**Abstract**—The TMEM16A protein is an important component of Ca\(^{2+}\)-dependent Cl\(^{-}\) channels (CaCCs) in vascular smooth muscle cells. A recent study showed that TMEM16A inhibits angiotensin II–induced proliferation in rat basilar smooth muscle cells. However, whether and how TMEM16A is involved in vascular remodeling characterized by vascular smooth muscle cell proliferation remains largely unclear. In this study, luciferase reporter, Western blotting, and qRT-PCR assays were performed. The results suggested that myocardin promotes TMEM16A expression by forming a complex with serum response factor (SRF) on the TMEM16A promoter in human aortic smooth muscle cells (HASMCs). In turn, upregulated TMEM16A promotes expression of myocardin and vascular smooth muscle cell marker genes, thus forming a positive feedback loop that induces cell differentiation and inhibits cell proliferation. Angiotensin II inhibits TMEM16A expression via Krüppel-like factor 5 (KLF5) in cultured HASMCs. Moreover, in vivo experiments show that infusion of angiotensin II into mice causes a marked reduction in TMEM16A expression and vascular remodeling, and angiotensin II–induced effects are largely reversed in KLF5 null (KLF5\(^{-/-}\)) mice. KLF5 competes with SRF to interact with myocardin, thereby limiting myocardin binding to SRF and the synergistic activation of the TMEM16A promoter by myocardin and SRF. Our studies demonstrated that angiotensin II induces KLF5 expression and facilitates KLF5 association with myocardin to disrupt the myocardin–SRF complex, subsequently leading to inhibition of TMEM16A transcription. Blocking the positive feedback loop between myocardin and TMEM16A may be a novel therapeutic approach for vascular remodeling. (Hypertension. 2015;66:412-421. DOI: 10.1161/HYPERTENSIONAHA.115.05280.) • Online Data Supplement

**Key Words:** Ang II ■ KLF5 ■ myocardin ■ TMEM16A ■ vascular remodeling

**Renin–Angiotensin System**

TMEM16A and Myocardin Form a Positive Feedback Loop That Is Disrupted by KLF5 During Ang II–Induced Vascular Remodeling

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vascular remodeling characterized by VSMC proliferation remain largely unclear.

Myocardin is a potent transcriptional coactivator expressed exclusively in contractile smooth and cardiac muscle cells. Myocardin does not bind DNA directly. It associates strongly with serum response factor (SRF), a transcription factor that binds to conserved CArG box [CC (A/T)₆ GG] DNA sequences present within the promoters of SMC genes and acts as a weak activator of gene expression, to selectively induce expression of CArG-dependent SMC genes, including SM22α and SMα-actin.²⁴,²⁵

In this study, we show that myocardin promoted TMEM16A expression by forming a complex with SRF bound to the CArG box of the TMEM16A promoter in human aortic smooth muscle cells (HASMCs). In turn, upregulated TMEM16A promoted expression of myocardin and VSMC marker genes, thus forming a positive feedback loop that induced cell differentiation and inhibited cell proliferation. Angiotensin II (Ang II) induced Krüppel-like factor 5 (KLF5) expression and facilitated KLF5 association with myocardin to disrupt the myocardin–SRF complex, subsequently leading to blockage of this positive feedback loop and inhibition of TMEM16A transcription, thus influencing cellular proliferation.

**Methods**

An expanded Methods section can be found in the online-only Data Supplement.

**Results**

**Myocardin Upregulates TMEM16A Expression**

To identify transcription factors that regulate expression of TMEM16A, we constructed a TMEM16A promoter-luciferase reporter construct, including the region spanning from −2000 to +1 of the human TMEM16A promoter and performed a luciferase assay in 293A cells. Our results indicated that overexpression of SRF or myocardin, but not KLF4, concentration-dependently increased TMEM16A promoter activity (Figure 1A). Truncating the TMEM16A promoter sequence from −2000 to −536 did not affect activation of the promoter by SRF. However, further deletion between −536 and −349 dramatically decreased the activation of this reporter by SRF (Figure 1B, left panel), indicating that the region between −536 and −349 is critical for conferring the capacity to respond to SRF. Indeed, this region contains one TGF-β control element and one SRF-responsive element CArG box (Figure 1B, right panel). Coexpression of myocardin and SRF increased activation of the TMEM16A promoter (−2000 to +1 and −536 to +1) ≈2-fold over that seen with myocardin or SRF alone. Truncating the TMEM16A promoter to −349 abolished the synergistic activation of the promoter by myocardin and SRF (Figure 1C). Moreover, knockdown of myocardin in HASMCs led to a decrease in TMEM16A protein (Figure 1D, left panel) and mRNA expression (Figure 1D, right panel). Taken together, these results indicate that myocardin and SRF are necessary for TMEM16A expression in HASMCs.

