Control of Phenotypic Plasticity of Smooth Muscle Cells by Bone Morphogenetic Protein Signaling through the Myocardin-related Transcription Factors

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Vascular smooth muscle cells (VSMCs), unlike other muscle cells, do not terminally differentiate. In response to injury, VSMCs change phenotype, proliferate, and migrate as part of the repair process. Dysregulation of this plasticity program contributes to the pathogenesis of several vascular disorders, such as atherosclerosis, restenosis, and hypertension. The discovery of mutations in the gene encoding BMPRII, the type II subunit of the receptor for bone morphogenetic proteins (BMPs), in patients with pulmonary arterial hypertension (PAH) provided an indication that BMP signaling may affect the homeostasis of VSMCs and their phenotype modulation. Here we report that BMP signaling potently induces SMC-specific genes in pluripotent cells and prevents dedifferentiation of arterial SMCs. The BMP-induced phenotype switch requires intact RhoA/ROCK signaling but is not blocked by inhibitors of the TGF-β receptor and with a general repression of BMP signaling (13–15). Despite these overwhelming correlations, the etiological mechanism of action of the BMP signaling pathway in PAH remains unclear.

BMPs represent the largest group in the transforming growth factor β (TGFβ) superfamily of growth factors (16–18). During embryonic development, the BMP pathway plays multiple essential roles in the induction of ventral mesoderm, cardiac myogenesis, and vasculogenesis (19). Targeted inactivation of the BMP signal transducers Smad1 and Smad5 display a severe vascular phenotype (20). However, the effects of BMPs on adult VSMCs are not completely understood. It has been reported that BMPs inhibit proliferation and induce apoptosis in serum-starved pulmonary artery smooth muscle cells (PASMCs) (10, 21). It is also known that BMP7 stimulates the maintenance of the SMC phenotype in aortic SMCs, while BMP2 and BMP4 appear to have opposing effects on expression of SMC-specific genes in VSMCs (22–27). These results suggest that the effects of BMP signaling on VSMCs are complex and possibly depend-

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‡§ The abbreviations used are: SMC, smooth muscle cell; TGF, transforming growth factor; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; ChIP, chromatin immunoprecipitation assay; PDGF, platelet-derived growth factor; DAPI, 4′,6-diamidino-2-phenylindole; PASMC, pulmonary artery smooth muscle cells; BMP, bone morphogenetic factor; HA, hemagglutinin; PAH, pulmonary arterial hypertension; SMA, smooth muscle α-actin.
ent on tissue, developmental and experimental variables (28). Here, we characterize the effect of BMP signaling on SMC phenotype modulation and show that BPs effectively induce a contractile phenotype and up-regulate SMC-specific gene expression in SMCs. The RhoA/Rho kinase (ROCK) pathway is required for SMC differentiation in response to BMP. The BMP pathway activates transcription of SMC genes by inducing nuclear translocation of the transcription factors MRTF-A and MRTF-B, which are known to bind the CARG box found within many SMC-specific gene promoters. Thus, the BMP pathway converges on MRTF-A/B to regulate the differentiation state of vascular SMCs.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and DNA Transfection**—PAC-1 (a gift from Dr. Joyce Li) and C3H10T½ cell lines (American Type Culture Collection) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (Sigma). These cells were transfected using FuGENE 6 (Roche Applied Science) according to the manufacturer’s instructions. Human primary SMC derived from the pulmonary artery (PASMC), the umbilical artery (UASM), or the aorta (AoSMC) were purchased from Cambrex and were maintained in Sm-GM2 media (Cambrex). Human SMCs were transfected as described (29). MRTF-A and MRTF-B expression constructs were a gift of Dr. D.-Z. Wang.

**Antibodies, Growth Factors, and Inhibitors**—The antibodies used in this study were: HA epitope tag (clone Y11, SC-805, Santa Cruz Biotechnology or 12CA5, Roche Applied Science), Myc epitope tag (clone 9E10, Tufts Core facility), anti-smooth muscle α-actin (SMA) (clone 1A4, Sigma), calponin (clone hCP, Sigma), anti-phospho-Smad1/5/8 (Cell Signaling), and anti-MRTF-A (anti-MAL, a kind gift of Dr. Treisman). Recombinant PDGF-BB, human TGFβ1, BMP2, BMP4, and BMP7 proteins were purchased from R&D Systems. The inhibitors used were SB-431542 (Tocris), Y-27632, Wortmannin, and inhibitors purchased from Sigma. Nuclei were stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, Invitrogen).

