The Four-and-a-half LIM Domain Protein 2 Regulates Vascular Smooth Muscle Phenotype and Vascular Tone

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In response to vascular injury, differentiated vascular smooth muscle cells (vSMCs) undergo a unique process known as “phenotype modulation,” transitioning from a quiescent, “contractile” phenotype to a proliferative, “synthetic” state. We have demonstrated previously that the signaling pathway of bone morphogenetic proteins, members of the transforming growth factor β family, play a role in the induction and maintenance of a contractile phenotype in human primary pulmonary artery smooth muscle cells. In this study, we show that a four-and-a-half LIM domain protein 2 (FHL2) inhibits transcriptional activation of vSMC-specific genes mediated by the bone morphogenetic protein signaling pathway through the CArG box-binding proteins, such as serum response factor and members of the myocardin (Myocd) family. Interestingly, FHL2 does not affect recruitment of serum response factor or Myocd, however, it inhibits recruitment of a component of the SWI/SNF chromatin remodeling complex, Brg1, and RNA polymerase II, which are essential for the transcriptional activation. This is a novel mechanism of regulation of SMC-specific contractile genes by FHL2. Finally, aortic rings from homozygous FHL2-null mice display abnormalities in both endothelial-dependent and -independent relaxation, suggesting that FHL2 is essential for the regulation of vasomotor tone.

Unlike skeletal and cardiac muscle cells, vSMCs never terminally differentiate, but are capable of transitioning to a synthetic phenotype characterized by decreased vSMC marker gene expression, increased matrix deposition, and responsiveness to signals that lead to increased migration and growth (1).

This plasticity is crucial for the normal development of vessels, homeostasis of blood pressure, and repair of injury, but it also contributes to the development of various vascular pathologies. Therefore, it is critical to understand the signaling pathways that modulate the phenotype of vSMCs to develop agents that can regulate aberrant vSMC responses in diseased vessels.

Recently we demonstrated that the BMP signaling pathway plays an important role in the phenotype modulation of PASMCs. Activation of the BMP pathway is required not only for maintenance of the contractile phenotype in PASMCs, but also for induction of vSMC marker gene expression in non-SMCs, such as mouse mesenchymal C3H10T½ (10T1/2) cells (2–4). BMPs are the largest group in the TGF family, play a crucial role in various physiological processes (5–7). During embryonic development, the BMP pathway participates in the induction of ventral mesoderm, cardiac myogenesis, and vasculosclerosis (8). Heterozygous mutations in the gene encoding BMPRII, the type II subunit of the BMP receptor, were identified in patients with both familial and sporadic idiopathic pulmonary arterial hypertension (IPAH) (9, 10). IPAH is characterized by elevated pulmonary vascular resistance and pulmonary arterial pressure with increased muscularization of small arteries, thickening or fibrosis of the intima, and presence of plexiform lesions and leads to right ventricular heart failure (11, 12). Loss of expression of either the type I or type II subunits of the BMP receptor has been observed in sporadic and secondary forms of PAH (13–15). Transgenic mice expressing a catalytically inactive BMPRII mutant in SMCs are predisposed to develop PAH in response to different stimuli (16, 17). These results suggest that BMP signaling plays an essential role in normal homeostasis of vascular cells and in the pathogenesis of IPAH (18). It is unclear why dysregulation of BMP signaling causes pulmonary hypertension but not systemic hypertension, because BMP is able to modulate the phenotype of SMCs derived from tissues other than the pulmonary vasculature, including myometrial, aortic, and umbilical cord SMCs.3

Nearly all vSMC-specific contractile genes studied, including smooth muscle α-actin (SMA), smooth muscle calponin (CNN1), and SM22α (SM22), require a conserved cis-acting element called the “CArG box” (CC(AT)6GG) found within their promoter or intronic sequences (19, 20). CArG boxes bind the transcription factor SRF, first identified and named for its ability to confer serum inducibility to the c-fos gene. A variety of cofactors are known to modulate SRF transcription activity and

3 B. N. Davis and A. Hata, unpublished results.
affinity for the CArG box, including proteins of the Myocd family, such as Myocd, MRTF-A, and MRTF-B. These are all extremely potent coactivators of SRF and have been shown to induce expression of all CArG-dependent vSMC marker genes (19–21). Our previous study shows that BMP signaling mediates nuclear translocation of MRTF-A and MRTF-B and activates the expression of vSMC-specific genes through the CArG box (3).

