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Functional Characterization of Bone Morphogenetic Protein Binding Sites and Smad1/5 Activation in Human Vascular Cells[S]

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ABSTRACT

Mutations in the bone morphogenetic protein (BMP) type II receptor (*BMPR2*) gene cause familial pulmonary arterial hypertension (FPAH), a disease characterized by excessive smooth muscle and endothelial cell proliferation. However, the specific receptors mediating responses to BMPs in human vascular cells are not known. We show that human pulmonary artery smooth muscle cells (HPASMCs) express high specific ^{125}I-BMP4 binding, whereas human microvascular endothelial cells (HMEC-1) and human pulmonary artery endothelial cells (HPAECs) exhibit low binding. BMP4 competes for both high- and low-affinity ^{125}I-BMP4 binding sites on HPASMCs, yet BMP2 competes only at the low-affinity binding sites. In addition, BMP4, but not BMP2, induced Smad1/5 phosphorylation at low concentrations in HPASMCs. Conversely, HMEC-1 cells exhibited a single binding site population with equal affinity for BMP2 and BMP4. In both cell types, growth differentiation factor-5 (GDF5), BMP6, and BMP7 stimulated Smad1/5 phosphorylation and competed for ^{125}I-BMP4 less efficiently than BMP2 or BMP4. HPAECs exhibited weak Smad responses to BMPs. Expression analysis suggested the low binding in endothelial cells corresponded to lower ALK3 and ALK6 expression. Although transfection of small interfering RNAs (siRNAs) for ALK3 and BMPR-II abrogated Smad1/5 phosphorylation to BMP4, BMP2, and GDF5 in HMEC-1 and HPASMCs, they had little effect on ^{125}I-BMP4 binding. ALK6 siRNA did not alter binding or Smad1/5 responses, even to GDF5, a reported ALK6 selective ligand. Therefore, ALK3/BMPR-II is the BMP4/BMP2/GDF5-responsive receptor in human vascular cells, but these studies suggest that a BMP4/GDF5 selective binding protein exists in HPASMCs. These cell-specific differences in BMP responses are important for understanding the pathogenesis of FPAH.

Members of the transforming growth factor (TGF) receptor superfamily have vital roles in mammalian development. Their ligands include TGFβ_{1-3}, the bone morphogenetic proteins (BMPs), activins, and growth differentiation factors (GDFs). TGF receptor superfamily members transduce signals by formation of heterotetrameric signaling complexes of relevant type I and II receptors, although the mechanisms of binding and complex formation by TGFβ, activins, and BMPs differ. TGFβ and activins bind to their higher affinity type II receptors, leading to recruitment of a relevant lower affinity type I receptor (ten Dijke et al., 1994a; Wrana et al., 1994). In contrast, BMPs were thought to bind the higher affinity type I receptors, leading to recruitment of a low-affinity type II receptor (Koenig et al., 1994; ten Dijke et al., 1994b; Macias-Silva et al., 1998; Kirsch et al., 2000). BMP2 and BMP4 are considered to bind to the same receptors, comprising ALK2, ALK3, or ALK6 in combination with BMPR-II, Act-RII, or ActR-IIB (ten Dijke et al., 1994b; Macías-Silva et al., 1998). However, the BMP5/6/7/8 subfamily may bind their type II receptors with higher affinity (Barbara et al., 1999; Aoki et

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ABBREVIATIONS: TGF, transforming growth factor; BMP, bone morphogenetic protein; GDF, growth differentiation factor; HMEC-1, human microvascular endothelial cell; HPAEC, human pulmonary artery endothelial cell; HPASMC, human pulmonary artery smooth muscle cells; BAMBI, BMP and activin membrane-bound inhibitor; RGM, repulsive guidance molecule; MAP, mitogen-activated protein; PAH, pulmonary arterial hypertension; FPAH, familial pulmonary arterial hypertension; *BMPR2*, bone morphogenetic protein type 2 receptor gene; BMPR-II, bone morphogenetic protein type II receptor; ActR-II, activin receptor type II; ActR-IIB, activin receptor type IIB; ALK, activin-like kinase; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; BSA, bovine serum albumin; TBS-T, Tris-buffered saline/Tween 20; 5% B, 5% (w/v) BSA; 5% M, 5% (w/v) nonfat milk; HRP, horseradish peroxidase; PCR, polymerase chain reaction; RT, reverse transcriptase; QPCR, quantitative polymerase chain reaction; C_T, threshold cycle; RISC, RNA-inducing silencing complex.

al., 2001). GDF5 binds to ALK6 and is reported to signal via ALK6/BMPRII or ALK6/ActRII (Nishitoh et al., 1996).

Although ALKs and type II receptors comprise the core signaling receptor complex for BMPs, an emerging array of coreceptors may modulate binding of BMPs to the core complex. BMPs have been shown to bind to the accessory receptors endoglin and betaglycan, and the inhibitory pseudoreceptor BAMBI (Barbara et al., 1999; Onichtchouk et al., 1999). Furthermore, the repulsive guidance molecules RGMa, RGMb and RGMc have been shown to function as accessory receptors that bind BMPs and enhance the BMP-responsiveness of the ALK/type II receptor complex (Babitt et al., 2005; Samad et al., 2005; Babitt et al., 2006)

Upon ligand-induced activation of the BMP receptor complex, the constitutively active domain of the type II receptor phosphorylates the type I receptor, which then directly phosphorylates and activates the specific intracellular signaling proteins Smad 1, Smad 5, and Smad 8 (Liu et al., 1995; Miyazono et al., 2001). Upon phosphorylation, these Smads associate with the co-Smad, Smad4, and translocate to the nucleus. There, in complex with a variety of other transcription factors, they bind to specific promoters to alter the expression of a wide range of genes. However, BMP receptors can also activate MAP kinase pathways such as p38 and extracellular signal-related kinase 1/2 (Miyazono et al., 2001). Activation of Smad or MAP kinase pathways is reported to be dependent on the mode of receptor interaction. Binding of ligand to type i receptor homodimers leads to recruitment of the type II receptors, leading to Smad activation. In contrast, if the ligand binds to preformed complexes of type I and II receptors, the MAP kinase pathways are preferentially activated (Nohe et al., 2002).

The reported disease-causing mutations in the bone morphogenetic protein type 2 receptor (*BMPR2*) gene in familial pulmonary arterial hypertension (FPAH) exposed a hitherto unknown role for BMPs in the human pulmonary circulation (Lane et al., 2000). We have shown that *BMPR2* mutations in FPAH result in impaired trafficking and coupling of BMPR-II protein to signal transduction pathways (Rudarakanchana et al., 2002). Moreover, BMPR-II protein expression is markedly reduced in the lungs of patients with nonfamilial PAH, implying that reduced BMPR-II function is important in the pathobiology of other forms of PAH (Atkinson et al., 2002).

Although the intracellular mechanisms mediating the functional responses of human vascular cells to BMPs are becoming clearer, the contributions of BMPR-II and the BMP type I receptors, ALK3 and ALK6, to ligand binding and signaling are not well characterized. Here we show that ALK3/BMPR-II is the dominant complex mediating Smad1/5 phosphorylation in response to BMP2 and -4 and, contrary to expectation, GDF5 in human vascular cells. In human pulmonary artery smooth muscle cells (HPASMCs), BMP4 and GDF5 competed with high affinity for ^{125}I-BMP4 binding sites that were insensitive to BMP2. Consistent with this, low concentrations of BMP4, but not BMP2, stimulate Smad1/5 phosphorylation in HPASMCs. In HMEC-1 cells, BMP2 and BMP4 exhibited equal potency in terms of ^{125}I-BMP4 competition and Smad1/5 phosphorylation. It is noteworthy that knockdown of ALK3, ALK6, or BMPR-II had no significant effect on ^{125}I-BMP4 binding in all cell types. This observation suggests the existence of accessory proteins mediating BMP4 binding, particularly in HPASMCs. These data highlight important functional differences in BMP responsiveness between vascular smooth muscle and endothelium, which may have important implications for our understanding of how BMPR-II mutation affects vascular cell function in FPAH.

Materials and Methods

Reagents. DMEM, Opti-MEM I, type II collagenase, fetal bovine serum (FBS), antibiotic/antimycotic solution, and trypsin-EDTA were purchased from Invitrogen (Paisley, Renfrewshire, UK). Recombinant human TGFβ_1, BMP2, BMP4, BMP6, BMP7, Activin A, mouse GDF5, ALK3-Fc, ALK6-Fc, BMPR-II-Fc, and human BMP4 enzyme-linked immunosorbent assay were from R&D Systems (Abingdon, Oxon, UK). Na^{125}I was from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK). Antibodies to phospho-Smad1/5, Smad 1, and phospho-p38 were from Cell Signaling Technology (Danvers, MA). The BMPR-II antibody was from BD Transduction Laboratories (BD Biosciences Pharmingen, Cowley, Oxon, UK). Monoclonal anti-human β-actin antibody was from Sigma (Poole, Dorset, UK). siFectamine was from IC-VEC Ltd. (London, UK) DharmaFECT1, Dharmacon BMPR-II siGenome Smartpool, Dharmacon ALK3 and ALK6 On-TARGETplus, Smartpool siGLO RISC-free siRNA, and On-TARGETplus siControl Nontargeting Pool were from Perbio Science UK Ltd. (Chester, Cheshire, UK). All other chemicals were from Sigma or Merck (Lutterworth, Leicestershire, UK).

Primary Cell Culture and Cell Lines. The isolation and characterization of the explant-derived HPASMC from lung resection specimens that were used in this study has been described previously (Morrell et al., 2001). Papworth Hospital ethical review committee approved the use of these tissues. The human microvascular endothelial cell line, HMEC-1 (Ades et al., 1992), was obtained from the Centers for Disease Control and Prevention (Atlanta, GA). HPAECs were purchased from Lonza Wokingham (Wokingham, Berkshire, UK). NIH-3T3 fibroblasts were purchased from the European Collection of Cell Cultures (Salisbury, Shropshire, UK). Cells were propagated according to the instructions supplied.

Iodination of Human BMP4 and BMP6. Human recombinant BMP4 and BMP6 were iodinated using the Chloramine T method as described previously for TGFβ_1 (Frolik et al., 1984). In brief, 500 ng (5 μl) of carrier-free human BMP4 or BMP6 was mixed with 10 μl of 0.5 M potassium phosphate buffer and 0.5 mCi of Na^{125}I. For BMP4, three 5-μl aliquots of 100 μg/ml Chloramine T were added sequentially, followed by incubations for 120, 90, and 60 s, respectively. For BMP6, only the first two additions were made. Reactions were terminated by addition of 50 mM *N*-acetyl-L-tyrosine (20 μl), 60 mM potassium iodide (200 μl), and 0.66 M urea (200 μl, dissolved in 1 M acetic acid) followed by fractionation on a G-75 Sephadex column equilibrated with elution buffer [4 mM HCl, 75 mM NaCl, and 0.1% (w/v) BSA], and 0.5-ml fractions were collected. The sizes of the iodinated products were confirmed using SDS-polyacrylamide gel electrophoresis and autoradiography. Elution buffer containing 4% (w/v) BSA (167 μl) was added to each peak fraction and binding tested on NIH-3T3 cells. The concentration of ^{125}I-BMP4 in the active fraction was measured with a specific enzyme-linked immunosorbent assay. The specific activity of the ^{125}I-BMP4 radioligand ranged from 990 to 1889 Bq/fmol (actual molecular mass = 36 kDa) between iodinations.

