Endothelial β-arrestins regulate mechanotransduction by the type II bone morphogenetic protein receptor in primary cilia

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Abstract

Modulation of endothelial cell behavior and phenotype by hemodynamic forces involves many signaling components, including cell surface receptors, intracellular signaling intermediaries, transcription factors, and epigenetic elements. Many of the signaling mechanisms that underlie mechanotransduction by endothelial cells are inadequately defined. Here we sought to better understand how β-arrestins, intracellular proteins that regulate agonist-mediated desensitization and integration of signaling by transmembrane receptors, may be involved in the endothelial cell response to shear stress. We performed both in vitro studies with primary endothelial cells subjected to β-arrestin knockdown, and in vivo studies using mice with endothelial specific deletion of β-arrestin 1 and β-arrestin 2. We found that β-arrestins are localized to primary cilia in endothelial cells, which are present in subpopulations of endothelial cells in relatively low shear states. Recruitment of β-arrestins to cilia involved its interaction with IFT81, a component of the flagellar transport protein complex in the cilia. β-arrestin knockdown led to marked reduction in shear stress response, including induction of NOS3 expression. Within the cilia, β-arrestins were found to associate with the type II bone morphogenetic protein receptor (BMPR-II), whose disruption similarly led to an impaired endothelial shear response. β-arrestins also regulated Smad transcription factor phosphorylation by BMPR-II. Mice with endothelial specific deletion of β-arrestin 1 and β-arrestin 2 were found to have impaired retinal angiogenesis. In conclusion, we have identified a novel role for

Abbreviations: Arrb1/Arrb2 ECKO, endothelial specific, conditional β-arrestin1 and β-arrestin2 double knock-out; BMPR-II, type II bone morphogenetic protein receptor (protein); BMPR2, type II bone morphogenetic protein receptor (gene); eNOS, endothelial nitric oxide synthase; GPCR, G protein-coupled receptor; HUVEC, human umbilical vein endothelial cell; IFT81, intraflagellar transport protein 81.

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endothelial β-arrestins as key transducers of ciliary mechanotransduction that play a central role in shear signaling by BMPR-II and contribute to vascular development.

**KEYWORDS**

beta-arrestin, BMPR2, endothelial cells, primary cilia, shear stress

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**INTRODUCTION**

Sensing of the environment by endothelial cells (ECs) is a complex phenomenon that regulates the development, physiology, and pathophysiology of vascular and lymphatic structures. These environmental stimuli include vasoactive mediators and mechanical stress that are central to EC biology. ECs are under constant mechanical stress and sense variations in blood flow-induced shear stress. Shear stress induces the expression of thousands of genes in ECs, with differing effects on cellular phenotype depending on the direction and the magnitude of the flow pattern. For example, unidirectional laminar flow promotes elongation and alignment of ECs and the expression of anti-inflammatory genes, while turbulent flow stimulates disorganized cell proliferation with the expression of inflammatory and oxidative genes. The underlying mechanism for such differential response remains to be fully elucidated. These phenotypic changes in ECs are controlled by their sensing and transduction of mechanical signals during vascular remodeling. Multiple signaling pathways are regulated by shear stress, include MAP kinases, Rho family GTPases, reactive oxygen species, nitric oxide (NO), and receptors such as G protein-coupled receptors (GPCRs), bone morphogenetic protein receptors (BMPRs), and vascular endothelial growth factor receptors.

Primary cilia are microtubule-based organelles found in most mammalian cell types that sense extracellular stimuli using sensors of the external environment such as GPCRs, receptor tyrosine kinases, and BMPRs. The primary cilia contain proteins critical for protein trafficking, structure, and signaling, such as adenyl cyclases, protein phosphatases, and protein kinases. Primary cilia have been shown to respond to blood flow and are required for flow sensing. Receptors have been shown to be specifically trafficked to the primary cilia, resulting in a unique signaling environment with increased levels of local second messengers such as cyclic AMP that result in distinct outcomes from extraciliary messengers. Flow or shear stress can activate the primary cilium, triggering calcium signaling and NO production. Primary cilia play a role in vascular development, with expression in mouse retinal endothelia where they promote vessel stabilization through increased sensitivity of the BMP-ALK1 pathway. They have been shown to be essential for appropriate vascular patterning and responsible for enhancing BMPR-II signaling. Diseases of the primary cilia, known as ciliopathies, have important cardiovascular phenotypes related to development, hypertension, and atherosclerosis. However, the precise signaling pathway of mechanotransduction by primary cilia have not been fully elucidated, despite their physiological importance in vascular biology.

With their known role in trafficking ciliary proteins and regulating GPCR signaling, we sought to elucidate the role of β-arrestin adaptor proteins in ECs. We conducted an unbiased interactome screen to identify proteins physically associated with β-arrestins, and found multiple ciliary proteins to be associated with β-arrestin. We found disruption of β-arrestin expression in ECs led to marked impairment of EC response to shear stress. Moreover, we found that ciliary shear response is at least in part mediated by BMPR-II, whose downstream signaling cascade also requires β-arrestin. Lastly, mice with conditional, endothelial specific deletion of β-arrestin 1 and β-arrestin 2 were found to have marked impairment in their retinal angiogenesis, providing in vivo evidence for the role of β-arrestin in EC function and vascular development.