It has recently been reported that the LIM domain proteins CRP1, CRP2, and Leupaxin are also involved in the regulation of vSMC marker genes. CRP1 and CRP2 act as adaptor proteins that tether SRF and GATA4/5/6 to confer activation of SMC genes in cardiac myocytes (22). Leupaxin forms a complex with SRF, binds to the CArG box, and activates CArG-containing gene transcription as a cofactor of SRF (23). FHL2 (also known as DRAL or Slim3) is also a LIM-containing protein. FHL2 interacts with both cytoplasmic and nuclear proteins and plays a role in modulation of various cellular processes, such as cell proliferation, transcription, and signal transduction (24). FHL2 is highly expressed in early cardiac precursor cells and in the heart in adults (25), but low levels of expression were detected in other tissues, including the vasculature (24, 26). It has been shown that transcription of the FHL2 gene is up-regulated in an SRF-dependent manner (27). Furthermore, FHL2 antagonizes RhoA-mediated induction of SMA and SM22 (27). In this study we examined the role of FHL2 in BMP-mediated vSMC phenotype modulation. Genetic evidence that patients with PAH carry truncation mutations of the tail domain of BMPRII suggests an important role of the tail domain in the BMP signaling function. FHL2 was cloned by yeast two-hybrid screen using BMPRII tail domain as bait. Here, we show that FHL2 translocates to the nucleus upon stimulation with BMP and antagonizes the BMP-mediated transcriptional activation of vSMC-specific contractile genes in PASMcs. We demonstrate that FHL2 does not inhibit recruitment of SRF, or of its cofactor MRTF-A, to the CArG sequence, but inhibits recruitment of the chromatin remodelling SWI/SNF complex protein Brg1 and RNA polymerase II. Furthermore, aortic vessels from FHL2-null mice are resistant to relaxation upon vasodilator stimuli. Altogether these data indicate that the essential role of FHL2 in the regulation of vascular tone and contractile responses mediated by contractile stimuli, such as BMP signaling.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—C3H10T½ (American Type Culture Collection) were maintained in Dulbecco’s modified Eagle’s media supplemented with 10% fetal bovine serum (FBS, Sigma). Human primary pulmonary artery smooth muscle cells (PASMcs) were purchased from Lonza (CC-2581) and were maintained in Sm-GM2 media (Lonza) containing 5% FBS.

**Antibodies and Growth Factors**—Antibodies used in this study are: FHL2 (#38–7900, Zymed Laboratories Inc.), FLAG-epitope tag (clone M2, A2220, Sigma), HA-epitope tag (clone Y11, SC-805, Santa Cruz Biotechnology or 12CA5, Roche Applied Science), Myc epitope tag (clone 9E10, Tufts Core facility or ab9106, Abcam), anti-SMA (clone 1A4, Sigma), MRTF-A (clone C-19, sc-21558, Santa Cruz Biotechnology), or SRF (clone G-20, sc-335X, Santa Cruz Biotechnology). Recombinant human BMP4 and Alexa Fluor-568-conjugated phalloidin were purchased from R&D Systems and Invitrogen, respectively.

**Plasmid DNA Construct and Transfection**—A full-length human FHL2 cDNA was cloned into the CS2 vector and subcloned into a C-terminal myc tag-containing pCR3 vector. Plasmid DNAs were transfected into 10T½ cells with FuGENE 6 (Roche Applied Science), as described (3, 28).

**Adenoviral Constructs and Transduction**—The recombinant adenoviruses carrying myc-FHL2 or dnALK6 cDNA were generated and purified by standard procedures. Transduction of adenoviruses was performed at 100 multiplicity of infection. There was no detectable toxicity to the cells under these conditions.

**RNA Interference**—Synthetic small interference RNA (siRNA) against human FHL2 and Brg1 was purchased from Dharmacon (ON-TARGETplus SMARTpool #L-015862-00) and Santa Cruz Biotechnology (sc-29827), respectively. The siRNA sequence targeting mouse FHL2 is 5'-GGAUCGGCA-CUGGCAUGAUAU-3'. A siRNA with a non-targeting sequence (Dharmacon) was used as a negative control. The siRNAs were transfected by Oligofectamine (Invitrogen) (3, 28).