Receptor Binding Studies. For competition and saturation binding studies, cells were grown to confluence in 24-well plates. ^{125}I-BMP4 competition binding was performed as described previously except that cells were maintained in DMEM/10% (v/v) FBS (Jeffery et al., 2005). In brief, cells pre-equilibrated in binding buffer (DMEM/0.5% BSA containing 25 mM HEPES) for 60 min at 4°C were incubated at 4°C for 3 h with binding buffer containing ^{125}I-BMP4 (~6 pM or 0.22 ng/ml) in the absence or presence of unlabeled BMP4 (0.01–300 ng/ml), BMP2,

BMP6, BMP7, TGFβ_1, or Activin A (all 0.3–300 ng/ml) or GDF5 (0.3–1500 ng/ml). Cells were then washed three times with ice-cold binding buffer and solubilized in lysis buffer [20 mM HEPES, pH 7.4, containing 10% (v/v) glycerol, 1% (v/v) Triton X-100, and 0.05% (w/v) BSA]. Each point was determined in triplicate for each experiment. Lysates were transferred to polystyrene tubes, and radioactivity measured with a gamma counter (PerkinElmer Life and Analytical Sciences, Waltham, MA).

For saturation binding studies, cells were incubated at 4°C for 3 h with binding buffer containing ^{125}I-BMP4 (0.1–100 pM or 3.67–3670 pg/ml) in the absence or presence of 500 ng/ml unlabeled BMP4. Each point was determined in triplicate for each experiment. Cells were washed three times with ice-cold binding buffer and lysed for 20 min in lysis buffer. Lysates were counted as described above.

Binding data were analyzed by an F test using Prism 3.0 (GraphPad Software, San Diego, CA) to determine whether binding was best explained by a one- or two-site model. The degree of competition by 300 ng/ml unlabeled BMP4 was defined as 100% specific binding. The relevant nonlinear regression analysis was used to calculate IC_{50} values for competitors.

Western Blotting. HPASMCs, HPAECs, and HMEC-1 cells were grown to confluence in six-well plates. Cells were washed and incubated in DMEM/0.1% FBS overnight. DMEM/0.1% FBS (pre-equilibrated to 37°C) either alone or containing BMP2, BMP4, BMP6, BMP7, or GDF5 was added for 1 h at 37°C. Cells were then snap-frozen on dry ice/ethanol and lysed in 100 μl of ice-cold lysis buffer [125 mM Tris-HCl, pH 7.4, 10% (v/v) glycerol, and 2% (w/v) SDS containing an EDTA-free protease inhibitor cocktail (Roche Diagnostics Ltd., Lewes, East Sussex, UK)]. Lysates were sonicated and frozen at −20°C until protein assay and Western blot analysis.

For Western blotting, lysates (30–50 μg total protein) were separated on 12% resolving SDS-polyacrylamide gels, and proteins were transferred to nitrocellulose by semidry blotting. For phospho-Smad1/5 and Smad 1 detection, membranes were blocked in TBS-T [50 mM Tris-HCl, pH 7.4, 137 mM NaCl, 0.05% (w/v) Tween 20] containing 5% (w/v) BSA (5% B) and 5% (w/v) nonfat milk (5% M) for 1 h at RT. Membranes were briefly rinsed with TBS-T and incubated with primary antibody against either phospho-Smad1/5 (1:1000) or Smad 1 (1:750) in TBS-T/5% B overnight at 4°C. Blots were washed with TBS-T and incubated with goat-anti-rabbit IgG-HRP (Dako UK Ltd., Ely, Cambridgeshire, UK) at 1:2000 dilution in TBS-T/5% M. For BMPR-II detection, blots were blocked in TBS-T/5% M and incubated with a mouse anti-BMPR-II monoclonal antibody (BD Biosciences) at 1:250 dilution in TBS-T/5% M. Blots were washed with TBS-T and incubated with goat anti-mouse IgG-HRP (Dako UK Ltd.) at 1:2000 dilution in TBS-T/5% M. For β-actin detection, membranes were blocked in TBS-T/5% M for 30 min at room temperature followed by incubation with mouse anti-human β-actin antibody at 1:5000 in TBS-T/5% M for 30 min at room temperature. Blots were rinsed with TBS-T and then incubated with rabbit-anti-mouse IgG-HRP at 1:5000 dilution in TBS-T/5% M for 30 min at RT. Blots were then washed with TBS-T and bound complexes detected using enhanced chemiluminescence (GE Healthcare).

RT-PCR for TGF Receptor Superfamily Members. Total RNA was extracted from cells incubated in either DMEM/0.1% FBS or DMEM/10% FBS for 24 h using QIAGEN RNeasy Mini columns with the DNase digestion protocol (QIAGEN Ltd., Crawley, West Sussex, UK) according to the manufacturer's instructions. Total RNA was reverse transcribed and amplified by PCR using a one-step RT-PCR kit (Access RT-PCR System; Promega, Southampton, Hampshire, UK) in a total volume of 20 μl with 0.8 μg of total RNA, 0.25 μM concentrations of each of the relevant upstream and downstream primers (Supplemental Table), and 1.5 mM magnesium sulfate. RT-PCR reactions were amplified on a PCR Express Thermocycler (Thermo Fisher Scientific, Basingstoke, Berkshire, UK). Reactions were incubated at 48°C for 45 min and heated to 95°C for 5 min. This was followed by 35 cycles of denaturation at 95°C for 60 s, annealing at the specified temperature (Supplemental Table) for 90 s, and extension at 72°C for 60 s. A final extension was performed at 72°C for 7 min. Each RT-PCR was performed on at least two separate occasions to demonstrate reproducibility. To ensure that genomic DNA was not being amplified, control reactions were run replacing the reverse transcriptase with nuclease-free water. RT-PCR products (10 μl) were analyzed by fractionation on a 2% agarose gel containing 0.35 μg/ml ethidium bromide.

For semiquantitative RT-PCR for ALK3, ALK6, BMPR-II, and β-actin, 15-μl reaction mixes were prepared using the relevant primers described above (Supplemental Table) and either 100 ng (ALK3 and ALK6) or 10 ng (BMPR-II or β-actin) of DNase-digested RNA. Reactions were amplified according to the protocol described above, except that products were amplified for 20 cycles (β-actin), 24 cycles (ALK3 and BMPR-II), or 26 cycles (ALK6) at the relevant temperatures (Supplemental Table).

Quantitative RT-PCR for BMP Receptors and Their Associating Proteins. DNase-digested total RNA (450 ng) was reverse-transcribed using Superscript III First Strand Supermix (Invitrogen) as described in the manufacturer's instructions. RNA was removed by RNase H treatment. QPCR reactions were prepared with 45 ng of cDNA using the SYBR Green Jumpstart *Taq* Readymix (Sigma) containing 200 nM concentrations of the relevant sense and antisense primers and 10 nM fluorescein (Invitrogen). Specific primers were used for ALK3 (sense: 5′-TTCGTATGACGGATCACTCG-3′; antisense: 5′-AGCCCTACATCATGGCTGAC-3′), BMPR-II (sense: 5′-CAAATCTGTGAGCCCAACAGTCAA-3′; antisense: 5′-GAGGAAGAATAATCTGGATAAGGACCAAT-3′), and β-actin (sense: 5′-GCACCACACCTTTCTACAATGA-3′; antisense: 5′-GTCATCTTCTCGCGGTTGGC-3′). Quantitect Primers for ALK2, ALK6, ActR-II, ActR-IIB, BAMBI, betaglycan, endoglin, RGMa, RGMb, RGMc, and 18S ribosomal RNA were purchased from QIAGEN. Reactions were amplified on an iCycler (Bio-Rad Laboratories, Hemel Hempstead, Hertfordshire, UK). The efficiency of each primer set was confirmed to be between 90 and 110% for HPASMC and HMEC cDNA before determination of relative mRNA expression patterns. For each QPCR plate, cDNA samples from HPASMCs, HMEC-1, and HPAECs were compared for the same gene set, and the housekeeping genes 18S rRNA and β-actin were included on every plate as a reference. Using the GeNorm program, we determined that expression of the housekeeping genes 18S rRNA and β-actin between equivalent amounts of cDNA from HPASMCs, HMEC-1, and HPAECs (n = 3 of each), were stable (M value = 0.687 for each gene) (Vandesompele et al., 2002). We applied the normalization factors generated in GeNorm to compare the relative expression of each gene between cell types. The relative expression levels of specific mRNAs in HMEC-1 and HPAECs were compared with their expression in HPASMCs using the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001). As we had demonstrated stability of both 18S rRNA and β-actin, we applied the assumption of equal PCR threshold values for equal transcript numbers of different genes, and used the $\Delta\Delta C_T$ method to calculate the relative expression of each gene to β-actin after normalizing to 18s rRNA (Livak and Schmittgen, 2001; Vandesompele et al., 2002). For quantifying altered receptor expression in siRNA experiments, 200 to 500 ng of RNA, depending on the yield for each experiment, was reverse transcribed, and 20 to 50 ng of cDNA was used in the final reaction.

Receptor-Fc Radioligand Binding. Soluble recombinant ALK3-Fc, ALK6-Fc, or BMPR-II-Fc proteins were diluted in TBS containing 1× casein (TBS/casein) and incubated overnight (100 μl/well) on protein A-coated 96-well plates (Perbio Science UK Ltd.) at 4°C. Plates were then washed once with TBS/casein and blocked with TBS/casein for 2 h at 4°C. The blocking buffer was removed, and 50 μl of TBS/casein was added alone or containing competitors (200–6000 ng/ml). ^{125}I-BMP4 (50 μl; ~12 pM or 0.44 ng/ml) was added to each well, and plates were incubated overnight at 4°C. Plates were then washed three times with TBS/casein. Individual wells were then separated and placed in polystyrene tubes followed by counted in a gamma counter.

siRNA for BMP Receptors. To determine the contributions of ALK3, ALK6, and BMPR-II to receptor binding, HPASMCs and

HMEC-1 cells were seeded in 24-well plates for binding studies or six-well plates for protein extraction and grown to approximately 70% confluence after 2 days. Cells were incubated in Opti-MEM I for 3 h followed by treatment with Opti-MEM I containing siFectamine alone or with siRNA to BMPR-II. Cells were transfected with either RISC-free siGlo or On-TARGETplus siControl Nontargeting Pool as stated. Transfection mixes, containing 10 nM siRNA where appropriate, were added in a final volume of 2 ml/well of Opti-MEM containing 2.22 μg of siFectamine for six-well plates and 364 μl Opti-MEM/well containing 0.404 μg of siFectamine for 24-well plates. Cells were incubated with the transfection mixtures for 4 h at 37°C, followed by replacement with DMEM/10% FBS for 24 h. Cells were then incubated for a further 24 h in DMEM/10% FBS for binding or DMEM/0.1% FBS for protein. Because IC-VEC Ltd. ceased to exist during this study, later transfections were achieved using Dharmafect 1 (4 μl/well in 2 ml for six-well plates or 1 μl/well in 500 μl for 24-well plates). For every experiment, parallel wells were transfected and incubated in the relevant medium for RNA extraction as described above. Specific reduction of the relevant RNA was quantified using QPCR.

