The lysosomal inhibitor, chloroquine, increases cell surface BMPR-II levels and restores BMP9 signalling in endothelial cells harbouring BMPR-II mutations

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Pulmonary arterial hypertension (PAH) is characterized by dysregulated pulmonary artery endothelial cell (PAEC) proliferation, apoptosis and permeability. Loss-of-function mutations in the bone morphogenetic protein receptor type-II (BMPR-II) are the most common cause of heritable PAH, usually resulting in haploinsufficiency. We previously showed that BMPR-II expression is regulated via a lysosomal degradative pathway. Here, we show that the antimalarial drug, chloroquine, markedly increased cell surface expression of BMPR-II protein independent of transcription in PAECs. Inhibition of protein synthesis experiments revealed a rapid turnover of cell surface BMPR-II, which was inhibited by chloroquine treatment. Chloroquine enhanced PAEC expression of BMPR-II following siRNA knockdown of the BMPR-II transcript. Using blood outgrowth endothelial cells (BOECs), we confirmed that signalling in response to the endothelial BMPR-II ligand, BMP9, is compromised in BOECs from patients harbouring BMPR-II mutations, and in BMPR-II mutant PAECs. Chloroquine significantly increased gene expression of BMP9-BMPR-II signalling targets Id1, miR21 and miR27a in both mutant BMPR-II PAECs and BOECs. These findings provide support for the restoration of cell surface BMPR-II with agents such as chloroquine as a potential therapeutic approach for heritable PAH.

INTRODUCTION

Pulmonary arterial hypertension (PAH) is a progressive disease characterized by dysregulated endothelial cell proliferation, apoptosis and vascular permeability as well as smooth muscle cell proliferation in the pulmonary circulation (1). The transforming growth factor-β (TGFβ) superfamily, especially the bone morphogenetic proteins, plays a key role in the pathobiology of PAH (2,3). Mutations in bone morphogenetic protein receptor type-II (BMPR-II), the gene encoding the bone morphogenetic protein type II receptor (BMPR-II), underlie at least 70% of heritable and 10–40% of apparently sporadic PAH cases (4–6). In pulmonary artery smooth muscle cells, truncating or missense mutations result in reduced BMP-induced Smad1/5 signalling and reduced transcriptional induction of the inhibitors of DNA binding transcription factors (Id) (7,8). The majority of mutations reported in BMPR-II lead to a state of haploinsufficiency (6).

Endothelial cells from patients with mutations exhibit increased proliferation and an inability to form vascular networks (9). Even in the absence of a BMPR-II mutation, deficiency of the receptor contributes to the pathobiology of non-genetic forms of PAH (10,11). In addition, commonly used animal models of PAH, including chronic hypoxia in mice or monocrotaline exposure in rats, reveal a marked reduction in BMPR-II levels in the lung (12,13). Moreover, targeted gene delivery of BMPR-II to the pulmonary vasculature prevents pulmonary hypertension in these models (14).

Recent studies from our laboratory have suggested the possibility that lysosome inhibitors might increase cell surface
RESULTS

Chloroquine increases BMPR-II protein levels in endothelial cells

We sought to investigate whether lysosomal blockade by chloroquine could significantly increase BMPR-II levels in endothelial cells. Treatment of PAECs for 16 h with either concanamycin A (50 nm) or chloroquine (100 μM) significantly increased BMPR-II protein levels by 5.6- and 3.5-fold, respectively (Fig. 1A). Transcription of BMPR-II mRNA was not significantly elevated suggesting that the increase in BMPR-II was likely due to lysosomal pathway inhibition (Fig. 1B). We also sought to determine whether the targeting of BMPR-II for lysosomal degradation by K5 mediated ubiquitination could be inhibited by chloroquine (15). Although not as marked as concanamycin A, chloroquine treatment of HeLa cells stably expressing K5 partially restored BMPR-II protein levels (Fig. 1C). Furthermore, in a microvascular endothelial cell line BMPR-II expression levels were strikingly increased by chloroquine (Fig. 1C). Again, chloroquine had no effect on BMPR-II gene expression in any of the cell lines (Fig. 1D).