**qRT-PCR Assays**—qRT-PCR assays were performed as described (3). Data were analyzed by the comparative C T method through software by Bio-Rad. PCR primers for mouse FHL2 (upstream: 5'-TCAGCATGACTGAACGTTCGTAC-3'; downstream: 5'-GCCTGATCATCCGGATGAGGG-3') and human FHL2 (upstream: 5'-GGTGGTGTGGCTTTGAGA-3'; downstream: 5'-GAAGCGATTCCGAGCACTGAG-3') were synthesized by Sigma. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), human SMA, calponin1 (CNN), and SM22α were described before (15, 29).

**Luciferase Assay**—Luciferase reporter constructs containing promoter of SMA (−2560/+2784), SM22 (−445/+1st exon), SM22(MUT) (SM22 with mutations in two CArG boxes), and CNN (−549/+1700) were obtained from Drs. G. K. Owens, L. Li, and J. Miano (30–33). Xvent-2-luc construct was described previously (29). After transfection of the reporter construct together with LacZ plasmid (internal control), 10T½ cells were resedeed onto 12-well plates and treated with 3 nm BMP3 or 100 pm TGFβ1 for 16–20 h in Dulbecco’s modified Eagle’s media/0.2% FBS. Luciferase assays were carried out using a Promega Luciferase assay system. Luciferase activity was normalized with β-galactosidase activity.

**Immunofluorescence Staining**—PASMcs or 10T½ cells were fixed and permeabilized in a 50% acetone-50% methanol or 4% paraformaldehyde/phosphate-buffered saline solution and subjected to staining using Alexafluor-568-conjugated phalloidin, anti-SMA or anti-Myc antibodies, and nuclear staining with 4’-6-diamidino-2-phenylindole (DAPI, Invitrogen). Immunofluorescence was indirectly observed by staining with Alexafluor-488-conjugated goat anti-mouse secondary antibodies (Invitrogen #A11029) and Zymax Cy3-conjugated goat anti-rabbit secondary antibodies (Invitrogen #81-6115).

**ChIP Assay**—ChIP assay was performed as described (34). Briefly, soluble chromatin was prepared from 10T½ following a cross-linking reaction with 5 mm dimethyl adipimidate and 1% formaldehyde (35). Chromatin was then incubated with either
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anti-FHL2 (clone 11-134, #K0055-3 MBL International), anti-RNA Polymerase II (clone CTD4H8, Upstate/Millipore, #05-623), anti-MRTF-A (clone C-19, sc-21558, Santa Cruz Biotechnology), anti-SRF (clone G-20, sc-335X, Santa Cruz Biotechnology) antibodies, or rabbit nonspecific IgGs as a negative control. PCR primers specific for the mouse CTGF gene and SMA gene promoter were described previously (3, 36).

Collagen Matrix Contraction Assay—Collagen matrix contraction assay was performed as described (37). Briefly, PASMCs transfected with siFHL2 or control siRNA (siControl) were embedded in attached collagen matrices, followed by 3 nm BMP4 treatment for 24 h. Upon releasing the collagen lattice from the culture dish, the embedded cells become free to contract the deformable collagen lattice and the surface area of collagen lattice becomes smaller (38). Twenty-four hours after the detachment of gel from the dish, the gel surface area was measured and demonstrated as percentage of the gel surface area at the time of detachment.

Murine Vascular Ring Studies—Vascular ring studies were performed with mouse thoracic aortas. Briefly, aortic rings were prepared, equilibrated, and mounted on force transducers in bioassay chambers as described previously (39, 40). Contractile responses to KCl were performed in all vascular ring assays. There was no difference between WT (0.148 ± 0.02 g) and FHL2(KO) (0.135 ± 0.03) mice (n = 6 each) in response to KCl (30 mm). Following maximal contraction with phenylephrine in the dose range of 10⁻⁸ m to 3 × 10⁻⁶ m, increasing concentrations (10⁻⁸ m to 3 × 10⁻⁶ m) of acetylcholine (Ach) was applied. Aortic rings were further relaxed by a treatment with two doses of S-nitroprusside (SNP) (10⁻⁸ m or 3 × 10⁻⁸ m). For Ach and SNP relaxation, total rings studied were as follows: WT, n = 10; FHL2(KO), n = 6. For phenylephrine studies, 18 rings were studied.