Results

^{125}I-BMP4 Binding Sites Exhibited Cell Specific Pharmacological Profiles. We sought to characterize ^{125}I-BMP4 binding sites on human vascular cells by examining the relative abilities of BMP2, BMP4, BMP6, BMP7, GDF5, activin A, and TGFβ_1 to compete for ^{125}I-BMP4 binding. Based on previous reports, we expected that BMP2, BMP4, and (to a lesser extent) BMP6 would identify ALK3 and ALK6 binding (ten Dijke et al., 1994b). Likewise, BMP6 and BMP7 were used to identify BMPR-II and ALK2; GDF5 was used to identify ALK6, and we used activin A and TGFβ_1 to confirm specificity (Nishitoh et al., 1996; Macías-Silva et al., 1998). As expected, unlabeled BMP4 competed for ^{125}I-BMP4 binding in all cell lines studied (Table 1, Fig. 1, A–C). Specific ^{125}I-BMP4 binding was similar in NIH-3T3 and HPASMCs but much lower in HMEC-1 cells and HPAECs (Table 1). The level of binding to HMEC-1 cells enabled construction of competition curves, whereas ^{125}I-BMP4 binding to HPAECs was too low to allow accurate curves to be constructed.

BMP4 and BMP2 competed for ^{125}I-BMP4 binding with equal affinity on NIH-3T3 and HMEC-1 cells (Table 1, Fig. 1, A and C). Furthermore, ^{125}I-BMP4 binding to NIH-3T3 (Fig. 1A) and HMEC-1 cells (Fig. 1C) competition curves demonstrated a single population of binding sites. We confirmed this in NIH-3T3 by saturation binding (Fig. 1A, inset).

In stark contrast to the other cell types examined, HPASMCs exhibited two populations of binding sites with high (28.2 ± 16.9% of the total specific binding) and low affinity in both competition and saturation binding assays (Fig. 1, B and inset; Table 1). Although BMP4 competed for binding to both populations, BMP2 was unable to compete at the high-affinity population, suggesting BMP4 specificity. BMP6, which is reported to bind to receptor complexes containing ALK2, ALK3, and ALK6, competed with lower affinity for ^{125}I-BMP4 binding than unlabeled BMP4 or BMP2, again only at the lower affinity binding sites. Furthermore, BMP7, which selectively binds complexes containing ALK2, competed only weakly at the highest concentration used (300 ng/ml) (Table 1, Figs. 1, A–C), suggesting that ALK2 does not contribute to our observed binding. As we expected, TGFβ_1 and activin A did not compete for ^{125}I-BMP4 binding (Table 1). GDF5 competed for ^{125}I-BMP4 binding in all cell lines with relatively high affinity (Table 1). However, 300 ng/ml GDF5 only competed for a proportion of the specific binding (54.7 ± 4.9% in NIH-3T3, 23.1 ± 5.6% in HPASMCs, and 54.9 ± 15.0% in HMEC-1). We suggested that this reflected the specificity of GDF5 for ALK6. Our data suggested that functional differences in BMP binding exist between different human vascular cell types and implied involvement of both ALK3 and ALK6.

Smad 1/5 Phosphorylation by BMPs and GDF5 Differed in Vascular Cells. We sought to determine whether our observed differences in ^{125}I-BMP4 binding between cell types reflected functional differences in Smad1/5 phosphorylation profiles. Thus, we compared the relative potencies of BMP2, BMP4, BMP6, BMP7, and GDF5 to stimulate Smad 1/5 C-terminal phosphorylation in vascular cells. To enable direct comparison of ligand selectivities between cell types, HPASMCs, HMEC-1, and HPAECs were treated with BMP2, BMP4, and GDF5, or BMP6 and BMP7, in the same experiment. In HPAECs, BMP2, BMP4, and GDF5 weakly induced Smad1/5 phosphorylation at concentrations up to 100 ng/ml (data not shown). In contrast, robust Smad1/5 phosphorylation was elicited in HPASMCs and HMEC-1 (Fig. 2). Fur-

TABLE 1

Competition for ^{125}I-BMP4 binding by BMP and TGF ligands

IC_{50} values are expressed as geometric mean and 95% confidence limits derived from at least three experiments for each competitor. Specific binding is expressed as mean ± S.E.M. for at least three experiments. Binding in HPAECs was too low to construct competition curves

Competitor	^{125}I-BMP4 Competition Mean IC_{50} (95% Confidence Interval)			
	NIH-3T3	HPASMCs	HMEC-1	HPAECs
BMP4 (site 1)	3.24 (2.43–4.31)	0.35 (0.06–1.96)	24.3 (12.8–46.2)	
BMP4 (site 2)		12.8 (5.33–30.7)		
BMP2	4.81 (1.51–15.3)	30.4 (19.9–46.4)	34.7 (15.6–77.3)	
BMP6	133 (38.4–464)	115 (35.4–373)	>300	
BMP7	54.7 (5.95–502)	94.6 (23.6–380)	>300	
GDF5	29.3 (7.29–118)	21.1 (4.26–104)	12.0 (1.22–117)	
Act A	>300	>300	>300	
TGF-β_1	>300	>300	>300	
Specific binding (% of total binding)	61.9 ± 3.2%	60.8 ± 8.0%	22.7 ± 4.4%	15.5 ± 2.1%

thermore, cell-specific differences in concentration responsiveness were consistently observed that broadly reflected ligand binding. Thus, in HMEC-1 cells, BMP4 and BMP2 induced Smad1/5 phosphorylation with similar potencies (Fig. 2, A and B). In HPASMCs, BMP4, but not BMP2, stimulated Smad1/5 phosphorylation at lower concentra-

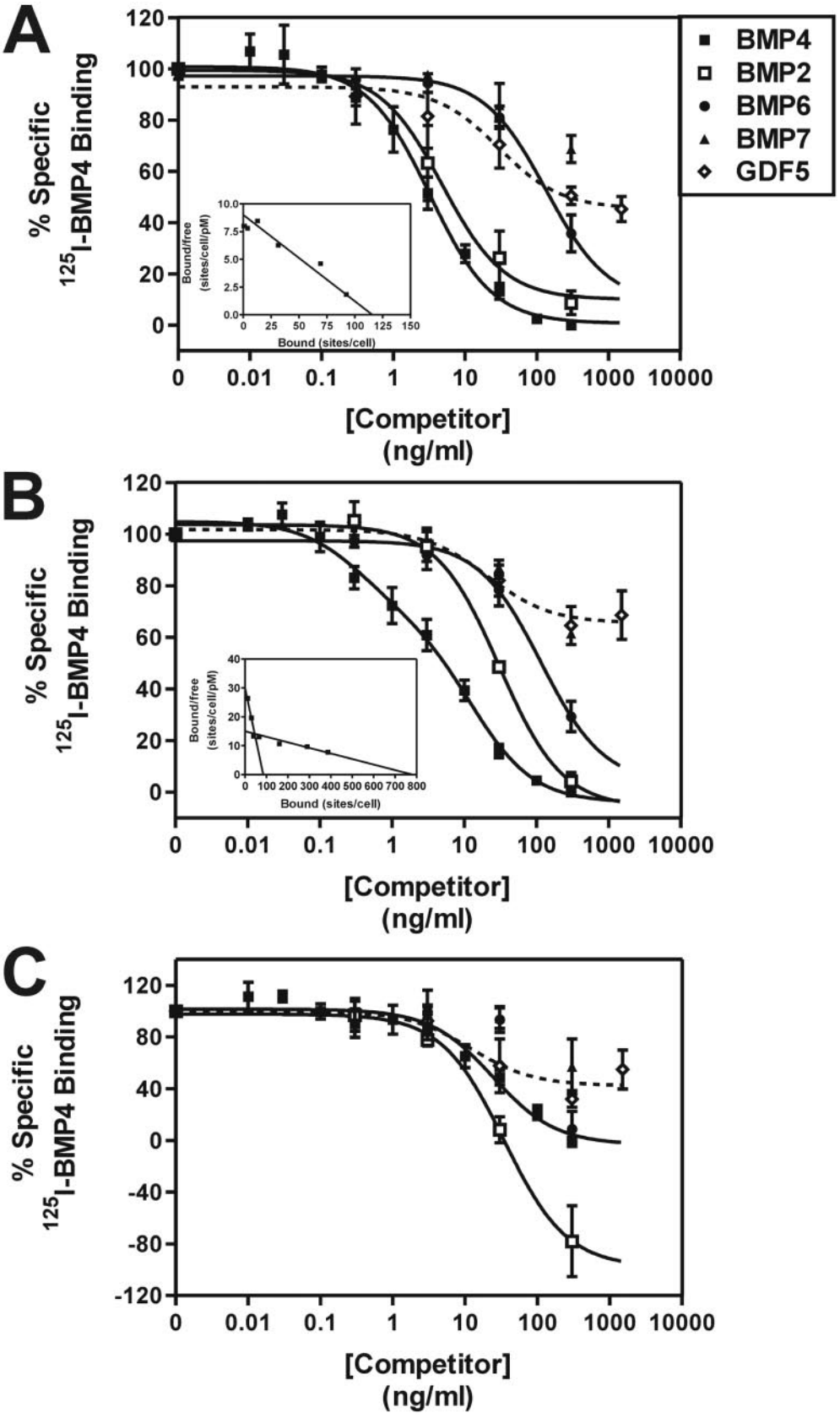

Fig. 1. Competition curves for ^{125}I-BMP4 binding to NIH-3T3 (A), HPASMCs (B), and HMEC-1 (C). Binding assays were performed as detailed under *Materials and Methods*. Cells were incubated with ^{125}I-BMP4 in the presence or absence of increasing concentrations of unlabeled BMP2 (□), BMP4 (■), BMP6 (●), BMP7 (▲), and GDF5 (◇). TGFβ_1 and activin A did not compete and so have been omitted for clarity. IC_{50} values were calculated using GraphPad Prism (Table 1). Inset, saturation binding of ^{125}I-BMP4 to NIH-3T3 cells (A) and HPASMCs (B) confirms that ^{125}I-BMP4 binding sites were best explained as a single population on NIH-3T3 cells, whereas two populations were evident on HPASMCs.

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tions, whereas BMP2 and BMP4 exhibited equal potencies at 10 ng/ml and above (Fig. 2, A and B). GDF5 was a weaker agonist in both cell types. In addition, BMP6 and BMP7 stimulated pSmad1/5 phosphorylation with equal potency in HMEC-1, whereas BMP7 was less potent than BMP6 in HPASMCs (Fig. 2C).

Expression of BMP Receptors and Their Associating Proteins By Vascular Cell Lines. The above studies showed potentially important functional differences in BMP responsiveness and binding between HMEC-1 cells and HPASMCs. We first considered the possibility that these differences were due to cell-specific BMP receptor expression. By RT-PCR, HMEC-1 and HPASMCs expressed mRNA for ALK1, ALK2, ALK3, ALK4, ALK5, ALK6, ActR-II, ActR-IIB, BMPR-II, TGFβ-RII, betaglycan, and endoglin (Supplemental Figure).