**METHODS**

**Materials**

Human recombinant BMP-2 and BMP-4 were purchase from R&D Systems. Anti-Myc and anti-HA antibodies were purchased from Cell Signaling Technology. FLAG-β-arrestin 1 and 2 plasmids and rabbit polyclonal anti-β-arrestin1 and 2 antibodies (A1CT and A2CT) were provided by Dr. Robert Lefkowitz (Duke University).
Plasmids encoding HA or Myc-tagged BMPR2, ALK3, and ALK6 were provided by Dr. Gerard Blobe (Duke University), the HA and Myc-tagged S532X BMPR2 plasmids were provided by Dr. Nicholas Morrell (Cambridge University), the bre-loc reporter was provided by Dr. Peter ten Dijke (Netherlands Cancer Institute), and parental and β-arr2 CRISPR KO HEK293 cells were provided by Asuka Inoue (Tohoku University).

Cell culture

Human umbilical vein endothelial cells (HUVECs) (Lonza) were cultured in EGM-2 medium (Lonza) at 37°C in a 5% CO₂ incubator. The HUVECs (passages 4–9) were grown to 80%–90% confluence for experimental treatments. HEK293 cells (WT and β-arr2 CRISPR KO) were grown in MEM with 10% FBS and 1% penicillin/streptomycin. Starvation medium consisted of MEM supplemented with 0.1% BSA, 10 mM HEPES, and 1% penicillin/streptomycin.

Transient transfection and siRNA knockdown

Transient transfection of plasmids was performed with Fugene HD (Promega) according to the manufacturer's instructions. For RNA silencing in HUVECs, short interfering RNAs (siRNAs) for both targeting sequences and nontargeting controls (GE Dharmacon, 50 nM, 72 h) were transfected with Lipofectamine RNAiMAX (Invitrogen) in HUVECs according to the manufacturer's instructions. siRNA knockdown of β-arrestins in HEK293s was performed as previously described.20 siRNA sequences targeting β-arrestin1 (5′-0AAAGCCUUCUGCGCGGAGAAU-3′) and β-arrestin2 (5′-AAGGACCGCAAGUGUUUGUG-3′) along with a control non-silencing RNA duplex (5′-0AAUUCU CCGAACGUGUCACGU-3′) were used. Cells were transfected using the GeneSilencer transfection reagent (Genlantis) as previously described.20

Shear stress condition and cell alignment assay

To subject HUVECs to orbital shear stress, calculated at 10.7 dynes/cm², an orbital shaker positioned inside the cell incubator was used. All shear stress experiments were performed for 24 h. To observe cell alignment under shear stress, the HUVECs were grown on 6 mm culture dishes and the cells 2 mm away from the edge of the well were taken as images using a brightfield microscope.

Proteomic analysis

HUVECs were subjected to 10.7 dynes/cm² shear stress for 24 h (shear samples) or maintained under static conditions (static samples), washed once in PBS and collected with NP-40 lysis buffer (Boston Bioproducts) with protease and phosphatase inhibitors (Roche). The cell lysates from the shear and static samples were immunoprecipitated separately with rabbit anti-β-arrestin1/2 antibody (CST) overnight at 4°C and protein A/G agarose beads (Santa-Cruz) for 2 h with rotation at 4°C. After two rinses in lysis buffer and one in PBS, the shear and static samples were profiled by tandem mass spectrometry and ultraperformance liquid chromatography (Bioproximity; LLC). Results were reported for proteins observed in the shear but not the static conditions.

Immunofluorescence staining

HUVECs grown on eight-well cell culture slides (Mat-Tek) coated with Fibronectin (Corning; Sigma-Aldrich) were washed with warmed PBS (37°C) twice, fixed in warm 4% paraformaldehyde (Thomas Scientific; 37°C) for 5 min and washed with PBS three times. The cells were permeabilized with 1% Triton X-100 in PBS for 5 min and washed again with PBS three times. The cells were blocked with 1% bovine serum albumin (Sigma-Aldrich) in PBS for 30 min. Anti-acetylated-tubulin (T6793; Sigma-Aldrich; 1:1000), anti-Arl13B (17711-1-AP; Proteintech; 1:500), anti-IFT81 (11744-1-AP; Proteintech; 1:500), and anti-BMPR-II (612292; BD Biosciences; 1:500) antibodies were incubated overnight at 4°C, and then detected with Alexa Fluor 568 donkey anti-mouse IgG (A10037; Invitrogen; 1:500), Alexa Fluor 568 goat anti-rabbit IgG (A11011; Invitrogen; 1:200), Alexa Fluor 488 goat anti-mouse IgG (A11001; Invitrogen; 1:200), and/or Alexa Fluor 647 goat anti-rabbit IgG (A21244; Invitrogen; 1:200). 0.5 µg/ml DAPI (Sigma-Aldrich) was used to stain the nuclei. The cells were mounted using Vectashield (Vector Labs) and imaged using fluorescence microscopy.