**Statistical Analysis**—Statistical significance was calculated using analysis of variance (ANOVA) and Fisher’s Projected Least Significant Difference (LSD) test, or by Student’s t test analysis (p < 0.05), as appropriate. All data are plotted as the mean ± S.E.

**RESULTS**

**BMP Signal Is Required for Maintenance of SMC Contractile Markers**—Recent studies have shed light on the pathophysiological pathways that lead to proliferative and obliterative vascular disorders, such as atherosclerosis and PAH. A characteristic change found in atherosclerotic lesions and pulmonary arteries (PAs) of patients affected by PAH is the loss of the SMC contractile phenotype. To investigate the possible role of BPs in the maintenance of a differentiated SM phenotype, we examined whether BMP can oppose the decrease of SM markers expression in dedifferentiating human pulmonary artery SM cells (hPASMCs). Prolonged culture of aortic SMCs in growth medium has been shown to lead to a partial dedifferentiation...
Quantitative RT-PCR analysis shows that human PASMCs express the BMP ligands BMP2–6 (but not BMP7), BMP receptors (ActRI, BMPRIA, BMPRIIB, and BMPRII), and downstream signal transducers (Smad1–7) (data not shown) (10). Although PASMCs do not synthesize BMP7, it may be produced by another type of pulmonary cell and subsequently secreted to affect PASMCs (37). Nevertheless, because specific ligand-receptor combinations might transmit different signals to PASMCs, we first tested the role of the PASMC-expressed BMP4 on pulmonary SMCs under the conditions previously used to study BMP7 (22).

**FIGURE 1.** BMP pathway prevents dedifferentiation of human primary PASMCs. A, confluent hPASMCs (passage 5) were cultured in growth media containing 3 nM BMP4 or vehicle. Total RNAs were collected at 0, 5, and 8 days after the treatment and were subjected to RT-PCR analysis of human SMA and GAPDH (loading control). B, hPASMCs at passage 7 (high passage) or passage 4 (low passage) were treated with 3 nM BMP4 or 400 pm TGFβ1 for 72 h in DMEM/0.1% FCS. Cells were then subjected to immunofluorescence staining with FITC-conjugated anti-SMA antibody (green) and nuclear staining with DAPI (blue). C, hPASMCs (passage 5) were treated with 20 ng/ml PDGF-BB and/or 3 nM BMP4 or 400 pm TGFβ1 for 72 h in DMEM/0.1% FCS. Cells were then subjected to immunofluorescence staining with anti-SMA antibodies and nuclear staining with DAPI. D, rat PAC-1 cells were treated with 3 nM BMP4 and/or 100 pm TGFβ1 for 48 h in DMEM/10% FCS. Cells were subjected to immunofluorescence staining with FITC-conjugated anti-SMA antibodies (green) and nuclear staining with DAPI (blue). E, hPASMCs at passage 5 were treated with 20 ng/ml PDGF-BB and/or 3 nM BMP4 or infection with recombinant adenovirus carrying HA epitope-tagged constitutively active (CA) ALK6 (m.o.i 200). Cells were subjected to immunofluorescence staining with FITC-conjugated anti-SMA antibody (green) and nuclear stain with DAPI (blue) (top panels). Total cell lysates prepared from hPASMCs treated with or without 3 nM BMP4 for 48 h were subjected to immunoblot with anti-SMA antibody or GAPDH (loading control) (bottom panel). F, hPASMCs at passage 5 were infected with recombinant adenovirus expressing HA-tagged dominant negative (DN) ALK2, ALK3, ALK6, or BMPRII (m.o.i, 200). Cells were subjected to immunofluorescence staining with anti-SMA (green) and anti-HA antibody (for ALK2, 3, 6, and BMPRII; yellow) and nuclear stain with DAPI (blue).
BMP4. In the absence of PDGF, BMP4 or TGFβ1 increased the basal level of SMA staining (Fig. 1C). A similar inhibitory activity on the PDGF effect was observed in PASMCs stimulated with the related factors BMP2 or BMP7 (Supplemental Fig. S1). Furthermore, similar results were obtained using the PAC-1 cell line, derived from rat pulmonary artery SM cells and capable of undergoing a phenotype switch upon serum deprivation or TGFβ treatment (40). Treatment of PAC-1 cells with 3 nM BMP4 induced the expression of SMA, suggesting that the effect of BMP signaling on SM gene expression is neither species-specific nor limited to primary cells (Fig. 1D). Stimulation of PAC-1 cells with TGFβ and BMP4 together induced a synergistic activation of SMA, pointing to a potentially distinct mechanism of induction of SM genes by the TGFβ and BMP4 signaling pathways (Fig. 1D). In agreement with the immunofluorescence data, BMP4 stimulation of PDGF-treated hPASMCs induced SMA mRNA to the level of untreated cells (Fig. 1E).

