMPRII retains the three-finger toxin fold of the TGFß superfamily receptor extracellular domains; it also shares activin type II receptors (ActRII) main hydrophobic patch, which is used to bind ligands (figure 7) (71). Comparisons of BMPRII crystal structure to other TGFß family receptors show many similiarities and differences. By use of these comparisons it is believed that His87 of BMPRII plays an important role in ligand binding and recognition it is unique amongst this family of receptors (71).

To date there have been 144 BMPR-II mutations associated with presumably heritable PAH. (68). All of these mutations cause a loss of receptor function; none cause a gain of function (9). The mutations induce failure of accurate transcription, including nonsense (early stop in transcription), missense (wrong amino acids), and frameshift (where everything downstream is miscoded) defects. Splicing mutations, which result in the absence or duplication of portions of the receptor, have also been described. Thirty percent of the mutations are missense alterations of the highly conserved regions of the receptor such as the kinase domain or the binding domain. The balance of mutations is mostly frame shift or nonsense, which would predictably lead to mRNA decay. This has led to the proposal that haploinsufficiency is the common final result of these mutations.

1.4.3 BMP signaling

The BMP receptors have several different downstream signaling methods, some of which have recently been discovered and are not fully understood. The classic downstream signaling cascade common to the TGFß superfamily of receptors is via the Small Mothers against Decapentaplegic or SMAD pathway. The receptor associated SMADs (R-SMADs) are SMAD1, 2, 3, 5, and 8. While some receptors of the superfamily signal through SMADs 2 and 3 the BMP receptors utilize SMADS 1, 5, and 8 (68;78). These SMADs then associate with the co-SMAD, SMAD4, and translocate to the nucleus. SMADs 6 and 7 are inhibitory SMADs (I-SMADs), which counteract the effects of binding to the receptor (79).

The SMADs are a group of well-conserved proteins which contain two highly conserved regions Mad-homology 1(MH1) and Mad homology 2(MH2) (80). The MH1 domain is found on the N terminus while the MH2 is on the C terminus; they are connected by a poorly conserved linker region (80). The N terminus interacts with C terminus inhibiting its actions and preventing it from interacting with co-SMAD4 (78). Receptor mediated phosphorylation of the C terminus in r-SMADs ends this hindrance. The N terminus MH1 is responsible for binding to the DNA at specific sequences 5-GTCT-3 or 5-AGAC-3 the MH2 domain interacts with other proteins such as other SMADs and activated receptors (81;82). This binding is weak at best and as a result the SMAD complexes interact with transcription factors such as FoxH1 in the nucleus which allow them to interact with their target sequences *(83).*

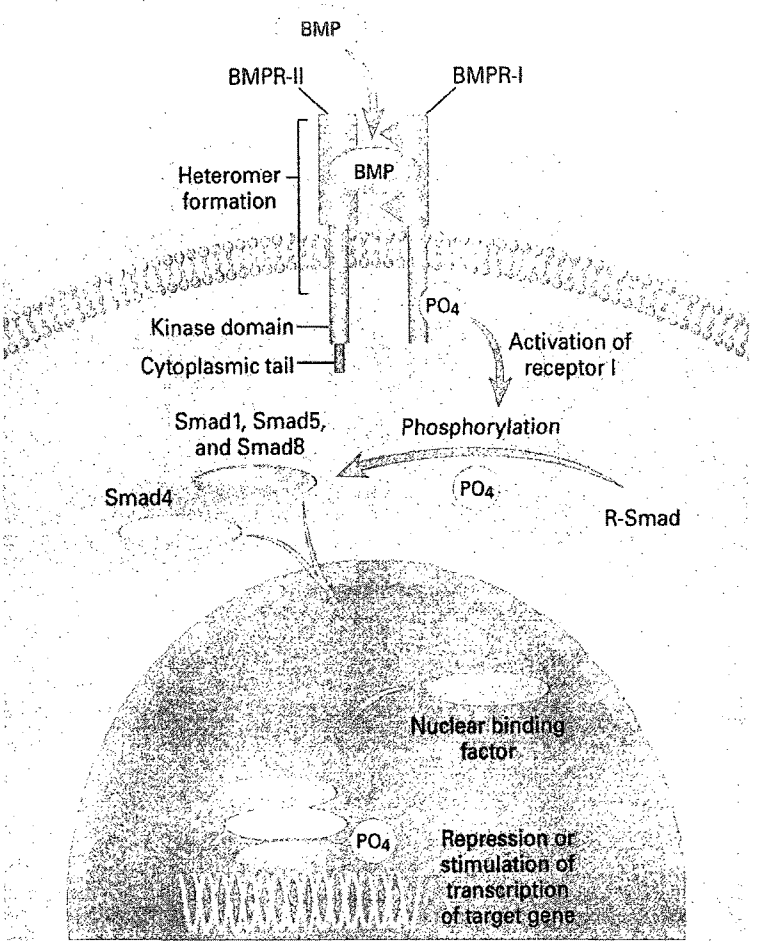

Figure 8: Image demonstrating the SMAD1/5/8 pathway used by the BMP receptors. From Newman et al. 2001 (84). Reprinted by permission of the Massachusetts Medical Society.

Recent studies have suggested that BMP signaling also occurs through mitogen activated protein kinases (MAPKs)(68;79;85;86) and that the BMP receptors behave somewhat differently than others in the TGF superfamily. All other TGFß receptor groups produce a heterodimer of type 1 and type 2 only when a ligand is bound. However, the BMP receptor may form a variety of dimer complexes, found

with or without a bound ligand (68;87). The vast majority of these preformed complexes contain one BMPRII and either a type I BMP receptor BMPRIA or BMPRIB. For the complexes that are not preformed, upon binding of a BMP, a pattern of dimerization identical to that of the preformed complexes occurs on the cell surface. The individual complexes signal differently upon binding of ligands (68;87). The preformed complexes signal via the traditional SMAD pathway as with other members of the TGF-B superfamily. By contrast, the ligand binding induced signaling complex signals via MAPKs including p38MAPK and p42/44MAPK (ERK1/2) as well as c-Jun-N-terminal kinase/stress activated protein kinase (JNK/SAPK) (86;88). Transfection of many of the known mutations of BMPRII found in IPAH and FPAH into mouse epithelial cells resulted in a ligand independent initiation of p38MAPK signaling. This had led to a hypothesis that reduced surface appearance of functioning BMPRII leads to preferential activation of the p38MAPK pathway in PAH (89).

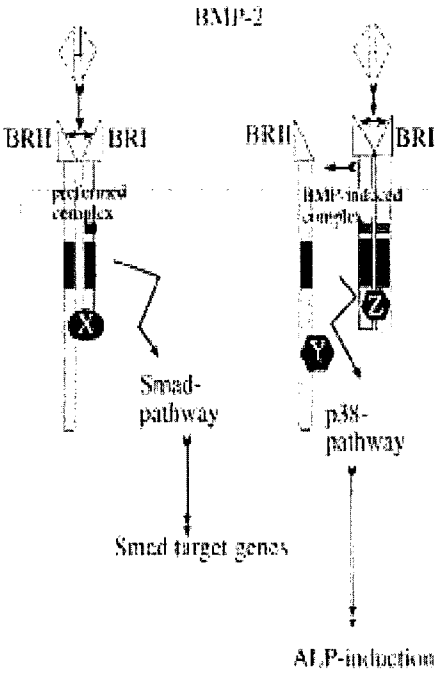

Figure 9: Depending on the preformed complex on the cell surface the BMP system signals through either the MAPK system or SMAD pathway. From Mohe et al. 2002 (87)

There may be some overlap between these two separate pathways since, in addition to the regular phosphorylation site SXS on SMAD1, 4 additional phosphorylation sites have been identified which are Phospho-MAPK/CDK Substrates (PXSP) sites (68;90). These latter sites are responsible for basal phosphorylation levels of SMAD-1 and are consensus sites for MAPK. Upon phosphorylation of the PXSP sites, nuclear localization of SMAD1 is prevented (68).

1.4.4 BMP Signaling Control

Evidence exists for self-control of BMP expression; however this does not appear to be the primary method of BMP signaling control. Several BMP-specific antagonists have been identified. Many are secreted extracellular antagonists which bind the BMPs, preventing them from binding to their receptors. These include: Noggin, the Chordin family of proteins, Twisted Gastrulation (Tsg), and the Dan family. Each has their own specificity in terms of which BMPs they act upon. Noggin is capable of binding BMPs 2 and 7 as well as growth differentiating factor (GDF) 5 and 6 and vegetally localized protein (Vg-1), but no other members of the TGFß superfamily. Chordin binds BMPs –2, 4, and 7 but no other members of the superfamily. Tsg binds to the chordin BMPs 2 and 4 complexes to enhance its inhibition strength. However, Tsg also acts as an agonist by increasing the cleavage of chordin, thereby preventing its inhibition of signaling. The Dan family has many members which bind to a variety of BMPs; this family is thought to play a role primarily in early development and not in adults.

On the cell surface a pseudoreceptor, BMP and activin bound protein (BAMBI), modulates BMP signaling. It is a transmembrane glycoprotein with an extracellular domain similar to that of other receptors of TGFß superfamily. BAMBI associates with BMPRIA and BMPRIB and inhibits their activation, even in the absence of BMP ligand (91;92). There are several intracellular inhibitory mechanisms for

BMP receptor signaling. The iSMADs, SMAD6 and SMAD7, have several actions: they compete with rSMADs for TGF and BMP receptor binding; they bind to the type 1 receptors and recruit E3-ubiquitin ligases known as SMAD ubiquitination regulatory factor 1 and 2 (Smurf1, Smurf2). When the Smurfs bind to the receptors, ubiquitination and degradation of the receptors occur thus decreasing the receptor level available for signaling *(93).* Smurfs 1 and 2 are also able to ubiquitinate SMADs; specifically 1 and 5. SMAD7 also recruits a complex, GADD34 the catalytic unit of protein phosphatase 1, to activated TGFß type I receptor and dephosphorylates and inactivates it. *(94).* The actions of SMAD6 are inhibited by associated molecules containing the SH3 domain or STAM (AMSH) which binds to the iSMADs and inhibits its interactions with BMPRI upon BMP2 stimulation. The alternate BMP signaling pathway via MAPK has been shown to phosphorylate AMSH and prevent it from binding to SMAD6.

Other intracellular proteins may act as co-activators and repressors of transcription. Ski is an important TGFß negative regulator, preventing the inhibition of growth normally mediated by TGFß. Increased levels of Ski have been found in tumour cells *(95;96).* Although its affinity to BMP SMADs 1/5/8 is weak, Ski is still able to decrease their signaling via other mechanisms, including stabilizing SMAD/ DNA complexes preventing newly phosphorylated SMADs from gaining access to SMAD binding elements on the DNA. Knockout of Ski in utero is lethal *(97).*

1.4.5 TGFß$_1$

TGFß$_1$ is the prototypical molecule of the TGFß superfamily which includes inhibins, activin, anti-müllerian hormone, bone morphogenetic proteins, decapentaplegic and Vg-1. It is a protein secreted by many cells of the body and affects cell proliferation and survival. Like other members of the superfamily it has type I and type II receptors (TβRI, TβRII) which dimerize to produce signals downstream upon binding. The type II receptor is found ubiquitously, as is the type I activin like kinase 5 (ALK5). However, another type I activin like kinase I (ALK1) is not found ubiquitously, but is abundantly present on endothelial cells. ALK-1 has recently been described on chondrocytes, (98) fibroblasts (99), myoblasts (100) as well as hepatic stellate cells *(101)*. ALK1 has two chromosome 12 transcript isoforms, which differ in their 5'UTR: a longer 4263bp mRNA transcript and a shorter 4126bp transcript. They both produce the same isoform of mature protein which is 503 amino acids in length and has great homology with other type I activin like kinase receptors, including in he serine-threonine kinase subdomains, a glycine and serine-rich region (called the GS domain) preceding the kinase domain, and a short C-terminal tail (source NCBI).