Confocal microscopy of β-arrestin 2 recruitment

HEK293 cells were transiently transfected with vectors encoding red fluorescent protein (RFP)-labeled β-arrestin2, BMPR2, and either of its coreceptors ALK3 or ALK6. Two micrograms of each plasmid was combined with Fugene transfection reagent (Roche Applied Science) at a ratio of 5 µl of transfection reagent
to 1 µg DNA in 500 µl of MEM and incubated at room temperature for 30 min before addition to a 10 cm dish of HEK293 cells at 50% confluence. Twenty-four hour after transfection, the cells were split into 35 mm glass-bottom dishes (MatTek). After a further 24–48 h of growth, the cells were starved for at least 1 h before fixation or stimulation with 50 ng/ml BMP-2 for 30 min followed by fixation. Cells were fixed in 1 ml 4% paraformaldehyde in PBS for 10–15 min. RFP-labeled β-arrestin2 was visualized on a Marianas spinning disk confocal microscope (Intelligent Imaging Innovations, Inc.).

Western blot in HUVECs

β-arrestin1 and β-arrestin2 siRNA (50 nM) or nontargeting siRNA (50 nM) were transfected into HUVECs for 24 h and the proteasome inhibitor MG132 (0.2 µM; Thermo Fisher Scientific) was added to the HUVECs for 48 h. The HUVECs were washed with PBS and lysed in NP-40 lysis buffer (Boston Bioproducts) including protease and phosphatase inhibitor (Roche). The cell lysates were incubated/rotated with mouse anti-β‐arrestin1, or 2 siRNA with 60 µl of Lipofectamine 2000 (Invitrogen). After 48 h of transfection, cells were scraped and lysed in lysis buffer of Pierce Crosslink IP Kit (Thermo/Pierce) with complete protease inhibitor cocktail tablet (Roche Applied Sciences). Cell lysates were incubated/rotated with mouse anti-β‐arrestins antibody (CST; 1:100) at 4°C overnight. Protein A/G agarose beads (Santa‐Cruz) were added to immunoprecipitated samples and rotated for 2 h with rotation at 4°C. After two washes in lysis buffer and one in PBS at 4°C, the agarose beads were boiled in Laemmli SDS sample buffer (Alfa Aesar) for western blot analysis with rabbit anti-β‐arrestin1, or 2 or nontargeting antibody (CST; 1:1000), mouse anti-β‐arrestin1/2, phospho‐β-arrestin1/2, or GAPDH antibody (CST; 1:1000).

Protein extraction and western blot analysis

HUVECs were lysed in NP40 lysis buffer (Boston Bioproducts) including cOmplete™ Mini EDTA-free Protease Inhibitor Cocktail (Roche; Sigma‐Aldrich) and PhosSTOP™ Phosphatase Inhibitor tablets (Roche; Sigma‐Aldrich). The lysed supernatant was obtained by centrifugation at 4°C for 10 min at 13,000 rpm and the total protein content was quantified by a Micro BCA Protein assay kit (Thermo Fisher Scientific). The proteins were boiled in Laemmli SDS sample buffer (Alfa Aesar) for 10 min and separated by Mini-Protean TGX Gels 4%–20% (Bio‐Rad), transferred onto an Immobilon‐PSQ PVDF Membrane (EMD Millipore). Membranes were probed with antibodies against β‐arrestin1/2, phospho‐eNOS (Ser1177), total‐eNOS, Myc‐tag, Erk5, GAPDH (CST; 1:1000), IFT81, IFT74 (Proteintech; 1:1000), GFP (Sigma‐Aldrich), and BMPR‐II (BD Bioscience; 1:1000). Bound primary antibodies were detected by secondary anti‐rabbit or anti‐mouse IgG‐HRP (CST, 1:2000). Blots were developed with Supersignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific).

Coimmunoprecipitation (CoIP) of β-arrestin1 and IFT81

After cotransfection with GFP‐tagged β‐arrestin1 or GFP control constructs and Myc‐tagged IFT81 constructs, HEK293 cells were washed with PBS and lysed in NP-40 lysis buffer (Boston Bioproducts) including protease and phosphatase inhibitor (Roche). The cell lysates were incubated/rotated with mouse anti-GFP antibody (Sigma‐Aldrich; 1:20) at 4°C overnight. Protein A/G agarose beads (Santa‐Cruz) were added to immunoprecipitated samples and rotated for 2 h with rotation at 4°C. After two washes in lysis buffer and one in PBS at 4°C, the agarose beads were boiled in Laemmli SDS sample buffer (Alfa Aesar) for western blot analysis with rabbit anti-Myc antibody (CST; 1:1000), mouse anti-GFP antibody (Sigma‐Aldrich; 1:1000), and GAPDH antibody (CST; 1:1000).

CoIP of BMPR‐II and β‐arrestins

For CoIP in HEK293s, cells were grown in six‐well plates to 50% confluency and cotransfected with Myc‐BMPR2 or Myc‐BMPR2‐S532X and HA‐β‐arrestin1/2 using Lipofectamine 2000 (Invitrogen). After 48 h of transfection, cells were scraped and lysed in lysis buffer of Pierce Crosslink IP Kit (Thermo/Pierce) with complete protease inhibitor cocktail tablet (Roche Applied Sciences). Cell lysates were incubated overnight at 4°C with anti‐Myc antibody (Cell Signaling) and Pierce Crosslink IP Kit (Thermo/Pierce). Sepharose beads were then eluted with 0.5 mg/ml c‐Myc peptide (Genscript) in TBS. The elution samples were then detected by immunoblotting.