Isolation of vSMCs from Mice—Carotid artery explants were harvested from wild-type and FHL2(KO) mice. Adventitia were dissected from media, which was cultured to yield vSMCs, whose phenotype was confirmed by typical morphology and immunohistochemical staining for SMA. Seven separate isolates of vSMCs were obtained and characterized, derived from a pool of aortas from three individual mice per each genotype.

Statistical Analysis—The results presented are average of at least three experiments each performed in triplicate with standard errors. For pairwise analysis, Student’s t tests were used. Multiple group comparisons were made by one-way or two-way analysis of variance, and multiple pairwise comparisons were made with the Student-Newman-Keuls method. For all statistical comparisons, p < 0.05 was considered significant and the values are indicated with asterisks.

RESULTS

We sought to identify tail domain-interacting proteins by a yeast two-hybrid screen. We used the full length of BMPRII tail domain (TD, amino acids 501–1038) or truncation mutation of tail domain found in IPAH, which contains amino acids 501–860, fused to the yeast Gal4 DNA binding domain as bait and screened a mouse embryonic fibroblast NIH3T3 library. Among the eight positive clones obtained from the screen with the full-length tail domain bait, one clone encoding a full-length FHL2 failed to interact with the truncated tail domain bait suggesting a possible involvement of FHL2 in pathogenesis of PAH. The interaction between FHL2 and BMPRII was confirmed in mammalian cells. The mammalian expression plasmid pCMV5 encoding Myc-epitope-tagged full-length mouse FHL2 was co-transfected into COS7 cells with expression plasmids encoding FLAG-epitope-tagged BMPRII. The interaction between FHL2 and BMPRII was detected by immunoprecipitation followed by immunoblot (Fig. 1A). Interaction between FHL2 and BMPRII was slightly decreased after BMP4 stimulation (Fig. 1A). Next we examined whether FHL2 colocalizes with BMPRII at the plasma membrane by immunofluorescence staining. When the wild-type BMPRII receptor (BMPRII(WT)) was coexpressed, FHL2 localized, in part, with BMPRII at the plasma membrane (Fig. 1B, top panels). The ectopically expressed tail-deletion mutant BMPRII (ΔT) (Leu-530 to stop codon), which is properly expressed at the plasma membrane, was not able to localize FHL2 on the cell surface (Fig. 1B, middle panels). These results suggest that FHL2 colocalizes with BMPRII on the cell surface through interaction with the BMPRII(TD). We also examined the ability of FHL2 to interact with a partial TD deletion mutant of BMPRII found in IPAH, BMPRII(Δ860) (Asn-861 to stop codon). Consistent with an observation in yeast two-hybrid assay, an immunofluorescence stain showed no colocalization between FHL2 and BMPRII(Δ860) (Fig. 1B, bottom panels), indicating that the N-terminal region of the BMPRII(TD) located between amino acids 530 and 860 is not sufficient for FHL2 interaction. Subcellular localization of endogenous FHL2 was examined in human primary pulmonary artery smooth muscle cells (PASMCs). Weak and ubiquitous localization of FHL2 was observed at a basal state, however, FHL2 was enriched in the nucleus upon BMP4 stimulation similar to FBS treatment (27) (Fig. 1C). These results indicate that BMP stimulates nuclear accumulation of FHL2, possibly by facilitating translocation of a plasma membrane-associated population of FHL2 to the nucleus.

