Functional Characterization of Novel Human Smad8 Isoform Cloned from the Human Lung

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Abbreviations

AA	Amino acid
ActR	Activin receptor
ALK	Activin like receptor kinase
APS	Ammonium persulfate
BMP	Bone morphogenetic protein
BMPR	Bone morphogenetic protein receptor
BRE	BMP responsive element
BSA	Bovine serum albumin
bp	Base pair
cDNA	Complementary deoxyribonucleic acid
CMV	Cyto megalie virus
Co-Smad	Common Smad
Co-IP	Co-Immuno precipitation
dNTP	deoxyribose nucleotide triphosphate
DMSO	Dimethylsulfoxide
ds	Double strand
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylendinitrilo-N,N,N',N',-tetra-acetate
FCS	Fetal calf serum
HRP	Horseradish peroxidase
HSC	Heat shock protein 70
IgG	Immunoglobulin G
IPTG	Isopropyl β-D-thiogalactoside
I-Smad	Inhibitory Smad
kb	Kilo base
kDa	Kilo Dalton
LB	Luria Bertani
LMW	Low molecular weight
Luc	Luciferase
MW	Molecular weight
MCS	Multiple Cloning Site

MIS	Mullerian inhibiting substance
NaOH	Sodium hydroxide
NES	Nuclear export signal
NLS	Nuclear localization signal
OD	Optical density
PCR	Polymerase chain reaction
RLU	Relative luciferase units
RNase	Ribonuclease
R-Smad	Receptor Smad
RT	Room temperature
RT-PCR	Reverse transcriptase PCR
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SS	Single strand
TAD	Transactivation domain
TAE	Tris acetate EDTA buffer
TβR1	Transforming growth factor-β receptor I
TβR2	Transforming growth factor-β receptor II
TEMED	N',N',N'-Tetra methyl diamine
TGF-β	Transforming growth factor-β
Tris	Tris-(hydroxy methyl)-amino methane
UV	Ultra violet

1. Introduction

1.1 Pulmonary hypertension

Idiopathic pulmonary arterial hypertension (IPAH) is associated with structural changes in the pulmonary vasculature ultimately leading to right heart failure if untreated. The changes in vascular structure, also known as vascular remodeling, comprise dilatation and atheroma of elastic arteries, medial hypertrophy, muscularization of arterioles, and intimal proliferation [1-6]. Despite variations in their distribution and severity, these lesions have been included in the description of most forms of pulmonary hypertension, including that associated with Chronic Obstructive Pulmonary Disease (COPD) and in populations living at high altitude [7].

Characteristic of chronic pulmonary hypertension are changes in the structure and function of endothelial cells, smooth muscle cells, and fibroblasts, as well as heterogeneity among cell phenotypes, resulting in vascular remodeling, altered tone, and vasoreactivity [8-11]. This disease is characterized by vascular cell proliferation and obliteration of small pulmonary arteries, which leads to sever pulmonary hypertension and right ventricular failure. Typical morphological appearances include increased muscularization of small arteries and thickening or fibrosis of the intima. The term plexiogenic arteriopathy is also used for this disease because of the existence of plexiform lesions (200 to 400 µm in diameter), which are capillary-like channels adjacent to small pulmonary arteries. A significant breakthrough in understanding of the pathogenesis of IPAH has emerged recently from genomic analysis. IPAH is rare, with an estimated prevalence of 1-2 cases per million, and is twice as common in women as in men [12].

Approximately 6% of all IPAH cases have a known family history of the disease. Linkage studies in families with multiple affected members have mapped the disease locus to a 3cM intercal on chromosome 2q31-32 (locos IPAH) [13]. Examination of candidate genes within this interval led to the identification of

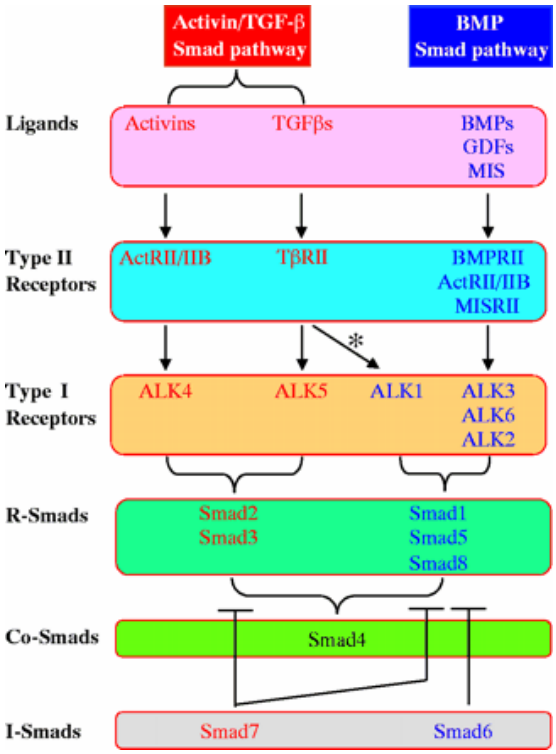
mutations in the Bone Morphogenetic Protein Receptor 2 (BMPRII) gene that encodes a disrupted protein and which tracks with the disease. In reports to date, inactivating heterozygous mutations have been found distributed throughout the BMPRII gene in approx sixty percent of patients with a family history and 26% of so-called sporadic cases of IPAH [14;15]. These mutations include frame shifts, nonsense and missense mutations, splice site variations and deletions that would be predicted to truncate the protein or alter highly conserved regions and interfere with ligand binding or kinase activity [16-23].

The mechanism by which this loss of function leads to the disease is presently the focus of intense research. The precise molecular mechanisms of disease pathogenesis remain to be elucidated but are likely to involve altered BMPRII function. Recent studies also show that compared with cells from control subjects or patients with secondary PH, Pulmonary artery smooth muscle cells from patients with IPAH fail to respond to the growth-suppressive effects of bone morphogenetic proteins (BMPs). Members of BMPs 2, 4, and 7 inhibit vascular smooth muscle cell proliferation and promote apoptosis [14,24;25]. Thus, disruption of BMPRII signaling might permit unrestricted cellular proliferation, providing the basis for vascular remodeling.

1.2 Bone morphogenetic proteins

BMPs were originally identified as molecules that can induce ectopic bone and cartilage formation in rodents [25]. With the exception of BMP-1, a metalloprotease, they are all members of the transforming growth factor β super family of secreted signaling molecules. BMPs are conserved broadly across the animal kingdom, ranging from vertebrates to arthropods to nematodes. In vertebrates, BMPs also play role in dorsoventral patterning of the early embryonic mesoderm and formation of epidermis. In Xenopus, BMP-2, -4, and -7 ventralize early mesoderm and act as negative regulators of neutralization [25-29]. Vertebrate BMPs also have roles in limb development, generation of primordial germ cells, tooth development, and the regulation of apoptosis [24;30-35].

All BMP ligands are translated as precursor proteins, consisting of an amino-terminal pro-region and a carboxyl-terminal ligand. This precursor forms a disulfide-linked homodimer in the cytoplasm, and the pro-region is then cleaved from the ligand. The pro-region disassociates, and the mature ligand is secreted from the cell. How do BMPs elicit such wide biological responses in different biological contexts? This diversity appears to be partly due to intracellular cofactors that participate in BMP signal transduction, as well as crosstalk between BMPs and other signaling pathways.



Aristidis Moustakas et al., JCS 114, 2001.

Figure 1: Signaling specificity in the TGF-β super family: Classification of the mammalian Smad signaling cascade into activin- TGF-β (maroon) and BMP (blue) pathways. Representative examples of mammalian ligands (pink shading), type II receptors (red shading), type I receptor (orange shading), R-Smads (green shading), Co-Smads (bright green shading) and I-Smads (grey shading) are depicted in pathways linked by arrows or signs of inhibition. Bifurcation of the TGF-β pathway at the level of type I receptors towards both TGF-β and BMP Smads is marked by an asterisk. Nomenclature of proteins not detailed in the text are growth and differentiation factors (GDFs), Mullerian inhibiting substance (MIS), activin type II and typeIIB receptor(ActRII/IIB) , TGF-β type II receptor (TβRII), BMP type II receptor (BMPRII), MIS type II receptor (MISRII), activin receptor-like kinases1 to 6 (ALK1-6).

1.3 BMP signal transduction

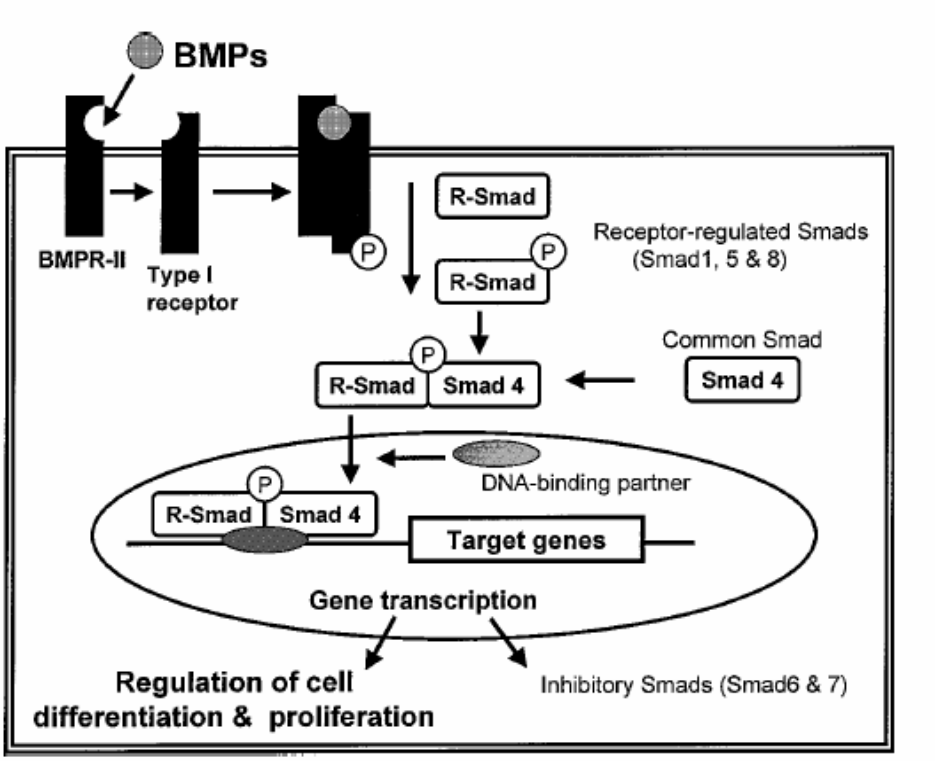
Several BMP genes and their downstream signal transducers are expressed in early mouse embryos before and during the process of gastrulation. These include BMP-2, BMP-4, BMP-5, BMP-7, ALK2, ALK3, ALK6, Smad1, Smad5, Smad4, and Smad8 (Figure 1) [36]. The BMPRII gene encodes the cell surface Type II BMP receptor, and is a member of the Transforming Growth Factor-β (TGF-β) family [36].

BMP receptors signal by forming heteromeric complexes of the two types of receptor serine/threonine kinases. Sequential binding is observed whereby a ligand binds to a Type II receptor, which in turn recruits a Type I, receptor. The intracellular serine/ threonine kinase domain of the Type I receptor then initiates phosphorylation of cytoplasmic transcription factors known as Smads [36-41]. The ligands for the BMPRII receptor comprise BMPs -2, -3 -4,-5, -6, -7, -8, -8b, -10, -15 and -17 (Figure 1).

The ligands induce downstream signaling through phosphorylation of specific receptor-regulated Smad proteins (RSmad 1, 5, and 8) that form a complex with the common mediator Smad4 and translocate to the nucleus to regulate gene transcription (Figure 2). The ligand-induced interaction of R-Smads with activated Type I receptors results in direct phosphorylation of the two distal serines of the C-terminal SSXS motifs by Type I receptor kinases. This interaction is specified by sequences in both the receptor and the R-Smad. In BMP signal transduction, the ligand first binds to its Type I receptor, which in turn activates BMPRII. The activated BMPRII again phosphorylates its Type I receptor by trans-phosphorylation (Figure 2).

The Type I, but not Type II, receptors contain a characteristic SGSGSG sequence, termed the glycine-serine (GS) domain, immediately N-terminal to the kinase domain. The activation of the Type I receptor involves the phosphorylation of its GS domain by the Type II receptor (Figure3). The activated Type I receptor kinase then transduces the BMP signaling into the cell by R-Smad (Smad1, 5 and 8) phosphorylation. L3 loop, a 17 amino acid region that protrudes from the core of the conserved SMAD C-terminal domain. The L3 loop sequence is invariant

among TGF-β and BMP-activated SMADS, but differs at two positions between these two groups which enable them to distinguish BMP and TGF-β Smads. The nine-amino-acid L45 loop in the Type 1 receptor kinase domain is the main determinant of receptor signaling and Smad binding specificity, and interacts directly with the L3 loop in the MH2 domain of the R-Smad [41;42]. Sequences downstream from the L3 loop also contribute to receptor-binding specificity (Figure 3). The activated or phosphorylated receptor Smads then bind to a co-Smad (Smad4), causing the whole complex to move towards the nucleus. The R-mad/Co-Smad complex then recruits more cofactors in the nucleus to bind the BMP-inducible gene promoters to start transcription (Figure 2) [43].



Julian W. Strange et al., Clinical Science, 102 (2002).

Figure 2: BMP signaling

1.4 Smad proteins

Major families of downstream targets of the TGF-β super family are Smad proteins. The first intracellular mediator of TGF-β signaling, mothers-against-decapentaplegic (MAD) was identified in Drosophila, quickly followed by the discovery of orthologs in worm and vertebrates which were named “Smad”. [44-46].

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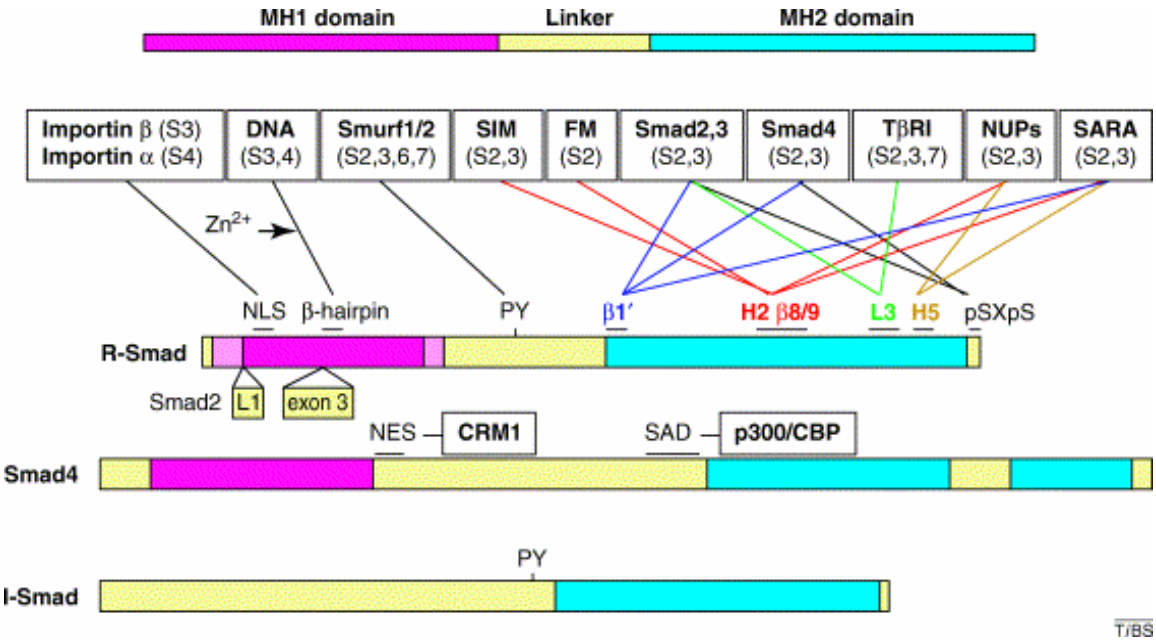


Peter Ten Dijke et al., TiBS 29, 2004.

Figure 3: Structure of the R-Smads (Smad2 and Smad3), Smad4 and the I-Smads (Smad6 and Smad7): The MH1 (dark pink) and MH2 (cyan) domains are conserved among Smads. Two regions that are conserved among R-Smads but not other Smads are indicated by pale pink boxes. Non-conserved regions (including the linker) are shown in yellow. Interactions between partner proteins and particular structural motifs on Smad2 and Smad3 (some of which are mutually exclusive) are indicated. The motifs shown are the b10-strand (b10, dark blue), a-helix 2 (H2, red), b-strands 8 and 9 (b8/9, red), L3 loop (L3, green), a-helix 5 (H5, brown), and pSXpS, the phosphorylated C-terminal SxS motif of the R-Smads (black). Both the MH1 and the MH2 domain interact with transcription factors, but only interactions with transcription factors containing a SIM or FM are indicated. FM, Fast or FoxH1 motif; MH, Mad homology; NES, nuclear export signal; NLS, nuclear.

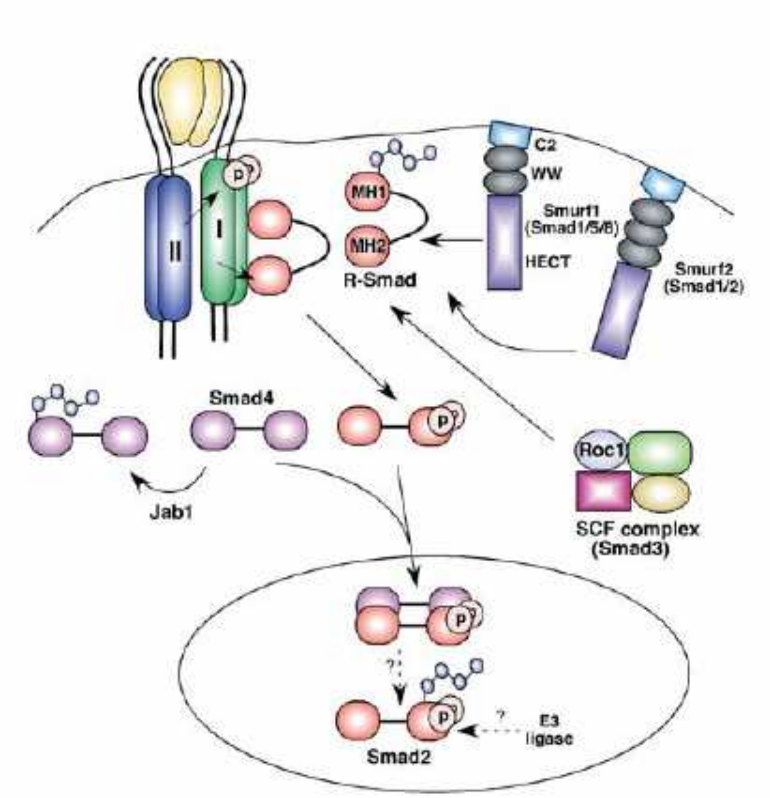
Subsequently, several genes in Caenorbabditis elegans (Sma2, Sma3, and Sma4) have been shown to be homologous to Mad and mutations in these genes result in a small phenotype of larval stage embryos. Several vertebrate Smad genes (Smad-Mad) have been characterized and different Smad proteins transduce signals of different families of the TGF-β -related proteins [44].

To date, there are eight vertebrate Smads identified: Smad1 to Smad8. The Type I receptors specifically recognize the Smad subgroup known as receptor-activated Smads (R-Smads) [45;47-50]. These include Smad2 and Smad3, which are recognized by TGF-β and Activin receptors, and Smad 1, 5, and 8, recognized by the BMP receptors. The R-Smads consist of two conserved domains that form globular structures separated by a linker region (Figure 3) [51]. The N-terminal MH1 domain has DNA-binding activity whereas the C-terminal MH2 drives translation into the nucleus and has transcription regulatory activity. The C-terminal region also contains a serine-rich domain known as SSXS motif, which is phosphorylated by the receptors (Figure 3). The linker appears to keep these two domains (MH1 and MH2) from a mutually inhibitory interaction before activation by phosphorylation. Phosphorylation of the R-Smads leads to their activation, Co-Smad interaction, and accumulation in the nucleus [51].

