An epigenetic regulatory loop controls pro-osteogenic activation by TGF-β1 or bone morphogenetic protein 2 in human aortic valve interstitial cells

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Rui Song1, David A. Fullerton1, Lihua Ao1, Ke-seng Zhao5, and Xianzhong Meng†1

From the 1Department of Surgery, University of Colorado Denver, Aurora, Colorado 80045 and the 5Department of Pathophysiology, Guangdong Key Laboratory of Shock and Microcirculation Research, Southern Medical University, Guangzhou 510515, China

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Calcific aortic valve disease (CAVD) is common in the elderly population, but pharmacological interventions for managing valvular calcification are unavailable. Transforming growth factor β1 (TGF-β1) and bone morphogenetic protein 2 (BMP-2) induce pro-osteogenic activation of human aortic valve interstitial cells (AVICs) that play an important role in valvular calcification. However, the molecular mechanism underlying pro-osteogenic activation in AVICs is incompletely understood. Here, we investigated an epigenetic regulatory mechanism in human AVICs in the presence or absence of TGF-β1 stimulation, several studies found that altered miR expression is associated with prolongation of the human life span and unavailability of pharmacological interventions for prevention of this disease progression. Investigation of the molecular mechanism by which TGF-β1 and BMP-2 stimulate AVICs may play an important role in aortic valve calcification and CAVD progression. Investigation of the molecular mechanism by which TGF-β1 and BMP-2 induce pro-osteogenic changes in human AVICs will improve understanding of the mechanism underlying CAVD progression.

MicroRNAs (miRs) are a group of small non-coding, 21- to 23-nt-long RNA molecules (9). miRs regulate the expression of protein-coding genes and thus play important roles in the epigenetic regulation of gene expression. Emerging evidence demonstrates that miRs modulate a wide range of developmental and physiological processes, including cell proliferation and differentiation (10). Recently, miRs are implicated in the pathophysiology of various cardiovascular diseases and have become intriguing targets for therapeutic interventions (11). In addition, several studies found that altered miR expression is associated with aortic valve calcification (12, 13). However, the mechanistic role of altered miRs in AVIC pro-osteogenic activity is incompletely understood.

Calcific aortic valve disease (CAVD)2 is one of the leading cardiovascular diseases in the United States (1, 2). CAVD is an active process involving chronic valvular inflammation and calcification (3). Severe aortic valve calcification causes morbidity and mortality and results in the second most common cardiovascular surgery performed (4). The growing prevalence of this disease associated with prolongation of the human life span and unavailability of pharmacological interventions for prevention of this disease progression emphasize the importance of understanding of the pathobiological mechanisms of this disease. Particularly, it is critical to elucidate the cellular and molecular mechanisms by which aortic valve leaflets become calcified.

TGF-β1 and bone morphogenetic protein 2 (BMP-2) are recognized as important pro-osteogenic factors involved in vascular and aortic valve calcification (5, 6). Previous immunohistochemical studies revealed the presence of higher levels of TGF-β1 and BMP-2 in calcified human aortic valve leaflets compared with non-calcified aortic valve leaflets (7). Both TGF-β1 and BMP-2 have been shown to up-regulate alkaline phosphatase expression and activity in human aortic valve interstitial cells (AVICs), the primary cells involved in aortic valve calcification (8). Thus, the pro-osteogenic effects of TGF-β1 and BMP-2 on AVICs may play an important role in aortic valve calcification and CAVD progression.

Targeting this regulatory loop may have therapeutic potential for suppressing aortic valve calcification.

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This article contains supplemental Table S1.

1 To whom correspondence should be addressed: Box C-320, 12700 E. 19th Ave., Aurora, CO 80045. Tel: 303-724-6303; Fax: 303-724-6330; E-mail: Xianzhong.meng@ucdenver.edu.

2 The abbreviations used are: CAVD, calcific aortic valve disease; BMP-2, bone morphogenetic protein 2; AVIC, aortic valve interstitial cell; miRNA, microRNA.
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The effects of TGF-β1 and BMP-2 remains unclear. Studies in this line will provide insight into the mechanism underlying CAVD pathogenesis and may identify therapeutic targets for suppression of CAVD progression.