The BMP4 effect on PASMC can be recapitulated by overexpression of constitutively active (CA) type I BMP receptors. Low-passage, PDGF-treated hPASMCs infected with adenovirus expressing ALK6 (CA) efficiently expressed SMA (Fig. 1F, upper panel). Activated mutants of other BMP type I receptors, such as ALK2 (CA) and ALK3 (CA), also mediated induction of SMA (data not shown). Finally, an increase in SMA protein upon BMP4 stimulation was also detectable by Western blot (Fig. 1F, lower panel). Thus, activation of the BMP pathway prevents hPASMC dedifferentiation caused by three distinct processes: 1) prolonged confluent culture, 2) high passage, and 3) PDGF treatment.

SMCs express BMP ligands, receptors and signal transducers both in vivo and in vitro (10, 21). Therefore, an autocrine BMP signaling loop may contribute to the maintenance of SMC differentiation. To test this hypothesis, hPASMCs were infected with adenovirus expressing dominant negative (DN) BMP type I [ALK2(DN), ALK3(DN), and ALK6(DN)], or type II [BMPRII(DN)] receptors. BMP-induced expression of SMA was blocked by the DN ALK receptors as well as the DN BMPRII (Fig. 1G). Furthermore, the basal level of SMA was also dramatically repressed by the DN-ALKs, indicating that interruption of an autocrine BMP signaling loop may lead to SMC dedifferentiation.

*Different BMP Ligands Exhibit a Similar Effect on Expression of SMC-specific Genes in Different Types of SMCs*—To examine a possible difference in activities of different BMP ligands on the expression of SMC-specific genes, low passage hPASMCs were treated with different concentrations of BMP4, BMP2, or BMP7, followed by RT-PCR analysis of the expression of three SMC-specific markers, SMA, calponin (CNN), and SM22α (SM22) (Fig. 2A). Both BMP2 and BMP7 induced all three SM markers in a dose-dependent manner in a range of concentrations between 0.3 nM and 3 nM, suggesting that induction of SM genes by BMP is not limited to BMP4 (Fig. 2A). BMP2 induced SMA and CNN about ~10-fold at 3 nM, in comparison with a ~4-fold induction by BMP4 and BMP7 (Fig. 2A). We also examined the BMP effect on SM-marker induction in vascular SMCs of different origin, such as human aortic SMCs (AoSM) and umbilical artery SMCs (UASM) (Fig. 2, B and C). All three BMP ligands strongly induced SMA and CNN in AoSM (Fig. 2B), but triggered a weaker effect in UASM (Fig. 2C). Overall, these results suggest that activation of the BMP pathway by all three BMP ligands tested results qualitatively in an induction of SM markers in vascular SMCs from three different tissues.

*BMP-mediated Activation of SM Genes Is Smad-dependent*—Canonical TGFβ/BMP signaling requires receptor-specific signal transducers named Smads, which act as substrates of the type I receptor serine/threonine kinase. Upon phosphorylation, Smad proteins translocate to the nucleus and regulate transcription by binding to a target gene promoter. To examine whether Smads are involved in the activation of SM genes by BMP4, hPASMCs were transfected with siRNAs against the two primary BMP-specific Smad proteins (Smad1 and Smad5) prior to BMP4 stimulation. Compared with mock or control siRNA-transfected cells, transfection of siRNA against Smad1 and Smad5 greatly reduced BMP induction of the SM markers SMA and calponin and of the Smad-dependent gene Id3 (Fig. 3). The residual BMP4 response observed in all three genes may be caused by incomplete repression of Smad1 and Smad5 expression, or to other BMP-specific Smads not targeted by the siRNAs (such as Smad8 and Smad9). These results indicate that BMP-mediated induction of SM genes relies, at least in part, on a Smad-dependent pathway.