Semiquantitative analysis of ALK3, ALK6, and BMPR-II mRNA expression showed that HPASMCs, HMEC-1, and HPAECs all expressed relatively high levels of BMPR-II, which is accepted as the low-affinity BMP receptor (Fig. 3A). HPASMCs expressed ALK3 and ALK6, possibly consistent with the high-level ^{125}I-BMP4 binding in these cells. In addition, HMEC-1 cells expressed ALK3 at levels similar to those of HPASMCs, but their expression of ALK6 was very low. Finally, both ALK3 and ALK6 expression were low in HPAECs, consistent with the very low binding and lack of responsiveness to BMP2/4. We confirmed these cell-specific expression patterns of ALK3, ALK6, and BMPR-II by quantitative PCR (Fig. 3, B and C).

Although ALK3 and ALK6 were the most likely candidates mediating BMP4 binding, we examined the expression patterns of other BMP receptors and accessory receptor proteins in HPASMCs, HMEC-1, and HPAECs (Fig. 3B). We show that endoglin, BMPR-II, and ActR-IIB are expressed at higher levels in endothelial cells than in HPASMCs (Fig. 3B). In terms of relative transcript abundance, ActR-IIB expression is much lower (approximately 100-fold) than BMPR-II (Fig. 3C). In contrast, RGMa, ALK6, ActR-II, RGMb, and BAMBI are expressed at lower levels in endothelial cells than in HPASMCs (Fig. 3B). The transcript abundance of RGMa and ALK6 are low in all cell types (Fig. 3C). ALK2 expression is high, but similar in all cell types. Betaglycan is expressed at higher levels in HMEC-1 cells. We could not detect RGMc in vascular cells, despite confirming its known expression in human skeletal muscle RNA (not shown) (Babitt et al., 2005; Babitt et al., 2006).

Effect of siRNA for BMP Receptors on ^{125}I-BMP4 Binding. To explore the roles of ALK3, ALK6, and BMPR-II

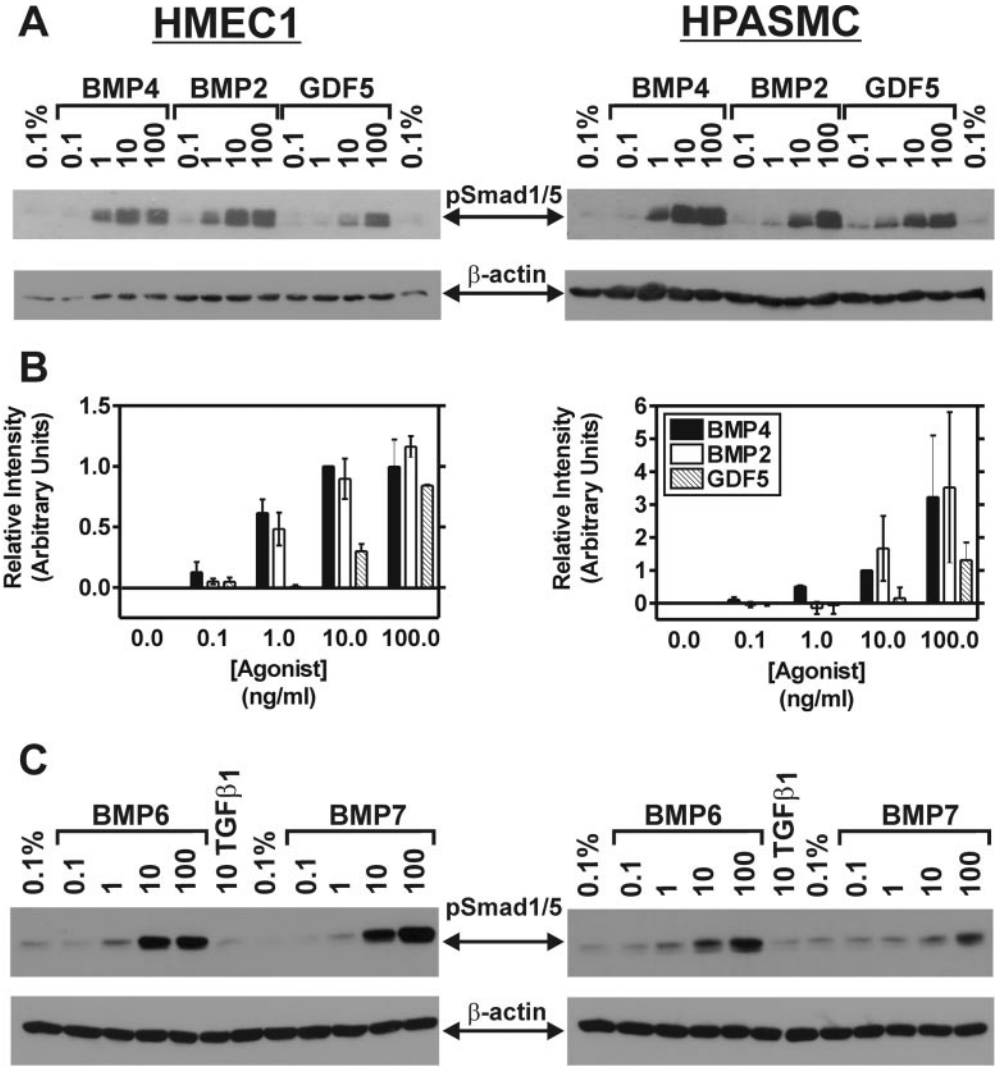

Fig. 2. Smad1/5 phosphorylation by BMP ligands in HMEC-1 and HPASMCs. All experimental repeats were performed on HPASMCs and HMEC-1 cells in parallel, using the same BMP/GDF serial dilutions to enable direct comparison. A, confluent cells were stimulated with 0.1 to 100 ng/ml BMP4, BMP2, or GDF5 in DMEM/0.1% FBS (0.1%) for 60 min, followed by lysis for total protein. Western blots are shown for Smad1/5 phosphorylation and a blot re-probed for β-actin to show equal loading. B, densitometry of pSmad1/5 bands from Western blots of HMEC-1 and HPASMCs treated in parallel. The band intensity value for unstimulated cells (0.1%) was subtracted from the intensity value for each band and the relative increases normalized to the value for 10 ng/ml BMP4. Films were used for exposure times where the 10 ng/ml BMP4 band was not overexposed. Data are presented as mean ± S.D. for three separate experiments. C, representative Western blots for phospho-Smad 1/5 and β-actin from confluent cells stimulated with 0.1 to 100 ng/ml BMP6 or BMP7 or 10 ng/ml TGFβ_1 in DMEM/0.1% FBS (0.1%) for 60 min. All blots shown are representative of three separate experiments.

in more detail, we examined the effect of siRNAs for BMPR-II, ALK3, and ALK6 on ^{125}I-BMP4 binding. siRNA knockdown of BMPR-II had no significant effect on specific ^{125}I-BMP4 binding in HMEC-1 and HPASMCs (Fig. 4A), consistent with the notion of BMPR-II as a low-affinity receptor for this ligand. More unexpectedly, siRNA for ALK3 and ALK6 did not significantly alter specific ^{125}I-BMP4 binding (Fig. 4A). Moreover, siRNA for ALK3, ALK6, or BMPR-II did not significantly alter the pharmacology of BMP4 competition for ^{125}I-BMP4 in HPASMCs (Fig. 4, B–D). Quantitative RT-PCR analysis confirmed that ALK3 siRNA did not alter ALK6 expression, and ALK6 siRNA did not alter ALK3 expression (data not shown).

Characterization of ^{125}I-BMP4 Binding to Receptor Extracellular Domain-Fc Fusion Protein. Having shown the lack of effect of ALK3 or ALK6 knockdown on ^{125}I-BMP4 binding in intact cells, we wished to confirm the binding characteristics of this ligand to the extracellular domains of BMP receptors fused to the Fc region of IgG. ^{125}I-BMP4 binding to ALK3-Fc (16.5–11,000 ng/well) or ALK6-Fc (15.75–10,500 ng/well) coated on Protein A plates, increased with the amount of ALK-Fc (Fig. 5A). In contrast, BMPR-II-Fc exhibited little capacity for ^{125}I-BMP4 binding but did bind ^{125}I-BMP6 (data not shown).

We further characterized competition of BMPs and GDF5 in the receptor-Fc system. At ALK6-Fc, BMP4 competed with