Receptor-Smads are directly phosphorylated by the activated Type I receptors. The structure of the MH2 domain comprises a central β sandwich, capped on one end by a three-helix bundle and on the other end by a collection of three surface loops and two auxiliary α-helices. In the crystal structures of the MH2 domain from the unphosphorylated R-Smads, the C-terminal 10 residues, including the characteristic SSXS motif at the extreme C terminus, are completely flexible and disordered. Phosphorylation destabilizes Smad interaction with Smad anchor for receptor activation (SARA), allowing dissociation of Smad from the complex and the subsequent exposure of a nuclear import region on the Smad MH2domain. In addition, R-Smad phosphorylation augments its affinity for Smad4. The association of these two proteins nucleates the assembly of transcriptional regulation complexes.

1.5 Inhibitory Smads (I-Smads) as adaptors for Smurfs

The regulation of cellular processes requires the activation of specific signaling pathways. However, equally important is the down-regulation of the signal. In most cell types, the expression of I-Smads (Smad6 and Smad7) is low at the basal state. Once the cells are stimulated with BMP or TGF-β, expression of inhibitory Smads is induced [45;47-50;59;60]. After their expression, the inhibitory Smads play a pivotal role in regulating the signaling by a feedback mechanism, binding directly to ser/thr Kinase receptors and thereby blocking R-Smad access to the receptor.



Luisa Izzi et al., Oncogene (2004)

Figure 4: Ubiquitin-dependent degradation of Smads: The ubiquitin–proteasome pathway regulates both the basal level of Smads as well the turnover of Smads upon the activation of the signaling pathway. Smad degradation is mediated at least in part by E3 ligases including Smurf1, Smurf2, and SCF/Roc1

The demonstration that I-Smads bind the Smurf (Smad ubiquitin regulatory factors) family of E3 ligases revealed an additional mechanism whereby I-Smads can interfere with TGF-β signaling (57, 58, 59).Specifically, I-Smads can function as adaptors to recruit Smurfs to the receptor complex and thereby mediate receptor degradation and down-regulation of TGF-β signaling [61;62]. The expression of Smad7 is regulated by a number of extra cellular signals, and both TGF-β and BMPs have been shown to increase Smad7 protein levels, particularly in the nucleus where Smad7 is preferentially localized. Smurf2 resides in the nucleus in unstimulatd cells; thus, the increase in Smad7 protein levels results in the association of Smad7 with Smurf2 [63;64]. This interaction is mediated by the PY motif in Smad7 and the WW2/WW3 domains of Smurf2.

The Smad7/Smurf2 complex is then exported from the nucleus to the cytoplasm, where Smad7 then recruits Smurf2 to the TGF-β receptor complex at the cell surface (Figure 4). Once bound to the receptor complex, the Smurfs ubiquitinate Smad7 and cause degradation of both Smad7 and the receptor complex. The mechanism of Smad6 and 7 associations with Smurf1 and/or Smurf2 to target either TGF-β or BMP receptor complex turnover has not been resolved, but it may be that different I- Smad/Smurf combinations serve similar functions in different cell types or tissues. An additional level of complexity in Smad/Smurf-dependent regulation of TGF-β signaling was recently revealed with the demonstration that Smad7 serves to protect Smad7 from Smurf1-mediated ubiquitination [65].

One of the key domains for the regulation of TGF-β or BMP signaling appears to be PY motif, as this domain helps the regulatory and inhibitory Smads binding to Smurfs, thereby undergoing degradation of major signaling molecules. So the presence of this domain in any signal transducer could cause big changes in the BMP and TGF-β signal transduction. In the present study, we identified and characterized in humans a novel isoform of Smad8 that has this special motif (PY) [51]. Here, we analyzed the functional role of new Smad8 isoform in the regulation of both BMP and TGF-β signaling.

1.6 Smad8

To date, eight human homologues of the Smad genes have been identified and are classified into three distinct classes based on their structures and biological functions. The first category consists of pathway-restricted or receptor-regulated Smads (R-Smads): Smad1, Smad5, and Smad8, which are involved in bone morphogenetic protein (BMP) signaling and Smad2 and Smad3, which are TGF-β/actin pathway restricted. The Smad8 gene, which displays a high degree of homology to the Smad1 and Smad5 genes, was originally described as MADH6 in human, often referred to as Smad9 and currently listed as MADH9 in EnsEMBL at the genomic location 35220321 to 35292902 bp on chromosome 13.

According to previous publications, human Smad8 contains 430 aa, lacking exon3 which codes for 37 amino acids. This molecule is shown to be phosphorylated by ALK2, ALK3, and ALK6 upon stimulation with BMP ligands [52-57]. The phosphorylated Smad8 moves towards the nucleus along with Smad4 to activate transcription of BMP responsive genes. Smad8 has an isoform known as Smad8B. This isoform does not possess an SSXS motif; therefore, it cannot be phosphorylated. Smad8B is also known to inhibit BMP signaling by inhibiting Smad8 by direct interaction and further degradation [58]. No reports currently exist regarding the expression and function of the full length of human Smad8. Therefore, in this study we analyze the functional characterization of the full length of Smad8, which codes for 467 aa.

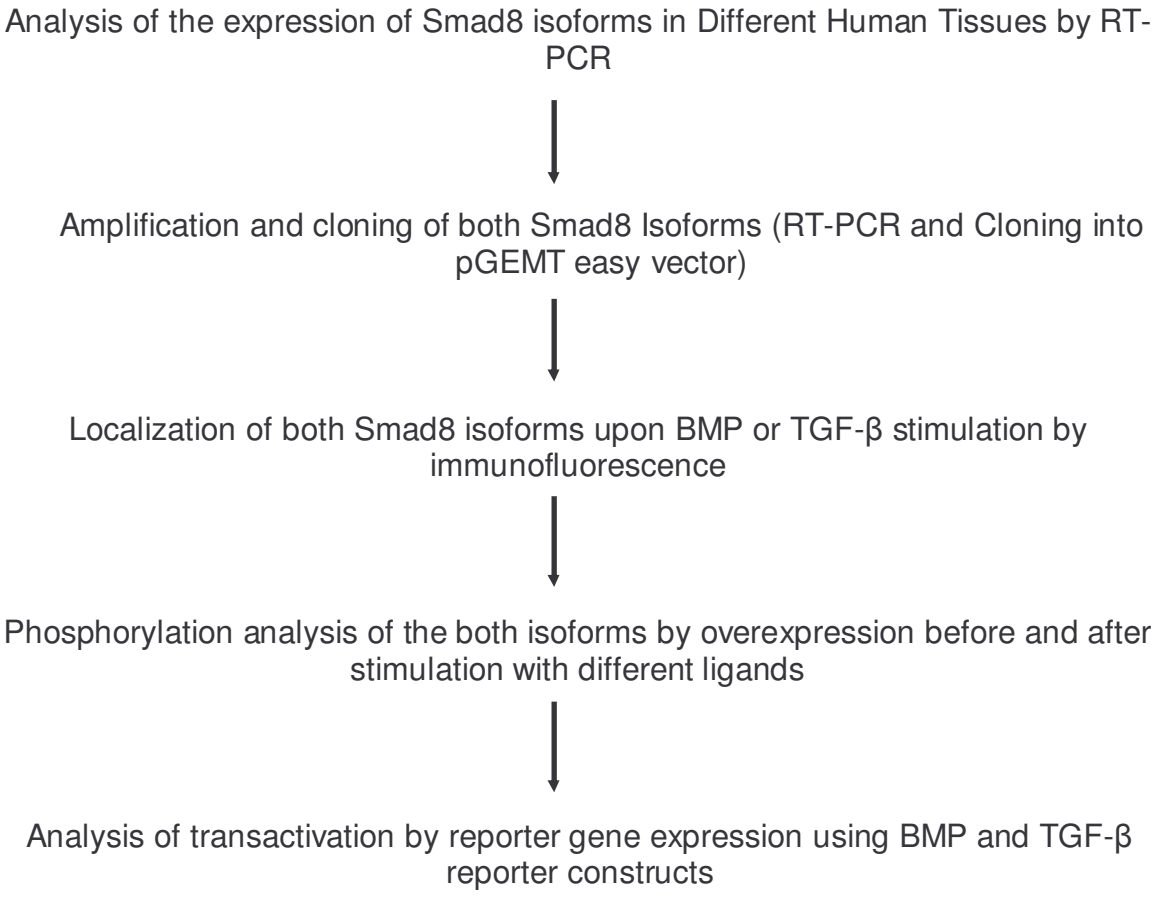
1.7 Aim of the work

Mutations in BMPRII and its reduced expression are discussed to be a cause for idiopathic pulmonary arterial hypertension. Smad 1, 5 and 8 are the major signaling molecules for BMP ligand. There are no detailed reports for expression patterns of BMP and TGF-β signaling molecules (receptors and Smads) during IPAH. In this study we analyzed the expression of Smads and TGF-β and BMP receptor expression in IPAH. During expression analysis, we discovered a novel Smad8 isoform expressed in the lung. As already described Smad8 isoform is called as Smad8B, we call this new Smad8 isoform as Smad8C.

Thus, in the present study we addressed the following aims:

- Characterization of expression of BMP signaling molecules in the lungs of IPAH and control patients
- Cloning, expression and functional characterization of human Smad8 isoforms

1.8 Experimental Design (FLOWCHART)



2. Materials and Methods

2.1 Bacterial strains and vectors

2.1.1 Bacterial strains

DH5α bacterial strain from Invitrogen was used for plasmid transformation. The genotype of the strain is F-80*lac*Z.M15. (vlacZYA-argF) U169 *rec*A1 *end*A1 *hsd*R17 (rk-, mk+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1.

2.1.2 Vectors

2.1.2.1 pGEM®-T Easy vector

The vector pGEM®-T Easy (Promega, Madison USA) was used for the cloning and sequencing of PCR products. The vectors are prepared by cutting Promega's pGEM®-T Easy Vectors with EcoRV and adding a 3´ terminal thymidine to both ends. These single 3'-T overhangs at the insertion site greatly improve the efficiency of ligation of a PCR product into the plasmids by preventing recircularization of the vector and providing a compatible overhang for PCR products generated by certain thermo stable polymerase. These polymerases often add a single deoxyadenosine, in a template-independent fashion, to the 3'-ends of the amplified fragments. The vector contains ColE1 ori for the replication in E coli, an Ampicillin-resistance gene for antibiotic selection, f1 ori for single-strand DNA production, and LacZ gene encoding β-galactosidase, which provides the possibility for blue/white color selection of recombinant clones. A multiple cloning site (MCS), T7, and SP6 RNA polymerase promoters for DNA sequencing are also present.

2.1.2.2 pCDNA3.1- vector

The vector pCDNA3.1- (Invitrogen) was used in the experiments for the cloning and expression of PCR products. Similar to pGEM-T easy, pCDNA3.1- also contains ColE1 ori, f1 ori, and MCS. The difference is that pCDNA3.1 – contains a strong mammalian CMV promoter for in vivo expression of the cloned PCR product, whereas pGEM-T easy is just a cloning vector to store the cDNA of any gene.

2.1.2.3 pCMV-2B vector

The pCMV-2B vector was used for tagging the protein of interest with an N-terminal FLAG epitope. This vector contains ColE1 eukaryotic origin of replication, f1 bacterial ori, Kanamycin resistance gene, and the FLAG epitope sequence followed by a multiple cloning site (MCS).

2.2 Oligonucleotides

The primers were designed against the sequences already published in NCBI. The oligonucleotides were designed by an Oligo MS-DOS program and produced by Qiagen, Germany, at the synthesis scale of 0.01 µmol.

2.2.1 Oligonucleotides for sequencing of the plasmids

pGEM®-T Easy vector

T7 Forward: 5’ TAATACGACTCACTATAG 3’

SP6Reverse: 5’ ATTTAGGTGACACTATAGAA 3’

pcDNA3.1+ vector

T7 Forward: 5’ TAATACGACTCACTATAG 3’

3.1 reverse: 5’ TAGAAGGCACACTCGAGG 3’

pCMV-Flag vector

Forward: 5’ AGTGTTACTTCTGCTCTAAAAGCTGC 3’

Reverse: 5’ CACTGCATTCTAGTTGTGGTTTGT 3’

2.2.2 Oligonucleotides for PCR reaction

(Recognition sequence of restriction enzymes in the primers are underlined)

To clone Human Smads into pGEMT-Easy vector and also to analyze their expression by semi quantitative RT-PCR

Smad1

Forward: 5’ GGAGACAGCTTTATTTCACCATATC 3’

Reverse: 5’ CAATAGTTTTCCAGAGGCAGATG 3’

Smad2

Forward: 5’ GGGAGGTTCGATACAAGAGGCT 3’

Reverse: 5’ GGACCACACACAATGCTATGACA 3’

Smad3

Forward: 5’ AGCCATGTCGTCCATCCTG 3’

Reverse: 5’ CTTCTTCCTTGACAACAATGGG 3’

Smad4

Forward: 5’ TTCACTGTTTCCAAAGGATCAAAA 3’

Reverse: 5’ GTATATCTGGGGGGTTTTGT 3’

Smad5

Forward: 5’ CTGTTCTTTCGGTAGCCACTGAC 3’

Reverse: 5’ GCATTATGAAACAGAAGATATGGGG 3’

Smad7

Forward: 5’ GACTTCTTCATGGTGTGCGG 3’

Reverse: 5’ TAGTTTGAAGTGTGGCCTGCTC 3’

Smad8 (to amplify full length of both Smad8 isoforms)

Forward (P1): 5’ GGCCTCTTATGCACTCCACC 3’

Reverse (P4): 5’ GGAAATGCAGCTTAAGACATGAC 3’

Smad8 (N-terminus of Smad8)

Forward (P1): 5’ GGCCTCTTATGCACTCCACC 3’

Reverse (P3): 5’ CTATGAGCACACTTCGGGAG 3’

Smad8 (C-terminus of Smad8)

Forward (P5): 5’ GAAGCCTCTGAGACCCAGAGTG 3’

Reverse (P4): 5’ GGAAATGCAGCTTAAGACATGAC 3’

Smad8C (Forward primer, P2 inside the exon 3 of Smad8)

Forward (P2): 5’ CTCCCGAAGTGTGCTCATAG 3’

Reverse (P4): 5’ GGAAATGCAGCTTAAGACATGAC 3’

2.2.3 Primer sequences to amplify human BMP and TGF-β receptor cDNAs by RT-PCR

ALK1

Forward: 5’ CATAGTCGACTTGAATCACTTTAGGC 3’

Reverse: 5’ ATATGATATCCACCATGACCTTGGGCT 3’

ALK2

Forward: 5’ GTGACCAAGAGCCTGCATTAAGTTG 3’

Reverse: 5’ CTGGACAATGACAACAACGTCAAATC 3’

ALK3

Forward: 5’ GCAAGACCAATTATTAAAGGTGACAG 3’

Reverse: 5’ CTAGAGTTTCTCCTCCGATGGTTTAAC 3’

ALK5

Forward: 5’ CTACTGTAAAGTCATCACCTGGCC 3’

Reverse: 5’ GTACAAGATCATAATAAGGCAGTTGG 3’

ALK6

Forward: 5’ CTTCCTTGATAACATGCTTTTGCG 3’

Reverse: 5’ GAAGAGTACCTGTTGGCTTTCTGCAG 3’

BMPRII (to amplify both short and long form of BMPRII)

Forward: 5’ CCCATATTTCTTTTCTTTGCCCTCC 3’

Reverse: 5’ GAAAACATTTCACAGACAGTTCATTCC 3’

2.2.4 Human Smads in pCMV-2B, Flag epitope tagged vector

Smad1

Forward (BamHI): 5’ GGATCCAATGTGACAAGTTTA 3’

Reverse (HindIII): 5’ AAGCTTTTAAGATACAGATGA 3’

Smad4

Forward (BamHI): 5’ GGATCCGACAATATGTCTATT 3’

Reverse (HindIII): 5’ AAGCTTTCAGTCTAAAGGTTG 3’

Smad8 and Smad8C

Forward (HindIII): 5’ AAGCTTCACTCCACCACCCCCATC 3’

Reverse (XhoI): 5’ CTCGAGATTAGACACTGAAGAAAT 3’

2.2.5 Human Smads into pCDNA 3.1- vector

Smad1

Forward (BamHI): 5’ GGATCCATGAATGTGACAAGT 3’

Reverse (HindIII): 5’ AAGCTTTTAAGATACAGATGA 3’

Smad8 and Smad8C

Forward (BamHI): 5' GGATCCATGCACTCCACCACC 3'

Reverse (HindIII): 5' AAGCTTTTAAGACACTGAAAG 3'

2.3 Enzymes

2.3.1 Platinum taq DNA polymerase (Invitrogen)

Platinum® *Taq* DNA Polymerase High Fidelity is an enzyme mixture composed of recombinant *Taq* DNA polymerase, *Pyrococcus species* GB-D polymerase, and Platinum® *Taq* Antibody. *Pyrococcus species* GB-D polymerase possesses a proofreading ability by virtue of its 3' to 5' exonuclease activity. An anti-*Taq* DNA polymerase antibody complexes with and inhibits polymerase activity. Activity is restored after the denaturation step in PCR cycling at 94°C, thereby providing an automatic hot start for *Taq* DNA polymerase in PCR.

2.3.2 Improme reverse transcriptase (Promega)

The ImProm-II™ Reverse Transcription System is a convenient kit that includes a newly formulated reverse transcriptase and an optimized set of reagents designed for efficient synthesis of first-strand cDNA in preparation for PCR amplification. The components of the ImProm-II™ Reverse Transcription System can be used to reverse transcribe RNA templates starting with either total RNA, poly(A) +mRNA or synthetic transcript RNA.

2.3.3 Restriction endonucleases

All restriction endonucleases were obtained from Promega Corporation, USA. Their activity was optimized according to buffers provided by the company.

The characters of restriction endonucleases were:

Enzyme	Sequence	Buffer
BamHI	G^GATCC	B
XbaI	T^CTAGA	H
HindIII	A^AGCTT	B
Xho1	C^TCGAG	H

2.3.4 T4 DNA ligase

T4 DNA ligase was purchased from Promega, USA. This enzyme catalyzes formation of a phosphodiester bond between the 5' phosphate of one strand of DNA and the 3' hydroxyl group of the other. This enzyme is used to covalently link or ligate fragments of DNA together. Most commonly, the reaction involves ligating a fragment of DNA into a plasmid vector.

2.4 RNA isolation from mammalian cells and tissues

A549 cells were washed twice with PBS, and then the protocol was followed for RNA preparation with a Qiagen RNA Mini/Midi Kit. The quality of the RNA was measured in a UV spectrophotometer by taking a 260/280 ratio. For RNA extraction from IPAH lungs, frozen lung tissue samples were collected from Medical Clinic II, University of Giessen. The patient details were listed in Table1. These tissues were homogenized with liquid nitrogen, mortar and pistol. The extracts were lysed, according to the protocol from Qiagen midi prep RNA extraction kit. The quality of the RNA was measured in a UV spectrophotometer by taking a 260/280 ratio. Human tissue RNA panel and human total embryonic RNA were purchased from R&D Systems, Germany.