*BMP Signal Induces Expression of SMC-specific Genes*—Because BMP signaling maintains SMC differentiation, we hypothesized that it may also induce the differentiation of precursors into a SMC type. It has been reported that TGFβ signaling can induce the expression of SM markers in pluripotent mouse embryonic C3H10T½ (10T½) cells (41–43). We investigated whether BMPs can mimic the effect of TGFβ and supply an instructive signal to 10T½ cells. BMP4 strongly induced expression of the SMC-specific marker SMA and a morphological change toward an elongated SMC-like morphology in 10T½, as detected by immunofluorescence staining (Fig. 4A, left panels) and immunoblot (Fig. 4A, left panel). A robust induction of SMA protein by BMP4 stimulation was observed in both immunofluorescence stain and immunoblot, suggesting that BMP is able to induce SM differentiation in 10T½ cells (Fig. 4A). In the presence of increasing concentrations of BMP4, the number of SMA-positive 10T½ cells gradually increased (Fig. 4B). An induction of mRNAs encoding SMC-specific genes, including SMA and calponin, was measured by quantitative RT-PCR analysis in BMP4-treated 10T½ cells (Fig. 4C). Expression of Id3, a non-SM-specific gene transcriptionally regulated by BMP (44), was monitored as a control. The result underscores the ability of BMP signaling to induce multiple SM markers, including SMA (2.5-fold) and calponin (3.1-fold), alongside Id3 (4.9-fold), in undifferentiated cells (Fig. 4C). A time course of induction of the SM markers SMA, CNN, and SM22 upon BMP4 treatment in 10T½ cells showed a similarly transient pattern of early exponential induction (up to 10 h after stimulation), followed by a plateau and a gradual decrease (between 16 and 48 h after induction) (Fig. 4D, left panel). Interestingly, induction of the same SM markers in PASMC was sustained and followed a different temporal pattern, with the expression of SM22 and SMA peaking at 24 and 48 h, respectively, while CNN continued to increase up to 72 h after induc-
tion. Changes occurring in transformed and immortalized cells such as 10T½ may prevent them from acquiring a stable differentiated SM phenotype. Alternatively, SM differentiation inhibitors may also be induced by BMP in 10T½ cells, but not in PASMCs (see “Discussion”).

The induction of several SM-specific mRNAs within 4 h after BMP4 treatment raises the possibility of a direct transcriptional activation of SM-specific genes by BMP4. To explore this possibility, 10T½ cells were transfected with luciferase reporter constructs driven by SMC-specific promoters from the SMA, SM-myosin heavy chain (MHC), SM22α, and CNN genes. All four promoters were activated by BMP4, suggesting that the BMP signal elicits a direct transcriptional activation of SMC-specific promoters in 10T½ cells (Fig. 4E).

**BMP-mediated Induction of SMC Genes Is ROCK-dependent but TGFβ Receptor-independent**—TGFβs are known modulators of VSMC growth and differentiation (41–43) and can directly induce the expression of SM markers. Therefore, BMPs might stimulate SMC differentiation indirectly by inducing the expression of TGFβ ligands, or otherwise activating the TGFβ type 1 receptors. This model was tested by treating hPASMCs with a specific inhibitor of the type I TGFβ receptor kinases (SB-431542) (45) prior to BMP4 or TGFβ1 stimulation, followed by immunostaining for SMA (Fig. 5A). SB-431542 treatment effectively blocked the TGFβ1-mediated induction of SMA, but did not inhibit SMA induction by BMP4, suggesting that BMP induces SMA independently of the TGFβ pathway.

The Rho GTPases RhoA, Cdc42, and Rac1 are important regulators of cytoskeletal remodeling (46, 47). RhoA signaling is known to affect contractility of pulmonary SMCs from rats with chronic PAH (48), and play a general role in maintenance of SMC differentiation (49). To examine a possible role of RhoA in SMC differentiation upon BMP treatment, 10T½ cells transfected with the SMA promoter-luciferase reporter were treated with a specific inhibitor of Rho-associated kinases (ROCK), Y-27632 (50), and then exposed to BMP4 (Fig. 5B). Treatment with Y-27632 significantly reduced the BMP-mediated induc-
In contrast, the phosphoinositide 3-kinase (PI-3K) inhibitors induction of SMA was decreased in a dose-dependent manner. Concentrations of ROCK inhibitor Y-27632, BMP4-mediated hPASMCs. When these cells were treated with increasing concentrations of ROCK inhibitor Y-27632, BMP4-mediated induction of SMA was decreased in a dose-dependent manner. In contrast, the phosphoinositide 3-kinase (PI-3K) inhibitors strongly inhibited both basal and induced transcription of the reporter, while constitutively active ROCK inhibitor Y-27632 decreased the basal level without affecting significantly BMP stimulation (Fig. 5B). Similar results were obtained in hPASMCs. When these cells were treated with increasing concentrations of ROCK inhibitor Y-27632, BMP4-mediated induction of SMA was decreased in a dose-dependent manner. In contrast, the phosphoinositide 3-kinase (PI-3K) inhibitors Wortmannin (Fig. 5C) and LY-29400 (data not shown) had no effect on BMP-induced SMA activation. These results indicate that activation of the RhoA/ROCK pathway in response to BMP4 stimulation is essential for expression of the SMA gene in both non-SMCs and SMCs.