Clinicopathological and conventional diagnostic imaging studies of calcified human aortic valves demonstrate that AVICs express osteopontin, osteocalcin, and osteogenic transcription factors such as Osterix (Osx) and Runx-related transcription factor 2 (Runx2)/Cbfal (14). Runx2 is required for osteoblast differentiation (15). Osx is identified as a BMP2-specific transcription factor involved in bone formation (16). Importantly, Osx knock-out mice display severe impairment of bone formation and osteoblastogenesis, indicating that this transcription factor is essential for osteoblast differentiation (16, 17). The significance of Runx2 and Osx in CAVD is highlighted by the observation that the expression of these two osteogenic transcription factors is elevated in AVICs from calcified human aortic valves (18).

Smad signaling plays a critical role in mediating the effect of TGF-β1 and BMP-2. Smad1 is required for induction of Osx by BMP-2 (19). We have observed that Smad1 and Smad3 are involved in mediating the expression of Runx2 and alkaline phosphatase in human AVICs induced by biglycan that up-regulates the production and release of BMP-2 and TGF-β1 (20). In contrast, Smurf2 is a negative regulator of Smad signaling and plays a role in the control of cellular response to TGF-β and BMP (21, 22). Currently, the knowledge of miR modulation by TGF-β1 and BMP-2 in human AVICs is limited. The roles of the Smad pathways and Smurf2 in regulation of AVIC expression of miRs remain unclear.

We hypothesized that TGF-β1 and BMP-2 induce pro-osteogenic activation in human AVICs via a miR-osteogenic transcription factor axis. The purpose of this study was to determine: 1) the effects of TGF-β1 and BMP-2 on the expression of miRs in human AVICs, 2) the role of Smad pathways in the mechanism by which TGF-β1 and BMP-2 exert their effects on miR expression, 3) the role of miRs in modulation of the expression of osteogenic transcription factors and pro-osteogenic activity in human AVICs, and 4) the effect of miRs on Smurf2 expression and the role of Smurf2 in modulation of AVIC response to TGF-β1 and BMP-2.

Results

TGF-β1 and BMP-2 up-regulate miR-486 and down-regulate miR-204 in human AVICs

Microarray analysis revealed that increased levels of miR-486 and decreased levels of miR-204 were the common changes in normal human AVICs exposed to TGF-β1 and BMP-2 (supplemental Table S1). TGF-β1 and BMP-2 reduced miR-204 levels by 75 and 63%, respectively, and these two pro-osteogenic factors increased miR-486 levels by 68 and 72%, respectively. Real-time PCR data confirmed significant changes in miR-204 and miR-486 levels in cells exposed to TGF-β1 or BMP-2, and showed that altered levels of these two miRs were comparable with those in AVICs from calcified human aortic valves (Fig. 1). Thus, both TGF-β1 and BMP-2 reduce miR-204 and increase miR-486 in human AVICs.

miR-204 is a negative regulator of osteoblastic differentiation and miR-204 down-regulation plays a role in AVIC and vascular smooth muscle cell calcification in vitro (23–25). To examine whether miR-486 is involved in miR-204 down-regulation induced by TGF-β1 and BMP-2, we determined the effect of lentiviral expression of miR-486 antagonist. We observed that expression of the miR-486 antagonist attenuated the negative effect of TGF-β1 and BMP-2 on miR-204 expression (Fig. 2A). Furthermore, we observed that attenuation of miR-204 down-regulation by the miR-486 antagonist correlated with a marked reduction of calcium deposit formation following prolonged stimulation with TGF-β1 or BMP-2 (Fig. 2B). Therefore, up-regulation of miR-486 by TGF-β1 and BMP-2 contributes to the mechanism by which these two pro-osteogenic factors down-regulate miR-204 and promote pro-osteogenic activity in human AVICs.

Expression of miR-486 antagonist attenuates miR-204 down-regulation and calcium deposit formation induced by TGF-β1 or BMP-2

TGF-β1 and BMP-2 down-regulate miR-204 via the Smad pathways

To understand how TGF-β1 and BMP-2 down-regulate miR-204 expression, we applied siRNAs to knockdown Smad isoforms. The results in Fig. 3A show that knockdown of Smad3, not Smad1, preserved miR-204 levels in cells exposed to TGF-β1. Conversely, knockdown of Smad1, not Smad3, exerted a similar effect on miR-204 levels in cells exposed to BMP-2 (Fig. 3A).