We have reported previously that BMP signaling induces vSMC-specific genes in both mouse pluripotent C3H10T½ (10T1/2) and PASMCs cells through activation of the Smad pathway and the RhoA/Rho kinase pathway and mediates differentiation into a SMC-like phenotype (3). Therefore, we examined the potential role of FHL2 on the BMP-mediated transcriptional activation of vSMC genes in 10T1/2 cells. The luciferase constructs containing the promoter region of SM22α (SM22-luc), smooth muscle α-actin (SMA-luc), or smooth muscle calponin (CNN-luc), which contain a CArG box sequence, were transfected with increasing amounts of FHL2 expression plasmid (Fig. 2). FHL2 inhibited the BMP4-mediated activation of all three vSMC reporters in a dose-dependent manner (Fig. 2). A mutant SM22-luc construct, which is mutated in critical residues of the CArG box, was not affected by FHL2 (Fig. 2, SM22(MUT)-Luc). Interestingly, FHL2 did not affect the activity of the non-vSMC gene Vent-2, a known target of the BMP-Smad pathway (Xvent2-luc) (29), suggesting that the inhibitory action of FHL2 is restricted to vSMC-specific genes (Fig. 2). These results unveil an antagonistic effect of FHL2 on BMP-mediated vSMC-specific gene expression.
The antagonistic activity of FHL2 was confirmed by examining endogenous vSMC-specific gene expression in human primary vSMCs, PASMCs. PASMCs were infected with recombinant adenovirus carrying cDNAs encoding FHL2 or GFP (control). qRT-PCR analyses indicate that BMP4-mediated induction of vSMC genes, SMA and CNN, was reduced to 40% in FHL2-expressing cells compared with control GFP-expressing cells (Fig. 3A, top). Consistent with the Xvent2-luc result (Fig. 2), FHL2 showed no effect on the non-vSMC-specific BMP-target gene Id3 (Fig. 3A). FHL2-mediated reduction in SMA protein expression was confirmed by immunofluorescence staining in PASMCs (Fig. 3A, bottom). To assess the role of endogenous FHL2 in the BMP-mediated activation of vSMC genes, FHL2 was downregulated by transfection of siRNA (siFHL2) in PASMCs. qRT-PCR analysis indicates that siFHL2 transfection reduced FHL2 mRNA expression to ~50% in comparison with cells transfected with non-targeting, control siRNA (siCtr, Fig. 3B). Upon down-regulation of FHL2, both the basal and BMP4-induced expression of all three vSMC genes examined was significantly elevated compared with siCtr-transfected cells (Fig. 3B). Similarly, increased level of SMA protein by siFHL2 was confirmed by immunofluorescence staining (Fig. 3C). Similar results were obtained in aortic SMCs (data not shown). Additionally, the up-regulation of vSMC genes by knockdown of FHL2 was blocked by expression of a dominant-negative BMP receptor type 1B (dnALK6), suggesting that this effect is dependent on endogenous autocrine BMP signaling (Fig. 3D). Taken together, these results confirm that FHL2 is a negative regulator of BMP-mediated transcriptional activation of vSMC genes. A role of FHL2 in the regulation of contractile response of vSMCs as a result of change in contractile gene expression was examined by the collagen gel lattice contraction assay (37, 38). It has been previously shown that the level of deformation of collagen lattices can be affected by reorganization of actin or in response to contractile stimuli, such as sphingosylphosphorylcholine in vSMCs (38). PASMCs transfected with siFHL2 or control siRNA (siControl) were embedded in attached collagen matrices and pretreated with BMP4 for 24 h. After the detachment of gel from the dish, the gel surface area was measured and demonstrated as the percentage of the gel surface area at the time of detachment (Fig. 3E). In control siRNA (siControl) transfected cells, BMP4 treatment reduced the size of collagen lattice from 93% to 77%, suggesting that BMP4 elevates contractility of cells presumably through increased expression of contractile proteins (Fig. 3E, open bars). Down-regulation of FHL2 by siRNA reduced the size of collagen lattice to 74% in comparison with control cells (93%) and BMP4 treatment margin-
FIGURE 2. **FHL2 inhibits the transcriptional activation of vSMC promoters.** Reporter constructs containing the luciferase gene fused to the vSMC gene promoters (SM22, SMA, and CNN) or the BMP-Smad target gene promoter Xvent2 were transiently transfected with or without a FHL2 expression construct (0.2, 0.4, or 0.6 µg) into 10T1/2 cells (for SMA, CNN, or SM22) or P19 cells (for Xvent2) as indicated. SM22-Luc with mutations in the CArG sequence reporter (SM22(MUT)-Luc) was also tested. The cells were treated with or without 3 nM BMP4 for 20 h, followed by luciferase assay. All reporter activities were normalized to β-galactosidase activity, which serves as internal transfection control.
ally decreased the gel size to 69% (Fig. 3E, solid bars). A small but significant difference was observed between siControl-transfected cells and siFHL2-transfected cells in the presence of BMP4 (77% versus 69%; Fig. 3E). These results confirm that FHL2 plays a role in the regulation of proteins involved in the contractile response in vSMCs.