**TMEM16A Induces VSMC Differentiation and Inhibits VSMC Proliferation**

To investigate the function of TMEM16A in VSMCs, adenoviruses encoding TMEM16A (Ad-GFP-TMEM16A) or GFP control (Ad-GFP) were transduced into HASMCs and mouse aortic smooth muscle cells. Voltage patch clamp experiments showed that HASMCs infected with Ad-GFP-TMEM16A exhibited much larger CaCC currents than those infected with Ad-GFP (Figure 2A). Western blot analysis showed that overexpression of TMEM16A in HASMCs significantly increased the expression of the VSMC differentiation markers SM22α and SMα-actin and decreased proliferating cell nuclear antigen (PCNA) and cyclin D1 levels in a time- and dose-dependent manner (Figure 2B). Similar results were obtained in mouse aortic smooth muscle cells (Figure 2C). By contrast, using 2 distinct siRNAs to silence TMEM16A expression led to elevated protein levels of PCNA and cyclin D1 and decreased protein levels of SM22α and SMα-actin in HASMCs (Figure 2D). Moreover, forced expression of TMEM16A in HASMCs also markedly increased SM22α and SMα-actin mRNA expression and inhibited cyclin D1 mRNA expression and cell proliferation (Figure 2E). To further validate the effect of TMEM16A on VSMC proliferation in vivo, we re-expressed GFP or TMEM16A using adenovirus-mediated gene transfer. Mice were subjected to endothelial denudation by wire injury to the femoral artery followed by intraluminal instillation with adenoviruses for 20 minutes and were examined 14 days later. As shown in Figure 2F and Figure S1 in the online-only Data Supplement, overexpression of TMEM16A largely reduced the neointimal area and medial proliferative cell number compared with the Ad-GFP-treated control mice. Because VSMC proliferation plays a key role in neointima formation after vascular injury, we conclude that expression of TMEM16A can inhibit VSMC proliferation in vivo, thus leading to reduced neointima formation after vascular injury.

**TMEM16A Induces VSMC Differentiation and Inhibits VSMC Proliferation via Upregulating Myocardin Expression**

To understand how TMEM16A regulates VSMC differentiation and proliferation, we detected the expression of a series of transcription factors responsible for VSMC differentiation and proliferation in HASMCs infected with Ad-TMEM16A. Interestingly, overexpression of TMEM16A markedly induced the mRNA and protein expression of myocardin but not KLF4, KLF5, and SRF (Figure 3A and 3B). Conversely, knockdown of TMEM16A by either of the 2 siRNAs targeting TMEM16A decreased expression of myocardin (Figure 3C). These data indicate that TMEM16A specifically induces the expression of myocardin and that myocardin forms a positive feedback loop with TMEM16A to induce VSMC differentiation and inhibit VSMC proliferation (Figure 3D).

**Ang II Inhibits TMEM16A Expression and Disrupts the Positive Feedback Loop Between TMEM16A and Myocardin**

Because Ang II is known to inhibit TMEM16A expression in basilar smooth muscle cells (BASMCs),²² we sought to determine whether Ang II also affected TMEM16A expression in HASMCs and, in turn, regulated the positive feedback loop between TMEM16A and myocardin. As shown in Figure 4A, treatment of HASMCs with Ang II significantly decreased...
TMEM16A protein expression while increasing the protein expression of its known target genes KLF5, cyclin D1, and NOX-1 in a time- and dose-dependent manner. Similarly, Ang II also decreased TMEM16A mRNA expression in a time- and dose-dependent manner in HASMCs (Figure 4B).

Ang II treatment also significantly inhibited TMEM16A protein expression in mouse aortic smooth muscle cells but not in human cardiac microvascular endothelial cells (Figure 4C).

Consistent with these results, Western blot analysis also showed decreased expression of TMEM16A in the thoracic aorta of human angiotensinogen transgenic mice compared with wild-type (WT) mice (Figure 4D). In addition, overexpression of TMEM16A largely blocked Ang II–induced upregulation of cyclin D1 and PCNA and abrogated Ang II–induced downregulation of SM22α and SMα-actin (Figure 4E, left panel). BrdU incorporation also showed that overexpression of TMEM16A largely abolished Ang II–induced cell proliferation (Figure 4E, right panel). These findings demonstrate that Ang II downregulates TMEM16A expression and disrupts the positive feedback loop between TMEM16A and myocardin, subsequently leading to cell proliferation.

Ang II Inhibits TMEM16A Expression via KLF5

Because KLF5 is known to mediate Ang II–induced gene expression,26,27 we sought to investigate whether KLF5 mediated Ang II–induced suppression of TMEM16A expression. The overexpression of KLF5 in HASMCs infected with adeno virus encoding KLF5 (Ad-GFP-KLF5) significantly decreased TMEM16A protein expression and increased cyclin D1 protein expression in a time- and dose-dependent manner (Figure 5A, left panel). Accordingly, KLF5 overexpression also markedly inhibited TMEM16A mRNA expression (Figure 5A, right panel). When HASMCs were transfected with KLF5-specific siRNA (siKLF5) to block endogenous KLF5 expression, Ang II–mediated suppression of TMEM16A protein and mRNA