Bre‐Luc reporter

The bre‐Luc luciferase reporter of Smad activity was obtained as a gift from Peter ten Dijke.21 A 10 cm dish of HEK293 cells of ~50% confluence was transiently transfected with 2 µg of the reporter and 20 µg of control, β‐arrestin1, or 2 siRNA with 60 µl of Lipofectamine 2000 (see above). After 24 h, cells were split into 96‐well plates at 50,000–100,000 cells/well. Twenty‐four hour later, cells were starved for 4 h and treated overnight with
Receptor biotinylation

HEK293 cells transfected with expression plasmids for HA-BMPR2 and FLAG-β-arrestin1/2 were surface biotinylated at 4°C for 20 min using EZ-link Sulfo-NHS-SS-Biotin (Pierce/Thermo). Unreacted biotin was quenched with 50 mM glycine in PBS at 4°C. For the endocytosis assay, cells were incubated with 100 ng/ml BMP-2 at 37°C for indicated time and remaining surface biotin was cleaved on ice with glutathione cleavage buffer (50 mM glutathione, 75 mM NaCl, 75 mM NaOH, 10 mM EDTA, 1% BSA in H2O). Excess glutathione was then quenched with 0.5% BSA in PBS for 30 min at least eight times at room temperature and incubated with anti-IB4 (121412; Invitrogen; 1:200) and Alexa Fluor 488 goat anti-rabbit IgG (A11008; Invitrogen; 1:500) as a secondary antibody for eNOS with 1% Triton X-100 in PBS overnight at 4°C in the dark. The retinas were washed in PBS for 30 min at least eight times at room temperature in the dark, postfixed in 1% paraformaldehyde in PBS for 5 min and washed with PBS three times. The retinas were mounted using Vectashield (Vector Labs) and imaged using fluorescence microscopy.

Statistical analysis

To analyze significant differences between two groups, two-tailed Student t-test or one-way ANOVA was used through GraphPad Prism 9.0. All data were reported as mean ± SEM, and a value of \( p < 0.05 \) was considered statistically significant.

RESULTS

β-arrestins interacts with primary ciliary proteins under shear stress and are required for the formation of primary cilia in HUVECs

β-arrestins 1 and 2 regulate multiple aspects of GPCR signaling, trafficking, and endocytosis and are ubiquitously expressed, including in ECs. To identify β-arrestin-interacting partners in ECs under differing hemodynamic states, we exposed HUVECs to either shear stress or a static environment. We then performed proteomics analysis on cell lysates pulled down by β-arrestin1/2 antibody under shear stress and static conditions. Interestingly, we found that β-arrestin binding increased to several primary ciliary proteins, such as IFT81, Kif13A, and Kif17 under shear stress compared to static conditions (Table 1). While β-arrestins have been associated with primary cilia in other cell types, their association with cilia in ECs have not been previously demonstrated. Primary cilia are prominent mechanosensors with the ability to physical bend as a result of shear stress. To explore if β-arrestins can colocalize with primary cilia in HUVECs, we transfected a GFP-tagged β-arrestin1 construct to HUVECs and stained cells with the primary cilia marker Ac-α-tubulin.
TABLE 1 β-arrestin-interacting partners in endothelial cells under shear stress

| Protein          | Hits | Description                                      |
|------------------|------|--------------------------------------------------|
| SEPT8            | 1    | SEPTIN-8, ciliary subcompartments                |
| DNAH17           | 2    | Dynein heavy chain 17, axonemal                 |
| DNAH6            | 1    | Dynein heavy chain 6, axonemal                  |
| FNIP1            | 3    | Folliculin-interacting protein                   |
| GOLGB1           | 3    | Golgin subfamily B member 1                     |
| IFT81            | 6    | Intraflagellar transport protein 81 homolog     |
| IPO4             | 5    | Importin-4                                       |
| KIF13A           | 2    | Kinesin-like protein KIF13A                     |
| KIF17            | 3    | Kinesin-like protein KIF17                      |
| NINL             | 1    | Ninein-like protein                              |
| PACSIN2          | 3    | Protein kinase C and casein kinase substrate in neurons protein 2 |
| PCNT             | 1    | Pericentrin                                      |
| PRC1             | 1    | Protein regulator of cytokinesis                 |
| PTCH1            | 1    | Protein patched homolog 1                        |

Note: Human umbilical vein ECs (HUVECs) were exposed to either shear stress or a static environment, and proteomics were performed analysis on cell lysates pulled down by β-arrestin1/2 antibody. Peptides identified more under shear stress conditions than static conditions are listed below.

While β-arrestin was detected throughout the cell, we found increased localization of β-arrestin1 to the primary cilia in HUVECs (Figure 1a). We further evaluated whether formation of primary cilia in ECs may be affected by disrupted expression of β-arrestins. We found siRNA mediated knockdown of β-arrestin1 and β-arrestin2 led to significant decrease in the % of ECs with primary cilia (Figure 1b,c, Supporting Information: Figure 1), suggesting that β-arrestins play an important role in primary cilia formation or maintenance.