The two different receptors act through the different SMAD pathways with ALK5 activating SMADs 2 and 3, while ALK1 uses SMADs 1, 5, and 8. ALK1 and ALK5 are only activate upon binding of their ligands, with the type II receptor then

phosphorylating the type I receptor, allowing it to produce its downstream effects. The exact stoichiometry of the receptors upon binding is not yet fully understood. In most cell types where ALK5 is the only type I receptor for TGFß, the TβRI and TβRII are found as homodimers in the cytoplasm, and the crystal structure of cell-surface TβRII suggests the same arrangement occurs on the plasma membrane. The TβRI/ TβRII complex is most likely made up of at least two TβRII receptors and two TβRI receptors *(83)*. As with BMP signaling, TGFß signaling may also employ MAPK activation (102) (103). Depending on their concentration, the receptors involved, and the type of TGF superfamily molecule being studied, opposing effects may be seen in the same cell type. It is thought that signaling via ALK5 suppresses proliferation. ALK5 is the more common receptor in mature endothelial cells. By contrast, ALK1 signaling has been found to be pro-proliferative and is highly active in developing vessels (104;105). Thus, the reaction of an endothelial cell to TGFß stimulation is very much dependant on the balance of ALK1 or ALK5 stimulation. Each receptor has a different pattern of gene stimulation. ALK1 stimulation promotes pro-angiogenic genes such as Id1, and IL1RL1 while ALK5 stimulates expression of maturation genes such as connexin 37, IG-H3, and plasminogen activator inhibitor-1 *(106) (107) (104)*

Endoglin (cd105), a co-receptor of TGFß also found predominantly on endothelial cells, interacts more tightly with ALK1 then ALK5. Mutations in endoglin also lead to the development of HHT. Ectopic expression of endoglin represses signals from ALK5 *(108)*. There appear to be two splice variants of endoglin, the

predominant long form L-endoglin, and a less common short form, S-endoglin. Recent findings in L_6E_9 myoblasts indicate that the more common long splice variant increases signaling via ALK1, therefore interfering with ALK5 signaling, while the less common short form interacts with ALK5 *(100)*

1.4.6 Balance of TGF and BMP signaling in the Pulmonary Vasculature

BMP and TGF signaling has been associated with control of cell growth, proliferation, and apoptosis. Derangement of these functions may be critical to the development of PAH. Abnormal growth responses in cells from patients with PAH to these molecules are described below. Pulmonary vascular smooth muscle from normal lungs, or from lungs of patients with idiopathic PAH or PH of other causes were cultured in the presence of various BMPs. TGFß decreased the proliferation of cells from normal and non-idiopathic PH lungs. However, the IPAH-lung derived PASMC showed increased proliferation at all TGFB concentrations. BMPs also decreased the proliferation of normal and non-IPAH lung -derived PASMC; however, the IPAH PASMC did not show this decrease in proliferation. This suggests that BMP signaling normally has an anti-proliferative effect on PASMC and this effect is lost in lungs affected by IPAH, and this may permit an exaggerated, paradoxical proliferative effect of TGFB. *(73)*

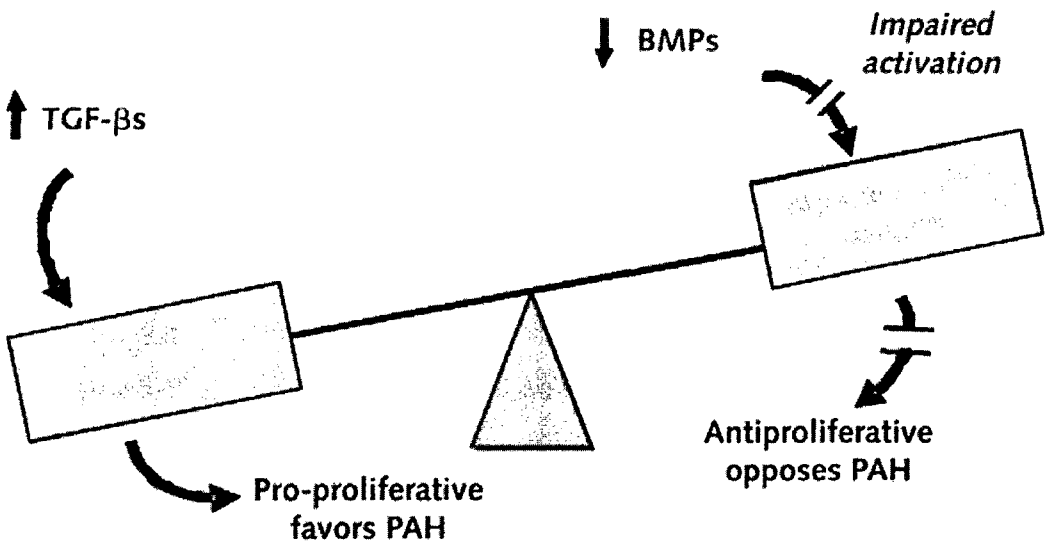

Figure 10: The loss of the normal suppressive effects of the BMPs and enhancement of the stimulatory effects of the TGFß molecules, creating an imbalance has been proposed to be the cause of PAH Figure from Newman et al. 2008 (9). Reprinted by permission of Annals of Internal Medicine

The effects of the TGFß/BMP system have been studied in various endothelial cell types. When the BMPRII gene is knocked down using siRNA, pulmonary artery endothelial cells (PAEC) developed increased apoptosis (11). Thus, some BMPs may normally act as survival factors for endothelial cells. The size and site of the vessel may also be important to the effects of BMPs, as well as the type of BMP. When the effects of BMP4 on endothelial cells derived from capillaries, veins and arteries derived from various different tissues were compared, human coronary and aortic endothelial cells were completely resistant to BMP4-induced apoptosis whereas the dermal microvascular, human umbilical artery and vein were sensitive to BMP4 induction of apoptosis. This study also demonstrated

increased levels of the iSMADs SMAD 6and 7 in large artery derived endothelial cells. The iSMADs have been shown to be particularly effective at down regulating SMAD1, 5, 8 signaling.

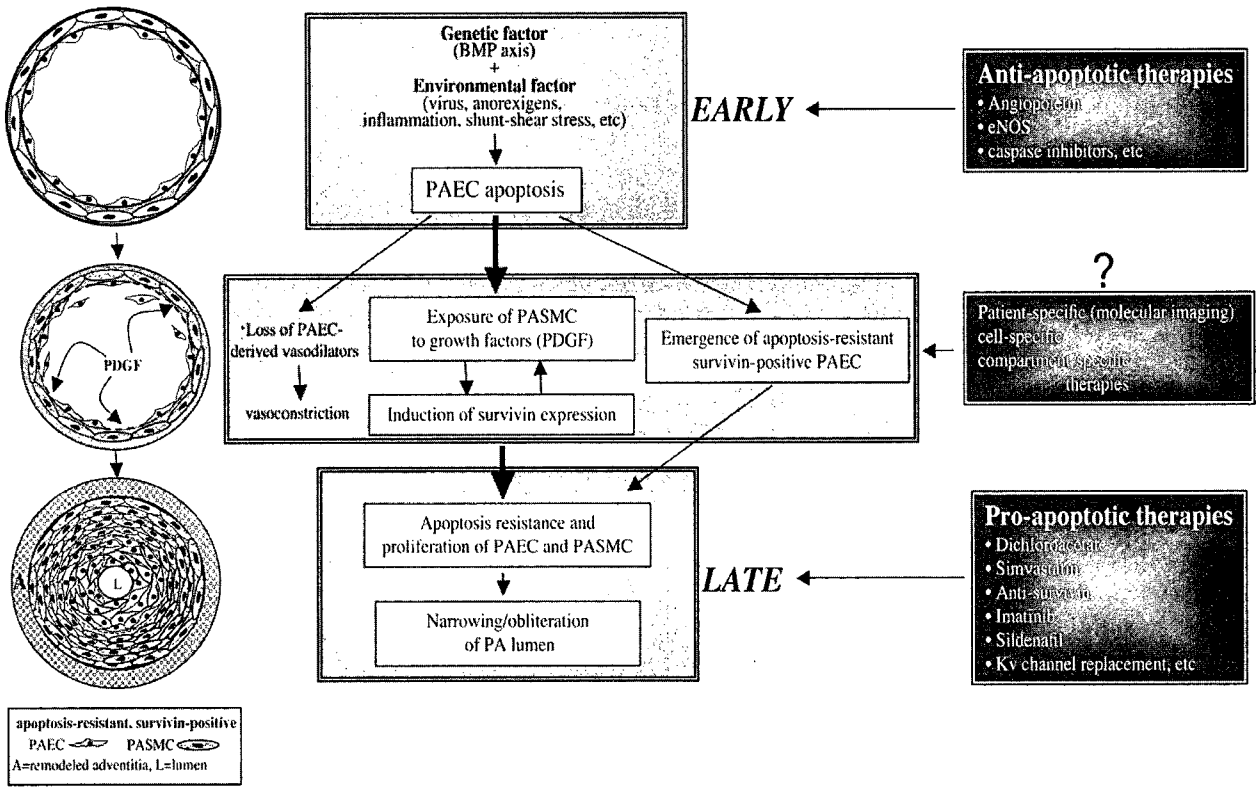

Figure 11: A diagram of the hypothetical development and disease progression of PAH. From Michelakis et al. 2006 (12). Reprinted by permission of Wolters Kluwer Health

1.4.7 The TGF/BMP System and its effects on the Endothelin System

Very quickly after the initial discovery of ET-1, it was recognized that TGFß affected ET-1 production. TGFß increases prepro-ET-1 mRNA expression increases in a dose and time-dependent manner in porcine aortic endothelial cells *(109)*. This effect seems to signal via the ALK5 receptor, because the specific ALK5 inhibitor SB-431542 prevents induction, while siRNA knockdown of the alternate primarily endothelial type 1 TGFß receptor ALK1 does not prevent the increased ET-1 production. In addition, TGFß was shown to hinder endothelial cell proliferation and mobility, and this was also reversed by the ALK5 inhibitor. Moreover, the effects of TGFB were partially inhibited by the ET-1 receptor antagonist bosentan, implying that part of the TGFß effect results from ET-1's autocrine effects on endothelial cells. *(110)*. BMP7, another member of the TGFβ superfamily, increases ET-1 production in Rat Calvarial Osteoblasts *(111)*. TGFß down regulates endothelin-1 selective binding sites in smooth muscle cell-derived A617 cells. (32). These sites are most likely ET-A receptors. A similar effect occurs in hepatic stellate cells which, like endothelial cells, express ALK1 (101;112).