Figure 1. Myocardin upregulates TMEM16A expression. A, A luciferase reporter controlled by the TMEM16A promoter was transfected into 293A cells with a serum response factor (SRF; left panel), myocardin (Myoc; middle panel), or Krüppel-like factor 4 (KLF4; right panel) expression plasmid. B, 293A cells were transfected with the indicated TMEM16A promoter luciferase reporters either in combination with or without a SRF expression plasmid (left panel). Diagram of the −536 to +1 region of the TMEM16A promoter. The −536 to −349 region contains one KLF-binding site (named TGF-β control element [TCE]) and one CArG box (right panel). C, 293A cells were transfected with the indicated TMEM16A promoter luciferase reporters alone or in combination with SRF and Myoc expression vectors. Cells were lysed, and the luciferase activity was measured using the dual luciferase reporter assay system. The data represent the relative TMEM16A promoter activity normalized to Renilla luciferase. Error bars represent the SD of 3 independent experiments. P<0.05 vs the pcDNA3.1 group. D, Human aortic smooth muscle cells (HASMCs) were mock-transfected (−) or transfected with siRNA targeting Myoc (siMyoc) or a control siRNA (siCon). 24 hours after transfection, Myoc and TMEM16A levels were determined via immunoblotting (left panel). GAPDH was used as a control for equal loading in all experiments. The relative expression of TMEM16A mRNA was examined using qRT-PCR and presented after normalizing to GAPDH (means±SD; n=3; right panel). P<0.05 vs the mock-transfected group.
expression was abrogated (Figure 5C). Simultaneously, the inhibitory effect of Ang II on TMEM16A protein expression was increased by KLF5 overexpression (Figure 5D). Further studies were performed in KLF5 null (KLF5−/−) and WT mice infused with or without Ang II. As shown in Figure 6A, a marked medial expansion was observed in the thoracic aorta of WT mice 28 days after Ang II infusion, and this expansion was strongly reduced in KLF5−/−mice. In addition, Ang II infusion promoted a marked increase in the density of nuclei and the number of proliferating Ki-67+ SMCs in the media.

Figure 2. TMEM16A induces vascular smooth muscle cells (VSMC) differentiation and inhibits VSMC proliferation. A, Representative traces of whole-cell current recorded from human aortic smooth muscle cells (HASMCs) infected with Ad-GFP (left panel) or Ad-GFP-TMEM16A (middle panel) with an intracellular solution containing 0.5 μM Ca2+. The current densities vs holding voltage relationship from experiments shown in left and middle panels (right panel; n=5–8). B, Ad-GFP-TMEM16A were used to infect HASMCs at the indicated doses and for the indicated times. Protein was isolated, and NOX-1, p47phox, cyclin D1, SM22α, SMα-actin, proliferating cell nuclear antigen (PCNA), and GAPDH expression levels were measured via immunoblotting. C, Mouse aortic smooth muscle cells (MASMCs) were infected with Ad-GFP or Ad-GFP-TMEM16A for the indicated periods of time. Protein was isolated, and cyclin D1, SM22α, SMα-actin, PCNA, and GAPDH expression levels were measured via immunoblotting. D, MASMCs were transfected with 2 independent siRNAs targeting TMEM16A. 24 hours after transfection, cyclin D1, SM22α, SMα-actin, PCNA, TMEM16A, and GAPDH protein levels were determined via immunoblotting. E, HASMCs were infected with Ad-GFP or Ad-GFP-TMEM16A for the indicated periods of time. SM22α, SMα-actin, and cyclin D1 mRNA levels were assessed using qRT-PCR, and cell proliferation was tested using the bromodeoxyuridine (BrdU) incorporation assay. Error bars represent the SD of 3 independent experiments. *P<0.05 vs the group infected with Ad-GFP. F, Hematoxylin and eosin (HE) staining of representative sections of the femoral arteries of mice 14 days after wire injury, from mice transduced with Ad-GFP (n=4) or Ad-GFP-TMEM16A (n=6). White arrowheads, internal elastic lamina; Black arrowheads, external elastic lamina, defining the borders of the intima and media (left panel). Quantification of cross-sectional neointimal area and media/total vessel area ratio (right panel). Data are presented as mean±SD. *P<0.05 vs the group infected with Ad-GFP.
of the thoracic aorta, and KLF5 deficiency ablated the stimulatory effect of Ang II on cell proliferation (Figure 6B and Figure S2). Moreover, Ang II infusion resulted in a marked decrease in TMEM16A expression in the thoracic aorta of WT mice, and this inhibitory effect was blocked by KLF5 deficiency (Figure 6C). By contrast, KLF5 expression was strongly induced by Ang II infusion in the thoracic aorta of WT mice and was undetected in KLF5−/− mice (Figure 6D).

Overexpression of KLF5 significantly increased cyclin D1 and PCNA expression and decreased SM22α and SMα-actin expression in HASMCs. However, the effects of KLF5 overexpression were completely abrogated by TMEM16A overexpression (Figure 6E). These data suggest that Ang II inhibits TMEM16A expression via KLF5 and that TMEM16A reduction is responsible for Ang II–induced vascular remodeling.

**Ang II Inhibits TMEM16A Expression by Promoting the Binding of KLF5 to Myocardin**

As shown in Figure 7A, KLF5 overexpression dose-dependently inhibited TMEM16A promoter activity. Progressive deletion of the TMEM16A promoter sequence from −2000 to −536 significantly suppressed activation of the TMEM16A promoter by KLF5, whereas truncating the TMEM16A promoter from −2000 to −349 abolished the inhibitory effect of KLF5 on the promoter (Figure 7B), suggesting that the region between −536 and −349 is necessary for KLF5 function. These results are consistent with the location of an SRF-responsive region within this promoter. Further, cotransfection of KLF5 with SRF and myocardin significantly inhibited the activation of the TMEM16A promoter reporter by SRF alone and the synergy between SRF and myocardin (Figure 7C, left panel).