Plasma levels of chloroquine in patients treated for rheumatoid arthritis vary from 36.6 to 3895 ng/ml (18). We conducted a concentration response assay encompassing this range. PAECs were treated for 16 h with the following concentrations 0, 0.5, 1, 5, 10 and 50 μM. Treatment with 0.5–50 μM chloroquine, increased BMPR-II protein expression in a dose response manner, compared with the no treatment control (Fig. 2A and Supplementary Material, Fig. S1). Even at the higher concentrations of chloroquine, expression of the endothelial specific type I receptor, Alk-1, was not significantly affected (Fig. 2A, grey bars).

Chloroquine increases cell surface localization of BMPR-II

We next sought to investigate whether lysosomal inhibition affected BMPR-II cell surface localization. A MCR5 lung fibroblast cell line stably expressing GFP-tagged BMPR-II was cultured in glass chamber slides and treated with the relevant vehicle, concanamycin A (50 nm) or chloroquine (100 μM) for 16 h. Using confocal microscopy, GFP-tagged BMPR-II was localized at the cell surface in the vehicle control (Fig. 2C–F). After treatment with concanamycin A, the GFP-tagged receptor co-localized with the lysosomal marker, LAMP-1 (Fig. 2G–J). Under these conditions, very little receptor was observed at the cell surface suggesting rapid turnover of the receptor and accumulation in the lysosome. Treatment with chloroquine also resulted in lysosomal accumulation, but also appeared to increase GFP-tagged BMPR-II at the cell surface (Fig. 2K–N; arrowheads).

To determine BMPR-II cell surface localization, biotinylation of cell surface receptors was utilized. Cells were treated for 16 h with vehicle, concanamycin A (50 nm) and chloroquine (100 μM). The non-biotinylation control showed basal expression of BMPR-II in the total lysate, but no surface expression with the avidin-agarose beads alone. Both concanamycin A and chloroquine increased total cell lysate BMPR-II expression compared with the vehicle control, but only chloroquine appeared to increase surface expression of BMPR-II (Fig. 3A). Biotinylation was also utilized to determine surface expression...
of endogenous BMPR-II in PAECs after a chloroquine concentration response. Biotinylation analysis revealed that all chloroquine concentrations (0.5–50 \(\mu M\)) increased surface expression of BMPR-II (Fig. 3B; arrowhead). Therefore, a concentration of 10 \(\mu M\) chloroquine, which reflects high steady-state blood concentrations in patients, was chosen for further experiments.

We next sought to determine the time course of chloroquine action on BMPR-II expression. PAECs were treated with chloroquine at 10 \(\mu M\) for 0.5, 1, 2, 4 and 16 h. BMPR-II protein levels were increased after 2–4 h chloroquine treatment (Fig. 3C). No significant increases in BMPR-II mRNA levels were observed at these time points (Fig. 3D).
Chloroquine inhibits the degradation of cell surface BMPR-II

In order to determine the effects of chloroquine on BMPR-II expression, the understanding of turnover of endogenous receptor is critical. The dynamics of BMPR-II regulation were investigated by blocking protein synthesis with cycloheximide over a time course. PAECs were treated with 20 μg/ml cycloheximide at 0, 0.5, 1, 2 and 4 h and assessed for BMPR-II protein and gene expression. Loss of total receptor expression was observed as early as 1 h (Fig. 4A). BMPR-II gene expression was unaffected across the time course (Fig. 4B; white bars).
Blockade of protein synthesis had no effect on Alk-1 protein and gene expression (Fig. 4A and B; grey bars). To determine whether chloroquine alters the loss of BMPR-II following protein synthesis inhibition, PAECs were pre-treated with chloroquine (10 μM) for 16 h prior to cycloheximide treatment. As observed previously, BMPR-II expression was lost rapidly after 1 h, but treatment with chloroquine preserved expression after 2 h (Fig. 4C). Again no significant effect on BMPR-II gene expression was observed (Fig. 4D). Rapid loss of BMPR-II was also seen in PASMCs after 1–2 h (Supplementary Material, Fig. S2). Furthermore, 16 h chloroquine treatment of PASMCs maintained BMPR-II expression over 2–4 h of protein synthesis blockade.

Since the pre-treatment with chloroquine for 16 h inevitably increased BMPR-II expression, we also conducted experiments in which the level of BMPR-II protein was similar to time 0 by pre-treating PAECs for only 1 h with chloroquine (10 μM) (Fig. 5A). Normalization of chloroquine-treated BMPR-II
expression prior to protein synthesis inhibition revealed that chloroquine increased and maintained BMPR-II expression significantly until 2 h (Fig. 5B).