Table 1: Patient details

Age	Sex	Disease type
53	M	Donor
36	F	Donor
43	M	Donor
49	F	Donor
28	M	Donor
48	F	Donor
42	F	IPAH
42	F	IPAH
29	M	IPAH
52	F	IPAH
44	M	IPAH
19	F	SPH

2.5 Reverse transcriptase polymerase chain reaction (RT-PCR)

2.5.1 Complementary DNA synthesis by reverse transcriptase

Reverse transcriptase reaction was conducted by Improme Reverse transcriptase from Promega, USA. 1µg of RNA was used for each RT reaction. 10mM oligo dT was added to the RNA in 5µl reaction with DEPC water and heated at 70°C for 5 min. The reaction tube was snap-chilled on ice after the time duration to allow the oligo dT to anneal to the poly A tail of the RNA before the RNA secondary structure reformed. A master mix containing buffer, Mgcl2, dNTPs, RT, and RNAase inhibitor was added to the RNA-Oligo dT mixture. The tubes were kept in a PCR machine and programmed as follows:

Reaction	**Temperature**	**Time**
Linearization of RNA	25°C	5min
cDNA synthesis	42°C	1hr

RT reaction components (for 20µl reaction)

RT reaction component	Volume
5x buffer	4 µl
MgCl2 (25 mM)	2 µl
dNTPs (10 mM)	1 µl
RNase inhibitor	1 µl (10 units)
Reverse transcriptase	1 µl (5 units)
RNA-Oligo dT mix	5 µl
DEPC water up to	20µl

2.5.2 Polymerase chain reaction

The polymerase chain reaction (PCR) allowed amplification of DNA fragments, due to the repetitive cycles of DNA synthesis. The reaction used two specific oligonucleotides (primers), which hybridized to sense and antisense strands of the template DNA; four deoxyribonucleotide triphosphates (dNTPs); and a heat-stable DNA polymerase. Each cycle consisted of three reactions that took place under different temperatures. First, the double-stranded DNA was converted into two single strands (denaturation at 94°C), which functioned as templates for the synthesis of new DNA. Second, the reaction was cooled (50-60°C) to allow the annealing (hybridization) of primers to the complementary DNA strands. Third, DNA polymerase extended both DNA strands at 72°C (DNA synthesis), starting from the primers. Annealing temperature, product length and extension time for each primer used were given in Table-2.

Table 2: List of annealing temperatures and extension times for PCRs

PCR product	Product length	Annealing temp	Extension time
Smad1	1.4 kb	56°C	2.5 min
Smad2	1.5 kb	57°C	2.5 min
Smad3	1.4 kb	56°C	2.5 min
Smad4	1.6 kb	58°C	3 min
Smad5	1.5 kb	58°C	2.5 min
Smad7	1.4 kb	55°C	2.5 min
Smad8	1.4 kb	61°C	2.5 min
ALK2	1.7 kb	57°C	2.5 min
ALK3	1.7 kb	57°C	2.5 min
ALK6	1.7 kb	57°C	2.5 min
TβRII	1.0 kb	58°C	2 min
β-Glycon	0.5 kb	58°C	1 min
Endoglin	1.0 kb	57°C	2 min
BMPRII	3 kb	56°C	4 min
ALK1	0.5 kb	55°C	1 min

Steps during the PCR program

PCR step	Temperature	Time duration
1st denaturation	95°C	2min
2nd denaturation	95°C	1min
Annealing	Table 1	1min
Extension	72°C	Table 1
Cycles (35)	---	---
Final extension	72°C	10min

After amplification, PCR products (10 µl) were electrophoretically analyzed in a 1% agarose gel with 0.2 µg /100 ml ethidium bromide, and purified if required.

PCR reaction (per 50 µl)

PCR reaction component	Amount
Template DNA	10 ng
Forward primer	10 pmole
Reverse primer	10 pmole
40 mM dNTP mix	1µl
10 x PCR buffer	5µl
Platinum Taq polymerase	0.25 µl
Water to 50µl	38.75µl

2.5.3 DNA electrophoresis and purification from agarose gel

The DNA sample was mixed with loading buffer and loaded onto a 1% agarose gel. Electrophoresis was performed for 45-60 min with 5 V/cm. (Biorad, electrophoresis apparatus, USA). The negatively charged DNA migrated from the cathode (-) to the anode (+). To visualize DNA, the gel was treated with ethidium bromide (0.5µg/ml), which intercalated between the bases of DNA double strands, forming a complex fluorescent under UV light. The size of DNA fragments was determined by a DNA molecular weight standard. The composition of the DNA sample loading buffer is given below.

Loading buffer final concentration

Component of the buffer	Final concentrations
Bromophenol blue	0.01 g /100 ml (0.01%)
Glycerol	40 ml /100 ml (40%)
10 x TAE buffer	10 ml / 100 ml (1x)

The corresponding DNA fragment was excised from the gel and purified using the QIAEX II kit (Qiagen, Germany). Three volumes of binding and solubilization buffer (QX1) and 10µl QIAEX II solution were added to 1 volume of

gel. To extract the DNA from the agarose gel the sample was incubated at 50°C for 10min with occasional mixing. The suspension was carefully applied to the column and centrifuged at 20800 G for 30s. After centrifugation the column was washed once with QX1 buffer and once with PE buffer. After removal of the last washing buffer, the column was completely dried by spinning the column at room temperature at high speed for 1min. After the drying procedure 20µl of H_2O was applied and column was incubated for 5min at room temperature. The DNA was eluted by centrifugation of the column at 20800 G for 1min. The supernatant, which contained the DNA fragments, was collected into a new tube.

2.6 Cloning

2.6.1 Ligation

The purified DNA fragments were ligated into the linearized plasmids by T4 ligase. The ligation reaction was incubated overnight at 16°C and then transformed into competent E.Coli cells. The components of the ligation reaction are given below.

Ligation mixture

DNA ligation component	Amount
DNA fragment	100 ng
Linearized plasmid	35 ng
2xligase buffer	5 µl
T4 DNA ligase	1 µl
DD water to	Up to 10µl

2.6.2 Isolation of plasmid DNA

The maxi-preparation of plasmid DNA was performed using a Maxi Prep Kit (Qiagen, Germany), according to the manufacturer's instructions. The transformed E.Coli DH5α cells were cultured in 250 ml LB medium to a density of 109 per ml (OD of 1 - 1.5 at 600 nm). The cells were pelleted by centrifugation at 4℃, 5860 g (6000 rpm, GSA rotor) for 30 min. The pellet was re-suspended in 10 ml of buffer P1, which contained 100 µg/ml of RNase. Then 10 ml of buffer P2 (with NaOH and SDS for bacterial lysis) was added and mixed gently four to six times (the mixture was not vortexed, to avoid shearing of genomic DNA). After 5 min incubation at RT (longer incubation could lead to irreversible denaturation of plasmid DNA), 10ml of buffer P3 was added for neutralization of the solution. The solution was filtered with the use of the column filters provided in the kit. Buffer ER was added to clear antitoxins from the filtrate. The filtrate was carefully applied to the column, which was equilibrated with 10 ml of buffer QN. When the lysate has been completely run by gravity flow through the column, it was washed twice with 20 ml of buffer N3 to remove single-stranded DNA, RNA, and all other impurities such as proteins, metabolites, polysaccharides, and NTPs. Afterwards, the double-stranded plasmid DNA was eluted with 15 ml of buffer EB and precipitated by adding 10.5 ml of isopropanol. Plasmid DNAs were pelleted by centrifugation at 4℃, 27000 g (5000 rpm, SS34 rotor) for 30 min. The DNA pellet was washed with 70% ethanol to remove salts, air-dried for 30 min, and dissolved in 200 µl H_2O. To determine the DNA concentration and the presence of protein in the probes, the OD at 260 nm (DNA) and 280 nm (protein) was measured. The prepared plasmids were checked by restriction analysis as described above.

2.6.3 Restriction digestion

The DNA fragments (Plasmid and PCR products) were restriction digested, following protocol. The mixtures were incubated at optimum temperatures for maximum activity of the specific enzymes. Reagents used in the restriction digestion reaction are given below.

Restriction digestion reaction

Restriction digestion component	Volume (20 µl)
Plasmid	5 µg / 5µl
Enzyme	1 µl
Buffer (10x)	2 µl
DD Water to 20 µl	12 µl

2.6.4 Construction of the Smad1, Smad8, and Smad8C in pCDNA 3.1 expression vector

Smad1, Smad8, and Smad8C in pcDNA 3.1- vector were cloned using oligonucleotides. Sub-cloning of Smad1, Smad8, and Smad8C fragments from pGEMT-Easy vectors to pcDNA 3.1- vector was done using BamH I and Hind III restriction enzymes. These sites were introduced into the fragments and then cloned into pGEMT-Easy by PCR from the original pGEMT-Easy vector as a template. The pGEMT-easy vector with restriction sites and the fragments were restriction digested with the enzymes and ligated into pCDNA3.1 after purification from the agarose gel using Qiagen gel purification columns followed by transformation into competent DH5α E.Coli. The colonies were screened for positive clones and sequenced for confirmation of the sequences.

2.6.5 Construction of the Smad1, Smad8, and Smad8C in N-terminal FLAG-tagged pCMV-2B eukaryotic expression constructs

Smad1, Smad8, and Smad8C in N-terminal FLAG-tag vector were cloned using oligonucleotides. As Smads has the phosphorylation domain (SSXS) on the C-terminus, we took N-terminal fusion, which might not disturb the activity of the receptor Smads. Sub-cloning of Smad1, Smad8, and Smad8C fragments from pGEMT-easy vector to pCMV-2B vector was conducted with the use of BamH I and Hind III restriction enzymes for Smad1 and Hind III and Xho1 for Smad8 and Smad8C. These sites were introduced into the fragments and then

cloned into pGEMT-Easy by PCR from the original pGEMT-easy vector as a template. The pGEMT-easy vector with restriction sites and the fragments were restriction digested with the enzymes and ligated into pCMV-2B after purification from the agarose gel, using a gel purification columns (Qiagen) followed by transformation into competent DH5α E.Coli. The colonies were screened for positive clones and sequenced for confirmation of the inframe sequences.

2.6.6 Preparation of competent E.coli

A single bacterial colony from the E.Coli DH5α glycerol culture was cultured in 5 ml LB medium at 37°C overnight. The following day, the bacterial suspension was diluted into 500 ml LB medium and kept for shaking in a 37°C shaker incubator until the OD reached to 0.3-0.4 at 550 nm (3-6 h). The suspension was centrifuged at 4°C, 5000 g for 15 min. The pellet was re-suspended in 100 ml (1/5 vol) of ice-cold 50 mM $CaCl_2$ and kept on ice for 5 min. The cells were again centrifuged at 4°C, 5000 G for 15 min. The pellet was re-suspended in 25 ml (1/20 vol) of 50 mM $CaCl_2$.

The cells were again centrifuged at 4°C, 5000 g for 15 min, and the pellet was re-suspended in 5 ml of 50 mM $CaCl_2$ in 20% glycerol. Suspension was divided into 50 µl aliquots and stored at -70°C. The competence of the bacterial cells was checked by the transformation of an Ampicillin or Kanamycin resistant plasmid.

2.6.7 Luria Bertani medium (LB)

LB medium in the form of dehydrated mixture was purchased from Invitrogen, USA; 25g of the mixture was mixed in 1000ml of distilled water and autoclaved.

2.6.8 Transformation of E.coli

Transformation of E.coli was performed with the heat shock method. The ligation mixture or the plasmid DNA was gently mixed with one aliquot of the competent cells and incubated at 4℃ for 30 min. Then the mixture was heated to 42℃ for exactly 1 min, followed by immediate cooling on ice. Thereafter, the bacterial cells were cultured in 200 µl SOC medium without antibiotic at 37℃ for 1 hr. An aliquot of 100 µl was spread over an Ampicillin or a Kanamycin containing agar dish and incubated overnight at 37℃. The composition of the SOC medium is given below.

SOC medium Final concentration

Components of the media	**Final concentration**
Tryptone	2%
Yeast extract	0.5%
NaCl	10mM
KCl	2.5mM
$MgCl_2$	10mM
$MgSO_4$	10mM
Glucose	20mM
pH	7.3

2.6.9 Ampicillin/Kanamycin agar dishes

For preparing agar plates, 500 ml LB medium containing 7.5 g bactoagar was autoclaved. After cooling to 50℃, 500 µl Ampicillin stock solution (final concentration 1% w/v) or 500l Kanamycin stock solution (final concentration 30 mg/ml) was added and mixed; 20 ml of the solution was poured into each sterile culture dish. The dishes were left for drying at room temperature overnight and then stored at 4℃ in the dark. Ampicillin stock solution 0.1 g/ml and Kanamycin stock solution 30 mg/ml were used for the transformed bacterial colony selection.

The bacterial colonies transformed with a ligation mixture or with a plasmid were picked and cultured in 5 ml LB medium with ampicillin (100 µg/ml) or Kanamycin (30 µg/ml) at 37°C overnight. Ten µl of the each bacterial culture was re-suspended in 90 µl of H2O, heated at 95°C for 10 min, and cooled on ice. 1 µl of this suspension was used as a template in setting the 25 µl PCR reaction using the primers specific for the fragment in question.

2.7 Cell biological methods

2.7.1 Cell culture

Culturing of the human pulmonary epithelial cell line A549 (lung carcinoma cell line) was performed according to the protocol recommended by the American Type Culture Collection. The cells frozen in DMSO at -70°C (app. 5 x 10^6 cells) were thawed at 37°C and then poured onto a 100 mm dish containing 12 ml of DMEM F12 1:1 supplemented with 10% FCS, 1% antibiotics (Penicillin and Streptomycin), 1% vitamins, 1% glutamate, glucose (1000 mg/l), and 1% non-essential amino acids. When the cells became confluent, they were trypsinized (2 ml 1 x trypsin per 100 mm plate for app. 5 min at 37°C). The reaction was stopped by adding 10 ml of medium with 10% FCS, which contained trypsin inhibitors.

For further culturing of the A549 cells, which were transferred to a new plate, the cells were usually 80% to 90% confluent again after 3 days. For transfection, protein or RNA isolation, 2 x 10^5 cells were plated in 6-well plates. For reporter assay, 5 x 10^4 cells were plated on a 48-well plate. A549 cells were cultured in gas-controlled incubators in the water vapor-saturated atmosphere with 1% O_2 (v/v) or atmospheric O_2 (v/v), 5% CO_2, and 94% (v/v) or atmospheric N_2 at 37°C in norm baric conditions.

2.7.2 Transfection of A549 cells

The liposome-mediated transfection method was employed for transfection of A549 cells. Lipofectamine 2000 transfection reagent (Invitrogen) was used for transfection. The principle of the method is that DNA gets trapped in the lipid mixture, making a liposome that has DNA in it, which is taken up by cells via endocytosis. One day before transfection, A549 cells (5 x 10^4 cells in 250 µl for 48-well plates and 3 x 10^5 cells in 2 ml for 6-well plate) were plated on respective culture dishes of growth medium without antibiotics, so that they were 85-90% confluent at the time of transfection (as per the protocol suggested for transfections with Lipofectamine 2000 reagent). DNA was diluted (4 µg in 250 µl for 6-well and 0.3 µg in 25 µl for 48-well) in Opti-MEM® medium (without) serum and mixed gently. Lipofectamine 2000 was mixed gently before use and then diluted in the appropriate amount (10 µl for 6-well plate and 1.2 µl for 48-well plate) in 250 µl of Opti-MEM® medium for 6-well and 25 µl for 48-well plate. Solutions were mixed gently and incubated for 5 min at room temperature. After 5 min incubation, the diluted DNA and lipofectamine2000 were mixed and incubated for 40 min at room temperature, to allow the DNA-Lipofectamine 2000 complexes to form. DNA-Lipofectamine complexes were added to each well containing cells and medium (200 ml for 48-well plate and 1.5 ml for 6-well plate), and mixed gently by rocking the plate back and forth. Cells were incubated for 12 to 16 hr in CO_2 incubators as per the experiment.

2.7.3 BMP and TGF-β ligand stimulation

BMP-2, BMP-4, and TGF-β1 were used as the ligands for all the studies in this project. All the ligands were obtained from R&D systems. Both BMP-2 and BMP-4 were used at concentrations of 10ng/ml and TGF-β1 was used at 2 ng/ml. The cells were stimulated after reaching 70% confluency for one day to analyze gene expression. In case of transfected cells the cells were stimulated after 12 hours of transfection procedure.

2.8 Reporter gene assays for transcriptional activity

The detection of luciferase activity in the cells transfected with reporter vectors containing the firefly was performed with a Luciferase Reporter Assay Kit (Promega). The luciferase assay is based on enzyme-catalyzed chemiluminescence. Luciferin present in the luciferase assay reagent is oxidized by luciferase in the presence of ATP, air oxygen, and magnesium ions. This reaction produces light with a wavelength of 562 nm that can be measured by a luminometer. Transfections were performed in a 48-well dish. The cells were incubated for 14 to 16 hr for protein expression after transfection. The cells were washed once with 1 x PBS. The transfected cells were shaken for 15 min in 100 µl of 1 x lysis buffer (Promega). For measurement of firefly luciferase activity, 100µl of the lyste were mixed in black and flat bottom 96-well plates containing 50 µl luciferase assay reagent, which was freshly prepared by mixing substrate and the luciferase assay buffer. The luminescence was measured in a luminometer for firefly luciferase activity. Constitutively active ALK2 receptor (Q207D) was a kind gift from Jeffrey L. Wrana, department of molecular and medical genetics, University of Toronto, Toronto, Canada.

2.9 Western-blot analysis

2.9.1 Total protein isolation from the cultured cells

Total protein isolation was conducted using two methods. The first method involved adding 1x Lamelli protein loading buffer to the PBS washed cells and extracting the protein. The cells were vortexed vigorously five times for 10 sec each, and the samples were boiled for 10 min at 100°C. The second method involved using RIPA cell lysis buffer (composition given below); 200 µl was added to a 6-well dish for western blot analysis, and 500 µl was added for immunoprecipitation.

2.9.2 Antibodies

Antibodies (primary and secondary) used in the experiments are all commercially available. Their parameters (dilutions) are given in Table-3.

Table 3: Primary antibodies

Antibody	Company	Dilutions
Anti-phospho Smad1/5/8 (Rabbit Polyclonal)	Cell signaling	1:1000
Anti-Smad1/2/3 (Rabbit Polyclonal)	Santa Cruz Biotechnology	1:1000
Anti-alpha tubulin-(Mouse monoclonal)	Santa Cruz Biotechnology	1:1500
Anti-P-Smad2(Rabbit Polyclonal)	Cell signaling solutions	1:1000
Anti-FLAG (M5-Mouse monoclonal)	Sigma	1:1500
Anti-Human Smad8 (Goat polyclonal)	Sigma	1:1000
Anti-Smad1 (Rabbit Polyclonal)	Upstate	1:1000
Anti-Smurf (Rabbit polyclonal)	Santa Cruz Biotechnology	1:1000
Anti-Smurf2 (Rabbit polyclonal)	Santa Cruz Biotechnology	1:1000

HRP conjugated secondary antibodies

Secondary antibody	Company	Dilutions
Anti-mouse IgG antibody	Pierce	1:2500
Anti-rabbit IgG antibody	Pierce	1:2500
Anti-goat IgG antibody	Pierce	1:2500

2.9.3 Poly Acrylamide Gel Electrophoresis of proteins (SDS-PAGE)

In SDS-PAGE the denatured proteins bind SDS and become negatively charged. The amount of SDS bound is always proportional to the molecular weight of the protein and is independent of its sequence; therefore, SDS-protein complexes migrate through polyacrylamide gels in accordance with the size of the protein. By using markers of known molecular weight, it is possible to estimate the molecular weight of the proteins. Protein sample from cell extract was denatured by heating to 95℃ for 5 min in 1 x loading buffer, and then cooled on ice immediately. The samples were collected by brief centrifugation and then loaded onto 10% Bis and polyacrylamide gel. The electrophoresis was performed with 200 V constant, and the gel was run until the bromophenol blue reached the bottom of the resolving gel (for about 1 hr).

2.9.4 Electro-blotting of immobilized proteins

The separated proteins in the SDS-polyacrylamide gel were electrically transferred to a nitrocellulose membrane by electro-blotting. To prepare the transfer equipment, two layers of Whatmann 3MM filter paper with transfer buffer followed by gel with transfer buffer were placed onto the electro-blotting chamber. The nitrocellulose membrane and the other two layers of filter paper with transfer buffer were placed on the gel. The cathode and anode from the power supply were connected with the electro-blotting chamber. Electro-blotting was performed at constant current (2mA / cm2) for approximately 90 min. Buffers and their compositions used in the western blot analysis are given below.