Rationale for Studying the Possible Relationship Between the TGFß System and ET-1 Synthesis

Increased expression and synthesis of endothelin-1, the highly potent vasoconstrictor and smooth muscle mitogen, has been found in the lungs of PAH patients. While this may be part of the greater endothelial dysfunction seen in PAH, including increased proliferation, loss of apoptosis, and abnormal synthesis of other vasoactive products, there may be specific triggers for the excess ET-1 production. Increasingly, the pulmonary microvascular endothelial cell seems central to the initiation and progression of PAH. The normal behaviour of these cells may be very different from endothelial cells of other origins, and findings in other cells may not be extrapolated to the lung microvasculature. The relatively recent connection between the bone morphogenic system and familial and idiopathic PAH as well as the closely associated TGFß system mutations in HHT suggest that these molecular signaling mechanisms are also central to PAH, and may be a true initiating factor, with a secondary trigger at times being present. The TGFB system affects ET-1 synthesis. Thus a connection between the TGFB system abnormalities and heightened ET-1 synthesis may be proposed.

Aim of Study

The purpose of this study was to determine which members of the TGFß family, if any had an effect on the ET-1 production levels of normal human microvascular endothelial cells of the lung.

Chapter 2:

Effects of Bone Morphogenic Proteins and Transforming Growth Factor-BETA on *In-vitro* Production of Endothelin-1 by Human Pulmonary Microvascular Endothelial Cells.

Gregory Star, Michele Giovinazzo, Dr. David Langleben

Submitted to the journal of Vascular Pharmacology © currently under review

2.1 INTRODUCTION

Pulmonary arterial hypertension (PAH) is a group of disorders that cause vascular remodeling of the pre-capillary pulmonary microvasculature, leading to increased pulmonary vascular resistance, right heart failure, and ultimately death (7;8). Endothelial cell (EC) dysfunction is a hallmark of PAH, manifesting most significantly as abnormal EC growth that occludes the microvascular lumen, but also via reduced levels of endothelium-derived mediators that normally help maintain vascular homeostasis, including prostacyclin and nitric oxide (7;13;14;113-115). Moreover, increased plasma and tissue levels of the endothelium-derived vasoconstrictor and mitogen, ET-1, have been described (16). Identification of increased ET-1 levels has led to the development of endothelin receptor antagonists that have improved the course of the disease for many PAH patients (116-118)

Whether the increased levels of ET-1 in PAH were related to excess local synthesis, or to a reduction in the normal pulmonary clearance of circulating ET-1 from the bloodstream, or both, had been unclear. Histologic study of lungs from patients with PAH established that excess local ET-1 expression and synthesis was a major factor (119). Physiologic measurements of pulmonary ET-1 extraction in patients with PAH subsequently established that the majority of patients have normal or near-normal levels of ET-1 extraction (120). Thus, the increased ET-1 levels in PAH result mainly from excess synthesis. There is at present only limited evidence that ET-1 synthesis can be reduced by therapy (121;122). The stimuli for this excess synthesis are unknown. Numerous factors

that can alter ET-1 production have been described, including transforming growth factor beta (TGFß) (123;124). *In vitro*, TGFß stimulates ET-1 synthesis in endothelial cells from several vascular origins (109;110;125;126). Its effects have not been studied in pulmonary microvascular endothelial cells.

Identification of families with PAH has led to the description of genetic mutations in components of receptors for the TGFß superfamily of molecules (87);(127). These components include a receptor for bone morphogenic proteins, BMPR-II (63;64;68) and the TGFß receptor components activin-like kinase-I (ALK-1) and endoglin (65). The end-effect of the mutations seems to be a reduction in signaling via the receptor type that is expressed by the mutated gene (haplotypic insufficiency) (128). Activation of these receptors affects growth, apoptosis and differentiation in many cell types (129) and BMP signaling promotes pulmonary endothelial cell survival (11). The loss of this survival factor may contribute to the emergence of apoptosis resistant endothelial clones that narrow the microcirculation in familial PAH (11). Moreover, ALK-1 is expressed in the circulation solely on endothelial cells, and a mutation in ALK-1 causes hereditary hemorrhagic telangiectasia (HHT), and PAH that is clinically indistinguishable from familial PAH (65). Thus, there is compelling evidence for involvement of the TGFß superfamily of molecules in the pathogenesis of several types of PAH (9). Given normally low ET-1 levels in humans, but high ET-1 levels in PAH, we hypothesized that the TGFß

superfamily of molecules would alter ET-1 production by pulmonary microvascular endothelial cells, the cell critical to the pathogenesis of PAH.

2.2 MATERIALS AND METHODS

2.2.1 Cells and reagents:

Lung-derived normal human microvascular blood vessel endothelial cells HMVEC-LBI (Lonza, Walkersville MD) were cultured in EGM-2MV medium (supplemented with 5% fetal bovine serum, 0.04% hydrocortisone, 0.4% human fibroblast growth factor, 0.1% vascular endothelial growth factor, 0.1% insulin like growth factor, and 1% GA-1000). These cell lines are purified by double staining and flow cytometry and are 90% pure for cells of vascular origin; they express CD31, but do not express podoplanin, a marker for cells of lymphatic origin (HMVEC - LBI Lung Blood MV Endothelial Cell - Technical Sheet, Lonza, February 2008). BMPs 2,4,7 and TGFß1 were purchased from Peprotech (Rocky Hill, NJ).

2.2.2 Experimental techniques:

HMVEC-LBI cells (passage 5 only) were grown on 24 well plates until they reached confluence. Twelve wells were used for each experimental condition, including each concentration at each time point. The medium was collected post exposure to the peptides and it was frozen at -70° C. for subsequent

measurement of ET-1 levels. At each time point, after aspiration of the medium, the cells were lysed with Reporter Lysis Buffer (Promega) and the protein concentration in the lysate was measured by the BCA method (Pierce, Rockford IL). ET-1 levels in the supernatant were measured with a commercial ELISA kit (Assay Designs, Ann Arbor, Michigan) with antibodies specific for human ET-1. The intra-assay coefficient of variation was 5.8%, with an inter-assay variation of 4.7%. Cross reactivity to ET-2, ET-3 and Big ET was < 0.1%. The ET-1 levels we measured in experiments fell within the mid-range of the curve generated by the standard concentrations provided with the ELISA kit - therefore the kit was capable of measuring a reduction in ET-1 levels as compared to CONTROLS, should have occurred in an experiment. The complete medium had undetectable levels of ET-1.

2.2.3 Experimental design:

In the first experiment, once the HMVEC-LBI cells were confluent, the complete medium was replaced with complete medium containing 0, 0.156, 0.3125, 0.625, 1.25, 2.5, 5, or 10 ng/ml of TGF-ß1, or 0,1,10,or 100 ng/ml BMP-2, 4 or 7. The supernatant was then collected and the cells processed as discussed above. To examine the effects at earlier time points, a time curve study of the response to TGFß (2.5 ng/ml) was performed - at time 0, after the EGM-2MV complete medium was replaced with complete medium containing the TGFß. One, 4, 8, and 24 hours later the supernatant was collected and the cells processed as discussed above. In a third experiment, to study the effects of

basal medium without serum and growth supplements, cells were exposed to basal medium containing 0.1% BSA for 16 hours, then the medium was removed and the cells were exposed to basal medium with BSA, with or without TGFß (2.5 ng/ml) or BMP 2, 4, or 7 (100 ng/ml) for 8 hours. The supernatant was then collected and the cells processed as discussed above.

2.2.4 SMAD signaling:

HMVEC-LBl cells were grown to confluence on 6-well plates in EGM-2MV medium. The cells were then serum starved in basal medium (EGM medium + 0.1% BSA) overnight. Next, the medium was replaced with either basal medium, complete EGM-2MV medium, or basal medium containing BMP 2, 4, or 7 (100 ng/ml) or TGFß (2.5 ng/ml) for 5 minutes. The cells were then lysed directly in sample buffer (2.5 mM Tris-HCl, pH 6.8, 25° C., 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% w/v bromophenol blue), homogenized by syringe aspiration through a 26-gauge needle, and separated on a 10% polyacrylamide gel. The proteins were transferred to a PVDF membrane (Pierce, Rockford IL) and blocked in TBST (0.02 M Tris, 0.14 M NaCl, 0.1% v/v Tween) with 5% powdered skim milk. The membranes were incubated overnight at 4°C with anti-SMAD5 (Cell Signaling, Danvers, MA), anti-phosphorylated SMAD1/5 (Cell Signaling, Danvers, MA), and anti-GAPDH (Fitzgerald, Concord, MA), all in TBST with 5% BSA. The membranes were then washed in TBST and incubated at room temperature for 1 hour with anti-rabbit-HRP (Cell Signaling, Danvers, MA) and anti-mouse-HRP (Pierce, Rockford, IL). After another wash,

the membranes were incubated with Western lightning enhanced luminol (Perkin-Elmer, Waltham, MA) and then exposed to photographic film. Density of the bands on the developed film was analyzed using ImageJ software (National Institutes of Health, Bethesda, MD).

In a separate experiment, the time course of SMADs 1/5 phosphorylation was studied. The cells were serum starved overnight in endothelial basal media (EBM, Lonza) containing 0.1% BSA. The medium was then replaced with fresh EBM alone or EBM containing 100ng/ml of BMP 2, 4, or 7, or 2.5ng/ml of TGFß. The cells were exposed to the experimental conditions for 15 minutes, 45 minutes and 7 hours, after which the cells were lysed in sample buffer. The samples were then processed and analyzed as above.

2.2.5 Statistical methods:

Data were expressed as mean ± SD. For each experimental group, at 24 or 48 hours, to detect differences in group means, one-way analysis-of-variance was used, followed where appropriate by the Tukey-Kramer multiple comparison test. Two-tailed p values < 0.05 are considered significant

2.3 RESULTS:

2.3.1 Effect of BMPs on ET-1 levels in complete medium (Figure 13A):

At the concentrations used, after 24 hours of exposure in complete medium, neither BMP-2, BMP-4 nor BMP-7 altered absolute ET-1 levels, normalized ET-1 levels or cell protein levels as compared to controls. Similarly, BMP-2 and BMP-4 did not raise absolute ET-1 levels after 48 hours of exposure. However, after 48 hours, BMP-2 (10ng/ml) slightly increased cell protein levels, by 19% over controls ($p<0.05$). By contrast, BMP-4 (1 ng/ml) slightly reduced cell protein levels (12% less than controls) and this resulted in an 18% increase in normalized ET-1 levels versus controls ($p<0.05$). At 48 hours, BMP-7, only at a dose of 100 ng/ml, increased absolute ET-1 levels by 32% as compared to control ($p<0.05$), and it also increased ET-1 levels normalized for cell protein by 27% versus controls.

2.3.2 Effect of TGFß1 on ET-1 levels in complete medium (figure 13B):

TGFß1 stimulated ET-1 production by the pulmonary microvascular endothelial cells. At 24 hours, as compared to controls it significantly increased ET-1 levels at all TGFß1 concentrations except 10 ng/ml, with a 47% increase at 2.5 ng/ml, and when normalized to cell protein, ET-1 levels were increased at all

concentrations except 5 and 10 ng/ml (40% increase at 2.5 ng/ml). Exposure to TGFß1 did not cause any alteration in cell protein levels. A similar pattern was observed after 48 hours of exposure to TGFß1, where protein levels were not increased, but ET-1 levels compared to controls were increased at 0.625, 2.5 5 and 10 ng/ml (30% increase at 2.5 ng/ml), and ET-1 levels normalized to cell protein were increased compared to controls at all TGFß1 concentrations between 0.625 and 10 ng/ml inclusive (31% increase at 2.5 ng/ml).