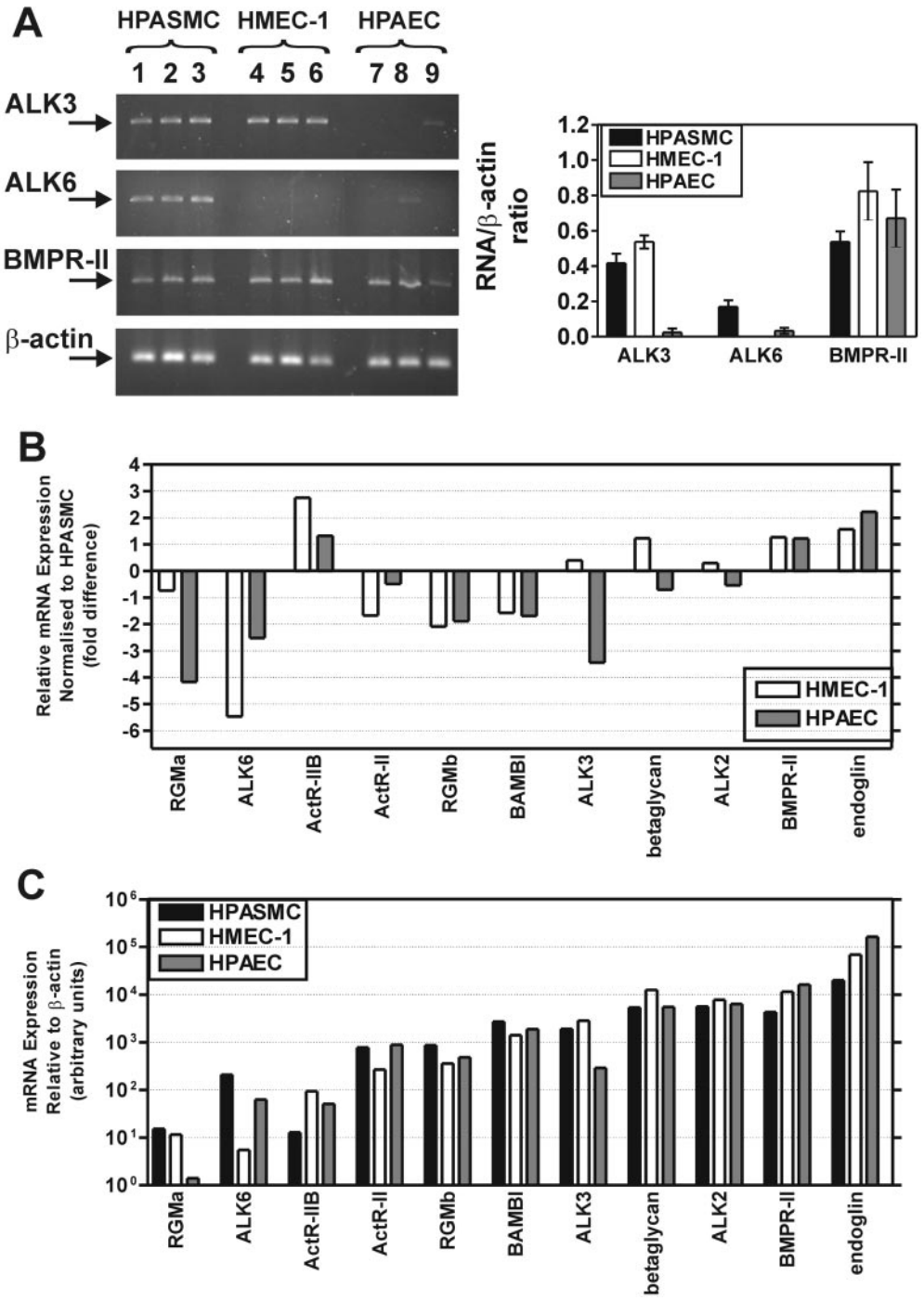

Fig. 3. Expression of BMP receptors and accessory binding proteins in HPASMCs, HMEC-1 and HPAECs. A, expression of ALK3, ALK6, and BMPR-II by semiquantitative RT-PCR. DNase-digested total RNA was extracted from confluent cells on three separate occasions for each cell line. Total RNA was amplified for ALK3, ALK6, BMPR-II, or β-actin using the semiquantitative protocols described under *Materials and Methods*. Products were separated on 1.5% agarose gels (panels), and the band intensities were quantified from digital images. The graph shows the mean of the band intensities normalized to β-actin for the gels shown. Data are representative of three separate experiments. B, expression of mRNA for BMP receptors and accessory proteins in HMEC-1 and HPAECs relative to their expression in HPASMCs. cDNA samples were prepared from equivalent amounts of mRNA from each cell line and aliquots of cDNA from HPASMC, HMEC-1, and HPAECs analyzed in parallel. Threshold cycle data were normalized to the two reference genes, 18s RNA and β-actin, using the GeNorm program. The expression level of each individual gene in HMEC-1 and HPAECs was normalized to the expression of that gene in HPASMCs. C, relative abundance of mRNA transcripts for BMP receptors and accessory proteins in HPASMCs, HMEC-1, and HPAECs. Based on the stability of 18s RNA and β-actin expression between the cell types, we applied the assumption of equal PCR threshold values for equal transcript numbers of different genes, and used the $\Delta\Delta C_T$ method to calculate the relative expression of each gene to β-actin after normalizing to 18s rRNA.

higher affinity than at ALK3-Fc (IC_{50} mean ± S.D.: ALK6-Fc = 605 ± 189 ng/ml versus ALK3-Fc = 2125 ± 216 ng/ml). BMP2 competed with lower affinity than BMP4 at both ALK3-Fc and ALK6-Fc. As we expected, GDF5 competed with equal potency to BMP4 at ALK6-Fc (IC_{50} mean ± S.D. = 395 ± 215 ng/ml; Fig. 5C), but did not compete at ALK3-Fc. BMP6 and BMP7 were weak competitors at ALK6-Fc but did not compete for ^{125}I-BMP4 binding to ALK3-Fc. We thus confirmed that ^{125}I-BMP4 radioligand exhibits intact binding in a cell-free system.

siRNA for BMP Receptors and Signal Transduction. Having shown that ^{125}I-BMP4 binding was unaffected by siRNA knockdown of BMP receptors and having confirmed the validity of that assay in a cell free system, we sought to further confirm the identity of the functional BMP receptors mediating Smad1/5 signaling in HMEC-1 and HPASMC. Transfection of BMPR-II siRNA into HMEC-1 cells or HPASMCs led to an attenuation of Smad1/5 phosphorylation in response to BMP4 and BMP2 and to abolition of the response to GDF5 (Fig. 6A). By QPCR, BMPR-II RNA was reduced by 75 to 90% by the BMPR-II siRNA compared with siFectamine or siGLO. We confirmed knockdown of BMPR-II protein by Western blotting (Fig. 6B). We also examined the effect of BMPR-II siRNA on Smad1/5 phosphorylation in response to BMP6 and BMP7 (Fig. 7). BMPR-II siRNA transfection resulted

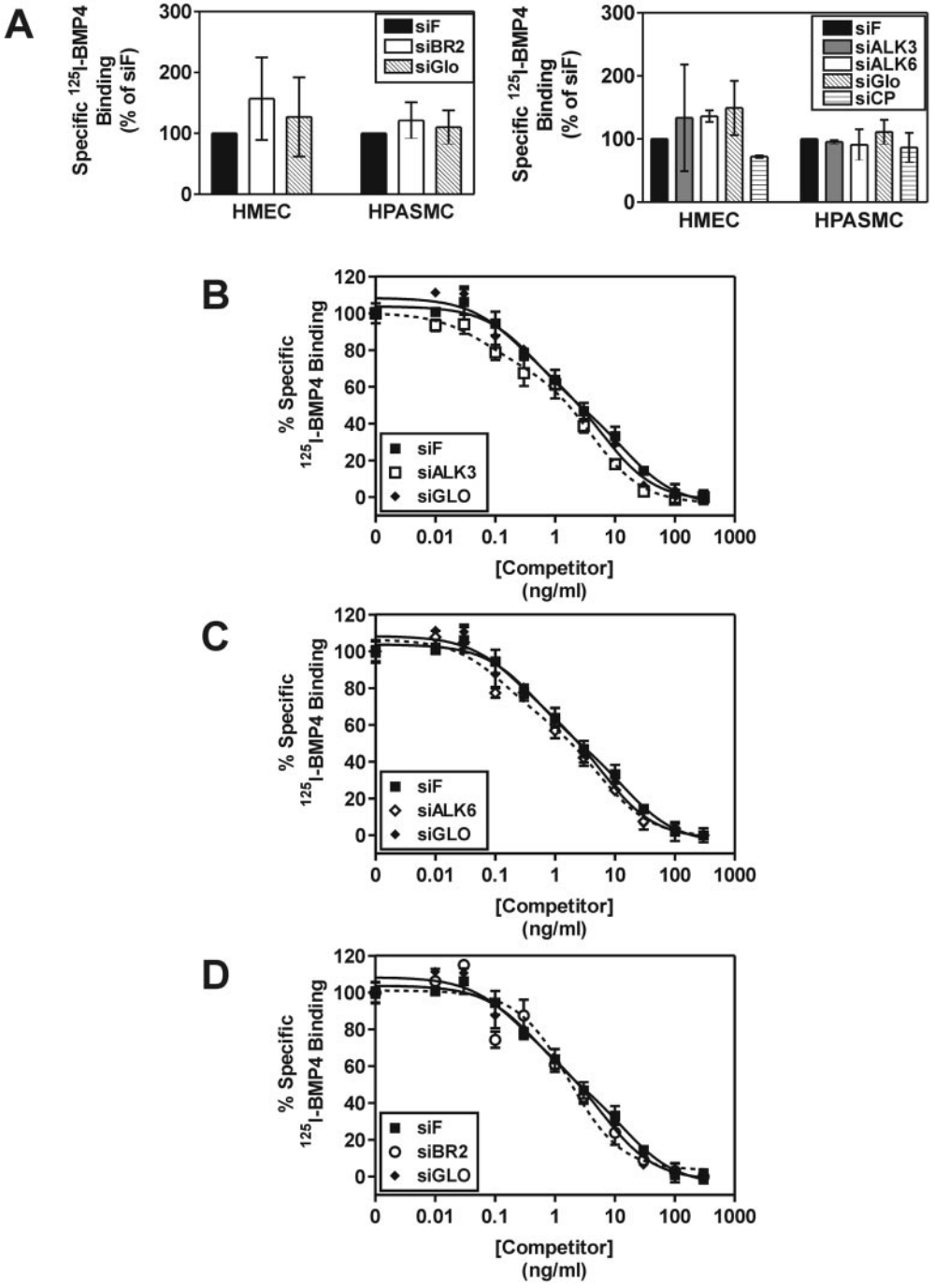

Fig. 4. Effect of siRNA for ALK3, ALK6, and BMPR-II on ^{125}I-BMP4 binding. Cells were transfected with siRNAs for ALK3 (*siALK3*), ALK6 (*siALK6*), or BMPR-II (*siBR2*), and parallel plates were transfected with either the RISC-free siGLO control or the On-TARGETplus siControl Non-targeting Pool (10 nM each siRNA). In each transfection, parallel wells were transfected, and RNA was extracted for quantitative RT-PCR analysis. A, specific binding of ^{125}I-BMP4 to HMEC-1 or HPASMCs after transfection with siRNA for BMPR-II (left) and ALK3 or ALK6 (right). Cells were incubated with ^{125}I-BMP4 in the presence of 100 ng/ml BMP4 to determine nonspecific binding or in the absence of competitor to determine total binding. The percentage of specific binding was then calculated. siRNA for BMPR-II, ALK3, and ALK6 had little effect on total binding. The competition graphs show ^{125}I-BMP4 binding competition by unlabeled BMP4 in HPASMCs transfected with siRNA for ALK3 (B), ALK6 (C), or BMPR-II (D). Curves were then compared with cells treated with transfection reagent alone (siF) or RISC-free siGLO (siGLO). ^{125}I-BMP4 binding was best explained as two sites. siRNA for ALK3, ALK6, and BMPR-II siRNAs did not significantly alter the ^{125}I-BMP4 competition curves. Quantitative RT-PCR analysis confirmed that BMPR-II was reduced by 77%, ALK3 by 54%, and ALK6 by 65% in the experiment shown.