Chloroquine restores BMPR-II protein levels following siRNA knockdown

Both genetic and non-genetic forms of PAH are often characterized by reduced BMPR-II protein expression (10). We therefore sought to recreate the diminished receptor expression seen in the disease using RNA interference. In our previous research, silencing of BMPR-II with 10 nM siRNA results in over 90% knockdown efficiency (26). Therefore, serial dilutions of siRNA were used to recreate the ‘haploinsufficient’ levels of BMPR-II protein expression. PAECs were transfected with 0.01, 0.1, 1, and 0.01 nM of BMPR-II. After 48 h, protein and transcript levels were assessed for knockdown efficiency. A concentration of 0.1 nM siRNA decreased BMPR-II protein expression by ≏50% compared with the transfection control. To allow for variability between PAEC donors, concentrations of 0.1 and 1 nM siRNA were used in subsequent experiments to determine whether chloroquine could rescue BMPR-II protein expression. Knockdown of BMPR-II was conducted as before except that after 32 h cells were treated with chloroquine for 16 h. BMPR-II expression was reduced to ≏50% levels in the siBMPR-II knockdown at a concentration of 1 nM. Densitometry of three independent experiments revealed that chloroquine treatment increased BMPR-II levels by 2.2- and 6.5-fold following knockdown with 0.1 and 1 nM of siRNA, respectively (Fig. 6C).
Chloroquine does not inhibit BMP-mediated Smad signalling and transcription of target genes

Since chloroquine inhibits the internalization of BMPR-II, it is possible that signalling downstream of the receptor could be adversely affected by this intervention. In addition, off-target effects of chloroquine might negatively impact BMP signalling. To assess this, PAECs were stimulated with BMP9 (1 ng/ml) in the presence of chloroquine (10 μM). Downstream signalling was assessed by examining canonical Smad1/5/8 protein phosphorylation and Id1 transcription. Cells were pre-treated with chloroquine for 16 h prior to stimulation with BMP9 for 1 and 4 h. As previously observed, BMP9 stimulation increased BMPR-II protein and gene expression (26) (Fig. 7A and B). As previously described in this study, chloroquine substantially increased BMPR-II protein expression, but not mRNA (Fig. 7A and B). Phosphorylation of Smad1/5/8 was increased by BMP9 treatment at both 1 and 4 h, which was unaffected by chloroquine (Fig. 7A). BMP9 potently increased Id1 gene expression particularly after 1 h stimulation (Fig. 7C). Again, this was unaffected by chloroquine treatment suggesting no detrimental effect of blocking the lysosome on BMP signalling in endothelial cells. In fact, Smad1/5/8 phosphorylation and Id1 expression remained unaffected by chloroquine for up to 8 h of BMP9 exposure (Supplementary Material, Fig. S3A–C). Furthermore, no discernible increase in Smad-independent signalling was observed when examining p38MAPK phosphorylation after BMP9 treatment in the presence of chloroquine (Supplementary Material, Fig. S4).

Chloroquine increases BMPR-II protein expression and improves downstream signalling in endothelial cells harbouring BMPR-II mutations

Initially, we confirmed that endothelial cells derived from patients with BMPR-II mutations are deficient in BMP9-mediated signalling. After quiescence, control and BMPR-II haploinsufficient blood outgrowth endothelial cells (BOECs) were treated with BMP9 (1 ng/ml) for 4 and 12 h. After 12 h, mutant BOECs showed a significantly reduced Id1 expression compared with controls (Fig. 8A). Furthermore, Id1 expression in a haploinsufficient BMPR-II PAECs, after 20 h BMP9 stimulation, was also significantly reduced (Fig. 8B).

Drake et al. (30) recently observed that miR21 and miR27a expression are regulated by BMP9. In that study, BMP9-stimulated miR21 and miR27a levels were completely abrogated by RNA interference of BMPR-II. We therefore assessed the effect of chloroquine on the processing of these microRNAs and Id1 gene expression. Control and BMPR-II haploinsufficient PAECs were pre-treated with chloroquine for 4/16 h prior to stimulation with BMP9 for 20 h. BMP9 stimulation of control PAECs induced miR21 and miR27a expression (Fig. 8B). As expected, haploinsufficient PAECs showed very little induction of these targets in the presence of BMP9. Chloroquine exerted no effect on the processing of miR21 and miR27a in the control cells after 16 h chloroquine pre-treatment. PAECs from a PHH patient with a BMPR-II mutation (deletion of exons 1–8) showed a significant reduction in BMP9 stimulated miR21, miR27a and Id1 levels (Fig. 8C). Chloroquine treatment of haploinsufficient PAECs significantly increased the expression of Id1, miR21 and miR27a in the presence of BMP9 (Fig. 8C). Similar significant results were observed after 4 h chloroquine pre-treatment and subsequent exposure to BMP9 (Supplementary Material, Fig. S5).