In silico analysis (UCSC Genome Bioinformatics) indicated that a potential Smads-binding site (CAGAC) might be present in the miR-204 promoter region. To elucidate the mechanism by which Smads mediate the effect of TGF-β1 and BMP-2 on miR-204 expression, a ChIP assay was performed to examine the interaction of Smads with the miR-204 promoter region. Antibody-mediated immunoprecipitation of fragmented chromatin from formaldehyde cross-linked cell lysates with a subsequent PCR amplification revealed binding of the Smad1 and Smad3 to the miR-204 promoter region from −1056 to −1223

Figure 1. TGF-β1 and BMP-2 down-regulate miR-204 and up-regulate miR-486 in human AVICs. Real-time quantitative RT-PCR analysis shows that miR-204 is down-regulated and miR-486 is up-regulated in normal AVICs treated with TGF-β1 (0.005 μg/ml) or BMP-2 (0.100 μg/ml) for 24 h. The levels of these two miRs in cells treated with TGF-β1 or BMP-2 are comparable with those in AVICs from calcified valves (n = 6 separate experiments using distinct cell isolates; *, p < 0.05 versus untreated normal AVICs).

| miRNA levels (%) |
|------------------|
| untreated        |
| normal           |
| calcified        |
| miR-486          |
| miR-204          |

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(Fig. 3B). The result suggests that the TGF-β1-Smad3 and BMP-2-Smad1 pathways modulate miR-204 transcription.

**miR-486 reduces Smurf2 to enhance AVIC response to TGF-β1 and BMP-2**

Smurf2 is a Smad inhibitor and plays a role in modulating TGF-β1 and BMP-2 signaling (21, 22). Interestingly, miR-486 mimic reduced and miR-486 antagonist increased Smurf2 levels in human AVICs (Fig. 4A). However, miR-204 mimic and antagonist had no effect (Fig. 4A). Furthermore, the miR-486 antagonist increased Smurf2 levels in AVICs exposed to TGF-β1 or BMP-2 (Fig. 4B).

To determine the role of Smurf2 in modulating miR-204 expression in human AVICs exposed to TGF-β1 and BMP-2, we treated cells with Smurf2 shRNA. As shown in Fig. 5A, Smurf2 shRNA effectively knocked down Smurf2 protein.

Knockdown of Smurf2 further reduced miR-204 levels in cells exposed to TGF-β1 or BMP-2, whereas treatment with lentiviral control shRNA had no effect (Fig. 5A).

In investigation of the role of miR-204 down-regulation in Osx and Runx2 expression induced by TGF-β1 and BMP-2, we applied miR-204 mimic or miR-204 antagonist to AVICs exposed to TGF-β1 or BMP-2. As shown in Fig. 5B, miR-204 mimic reduced Osx and Runx2 expression induced by either TGF-β1 or BMP-2. Conversely, miR-204 antagonist enhanced the effect of TGF-β1 and BMP-2 on the expression of these two osteogenic transcription factors. However, control miRNA had no effect.

To understand whether Smurf2 functions as a negative regulator in the expression of Osx and Runx2 induced by TGF-β1 and BMP-2, we treated AVICs expressing Smurf2 shRNA with TGF-β1 or BMP-2 and examined Osx and Runx2 levels. Smurf2...
knockdown enhanced the expression of Osx and Runx2 following exposure to TGF-β1 or BMP-2 (Fig. 5C). Together, the results show that reduction of the Smurf2 level exaggerates miR-204 down-regulation to enhance Osx and Runx2 expression following TGF-β1 and BMP-2 stimulation, and suggest that the effect of miR-486 on Smurf2 levels accounts for its impact on miR-204 down-regulation and the pro-osteogenic activity in human AVICs exposed to TGF-β1 or BMP-2.