Activation of SM Genes by the BMP Signaling Pathway Is CArG Box-dependent—DNA-binding proteins such as serum response factor (SRF) regulate transcription of SMC-specific genes (51, 52). SRF, in association with its cofactors, binds a specific DNA sequence called the CArG box [CC(AT)6GG] and activates transcription (52). To test whether the BMP pathway regulates SMC gene transcription through the CArG element, SM22α or calponin promoter-luciferase reporter constructs, with or without mutations in the CArG sequences, were transfected into 10T½ cells (Fig. 6A). Mutation in the CArG elements decreased (SM22α) or abolished (calponin) the BMP-dependent activation of these reporters, suggesting that the BMP pathway acts, at least in part, through regulation of CArG box binding factors (Fig. 6A). BMP4-dependent activation of a CArG-mutated calponin reporter construct was also completely abolished in PAC-1 cells, indicating that the requirement of a functional CArG box for BMP stimulation is not cell type-specific (Fig. 6B). In agreement with previous reports (53), the CArG box-mutated calponin promoter was still partially inducible by TGFβ1 stimulation, confirming that TGFβ regulates the calponin promoter through a cis-acting element other than the CArG box (Fig. 6B). These results indicate that the CArG box is specifically required for BMP-mediated transcriptional activation of calponin.

Involvement of the SRF Cofactors MRTF-A and MRTF-B in the Regulation of SM Genes by BMP—The CArG sequence binds to a complex of transcription factors comprising SRF and its coactivators, such as myocardin, MRTF-A (also called MAL, MKL-1, and MSAC), or MRTF-B (also called MKL-2) (51). Unlike myocardin, which is expressed only in the cardiovascular system and is absent in 10T½ cells, MRTF-A and MRTF-B are widely expressed (54, 55), raising the possibility that they may be involved in mediating the CArG-dependent BMP stimulation of SMC-specific promoters in 10T½, and possibly primary SMCs (51). Furthermore, the MRTFs have been proposed to mediate SMC phenotype regulation by the RhoA pathway (56), making them also good candidates as effectors of the ROCK-dependent BMP stimulation of the SMC phenotype. Transfection of 10T½ cells with the SMA-reporter construct and increasing amounts of MRTF-A showed a gradual stimulation of both basal and BMP-induced transcription (Fig. 7A). The increase of basal activity of the SMA-reporter at higher amounts of MRTF-A is possibly caused by protein overexpression, which causes localization of some MRTF-A in the nucleus (Fig. 7A). Thus, MRTF-A is able to amplify the activation of the SMA promoter in response to BMP4 in 10T½ cells, suggesting that the intracellular concentration of MRTF-A is a limiting factor for BMP stimulation of SMA transcription. MRTF-B was also able to induce the SMA-reporter upon BMP stimulation, but with an overall weaker activity compared with MRTF-A, which might be caused by a difference in the intrinsic transcription activation potential between MRTF-A and MRTF-B (55) (Supplemental Fig. S2).

To investigate the requirement of MRTF-A and MRTF-B for the BMP-mediated regulation of SMA expression, 10T½ cells were transfected with siRNA directed against MRTF-A or MRTF-B. The specificity of the siRNA duplexes was confirmed by quantitative RT-PCR analysis of MRTF-A and MRTF-B mRNAs in siRNA-transfected cells (Fig. 7B). 10T½ cells treated with MRTF-A siRNA showed a dramatic reduction in both SMA reporter activity (Fig. 7C) and SMA staining after BMP4 stimulation (Fig. 7D). The MRTF-B siRNA had no effect on SMA reporter activity compared with the effect of MRTF-A siRNA (Fig. 7C), but it markedly reduced the BMP-mediated expression of endogenous SMA gene expression (Fig. 7D), possibly reflecting a different ability of MRTF-B to activate episomal versus genome-embedded promoters. Consistent with the observation that the CArG box mutant calponin reporter is still partially responsive to TGFβ treatment (Fig. 6B), the TGFβ-induced expression of SMA was only weakly affected by MRTF-A or MRTF-B siRNAs (Fig. 7D). These results suggest that both MRTF-A and MRTF-B are essential for the BMP-mediated activation of SMA in 10T½ cells.