Control and mutant PAECs were pre-treated with chloroquine for 4 and 16 h and BMPR-II protein expression was measured.
An increase in BMPR-II levels was observed in control cells after 4 and 16 h chloroquine treatment (Fig. 8D). Significantly, an increase in BMPR-II protein expression in haploinsufficient PAECs was observed in cells treated for 4 and 16 h with chloroquine (Fig. 8D). We further assessed the effect of chloroquine on BOECs from two patients with a BMPR-II mutation (R320X and R548X). Chloroquine treatment significantly increased total BMPR-II expression after 4 and 16 h in mutant BOECs (Fig. 9A and Supplementary Material, Fig. S6). Densitometry from the mutant PAEC line and haploinsufficient BOEC lines confirmed a significant increase in BMPR-II expression in the presence of chloroquine (Fig. 9B). The BMPR-II mutant R320X BOECs demonstrated significantly lower Id1 expression compared with controls after 20 h of BMP9 stimulation (Fig. 7C). Furthermore, 16 h chloroquine pre-treatment significantly increased Id1 expression in the presence of BMP9 (Fig. 9C).

DISCUSSION

The type II bone morphogenetic protein receptor plays a critical role in PAH. In those cases where a mutation is identified, reduced expression of BMPR-II is observed in the lung. Furthermore, idiopathic cases of the disease in the absence of BMPR-II mutation, and animal models of disease are all associated with a marked reduction in lung BMPR-II protein levels (10–13). Therefore, the ability to maintain the cell surface expression of BMPR-II is of particular interest in the treatment of PAH.

Our recent study highlighted the potential in targeting the regulation of BMPR-II by inhibiting the lysosome (15). We have also recently reported that treatment of monocrotaline exposed rats with chloroquine not only inhibited the development of PAH in this model but also increased lung BMPR-II protein levels consistent with our observation that lysosome blockade increases endogenous expression of the receptor.
The potential mechanisms involved in the regulation of BMPR-II expression levels remain poorly understood.

Here, we report that chloroquine was able to partially restore K5 mediated ubiquitination and downregulation of BMPR-II in HeLa cells, similar to our previous findings with concanamycin A (15). Although both inhibitors block the lysosomal degradation pathway by affecting lysosomal acidification, their effect on BMPR-II localization differed. Concanamycin A is a selective inhibitor of vacuolar ATPases. Vacuolar ATPases are proton pumps localized to the membranes of many intracellular organelles and primarily regulate intracellular pH (31). Inhibition by concanamycin A immediately increases luminal pH (32). It is worth noting that bafilomycin A1, another macrolide antibiotic, has been shown to completely abolish EGF lysosomal degradation but had no affect on ligand internalization and endocytosis (33). The high toxicity of concanamycin A and other macrolides precludes their use in clinical therapy. The mode of action of chloroquine is as a weak lipophilic base where the free base passes easily through membranes (34).

**Figure 9.** Chloroquine treatment significantly increases BMPR-II protein expression and rescues Id1 gene expression in mutant BOECs. (A) Human BOECs were treated with chloroquine (10 μM) or vehicle for 4 or 16 h. Immunoblotting was performed with a BMPR-II antibody and blots were reprobed with α-tubulin to ensure equal loading. (B) Immunoblots of BMPR-II expression were quantified and expression represented as the ratio of BMPR-II:loading control. Bar graph showing BMPR-II fold change after chloroquine treatment relative to control (n = 3). *P < 0.05 compared with control. (C) Human BOECs were pretreated with chloroquine (10 μM) for 16 h prior to BMP9 (3 nM) stimulation for 20 h. Total RNA was extracted and expression of Id1 determined by qPCR. Expression was normalized to β-actin and expressed as fold change relative to control. *P < 0.05 and **P < 0.01 compared with control. Data are mean ± SEM (n = 3).