Transfer Buffer (pH = 8.3) Final concentration

Buffer component	Final concentration
Tris	25 mM
Glycine	192 mM
Methanol	20%

1X PBS-Tween buffer

Buffer component	Final concentration
Tween 20	0.1%
PBS	1x

Blocking buffer

Buffer component	Final concentration
PBS-Tween	1X
Nonfat dry milk	5%

2.9.5 Immunological detection of immobilized proteins

The transformed membrane proteins were blocked with 10% nonfat dry milk in 1 x PBS-Tween buffer at room temperature for 1 hr, followed by incubation with primary antibody at 4°C overnight. After washing with 1 x PBS-Tween three times for 20 min each, the membrane was incubated with the respective secondary antibody at room temperature for 1 hr, followed by washing three times with 1 x PBS-Tween buffer for 10 min each. Proteins were detected by ECL (Enhanced Chemi-Luminescence) treatment, followed by exposure of the membrane in a Kodak chemi-luminescence imager.

2.10 Immunostaining

The A549 cells cultured on chamber slides were transfected with human Smad8 and Smad8C in a pCDNA 3.1 vector. After 20 hr of transfection, cells were treated with BMP-2 and BMP-4 (10 ng/ml each) for 1 hr. Cells were washed twice with ice-cold PBS and then fixed with ice-cold methanol/acetone (1:1) for 10 min. Slides were blocked with 5% FCS in PBS for 30 min. Then the cells were

incubated with an anti-human Smad8 antibody (1:250 in 2.5% FCS) for 1 hr. After washing three times with 0.2% BSA in 1 x PBS, a FITC-conjugated rabbit anti-goat IgG secondary antibody (1:400) was applied to detect the binding of the primary antibody. The glass chamber slides were observed under a fluorescent inverted microscope (Leica, Germany)

2.11 Statistics

Statistical analysis was performed using student t test and a *P* value less than 0.05 was considered significant.

2.12 Densitometric analysis

Relative band intensity was measured by densitometric analysis using the Quantity one software (Biorad)

3. Results

3.1 Expression of BMP and TGF-β signaling molecules in the human lung

3.1.1 Expression of Smads in human lung RNA

To characterize the expression of Smads in the human lung, RT-PCR was performed. cDNA was produced by Reverse Transcriptase (RT) reaction with the use of total human lung RNA as a template, and full-length PCR products were obtained for all the Smads (R-Smad, Co-Smad, and I-Smads), using their respective gene-specific primers. All the Smads except I-Smads were found to be expressed in the human lung (Figure 5). An additional band at the expected size was observed for Smad8 PCR product (Figure 5).

Figure 5: RT-PCR analysis of different Smad mRNA expression in the human lung: The cDNA was prepared from human lung RNA, and PCR products were obtained by using gene-specific primers for full-length human Smads (S1 – S8).

3.1.2 Expression of BMP and TGF-β receptors in human tissues

Members of the transforming growth factor β family of proteins (TGF-β, BMP, and Activin) signal through cell surface transmembrane serine/threonine protein kinases known as Type I and Type II receptors. The expression of these receptors on the cells controls various cellular activities (e.g., cell proliferation, cell death, and polarity). To analyze these receptor expressions in different human tissues, we chose heart, brain, kidney, liver, testis, spleen, skeletal

muscle, and lung RNAs. All the receptors were expressed in several adult human tissues (Figure 6). With the designed primers, we observed both isoforms for BMPRII (long and short). Full-length cDNA sequences of ALK2, ALK3, ALK6, and BMPRII isoforms were characterized by cloning and sequencing alignment.

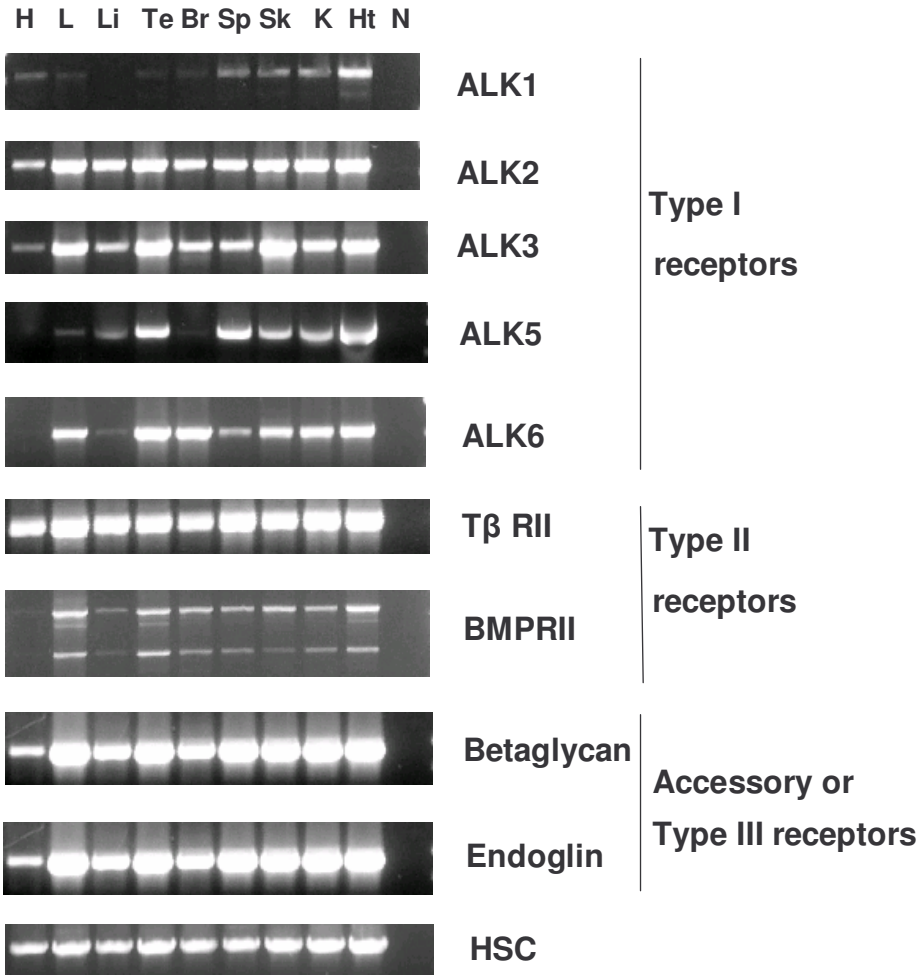
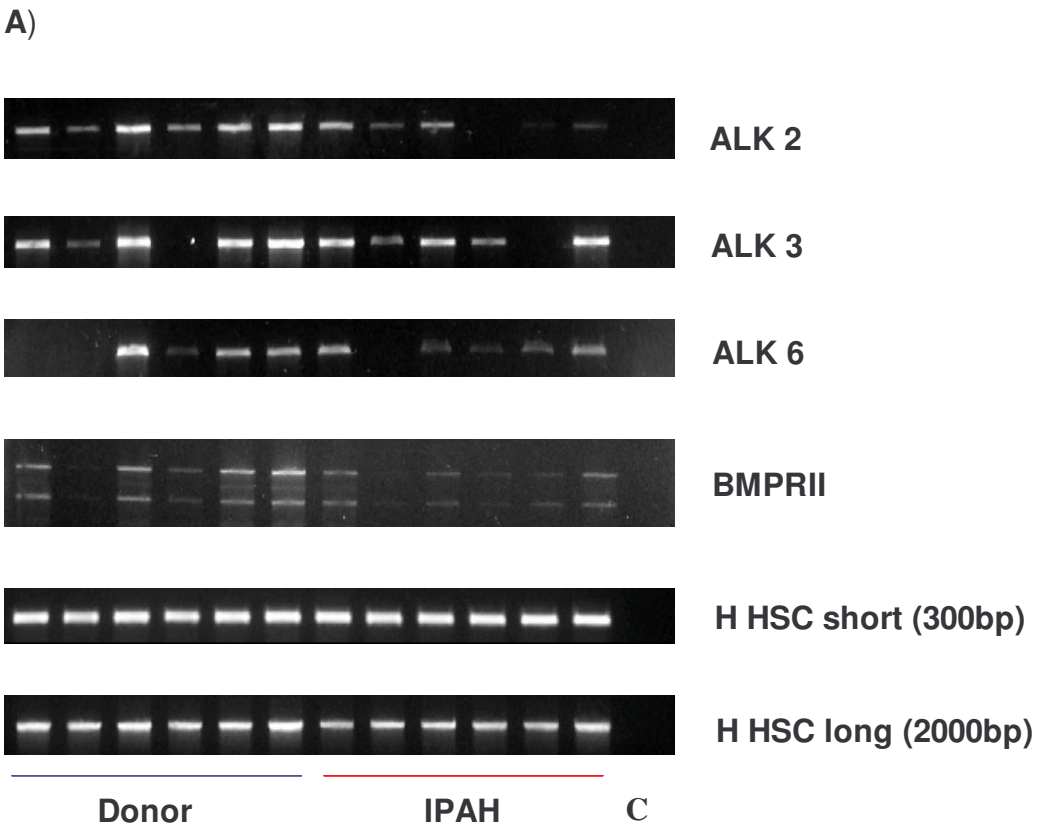


Figure 6: Expression of BMP and TGF-β receptors in various human tissues: BMP and TGF-β receptor expression was analyzed in various human tissues: heart (H), lung (L), liver (Li), kidney (K), testis (T), brain (B), spleen (Sp), skeletal muscle (Sk), and total human embryonic RNA (Ht). N represents water control.

3.1.3 Expression analysis of BMP receptors in donor and IPAH lungs

BMPRII is known to have reduced expression during pulmonary hypertension (76). So we analyzed the mRNA expression of other Type I BMP receptors (ALK3, and ALK6) along with ALK2 and BMPRII in lungs from donors and IPAH. We observed no differences in BMPRII isoforms (long and short)

along with ALK2, ALK3 and ALK6 (Figure 7A). Human HSC was used as an equal loading control. The densitometric analysis of Figure 7A is shown in Figure 7B.



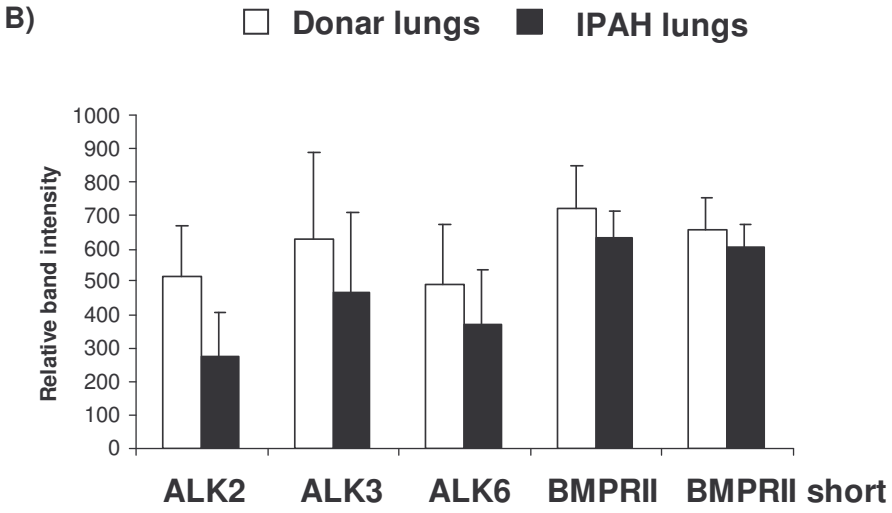


Figure 7: Expression of BMP receptors in donor and IPAH lungs: A) RNA isolated from donor and IPAH human lungs were analyzed for the expression of human BMP receptors (Type I and Type II). 'C' represents water control. HSC short and HSC long served as loading controls. **B** shows densitometric analysis of A. Values on the Y axis correspond to the band intensity ratio between specific receptor to HSC short.

3.1.4 Expression analysis of Smads in donor and IPAH human lungs

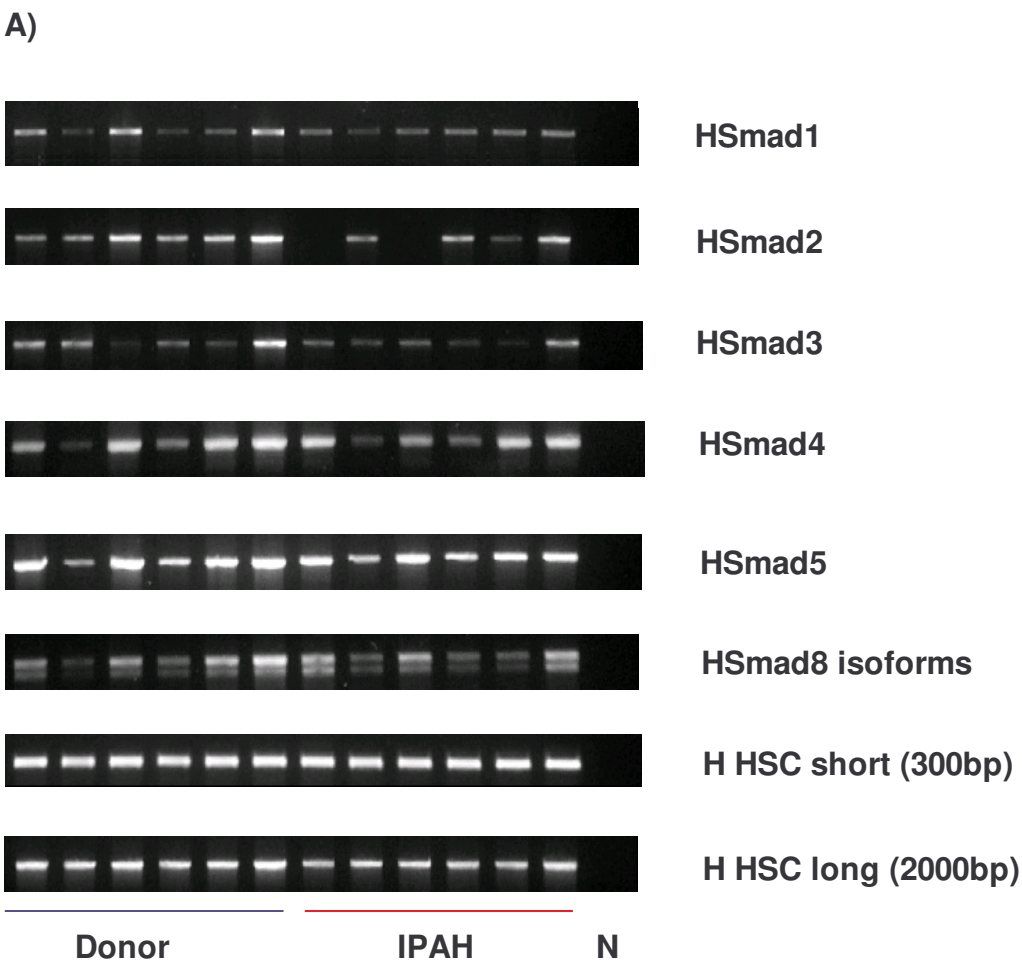


Figure 8: Expression profile of Smads in donor and IPAH lungs: A) RNA from donors and idiopathic pulmonary arterial hypertension (IPAH) patient's lungs was analyzed for human (HSmad1, 2, 3, 4, 5, and 8 expressions by RT-PCR. 'N' represents water control. HSC short and HSC long PCRs were performed as control for equal loading. **B** shows the relative band intensity by densitometric analysis of A. Values on the Y axis correspond to the band intensity ratio between specific Smad to HSC.

As BMP receptors are known to be down-regulated in IPAH, we proceeded to investigate whether the same applies for Smad expression pattern. RNA was extracted from lungs of six donors and six IPAH patients, and analyzed for the mRNA expression of Smads by RT-PCR. All Smads showed almost equal expression between the two groups (Figure 8).

3.2 Cloning and expression analysis of Smad8 isoforms

3.2.1 Cloning of human Smads

In order to study the function of Smads in more detail, Smad1, Smad4, Smad8 and Smad8C were amplified from total human lung RNA by RT-PCR and were cloned into pGEMT-easy. As explained earlier, this vector contains thymidine ”T” overhangs which are designed for cloning of PCR product with adenine ”A” overhangs.

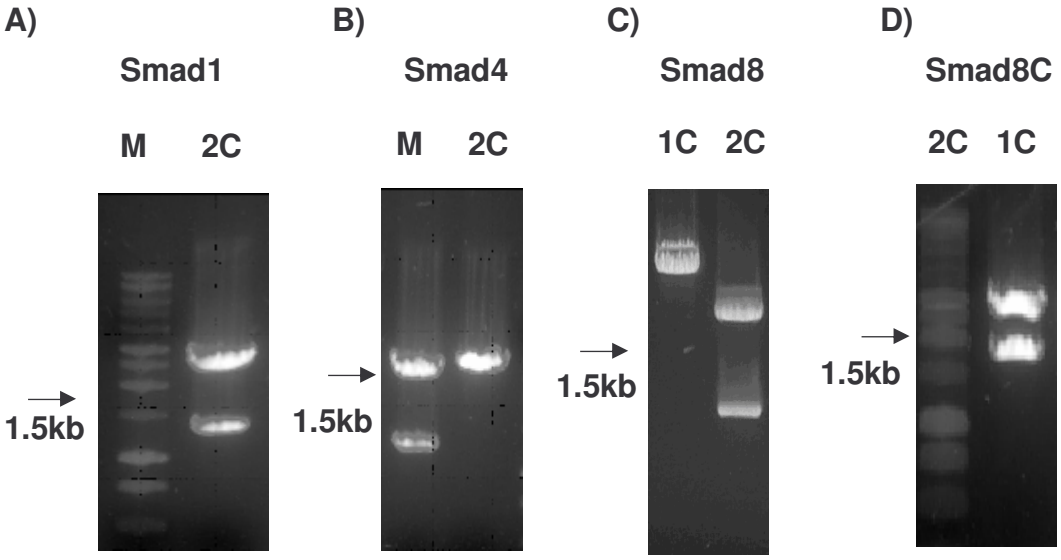


Figure 9: Cloning of human Smads: Smad1, Smad4, Smad8 and Smad8C were cloned into pGEMT-easy and analyzed by restriction digestion for the insert release from the vector. A) Human Smads cloned into pGEMT-easy were digested with EcoR I Smad4 and Smad8) with Not I (Smad1. Restriction digestion of Smad1 is in panel A; Smad4 is in panel B; Smad8 is in panel C and Smad8C is in panel D. All the Smads were screened for single clone. 1C represents the restriction digestion with 1 enzyme to linearize the vector, and 2C represents restriction digestion with 2 enzymes to release the insert from the vector (EcoRI Smad8C).

Clones were analyzed for the presence of the insert by blue/white screening (based on the β-galectosidase expression in E.coli), restriction digestion (Figure 9), and full-length cDNA sequencing. The DNA sequencing reports were compared to the published sequences from NCBI to confirm the right clones. Restriction digestion of the plasmid DNA from white colonies showed an insert release at expected sizes (Figures 9A, 9B, 9C and 9D), confirming the right clones

3.2.2 Identification, cloning and sequence confirmation of novel human Smad8 isoform

During initial PCR amplification (Figure 1) of the full length of the Smad8 from total human lung RNA, we identified two specific bands on an agarose gel electrophoresis after RT-PCR. Cloning and sequencing analysis of both bands revealed that both molecules were alternatively spliced variants of Smad8. It is known from NCBI genome nucleotide database (GenBank accession numbers **BC011559** and **BC104760** for Smad8 and Smad8C respectively) that these two isoforms differ by 111bp, corresponding to 37 amino acids. Genome analysis confirmed that the region corresponded to exon3 of Smad8. Structurally, exon3 is in the linker domain of the Smad8, which connected both MH1 and MH2 domains (Figure 10). These results showed that we could amplify and clone the full length of Smad8, which we called Smad8C.