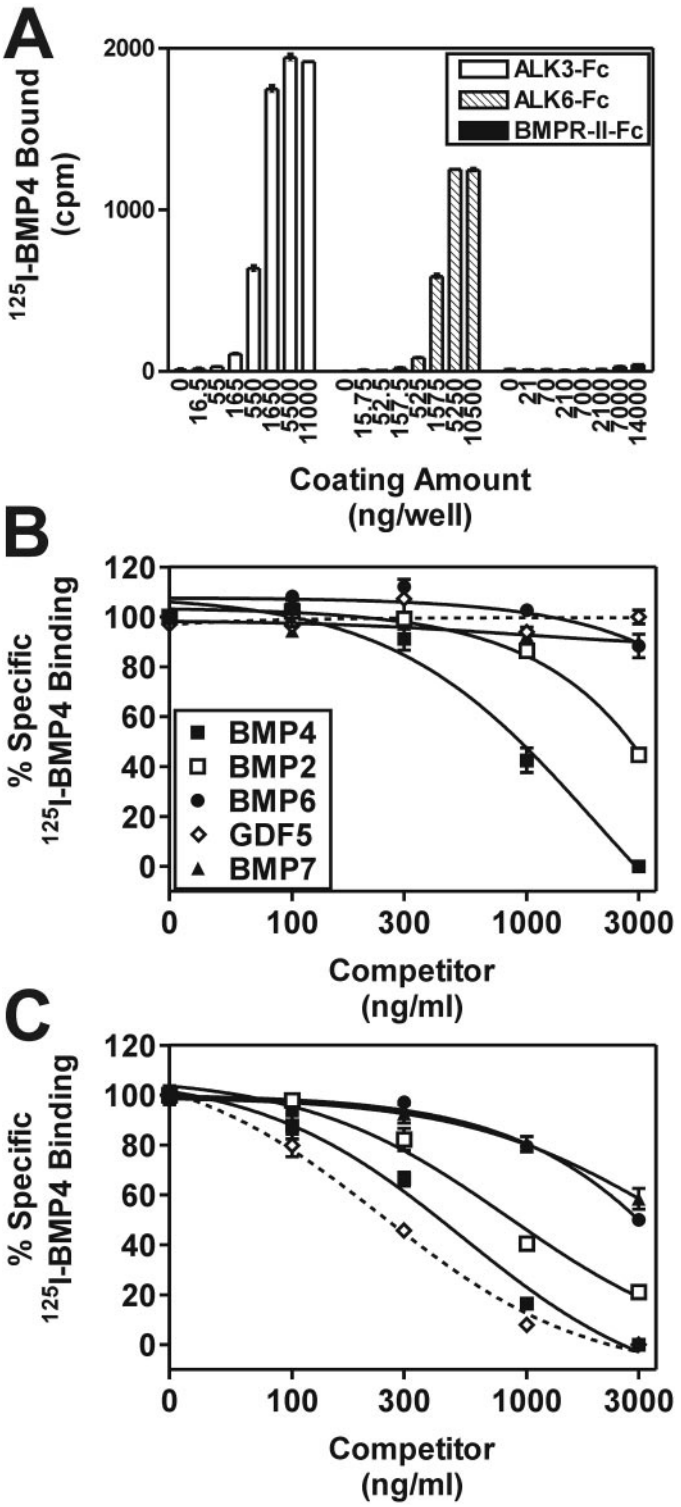

Fig. 5. Binding of ^{125}I-BMP4 to Receptor-Fc fusion proteins immobilized on Protein A plates. A, plates were coated with ALK3-Fc (16.5–1100 ng/well), ALK6-Fc (15.75–10,500 ng/well), or BMPR-II-Fc (21–14,000 ng/well) and ^{125}I-BMP4 binding analyzed. ALK3-Fc (■) and ALK6-Fc (□) bound ^{125}I-BMP4 whereas BMPR-II-Fc exhibited little binding. Competition binding for ^{125}I-BMP4 to ALK3-Fc (1650 ng/well) (B) or ALK6-Fc (5250 ng/well) (C) by 100 to 3000 ng/ml of either BMP4, BMP2, BMP6, BMP7, or GDF5 (dotted line) indicates the higher affinity for BMP4 of ALK6-Fc compared with ALK3-Fc, and the selective competition of GDF5 at ALK6-Fc only.

in an abrogation of BMP4-induced Smad1/5 phosphorylation in both HMEC-1 cells and HPASMCs. However, Smad1/5 phosphorylation responses to BMP7 were enhanced in HMEC-1 or HPASMCs treated with BMPR-II, whereas BMP6 responses were only enhanced in HPASMCs. This suggests fundamental differences in the receptors mediating BMP6 and BMP7 responses in these two cell types.

We then explored the contributions of ALK3 and ALK6 to functional BMP-mediated Smad1/5 responses in HPASMCs and HMEC-1 cells. Transfection of siRNA for ALK3 reduced Smad1/5 phosphorylation in response to BMP4, BMP2, and GDF5 in both HMEC-1 and HPASMCs (Fig. 8). This is not consistent with the accepted model of GDF5 selectively bind-

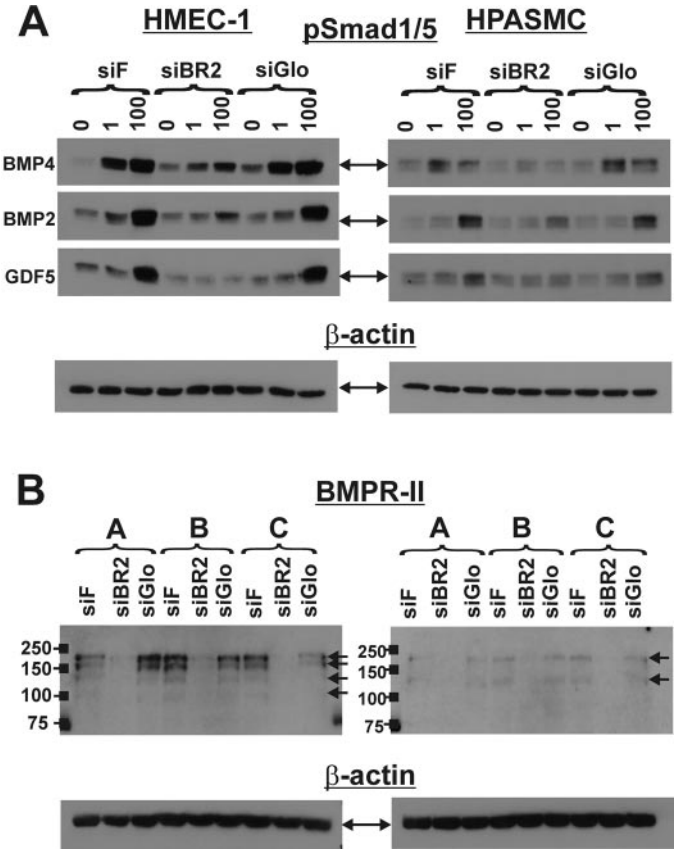

Fig. 6. Effect of siRNA for BMPR-II Smad1/5 phosphorylation by BMP ligands in HMEC-1 and HPASMCs. A, representative Western blots for phospho-Smad 1/5 in cells transfected with siFectamine alone (siF), or with 10 nM concentrations of either BMPR-II siRNA (siBR2) or RISC-free siGlo control (siGlo). Cells were treated with DMEM/0.1% FBS alone (0) or DMEM/0.1% FBS containing BMP4, BMP2, or GDF5 (1 or 100 ng/ml) followed by lysis for total protein. Blots were reprobed for β-actin to confirm equal protein loading. This experiment was performed twice. B, Western blot of cell extracts from HMEC-1 and HPASMCs transfected with siRNA for BMPR-II (siBR2) or the siFectamine (siF) or the RISC-free siGlo control (siGlo). These extracts are those from the "0" lanes for the BMP4 (lanes A), BMP2 (lanes B), and GDF5 (lanes C) blots shown in A. These confirm that BMPR-II protein was reduced by siRNA for BMPR-II (siBR2), but not by siFectamine (siF) or the RISC-free siGlo control (siGlo). The weights and positions of the protein markers are represented on the left of each blot. The arrows indicate that four bands were evident in HMEC-1, 2 major bands between 150 and 250 kDa and two minor bands of 130 kDa and ~100 kDa. In contrast, two major bands were observed in HPASMC, migrating at approximately 200 and 130 kDa. These data represent three separate Western blots from BMPR-II siRNA analysis. In each transfection, parallel wells were prepared from which RNA was extracted. QPCR analysis confirmed that BMPR-II was reduced by 77% in the HMEC-1 and 87% in the HPASMC blots shown.

ing to ALK6. In contrast, ALK6 siRNA had no effect in either cell type, suggesting that ALK3 is the core type I receptor mediating Smad responsiveness to BMP4, BMP2, and GDF5 in both cell types.

Radiolabeled ^{125}I-BMP4 Retains the Ability to Activate BMPR-II-Dependent Smad1/5 Phosphorylation. The studies reported above showed that ^{125}I-BMP4 could bind to ALKs in cell-free assays but had no direct evidence for this in cells. We sought to confirm ^{125}I-BMP4 was binding to a receptor complex containing BMPR-II, which we had shown does not bind ^{125}I-BMP4 directly. We transfected HMEC-1 and HPASMCs with siRNA for BMPR-II and examined Smad1/5 phosphorylation in response to BMP4 or ^{125}I-BMP4 (Fig. 9). Both unlabeled BMP4 and ^{125}I-BMP4 stimulated Smad1/5 phosphorylation in both cell types. Furthermore, the Smad responses to both ligands were abrogated by siRNA for BMPR-II but not by the siControl nontargeting pool. These data confirm that ^{125}I-BMP4 was binding to a cell-surface complex comprising BMPR-II.