(21). The ability to rescue reduced receptor expression could be a critical factor in the treatment of PAH. The majority of heritable or idiopathic PAH cases, where a mutation in BMPR-II is identified, are due to nonsense, frameshift and splice-site defects. This results in the premature termination of BMPR-II transcript and therefore loss of expression through nonsense-mediated decay (40). Haploinsufficiency for BMPR-II with a 50% reduction in BMPR-II expression is predicted in these individuals. We...
attempted to recreate this expression level by titration of the BMPR-II siRNA previously used (26). Using 10–100-fold dilutions of siRNA, we were able to mimic the reduction in expression levels associated with BMPR-II mutations (10). Treatment with chloroquine partially restored the receptor expression levels where BMPR-II levels were 50% or lower. More importantly, chloroquine treatment of endothelial cells from patients with BMPR-II mutations partially rescued BMPR-II protein expression.

Preventing the cell surface turnover of BMPR-II might impair BMP signalling. We determined the effect of chloroquine on BMP9 induced Smad signalling and its downstream transcription factor, Id1. In control PAECs, chloroquine was not detrimental to the canonical signalling processes mediated by BMP9. As previously reported by Drake et al. (30), we confirmed that PAECs and BOECs harbouring a BMPR-II mutation are deficient in the induction of Id1 expression and processing of microRNAs, miR21 and miR27a by BMP9. This contrasts with our previous report in which we employed siRNA knockdown of BMPR-II and ActR-IIa to show that induction of Id gene expression by BMP9 in PAECs was co-dependent on these receptors (26). This highlights important differences between the experimental knockdown of a receptor and study of the endogenous mutated receptor. Nevertheless, in cells harbouring BMPR-II mutations in the present study, we demonstrate that chloroquine treatment of BMPR-II mutant endothelial cells can rescue the expression of these downstream BMP9/BMPR-II targets. Our results suggest that enhancement of cell surface expression of BMPR-II in the normal endothelium does not further increase signalling in response to ligand, rather our findings suggest that BMPR-II limits BMP signalling at lower levels of receptor expression. Under these circumstances, in the presence of BMPR-II haploinsufficiency, chloroquine was capable of enhancing BMP signalling. This gene dosage effect of BMPR-II has been reported previously in vivo (41). At present, it remains uncertain whether the optimal point for intervention on the mutated BMPR-II pathway is at the level of the receptor itself, or the downstream signalling. Thus in a situation where BMP signalling was deficient downstream of the receptor, enhanced BMPR-II expression levels may be ineffective. Nevertheless, in in vivo rat models of pulmonary hypertension where several mechanisms likely mediate loss of BMP signalling, we have shown that chloroquine can prevent onset and progression of disease (21).

This study is the first report of the dynamic regulation of BMPR-II and the possibility to modulate cell surface expression with a widely used therapeutic agent. However, several questions remain regarding the mechanism of BMPR-II degradation and turnover in the endothelium, in addition to the potential non-specific effects of chloroquine. It is well known that 4-aminoquinolones have a wide range of effects. Other than modifying lysosomal acidification, they have been shown to target other molecules involved in endocytic degradation. Accumulation of chloroquine in the lysosome inhibits phospholipase A2 (16). It has recently been shown that antagonists of cytoplasmic phospholipase A2 inhibit multiple endocytic pathways (42). In this case, chloroquine could be maintaining BMPR-II at the cell surface via another mechanism other than lysosomal inhibition.

There have been several reports detailing the endocytic pathways involved in TGFβ superfamily receptor degradation. In particular, Hartung et al. (39) established roles for both clathrin and caveolae mediated endocytosis in the processing of BMPR-II. Furthermore, they identified that BMPR-II interacts with Eps15R, a key component of clathrin coated pits (CCPs), and caveolin-1, the main component of caveolae. BMPR-II has also been shown, using fluorescence resonance energy transfer, to bind another key component of CCPs, adaptor protein complex 2 (AP2). Disruption of CCPs increased Smad signalling and osteogenesis in C2C12s, suggesting that CCPs operate as an inhibitory membrane domain (43).

Recent research has highlighted the importance of caveolin-1 in vascular signalling and pulmonary hypertension. BMPR-II has been reported to localize to lipid rich membrane domains of PAECs and co-localize with caveolin-1 (44). Caveolin-1 has also been shown to be important in vascular smooth muscle cell signalling. Targeted reduction of caveolin-1 using RNA interference resulted in reduced BMP signalling (45). Furthermore, caveolin-1 knockout mice develop right ventricular hypertrophy and an increase in pulmonary artery pressure (46). Mutations in several members of the BMP/Smad signalling cascade have been identified in PAH patients, including Alk-1, endoglin and Smad8. Recently, Austin et al. (47) were the first to identify a frameshift mutation in caveolin-1 in a family with heritable PAH and an individual with idiopathic PAH. Thus, providing a link between the regulation of BMP signalling and endocytosis in pulmonary hypertension. Despite these considerations, chloroquine and hydroxychloroquine are widely used in clinical practise and would appear on the basis of our present and recent findings to be worth pursuing in clinical studies as potentiators of BMP signalling in PAH, where BMP signalling is compromised (21).