When the TGF-β control element box was mutated, KLF5 could still decrease the activation of the TMEM16A promoter (−536 to +1) by SRF alone and the synergy between SRF and myocardin (Figure 7C, middle panel). By contrast, following CArG box mutation, neither transfection of SRF alone nor cotransfection of SRF and myocardin, regardless of the presence or absence of KLF5, could activate the TMEM16A promoter (−536 to +1; Figure 7C, right panel). These data strongly suggest that the CArG box contained in the −536 to −349 region is required for the activation of the TMEM16A promoter by SRF alone and the synergy between myocardin and SRF and that the repression of the promoter activity by KLF5 also depends on this CArG box. In addition, a coimmunoprecipitation assay showed that Ang II time-dependently promoted the interaction of KLF5 with myocardin and disrupted the association of myocardin with SRF (Figure 7D and 7E). These studies indicate that Ang II–induced KLF5 competes with SRF to interact with myocardin, thereby limiting myocardin binding to SRF and synergistic activation of the TMEM16A promoter by myocardin and SRF.

**Discussion**

Sequence analysis of the human TMEM16A promoter region spanning from −2000 to +1 indicated that there are several potential binding sites for transcription factors, including SRF, Sp1, KLF, and NF-κB. We then performed a luciferase assay and found that the overexpression of SRF, but not KLF4, Sp1, and NF-κB, increased TMEM16A promoter activity (Figure 1A and data not shown). Myocardin, known
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as a coactivator of SRF, also concentration-dependently increased TMEM16A promoter activity (Figure 1A, middle panel) and dramatically increased the activation of this reporter by SRF (Figure 1C, left panel). Because myocardin is known to be a key transcription factor in SMC differentiation, discovering that myocardin upregulated TMEM16A expression prompted us to test whether TMEM16A affected VSMC proliferation and differentiation. We found that TMEM16A markedly increased the expression of the VSMC differentiation markers SM22α and SMα-actin and decreased PCNA and cyclin D1 levels and cell proliferation (Figure 2B–2E). Moreover, an in vivo animal experiment showed that TMEM16A largely attenuated the neointimal area and medial proliferative cell number after vascular injury (Figure 2F and Figure S1). These results suggest that the overexpression of TMEM16A can inhibit VSMC proliferation in vivo, thus leading to reduced neointima formation after vascular injury. Indeed, Cl− channels have been found to play a key role in the regulation of cell cycle progression and cell proliferation in a variety of cell types. In particular, a recent study showed that the TMEM16A CaCC acts as a negative regulator of cell proliferation by arresting the cell cycle at the G0/G1 phase via a reduction in cyclin D1 and cyclin E expression. However, how TMEM16A regulates VSMC

Figure 4. Angiotensin (Ang) II inhibits TMEM16A expression. A and B, Human aortic smooth muscle cells (HASMCs) were incubated in serum-free medium for 24 hours, followed by treatment with Ang II at the indicated doses for the indicated times. A, TMEM16A, TMEM16J, Kßul-plike factor 5 (KLF5), cyclin D1, NOX-1, and GAPDH protein expression levels were measured via immunoblotting. B, The relative expression of TMEM16A mRNA was examined using qRT-PCR and presented after normalizing to GAPDH (mean±SD; n=3). *P<0.05 vs the untreated group. C, Mouse aortic smooth muscle cells (MASMCs) or human cardiac microvascular endothelial cells (HCMECs) were incubated in serum-free medium for 24 hours, followed by treatment with Ang II for the indicated times. TMEM16A and GAPDH protein expression levels were measured via immunoblotting. D, Lysates were prepared from the thoracic aortic tissues of wild-type (WT) mice and human angiotensinogen transgenic (hAGT-Tg) mice (4 mice per group) and immunoblotted using the indicated antibodies. E, HASMCs were infected with Ad-GFP or Ad-GFP-TMEM16A for 24 hours and then exposed to 0 mol/L or 2×10−7 mol/L Ang II for another 24 hours. The expression levels of cyclin D1, SM22α, SMα-actin, proliferating cell nuclear antigen (PCNA), GAPDH, and GFP were measured via immunoblotting (left panel), and cell proliferation was tested using the BrdU incorporation assay (right panel). *P<0.05 vs the group infected with Ad-GFP alone. #P<0.05 vs the group treated with Ad-GFP and Ang II.
differentiation and proliferation remains unknown. We speculated that TMEM16A might regulate the expression of some transcription factors responsible for VSMC differentiation and proliferation. Interestingly, TMEM16A specifically induced the expression of myocardin in HASMCs (Figure 3A–3C). Thus, we concluded that myocardin and TMEM16A form a positive feedback loop that induces VSMC differentiation and inhibits VSMC proliferation (Figure 3D). Further studies are required to determine how TMEM16A regulates myocardin expression.

Consistent with the results of a previous study that showed Ang II inhibits TMEM16A expression in BASMCs, our study demonstrated that Ang II also decreased TMEM16A protein and mRNA expression in HASMCs (Figure 4). These findings demonstrate that Ang II downregulates TMEM16A expression and disrupts the positive feedback loop between TMEM16A and myocardin, subsequently leading to cell proliferation.

KLF5 belongs to the KLF family and plays a key role in VSMC proliferation by regulating the expression of many types of genes involved in cell proliferation and cardiovascular remodeling. Importantly, KLF5 is a target for Ang II signaling and acts as an essential regulator of cardiovascular remodeling induced by Ang II. In this study, in vitro and in vivo experiments showed that KLF5 mediated Ang II–induced suppression of TMEM16A expression (Figures 5 and 6). To further understand the mechanism by which KLF5 mediates Ang II–induced suppression of TMEM16A expression, we performed a luciferase assay. We found that KLF5 significantly inhibited the activation of the TMEM16A promoter.
by the synergy between SRF and myocardin through the CArG box located in the region between −536 and −349 of the TMEM16A promoter (Figure 7A–7C). We proposed that Ang II might trigger the displacement of myocardin from SRF by promoting the interaction of KLF5 with myocardin. As expected, coimmunoprecipitation assays showed that Ang II time-dependently increased the interaction of KLF5 with myocardin and decreased the interaction of myocardin with SRF (Figure 7D and 7E). These studies indicate that Ang II–induced KLF5 competes with SRF to interact with myocardin, thereby limiting myocardin binding to SRF and the synergistic activation of the TMEM16A promoter by myocardin and SRF.