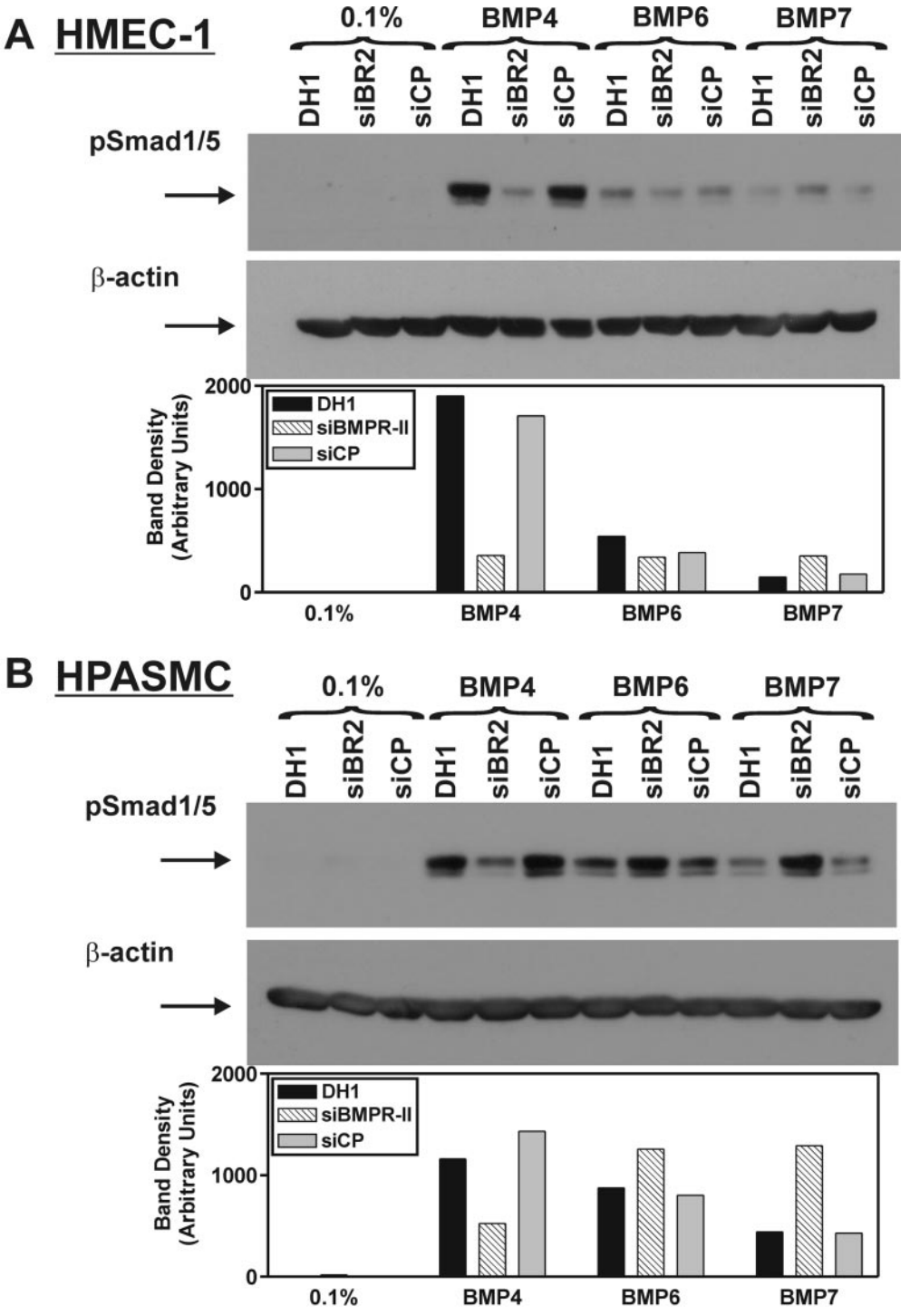

Fig. 7. Effect of siRNA for BMPR-II Smad1/5 phosphorylation by BMP4, BMP6, and BMP7 ligands in HMEC-1 (A) and HPASMCs (B). Representative Western blots are shown for phospho-Smad 1/5 in cells transfected with Dharmafect 1 (DH1), or with 10 nM concentrations of either BMPR-II siRNA (siBR2) or On-TARGETplus siControl Nontargeting Pool (siCP). Cells were treated with DMEM/0.1% FBS alone (0.1%) or DMEM/0.1% FBS containing 10 ng/ml BMP4, BMP6, or BMP7. Blots were reprobed for β-actin. The graphs show quantification of the Smad1/5 bands by densitometry. HMEC-1 cells treated with BMPR-II siRNA demonstrated an abrogation of Smad1/5 phosphorylation in response to BMP4 and BMP6 but a slight increase in response to BMP7. In HPASMCs treated with BMPR-II siRNA, there was an abrogation of Smad1/5 phosphorylation in response to BMP4, whereas BMP6 and BMP7 showed marked enhancement of the response. Representative blots from three experiments are shown. In each transfection, parallel wells were prepared from which RNA was extracted. Quantitative RT-PCR analysis confirmed that BMPR-II was reduced by 94% in the HMEC-1 and 93% in the HPASMC blots shown.

Discussion

Germ-line mutations in the *BMPR2* gene underlie the majority of cases of familial PAH, yet the receptors mediating BMP responsiveness in human lung vascular cells are not well characterized. Here, we show that ALK3/BMPR-II is the major receptor complex mediating Smad1/5 phosphorylation in human vascular cells in response to BMP2 and BMP4. Contrary to expectations, GDF5 responses were mediated by ALK3 rather than ALK6. We also showed that, although ^{125}I-BMP4 binds to ALK3 and ALK6 in a cell-free assay, these receptors are not the major components that determine cell surface binding to intact cells. These data suggest the existence of as-yet-uncharacterized accessory receptors or binding proteins for BMPs, especially in HPASMCs. In addition, our siRNA studies confirm that reducing BMPR-II expression by >90% leads to a gain of signaling in response to BMP6 and BMP7 in human vascular cells. These data provide a platform for the characterization of novel BMP-receptor accessory proteins mediating BMP selectivity in the human lung vasculature.

Although BMPR-II mutations underlie FPAH, the relative contributions of BMPR-II and the identities of the BMP type I receptors mediating vascular cell responsiveness to BMPs are not well characterized. Therefore, we sought to define the BMP receptors mediating BMP binding and signaling in human vascular cells. We first compared ^{125}I-BMP4 binding sites on HPASMCs and endothelial cells (HMEC-1 and HPAECs) and studied NIH-3T3 fibroblasts as a reference for well-defined BMP2/4 receptors (Koenig et al., 1994; Iwasaki et al., 1995; Rudarakanchana et al., 2002). We expected BMP2 and BMP4 to bind their higher affinity type I receptors, ALK3 and ALK6, in combination with the lower affinity type II receptors. BMP2 and BMP4 are reported to bind to ALK2/BMPR-II, ALK3/ActR-II, ALK3/ActR-IIB, ALK3/BMPR-II, ALK6/ActR-IIB, and ALK6/BMPR-II (ten Dijke et

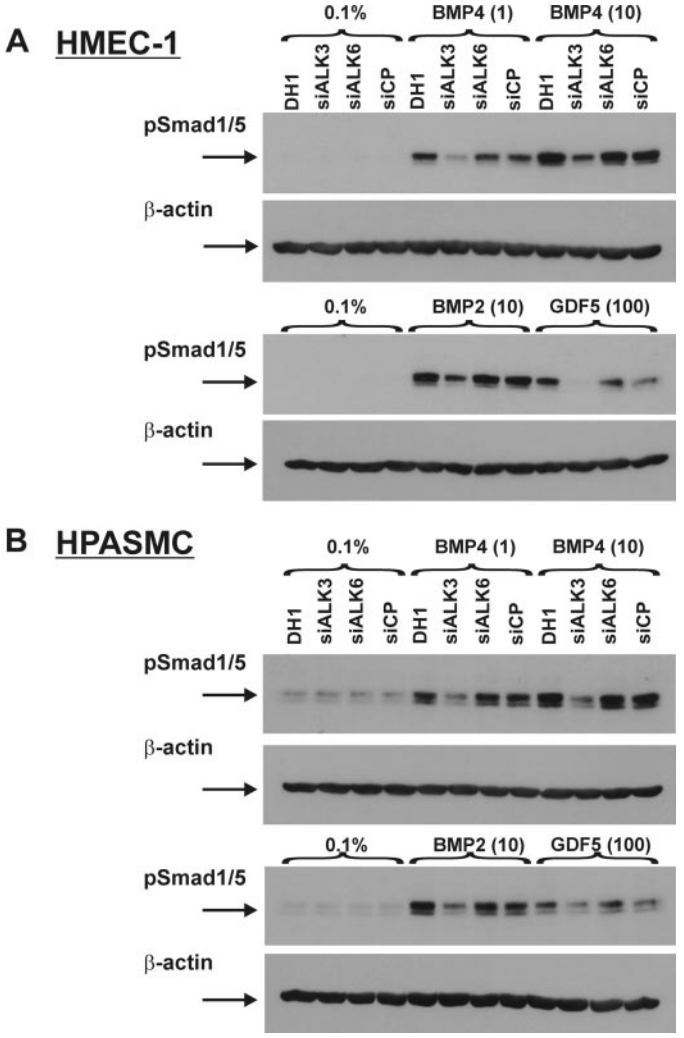

Fig. 8. Effect of siRNA for ALK3 and ALK6 on Smad1/5 phosphorylation by BMP4 and GDF5 in HMEC-1 (A) and HPASMCs (B). Representative Western blots are shown for phospho-Smad 1/5 in cells transfected with Dharmafect 1 (DH1) alone, or DH1 with 10 nM concentrations of either ALK3 siRNA (siALK3), ALK6 siRNA (siALK6), or On-TARGETplus siControl Nontargeting Pool (siCP). Cells were treated with DMEM/0.1% FBS alone (0.1%) or DMEM/0.1% FBS containing BMP4 (1 or 10 ng/ml), BMP2 (10 ng/ml), or GDF5 (100 ng/ml). Blots were then reprobed for β-actin. HMEC-1 and HPASMCs treated with ALK3 siRNA showed abrogation of their responses to BMP4 and GDF5, whereas those transfected with siRNA for ALK6 did not change compared with the controls. Representative blots from three experiments are shown. In each transfection, parallel wells were prepared from the RNA extracted. Quantitative RT-PCR analysis confirmed that ALK3 and ALK6 were reduced by 85 and 60%, respectively, in the HMEC-1 blots and 80 and 77%, respectively, in the HPASMC blots shown. Blots are representative of three separate experiments.

Although BMPR-II mutations underlie FPAH, the relative contributions of BMPR-II and the identities of the BMP type I receptors mediating vascular cell responsiveness to BMPs are not well characterized. Therefore, we sought to define the BMP receptors mediating BMP binding and signaling in human vascular cells. We first compared ^{125}I-BMP4 binding sites on HPASMCs and endothelial cells (HMEC-1 and HPAECs) and studied NIH-3T3 fibroblasts as a reference for well-defined BMP2/4 receptors (Koenig et al., 1994; Iwasaki et al., 1995; Rudarakanchana et al., 2002). We expected BMP2 and BMP4 to bind their higher affinity type I receptors, ALK3 and ALK6, in combination with the lower affinity type II receptors. BMP2 and BMP4 are reported to bind to ALK2/BMPR-II, ALK3/ActR-II, ALK3/ActR-IIB, ALK3/BMPR-II, ALK6/ActR-IIB, and ALK6/BMPR-II (ten Dijke et

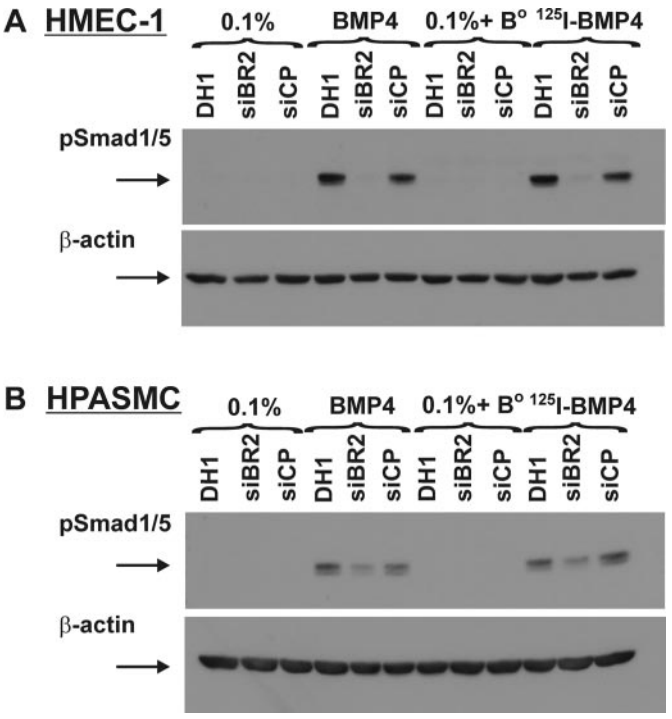

Fig. 9. Effect of siRNA for BMPR-II Smad1/5 phosphorylation by BMP4 and ^{125}I-BMP4 in HMEC-1 (A) and HPASMCs (B). Representative Western blots are shown for phospho-Smad 1/5 in cells transfected with Dharmafect 1 (DH1), or with 10 nM concentrations of either BMPR-II siRNA (siBR2) or On-TARGETplus siControl Nontargeting Pool (siCP). Cells were treated with DMEM/0.1% FBS alone (0.1%), or DMEM/0.1% FBS containing 10 ng/ml BMP4 (BMP4), iodination column buffer (1:40 dilution; 0.1% + B°), or ^{125}I-BMP4 (1:40, approximately 2.75 ng/ml, ^{125}I-BMP4). Blots were then reprobed for β-actin. In HMEC-1 and HPASMCs, BMP4 and ^{125}I-BMP4 both stimulated Smad1/5 phosphorylation, and this was abrogated by transfection of BMPR-II siRNA. In each transfection, parallel wells were prepared from which RNA was extracted. Quantitative RT-PCR analysis confirmed that BMPR-II was reduced by 96.5% in the HMEC-1 and 92.8% in the HPASMC blots shown. Blots are representative of three separate experiments.

al., 1994b; Macías-Silva et al., 1998). BMP6 was expected to bind to ALK2 and bind weakly to ALK3 and ALK6 (Aoki et al., 2001). BMP7 was expected to bind to Act-RII, ActR-IIB, or BMPR-II in combination with ALK2 (Barbara et al., 1999; Aoki et al., 2001). GDF5 was expected to bind to ALK6 in combination with type II receptors (Nishitoh et al., 1996).