Taken together, our findings provide proof of concept for targeting the degradation of the BMPR-II receptor in PAH associated with BMPR-II deficiency or mutation to restore downstream signalling and function. Further elucidation of the mechanisms by which BMPR-II is degraded from the cell surface via the lysosome may provide additional specific targets for intervention.

MATERIALS AND METHODS

Cell culture

Human PAECs were purchased from Lonza, Workingham, UK. Cells were maintained in complete endothelial cell growth medium-2 (EGM-2) and were used at passages 4–8. PAECs from a PAH patient with a germline mutation in BMPR-II (deletion of exons 1–8) were isolated as previously described (30,48,49). BOECs were isolated as previously described (9). Cells were maintained in complete EGM-2 and were used at passages 5–7. The transformed human microvascular endothelial cell line (HMEC-1) was obtained from the Centre for Disease Control (CDC, Atlanta, Georgia, USA) and maintained in the MDCB131 medium (Life Technologies, Paisley, UK) containing 15% fetal bovine serum (FBS) (Life Technologies), 10 ng/ml EGF, 1 ng/ml hydrocortisone (Sigma-Aldrich, Poole, Dorset, UK) and antibiotics. Human pulmonary artery smooth muscle cells (PASMCs) were isolated in our laboratory by explants and cultured as previously described (50). Immortalized MRC5 (SV40T) lung fibroblasts stably expressing
human wild-type BMPR-II-GFP (MRC5-BMPR-II-GFP) were cultured as previously described (51). HeLa cell lines were grown in DMEM supplemented with 10% FBS and antibiotics. The HeLa K5 stable cell line was created and cultured as previously described (15). Concanamycin A was dissolved in DMSO and used at a final concentration of 50 nM for 16 h. Chloroquine (N\(^{-4}\)(7-Chloro-4-quinolinyl)-N\(^{1}\),N\(^{1}\)-dimethyl-1,4-panethanediamine diphosphate salt) was dissolved in sterile nuclease-free water and used at differing concentrations and times (both Sigma-Aldrich). Cycloheximide was dissolved in sterile nuclease-free water and used at 20 µg/ml (Sigma-Aldrich). For BMP9 treatments, see relevant controls.

mRNA quantitative reverse transcriptase-PCR

Total RNA was extracted using the RNeasy mini kit (Qiagen, Crawley, UK) according to the manufacturer’s protocol along with on-column DNase digestion. Total RNA (1000 ng) was reverse transcribed using the high capacity reverse transcription kit (Applied Biosystems, Warrington, UK) as described in the manufacturer’s instructions. Synthesized complementary DNA was amplified using SYBR\(^\text{®}\)-Green JumpStart\(^{\text{TM}}\) Taq Ready-Mix\(^{\text{TM}}\) (Sigma-Aldrich) and the relevant sense and antisense primers along with ROX Reference dye (Life Technologies). The following primer sequences were used for BMPR-II (sense 5'-caaatctgtgaccecaactcga-3', anti-sense 5'-gaggaagtaattggataaggaccat-3'); β-actin (sense 5'-ggcacacaccttacaagta-3', antisense 5'-gtctctctctgcgttcgc-3'). QuantiTect Primer Assays were used for Alk-1, ID1, GAPDH and β2-microglobulin (B2M) (Qiagen). Reactions were amplified on a StepOnePlus\(^{\text{TM}}\) Real-Time PCR System (Applied Biosystems). Relative expression of target mRNA was normalized to GAPDH, β-actin or B2M using the ΔΔCT method (52) and expressed as fold change relative to the relevant control.

microRNA quantitative reverse transcriptase-PCR

Total RNA was extracted using miRNasy mini kit (Qiagen, Valencia, CA, USA). mir21 and miR27a were quantified using TaqMan microRNA kits (Applied Biosystems, Foster City, CA, USA) and normalized to RNU48 as previously described (30).