### β-arrestin1 localization to primary cilia is IFT81-dependent

Our proteomics analysis found that IFT81 was the most frequently pulled down protein by β-arrestin1/2 in HUVECs (Table 1). IFT81 is a member of the intraflagellar transport protein family, which is known to be critical in primary cilia formation. IFT81 belongs to the IFT-B group of proteins that are involved in anterograde transport with heterotrimERIC kinesin-2, which denotes transport from the base to the ciliary tip. Previous studies have shown that IFT81 accumulates both on the base and the tip of primary cilia in cells and is involved in elongation of primary cilia.27,28

Given IFT81’s interaction with β-arrestin1/2 and its importance in primary cilia function, we sought to further elucidate the relationship of IFT81 and β-arrestin1/2. We found that IFT81 is colocalized with β-arrestin1 on primary cilia (Figure 1d), and directly associates with β-arrestin1 in HUVECs as demonstrated by immunoprecipitation (Figure 1e). To assess the role of IFT81 and its interaction with β-arrestin1, we evaluated GFP-tagged β-arrestin1 localization in IFT81-silenced HUVECs. We found that β-arrestin1 localization to the primary cilia was significantly decreased in IFT81-deficient HUVECs (Figure 1f,g), suggesting that IFT81 is required for the recruitment of β-arrestin1 to primary cilia. While we expected that the formation of primary cilia would be suppressed in IFT81-deficient HUVECs, we found that IFT81 siRNA knockdown alone did not affect the formation of primary cilia in HUVECs, suggesting that IFT81 may be dispensable for primary cilia formation (data not shown).

### β-arrestins interact with BMPR-II, which is required for the shear stress response

β-arrestins are established downstream signaling partners of GPCRs. With the evidence of β-arrestins being recruited to primary cilia, we next tested whether GPCRs and GPCR cofactors known to be expressed in the vascular endothelium (Supporting Information: Table 1) were localized to the endothelial primary cilia. We found that GFP-tagged S1PR1, CXCR2, and CXCR7 were localized to primary cilia (Supporting Information: Figure 1a), while CXCR1, GPR4, GPR15, PAR1, and RAMP2 did not appear to have preferential ciliary localization (Supporting Information: Figure 1b,c, Supporting Information: Figure 1). We next conducted siRNA knockdown of S1PR1, CXCR2, and CXCR7 in ECs to test whether these GPCRs regulated the EC response to shear stress, such as induction of eNOS expression29 (Supporting Information: Figure 2). Despite robust knockdown of each GPCR, we did not observe any significant change in eNOS expression or phosphorylation in response to shear stress in ECs (Supporting Information: Figure 2), suggesting that these cytoplasmic GPCRs may not be critical to regulating EC response to shear stress.

Given our findings, we sought to identify additional cell surface receptors that may utilize β-arrestins and be involved in mechanotransduction from endothelial primary cilia. The type II bone morphogenetic protein receptor (BMPR-II) has previously been shown to have a key role in ciliary flow sensing.30,31 While β-arrestins have been shown to interact with BMPR-specific coreceptors,32-34 whether BMPR-interact with and signal through β-arrestins in the primary cilia has not been determined. We first tested whether β-arrestins are
FIGURE 1  (See caption on next page)
recruited to BMPR-I/BMPR-II complexes (Figure 2a). Under basal conditions, β-arrestin2 was found throughout the cytoplasm. After stimulation with BMP-2, β-arrestin2 redistributed to the endosomes, consistent with a ligand-dependent interaction with BMPR-II (Figure 2a, right panels).

We further investigated the role of BMPR-II in endothelial shear sensing. We found that eNOS expression under shear stress was decreased in HUVECs subjected to BMPR2 knockdown (Figure 2b). Consistent with this finding, physical alignment of cells induced by shear stress was largely absent in BMPR-II-deficient HUVECs (Figure 2c). Taken together, these findings are consistent with BMPR-II being critical for induction of eNOS expression and EC alignment in response to shear stress. To investigate whether localization of BMPR-II in primary cilia changed under shear stress, we analyzed the localization of BMPR-II in HUVECs with or without shear stress. Interestingly, we found that under static condition BMPR-II was widely distributed throughout the cell, but subjecting ECs to shear stress led to significant increase in BMPR-II localization to the primary cilia (Figure 2d,e). We next assessed whether BMPR-II may be involved in recruitment of β-arrestin1 to the primary cilia. We transfected GFP-tagged β-arrestin1 constructs to BMPR2-knockdown HUVECs and stained for primary cilia. We found that the localization of β-arrestin1 to primary cilia was markedly decreased in BMPR2-silenced HUVECs (Figure 2f,g). Our results demonstrate the importance of BMPR-II in regulation of shear stress response in ECs and its role in regulating β-arrestin translocation to the primary cilia.