2.3.3 Time course of ET-1 stimulation by TGFß in complete medium (figure 14):

As compared to CONTROL, exposure to TGFß (2.5 ng/ml) in complete medium significantly increased normalized ET-1 secretion at 4, 8, and 24 hours. The difference was greatest at 8 hours (27% increase), with a smaller difference (15%) at 24 hours. TGFß also increased absolute ET-1 levels at those time points, and it slightly (maximum 4%) increased cellular protein levels (data not shown).

2.3.4. Effects of BMPs and TGFß1 on SMAD levels (Figure 15):

At 5 minutes, levels of SMAD-5 remained unchanged by exposure to BMPs, TGFß1, or complete medium (figure 3, upper). However, after 5 minutes of exposure in basal medium, BMPs 2, 4 and 7 and TGFß1 as well as complete medium increased levels of phosphorylated SMAD-1/5. Phosphorylation of SMADs 1/5 in basal medium was rapid (figure 3 lower). It was apparent at 15

minutes, and still present at 45 minutes, with less phosphorylation by BMP-7 at that time. TGFß, still induced phosphorylation at 7 hours.

2.3.5 Effect on ET-1 levels of BMPs 2, 4, and 7, and TGFß in basal medium (figure 16):

In basal medium, an 8 hour exposure to BMPs 2, or 7 or TGFß significantly increased normalized ET-1 levels as compared to controls, with TGFß inducing a 51% increase and BMP-7 a 27% increase. BMP-4 had no effect on ET-1 levels. Absolute ET-1 levels were significantly increased by BMP-7 (24% increase) and TGFß (20%) as compared to controls, but not by BMP-2 (data not shown). None of the BMPs affected cellular protein levels, but TGFß decreased cellular protein by 20%.

2.4 DISCUSSION

The results of the present studies demonstrate that ET-1 can be modulated by some BMPs and by TGFß in human pulmonary microvascular endothelial cells in vitro. With TGFß, this stimulated increase in ET-1 occurs within 4 hours, remains at 24 and 48 hours and is seen in basal or complete medium. Despite inducing sustained SMADs 1/5 phosphorylation, BMPs 2 and 4 have minimal or none of the above effects, while BMP-7 at high doses has stimulatory effects

similar to TGFß at 48 hours in complete medium and at 8 hours in basal medium.

An imbalance, with loss of the normal suppressive effects of the BMPs and enhancement of the stimulatory effects of the TGFß molecules, has been proposed to lead to the vascular abnormalities in PAH (9). For example, unlike their normal growth-inhibitory effects seen in controls, BMPs did not inhibit proliferation of pulmonary artery smooth muscle cells derived from patients with idiopathic PAH (73). By contrast, TGFß1 stimulated proliferation in those same cells. These two effects were not apparent in smooth muscle cells from patients with other causes of pulmonary hypertension. Furthermore, BMPs appear to be essential survival factors for some cell types, preventing apoptosis in pulmonary vascular endothelial cells (11). Thus it is essential to characterize the effects of these peptides in the clinically relevant cell population. The pulmonary microvascular endothelial cell appears to be the primary source of the excess ET-1 in PAH (15), although there may be some lesser contribution from other cells in the vessel wall. In this study, we examined cells from individuals without pulmonary hypertension, to examine normal signaling pathways. Previously available commercial microvascular endothelial cell lines were significantly contaminated with endothelial cells of lymphatic origin. The line we used, HMVEC-LBI is vascular in origin. Thus, our results provide novel information about the effects of the TGFß superfamily of molecules on ET-1 synthesis in these cells most relevant to PAH.

The HMVEC-LBI are extremely dependent on adequate culture conditions, including the presence of complete medium. They do not survive well in basal medium for extended periods of time. Therefore, our initial experiments were all performed with complete medium as the control. In that medium, only BMP-7 and TGFß increased ET-1 levels. The time course experiment (figure 14) suggests that ET-1 levels can be stimulated to rise within 4 hours of exposure to TGFß, and that the levels continue to rise as compared to control levels. We chose 24 and 48 hour time points for our dose-response experiments, since differences at those times would be more clearly detectable. A previous study has also used the 24 hour time point (110). Due to the need for serum starvation prior to onset of the experimental period, the studies using basal medium could not be extended beyond 8 hours since cell mortality became an issue. However, in the experiments we performed in basal medium, the same pattern was seen, with BMP-7 and TGFß increasing ET-1. Complete medium clearly stimulates ET-1 levels by itself, and this may reduce the ability to detect earlier ET-1 increases induced by BMP-7, such as were seen in basal medium.

Activation of the BMP receptor, or the type 1 TGFß receptor ALK-1, results in phosphorylation of SMADs 1 and 5 (127). The effects of BMPs and TGFß on SMADs 1/5 phosphorylation were studied in basal medium. Complete medium induces significant phosphorylation by itself (figure 15), likely due to the endogenous cytokine and TGFß-family molecules present in the growth

supplement and fetal bovine serum. Our measurement of SMAD levels confirms that the different biologic effect between the BMPs studied and TGFß was not related to abnormal ligand-receptor interactions, or to defective BMP molecules. All the BMPs studied and TGFß1 caused rapid SMADs 1/5 phosphorylation, which would be the predicted signaling reaction for effective receptor-ligand interactions. Also, levels of SMAD 5 did not change, indicating that the peptides did not affect the levels available for phosphorylation, but this latter finding would have been important only if we had not demonstrated the phosphorylation event. The time course experiments show that phosphorylation is rapid, within 5 minutes, and sustained at 45 minutes. By 7 hours it has tapered off for TGFß or returned to basal levels for the BMPs. The appearance of increased ET-1 levels in the medium takes several hours. The timing of the phosphorylation and secretion events suggests that they are linked, but separated by the delay necessary for *de novo* ET-1 peptide synthesis. In future studies, siRNA inhibition of SMAD components or the TGFß family receptors, as has been reported elsewhere, might help clarify this issue in the cells we studied (110).

We studied the effects of BMPs 2, 4 and 7, which interact with BMPR-II, and TGFß1 which interacts with TGFß receptors, but not BMPR-II (73). The experiments in Figure 13 were not all performed simultaneously, and results for a given molecule should only be compared with its respective control, not to other molecules or time points. BMP-2 and 4 did not alter ET-1 levels. BMP-7

stimulated ET-1 production, but only at the highest concentration studied (100 ng/ml). Studies of BMPs as endothelial survival factors have employed concentrations of 200 ng/ml (11). It is noteworthy that in a previous study relevant to PAH, all these BMPs demonstrated effects on pulmonary vascular smooth muscle cells at concentration from 1 to 100 ng/ml (73). However, it is also noteworthy that some cells, such as osteoblasts, only increase ET-1 production when stimulated with BMP-7 concentration of 100 ng/ml or higher (111). Thus, the microvascular ECs seem to demonstrate a true response to BMP-7, and it may be that endothelial cells are much less sensitive to BMP effects than other vascular cells such as smooth muscle. Indeed, a previous study of endothelial cells of various origins showed origin-dependent variability in responses to a given BMP, in that case BMP-4 (130). It is unknown whether local pulmonary levels of BMP-7 reach these high concentrations *in-vivo* in patients with PAH. By contrast, TGFß did stimulate ET-1 production at most concentrations studied, and these concentrations overlap those that have previously shown biologic effects on pulmonary smooth muscle cells *in vitro* (73). That previous study, using cells from patients with idiopathic PAH, showed a paradoxical response to TGFß1 as compared to controls, leading the authors to suggest that abnormalities in TGFß superfamily signaling in PAH might be more widespread than just via BMP receptors, and might also involve TGFß receptors (73). If this concept is true, then one stimulus for increased ET-1 production in PAH might be via TGFß.

Our study has several limitations. First, the cells studied were not from patients with PAH. We chose to define "normal" behaviour in these experiments. Second, the cells were studied in isolation, without other vascular cell types being present, and they represent an imperfect simulation of events in a vessel wall. Third, the relevance of *in-vitro* findings to the clinical state is still unclear. Nonetheless, our study does show differing biologic effects of the various TGFß superfamily of molecules, and suggest a potential role for TGFß1 in controlling pulmonary microvascular ET-1 production *in-vivo*. Clinical studies will be needed to confirm the applicability of these findings to PAH.

2.5 CONCLUSIONS:

- BMPs 2 and 4 do not affect ET-1 production by human pulmonary microvascular cells *in vitro*. BMP-7 at high concentrations stimulates ET-1 production. The interaction of these BMPs with their receptor results in rapid phosphorylation of SMAD 1/5, indicating a functional receptor. - TGFß stimulates ET-1 production by human pulmonary microvascular cells *in vitro*, and the interaction of TGFß with its receptor results in phosphorylation of SMADs 1/5. This may have clinical relevance in PAH.

2.6 ACKNOWLEDGEMENTS:

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Hospital. Dr. Langleben is a Chercheur-Boursier Clinicien National (National Clinical Research Scholar) of the Fonds de la Recherche en Sante du Quebec.

2.8 FIGURE CAPTIONS

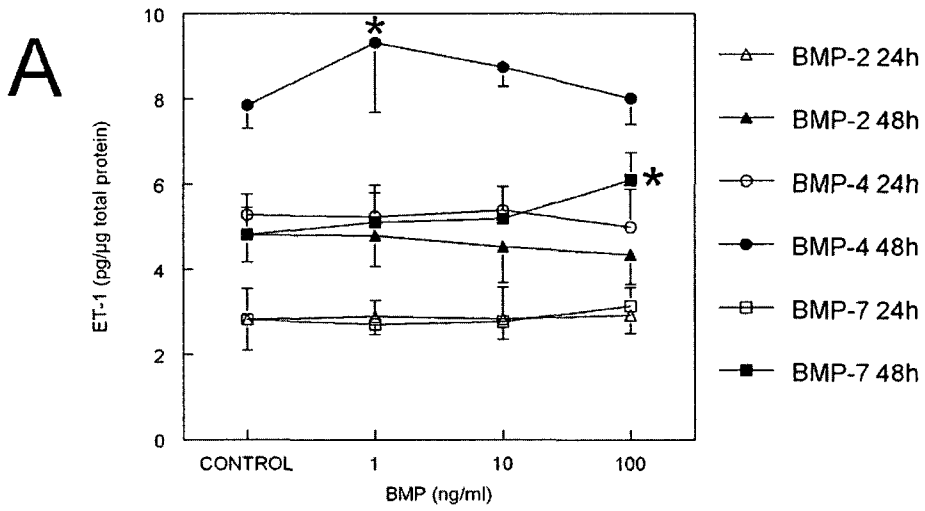
Figure 13. A: Cell culture medium levels of endothelin-1 normalized to cellular protein levels after 24 and 48 hours of exposure to bone morphogenic proteins (BMP) 2, 4, and 7 at various concentrations in complete medium. Bars are group (n=12) mean ± SD. B: Cell culture medium levels of endothelin-1 normalized to cellular protein levels after 24 and 48 hours of exposure to transforming growth factor-beta (TGFß) at various concentrations in complete medium. *, $p < 0.05$ versus respective CONTROL.

Figure 14. Cell culture medium levels of endothelin-1 normalized to cellular protein levels after exposure to transforming growth factor-beta (TGFß, 2.5 ng/ml) for various times in complete medium. Bars are group (n=12) mean ± SD *, $p < 0.05$ versus CONTROL at the same time point.