Nuclear Translocation of MRTF-A Mediated by BMP Signaling—To examine the potential mechanism of regulation of MRTF-A by BMP signaling, the subcellular localization of transfected Myc epitope-tagged MRTF-A in response to BMP4 treatment was examined by immunofluorescence in 10T½
cells. Myc-MRTF-A was predominantly localized in the cytoplasm (86% cytoplasmic localization) in cells grown in low-serum conditions (Fig. 7E, Control). In the presence of BMP4, MRTF-A translocated to the nucleus (66.4% nuclear) with an efficiency comparable to that achieved in high serum (56% nuclear), a condition that has been previously reported to stimulate nuclear localization of MRTF-A (Fig. 7E) (57). When cells were treated with both BMP4 and Latrunculin B, an inhibitor of actin polymerization, nuclear localization of MRTF-A was reduced (Fig. 7E, BMP4+LB), suggesting an important role of actin stress fibers for the translocation of MRTF-A (57). Latrunculin B also inhibits MRTF-A nuclear translocation upon stimulation of the RhoA pathway (57), providing further evidence of the requirement of a functional RhoA-dependent pathway for BMP-mediated induction of the SMC phenotype.

We examined whether MRTF-A, which is translocated to the nucleus upon BMP stimulation, is recruited to the CArG element of the SMA promoter by ChIP assay. 10T1/2 cells transfected with expression plasmids encoding Myc epitope-tagged myocardin, MRTF-A or MRTF-B were treated with BMP4 prior to Ch-IP with an anti-Myc antibody. Myocardin was constitutively recruited to the SMA promoter when overexpressed (Fig. 7F). Unlike myocardin, recruitment of MRTF-A and MRTF-B to the SMA promoter was strongly augmented by BMP4 stimulation (Fig. 7F). No MRTFs were recruited to the myocardin-independent CArG element of the c-fos promoter, with or without BMP stimulation (data not shown).

These results suggest that nuclear translocation of MRTF-A or MRTF-B by BMP4 stimulation leads to their recruitment to the SMC-specific CArG box and results in transcriptional activation.

**DISCUSSION**

The phenotypic switch of SMCs from a quiescent, “contractile” state to a proliferative “synthetic” state has been shown to play a key role in the repair of tissue damage and in the development of a variety of human SMC-linked diseases, including asthma, restenosis, atherosclerosis, and arterial hypertension. It is known that the phenotypic switch is caused by coordinate repression/activation of transcription of SMC-specific genes, such as SMA, SM22α, calponin, and SM-MHC.
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However, the mechanisms that regulate this process are not well understood. In this study, we report that the BMP signaling pathway induces SMC-specific genes by recruiting the members of the myocardin family of transcription factors MRTF-A and MRTF-B to SMC gene promoters. As MRTF-A is ubiquitously expressed in SMCs as well as non-SMCs (55), we speculate that BMP-mediated induction of SMC genes might be involved not only in maintenance of SMCs at a mature differentiated stage, but also in switching myofibroblasts and vascular SM precursor cells from a “synthetic” to a “contractile” state during vascular injury and repair.

Cross-talk between BMP and TGFβ Signals—Both BMPs and TGFβs are synthesized and secreted from vascular SMCs and endothelial cells. TGFβ directly inhibits SMC proliferation and migration and stimulates the expression of contractilic SMC markers (39). However, little is known about the role of the BMP signaling pathway in the modulation of the SMC phenotype (22, 27). TGFβ is a potent inducer of SMC gene transcription, partially through a cis-acting sequence (TGFβ control element, or TCE) to which TCE-binding transcription factor(s) are recruited, and partially through the CArG box (53). BMP4 appears to activate SMC genes through the CArG box by recruiting CArG-binding factors, SRF and MRTFs. In a typical SMC-specific promoter, such as that of the SMC gene, a TCE (−42) and two CArG boxes (−62 and −113) are found in close proximity (53). In PAC-1 cells, BMP4 and TGFβ synergize for SMC gene induction (Fig. 1D). This is the first evidence showing cross-talk between BMP and TGFβ pathways in the regulation of smooth muscle cell physiology. We speculate that the synergistic effect of TGFβ and BMP4 in the regulation of SMC might be caused by cooperation among transcription factors recruited to the TCE and the CArG box. It was reported that the TGFβ-specific signal transducer Smad3, in complex with myocardin, binds, and stimulates the SM22α promoter in a CArG-independent manner in response to TGFβ stimulation (58). We found that the responsiveness to BMP4 of the CarG mutant SM22α reporter was reduced but not completely abolished, raising the possibility that also the BMP pathway may be able to activate the SM22α promoter in part via a CArG-independent mechanism.