**Discussion**

TGF-β1 and BMP-2 are pro-osteogenic mediators in the development and progression of CAVD. In this regard, high levels of these two mediators have been found in the aortic valves explanted from patients with CAVD (8, 26). Furthermore, a number of studies demonstrate that TGF-β1 and BMP-2 induce the osteogenic responses in human AVICs (8, 27–29). Previous studies have shown that several miRs, including miR-322, regulate osteoblast differentiation induced by TGF-β1 and BMP-2 (30). More importantly, miR-141 is involved in porcine aortic valve cell calcification induced by BMP-2 (31). As TGF-β1 and BMP-2 have similar pro-osteogenic effects on human AVICs, it is likely that these two pro-osteogenic mediators modulate the expression of a specific group of miRs to exert their effects on AVIC osteogenic responses. Interestingly, our microarray data show that only miR-486 and miR-204 levels are altered in human AVICs by both TGF-β1 and BMP-2. Furthermore, either of these two pro-osteogenic mediators down-regulates miR-204 expression and up-regulates miR-486 expression to levels comparable with those seen in AVICs from calcified human aortic valves.

miR-204 is a known negative regulator of osteoblastic differentiation. Currently, it is unclear how TGF-β1 and BMP-2 down-regulate miR-204 expression in human AVICs. In addition, it is unknown whether miR-486 up-regulation plays a role in mediating osteogenic responses to TGF-β1 and BMP-2 in human AVICs and whether miR-486 and miR-204 interact in...
mediating the osteogenic responses. The findings of the present study provide mechanistic information.

**Smads binds to miR-204 gene promoter to modulate the expression of this negative regulator of pro-osteogenic activity**

Both TGF-β1 and BMP-2 utilize the Smad signaling pathways to exert their effects on human AVICs (20). Eight mammalian Smad proteins have been identified to date, and the Smad family of proteins have distinct roles in TGF-β1 and BMP-2 signaling (32). In this regard, Smad3 mediates the effect of TGF-β1, whereas Smad1 mediates the effect of BMP-2 in the up-regulation of pro-osteogenic activity in human AVICs (20). Using a ChIP approach, we found Smad1 and Smad3 can bind to the promoter region of miR-204. Knockdown of Smad3, not Smad1, reduces the effect of TGF-β1 in the down-regulation of miR-204 expression, whereas knockdown of Smad1 reduces the negative effect of BMP-2 on miR-204 expression. Thus, TGF-β1 and BMP-2 down-regulate miR-204 expression in human AVICs through the classical Smad signaling pathways, and Smad3 and Smad1 appear to mediate the effects of TGF-β1 and BMP-2, respectively, on miR-204 expression through modulation of gene transcription.

miR-204 modulates osteoblastic differentiation (23), and down-regulation of miR-204 promotes in vitro calcification in vascular smooth muscle cells and human AVICs (24, 25). To confirm the role of miR-204 down-regulation in mediating the pro-osteogenic effects of TGF-β1 and BMP-2, we evaluated the expression of osteogenic transcription factors Osx and Runx2 in human AVICs. The results show that TGF-β1 and BMP-2 increase the levels of both Osx and Runx2. Two lines of evidence indicate that miR-204 down-regulation plays a role in promoting the expression of Osx and Runx2 in human AVICs. First, miR-204 mimic suppresses and miR-204 antagomir increases cellular levels of Osx and Runx2 in human AVICs exposed to TGF-β1 or BMP-2. Second, treatment of human AVICs with miR-204 antagomir in the absence of TGF-β1 or BMP-2 causes an increase in the levels of Osx and Runx2. Thus, modulation of miR-204 levels may affect aortic valve pro-osteogenic activity.

miR-141 has been shown to mediate BMP-2-induced osteogenic responses in porcine aortic valve cells (31). However, neither TGF-β1 nor BMP-2 alters miR-141 levels in human AVICs. Our work demonstrates a significant down-regulation of miR-204 by either TGF-β1 or BMP-2 in human AVICs and an important role of miR-204 down-regulation in mediating the
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A

Smurf2

β-actin

Lenti-Control shRNA

Lenti-Smurf2 shRNA

miR-204 expression (% baseline)

B

Osx

β-actin

TGF-β1

BMP-2

miR-204 mimic

miR-204 antagonim

miR-C

Fold change in Osx levels

C

Runx2

β-actin

TGF-β1

BMP-2

miR-204 mimic

miR-204 antagonim

miR-C

Fold change in Runx2 levels

Fold change in Osx and Runx2 expression

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osteogenic responses to TGF-β1 and BMP-2. The difference between our observations and the previously reported finding could be due to a species-related difference in AVIC response to pro-osteogenic mediators.