Figure 15. Upper: Western blot SMAD 5 levels and phosphorylated SMADs1/5 levels after 5 minute exposures to basal medium, complete medium or basal medium containing bone morphogenic proteins (BMPs) 2, 4, or 7 (100 ng/ml) or transforming growth factor-beta (TGFß) (2.5 ng/ml).
A GAPDH loading control is provided. Lower: Western blot phosphorylated SMADs1/5 levels after 15 or 45 minute or 7 hour exposures to basal medium, or basal medium containing bone morphogenic proteins (BMPs) 2, 4, or 7 (100 ng/ml) or transforming growth factor-beta (TGFß) (2.5 ng/ml). A GAPDH loading control is provided.

Figure 16: Cell culture medium levels of endothelin-1 normalized to cellular protein levels after 8 hours of exposure to bone morphogenic proteins (BMP) 2, 4, and 7 (100 ng/ml) and transforming growth factor beta(TGFß, 2.5 ng/ml) in basal medium. Bars are group (n=12) mean ± SD. *, $p < 0.05$ versus CONTROL.

Figure 12:


A
ET-1 (pg/µg total protein)
10
8
6
4
2
0
CONTROL
1
10
100
BMP (ng/ml)
BMP-2 24h
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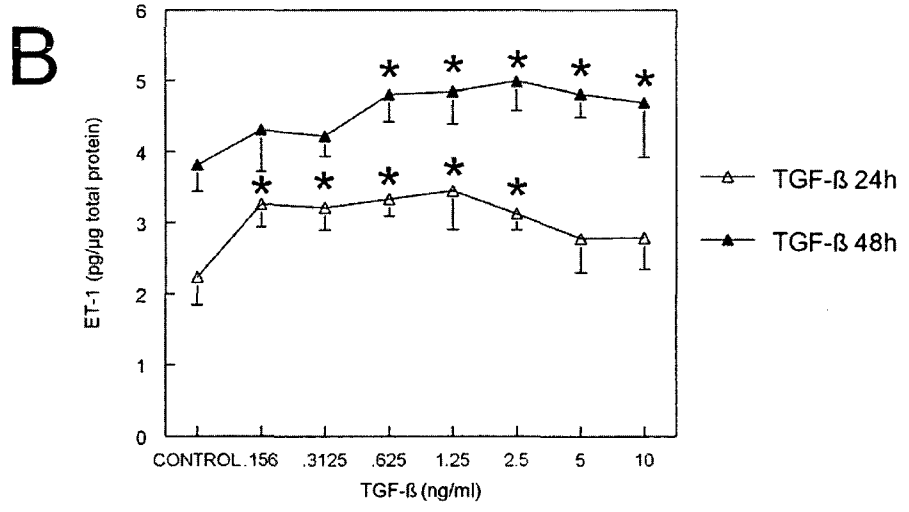
BMP-4 24h
BMP-4 48h
BMP-7 24h
BMP-7 48h


B
ET-1 (pg/µg total protein)
6
5
4
3
2
1
0
CONTROL
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2.5
5
10
TGF-ß (ng/ml)
TGF-ß 24h
TGF-ß 48h

Figure 13:

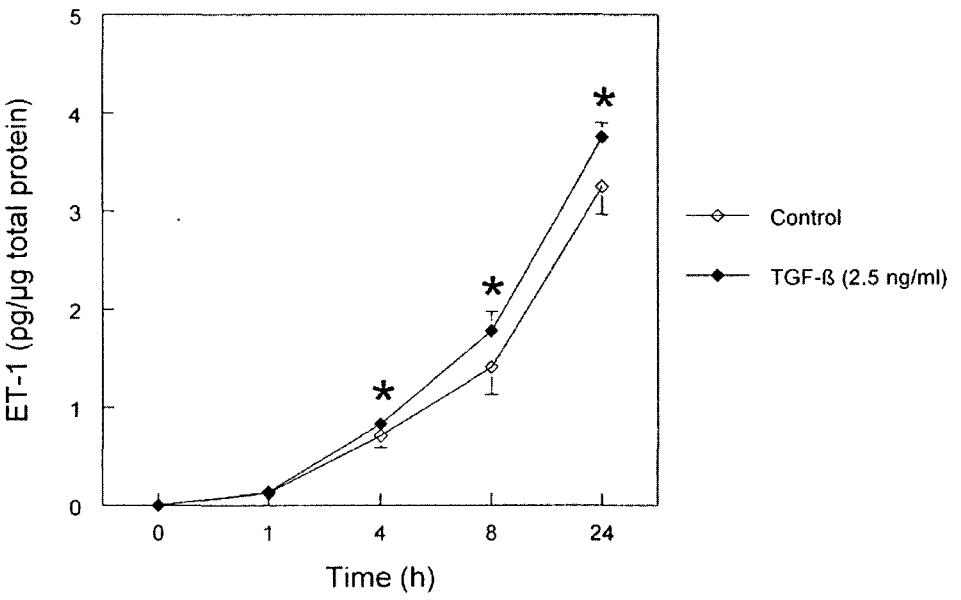
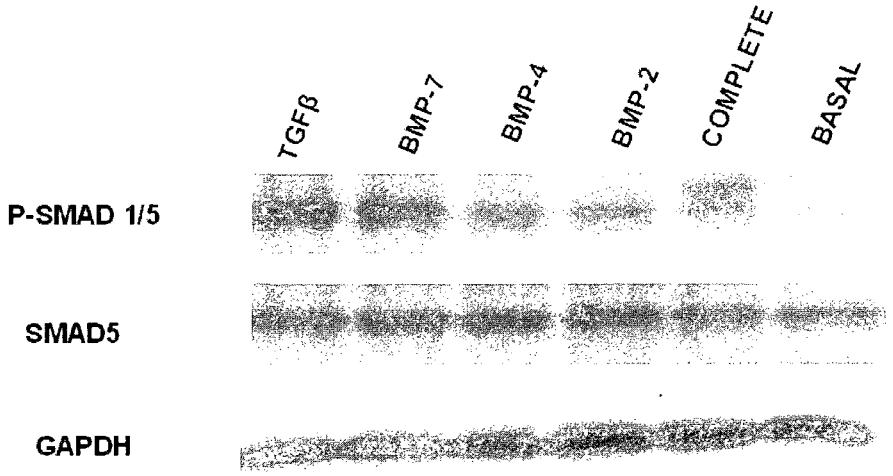

ET-1 (pg/µg total protein)
5
4
3
2
1
0
0
1
4
8
24
Time (h)
*
*
*
Control
TGF-ß (2.5 ng/ml)

Figure 14: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BMP-4
BMP-2
COMPLETE
BASAL
P-SMAD 1/5
SMAD5
GAPDH

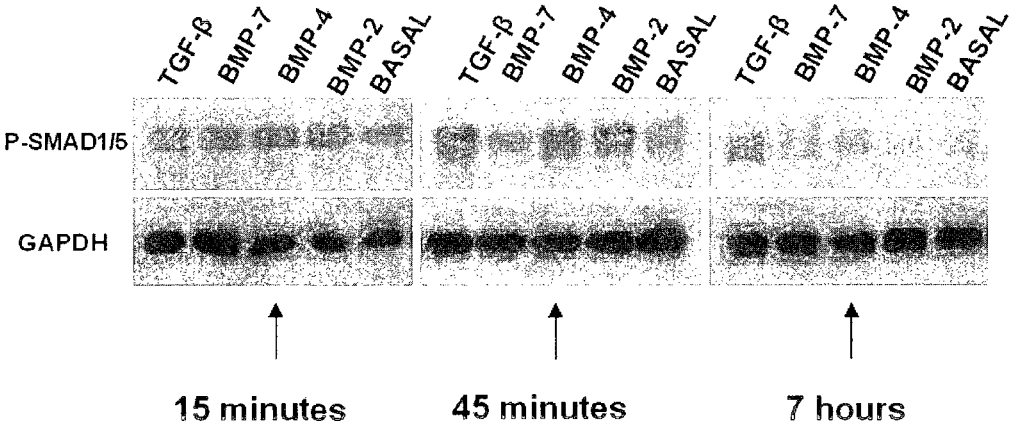
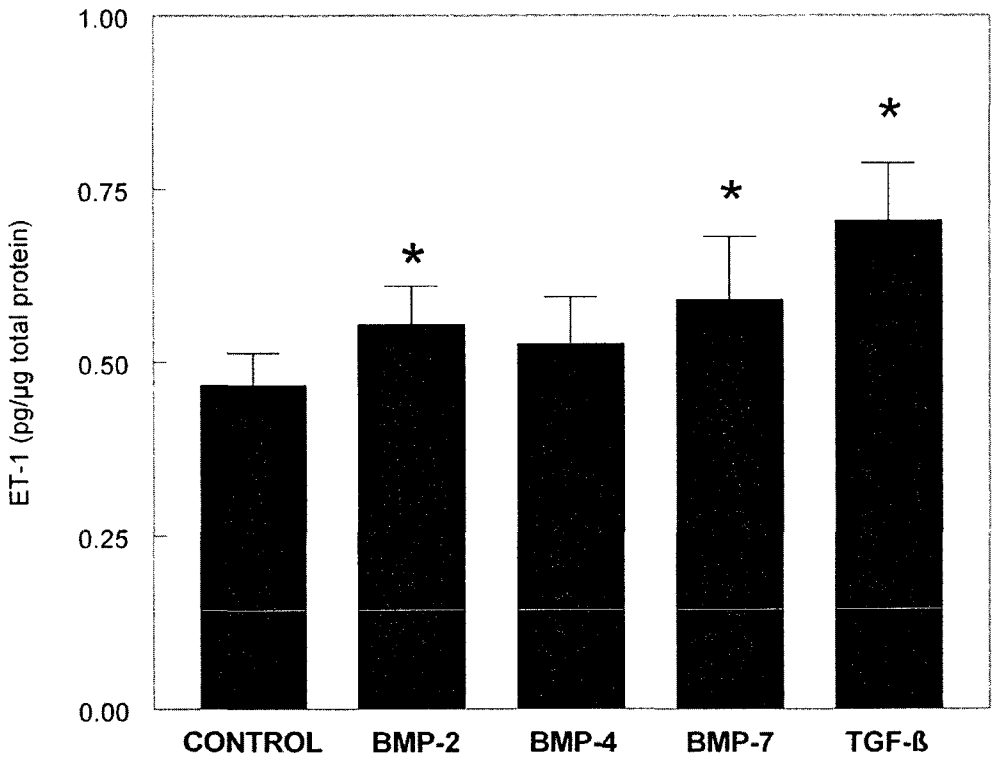

TGF-β
BMP-7
BMP-4
BMP-2
BASAL
P-SMAD1/5
GAPDH
15 minutes
45 minutes
7 hours

Figure 15:


1.00
0.75
0.50
0.25
0.00
ET-1 (pg/µg total protein)
*
*
*
CONTROL
BMP-2
BMP-4
BMP-7
TGF-ß

Chapter 3:

Summary and Conclusion

3.1 Conclusion and Discussion

Idiopathic and familial pulmonary arterial hypertensions are both rare forms of PAH. Both are characterized by occluded arterioles in the lung as well muscularization of these same arterioles. These diseases still remain very much a mystery, despite many advances in recent years. The studies conducted during the course of this thesis were basic, but essential to understanding the normal physiology and response of the blood microvascular endothelial cells of the lung to various members of the TGFß superfamily. It had long been known that there was a genetic link for PAH, but this link remained a mystery until 2000. BMPR2, the type two receptor of the BMP group of proteins, was found to be this link (63;64;68). As noted several times throughout this thesis, endothelial cell dysfunction is a hallmark of this disease. Significantly increased EC growth results in the occlusion of the microvascular lumen. Also, normal endothelium-derived vasoactive molecules are decreased; including prostacyclin and nitric oxide (7;13;14;113-115). Also, increased levels of endothelium-derived vasoconstrictor and mitogen, ET-1 have been described in plasma and tissue (16). With this knowledge, advanced treatments have become available, increasing life expectancy of patients after diagnosis

It is currently thought that BMP and TGFß signaling counteract each other in the cell creating a balance (9). Normally, BMPs have a growth-suppressive effect while TGFß has a stimulatory effect. The loss of the normal signaling of

either may lead to the vascular abnormalities in PAH (9). BMPs do not inhibit proliferation of pulmonary artery smooth muscle cells derived from patients with idiopathic PAH, unlike their action on normal smooth muscle cells (73). By contrast, TGFß stimulates proliferation in those PAH derived cells, unlike in cells derived from normal lungs (73). Furthermore, in pulmonary artery ECs BMPs appear to have an antiapoptotic effect (11). We chose to use HMVEC-Lbl cells because they are derived from the lung blood microvasculature and have been enriched for the vascular population ECs as opposed to lymphatic, by selecting against podoplanin, a lymphatic EC marker (131). It has been demonstrated that endothelial cells of different origin can have different responses to stimuli, and it has even been shown that they can respond differently BMPs (130). Thus it is essential to characterize the effects of these peptides in the most clinically relevant cell population. The endothelial cells of the lung microvasculature appear to be the primary source of the excess ET-1 in PAH (15). In this study, we examined cells from individuals without pulmonary hypertension, to examine normal signaling pathways. Our results provide novel information about the effects of the TGFß superfamily of molecules on ET-1 synthesis in these cells most relevant to PAH.

Our studies demonstrate that some BMPs and TGFß modulate ET-1 human pulmonary microvascular endothelial cells in vitro. TGFß effects on ET-1 levels occurred as early as 4 hours, and remained until the last time point tested, 48 hours. This stimulation occurred with or without the presence of serum and

other growth factors. BMP-7 at high doses has stimulatory effects similar to TGFß at 48 hours in complete medium and at 8 hours in basal medium. BMPs 2 and 4 had no significant effect on ET-1 levels which led us to consider that perhaps the proteins being used were not biologically active or binding abnormally to the receptors. However, they as well as BMP 7 and TGFß induced a sustained phosphorylation of SMADs 1/5, the specific SMADs of the BMPR system as well as ALK1 and therefore active. The HMVEC-LBI cells do not survive well in basal condition for extended periods of time. Therefore complete medium with no BMPs or TGFß was used as the control in initial experiments. The results indicate that as early as 4 hours, ET-1 levels increased by exposure to TGFß (figure 14). In basal conditions due to concern for cell viability, experiments could only be performed for a maximum of 8 hours, given need for overnight serum starvation prior to experiment. Under basal conditions a similar pattern was observed in terms of the proteins capable of stimulating increased production of ET-1, i.e BMP7 and TGFß. The one difference in this case was that it took only 8 hours to see a significant increase with BMP7, as opposed to 48 hours in complete media conditions. Complete medium clearly stimulated ET-1 levels by itself, and may have reduced the ability to detect earlier ET-1 increases induced by BMP-7, such as were seen in basal medium.

Phosphorylation of SMADs 1 and 5 was assayed as they are both phosphorylated by ALK1 and the BMP receptors (127). In order to do so these

studies had to be conducted under basal conditions as complete medium induces significant phosphorylation by itself (figure 15), likely due to the endogenous cytokine and TGFß-family molecules present in the growth supplement and fetal bovine serum. All the BMPs studied and TGFß caused a rapid and sustained SMADs 1/5 phosphorylation. This would be predicted in an effective receptor-ligand signaling reaction Phosphorylation is rapid, within 5 minutes, and sustained at 45 minutes. By the 7 hour time point it had returned to basal for all BMPs and tapered off for TGFß. The stagger between increased ET-1 levels and SMAD 1/5 phosphorylation suggests that they are linked, but separated by the delay necessary for de novo ET-1 synthesis. The experiments in Figure 13 were not performed simultaneously, and results should only be compared to their respective control, and not any other molecules or time points. BMP-2 and 4 did not alter ET-1 levels. BMP-7 stimulated ET-1 production, but only at the highest concentration studied (100 ng/ml). All concentration used throughout this study were based on previously published data (11;73). It has been demonstrated that osteoblasts only increase ET-1 production when stimulated with BMP-7 concentration of 100 ng/ml or higher (111). Thus, the microvascular ECs seem to demonstrate a true response to BMP-7. It is unknown whether local pulmonary levels of BMP-7 reach these high concentrations in-vivo in patients with PAH. By contrast, TGFß did stimulate ET-1 production at most concentrations studied, and these concentrations overlap those previously shown to have a biologic effect on pulmonary smooth muscle cells in vitro (73). That study used smooth muscle

cells isolated from IPAH patients and showed, as compared to controls, a paradoxical response to TGFß, suggesting that in IPAH there may be more widespread abnormalities in TGFß superfamily signaling than just via BMP receptors (73). If this concept is true, then one stimulus for increased ET-1 production in PAH might be via TGFß and inhibitors to this pathway may be a treatment option worth looking at in clinical studies.

This study has several limitations. Firstly, the cells studied were not from patients with PAH. Therefore the results of these experiments likely represent normal behaviour in microvascular ECs of the lung. Also all experiments were done with the cells in isolation; no co-culturing was done with other vascular cells. This represents an imperfect simulation of events in a vessel wall. Lastly, the relevance of in-vitro findings does not necessarily give a true representation of the clinical state. Nonetheless, our studies do suggest a potential role for controlling pulmonary microvascular ET-1 production in-vivo by TGFß.

Further studies

The obvious question is which receptors are responsible for the responses observed and via which pathway they are acting through. siRNA inhibition of various TGFß/BMP signaling components have been reported elsewhere, and this along with use of inhibitors might help clarify this issue in the cells we

studied (110). It would be important to see what effect the members of the TGFß family have on the levels of both endothelin receptor types and the ratio between them. Several new ligands for the primarily endothelial receptor ALK1 have been reported (132). We would like to see what effects these may have on our cells in terms of ET-1 production as well as apoptosis.

Appendix A: Final published paper

Star GP., Giovinazzo M., Langleben D. Effects of bone morphogenic proteins and transforming growth factor-beta on In-vitro production of endothelin-1 by human pulmonary microvascular endothelial cells. Vascular Pharmacology 2009; 50:45–50.

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Effects of bone morphogenic proteins and transforming growth factor-beta on *In-vitro* production of endothelin-1 by human pulmonary microvascular endothelial cells

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ABSTRACT

Background: Altered endothelial cell (EC)-derived mediator levels, including increased endothelin-1 (ET-1), are hallmarks of human pulmonary arterial hypertension (PAH). Gene mutations for receptors for bone morphogenic proteins (BMP), or transforming growth factor-β (TGF-β) cause heritable PAH. The effects of BMPs and TGF-β on ET-1 production by human pulmonary microvascular EC (HMVEC-LBl) are unknown.
Methods: HMVEC-LBl were exposed *in-vitro* to BMPs 2, 4, and 7 or TGF-β1 in basal or complete medium. ET production was measured, as well as total cellular protein. Levels of Smad 5 and phosphorylated Smads 1/5 were also measured.
Results: BMP-4 did not increase ET-1 while BMP-2 increased it minimally in basal medium. BMP-7 increased ET-1, but only at 100 ng/ml. By contrast, TGF-β increased ET-1 throughout most of the studied dose range. All BMPs and TGF-β increased levels of phosphorylated Smads 1/5 without depleting levels of Smad 5.
Conclusions: With the exception of BMP-7 at high-concentrations, the BMPs that interact with BMP receptor 2, the receptor implicated in heritable PAH, do not or minimally modulate in-vitro constitutive ET-1 production by HMVEC-LBl. TGF-β increases ET-1 synthesis, and this may have clinical relevance in PAH.

1. Introduction

Pulmonary arterial hypertension (PAH) is a group of disorders that cause vascular remodeling of the pre-capillary pulmonary microvasculature, leading to increased pulmonary vascular resistance, right heart failure, and ultimately death (Humbert et al., 2004; Simonneau et al., 2004). Endothelial cell (EC) dysfunction is a hallmark of PAH, manifesting most significantly as abnormal EC growth that occludes the microvascular lumen, but also via reduced levels of endothelium-derived mediators that normally help maintain vascular homeostasis, including prostacyclin and nitric oxide (Archer et al., 1998; Christman et al., 1992; Giaid and Saleh, 1995; Humbert et al., 2004; Pietra et al., 2004; Tuder et al., 1999). Moreover, increased plasma and tissue levels of the endothelium-derived vasoconstrictor and mitogen, ET-1, have been described (Giaid et al., 1993; Stewart et al., 1991). Identification of increased ET-1 levels has led to the development of endothelin receptor antagonists that have improved the course of the disease for many PAH patients (Barst et al., 2006; Langleben, 2007; Rubin et al., 2002).

Whether the increased levels of ET-1 in PAH were related to excess local synthesis, or to a reduction in the normal pulmonary clearance of circulating ET-1 from the bloodstream, or both, had been unclear. Histologic study of lungs from patients with PAH established that excess local ET-1 expression and synthesis was a major factor (Giaid et al., 1993). Physiologic measurements of pulmonary ET-1 extraction in patients with PAH subsequently established that the majority of patients have normal or near-normal levels of ET-1 extraction (Langleben et al., 2006). Thus, the increased ET-1 levels in PAH result mainly from excess synthesis. There is at present only limited evidence that ET-1 synthesis can be reduced by therapy(Langleben et al., 1999; Prins et al., 1994). The stimuli for this excess synthesis are unknown. Numerous factors that can alter ET-1 production have been described, including transforming growth factor beta (TGF-β) (Michael and Markewitz, 1996; Perez del Villar et al., 2005). *In vitro*, TGF-β stimulates ET-1 synthesis in endothelial cells from several vascular origins (Castanares et al., 2007; Kurihara et al., 1989; Lee et al., 2000; Rodriguez-Pascual et al., 2004). Its effects have not been studied in pulmonary microvascular endothelial cells.

Identification of families with PAH has led to the description of genetic mutations in components of receptors for the TGF-β superfamily of molecules (Nohe et al., 2002; Yamashita et al., 1996). These components include a receptor for bone morphogenic proteins, BMPR-II (Deng et al., 2000; Lane et al., 2000; Morrell, 2006) and the TGF-β receptor components activin-like kinase-1 (ALK-1) and endoglin (Trembath et al., 2001). The end-effect of the mutations seems to be a reduction in signaling via the receptor type that is expressed by the mutated gene (haplotypic insufficiency) (Machado et al., 2001). Activation of these receptors affects growth, apoptosis and differentiation in many cell types (Miyazono et al., 2005) and BMP signalling

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promotes pulmonary endothelial cell survival (Teichert-Kuliszewska et al., 2006). The loss of this survival factor may contribute to the emergence of apoptosis resistant endothelial clones that narrow the microcirculation in familial PAH (Teichert-Kuliszewska et al., 2006). Moreover, ALK-1 is expressed in the circulation solely on endothelial cells, and a mutation in ALK-1 causes hereditary hemorrhagic telangiectasia (HHT), and PAH that is clinically indistinguishable from familial PAH (Trembath et al., 2001). Thus, there is compelling evidence for involvement of the TGF-β superfamily of molecules in the pathogenesis of several types of PAH (Newman et al., 2008). Given normally low ET-1 levels in humans, but high ET-1 levels in PAH, we hypothesized that the TGF-β superfamily of molecules would alter ET-1 production by pulmonary microvascular endothelial cells, the cell critical to the pathogenesis of PAH.