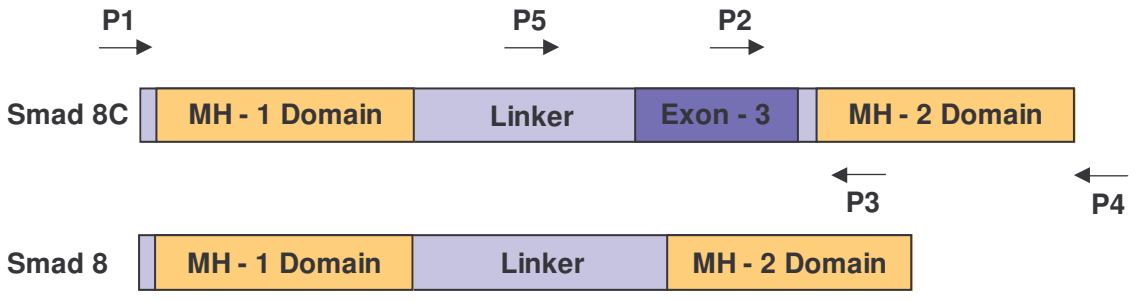


Figure 10: Domain alignment of Smad8 isoforms: The major domains of human Smad8, MH1, MH2 (orange), and the linker (light blue) are shown. Exon3 (dark blue), which is present in only Smad8C, is depicted in blue. P (1-5) indicates the primer positions designed to amplify different regions (Full length, N-terminus, C-terminus and only Smad8C) of Smad8 isoforms.

3.2.3 Expression profile of Smad8 isoforms in various human tissues

In order to analyze the expression profile of human Smad8 isoforms in different human tissues, RNA from human heart, kidney, brain, lung, testis, placenta, trachea, uterus, and thyroid, as well as total human RNA, were investigated. Primers P1 & P4 were designed to amplify the full length, P1 & P3 to amplify N-terminus of Smad8 isoforms, P5 & P4 to amplify C-terminus Smad8 sioforms (Figure 10).

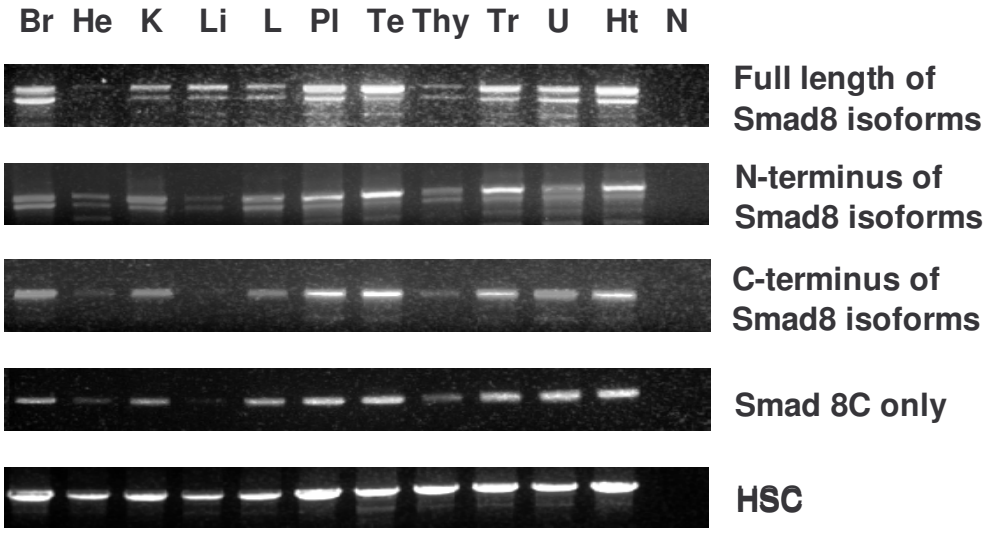


Figure 11: Expression profile of Smad8 isoforms in various tissues: Smad8 isoform expression was analyzed by RT-PCR from ten different human tissues. The PCR products were obtained using the primer pairs P1 + P4 for the first panel (full lengths of Smad8C and Smad8 with product length 1437 & 1326 bp, respectively), P1 + P3 for the second panel (N-terminus of Smad8C and Smad8 with product lengths 903 and 792 bp, respectively), P5 + P4 for the third panel (C-terminus of Smad8C and Smad8 with the same product length of 727 bp) and P2 + P4 for the fourth panel (only Smad8C with product length 554 bp). Various tissues used in this study are: B- Brain, He- Heart, K-Kidney, Li- Liver. L-Lung, Pl- Placenta, Te- Testis, Thy- Thyroid, U- Uterus, Ht-Human total, N- Water control. HSC served as a loading control

A forward primer (P2) was also designed inside the exon3 to amplify only the Smad8C. Both isoforms were expressed in almost all the tissues (Figure 11). Heart and Thymus showed lower expression of Smad8 isoforms, compared to that of other tissues.

3.3 Functional characterization of Smad8 isoforms

3.3.1 Cloning of Smad8 isoforms into N-terminal FLAG fusion vector

For further functional characterization of the Smad8C isoform, we cloned Smad1, Smad8, and Smad8C into N-terminal FLAG-tagged vector (pCMV 2B). The expression of these constructs was analyzed by western blot with anti-FLAG antibody. A549 cells were transfected with FLAG-tagged Smad1, Smad8, and Smad8C. The lysates were run on an SDS-PAGE gel and western blotted for FLAG-tagged proteins with anti-FLAG antibodies. We observed Smad1, Smad8, and Smad8C expression at the expected sizes (Figure 12).

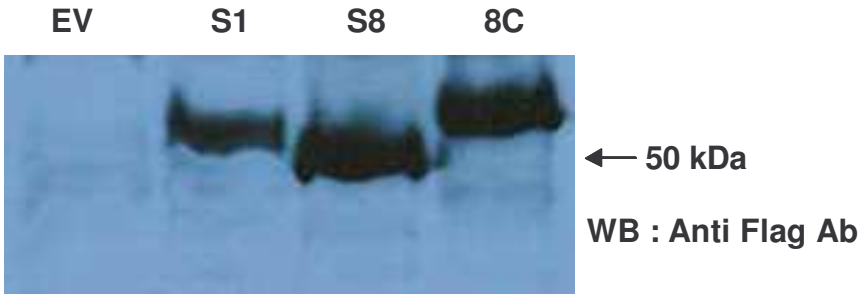


Figure 12: Cloning of Smads into eukaryotic expression vectors with FLAG tag: Smad1 (S1), Smad8, and Smad8 full length (8C) were cloned into N-terminal FLAG and tagged vector, and their expression was analyzed by western blotting with Anti-FLAG antibody. Empty vector (EV) was used as a no-transfection control.

3.3.2 Phosphorylation analysis of human Smad8 isoforms

3.3.2.1 Anti-Smad1/2/3 antibody can cross-react with Smad8 and Smad8C

As no good antibody was available for human Smad8 at this time, we screened several antibodies that could cross-react with human Smad8 isoforms. As Smad1 is more homologous to the sequence of Smad8, anti-Smad1/2/3 antibody was analyzed for its cross-reactivity with Smad8 isoforms. An immuno-

precipitation experiment was performed with Smad1, Smad8, and Smad8C cloned into an N-terminal FLAG tag vector.

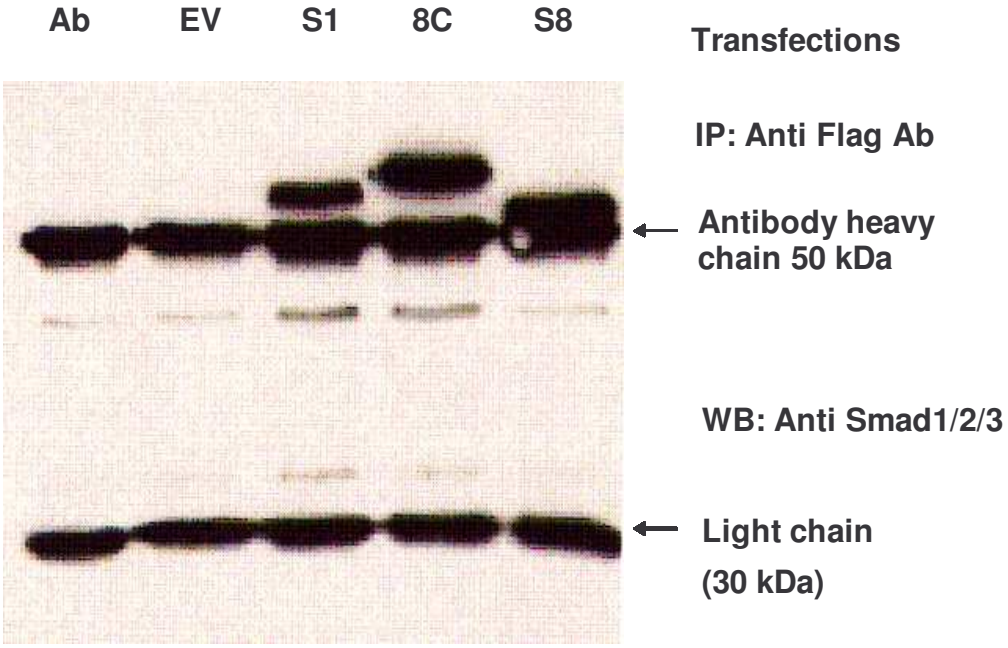


Figure 13: Cross-reaction of anti-Smad 1/2/3 antibody with Smad8 and Smad8C: A549 cells were transfected with FLAG-tagged Smad1 (S1), Smad8 (S8) and Smad8C (8C), and the cell lysates were immuno-precipitated (IP) with anti-FLAG antibody. The IP samples were analyzed by western blot (WB) with anti-Smad1, 2, and 3 antibody. Anti-FLAG antibody (Ab) and antibody with cell lysate transfected with empty vector (EV) were used as IP control.

A549 cells were transfected with Smad1, Smad8, and Smad8C with a FLAG tag and immuno-precipitated with anti-FLAG antibody. The samples after IP were analyzed by western blot with anti-Smad1/2/3 antibody. We observed specific bands corresponding to Smad1, Smad8C, and Smad8 (Figure 13). This result confirmed that we had found an antibody that could cross-react with Smad8 isoforms and be used in the following experiments.

3.3.2.2 Phosphorylation analysis of Smad8C

An important property of receptor Smads is the ability to be phosphorylated by Type I receptors in the presence of the ligand. So we analyzed the phosphorylation property of the full-length form of Smad8 (Smad8C). A549 (lung epithelial cell line) was transfected with Smad1, Smad8, and Smad8C in pCDNA 3.1-, a eukaryotic over-expression vector in a 6-well dish. Smad1 and Smad8 phosphorylation was also analyzed as a positive control for BMP stimulation. After 24 hr of transfection, the cells were stimulated with BMP-2 (10 ng/ml) and with TGF-β1 (5 ng/ml) for 1 hr. The protein extracts were analyzed for the presence of phosphorylated Smads by western blotting. The cell lysate was divided into two parts, and the first part was analyzed for phosphorylation of Smad1, 5, and 8 with anti-phospho Smad1/5/8 specific antibody. The second part of the lysate was analyzed for phospho-Smad2 as a positive control for TGF-β stimulation. As expected, we found an increased amount of phosphorylation for Smad1 when it was stimulated with BMP-2 ligand.

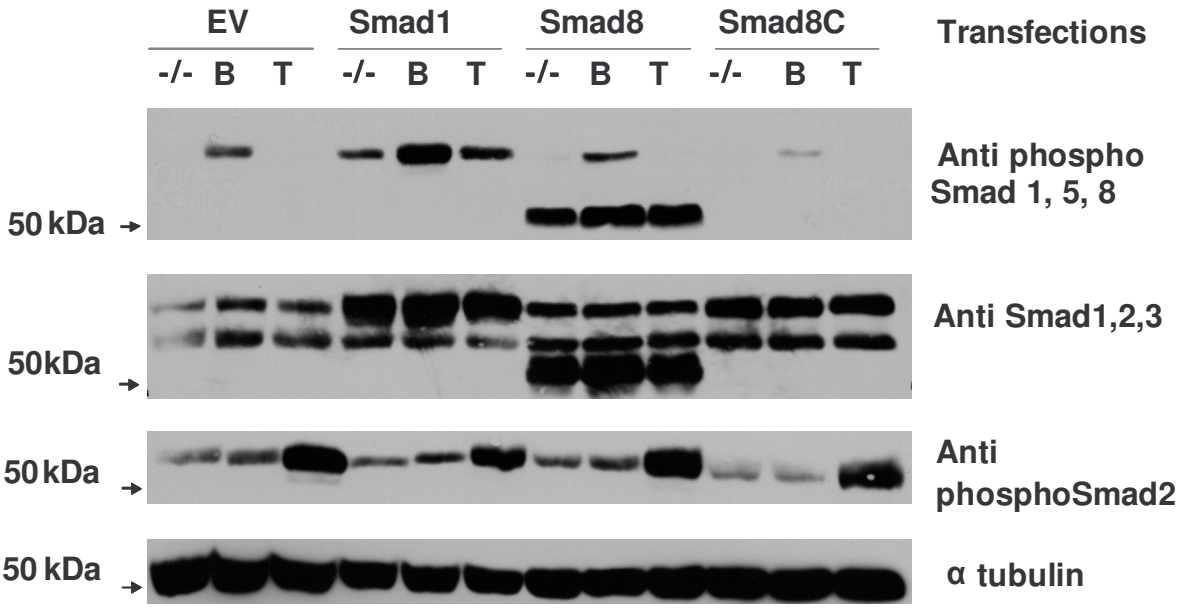


Figure 14: Differential phosphorylation of Smad8 isoforms: A549 cells were transfected with Smad1, Smad8, and Smad8C in pCDNA3.1-, along with the empty vector (EV) as a no-transfection control. After 20 hr of transfection, the cells were stimulated with BMP-2 (10 ng/ml) and TGF-β1 (5 ng/ml). The cell lysates were analyzed by western blot for phospho-Smad1 and 8 with anti-phospho Smad1/5/8 antibody. Anti-Smad1/ 2 /3 was used as a control of equal transfection. A-tubulin blot showed equal protein-loading.

Smad8 isoform, which lacked exon3, was found to be phoshorylated at a basal level (Figure 14). Even though there was a slight increase in the phosphorylation of Smad8 after BMP stimulation, basal phosphorylation was much greater than that of Smad1. Smad8C did not show any phosphorylation even after stimulation with BMP or TGF-β (Figure 14). The same results were found when the cells were stimulated with BMP-4 (data not shown).

Not only was Smad8C not phosphorylated, but it was also affected endogenous Smad1 phosphorylation. The band intensity for endogenous phospho-Smad1 decreased when the cell were transfected with Smads8C. The same membrane was stripped and blotted for Smad1, 2 and 3 which could cross-react with Smad8C and Smad8, to analyze the equal expression of transfected Smads (Smad1, Smad8 and Smad8C). It was observed that neither Smad8 nor Smad8C was responsive for TGF-β stimulation (Figure 14). This result shows that Smad8 was getting more basal phosphorylation, compared to Smad1 and Smad8C. Smad8C was not phosphorylated at any stage of stimulation; instead, it showed possible inhibitory action on Smad1 phosphorylation after BMP stimulation.

3.3.3 Inhibitory function of Smad8C

3.3.3.1 Smad8C inhibits Smad1 phosphorylation after BMP stimulation

We observed decreased BMP-induced endogenous Smad1 phosphorylation in A549 cells when co-transfected with Smad8C (Figure 15). To confirm this result, we analyzed Smad1 phosphorylation after transfection of Smad8C in a dose-dependent manner. A549 cells were transfected with 0.5 µg, 1 µg, 2 µg, 3 µg, and 4 µg of Smad8C (in pCDNA 3.1-) in a 6-well dish. During transfection, the DNA was equalized in all the wells with an empty vector of pCDNA 3.1-. Twenty-four hours after transfection, the cells were stimulated with BMP-4 (10 ng/ml), and proteins were extracted and analyzed for phospho-Smad1 with anti-phospho-Smad1/5/8 antibody.

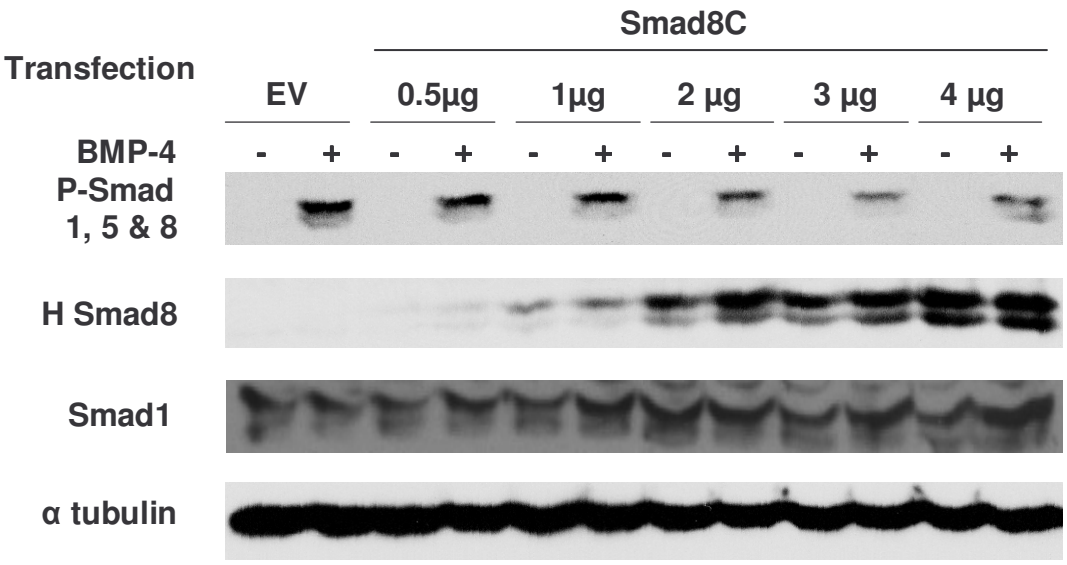


Figure 15: Effect of Smad8C on Smad1 phosphorylation: A549 cells were transfected with increasing amounts of Smad8C (0.5 µg, 1 µg, 2 µg, 3 µg, and 4 µg). BMP + and – indicates the presence and absence of BMP-4 stimulation (10ng/ml). The upper panel is the blot for phospho-Smad1/ 5/ 8. The middle panel is the blot for Smad8, and the last one is for α-tubulin. The EV (empty vector) was taken as no transfection control.

We observed a decrease in the amount of endogenous phosphorylation when the amounts of Smad8C were increased. The same blot was stripped and blotted for Smad8C over-expression with anti-Smad8 antibody (Figure 15). The lower band we observed in the Smad8 blot was believed to be a degradation product. The same membrane was stripped again and blotted for human ALK6 (the only available BMP receptor antibody at that time) to determine if the decrease in the phosphorylation of Smad1 was due to receptor degradation. No change in the expression of ALK6 was observed (Figure 15). These results showed that Smad8C inhibited BMP-induced phosphorylation of Smad1.

3.3.3.2 Smad8C inhibits BMP signal transduction

Analysis of the Smad8C transcriptional activity was the next interesting functional assay. So we performed a BMP reporter gene assay with a 4X BRE (BMP responsive element) (i.e., four times BRE sequence was cloned in front of a luciferase gene). A549 cells were transfected in a 48-well dish with empty vector, Smad1, Smad8, and Smad8C in two different experiments.