Taken together, our studies show that myocardin promotes TMEM16A expression by forming a complex with SRF bound to the CArG box of the TMEM16A promoter in HASMCs and, in turn, upregulated TMEM16A promotes expression of myocardin and VSMC marker genes, thus forming a positive feedback loop that induces cell differentiation and inhibits cell proliferation. Ang II induces KLF5 expression and facilitates KLF5 association with myocardin to disrupt the myocardin–SRF complex, subsequently leading to the inhibition of TMEM16A transcription and the interruption of this positive feedback loop and ultimately influencing cellular proliferation (Figure 7F). For the first time, our data provide insight into the molecular mechanism by which TMEM16A participates in Ang II–mediated VSMC proliferation and vascular remodeling.

**Perspective**

In these studies, we demonstrated that TMEM16A specifically induces the expression of myocardin and that myocardin

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**Figure 6.** Krüppel-like factor 5 (KLF5) mediates angiotensin (Ang) II–induced suppression of TMEM16A expression in vivo. A, Hematoxylin and eosin (HE) staining and medial thickness of representative sections of thoracic aorta from WT mice, WT mice with Ang II infusion for 28 days, or KLF5 null (KLF5−/−) mice with Ang II infusion for 28 days. B, nuclei density (normalized to an aortic section with a length of 100 μm) was reduced significantly in Ang II–treated KLF5−/− mice compared with Ang II–treated WT mice. C, Immunostaining and qRT-PCR for TMEM16A in the thoracic aorta of different groups of mice as indicated. D, Immunostaining and qRT-PCR for KLF5 in the thoracic aorta of different groups of mice as indicated. *P<0.05 vs the WT Con group; #P<0.05 vs Ang II–treated WT controls (n=5 in each group). The data represent the mean±SD. E, Human aortic smooth muscle cells (HASMCs) were infected with Ad-GFP-TMEM16A and Ad-GFP-KLF5. Protein was isolated, and cyclin D1, SM22α, SMα-actin, proliferating cell nuclear antigen (PCNA), and GAPDH expression levels were measured via immunoblotting.
forms a positive feedback loop with TMEM16A to induce VSMC differentiation and inhibit VSMC proliferation. Ang II downregulates TMEM16A expression and disrupts this positive feedback loop via a KLF5–myocardin–SRF pathway, subsequently leading to inhibition of TMEM16A transcription and vascular smooth muscle cells (VSMC) proliferation.

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Disclosures
None.

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What Is Relevant?

These studies unveil a potential target for the treatment and prevention of diseases involving vascular remodeling.
TMEM16A and Myocardin Form a Positive Feedback Loop That Is Disrupted by KLF5 During Ang II–Induced Vascular Remodeling
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TMEM16A and myocardin form a positive feedback loop that is disrupted by KLF5 during Ang II-induced vascular remodeling

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Short title: ANO1 mediates Ang II-induced vascular remodeling

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SUPPLEMENTAL MATERIAL

Expanded Methods

Animal Models

All animals were housed and handled according to the guidelines of the local Animal Care and Use Committee at Hebei Medical University. Eight- to -10-week-old male C57BL/6 mice were anesthetized with 1.5% isoflurane. To reproducibly induce vascular remodeling, we performed femoral artery wire injury as previously described. Briefly, we carefully separated the left femoral artery and the accompanying femoral nerve under anesthesia. A small branch of the femoral artery was isolated under the muscles. The femoral artery and the small branch were looped with 6-0 silk sutures to temporarily stop blood flow during the procedure. A spring wire (0.38-mm diameter; Cook Inc., Bloomington, IN) was inserted into the femoral artery more than 5 mm and moved in and out twice. The wire was then removed, and blood flow in the femoral artery and branch was restored by releasing the sutures, and the skin incision was closed with a 5-0 silk suture. For the TMEM16A-overexpression model, femoral artery wire injury was performed as described above. Immediately after injury, the femoral artery was cannulated, and the boclamped segment was incubated with 20 μl of adenovirus (1.5x10⁹ pfu/ml) encoding TMEM16A (n=6) or GFP (n=4) for 15 minutes. For the bromodeoxyuridine (BrdU) incorporation assay, femoral artery wire injury and adenovirus transduction were performed as described above. Alzet osmotic minipumps (model 2002, Durect Corp) were placed subcutaneously via a midback incision and loaded with BrdU (Sigma, catalog no. B5002) to deliver 25 mg/kg/d (n=5 per group). After 14 days, all animals were anesthetized and perfused with cold 0.9% NaCl, and the femoral arteries were harvested for analysis of hematoxylin eosin (HE) or BrdU staining.