**miR-486 modulates Smurf2 expression to augment miR-204 down-regulation**

Interestingly, the results of this study show that up-regulation of miR-486 also plays a role in mediating the pro-osteogenic activity in human AVICs exposed to TGF-β1 or BMP-2 because lentiviral expression of miR-486 antagonist markedly attenuates calcium deposition induced by TGF-β1 or BMP-2. To our knowledge, such a role for miR-486 has not been reported. In correlation to this effect, lentiviral expression of the miR-486 antagonist preserves cellular levels of miR-204 in human AVICs exposed to TGF-β1 or BMP-2. Like all RNAs, miRs are products of transcription. It is not surprising that the expression of some miRs are regulated by other miRs. An example of this type of miR-modulated miR expression is the cross-regulation between members of the “myomiR” family of miRs that are encoded within myosin heavy chain genes (33, 34). Our data show an interaction of miR-486 with miR-204 and suggest that up-regulation of miR-486 by TGF-β1 and BMP-2 plays a role in the mechanism by which they down-regulate miR-204.

It is likely that miR-486 contributes to the mechanism of elevated pro-osteogenic activity in human AVICs stimulated by TGF-β1 or BMP-2 through its negative impact on miR-204 expression. This raises the question of how miR-486 exerts its effect on miR-204 expression. The results of this study show that the miR-486 mimic decreases and miR-486 antagonist increases Smurf2 levels in human AVICs. TGF-β1 and BMP-2 reduce Smurf2 levels and antagonizing miR-486 elevates Smurf2 levels in human AVICs exposed to TGF-β1 and BMP-2. Therefore, miR-486 negatively regulates Smurf2 expression in human AVICs. Because Smurf2 is an intrinsic inhibitor of Smad signaling (22), it is reasonable to propose that up-regulation of miR-486 reduces Smurf2 expression to augment the osteogenic responses to TGF-β1 and BMP-2. Indeed, our data show that knockdown of Smurf2 exaggerates the induction of Osx and Runx2 expression by TGF-β1 and BMP-2.

Overall, this study found miR-204 down-regulation and miR-486 up-regulation as a common feature of AVICs from calcified human aortic valves and normal AVICs stimulated by pro-osteogenic mediators TGF-β1 and BMP-2. Furthermore, we identified a miR-486–Smurf2–Smad loop in modulation of miR-204 expression and pro-osteogenic activity in human AVICs exposed to TGF-β1 and BMP-2. Targeting this regulatory loop may have a therapeutic potential for suppression of aortic valve calcification.

**Figure 5. Smurf2 knockdown augments miR-204 down-regulation induced by TGF-β1 or BMP-2 and leads to enhanced expression of osteoblastic biomarkers.** A, AVICs were untreated or infected with lentivirus that expresses Smurf2 shRNA. A representative immunoblot shows that Smurf2 shRNA effectively knocks down Smurf2 protein. Knockdown of Smurf2 further reduced miR-204 levels in AVICs exposed to TGF-β1 or BMP-2. n = 5 separate experiments using distinct cell isolates; *, p < 0.05 versus TGF-β1 alone or BMP-2 alone. B, representative immunoblots and densitometric data show that the miR-204 mimic suppresses and miR-486 antagonist enhances the expression of Runx2 and Osx at 24 h following treatment with TGF-β1 or BMP-2. n = 6 separate experiments using distinct cell isolates; *, p < 0.05 versus control; #, p < 0.05 versus stimulant + miR-C (irrelevant oligonucleotide). C, representative immunoblots and densitometric data show that Smurf2 knockdown enhances the expression of Runx2 and Osx in AVICs exposed to TGF-β1 or BMP-2. n = 5 separate experiments using distinct cell isolates; *, p < 0.05 versus control; #, p < 0.05 versus TGF-β1 alone or BMP-2 alone; †, p < 0.05 versus stimulant + control shRNA.

**Figure 6. Schematic description of the miR-486-Smurf2-Smad loop in regulating AVIC osteogenic response to TGF-β1 and BMP-2.** Both TGF-β1 and BMP-2 up-regulate miR-486 and down-regulate miR-204 in human AVICs. The Smad pathways mediate down-regulation of miR-204 that functions as a negative regulator of Runx2 and Oxs expression. miR-486 down-regulates Smurf2 levels to enhance Smad signaling, leading to augmented miR-204 down-regulation and further elevated osteogenic activity in human AVICs.