A mechanism of antagonistic action of FHL2 in the regulation of BMP-mediated SMC gene expression was examined. Our previous study indicates that BMP signaling induces nuclear translocation of MRTF-A, the Myocd family member that regulates vSMC gene transcription through binding to CArG boxes in complex with SRF in 10T1/2 cells (3). We exam-
ined whether exogenous FHL2 affects the extent of nuclear translocation of MRTF-A after BMP4 stimulation; however, no significant change was observed in the presence or absence of exogenous FHL2 (Fig. 4A). We then examined the effect of FHL2 on the transcriptional activation of a vSMC-specific gene by proteins of the Myocd family. The SM22-luciferase reporter construct containing the CArG box region was transfected with a limiting amount of MRTF-A, MRTF-B, or Myocd and increasing doses of FHL2 expression construct in 10T½ cells. Upon stimulation with BMP4 for 24 h, a robust activation of SM22-luc activity was observed upon transfection of any of the three Myocd-family proteins (Fig. 4B), as previously reported (3, 41, 42). Both the basal and the BMP-induced SM22-luc activities mediated by MRTF-A, MRTF-B, or Myocd were strongly inhibited by FHL2 in a dose-dependent manner (Fig. 4B). These results suggest that FHL2 antagonizes the transcription activities of all three Myocd family proteins similarly. Together with the observation that endogenous FHL2 was enriched in the nucleus after BMP4 treatment (Fig. 1C), we speculate that FHL2 inhibits the Myocd-family protein function in the nucleus possibly at a step of association with the CArG box or transcriptional activation.

To understand the molecular mechanism of inhibition of BMP-mediated recruitment of CArG box-binding proteins, we first investigated which proteins are recruited to the SMA promoter upon BMP treatment. It has previously been demonstrated that Brg1, a component of the SWI/SNF ATP-dependent chromatin remodeling complex, is recruited to the SMA promoter and plays a critical role in MRTF-A/SRF-dependent transcriptional activation (22, 43). When endogenous Brg1 expression was reduced to 20% (Fig. 5A, left panel), the basal as well as BMP4-induced levels of SMA were significantly down-regulated in PASMCs (Fig. 5A, right panel), suggesting that Brg1 is essential for the transcriptional activation of SMA by BMP4. We then performed ChIP assays to examine a time-course recruitment of Brg1, RNA polymerase II (pol II), SRF, and MRTF-A to the SMA promoter (Fig. 5B). As previously demonstrated, SRF, unlike MRTF-A, which was recruited to the CArG box upon BMP stimulation, was constitutively bound to the SMA promoter (Fig. 5B, green line) (3). Brg1 and pol II, as well as MRTF-A, were recruited to the SMA promoter as early as 1 h after BMP stimulation, and the amount of Brg1 and MRTF-A was decreased gradually after 4 h (Fig. 5B). pol II was stably recruited for up to 4 h during stimulation with BMP4 (Fig. 5B). To examine whether FHL2 antagonizes the recruitment of MRTF-A or SRF to the CArG box of the SMA promoter.