Vascular smooth muscle cell (VSMC)-specific KLF5 knockout (VSMC KLF5−/−) mice were derived from breedings of floxed KLF5 mice, generously provided by Dr Huajing Wan, with heterozygous Tgln-cre mice (Jackson Laboratory, Bar Harbor, ME, USA). Genotyping was performed by PCR. At 8 to 10 weeks of age, wild-type (WT) mice or VSMC KLF5−/− mice were surgically implanted sc under isoflurane anaesthesia with Alzet osmotic mini-pumps (Model 2004, Durect Corp) infusing Ang II (1,000 ng/kg/min, Sigma, catalog no. A9525). After 28 days, all animals were anesthetized and perfused with cold 0.9% NaCl, and the thoracic aorta tissues were harvested for analysis of RNA, morphology, and histolog.

The human angiotensinogen transgenic (hAGT-Tg) mice were obtained from the Institute of Laboratory Animal Sciences (CAMS&PUMC, Beijing, China). Genotyping was performed by PCR. WT C57BL/6 mice and hAGT-Tg C57BL/6 mice (4 mice per group) were anesthetized and perfused with cold 0.9% NaCl, and the thoracic aorta was collected for protein isolation and Western blot analysis.

Morphology analysis

Mice were euthanized, perfused and then fixed with 4% paraformaldehyde in PBS for 3 min through the left ventricle under physiological pressure. The thoracic aorta and femoral arteries were harvested and embedded in paraffin. Ten consecutive 4-μm-thick sections were prepared for HE staining. Images were acquired using a Leica microscope (Leica DM6000B,
Measurement of the neointimal area, media/total vessel area ratio, medial thickness and nuclear density was performed in a blind manner. For each section, four random, noncontiguous microscopic fields were examined.

**Immunostaining**

Paraffin cross-sections (4-μm thick) from thoracic aorta and femoral arteries were deparaffinized with xylene and rehydrated in a graded ethanol series and endogenous peroxidase activity was inhibited by incubation with 3% H$_2$O$_2$. Sections were blocked with 5% goat serum in phosphate-buffered saline (PBS) and incubated overnight at 4°C with primary antibodies. After a PBS wash, the sections were incubated with secondary antibody at 37°C for 30 min. Immunohistochemical staining was visualized by use of a dianaminobenzidine kit (Zhongshan Goldenbridge Biotechnology, Beijing, China) according to the manufacturer’s instructions. Sections were counterstained with hematoxylin to visualize nucleus. The primary antibodies included anti-TMEM16A Ab (1:100 dilution, ab53212, Abcam) and anti-KLF5 Ab (1:200 dilution, GTX103289, GeneTex). BrdU immunohistochemistry was performed as above, except that sections were first pre-treated with 1 M HCl and 0.1% trypsin (each 30 min; 37°C) before blocking, and the anti-BrdU primary antibody (1:100 dilution, B8434, Sigma) was incubated for 1 h. Images were acquired using a Leica microscope (Leica DM6000B, LAS V.4.3, Switzerland). BrdU incorporation was recorded by blinded, manual count of immunoreactive nuclei.

Immunofluorescence staining was performed with 4 μm paraffin cross-sections from the thoracic aorta. After deparaffinized with xylene and rehydrated, the slides were pre-incubated with 10% normal goat serum and then incubated with primary antibodies anti-SM22α (ab14106, abcam) and anti-Ki-67 (ab6526, abcam). Secondary antibodies were fluorescein-labeled antibody to rabbit IgG (021516, KPL, USA) and rhodamine-labeled antibody to mouse IgG (031806, KPL, USA). In each experiment, DAPI (157574, MB biomedical) was used for nuclear counterstaining. Images were captured by confocal microscopy (DM6000 CFS, Leica) and processed by LAS AF software.

**Cell Culture and Treatment**

Human aortic smooth muscle cells (HASMCs) (ScienCell, no. 6110) were grown in Smooth Muscle Cell Medium containing apo-transferrin, insulin, fibroblast growth factor-2, insulin-like growth factor-1, hydrocortisone, and 2% foetal bovine serum (FBS) (ScienCell, no. 1101). Mouse aortic smooth muscle cells (MASMCs) were isolated from thoracic aorta of WT C57BL/6 mice. The aortas were carefully dissected to remove excess connective tissue, then minced and digested in type III porcine pancreatic elastase (Sigma, no. E1250), type I collagenase (Sigma, no. C0130), and soybean trypsin inhibitor (Sigma, no. T6522) at 37°C for 1 hour. The cells were plated in DMEM containing 10% FBS. Cells were maintained in 5% CO$_2$ at 37°C within a humidified atmosphere and determined to be SMCs by morphology and expression of SM α-actin. Human cardiac microvascular endothelial cells (HCMECs) were obtained from Sciencell (no. 6000) and maintained in Endothelial Cell Medium (ScienCell, no. 1001). Prior to stimulation with Ang II, cells were incubated in serum-free medium for 24 h. Human embryonic kidney 293A cells were obtained from ATCC (Manassas, VA) and maintained in high glucose DMEM supplemented with 10% FBS.
**Adenovirus Expression Vector and Plasmid Constructs**

The expression plasmids of KLF4 and KLF5 were created by the placement of human KLF4 and KLF5 cDNAs into the pEGFP-C2 vector. The full-length human SRF and myocardin cDNAs were cloned into the pcDNA3.1 vector. Expression plasmid for human TMEM16A (pcDNA3.1-TMEM16A) was a generous gift from Dr. Rainer Schreiber. The 5' regulatory region of human TMEM16A (-2000 to +1 bp) was amplified by PCR and cloned into the pGL3-Basic vector (Promega) in order to generate the TMEM16A promoter-reporter pGL3-TMEM16A-luc. Truncated TMEM16A luciferase reporters were generated by cloning the -1541, -1171, -805, -536, and -349 to +1 regions of the TMEM16A promoter into pGL3. The TCE (CACCC) and CArG (GGCACTTTCC) box in the -536 to -349 region of the TMEM16A promoter was mutated by site-directed mutagenesis using the QuikChangeTM site-directed mutagenesis kit (Agilent Technologies-Stratagene, La Jolla, CA) according to the manufacturer’s instructions. Adenoviruses encoding TMEM16A (Ad-GFP-TMEM16A), KLF4 (Ad-GFP-KLF4), KLF5 (Ad-GFP-KLF5) and GFP control (Ad-GFP) were entrusted to Invitrogen.