Additional Indirect Effects of BMPs—Long-term maintenance of the SM phenotype by BMPs may also be partially indirect, involving the transcriptional or post-transcriptional regulation of proteins/RNAs that modulate SMC genes expression. Transcription regulation via the CarG box and its binding factors is subject to repression by a variety of co-repressor proteins. For example, the hairy-related transcription factor 2 (HRT-2) (59) and GATA factors (60) associate with myocardin and repress its activity by an unknown mechanism. The four and half LIM protein 2 (FHL2) interacts with SRF, competes with MRTFs for SRF binding and represses SMC-specific gene transcription (61). Therefore, the BMP signal might negatively regulate the activity or the expression of these negative regulators, a hypothesis currently under investigation. Also, Krüppel-like factor 4 (KLF4) was identified as a factor induced in response to vascular injury and able to repress myocardin expression and block association of the SRF/myocardin complex to the CarG box (62). It is interesting to speculate that the decrease of expression of SM markers at 48 h after BMP stimulation in 10T½ cells (Fig. 4C) could be the consequence of an induction or accumulation of KLF4 upon BMP treatment. We indeed observed an induc-
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**A**

![Graph](image)

**B**

![Graph](image)

**FIGURE 6.** BMP-mediated transcriptional activation of SM gene is CARG box-dependent. *A*, 10T½ cells were transfected with wild-type or mutant SM22α or calponin reporter constructs, which are mutated in the CARG box sequence as indicated, treated with BMP4, and assayed for luciferase activity. *B*, PAC-1 cells were transfected with wild-type or mutant calponin reporter constructs, which are mutated in the CARG box sequence as indicated, treated with 3 nM BMP4 or 100 pM TGFβ1 and assayed for luciferase activity.

Mechanism of MRTF Translocation—It has been shown that the subcellular localization of MRTFs, which is responsible for regulation of SRF activity and SMC-specific gene expression, is sensitive to actin dynamics (57). Several regulatory mechanisms were proposed for this process. When actin filaments (F-actins) are destabilized, MRTFs are sequestered in the cytoplasm by direct binding to actin monomers (G-actins) via the N-terminal RPEL motifs. On the contrary, when members of the Rho family (RhoA, Cdc42, and Rac1) or the downstream kinase ROCK are activated, actin polymerization is stabilized. The increase in the F-actin/G-actin ratio triggers a release of MRTFs from the cytoplasm to the nucleus and activation of SM gene promoters. More recently, an F-actin-binding protein named Striated Muscle Activator of Rho Signaling (STARS) was found to promote nuclear translocation of MRTFs and activate SRF in a Rho-dependent manner. It is possible that molecules of the BMP signaling pathway, such as the BMP receptor kinase, might act through proteins similar to STARS or directly modulate the RPEL motifs of MRTFs in response to BMP4 and induce nuclear translocation. It is also possible that the nuclear translocation of MRTFs is a direct consequence of an increase in the F-actin/G-actin ratio through activation of the Rho/ROCK pathway by BMP signaling. The exact mechanism of regulation of the Rho/ROCK pathway by BMP4, however, will be the subject of a future study.

Role of the BMPRII C-terminal Domain—Several proteins were shown to associate with the C-terminal region of the cytoplasmic, non-enzymatic, “tail” domain of BMPRII (BMPRII-TD), including LIM kinase 1 (LIMK1) (64, 65). The association of LIMK1 with BMPRII-TD modulates the catalytic activity of LIMK1. The exact mechanism of regulation of LIMK1 by BMPRII-TD is yet to be studied. LIMK1 is known to phosphorylate ADF/cofilin and block actin de-polymerization (66). Therefore, it is plausible that the regulation of nuclear localization of MRTFs upon BMP4 stimulation is, at least in part, caused by a change in LIMK1 activity, possibly a stimulation of LIMK1 and stabilization of actin filaments. Some of the mutations in the BMPRII gene associated with PAH cause truncation of the BMPRII-TD, which might result in dysregulation of the interaction with LIMK1 or other BMPRII-TD-binding proteins. The characteristics of pathological changes found in the pulmonary arterioles of PAH patients include dedifferentiation of SMC phenotype in the media as well as proliferation and fibrosis of the intima. It is interesting to speculate the existence of a pathway by which down-regulation of LIMK1 activity in PASMCs as a result of truncation of the BMPRII-TD triggers a decrease in F-actin/G-actin ratio and causes cytoplasmic accumulation of MRTFs and reduction of SMC-specific gene expression. BMPRII-TD is also known to interact with dynein light chain Tctex1 (67), a microtubule motor protein; it is possible that BMPRII, in association with Tctex1, might be directly involved in transport of MRTFs or other proteins required for the regulation of SM gene expression.