antibodies. Cy3- and fluorescein isothiocyanate-conjugated secondary antibodies were used to identify MRTFA-myc- and FLAG-FHL2-expressing cells, respectively. The cells in which MRTFA, or both MRTF-A and FHL2, are coexpressed and MRTF-A localization was predominantly nuclear were counted and compared as percentage to the total number of transfected cells. n = 100 of each condition. B, SM22-luciferase reporter construct was transiently transfected with or without an FHL2 expression construct (200, 400, or 600 ng) and/or Myocd (5 ng), MRTF-A (15 ng), or MRTF-B (100 ng) expression plasmids into 10T½ cells as indicated. The cells were treated with or without 3 nm BMP4 for 20 h, followed by luciferase assay. All reporter activities were normalized to β-galactosidase, which serves as internal transfection control. Data represent means ± S.E. of triplicates.
cells infected with adenovirus carrying GFP (control) or FHL2 cDNA were subjected to ChIP assays. To our surprise, exogenous FHL2 did not decrease the level of SRF or MRTF-A recruitment to the CArG box (Fig. 5C). Interestingly, increased expression of FHL2 slightly elevated the level of recruitment of MRTF-A to the SMA promoter upon BMP4 treatment (Fig. 5C). This result suggests that FHL2 antagonizes transcriptional activation of SMA by affecting another component of the protein complex associated with the CArG box, such as Brg1. Indeed, recruitment of Brg1 to the SMA promoter by BMP4 was abolished in the presence of exogenous FHL2 (Fig. 5D, Brg1 and SMA). Consistently with Brg1, recruitment of pol II was abolished in the presence of exogenous FHL2 (Fig. 5D, pol II and SMA). Inhibition of Brg1 recruitment by FHL2 is promoter-specific, because FHL2 did not affect Brg1 recruitment to connective tissue growth factor (CTGF) gene promoter (36) (Fig. 5D, CTGF). All together these results suggest that FHL2 inhibits transcriptional activation of SMC-specific genes by inhibiting the recruitment of Brg1 and pol II.

To confirm our findings in 10T1/2 cells and in vSMCs, we established vSMC cells from the aorta of wild-type (WT) or FHL2(KO) mice. qRT-PCR demonstrated that basal expression of SMA and CNN mRNAs was elevated in FHL2(KO) cells, and little change was observed after BMP4 treatment (Fig. 6A). Consistently with the qRT-PCR result (Fig. 7A), both anti-SMA and phalloidin immunofluorescence staining were significantly elevated in FHL2(KO) vSMCs compared with WT vSMCs (Fig. 6B). Unlike WT vSMCs, abundance of SMA protein was not augmented by BMP4 (Fig. 6B). Finally, ChIP assays were performed to compare relative amount of recruitment of pol II in FHL2(KO) and WT vSMCs. Supporting the level of SMA mRNA expression in Fig. 6A, FHL2(KO) vSMCs indicated higher level of recruitment of pol II to the SMA promoter both at the basal and the BMP-stimulated condition in comparison with WT vSMCs, confirming that recruitment of pol II to the SMC promoter is antagonized by FHL2 in vSMCs (Fig. 6C).

To examine whether FHL2 has a physiological role in the regulation of vasomotor tone of large vessels, we isolated thoracic aorta from FHL2 homozygous-null mice (FHL2(KO)) or WT mice and measured the responses of ex vivo aortic rings to acetylcholine (Ach), an endothelial-dependent vasodilator that increases nitric oxide production and SNP, a nitric oxide donor that causes endothelial-independent vSMC relaxation. FHL2(KO) mice are viable and overall development, in
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FIGURE 6. Characterization of vSMC in WT and FHL2(KO) mice. A, qRT-PCR analysis of vSMC genes (SMA and CNN) and FHL2 expression normalized to GAPDH in vSMC lines from WT or FHL2(KO) mice. Three independent vSMC clones per genotype were tested at least in three independent experiments, and a representative result is shown. The level of expression of SMA, CNN, or FHL2 in WT cells without BMP4 stimulation was set to 1. Error bars represent the SEM of triplicate measurements. B, immunostaining with anti-SMA antibody (green) and nuclear stain with DAPI (blue) are shown in the left panels. Alexa Fluor 568-conjugated phalloidin was used to visualize filamentous actin (red) shown in the right panels. C, recruitment of endogenous pol II to the SMA promoter was examined by ChIP assay in FHL2(KO) and WT vSMCs. Results are shown as relative enrichment of the promoter after IP, and the basal level of binding without BMP4 stimulation was set to 1. Mean values are expressed ± S.E.

Our study demonstrates that FHL2 abolishes recruitment of both a chromatin-remodeling protein Brg1 and pol II to the SMA promoter. A number of studies have shown that the chromatin-remodeling proteins facilitate recruitment and binding of pol II by reorganizing nucleosomes in the proximal promoter region (45–47). Our result suggests that Brg1 is required for recruitment of basic machineries of the transcription initiation, such as pol II, to the proximal promoter of the vSMC genes by clearing nucleosomes in the region proximal to the CArG box. It is speculated that FHL2 might block (i) assembly of basic transcription factors at the initiation site and inhibits initiation/re-initiation of pol II, (ii) promoter clearance of pol II possibly by inhibiting phosphorylation of C-terminal domain of pol II, or (iii) elongation of the mRNA synthesis by causing premature termination of pol II. Further studies are necessary to elucidate the detailed mechanism of inhibition of pol II recruitment by FHL2. It is also known that Brg1 facilitates the recruitment of proteins that covalently modify histones, such as histone deacetylases and p300. Therefore, it is plausible that FHL2 might block potential histone modifications required for constitutive activation of vSMC genes.