**Electrophysiology**

Whole-cell patch-clamp recording was performed at room temperature on HASMCs infected with Ad-GFP or Ad-GFP-TMEM16A with Axon 700B and pClamp 10.2 (Axon Instruments and Molecular Device, USA). Data were acquired at 2 kHz and low-pass filtered at 3 kHz. The external solution contained the following (in mM): 140 N-methyl-d-glucamine (NMDG), 1 CaCl_2, 1 MgCl_2, 10 glucose, 10 HEPES and pH 7.4 adjusted with HCl. The internal solution contained the following (in mM): 130 CsCl, 10 EGTA, 1 MgCl_2, 10 HEPES and 8 mM CaCl_2 to obtain about 500 nM free Ca^{2+}. The pH of internal solution was adjusted with CsOH and ATP was added on the day of recording. Patch pipettes were pulled from thick-walled borosilicate glass capillaries with a horizontal micropipette puller (P-97, Sutter Instruments, USA) and fire polished. The recording pipettes had open-tip resistances of 3-5 MΩ when filled with internal solution. The cell was holding at -50 mV. The currents was elicited from a holding potential of 0 mV followed by voltage steps from -100 to +100 mV in 20 mV step for 750 ms. Membrane current densities were obtained by current amplitude dividing the cell capacitance.

**RNA Preparation and Quantitative Real-time PCR**

Total RNA was extracted by TRizol (Invitrogen) and 1 μg of RNA was subjected to reverse transcription using first-strand cDNA synthesis kit (Invitrogen) according to the manufacturer’s instructions. Real-time PCR analysis was done with the ABI 7500 FAST system, using the Platinum SYBR Green qPCR SuperMix UDG Kit (Invitrogen), according to the manufacturer’s instructions. As an internal control, GAPDH primers were used for RNA template normalization. All PCRs were performed in triplicate. The relative expression level was calculated using the following equation: relative gene expression = 2^{(ΔC_{control} - ΔC_{sample})}. The primer sequences were as follows: human GAPDH, 5'-AAGGTTAAGCTCGAGTAC-3' and 5'-GATTTTGGAGGATATCTCG-3'; human KLF4, 5'-CCACACCTACTGCTTCT-3' and 5'-CGTCCACGTCCAGTGAATT-3'; human
KLF5: 5'-AAACGACGCATCCACTACTGC-3' and 5'-TTGTATGGCTTTCACCAGTGTG-3'; human TMEM16A: 5'-TCCAAGGACTTCTGGGCGGCT-3' and 5'-TAGGCAATGGCTGGGCTMGG-3'; human SM α-actin: 5'-GCGTGGCTATTCCCTTTCACTGA-3' and 5'-ATGAAGGATGGCTGGAACAG-3'; human SM22α: 5'-TCCAAGGACTTCTGGGCGGCT-3' and 5'-TAGGCAATGGCTGGGCTMGG-3'; human cyclin D1: 5'-CCCTCGGTGTCTCCTACTC-3' and 5'-CCCTCGGTGTCTCCTACTC-3'; human myocardin: 5'-CTCGGCTTCCTTTGAACAAG-3' and 5'-CTCGGCTTCCTTTGAACAAG-3'; human SRF: 5'-CTTCCTCTTCTCCCACAAAG-3' and 5'-CTTCCTCTTCTCCCACAAAG-3'; human GAPDH: 5'-CGTCCCGTAGACAAAATGGT-3' and 5'-GAGGTCAATGAAGGGGTCG-3'; human TMEM16A: 5'-ATGAAGGATGGCTGGAACAG-3' and 5'-ATGAAGGATGGCTGGAACAG-3'; human KLF5: 5'-ACCAGACCGCGACGAATTGGACAC-3' and 5'-ATTTAGCGCGACGACGACGAG-3'.

**Immunoblotting**

Proteins were isolated from cells as previously described,\(^4\) electrophoresed on 10% SDS-PAGE gels and transferred onto PVDF membranes. Membranes were blocked with 5% dry milk in TTBS for 2 h at 37°C and incubated overnight at 4°C with the following primary antibodies: 1:1000 rabbit anti-TMEM16A (ab53212, Abcam), 1:1000 rabbit anti-myocardin (ab107301, Abcam), 1:1000 rabbit anti-SRF (Cell Signaling Technology, 5147), 1:1000 rabbit anti-KLF5 (GeneTex, GTX103289), 1:1000 rabbit anti-KLF4 (ab151733, Abcam), 1:1000 rabbit anti-TMEM16J (ab140087, Abcam), 1:1000 rabbit anti-SM22α (ab155272, Abcam), 1:1000 rabbit anti-α-actin (ab5694, Abcam), 1:10000 rabbit anti-cyclin D1 (AJ1209b, ABGENT), 1:5000 rabbit anti-NOX1 (NPB1-31546, NOVUS BIOLOGICALS), 1:1000 rabbit anti-p47phox (Cell Signaling Technology, 4312), 1:10000 rabbit anti-PCNA (ab92552, Abcam), 1:10000 rabbit anti-GAPDH (ab181602, Abcam), and 1:50000 rabbit anti-GFP (ab183734, Abcam). Following incubation with the appropriate secondary antibody, antibody-antigen complexes were imaged using the Chemiluminescence Plus Western immunoblot analysis kit (MILLIPORE).