Potential Role of ALK1—In addition to the existing link between BMPRII mutations and PAH, defects in the genes
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FIGURE 7. BMP4 signal mediates a recruitment of cofactor MRTFs to SMA promoter. A, 10T½ cells were transfected with SMA-reporter constructs and increasing amounts of Myc-MRTF-A expression plasmid (1, 2, 3, 4, or 5 ng) and assayed for luciferase activity. B, total RNAs were extracted from 10T½ cells transfected with siRNA against MRTF-A or MRTF-B or non-targeting (control) siRNA and were subjected to RT-PCR analysis to examine expression of MRTF-A or MRTF-B mRNA. RNA levels were quantified by real-time PCR analysis with normalization to GAPDH expression. C, 10T½ cells transfected with MRTF-A or MRTF-B siRNAs, or non-targeting (control) siRNA, together with the SMA reporter construct. The luciferase activity was measured after treating cells with or without 3 nM BMP4 for 48 h. There is no statistically significant change in SMA reporter activity in cells transfected with MRTF-A siRNA, unlike cells transfected with control siRNA or MRTF-B siRNA (Student’s t test, n.s., represents p > 0.05). D, 10T½ cells transfected with siRNAs as in panel C, and treated with or without 3 nM BMP4 or 400 pM TGFβ1, followed by immunostaining with FITC-conjugated anti-SMA antibody (green) and nuclear stain with DAPI (blue). As negative control, non-targeting siRNA was used. E, 10T½ cells were transiently transfected with Myc-MRTF-A expression plasmid. After being treated with 3 nM BMP4, 20% serum, or BMP4 and Latrunculin B (LB), an inhibitor of actin polymerization, cells were subjected to immunostaining with FITC-conjugated anti-Myc antibody (green) and nuclear stain with DAPI (blue). Cells in which MRTF-A localization was predominantly nuclear were counted and compared as percentage to the total number of cells. F, 10T½ cells were transfected with Myc-tagged myocardin-856 (smooth muscle isoform), MRTF-A, or MRTF-B and treated with 3 nM BMP4 for 24 h. Cells were then subjected to ChIP assay. A recruitment of these cofactors to the SMA promoter was examined by immunoprecipitation with an anti-Myc antibody, followed by quantitative real-time PCR analysis using primers specific for the SMA promoter. As control, untransfected cells were subjected to the ChIP assay using anti-Myc or anti-SRF antibody, followed by PCR using the same primers. An average of triplicate experiments is presented. The data show induction of SMA chromatin binding by BMP4 compared with cells treated with vehicle. In the inset, data are normalized to the background signal observed with the anti-Myc antibody immunoprecipitation in untransfected cells. The amount plotted in the input bar is 1:60 of the total used for each immunoprecipitation.
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for binding to the CArG box and thus inhibit expression of SMC-specific genes. Although apparently inconsistent with our findings, the conclusions drawn by Hayashi et al. can be reconciled with ours considering that, in their studies, Hayashi et al. (74, 75) use rat primary VSMCs cultured on laminin in media supplemented with insulin-like growth factor-1 (IGF-1), a cytokine that strongly activates the expression of SMC-specific genes and induces differentiation of VSMCs. An equivalent pro-differentiation effect of BMPs might be difficult to detect under their conditions. Conversely, the system employed by Hayashi et al. might be ideal for detecting down-regulation of SM genes and dedifferentiation of SM cells. Thus, we speculate that the different response to BMP signaling observed by Hayashi et al. might be due to different initial stages of smooth muscle phenotype at the time of BMP treatment. Consistently with this hypothesis, we did not find that the Msx genes are activated by BMP4 in human PASMCs in our recent comprehensive microarray analysis, while simultaneously SMC-specific genes such as SMA, smoothelin, calponin, and SM-MHC were strongly induced upon BMP4 stimulation (data not shown). Furthermore, we were unable to detect by real-time RT-PCR an induction of Msx1 or Msx2 mRNAs by BMP4 treatment in PASMCs (see Supplemental Fig. S3). Interestingly, we detected a stable ~2/3-fold induction of Msx1 in 10T½ cells (Supplemental Fig. S3), which may help explain, together with the induction of KLF4, the mechanism by which BMP-mediated induction of SM genes in 10T½, but not PASMCs, declines after 48 h. On the contrary, tissue- or species-specific effects cannot explain the differences between these studies, because we also examined the effect of BMP signals in VSMCs from other mammals, such as mouse and rat aortic SMCs, and non-vascular SMCs (human myometrial SMCs), and found induction of SM genes in these cells similar to PASMCs under our culture condition. Finally, it is conceivable that, depending on context, the same growth factor may be involved in both differentiation and dedifferentiation of SM cells. Further study is required to clarify the mechanism of this potential responsiveness switch.

In conclusion, our study sheds light on a fundamental role of BMP signaling during maintenance of arterial homeostasis and repair of vascular damage, and delineates a novel signaling response that may be amenable to a targeted pharmacological treatment of vascular disorders.

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