2. Materials and methods

2.1. Cells and reagents

Lung-derived normal human microvascular blood vessel endothelial cells HMVEC-LBl (Lonza, Walkersville MD) were cultured in EGM-2MV medium (supplemented with 5% fetal bovine serum, 0.04% hydrocortisone, 0.4% human fibroblast growth factor, 0.1% vascular endothelial growth factor, 0.1% insulin like growth factor, and 1% GA-1000). These cell lines are purified by double staining and flow cytometry and are 90% pure for cells of vascular origin; they express CD31, but do not express podoplonin, a marker for cells of lymphatic origin (HMVEC – LBl Lung Blood MV Endothelial Cell – Technical Sheet, Lonza, February 2008). BMPs 2,4,7 and TGF-β 1 were purchased from Peprotech (Rocky Hill, NJ).

2.2. Experimental techniques

HMVEC-LBl cells (passage 5 only) were grown on 24 well plates until they reached confluence. Twelve wells were used for each experimental condition, including each concentration at each time point. The medium was collected post exposure to the peptides and it was frozen at −70 °C for subsequent measurement of ET-1 levels. At each time point, after aspiration of the medium, the cells were lysed with Reporter Lysis Buffer (Promega) and the protein concentration in the lysate was measured by the BCA method (Pierce, Rockford IL). ET-1 levels in the supernatant were measured with a commercial ELISA kit (Assay Designs, Ann Arbor, Michigan) with antibodies specific for human ET-1. The intra-assay coefficient of variation was 5.8%, with an inter-assay variation of 4.7%. Cross reactivity to ET-2, ET-3 and Big ET was <0.1%. The ET-1 levels we measured in experiments fell within the mid-range of the curve generated by the standard concentrations provided with the ELISA kit – therefore the kit was capable of measuring a reduction in ET-1 levels as compared to CONTROLS, should have occurred in an experiment. The complete medium had undetectable levels of ET-1.

Experimental design: In the first experiment, once the HMVEC-LBl cells were confluent, the complete medium was replaced with complete medium containing 0, 0.156, 0.3125, 0.625, 1.25, 2.5, 5, or 10 ng/ml of TGF-β1, or 0, 1, 10, or 100 ng/ml BMP-2, 4 or 7. The supernatant was then collected and the cells processed as discussed above. To examine the effects at earlier time points, a time curve study of the response to TGF-β (2.5 ng/ml) was performed – at time 0, after the EGM-2MV complete medium was replaced with complete medium containing the TGF-β. One, 4, 8, and 24 h later the supernatant was collected and the cells processed as discussed above. In a third experiment, to study the effects of basal medium without serum and growth supplements, cells were exposed to basal medium containing 0.1% BSA for 16 h, then the medium was removed and the cells were exposed to basal medium with BSA, with or without TGF-β (2.5 ng/ml) or BMP 2, 4, or 7 (100 ng/ml) for 8 h. The supernatant was then collected and the cells processed as discussed above.

2.3. Smad signalling

HMVEC-LBl cells were grown to confluence on 6-well plates in EGM-2MV medium. The cells were then serum starved in basal medium (EGM medium +0.1% BSA) overnight. Next, the medium was replaced with either basal medium, complete EGM-2MV medium, or basal medium containing BMP 2, 4, or 7 (100 ng/ml) or TGF-β (2.5 ng/ml) for 5 min. The cells were then lysed directly in sample buffer (2.5 mM Tris–HCl, pH 6.8, 25 °C, 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% w/v bromophenol blue), homogenized by syringe aspiration through a 26-gauge needle, and separated on a 10% polyacrylamide gel. The proteins were transferred to a PVDF membrane (Pierce, Rockford IL) and blocked in TBST (0.02 M Tris, 0.14 M NaCl, 0.1% v/v Tween) with 5% powdered skim milk. The membranes were incubated overnight at 4 °C with anti-Smad5 (Cell Signalling, Danvers, MA), anti-phosphorylated Smad1/5 (Cell Signalling, Danvers, MA), and anti-GAPDH (Fitzgerald, Concord, MA), all in TBST with 5% BSA. The membranes were then washed in TBST and incubated at room temperature for 1 h with anti-rabbit-HRP (Cell Signalling, Danvers, MA) and anti-mouse-HRP (Pierce, Rockford, IL). After another wash, the membranes were incubated with Western lightning enhanced luminol (Perkin-Elmer, Waltham, MA) and then exposed to photographic film. Density of the bands on the developed film was analyzed using ImageJ software (National Institutes of Health, Bethesda, MD).

In a separate experiment, the time course of Smads 1/5 phosphorylation was studied. The cells were serum starved overnight in endothelial basal media (EBM, Lonza) containing 0.1% BSA. The medium was then replaced with fresh EBM alone or EBM containing 100 ng/ml of BMP 2, 4, or 7, or 2.5 ng/ml of TGF-β. The cells were exposed to the experimental conditions for 15 min, 45 min and 7 h, after which the cells were lysed in sample buffer. The samples were then processed and analyzed as above.

2.4. Statistical methods

Data were expressed as mean ± SD. For each experimental group, at 24 or 48 h, to detect differences in group means, one-way analysis-of-variance was used, followed where appropriate by the Tukey–Kramer multiple comparison test. Two-tailed p values <0.05 are considered significant.

3. Results

3.1. Effect of BMPs on ET-1 levels in complete medium (Fig. 1A)

At the concentrations used, after 24 h of exposure in complete medium, neither BMP-2, BMP-4 nor BMP-7 altered absolute ET-1 levels, normalized ET-1 levels or cell protein levels as compared to controls. Similarly, BMP-2 and BMP-4 did not raise absolute ET-1 levels after 48 h of exposure. However, after 48 h, BMP-2 (10 ng/ml) slightly increased cell protein levels, by 19% over controls ($p<0.05$). By contrast, BMP-4 (1 ng/ml) slightly reduced cell protein levels (12% less than controls) and this resulted in an 18% increase in normalized ET-1 levels versus controls ($p<0.05$). At 48 h, BMP-7, only at a dose of 100 ng/ml, increased absolute ET-1 levels by 32% as compared to control ($p<0.05$), and it also increased ET-1 levels normalized for cell protein by 27% versus controls (Fig. 1A).

3.2. Effect of TGF-β 1 on ET-1 levels in complete medium (Fig. 1B)

TGF-β1 stimulated ET-1 production by the pulmonary microvascular endothelial cells. At 24 h, as compared to controls it significantly increased ET-1 levels at all TGF-β1 concentrations except 10 ng/ml, with a 47% increase at 2.5 ng/ml, and when normalized to cell protein, ET-1 levels were increased at all concentrations except 5 and 10 ng/ml (40% increase at 2.5 ng/ml). Exposure to TGF-β1 did not cause any

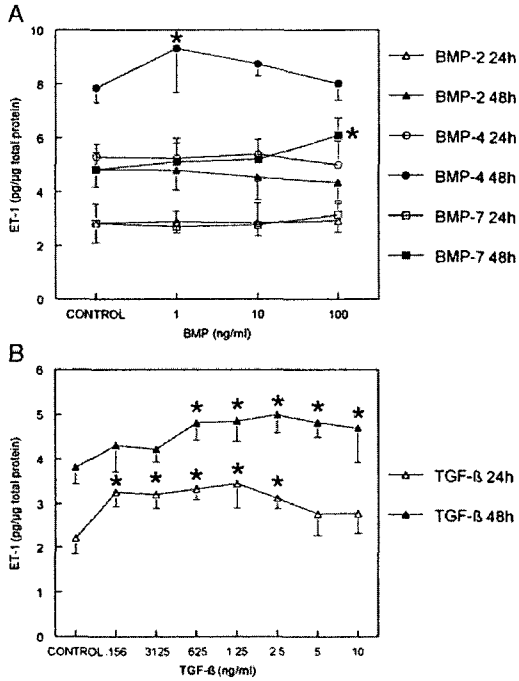

Fig. 1. A: Cell culture medium levels of endothelin-1 normalized to cellular protein levels after 24 and 48 h of exposure to bone morphogenic proteins (BMP) 2, 4, and 7 at various concentrations in complete medium. Bars are group (n = 12) mean ± SD. B: Cell culture medium levels of endothelin-1 normalized to cellular protein levels after 24 and 48 h of exposure to transforming growth factor-beta (TGF-β) at various concentrations in complete medium. *, $p<0.05$ versus respective CONTROL.

alteration in cell protein levels. A similar pattern was observed after 48 h of exposure to TGF-β1, where protein levels were not increased, but ET-1 levels compared to controls were increased at 0.625, 2.5 5 and 10 ng/ml (30% increase at 2.5 ng/ml), and ET-1 levels normalized to cell protein were increased compared to controls at all TGF-β1 concentrations between 0.625 and 10 ng/ml inclusive (31% increase at 2.5 ng/ml).

3.3. Time course of ET-1 stimulation by TGF-β in complete medium (Fig. 2)

As compared to CONTROL, exposure to TGF-β (2.5 ng/ml) in complete medium significantly increased normalized ET-1 secretion at 4, 8, and 24 h. The difference was greatest at 8 h (27% increase), with a smaller difference (15%) at 24 h. TGF-β1 also increased absolute ET-1 levels at those time points, and it slightly (maximum 4%) increased cellular protein levels (data not shown) (Fig. 2).

3.4. Effects of BMPs and TGF-β1 on Smad levels (Fig. 3)

At 5 min, levels of Smad-5 remained unchanged by exposure to BMPs, TGF-β1, or complete medium (Fig. 3, upper). However, after 5 min of exposure in basal medium, BMPs 2, 4 and 7 and TGF-β1 as well as complete medium increased levels of phosphorylated Smad-1/5. Phosphorylation of SMADs 1/5 in basal medium was rapid (Fig. 3 lower). It was apparent at 15 min, and still present at 45 min, with less phosphorylation by BMP-7 at that time. TGF-β, still induced phosphorylation at 7 h.

3.5. Effect on ET-1 levels of BMPs 2, 4, and 7, and TGF-β in basal medium (Fig. 4)

In basal medium, an 8 h exposure to BMPs 2, or 7 or TGF-β significantly increased normalized ET-1 levels as compared to CONTROLS, with TGF-β inducing a 51% increase and BMP-7 a 27% increase. BMP-4 had no effect on ET-1 levels. Absolute ET-1 levels were significantly increased by BMP-7 (24% increase) and TGF-β (20%) as compared to CONTROLS, but not by BMP-2 (data not shown). None of the BMPs affected cellular protein levels, but TGF-β decreased cellular protein by 20%.