**Co-immunoprecipitation Assay**

Co-immunoprecipitation was performed as previously described.\(^5\) In brief, cell extracts were precleared with 25 μl of protein A-agarose (50% v/v). The supernatants were immunoprecipitated with 2 μg of anti-KLF5, anti-myocardin, or anti-SRF antibodies for 1 h at 4°C, followed by incubation with protein A-agarose overnight at 4°C. Protein A-agarose-antigen-antibody complexes were collected using centrifugation at 12,000 rpm for 60 s at 4°C. The pellets were washed five times with 1 ml of IPH buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 0.1 mM phenylmethylsulfonyl fluoride) for 20 min at 4°C. Bound proteins were resolved using SDS-PAGE, followed by Western immunoblotting with anti-myocardin, anti-KLF5, or anti-SRF antibodies.

**Small Interfering RNA Transfection**
Small interfering RNAs (siRNAs) targeting human TMEM16A (siTMEM16A) and KLF5 (siKLF5) were designed and synthesized by GenePharma (Shanghai, China). The siRNA sequences were as follows: KLF5 siRNA, 5’-CUCCAGAGGUGAACAUAUTT-3’ and 5’-AUAUUGUCACCUCUGAGTT-3’; TMEM16A siRNA, 5’-GAGCCAAAGACAAUCGGAAUTT-3’ and 5’-AUUCCGAUGUCUUUUGGCUCTT-3’; 5’-GGGACUACAACGGUGAAATT-3’ and 5’-UUUCACCGUUGAGUAGUCCTT-3’. Non-specific siRNA (siControl) and siRNA specific for human myocardin (siMyoc) were purchased from Santa Cruz Biotechnology. Transfection was performed using Lipofectamine reagent (Invitrogen) following the manufacturer’s instructions. Twenty-hours following transfection, HASMCs were treated with Ang II. Cells were then harvested and lysed for western immunoblotting.

**Luciferase Assay for TMEM16A Promoter Activity**

Human embryonic kidney 293A cells were maintained as previously described. 6 3×10^4 cells were seeded into each well of a 24-well plate and grown for 24 h prior to transfection with reporter plasmids and the control pTK-RL plasmid. Cells were transfected using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions. Luciferase assays were performed after 24 h using a dual luciferase assay kit (Promega). Specific promoter activity was expressed as the relative ratio of firefly luciferase activity to Renilla luciferase activity. All promoter constructs were evaluated in a minimum of three separate wells per experiment.

**Cell Proliferation Assay**

HASMC proliferation assays were performed with the BrdU Cell Proliferation Assay kit (Millipore) according to the manufacturer’s recommendations. Cells were labeled for 6 h and OD readings were performed at 450 nm. All groups were evaluated in a minimum of four separate wells per experiment.

**Statistical Analysis**

Data are presented as bar graphs (means ± SD) of at least three independent experiments. Statistical analyses were performed using Wilcoxon (two samples) or Kruskal Wallis H test (multiple samples). The results were considered statistically significant at $p < 0.05$.

**References**

4. Zhang XH, Zheng B, Gu C, Fu JR, Wen JK. TGF-β1 downregulates AT1 receptor


Supplemental Figures

Figure S1

**Figure S1.** TMEM16A inhibits VSMC proliferation. A, Representative staining of bromodeoxyuridine (BrdU) of mouse femoral arteries 14 days after wire injury, from mice transduced with Ad-GFP (n=5) or Ad-GFP-TMEM16A (n=5). White arrowheads: internal elastic lamina; Black arrowheads: external elastic lamina, defining the borders of the intima and media. B, BrdU index was calculated as percentage of the ratio of BrdU-stained nuclei to the total number of cells in the media. Absolute BrdU positive cells of each group were also compared. *P<0.05 vs Ad-GFP.
Figure S2. KLF5 deficiency results in decreased VSMC proliferation induced by Ang II in the media. Immunofluorescence (IF) staining for DAPI, Ki-67, or SM22α on sections of thoracic aorta from WT mice, WT mice infused with Ang II for 28 days, or KLF5 null (KLF5−/−) mice infused with Ang II for 28 days. The ratio of proliferating VSMCs to the total cells in the media was determined by dividing the number of Ki-67 positive cells per section by the total cell number per section (n=5 per group). *P<0.05 vs WT Con, #P<0.05 vs WT+Ang II. M = media; L = lumen.
Figure S3. Full-width of membrane with a protein marker besides the each box. The molecular size is shown as indicated. A, Panels are the same as those in Figure 1D. B, Panels are the same as those in Figure 2B. C, Panels are the same as those in Figure 3B.
**Figure S4.** Full-width of original blots for Figures 1-3. Panels indicated in this supplemental figure correspond to those in the main article.
Figure S5. Full-width of original blots for Figure 4. Panels indicated in this supplemental figure correspond to those in the main article.
Figure S6. Full-width of original blots for Figures 5-7. Panels indicated in this supplemental figure correspond to those in the main article.