**β-arrestins interact with and regulate signaling by BMPR-II**

We next tested whether β-arrestins could directly interact with BMPR-II and regulate its signaling. CoIP of overexpressed BMPR-II and β-arrestins in HEK293 cells demonstrated a constitutive interaction between BMPR-II and β-arrestin1 and 2 (Figure 3a); this interaction was preserved in a BMPR2 truncation mutant (S532X) that removed the long C-terminal tail after the S/T kinase domain (Figure 3a). In the context of confocal microscopy that demonstrated β-arrestin redistribution after ligand stimulation (Figure 2a), this is consistent with β-arrestins interacting with BMPR-II in both a constitutive and ligand-induced manner. We next examined the effects of loss of β-arrestin1 and 2 on BMPR-II signaling as assessed by Smad1/5/8 (Smad) phosphorylation. HEK293 cells were transfected with β-arrestin1 or β-arrestin2 siRNA and stimulated with BMP-2. We observed that β-arrestin1 knockdown significantly decreased BMP-induced Smad phosphorylation while β-arrestin2 knockdown had no effect (Figure 3b), except for a modest increase at 15 min (Figure 3c). We then tested whether β-arrestin1 or 2 siRNA knockdown had any effect of transcription of a luciferase reporter with BMP-responsive elements from the Id1 promoter, providing a downstream readout of BMPR-II signaling. Although β-arrestin1 and β-arrestin2 had different effects on Smad phosphorylation, knockdown of either significantly decreased the activity of this reporter transfected in HEK293 cells stimulated with BMP-2 (Figure 3d). Taken together, these data demonstrate that β-arrestins regulate Smad phosphorylation by BMPR2 and its transcriptional activity, although the levels of Smad phosphorylation are not the sole determinant of transcriptional activity.

For many GPCRs, β-arrestins act as adaptor proteins for receptor endocytosis. We tested for such a role at BMPR2 by assessing receptor endocytosis in combination with either β-arrestin1/2 knockout (KO) by CRISPR/Cas9 or overexpression of β-arrestin1/2. Using a receptor biotinylation assay to selectively pulldown endocytosed receptor, we did not observe any change in receptor internalization in a β-arrestin1/2 KO HEK293 cell line compared to the parental cell line (Figure 3e). Restoration of β-arrestin1/2 expression in the KO cells did not impact internalization (Figure 3f).
FIGURE 2 (See caption on next page)
FIGURE 2 BMPR2 and β-arrestin1 are required for the shear stress response. (a) Confocal microscopy demonstrating BMP-2-induced internalization of RFP-labeled β-arrestin2 before (pre) and 30 min after stimulation (post) with 50 ng/ml of BMP-2 in HEK293 cells transiently transfected with (top panel) BMPR1A/BMPR2 and (bottom panel) BMPR1B/BMPR2. (b) Immunoblot detection of phosphorylated eNOS (p-eNOS), total eNOS (t-eNOS), β-arrestin1/2, BMPR-II and GAPDH levels extracted from BMPR2 siRNA (BMPR2 si), or control siRNA (Con si) treated HUVECs with or without shear stress. (c) Cell alignment of BMPR2 siRNA (BMPR2 si) and control siRNA (si Con) treated HUVECs. (d, e) Immunofluorescence staining and quantification of BMPR-II (green) in HUVECs with or without shear stress. The number of BMPR-II localized primary cilia was quantified as percentage based on the number of primary cilia (red; Ac-α-tubulin). n indicates number of primary cilia counted and ****p < 0.0001. (f, g) Immunofluorescence staining and quantification of β-arrestin1 (green) in BMPR2 siRNA (si BMPR2) and control siRNA (si Con) treated HUVECs. The number of β-arrestin1 localized primary cilia was quantified as percentage based on the number of primary cilia (red; Ac-α-tubulin). n indicates number of primary cilia counted and ***p < 0.001. BMPR, bone morphogenetic protein receptor; HUVECs, human umbilical vein endothelial cells; REP, red fluorescent protein.

FIGURE 3 β-arrestins interact with and regulate signaling by BMPR-II. (a) Interaction of β-arrestins with BMPR-II as assessed by coimmunoprecipitation of overexpressed FLAG-tagged β-arrestin1/2 with myc-tagged full length BMPR2 and its 532X truncation. (b, c) β-arrestin1 significantly reduces BMP-induced Smad1/5/8 phosphorylation by BMPR-II after stimulation with BMP-2 (50 ng/ml) with siRNA knockdown of β-arrestin1 or β-arrestin2. pSmad/tSmad levels were normalized to ctl siRNA signal at 30 min (*p < 0.05 compared to other siRNA treated samples for that time point by two-way ANOVA with Tukey’s multiple comparison test). (d) Both β-arrestin1 and 2 knockdown significantly decreases activity of a BMP reporter (Bre-luc) transfected in HEK293 cells stimulated with BMP-2 (10 ng/ml) (*p < 0.05 all groups significantly different; **p < 0.05 β-arrestin1 and ctl-siRNA significantly different by two-way ANOVA with Tukey’s multiple comparison test). (e) No difference in time course of BMP-2-induced endocytosis between WT and β-arrestin1/2 KO HEK293 cells. The endocytosed pool of BMPR-II was labeled with biotin (see Section 2) before pulldown with avidin beads. (f) No effect of β-arrestin1/2 overexpression on receptor internalization in β-arrestin1/2 KO cells. Endocytosis at 30 min was significantly decreased by the clathrin inhibitor Pitstop2 but not by the dynamin inhibitor dynasore. All experiments performed at least three times; shown are mean ± SEM. Blots and images shown are representative from at least three independent experiments. BMPR, bone morphogenetic protein receptor.
with β-arrestins not playing a role in BMP-induced receptor internalization. Receptor internalization did decrease significantly with clathrin inhibition, but was not affected by dynamin inhibition (Figure 3f). These findings are consistent with ligand-induced BMPR-II internalization requiring clathrin but neither β-arrestins nor dynamin.