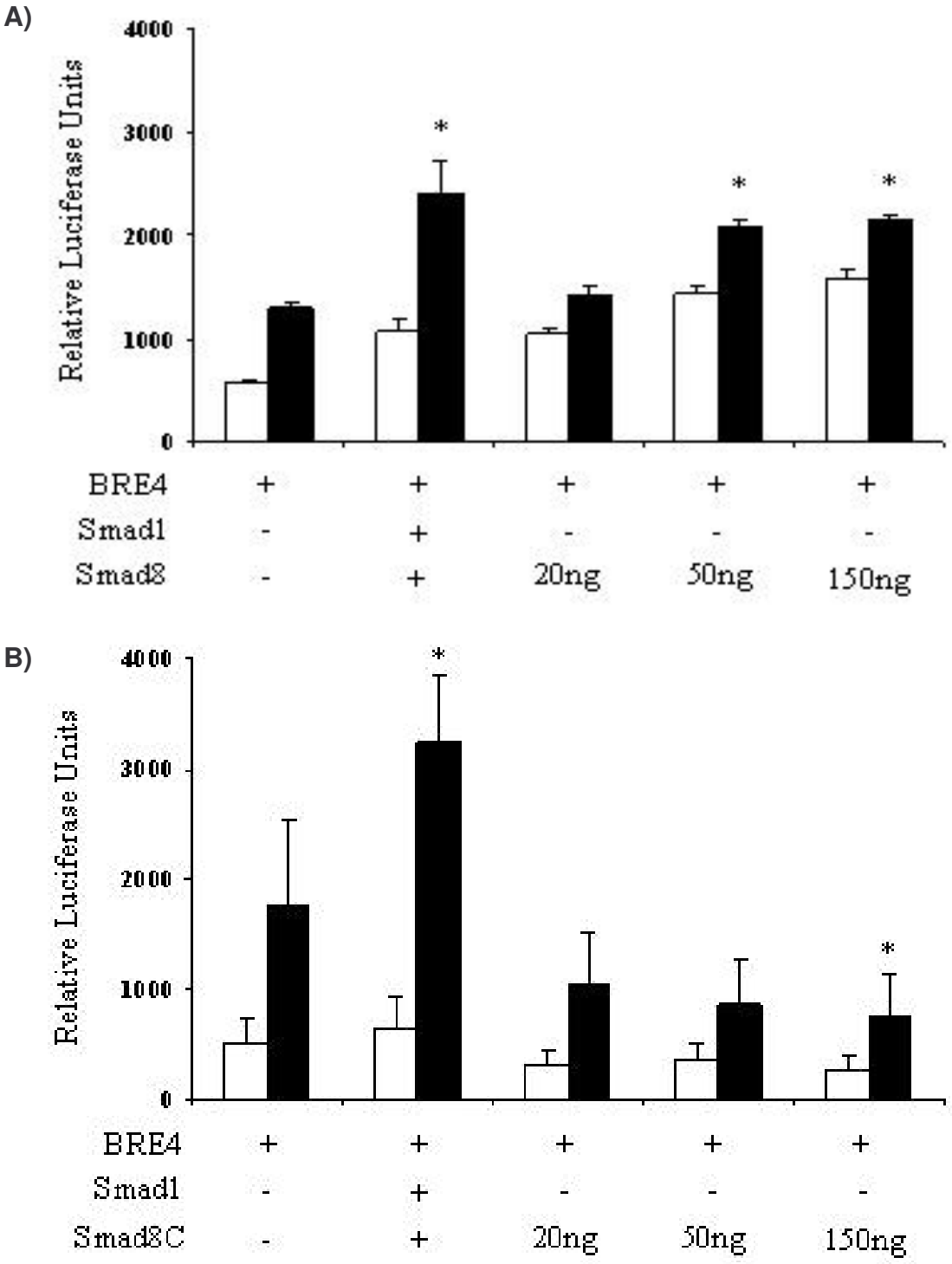


Figure 16: Smad8 and Smad8C show opposite effects: A) A549 cells were transfected with empty vector, Smad1, and Smad8, whereas Smad8 was transfected with increasing amounts (20 ng, 50 ng, 150 ng). 100 ng of 4XBRE and 200 ng of Smad1 were used for BMP-4 positive response. B) A549 cells were transfected with empty vector, Smad1, and Smad8C, whereas Smad8C was transfected with increasing amounts (20 ng, 50 ng, 150 ng). 100 ng of 4XBRE and 200 ng of Smad1 were used for BMP-4 positive response. The experiment was performed from 4 different samples and the error bars show the standard deviation. *, $P < 0.01$

In the first experiment, cells were transfected with empty vector, Smad1 (200 ng/well), and Smad8 in increasing amounts (20 ng, 50 ng and 150 ng) (Figure 16A). In the second experiment, cells were transfected with empty vector, Smad1 with constant, and Smad8C in increasing amounts (20 ng, 50 ng, and 150 ng) (Figure 16B).

The DNA in all the wells was normalized by the empty vector to avoid discrepancies in transfection efficiency. After 12 hr of transfection, the cells were stimulated with BMP-4 (10 ng/ml) for 12 hr. We observed an increase in BMP signal when Smad8 was transfected. The increase in the signal was gradual but significant, and it corresponded to increasing amounts of Smad8 (Figure 16A). In the case of Smad8C, the BMP signaling was inhibited significantly in a dose-dependent manner. Inhibition was observed at both the basal and the BMP-stimulated states (Figure 16B). This result supported the previous phosphorylation findings, indicating that Smad8C could be a potential inhibitor of BMP signaling.

3.3.3.3 Smad8C does not interfere in transcriptional activity of Smad8

Previous publications have indicated that there is another isoform of Smad8, known as Smad8B. This isoform does not possess an SSXS motif. Consequently, it is not phosphorylated even after BMP stimulation. Smad8B was also shown to be an inhibitor of Smad8. Since Smad8C also functions as an inhibitory Smad, we investigated whether Smad8C could inhibit Smad8: Smad8 was co-transfected at increasing amounts (20ng, 50ng, 150ng) in a 48-well dish in the presence of constant Smad8C.

After BMP-4 stimulation, we observed that Smad8 could gradually increase BMP signaling even in the presence of Smad8C (Figure 17). We also determined that the inhibitory effect of Smad8C on BMP signaling was not the result of inhibiting Smad8. Thus, it might interfere with some other molecule which could transmit BMP signaling (e.g., Smad1 or Smad5).

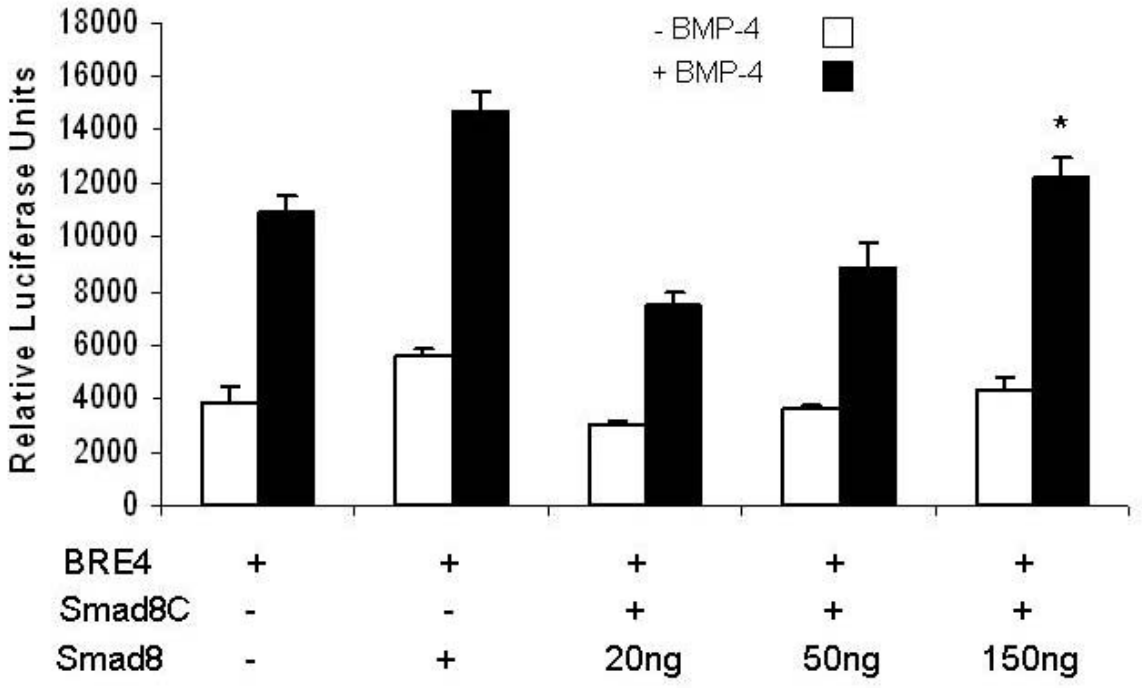


Figure 17: Coordination of BMP signaling by Smad8 isoforms: A549 cells were transfected with empty vector, Smad8C, and Smad8 in increasing amounts (20 ng, 50 ng, 150 ng). 100 ng of 4XBRE and 200 ng of Smad1 were used for BMP-4 positive response in the respective wells. BMP signaling increased when the cells were transfected with Smad8 (Smad8S), even in the presence of constant Smad8C (150ng). The experiment was performed from 4 different samples and the error bars show the standard deviation. *, $P < 0.01$

3.3.3.4 Effect of Smad8C on regulation TGF-β reporter activity

Some BMP signaling molecules, such as Smad1, are activated by TGF-β. Thus, we wanted to analyze transcriptional activity of Smad8C on TGF-β responsive reporter gene. For this purpose, we chose the (CAGA)x12 reporter, which contains 12 repeats of CAGA sequence cloned in front of a luciferase gene. A549 cells were transfected in a 48-well dish with empty vector, Smad1, Smad8, and Smad8C. After BMP-4 and TGF-β stimulation, the cells were lysed and analyzed for luciferase expression in a luminometer. We observed a tenfold increase of luciferase activity when the cells were stimulated with TGF-β1 (Figure 18). Almost no response was observed when the cells were stimulated with BMP-4 on this reporter. We also observed a slight decrease in the luciferase activity with TGF-β stimulation when Smad8C was transfected. This result

indicated that Smad8 could inhibit not only the BMP reporter gene assay but also the TGF-β reporter, to some extent.

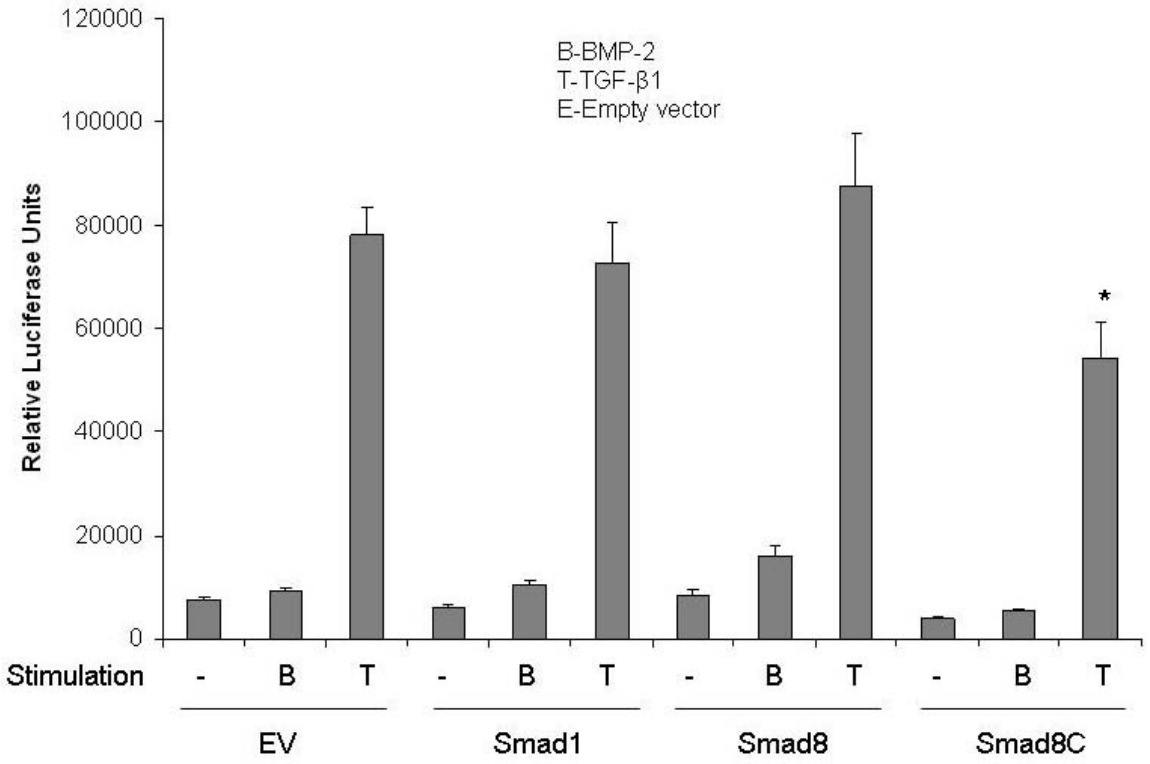


Figure 18: Regulation of TGF-β responsive reporter (CAGA) by Smad8 isoforms: A549 cells were transfected with empty vector, Smad1, Smad8 Smad8C in pCDNA 3.1-vector (300 ng) in each well of 48-well dish. Cells were stimulated with BMP-4 (10 ng/ml) and TGF-β1 (5 ng/ml) for 12 hr. Luciferase activity was measured 24 hr after transfection The experiment was performed from 4 different samples and the error bars show the standard deviation. *, $P < 0.02$

3.3.3.5 Smad8C can inhibit constitutively active ALK2

To confirm our previous phosphorylation and reporter gene results (Figure 14 &16) with BMP-4 ligand stimulation, we measured the effect of constitutively active ALK2 receptor (CaALK2) on Smad8 and Smad8C. CaALK2, when transfected along with Smad1/Smad8/Smad8C, its activity was inhibited by Smad8C as shown in the figure 19, where Smad1 or Smad8 enhanced CaALK2 activity to the empty vector control

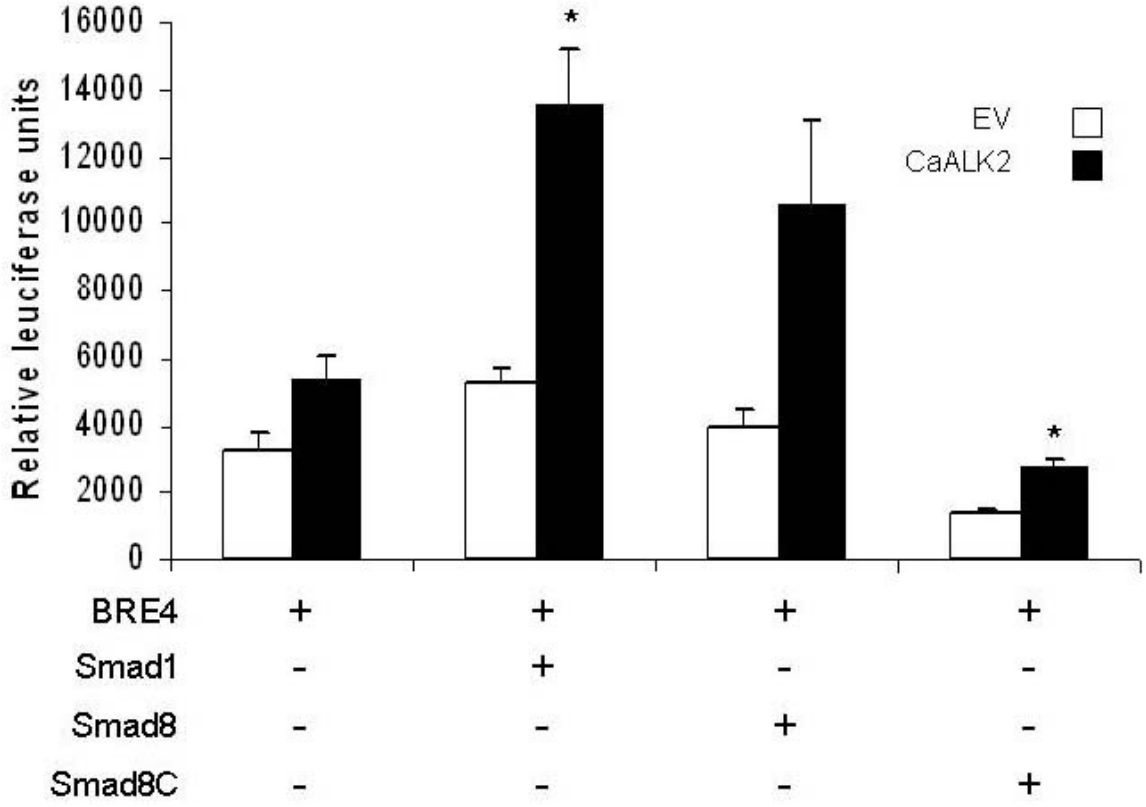


Figure19: Inhibitory effect of Smad8C: A549 cells were transfected with 100ng of CaALK2 along with 200ng of EV/Smad1/Smad8/Smad8C in 48 well cell culture dish. Empty vector was transfected as no BMP stimulation control in the first panel of the figure. Luciferase activity was measured 24 hours after transfection. The experiment was performed from 4 different samples and the error bars show the standard deviation. *, $P < 0.02$.

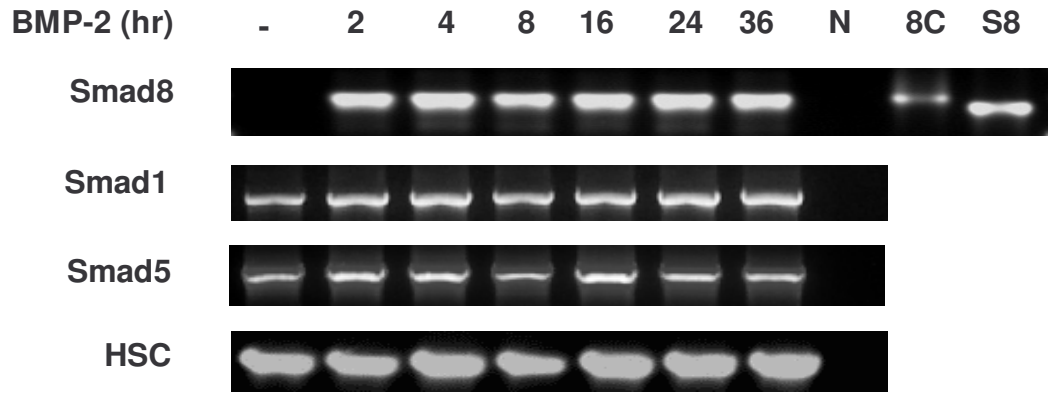
3.3.4 Smad8C is an early responsive gene for BMP-2 and BMP-4

3.3.4.1 Expression of Smad8C mRNA increases after BMP-2 and BMP-4 stimulation

It is known that Smad6 and Smad7 expression is low at the basal state but increases upon BMP and TGF-β stimulation. After expression they inhibit BMP and TGF-β signaling by the degradation of their concerned receptors with the help of Smad ubiquitine regulatory factor (Smurfs). So we wanted to investigate whether the Smad8C could act in the same way. A549 cells were stimulated with

BMP-2 (10 ng/ml) and BMP-4 (10 ng/ml) at different time points (2 hr, 4 hr, 8 hr, 16 hr, 24 hr, 36 hr), and the RNA was analyzed for Smad8 expression by RT-PCR. We found a massive increase in the RNA levels of Smad8C but not Smad8. Smad8 could not be observed in the gel as its expression was much less in A549 cells. We could observe an increased expression starting from 2 hr of stimulation until 36 hr (Figure 20A, B). From the same aliquot of RNA, RT-PCRs were also performed for human Smad1 and Smad5, but no change was observed in their expression after stimulation (Figures 20A & B)

A)



B)