Western blotting

Cells were grown to confluence in 60 mm dishes prior to the relevant treatment as detailed in the results section. Cells were lysed in 150 µl of ice cold lysis buffer (50 mM Tris–HCl, pH 8; 150 mM NaCl; 1% IGEpal CA-630; 0.5% deoxycholate; 0.1% SDS and 1x EDTA-free protease inhibitor cocktail) (Roche, West Sussex, UK) and centrifuged for 10 min at 10 000g. Protein concentration was determined using the Bio-Rad Lowry assay (Bio-Rad Laboratories, Hemel Hempstead, UK), using bovine serum albumin (BSA) as the standard. An equal amount of protein from each sample was diluted with 5 x sample loading buffer and boiled for 5 min. Cell lysates (40–80 µg of total protein) were separated by SDS–PAGE gels and proteins transferred to polyvinylidene fluoride membranes by semi-dry blotting. Blots were blocked and probed with the relevant antibodies. BMPR-II mouse monoclonal (BD Transduction Laboratories, NJ, USA); GFP mouse monoclonal (Roche); phospho-Smad1/5/8 rabbit polyclonal, total Smad1 rabbit polyclonal, phospho-p38MAPK and total p38MAPK (Cell Signaling Technology, Danvers, MA, USA). The Alk-1 rabbit polyclonal was a kind gift from Professor D Marchuk (Duke University, NC, USA). Blots were incubated with an appropriate horseradish-peroxidase-conjugated antibody and enhanced chemiluminescence reagent (GE Bioscience, Little Chalfont, UK). To confirm equal loading blots were incubated with an anti-α-tubulin antibody (Sigma-Aldrich).

Cell surface biotinylation

Method adapted from Rennolds et al. (53). PAECs or MRC5-BMPR-II-GFP cells were seeded in 60 mm dishes (4.38 × 10\(^5\) or 7 × 10\(^5\) cells/well, respectively) and cultured for 48 h prior to treatment. Cells were treated as described in the relevant results section. After treatment cells were placed on ice and washed twice with ice-cold PBS. Cells were washed once with PBS pH 8 containing 1 mM MgCl\(_2\) and 0.1 mM CaCl\(_2\) (PBS-pH8), and then incubated for 10 min at 4°C with freshly prepared EZ-Link\(^{\text{TM}}\) NHS-SS-biotin at 3 mg/ml (Pierce). To confirm equal loading blots were incubated with an anti-α-tubulin antibody (Sigma-Aldrich).

Immunofluorescence

MRC5-BMPR-II-GFP cells were seeded onto BD Falcon\(^{\text{TM}}\) glass chamber slides (BD Biosciences) and cultured for 48 h prior to treatment. After treatment, the chamber slides were washed with PBS and fixed and permeabilized with 1:1 acetonemethanol. Prior to staining cells were blocked with 10% FBS in

RNA interference

PAECs were seeded in 60 mm dishes (4.38 × 10\(^5\) cells/well) for protein or six-well plates (2 × 10\(^5\) cells/well) for RNA and cultured for 48 h in EGM-2. Before transfection, PAECs were incubated in Opti-MEM I (Life Technologies) for 3–4 h. PAECs were transfected with 0.01, 0.1, 1 or 10 nm siRNA (BMPR-II (Dharmacon siGENOME SMARTpool) or siControl non-targeting (both Dharmacon On-TARGETplus) (Perbio Science, Erembodegem-Aalst, Belgium) complexed with DharmaFECT1 (8.75 µl/well for 60 mm dish or 4 µl/well for six-well plate) diluted in Opti-MEM I. Cells were incubated with the siRNA:DharmaFECT1 complexes for 4 h at 37°C, and then replaced with EGM-2. Knockdown efficiency was confirmed by qPCR and western blotting where possible.
PBS. LAMP-1 was detected with the primary antibody mouse anti-human-LAMP-1 at 1:200 (Dako, Ely, UK) and a secondary rabbit anti-mouse TRITC (Dako) antibody at 1:500. Chamberslides were washed three times with PBS and mounted in glycerol/PBS solution DAPI (Vectorashield, Peterborough, UK). Cells were viewed and photographed using a confocal microscope (Leica TCS SPE) and images captured using Leica LAS AF software.

**Statistical analysis**

Statistical analysis was performed using Student’s t-test (*P < 0.05, **P < 0.01 and ***P < 0.001).

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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**Conflict of Interest statement.** None declared.

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