**β-arrestin is required for the EC response to shear stress and eNOS expression**

To investigate whether β-arrestin is required for EC signal transduction in response to shear stress, we tested for changes in HUVEC orientation and eNOS expression with or without shear stress in β-arrestin-deficient HUVECs. HUVEC cell alignment in response to shear stress was maintained in control siRNA treated cells but were lost in both β-arrestin1- or 1 + 2 siRNA-treated cells (Figure 4a). Consistent with this, we found that induction of eNOS expression in response to shear stress was lost in HUVECs subjected to β-arrestin knockdown (Figure 4b,c). These results were consistent with β-arrestins being required for the EC response to shear stress.

We next sought to determine the mechanism of disrupted shear response in β-arrestin deficient ECs. First, we found that while eNOS protein levels were not upregulated in response to shear stress in β-arrestin knockdown ECs, the increase in eNOS mRNA transcript by shear stress was not significantly affected by β-arrestin knockdown (Supporting Information: Figure 3). Moreover, transcription factors Krüppel-like factor 2 (KLF2) and KLF4, which are well known to be transcriptionally induced by shear stress was not significantly affected by β-arrestin knockdown (Supporting Information: Figure 3). These findings suggest that shear stress induced eNOS transcriptional upregulation is not affected by β-arrestin knockdown. To further explore the mechanism of β-arrestins in modulating eNOS protein expression, we investigated ubiquitination of eNOS. eNOS is regulated by Hsp90-based chaperone mediated ubiquitin-proteasome system. It is reported that MG132, which is a proteosomal inhibitor, increases eNOS expression and NO generation in HUVEC. Interestingly, we found that ubiquitination of eNOS was increased in response to β-arrestin knockdown (Figure 4d), which demonstrated that β-arrestins contribute to the shear response in HUVECs by inhibiting degradation of eNOS. We further evaluated whether ciliary proteins, namely the IFT proteins, can directly modulate eNOS induction in response to shear stress. We found that shear stress induced eNOS expression was significantly decreased in response to IFT81 knockdown, which was further decreased via IFT81 and IFT74 combined knockdown in HUVECs (Figure 4e). These findings are consistent with ciliary structural proteins being required for β-arrestin-mediated regulation of eNOS expression in HUVECs.

**Retinal vascular development is impaired in Arrb1/Arrb2 ECKO mice**

Primary cilia play a key role in vascular development, with expression in mouse retinal endothelium where they promote vessel stabilization through increased sensitivity of the BMP-ALK1 pathway. We generated endothelial-specific, conditional β-arrestin1 and β-arrestin2 double KO mice (Arrb1/Arrb2 ECKO mice) to determine whether β-arrestins could affect primary cilia and vascular development. Mouse pups were injected with tamoxifen on postnatal Day (P) 1 through P3 to induce endothelial Arrb1/Arrb2 deletion and were analyzed at P7 to assess for changes in retinal vascular development.

We found that vascular plexus of Arrb1/Arrb2 ECKO retinas was markedly reduced compared to that of control retinas (Figure 5a) with shorter radial expansion length compared to that of control retinas (Figure 5b), and a significant reduction in the number of branchpoints was observed in Arrb1/Arrb2 ECKO retinas compared to controls (Figure 5c). These findings suggest that endothelial β-arrestin1 and β-arrestin2 play an important role in vascular development. Notably, mouse retina vessels have been reported to have different numbers of primary cilia depending on the specific vessel bed, with increased numbers in the veins and plexus, and fewer in the arteries. Therefore, we tested whether there was a difference in the number of primary cilia between veins and arteries in mice retina and whether this differed in Arrb1/Arrb2 ECKO compared to wild-type retina. We quantified the number of primary cilia and normalized them to the area of vessels. We found no difference in the number of primary cilia between different vessels, nor any significant difference between Arrb1/Arrb2 ECKO and wild-type retinas (Supporting Information: Figure 4). To determine whether β-arrestin also regulates eNOS expression in vivo as we observed in vitro, we stained Arrb1/Arrb2 ECKO retinas for eNOS. Consistent with our in vitro findings, Arrb1/Arrb2 ECKO retinas displayed significant decrease in eNOS expression compared to control retinas, consistent with β-arrestins being critical for eNOS expression in the vasculature in vivo (Figure 5d).
DISCUSSION

The primary cilium is a sensory organelle that receives both mechanical and chemical signals from other cells and the environment. The integration of these different stimuli through primary cilia is central to many aspects of endothelial function. Primary cilia are present in almost all types of cells and essential signal transducers that dictate various cellular responses. For example, multiple components of the sonic hedgehog signaling cascade and many growth factors localize to the primary cilium, and loss of the cilium blocks ligand-induced signaling, leading to uncontrollable cell growth or disruptions in homeostasis. Primary cilia are largely...
absent in high laminar flow, athero-resistant regions of vessels but found in regions of disturbed flow\textsuperscript{43} or areas of high shear stress.\textsuperscript{44} Mechanosensing responses of primary cilia in ECs play important roles in many vascular functions such as in the pulmonary circulation.\textsuperscript{41} Many signaling pathways have been shown to contribute to endothelial mechanosensing in PAH. For example, vascular stiffness has been shown to promote YAP/TAZ-dependent glutaminolysis that contributes to PAH.\textsuperscript{45} TRPC6 and Piezo1 channels have been shown to transduce mechanosensitive cation channels in PA ECs,\textsuperscript{46} and loss of endothelial Piezo2 has been shown to impair NO synthesis and induce endothelial-tomesenchymal transition and PH.\textsuperscript{47} Here we identified a novel signaling mechanism in which β-arrestins regulate ciliary BMPR-II signaling that contributes to the endothelial response to shear stress.