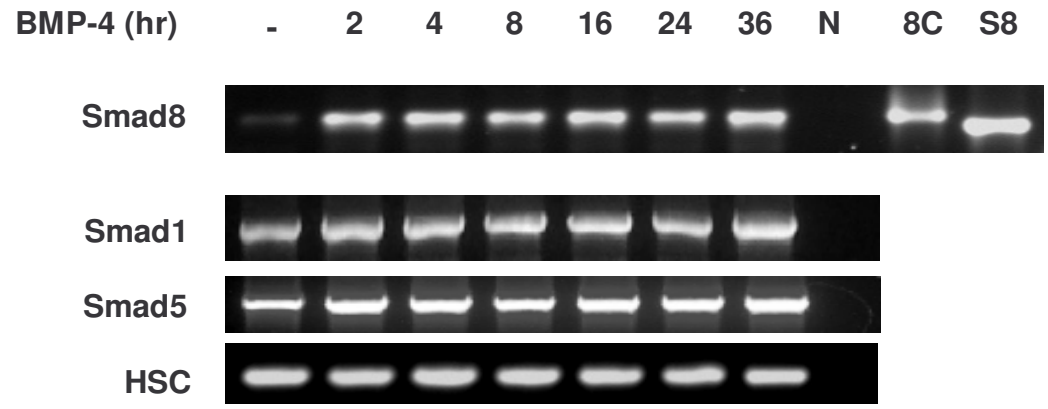


Figure 20: Smad8C expression is increased after BMP-2 and BMP-4 stimulation: A549 cells were stimulated with BMP-2 (10ng/ml) (A) and BMP-4 (10ng/ml) (B) at different time points, indicated in the figure. The RNAs were extracted from these cells and analyzed for expression of Smad8 isoforms, Smad1 and Smad5. HSC was used as a positive control of loading

3.3.4.2 Effect of BMP-2 and BMP-4 stimulation on Smad8C protein expression in A549 cells

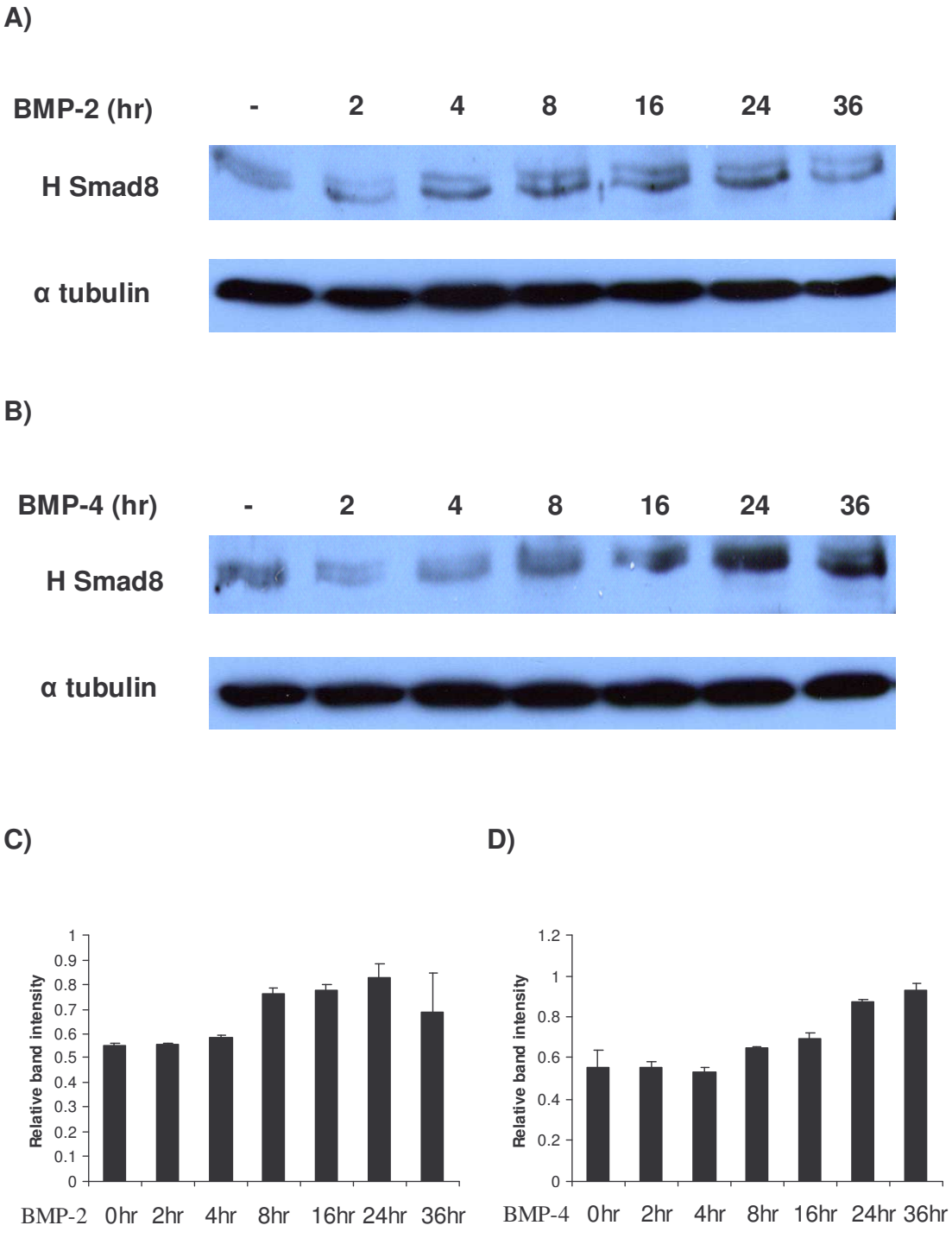


Figure 21: Smad8C expression at the protein level increases after BMP-2 and BMP-4 stimulation: Proteins were extracted from A549 cells after BMP-2 (**A**) and BMP-4 (**B**) stimulation at the time points. The cells were lysed with RIPA cell lysis buffer, and the lysates were analyzed for Smad8 expression by western blot. The same blot was stripped and blotted for α-tubulin as a protein loading control. **C** and **D** show the densitometric analysis of A and B respectively. Values on the Y axis correspond to the band intensity ratio between Smad8C to α-tubulin.

As mRNA of Smad8C showed increased expression, we also wanted to determine its protein levels after BMP-2 and BMP-4 ligand stimulation. Proteins were extracted from A549 cells before and after stimulation with BMP-2 (Figure 21A) and BMP-4 (Figure 21B) at different time points analyzed for Smad8C expression by western blot with anti-Smad8 antibody. We found a significant increase in the protein levels of Smad8C after 24 hr of stimulation. This result indicates that Smad8C expression was enhanced after BMP stimulation and suggests that Smad8C is an early BMP responsive gene. Figure 21C and Figure 21D show the relative fold intensity by densitometric analysis of Figure 21A and Figure 21B respectively.

3.3.4.3 Smad8C inhibits BMP signaling through the degradation of Smad1

There are two possibilities through which Smad8C can inhibit BMP signaling. One is by degradation of type I or type II receptors, and the other is by the degradation of BMP responsive Smads, Smad1, and Smad5. So we analyzed the expression of Smad1 and ALK6 in A549 cells after stimulation with BMP-2 and BMP-4 by western blot with gene-specific antibodies. We observed a decrease in the protein levels of Smad1 after BMP-2 and BMP-4 stimulation (Figures 22A & B). Our previous phosphorylation results and these results might indicate that the Smad8C inhibition of BMP signaling may be through the degradation of Smad1. Figure 22C and Figure 22D show the relative band intensity by densitometric analysis of the Figure 22A and Figure 22B respectively.

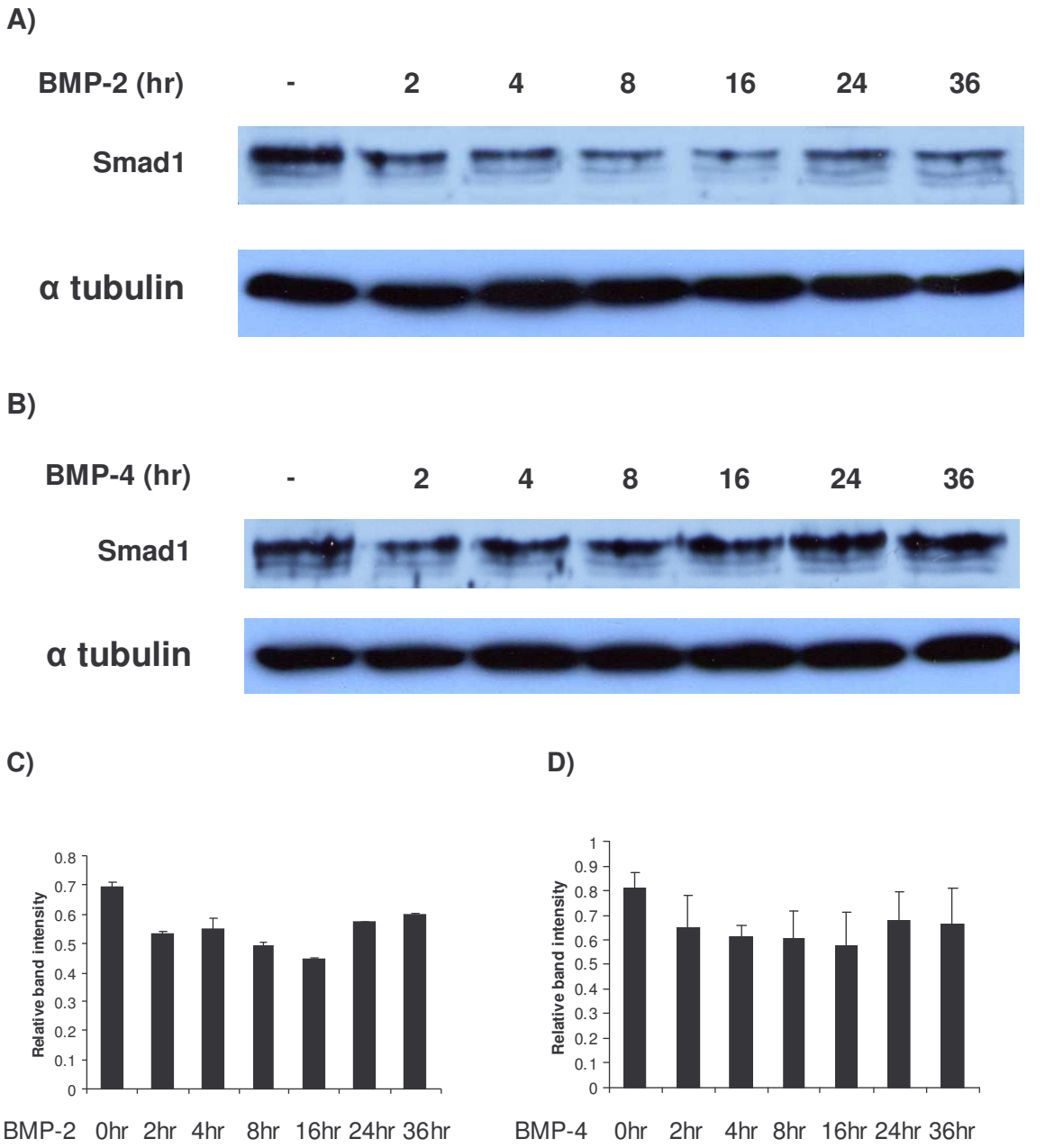


Figure 22: Smad1 is getting degraded after BMP stimulation: Western blot for native Smad1 was performed from A549 cell lysates after BMP-2 (**A**) and BMP-4 (**B**) stimulations at the time points indicated in the figure. The same membranes were stripped and blotted for α-tubulin for protein loading control. **C** and **D** show the densitometric analysis of the A and B respectively. Values on the Y axis correspond to the band intensity ratio between Smad1 to α-tubulin.

3.3.4.4 Cellular localization of Smad8 isoforms

As Smad function is also dependent on cellular localization, we analyzed the occurrence of Smad8C before and after stimulation with BMP. For this purpose, A549 cells were transfected with Smad8 and Smad8C. After 16hr of

transfection, the cells were treated with or with out BMP-4 (10 ng/ml) for 1 hr. Smad8 was mostly distributed in both cytoplasm and nucleus before stimulation and translocated towards cytoplasm when the cells were treated with BMP-4 (Figure 23). Smad8C was mostly located in the cytoplasm and translocated towards the nucleus after BMP stimulation.

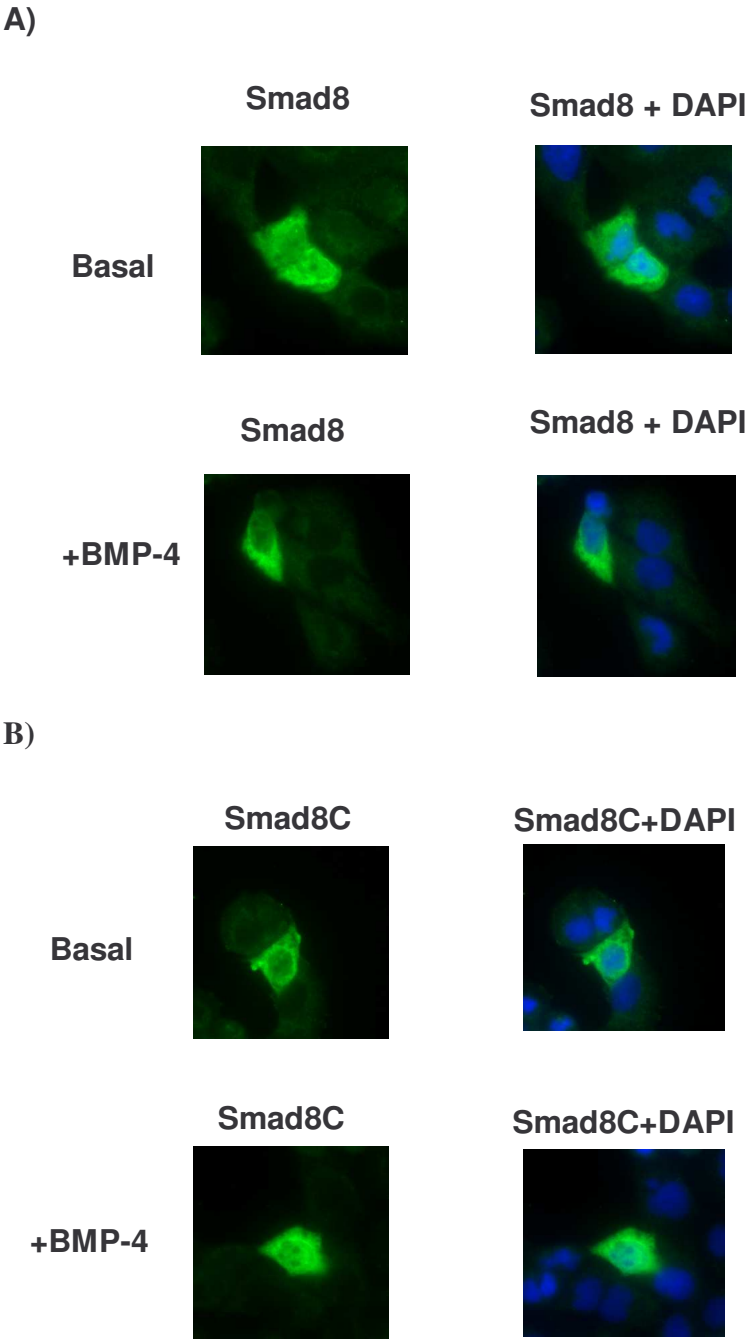


Figure 23: Cellular localization of Smad8 isoforms: Smad8 and Smad8C in pCDNA3.1- vector were transfected in A549 cells. Sixteen hr after transfection, the cells ere stimulated with BMP-4 (10 ng/ml) for 1 hr and stained for Smad8 with anti-human Smad8 antibody.

4. Discussion

In our present study, we analyzed the expression of BMP and TGF-β signaling molecules during IPAH and identified a novel human Smad8 isoform. The receptors for BMP and TGF-β are expressed in most of the human tissues. During IPAH, the expression of BMPRII and Type I receptors along with ALK2 was unchanged. During the expression analysis a novel isoform of Smad8 was identified with 37 additional amino acids in the linker region and was termed as Smad8C. Further characterization of this molecule revealed that Smad8C does not get phosphorylated upon BMP stimulation unlike Smad8. Over expression of Smad8C inhibited BMP induced Smad1/5 phosphorylation. Reporter gene analysis also revealed that Smad8C inhibits BMP signal transduction. Similar to inhibitory Smads, mRNA and protein expression of Smad8C was enhanced after BMP stimulation. We also observed decreased protein levels for Smad1 after prolonged BMP stimulation indicating that inhibitory action of Smad8C on BMP signaling might be through reduced protein levels of Smad1. In summary, we identified and functionally characterized a novel isoform of human Smad8.

4.1 Expression profile of human Smads

Idiopathic pulmonary arterial hypertension (IPAH) is associated with structural changes in both the pulmonary vasculature and the right ventricle, due to muscularization of arterioles and intimal proliferation [66-70]. Recent reports have shown that some mutations in the Bone morphogenetic protein (BMP) signaling molecules which could make them dysfunctional are one of the causes for this disease. BMP belongs to TGF-β super family and has major role in embryo development and cell proliferation [71;72]. It is known that Smads are expressed throughout embryonic development. Transforming growth factor-β (TGF-β)/Smad pathway can induce growth inhibitory and apoptotic responses. Inactivation of intracellular components of these pathways has been shown to contribute for tumorogenesis. Therefore, we analyzed the expression of Smads in total human lung RNA. Our results revealed that most of the Smads (receptor Smads and Co-Smad) were expressed in the normal human total lung RNA

where as the mRNA expression of Smad6 and Smad7 was not detectable by RT-PCR. Smad6 and Smad7 are the inhibitory Smads, and their expression depended on the other growth factor expression and activity [59;73-76]. BMP and TGF-β induced Smad6 and Smad7 expression in the cells after stimulation [77].

The TGF-β super family is composed of many multifunctional cytokines including TGF-βs, activins, and bone morphogenetic proteins (BMP). These proteins regulate a variety of biological responses such as proliferation, differentiation, apoptosis, and development [78]. Members of this family signal through cell surface transmembrane serine/threonine protein kinases are known as Type I and Type II receptors. Expression of these receptors in different cell types controls cell fate. In this study, we analyzed the mRNA expression of these receptors in different organs of humans. All the receptors were found to be expressed in most of the tissues suggesting a possible role of these molecules in the cellular functions during the adult stage.

IPAH is characterized by obstruction of pre-capillary arteries, leads to sustained elevation of pulmonary arterial pressure [43;71;72]. Even though the cause of the disease is not really understood, the histological features reveal proliferation of endothelial and smooth muscle cells with vascular remodeling. In order to explore the expression of BMP signaling molecules in these cell types, we analyzed expression of all human Smads in primary epithelial and endothelial cells. Despite a slightly decreased expression in epithelial cell fraction, most of the Smads were expressed in both cell types (data not shown).