**Conclusions**

TGF-β1 and BMP-2 promote pro-osteogenic activity in human AVICs through up-regulation of miR-486 and down-regulation of miR-204. The Smad pathways mediate the effects of TGF-β1 and BMP-2 in down-regulation of the expression of miR-204, a negative regulator of osteogenic responses. miR-486 down-regulates Smurf2 levels to enhance the negative effect of TGF-β1 and BMP-2 on miR-204 expression. The miR-486-Smurf2-Smad loop plays an important role in regulating AVIC osteogenic responses to TGF-β1 and BMP-2 (Fig. 6).

**Experimental procedures**

**Materials**

Antibodies against human Osx were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies against human Runx2, Smad1, Smad3, and Smurf2 were purchased from Cell Signaling, Inc. (Beverly, MA). miR mimics and antagonirs, control miR, HiPerFect® transfection reagent, other transfection-related reagents, and EndoFree Plasmid Maxi Kit were purchased from Qiagen (Valencia, CA). Lentivirus vector expressing miR mimic or antagonist and Block-it lentiviral pol II miR RNAi expression plasmids were purchased from Invitrogen. Lenti-Smurf2 shRNA and lentivirus non-target shRNA were purchased from the Functional Genomics Facility of the University of Colorado. Lipofectamine 2000 was pur-
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chased from Life Technologies, Inc. ChromaFlash™ Chromatin Extraction Kit and ChromaFlash™ One-Step ChIP Kit were purchased from Epigentek Group Inc. (Farmingdale, NY). TransDux transduction reagent was purchased from System Biosciences (Mountain View, CA). Medium 199 was purchased from Lonza (Walkersville, MD). All other chemicals and reagents were from Sigma.

**Isolation, culture, and treatment of human AVICs**

Normal aortic valve leaflets were collected from explanted hearts of 6 patients (4 males and 2 females, age 59.0 ± 8.1 years) undergoing heart transplantation due to late stage cardiomyopathy. These valve leaflets were thin and had no histological abnormality. Calcified aortic valve leaflets were from 6 patients (4 males and 2 females, age 63.0 ± 6.9 years) undergoing aortic valve replacement surgery due to CAVD. This study was approved by the University of Colorado Denver Institutional Review Board. All patients gave informed consent for the use of their aortic valves for this study.

AVICs were isolated and cultured using a previously described method (28, 35, 36). Briefly, a high concentration of collagenase (2.5 mg/ml) was used to remove endothelial cells, and the remaining tissue was treated with a low concentration of collagenase (0.8 mg/ml) to free the interstitial cells. Cells were collected by centrifugation and cultured in M199 growth medium supplemented with 10% fetal bovine serum, penicillin G (100 units/ml), streptomycin (100 mg/ml), and amphotericin B (0.25 μg/ml). Cells of passage 3 to 6 were used for the experiments.

Lentiviral expression of miR-486 antagonist was applied to determine its effect on calcium deposition induced by TGF-β1 (0.005 μg/ml) or BMP-2 (0.100 μg/ml). AVICs were treated with lentivirus expressing miR-486 antagonist for 72 h and then stimulated with TGF-β1 or BMP-2 in a conditioning medium (growth medium supplemented with 10 mmol/liter of β-glycerophosphate, 10 nmol/liter of vitamin D$_3$, 10 nmol/liter of dexamethasone, and 8 mmol/liter of CaCl$_2$) for 14 days. Alizarin Red staining was applied to evaluate calcium deposition.

To determine the role of miR-204 in modulating the effects of TGF-β1 and BMP-2 on AVIC expression of Osx and Runx2, cells were transfected with miR-204 mimic (5 nm), or control miR (5 nm) with or without stimulation with TGF-β1 or BMP-2.

Gene knockdown was applied to determine the role of the Smad-dependent pathway in mediating the effect of TGF-β1 and BMP-2 on AVIC expression of miR-204. Cells were pre-treated with Smad1 siRNA (50 nM), Smad3 siRNA (50 nM), or scrambled siRNA (50 nM) for 48 h, and then stimulated with TGF-β1 or BMP-2 for 24 h.

To determine the role of Smurf2 in modulating the effects of TGF-β1 and BMP-2 on AVIC expression of Osx and Runx2, cells were treated with lentiviral Smurf2 shRNA, or lentiviral control shRNA. Then cells were stimulated with TGF-β1 or BMP-2.