We found that β-arrestins, already known to regulate signal transduction, are required for formation and mechanosensing responses of primary cilia in ECs.

**FIGURE 5** Endothelial-specific knockout of β-arrestins results in a retinal vascular development defect. (a) IB4 stained p7 retinal flat-mounts of Arrb1/Arrb2 ECKO and wild-type (control) mice. (b) Quantification of vascular radial expansion in Arrb1/Arrb2 ECKO and wild-type (Ctl) mice. N = 7 wild-type and 4 Arrb1/2 ECKO. *p < 0.05. (c) The number of branchpoints in Arrb1/Arrb2 ECKO and wild-type (Ctl) mice. N = 7 wild-type and 3 Arrb1/2 ECKO. *p < 0.05. (d) eNOS (green) and IB4 (red) stained p7 retinal flat-mounts of Arrb1/2 ECKO and wild-type mice. A indicates arteries and V indicates veins.
β-arrestins associated with IFT81 to localize in the primary cilia and this complex likely plays role in formation of the primary cilia, as β-arrestin knockdown decreased the number of primary cilia. We also found that the eNOS-mediated shear response via primary cilia in ECs was regulated by β-arrestins and required BMPR2. We also demonstrated that β-arrestins were required for BMPR-II signaling through phosphorylation of Smad transcription factors, consistent with their role in regulating the endothelial mechanosensing response. A limitation of those biochemical studies was that they were performed in HEK293 cells, as we were unable to identify the specific BMPs to induce robust Smad phosphorylation in HUVECs. While β-arrestins have previously been shown to play a role in regulating signaling by TGF-β coreceptors, our study identified a role for β-arrestins in promoting signaling by a type II TGF-β receptor, BMPR-II. Notably, while β-arrestins regulate multiple aspects of GPCR signaling and endocytosis, we only found that β-arrestins were required for Smad phosphorylation and the downstream transcriptional response but not required for receptor endocytosis. While we did not elucidate the full mechanistic details that underlie β-arrestin regulation of BMPR-II, this suggests that the interaction of β-arrestins with BMPR-II relies on different mechanisms than β-arrestin regulation of GPCRs.

With the well-described role of β-arrestins in regulating GPCR signaling, we attempted to find GPCRs which regulate endothelial shear response via interacting with β-arrestins in primary cilia based on the fact that β-arrestin is a multifunctional regulator for most of GPCRs. We had eight potential GPCR candidates that are known to affect vascular development and functions, however, the candidates we tested did not appear to promote eNOS-mediated endothelial shear response through β-arrestins. It is likely that other GPCRs that are localized in primary cilium interact with β-arrestins to regulate endothelial mechanosensing. Based on the localization of other GPCRs, including S1PR1, CXCR2, and CXCR7, β-arrestins may play a role in integrating signals from a number of receptors in the primary cilium that regulate the endothelial response to shear stress.

Endothelial mechanosensing plays an important role not only in the response to shear stress in the adult, but also in vascular development. To address whether β-arrestins played a role in vascular development, we generated mice with inducible, endothelial specific KO of both β-arrestins (Arrb1/Arrb2 ECKO mice). We generated these mice because β-arrestin1 homozygous KO mice have essentially normal basal cardiovascular physiology including heart rate, blood pressure, and left ventricular ejection fraction but display abnormal response to β-adrenergic stimulation. Similarly, β-arrestin2 KO mice are phenotypically normal and produce viable progeny but display abnormal responses to physiologic stresses. In contrast, while mice deficient of either β-arrestin1 or β-arrestin2 develop normally and are fertile, β-arrestin1 and β-arrestin2 double KO mice die within few hours after birth due to pulmonary hypoplasia. This is consistent with β-arrestins compensating for one another in the setting of KO of the other isoform. Arrb1/Arrb2 ECKO mice allowed us to determine whether changes in the EC shear stress response mediated by β-arrestins result in a vascular phenotype. We found that Arrb1/Arrb2 ECKO mice displayed abnormal retinal vascular development, with decreased radial expansion and a loss of branchpoints. Consistent with this, we found that Arrb1/Arrb2 ECKO retinas displayed significantly less eNOS localization at arteries and veins than WT retinas. These findings are consistent with β-arrestins regulating the endothelial shear response through BMPR2, but may also reflect a role for β-arrestins in regulating GPCRs that are important in vascular development.

In conclusion, we report a novel function of endothelial β-arrestins in primary ciliary mechanosensing via BMPR-II signaling. Our findings contribute to an enhanced understanding of the regulation of ciliary signaling and how flow-induced shear stress regulates receptor signaling and vascular development. Future studies will be required to address the detailed biochemical mechanisms that underlie β-arrestin regulation of BMPR-II signaling and how signaling through different receptors in the primary cilium is integrated through β-arrestins and other signaling proteins.

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.
ETHICS STATEMENT

All animal experimental protocols were approved by the Institutional Animal Care and Use Committee at Duke University Medical Center.

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