As expected, BMP2 and BMP4 competed equally for ^{125}I-BMP4 at a single population of binding sites on NIH-3T3 fibroblasts (Koenig et al., 1994). In contrast, competition and saturation binding revealed two ^{125}I-BMP4 binding site populations on HPASMCs. Two populations of ^{125}I-BMP7 binding sites have been reported on bone cells, and we have reported two ^{125}I-BMP4 sites on HFL-1 cells (Malpe et al., 1994; Jeffery et al., 2005). Here, we showed that BMP4 and GDF5, but not BMP2 or BMP6, compete at the high-affinity sites, whereas BMP4, BMP2, and BMP6, but not GDF5, compete at the low-affinity sites. Smad phosphorylation was stimulated by BMP4 at lower concentrations than BMP2, suggesting the functional responses to BMPs reflected the ligand binding affinities. In contrast to HPASMCs, ^{125}I-BMP4 binding to HMEC-1 cells and HPAECs was low. We could construct competition curves in HMEC-1 cells, and showed that BMP2 competes for ^{125}I-BMP4 to a greater extent than BMP4. Again, the ligand affinities for radioligand competition reflected their abilities to stimulate Smad1/5 phosphorylation.

Both semiquantitative RT-PCR and QPCR analyses supported our basic hypothesis that the expression of ALK3 and ALK6 were associated with the high levels of ^{125}I-BMP4 binding to HPASMCs (high ALK3/ALK6) and low binding to HMEC-1 (high ALK3/low ALK6) and HPAECs (low ALK3/ALK6). Our data suggest that BMP4 may not be a major ligand in HPAECs. These cells express very low ALK3 and ALK6 and exhibit little binding and Smad responsiveness. In addition, HPAECs express low levels of RGMa and RGMb, two accessory receptors implicated in BMP2/4 responsiveness (Babitt et al., 2005; Samad et al., 2005). We suggest that BMPR-II in complex with ALK1 and endoglin, or with ALK2, form the main BMP receptors in HPAECs. Indeed, ALK1 and endoglin mutations cause hereditary hemorrhagic telangiectasia, and ALK1 mutations are also associated with hereditary hemorrhagic telangiectasia and PAH (McAllister et al., 1994; Harrison et al., 2003). Furthermore, it was recently demonstrated that ALK1/BMPRII/endoglin mediate BMP9 signaling in endothelial cells (Scharpfenecker et al., 2007).

We used siRNA to explore the contributions of endogenous BMP receptors to binding and responsiveness in HPASMCs and HMEC-1 cells. There was no significant effect of siRNA for ALK3, ALK6, or BMPR-II on total binding to either cell type. In addition, reduction of ALK6 or BMPR-II expression by as much as 90 to 95% did not alter the level of binding or the pharmacology of the binding sites in HPASMCs. We were concerned that ^{125}I-BMP4 binding was affected by chemical modification, yet the radioligand binds to the extracellular domains of ALK3 or ALK6 in a cell-free assay. In addition, we have confirmed that ^{125}I-BMP4 stimulates Smad1/5 phosphorylation in HMEC-1 and HPASMCs. Furthermore, siRNA for BMPR-II abrogates this response, confirming that the radioligand is binding to and activating functional receptors comprising BMPR-II.

In contrast to our binding data, BMPR-II siRNA abrogated Smad responses to BMP4, BMP2, and GDF5. We were surprised to find that ALK3 siRNA, but not ALK6 siRNA, abrogated the responses to BMP4, BMP2, and GDF5 in both cell types. Mutations in ALK6 or GDF5 cause brachydactyly in humans, showing their close functional relationship, and we show that GDF5 only competed at ALK6-Fc in the cell-free assay (Lehmann et al., 2003; Seemann et al., 2005). However, our siRNA studies support recent data showing a failure of ALK6 siRNA to alter Smad phosphorylation in response to BMP4 in mouse pulmonary artery smooth muscle cells (Yu et al., 2005). Thus, we suggest that GDF5 is activating Smad1/5 via ALK3/BMPR-II but that GDF5 or BMP4 binding requires an accessory receptor (Fig. 10).

Although ALK6 does not contribute to ^{125}I-BMP4 binding or Smad1/5 activation, alternative pathways may mediate functional BMP responses via ALK6. Elevated ALK6 expression was reported in HPASMCs from a patient with PAH with no identified BMPR-II mutation (Takeda et al., 2004). In these cells, ALK6 induced mitosis via p38 and p42/44 MAP

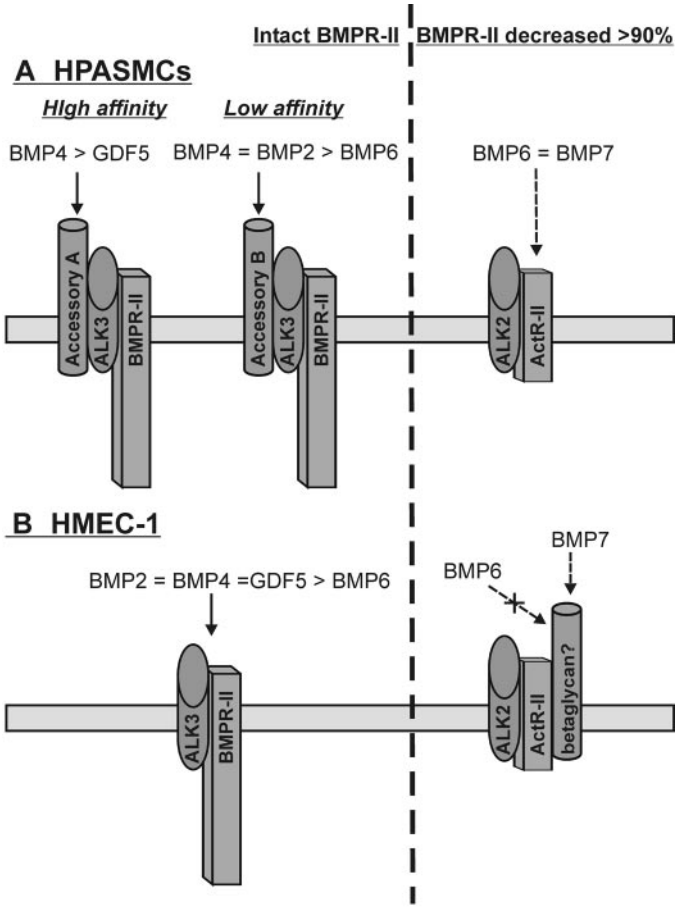

Fig. 10. Hypothetical model of the receptor complexes mediating BMP selectivity and Smad responses in HPASMCs and HMEC-1 cells. A, in a combination of our data with that from recent reports in the literature (dashed arrows), we suggest that ALK3/BMPR-II is the core receptor pairing mediating BMP responses in HPASMCs. When in complex with a putative protein (accessory protein A) contributing to 25% of the total binding sites, BMP4 and GDF5 can bind, whereas BMP2 and BMP6 are excluded. The other 75% of the sites bind BMP4, BMP2, BMP6, but not GDF5, and a second protein (accessory protein B) would be required for this high level of binding. Reduction of BMPR-II leads to a gain of BMP6/7 signaling, probably via ALK2/ActR-II (Yu et al., 2005). B, ALK3/BMPR-II is the core receptor pairing mediating BMP2, BMP4, and GDF5 responses in HMEC-1. Whether additional proteins are required has to be determined. In addition, BMP7 probably acts via ALK2/ActR-II in complex with betaglycan (Wiater and Vale, 2003), as BMPR-II reduction leads to a gain of BMP7 but not BMP6 signaling.

kinases (Takeda et al., 2004). Indeed, we reported that p38 and p42/44 MAP kinases mediate BMP4-induced proliferation in HPASMCs (Takeda et al., 2004; Yang et al., 2005). In our siRNA studies, we did not observe altered p38 MAP kinase phosphorylation (data not shown), but further investigation of p42/44 MAP kinase activation is required.

The failure of BMP7 to compete with high affinity for ^{125}I-BMP4 suggests that BMP4 is not binding to ActR-II or ActR-IIB, supported by the low expression of these receptors in HPASMCs (ten Dijke et al., 1994b; Macías-Silva et al., 1998). In addition, reduction of BMPR-II expression by ≥90% in HMEC-1 or HPASMCs enhances Smad1/5 phosphorylation by BMP7. The response to BMP6 is also enhanced in HPASMCs but not in HMEC-1. This correlates to a recent report that BMPR-II deletion in mouse pulmonary artery smooth muscle cells causes a gain of BMP6 and BMP7 signaling via Smad1/5/8 and p38 via ActR-II and ALK2 (Fig. 10) (Yu et al., 2005). The gain of BMP6 function may not occur in HMEC-1 cells because of exclusion of BMP6 from the ALK2/ActR-II receptor complex, possibly by betaglycan. Betaglycan, which we show is expressed at higher levels in HMEC-1 cells than HPASMC or HPAECs, can associate with Act-RII and ALK2 to mediate BMP7 binding (Wiater and Vale, 2003).

We considered other candidates that might contribute to the ALK3/ALK6-independent ^{125}I-BMP4 binding in HPASMCs. From our expression data and studies of mouse PASMCs, we thought it unlikely that ALK2, ActR-II, or Act-RIIB were directly binding ^{125}I-BMP4 (Yu et al., 2005). BAMBI, endoglin, and betaglycan are expressed at higher levels in endothelial cells, which have lower ^{125}I-BMP4 binding. In addition, these proteins require intact BMP receptors for ligand binding, so BMP receptor siRNA transfection would be expected to abolish binding (Barbara et al., 1999; Onichtchouk et al., 1999). Other potential candidates are RGMa and RGMb, which we show to be expressed at higher levels in HPASMCs than endothelial cells (Babitt et al., 2005; Samad et al., 2005). Our observation that RGMa and RGMb, but not RGMc, are expressed in human vascular cells correlates to the expression pattern reported in mouse pulmonary artery smooth muscle cells (Xia et al., 2007). RGMs directly bind ^{125}I-BMP4 with high affinity; this binding is competed for by BMP2 and BMP4, but not BMP7 and TGFβ_1 (Babitt et al., 2005; Samad et al., 2005; Xia et al., 2007). However, preliminary studies in our laboratory examining transfection of siRNAs for RGMa and RGMb suggest little effect on binding or Smad phosphorylation in HPASMCs (P. D. Upton, unpublished data), correlating to a recent report in normal mouse PASMCs (Xia et al., 2007). Other proteins, such as Megalin (LRP2), may also bind ^{125}I-BMP4 (Spoelgen et al., 2005). We are now investigating further potential candidates.

In conclusion, we showed that ALK3/BMPR-II was the receptor mediating Smad1/5 responses to BMP2/4 and GDF5 in HPASMCs and HMEC-1 cells. However, our data suggest that differences in cell selectivity for BMP ligands might require accessory proteins that we have yet to identify. These cell-specific differences in BMP receptor pharmacology that we have identified provide an important insight into the cell- and context-specific nature of BMP responses.

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