It has been suggested that Brg1 is recruited to vSMC gene promoters via the LIM only protein CRP2, which contains two LIM domains (22). Because CRP2 interacts with Brg1 through an LIM domain (22), which has high homology with the LIM domains of FHL2, it is plausible that FHL2 might compete with CRP2 for binding to Brg1 and sequester CRP2 from binding to vSMC gene promoters. It is known that the MRTF-A-SRF complex induces expression of vSMC-specific genes, but not mitogen-activated protein kinase-regulated immediate early genes.
such as c-fos, despite both sets of genes containing SRF binding sites in the promoter region. The mechanism by which the MRTF-A-SRF complex discriminates between two sets of target genes is unclear. Recently, it has been proposed that recruitment of the chromatin-remodeling protein Brg1 is critical for MRTF-A-SRF-dependent activation of vSMC-specific genes, but not immediate early genes (43). A mechanism wherein FHL2 inhibits Brg1 recruitment to vSMC-specific promoters, rather than recruitment of MRTF-A or SRF, provides a possible explanation for the reason why FHL2 antagonizes the expression of vSMC genes but not immediate early genes.

In contrast to our study in vSMCs, a study in skin fibroblasts suggests that FHL2 induces SMA expression in myofibroblasts that have invaded the injury site to facilitate the repair process after skin wounding (48). Furthermore, embryonic fibroblasts from FHL2-null mice exhibit decreased motility and reduced collagen contraction (48). This is in agreement with a study showing that FHL2 increases Myocd and MRTF-A protein levels by protecting these proteins from the ubiquitin-proteasome-dependent degradation pathway (26). We found no evidence that MRTF-A is ubiquitinated or degraded in a proteasome-dependent mechanism in PASMCs. We also examined MRTF-A protein expression in WT and FHL2(KO) vSMCs cells by immunoblot and did not find significant difference in MRTF-A levels in these cells (supplemental Fig. S1). Therefore, we speculate that the role of FHL2 in vSMC gene regulation is tissue- and context-dependent. As FHL2 functions through interaction with other proteins, it is likely that FHL2 has different functional effects in various tissues or stimulatory contexts, because expression of protein partners may vary. Recently, increased expression of FHL1, another member of the FHL-family proteins, was found in lung tissues from human PAH patients and hypoxia-induced PAH mice (49). Because FHL1 and FHL2 share high structural homology and some of the protein binding partners, it is plausible that FHL2 as well as FHL1 might be contributing to the pathogenesis of PAH by promoting PASMCs transition to highly proliferative and migratory phenotype, or through modulation of BMP-mediated regulation of contractile genes (49).

Finally, we demonstrated that aortic rings from FHL2-null mice exhibit resistance to vasodilators, suggesting that FHL2 is a key regulator of vasomotor tone. It is intriguing to speculate that a dysfunction of vasomotor tone may be derived from elevated contractile gene expression in vSMCs in response to contractile stimuli, such as BMP signaling. It is interesting to note that FHL2-null mice exhibit significant elevations in systolic, diastolic, and mean blood pressure.4 Molecular, cellular, and animal studies support that loss of function or loss of expression mutations of proteins in the contractile pathway, such as voltage-gated calcium channels, cGMP-dependent protein kinase, and the regulator of G protein signaling 2, give rise to abnormal vascular contraction and/or relaxation as well as hypertension independent of kidney dysfunction (40, 50). We cannot exclude a possibility that hypertension in FHL2-null mice originates from kidney dysfunction, however, it is intriguing to speculate that abnormality in vasomotor tone might play a role in elevation of blood pressure. In conclusion our study demonstrates that FHL2 is critically involved in vascular phenotype modulation, which is fundamental to the regulation of vascular motor function.

4 N. A. Neuman and A. Hata, unpublished observation.
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