4.2 Expression of BMP and TGF-β signaling molecules in normal and diseased (IPAH) human lungs

Signaling by TGF-β family members occurs through Type I and Type II serine/threonine kinase receptors. Five Type II and seven Type I receptors, also termed Activin Receptor-Like Kinases (ALKs), have been identified in vertebrates [79]. Members of the TGF-β family play crucial roles in development and tissue homeostasis therefore differences in these receptor expressions in the tissues

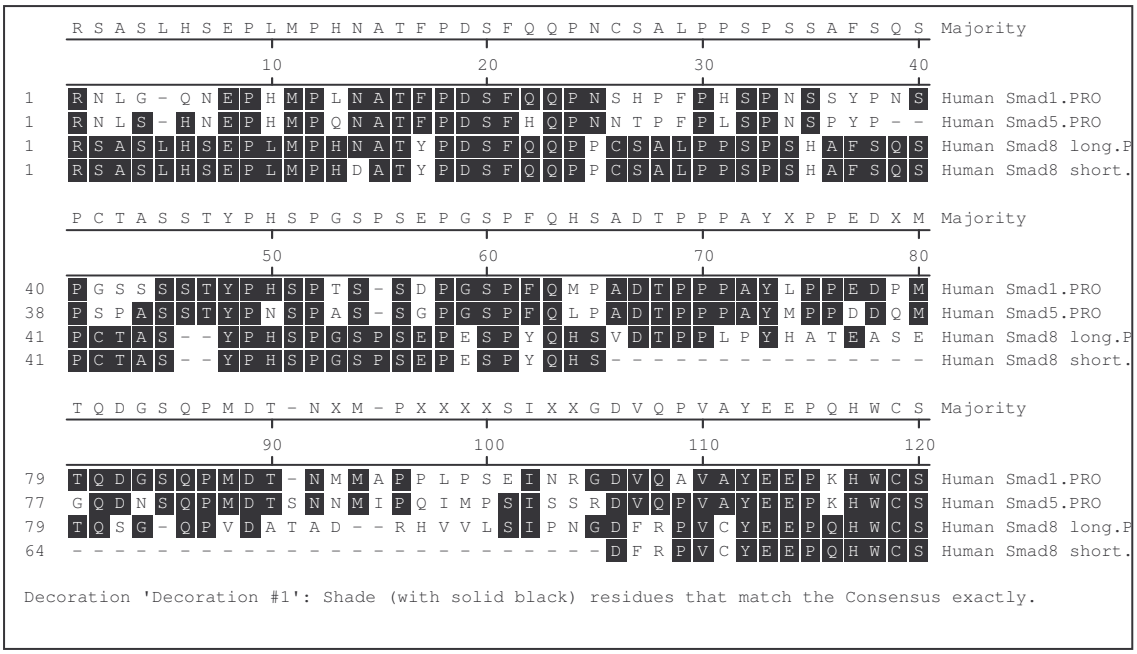
affects cell proliferation and cell death. Here, we analyzed receptor expression within various tissues of the human body. Most of the BMP and TGF-β receptors were expressed in the human tissues. This finding may suggest that receptors of BMP and TGF-β play a role not only during development but also in the functional maintenance of various tissues.

BMP ligands signal by binding the BMP Type II receptor (BMPRII) or the activin Type II receptors (ActRIIa and ActRIIb) in conjunction with Type I receptors to activate Smads 1, 5, and 8 as well as members of the mitogen-activated protein kinase family [80;81]. Loss of function of BMPRII through reduced expression and mutations in the gene is reported as a cause of IPAH. Here, we sought to determine the expression pattern of Smads during this disease. Though BMPRII is known to have reduced expression in the lung during IPAH [14] we did not see major differences in the expression of both BMPRII isoforms. Recent reports have confirmed that lack of tail in the BMPRII did not affect the kinase activity of the receptor. Therefore, we speculated that BMPRII long and short forms might have similar functions and reduction in either of these forms might lead to IPAH. We also observed that Type I receptors for BMP ligands (ALK3 and ALK6) along with ALK2 had no change in the expression during IPAH. Reduced expression or mutation in the BMP signaling molecules causes disturbance in cell division, leading to the disease.

4.3 Smad8 isoforms

Smad8 is a BMP-responsive Smad. Until now, the Smad8 isoform, which lacks the 3rd exon, is known as Smad8 [57;82]. An isoform of human Smad8 (Smad8B) has been functionally characterized and is known to lack the SSXS domain [58].

A)



B)

Smad2	**PETP*PPGY*ISED**
Smad3	**PETP*PPGY*LSED**
Smad4	----------
Smad1	**ADTP*PPAY*LPPE**
Smad5	**ADTP*PPAY*MPPD**
Smad8	----------
Smad8C	**VDTP*PLPY*HATE**
Smad6	**PESP*PPPY*SRLS**
Smad7	**LESP*PPPY*SRYP**

Figure 24: Smad8C possesses a PY motif in its linker domain: Sequence alignment of linker domain of Smad8C (Smad8C) with Smad8 and other BMP signaling molecules, Smad1 and 5 (**A**). Sequencing alignment of PY motif from Smad8C with other known PY motif in the receptor Smads and inhibitory Smads (**B**).

Another form of Smad8, which is Smad8C, has been revealed in this study. A difference of 111bp, which corresponds to 37 amino acids, distinguishes both forms of Smad8 (Figure 24 A). The initial finding of the Smad8C came from a PCR against the full length of Smad8. Several PCR primers were designed to

amplify different regions of Smad8. It has been found that both isoforms are expressed in most human tissues, but the expression of the Smad8C is slightly greater than that of the short form.

To determine the function of the additional sequence (37 amino acids) of Smad8C, the sequence of protein was closely observed for possible conserved domains that are common in the Smads. Interestingly, a domain known to be present in inhibitory Smads and receptor Smads, ”PY motif,” was found in this additional sequence of the Smad8C (Figure 24 B). The inhibitory Smads (Smad6 and Smad7) are known to bind to Smurf1 and 2 (Smad ubiqitination regulatory factor) to inhibit BMP and TGF-β signaling by degrading their receptors by proteosome pathway [65;83-86]. Thus, from the initial sequencing results we hypothesized that Smad8C might play an inhibitory role on BMP or TGF-β signaling by interaction with either of the Smurfs and thereby undergo receptors or receptor Smad degradation.

4.4 Phosphorylation of Smad8 isoforms

Mouse Smad8 phosphorylation is downstream of BMP receptors ALK 2, ALK 3, and ALK 6 [82]. Phosphorylation analysis for human Smad8 isoforms gave interesting new results. Smad8, which is known to be BMP responsive, showed basal phosphorylation without any stimulation. Though an increase in phosphorylation was evident after BMP-2 and BMP-4 stimulation, the basal phosphorylation was much higher, compared to Smad1 and Smad8C. There are several speculations regarding such behavior. The first possibility is a break in the linker region of the Smad8. Linker is known to keep the MH1 and MH2 domains of receptor Smads together and cause them to inhibit each other, preventing receptor Smads from interacting with Type 1 receptors or other transcription factors in the absence of the ligand. The absence of the linker may give MH2 domain more freedom to interact with BMP Type 1 receptor and to be phoshorylated better than any other receptor Smads at the basal level. Another interesting point is the lack of phosphorylation for Smad8C at the basal as well as the activated state. The reason could be that the phospho-Smad1/5/8 antibody

used to detect phosphorylation in the BMP-activated Smads detects phosphorylation in the SSXS domain. With this result, one can only say that no phosphorylation for Smad8C occurred at this major phosphorylation domain, but from the previous literature, it is known that Smads also can get phosphorylated at other structures (e.g., serine and tyrosine rich linker domain) [51]. Here, we speculate that the same could happen with the Smad8C.

4.5 Inhibitory function of Smad8C

Until now, Smad6 and 7 have been the only known inhibitory Smads [76]. Smad8 is known as the BMP responsive transcription factor carrying its signal from the cell membrane to the nucleus. In this study a novel isoform of Smad8 (Smad8C) showed an interesting and opposite effect to the normal receptor Smad. Even though Smad8C appears similar to a receptor Smad with MH1, MH2 domains along with an SSXS motif for phosphorylation, Smad8C could inhibit BMP responsive signal significantly and TGF-β responsive signal to some extent. The fact that an inhibitory Smad contains a phosphorylation domain is intriguing. Another isoform of Smad8, named Smad8B, acts opposite the BMP signaling pathway; however, its inhibition is limited to Smad8 [58].

The inhibitory function of Smad8C was first observed in phosphorylation analysis. Smad8C after BMP-2 and BMP-4 stimulation revealed no signal of phosphorylation indicating that Smad8C might not be a regular receptor Smad. Further, the endogenous Smad1 phosphorylation was reduced when Smad8C was over expressed in A549 cells. These findings may imply that Smad8C might inhibit BMP signal by inhibiting Smad1 phosphorylation. In our dose-dependent experiment when Smad8C was over expressed, there was a significant reduction in the BMP-4 induced phosphorylation of Smad1 which indicates that Smad8C can inhibit Smad1 phosphorylation, supporting our previous data. Though a weak signal for Smad5 was observed after BMP stimulation but not much difference was observed in its phosphorylation. Smad8 was not observed in the control blots because of its low endogenous expression in A549 cells.

Smad8 is known to induce signal transduction on BRE4, a BMP reporter upon BMP stimulation and act as a transcription factor. Where as, in this study Smad8C, which contains PY motif inhibited BMP induced luciferase expression on BRE4 reporter. Smad8C could also show its inhibitory action when co-transfected with constitutively active ALK2 which mimics BMP stimulation. The similar inhibitory action was also observed on Id-I, another reporter gene for BMP ligands (data not shown). CAGA, a TGF-β reporter was also screened for Smad8C activity, where a slight inhibition of TGF-β mediated transcriptional activity was observed. All these results showed Smad8C as possible inhibitory Smad.

As expected, Smad8 could increase BMP signal at the basal and stimulated states when overexpressed in A549 cells. In contrast, Smad8C did not show its inhibitory role when Smad8 was overexpressed along with Smad8C. This experiment implies that Smad8C may not inhibit BMP induced Smad8 transcriptional activity which is different from Smad8B. These results also suggested that Smad8C may not only inhibit Smad1 or Smad5, since Smad1 phosphorylation decreased in the presence of Smad8C in A549 cells. It is still difficult to speculate about the regulation of Smad5 transcriptional activity because of the lack of individual phospho-specific antibodies.

4.6 Increased expression of Smad8C upon BMP-2 and BMP-4 stimulation

Smad6 and Smad7 are known to increase their expression after BMP and TGF-β stimulation [87]. As Smad8C functions much similar to inhibitory Smads, we also analyzed its expression after BMP and TGF-β stimulation. BMP-2 and BMP-4 could dramatically increase Smad8C expression. TGF-β induction of Smad8 expression was not as significant as BMP. The induction in the expression of Smad8 has been shown both at RNA and protein level. This observation may suggest that Smad8C inhibition on BMP signaling is through a feedback mechanism. Interestingly, the increase in expression was found in only Smad8C but not in Smad8.

Expression of most genes depends on their promoter activity. As there was a significant change in the expression of Smad8 after BMP stimulation, the promoter of Smad8 was also analyzed for its activity by luciferase reporter gene assay. No significant change was observed in its promoter activity in A549 cells after BMP-2 and BMP-4 stimulation (data no shown). This result may indicate the fact that the real promoter for Smad8 stayed more upstream (before -2000bp) of the Smad8 open reading frame (ORF). We also speculate that an enhancer sequence may be needed for Smad8 gene expression, which might be lying very far from the Smad8 gene locus.

4.7 Smad8C can inhibit BMP signal transduction by degrading Smad1

Inhibition of BMP signaling through Smad8C could take place in several ways. Based on our results, there could be two ways of possible inhibition: One through the degradation of Smad1 or Smad5 and the second by blocking of the phosphorylation of Smad1 and Smad5. To determine if the Smad8C-induced inhibition of BMP signaling was through Smad1, Smad1 expression was analyzed after Smad8C overexpression after BMP-2 and BMP-4 stimulation. Interestingly, degradation of Smad1 at the initial stages (8-16 hours) of BMP stimulation was observed, but its levels were resumed to normal at the later stages (36 hours). This data may suggest that the Smad8C induced inhibition of BMP signaling is only through the degradation of phosphorylated Smad1 but not the native Smad1. Increased expression of Smad8C after BMP stimulation and its action inhibiting BMP signaling appeared to supports this hypothesis. Additionally, we did not observe any degradation of Type 1 receptor (ALK 6) after BMP stimulation.

Several reports indicate that phospho-Smad1 levels become low after 4 to 6 hr of BMP stimulation [88]. The reasons for this phenomenon are unclear but one possibility could be that a phosphatase that might get activated after BMP stimulation and inhibit Smad1 phosphorylation. Another possibility is that a protease that could degrade phospho-Smad1 might also get activated upon BMP stimulation. Recently it has been shown that receptor Smads can be degraded by Smurf1 or Smurf2 with the help of inhibitory Smads (Smad6 and Smad7) after

prolonged stimulation with BMP or TGF-β [86;89;90]. In this context, it is speculated that once Smad8C is expressed after BMP stimulation, it could easily access Smurf1 or 2 with its PY motif and degrade receptor Smads. Thus, Smad8C mediated inhibition of BMP signaling may occur through degradation of Smad1 with the help of Smurfs.

4.8 Cellular localization of Smad8 isoforms

Variation in cellular localization is a common feature in receptor Smads. Most of the receptor Smads stays in the cytoplasm in the native state and moving towards the nucleus once they get phoshorylated by their concerned receptor [79]. Smad8 is also known to localize in the cytoplasm and moves towards nucleus upon phosphorylation by Type 1 receptors [55]. In the immuno-staining experiments, it was observed that Smad8 was present in both cytoplasm and nucleus before stimulation and moving towards nucleus after stimulation. This result supports our previous phosphorylation data, as we observed Smad8 phosphorylation at the basal level.

In contrast, it was interesting to observe that most of the Smad8C was in the cytoplasm before and after stimulation. It was moving towards the nucleus once the cells were stimulated with BMP-2 or BMP-4. In this study movement of Smad8C was not expected after BMP stimulation as it does not contain any phosphorylation domain. With these results one can speculate that Smad8 once expressed may bind to receptor Smads and moves towards the nucleus after stimulation. Thus, Smad8C may possibly bind to Smad1 and move towards the nucleus and there after degrading it either in the nucleus or in the cytoplasm. This phenomenon might explain the way that Smad8C could inhibit BMP signaling.

4.9 Inhibition of BMP signaling by Smad8C is via Smads 1 or 5 or 8?

In this study, it has been shown that human Smad8 isoforms (Smad8, Smad8C) differ in 37 amino acids in the linker region, which enable them to act

differently at the functional level. The expression studies of both Smad8 isoforms in different tissues confirm their importance in the maintenance of the tissues. Even though both isoforms are expressed in most tissues, Smad8C expression is much higher than that of Smad8. In this case, the enzymes that can splice the Smad8C RNA and produce the short isoform may play a role in maintaining the homeostasis between the two isoforms. However, a balance between receptor Smads for BMP (Smad1, 5, and 8) is also necessary, since any of them could take BMP signaling from the membrane to the nucleus. During the functional studies it has been shown that Smad1 gets degraded by Smad8C, but the same is not true for Smad8. These findings show that Smad8C has a unique way on its inhibitory action of BMP signaling. So understanding the regulation of these molecules has a greater importance.

5. Future Directions

Crystallizing recombinant Smad8 and resolving its tertiary structure would help to understand constitutively active nature of Smad8. It would also resolve the structure of Smad8C and explains the reasons behind its non-phosphorylated state after stimulation even though it possesses the SSXS motif. Possible interaction of Smad8C with Co-Smad (Smad4) or other receptor Smads should be analyzed, as it plays an inhibitory role through degradation of Smad1. Interaction of Smad8C with Smurf1 and Smurf2 should be the focus of immediate analysis as Smurfs play a major role in the degradation of receptors and thereby inhibit BMP and TGF-β signaling. Promoter or enhancer of the Smad8 should be studied more closely, as Smad8 expression increases after BMP-2 and BMP-4 stimulation. Smad8 promoter should be cloned in front of the luciferase reporter gene, and its expression after BMP stimulation should be analyzed.

6. Summary

Idiopathic pulmonary arterial hypertension (IPAH) is associated with structural changes to the pulmonary vasculature that ultimately lead to right ventricular failure. A breakthrough in our understanding of the pathogenesis of IPAH was identification in IPAH patients of inactivating heterozygous mutations in the gene encoding the type II bone morphogenetic protein (BMP) receptor (BMPRII). Bone morphogenetic proteins and transforming growth factor (TGF)-β are a superfamily of polypeptide ligands that transduce signals via their cognate receptors and the Smad family of transcription factors. There are eight different Smads, of which Smads 1, 5 and 8 are BMP receptor-activated Smads. In this study we describe a novel splice-isoform of human Smad8 (Smad8C), and present its preliminary characterization.

Initially, the expression of BMP receptors and their associated Smads in healthy donor lungs and in lungs from patients with IPAH was assessed by reverse-transcription polymerase chain reaction (RT-PCR). No changes in mRNA expression were observed comparing lungs from donors and IPAH patients. However, during the Smad expression studies, a Smad8 splice-variant with a higher molecular mass (which we have called Smad8C) was identified. Sequencing of the full-length Smad8C transcript revealed that the transcript contained an insertion of an additional 111 nucleotides, encoding an additional 37-amino acid residue insertion in the Smad8C polypeptide chain. This additional coding sequence inserted a PY domain (which directs protein stability) into the important regulatory “linker” region of Smad8, located between the phosphorylation (MH1) and DNA binding (MH2) domains. Unlike all the receptor-activated Smads, Smad8C did not exhibit any phosphorylation after BMP or TGF-β stimulation, but rather dose-dependently inhibited the phosphorylation of endogenous Smad1 upon over-expression of Smad8C in human lung epithelial A549 cells. Furthermore, when Smad8C was over-expressed in A549 cells, the BMP-induced expression of the luciferase gene, when placed downstream of the BRE4 BMP-response element, was inhibited. These data demonstrated that Smad8C could attenuate BMP signaling.

In conclusion, we have identified a novel human Smad8 isoform (Smad8C) containing an additional insertion in its linker region. This novel Smad8 splice-isoform appears to negatively-regulate BMP-signaling in lung epithelial cells.

7. Zusammenfassung

Bei der primären pulmonalen arteriellen Hypertonie (IPAH) sind die Blutgefäße in der Lunge stark verengt, wodurch der Widerstand und somit der Druck in diesen Gefäßen steigt. Langfristig führt der erhöhte Druck auch zu Veränderungen in der Struktur der Blutgefäße in der Lunge, dem so genannten Remodelling. Durch eine zunehmende Obliteration der Lungenstrombahn kommt es zu einer progredienten Belastung des rechten Herzens, die in einem Rechtsherzversagen resultieren kann.

In einem Großteil der Patienten mit IPAH liegen Keimbahnmutationen im BMP Typ II Rezeptor (BMPR2) vor. Bone morphogenic proteins und Transforming Growth Factor-ß (TGF-ß) vermitteln ihre Signale über Membran-gebundene Serin/Threonin Kinase Rezeptoren, die nach Stimulation Smad Proteine phosphorylieren und dadurch deren nukleäre Translokation und transkriptionelle Aktivierung auslösen. Die Rezeptor-aktivierten Smads 1, 5 und 8 werden über BMP Rezeptoren phosphoryliert. In der vorliegenden Arbeit wird die funktionelle Charakterisierung der neuen Smad Isoform Smad 8 beschrieben.

Die Analyse der Genexpression von BMP Rezeptoren und deren assoziierten Smad Proteine mittels Polymerase-Kettenreaktion (PCR) ergab, dass in Lungenhomogenaten von IPAH Patienten keine Änderung der Expression von BMP Rezeptoren und den bisher bekannten Smad Proteinen zu beobachten ist. Jedoch konnte eine neue, bislang nicht charakterisierte Isoform von Smad8 identifiziert und vollständig sequenziert werden, die als Smad8C bezeichnet wurde. Die Basensequenz von Smad8C unterscheidet sich von Smad8 durch 111 zusätzliche Nukleotide, die für 37 zusätzliche Aminosäuren kodieren. Innerhalb dieser Aminosäuresequenz, die zwischen der Phosphorylierungs- und DNA-Bindungsdomäne von Smad8C lokalisiert ist, konnte ein als PY-Domäne bezeichnetes Sequenzmotiv nachgewiesen werden, welches die Proteinstabilität beeinflusst. Im Gegensatz zu Rezeptor-aktivierten Smads wird Smad8C nicht durch die BMP- oder TGF-ß vermittelte Stimulation phosphoryliert, sondern bewirkt eine dosisabhängige Inhibierung der

Phosphorylierung von endogenem Smad1 in der epithelialen Lungenzelllinie A549. Zudem resultierte die Überexpression von Smad8C in A549 Zellen in einer signifikanten Abnahme der Aktivität des BMP-abhängigen BRE4-Luciferase Reportergens. Die vorliegenden Daten geben somit Grund zu der Annahme, dass die identifizierte Smad8 Isoform eine BMP-vermittelte Signaltransduktion inhibitorisch moduliert.

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Erklärung

“Ich erkläre: Ich habe die vorgelegte Dissertation selbständig, ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten oder nicht veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der” Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis” niedergelegt sind, eingehalten.“

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