4. Discussion

The results of the present studies demonstrate that ET-1 can be modulated by some BMPs and by TGF-β in human pulmonary microvascular endothelial cells in vitro. With TGF-β, this stimulated increase in ET-1 occurs within 4 h, remains at 24 and 48 h and is seen in basal or complete medium. Despite inducing sustained SMADs 1/5 phosphorylation, BMP 2, only in basal medium, induced a slight increase in normalized ET-1 levels, and BMP-4 had no effect in basal medium, while BMP-7 at high doses had stimulatory effects similar to TGF-β at 48 h in complete medium and at 8 hours in basal medium.

An imbalance, with loss of the normal suppressive effects of the BMPs and enhancement of the stimulatory effects of the TGF-β molecules, has been proposed to lead to the vascular abnormalities in PAH (Newman et al., 2008). For example, unlike their normal growth-inhibitory effects seen in controls, BMPs did not inhibit proliferation of pulmonary artery smooth muscle cells derived from patients with idiopathic PAH (Morrell et al., 2001). By contrast, TGF-β1 stimulated proliferation in those same cells. These two effects were not apparent in smooth muscle cells from patients with other causes of pulmonary hypertension. Furthermore, BMPs appear to be essential survival factors for some cell types, preventing apoptosis in pulmonary vascular endothelial cells (Teichert-Kuliszewska et al., 2006). Thus it is essential to characterize the effects of these peptides in the clinically relevant cell population. The pulmonary microvascular endothelial cell appears to be the primary source of the excess ET-1 in PAH (Giaid et al., 1993), although there may be some lesser contribution from other cells in the vessel wall. In this study, we examined cells from individuals without pulmonary hypertension, to examine normal signaling pathways. Previously available commercial microvascular endothelial cell lines were significantly contaminated with endothelial cells of lymphatic origin. The line we used, HMVEC-LBl is vascular

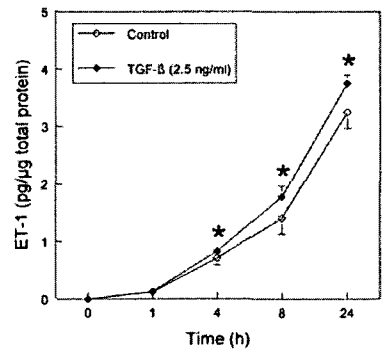

Fig. 2. Cell culture medium levels of endothelin-1 normalized to cellular protein levels after exposure to transforming growth factor-beta (TGF-β, 2.5 ng/ml) for various times in complete medium. Bars are group (n = 12) mean ± SD *, $p<0.05$ versus CONTROL at the same time point.

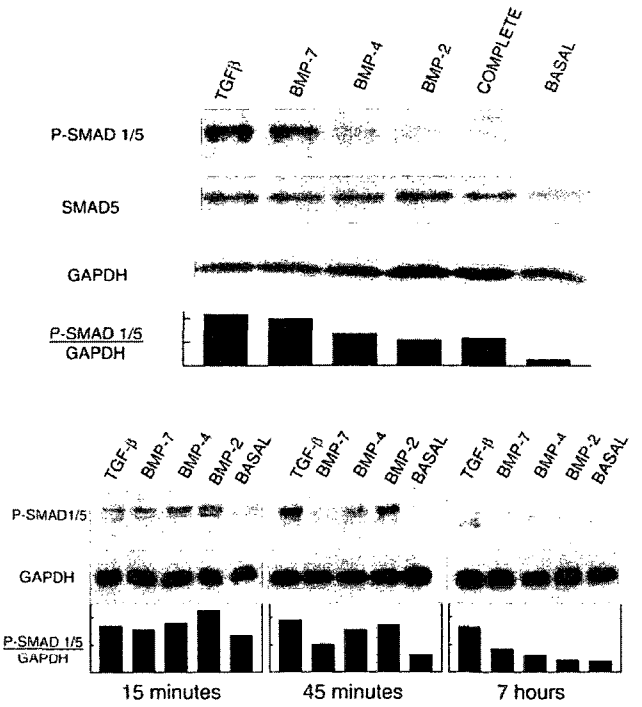

Fig. 3. Upper: Western blot Smad 5 levels and phosphorylated Smads1,5 levels after 5 min exposures to basal medium, complete medium or basal medium containing bone morphogenic proteins (BMPs) 2, 4, or 7 (100 ng/ml) or transforming growth factor-beta (TGF-β) (2.5 ng/ml). A GAPDH loading control is provided. The bar graph presents densitometric analysis of the Western blot. Lower: Western blot phosphorylated Smads1/5 levels after 15 or 45 min or 7 h exposures to basal medium, or basal medium containing bone morphogenic proteins (BMPs) 2, 4, or 7 (100 ng/ml) or transforming growth factor-beta (TGF-β) (2.5 ng/ml). A GAPDH loading control is provided. The bar graph presents densitometric analysis of the Western blot.

in origin. Thus, our results provide novel information about the effects of the TGF-β superfamily of molecules on ET-1 synthesis in these cells most relevant to PAH.

The HMVEC-LBl are extremely dependent on adequate culture conditions, including the presence of complete medium. Although

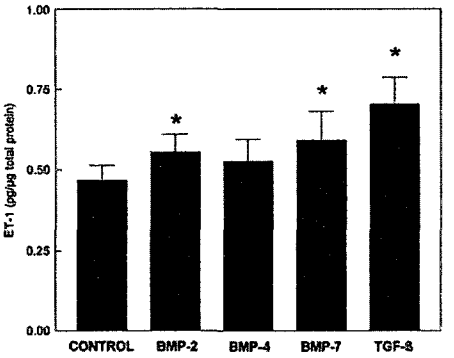

Fig. 4. Cell culture medium levels of endothelin-1 normalized to cellular protein levels after 8 h of exposure to bone morphogenic proteins (BMP) 2, 4, and 7 (100 ng/ml) and transforming growth factor beta (TGF-β, 2.5 ng/ml) in basal medium. Bars are group (n = 12) mean±SD. *, $p < 0.05$ versus CONTROL.

experiments in basal medium represent the ideal situation free of endogenous cytokines, the HMVEC-LBl do not survive well in basal medium for extended periods of time. Therefore, our initial experiments were all performed with complete medium as the control. In that medium, only BMP-7 and TGF-β increased ET-1 levels. The time course experiment (Fig. 2) suggests that ET-1 levels can be stimulated to rise within 4 h of exposure to TGF-β, and that the levels continue to rise as compared to control levels. We chose 24 and 48 h time points for our dose-response experiments, since differences at those times would be more clearly detectable. A previous study has also used the 24 h time point (Castanares et al., 2007). Due to the need for serum starvation prior to onset of the experimental period, the studies using basal medium could not be extended beyond 8 h since cell mortality became an issue. However, in the experiments we performed in basal medium, the same pattern was seen, with BMP-7 and TGF-β increasing ET-1, with a lesser effect for BMP-2. Complete medium clearly stimulates ET-1 levels by itself, and this may reduce the ability to detect earlier ET-1 increases induced by BMP-7, such as were seen in basal medium.

Activation of the BMP receptor, or the type 1 TGF-β receptor ALK-1, results in phosphorylation of Smads 1 and 5 (Yamashita et al., 1996). The effects of BMPs and TGF-β on Smads 1/5 phosphorylation were studied in basal medium. Complete medium induces significant phosphorylation by itself (Fig. 3), likely due to the endogenous cytokine and TGF-β-family molecules present in the growth supplement and fetal bovine serum. Our measurement of Smad levels confirms that the different biologic effect between the BMPs studied

and TGF-β, was not related to abnormal ligand-receptor interactions, or to defective BMP molecules. All the BMPs studied and TGF-β1 caused rapid Smads 1/5 phosphorylation, which would be the predicted signalling reaction for effective receptor-ligand interactions. Also, levels of Smad 5 did not change, indicating that the peptides did not affect the levels available for phosphorylation, but this latter finding would have been important only if we had not demonstrated the phosphorylation event. The time course experiments show that phosphorylation is rapid, within 5 min, and sustained at 45 min. By 7 h it has tapered off for TGF-β or returned to basal levels for the BMPs. The appearance of increased ET-1 levels in the medium takes several hours. The timing of the phosphorylation and secretion events suggests that they are linked, but separated by the delay necessary for *de novo* ET-1 peptide synthesis. In future studies, siRNA inhibition of Smad components or the TGF-β family receptors, as has been reported elsewhere, might help clarify this issue in the cells we studied (Castanares et al., 2007).

We studied the effects of BMPs 2, 4 and 7, which interact with BMPR-II, and TGF-β1 which interacts with TGF-β receptors, but not BMPR-II (Morrell et al., 2001). The experiments in Fig. 1 were not all performed simultaneously, and results for a given molecule should only be compared its respective control, not to other molecules or time points. BMP-2 and 4 did not alter ET-1 levels. BMP-7 stimulated ET-1 production, but only at the highest concentration studied (100 ng/ml). Studies of BMPs as endothelial survival factors have employed concentrations of 200 ng/ml (Teichert-Kuliszewska et al., 2006). It is noteworthy that in a previous study relevant to PAH, all these BMPs demonstrated effects on pulmonary vascular smooth muscle cells at concentration from 1 to 100 ng/ml (Morrell et al., 2001). However, it is also noteworthy that some cells, such as osteoblasts, only increase ET-1 production when stimulated with BMP-7 concentration of 100 ng/ml or higher (Kitten and Andrews, 2001). Thus, the microvascular ECs seem to demonstrate a true response to BMP-7, and it may be that endothelial cells are much less sensitive to BMP effects than other vascular cells such as smooth muscle. Indeed, a previous study of endothelial cells of various origins showed origin-dependent variability in responses to a given BMP, in that case BMP-4 (Kiyono and Shibuya, 2006). It is unknown whether local pulmonary levels of BMP-7 reach these high concentrations *in vivo* in patients with PAH. By contrast, TGF-β did stimulate ET-1 production at most concentrations studied, and these concentrations overlap those that have previously shown biologic effects on pulmonary smooth muscle cells *in vitro* (Morrell et al., 2001). That previous study, using cells from patients with idiopathic PAH, showed a paradoxical response to TGF-β1 as compared to controls, leading the authors to suggest that abnormalities in TGF-β superfamily signalling in PAH might be more widespread than just via BMP receptors, and might also involve TGF-β receptors (Morrell et al., 2001). If this concept is true, then one stimulus for increased ET-1 production in PAH might be via TGF-β.

Our study has several limitations. First, the cells studied were not from patients with PAH. We chose to define "normal" behaviour in these experiments. Second, the cells were studied in isolation, without other vascular cell types being present, and they represent an imperfect simulation of events in a vessel wall. Third, the relevance of *in vitro* findings to the clinical state is still unclear. Nonetheless, our study does show differing biologic effects of the various TGF-β superfamily of molecules, and suggest a potential role for TGF-β1 in controlling pulmonary microvascular ET-1 production *in vivo*. Clinical studies will be needed to confirm the applicability of these findings to PAH.

5. Conclusions

- BMP 2 and 4 have minimal effects on ET-1 production by human pulmonary microvascular cells *in vitro*. BMP-7 at high concentrations stimulates ET-1 production. The interaction of these BMPs with their receptor results in rapid phosphorylation of Smad 1/5, indicating a functional receptor.
- TGF-β stimulates ET-1 production by human pulmonary microvascular cells *in vitro*, and the interaction of TGF-β with its receptor results in phosphorylation of Smads 1/5. This may have clinical relevance in PAH.

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