**Lentiviral transduction**

Block-it lentiviral pol II miR RNAi expression plasmids were amplified using standard bacterial transformation and purified using EndoFree Plasmid Maxi Kit. Lentivirus expressing miR-486 mimic and antagonist and non-target miRNA was generated by Lipofectamine 2000 co-transfection of 293T cells. After 48 h, lentiviral supernatants were collected and concentrated. AVICs were infected with lentivirus expressing miRNA mimic and co-transfected with TransDux transduction reagent.

**Immunoblotting**

Immunoblotting was performed, as described previously (37), to analyze protein levels of Smad1, Smad3, Osx, Runx2, and Smurf2. β-Actin levels were examined for normalization of loading. In brief, whole cell lysates were prepared with a sample buffer (100 mmol/liter of Tris-HCl, pH 6.8, 2% SDS, 0.02% bromphenol blue, and 10% glycerol) after treatment of cells. Proteins in cell lysate were fractioned by 4–20% SDS-PAGE and subsequently transferred to PVDF membranes. After being blocked with 5% skim milk solution, membranes were incubated with primary antibodies, followed by peroxidase-linked secondary antibodies specific to the primary antibodies. Protein bands were visualized with ChemiDoc™ MP imaging System (Bio-Rad). Band density was analyzed using the ImageJ software (Wayne Rasband, National Institutes of Health, Bethesda, MD).

**Real-time RT-PCR analysis**

Total RNA was isolated using TRizol reagent and a Qiagen miRNeasy Mini Kit (Valencia, CA). Reverse transcription (RT) and PCR were performed using iScript™ cDNA Synthesis Kit (Bio-Rad), Qiagen miScript II RT Kit, iQ™ SYBR® Green Supermix, and Qiagen miScript SYBR® Green PCR Kit according to the manufacturer’s instructions. The following primers were used to amplify specific cDNA fragments: miR-486 (forward, 5'-CCCCCTCCTGTACTGAGCTGCCCCGAG-3'; reverse, miScript Universal Primer); miR-204 (forward, 5'-CCCCCTTCTTTTTGATGAGTGGGCACCACA-3'; reverse, miScript Universal Primer); miR-486 and miR-204 levels were quantified by real-time PCR using the IQ™ 5 Multicolor Real-time PCR Detection system (Bio-Rad). miR-486 and miR-204 levels normalized to U6 were calculated using the 2$^{-\Delta\Delta Ct}$ method (38).

**Chromatin immunoprecipitation**

Chromatin was prepared from human AVICs and ChIP was performed using ChromaFlash™ Chromatin Extraction Kit and ChIP Assay Kit according to the manufacturer’s instruction (Epigentek Group Inc.). The sheared chromatin were immunoprecipitated with antibodies against Smad1, Smad3, non-immune IgG (negative control), or RNA polymerase II (positive control) at room temperature for 90−120 min. DNA-protein complexes were collected and eluted. ChIP DNA was used as a template for PCR. Primers specific for detection of Smads-binding site (amplifying a region from −1223 to −1056 of the miR-204 promoter) were as follows: 5’-TGATTGAGGTGCTAAAGAAGATGG-3’; 5’-TGACACAGGTCAGATTTG-3’. PCR products were separated on a 1% agarose gel. Input chromatin isolated before the immunoprecipitation was used to control for equal amounts of input DNA (39).
Staining for calcium deposits

Alizarin Red staining was performed as described previously (28, 40). Briefly, cell monolayers and aortic valves were washed twice with phosphate-buffered saline (PBS) and fixed for 15 min in 4% paraformaldehyde. Specimens were incubated with 0.2% Alizarin Red solution (pH 4.2) to examine calcium deposits. Specimens were washed with distilled water. Alizarin Red stains were bleached with 10% acetic acid at 85 °C. Supernatant was spectrophotometrically analyzed at 450 nm (41).

Statistical analysis

All results are expressed as mean ± S.D. Comparisons between groups were performed using SPSS 13.0 software with one-way analysis of variance with the post hoc Bonferroni/Dunn test. Differences were confirmed with the Kruskal–Wallis H test. A difference was considered significant at p ≤ 0.05.

Author contributions—R. S. and X. M. conceived and designed the research. R. S. and L. A. performed experiments. R. S., L. A., D. A. F., K. Z., and X. M. analyzed data and interpreted results. R. S. prepared figures and drafted the manuscript. X. M. revised and finalized the manuscript. All authors read and approved the final version of this manuscript.

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