ORIGINAL ARTICLE

TMEM16A inhibits angiotensin II-induced basilar artery smooth muscle cell migration in a WNK1-dependent manner

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Abstract   Vascular smooth muscle cell (VSMC) migration plays a critical role in the pathogenesis of many cardiovascular diseases. We recently showed that TMEM16A is involved in hypertension-induced cerebrovascular remodeling. However, it is unclear whether this effect is related to the regulation of VSMC migration. Here, we investigated whether and how TMEM16A contributes to migration in basilar artery smooth muscle cells (BASMCs). We observed that AngII increased the migration of cultured BASMCs, which was markedly inhibited by overexpression of TMEM16A. TMEM16A overexpression inhibited AngII-induced RhoA/ROCK2 activation, and myosin light chain phosphatase (MLCP) and myosin light chain (MLC20) phosphorylation. But AngII-induced myosin light chain kinase (MLCK) activation was not affected by TMEM16A. Furthermore, a suppressed activation of integrinβ3/FAK pathway, determined by reduced integrinβ3 expression, FAK phosphorylation and F-actin rearrangement, was observed in TMEM16A-overexpressing BASMCs upon AngII stimulation. Contrary to the results of TMEM16A overexpression, silencing of TMEM16A showed the opposite effects. These *in vitro* results were further demonstrated *in vivo* in basilar arteries from VSMC-specific TMEM16A transgenic mice during AngII-induced hypertension. Moreover, we observed that the inhibitory effect of TMEM16A

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on BASMC migration was mediated by decreasing the activation of WNK1, a Cl⁻-sensitive serine/threonine kinase. In conclusion, this study demonstrated that TMEM16A suppressed AngII-induced BASMC migration, thus contributing to the protection against cerebrovascular remodeling during AngII-infused hypertension. TMEM16A may exert this effect by suppressing the RhoA/ROCK2/MLCP/MLC20 and integrinβ3/FAK signaling pathways *via* inhibiting WNK1. Our results suggest that TMEM16A may serve as a novel therapeutic target for VSMC migration-related diseases, such as vascular remodeling.

1. Introduction

Vascular smooth muscle cell (VSMC) migration plays a critical role in the pathogenesis of many cardiovascular diseases and its clinical manifestations such as vascular remodeling during hypertension, neointima formation after vascular injury, and atherosclerotic plague progression[1,2]. Generally, SMC migration is a polarized cellular process involving a protrusive cell front and a retracting trailing rear, which can be simply described as the following two key steps: (1) Lamellipodia and focal adhesion are formed in the front of cell; (2) Increase in actomyosin activity leads to retraction of the cell rear[3]. By the cycles of these steps, the motile cells are allowed to crawl on the extracellular matrix (ECM) and move forward. In this process, the importance of integrin in focal adhesions formation and myosin light chain (MLC) phosphorylation in actomyosin activation has been intensively highlighted[1,3]. Inhibition of integrin expression and myosin activation is associated with reduced neointima formation *in vivo*[4]. Improvement in understanding the signal regulation mechanism of VSMC migration will be very attractive to establish new therapeutic strategies to control VSMC migration-related cardiovascular diseases.

TMEM16A (also known as Ano 1), the first member of the ten-member family of "transmembrane protein 16" (including TMEM16A−H, TMEM16J, and TMEM16K), was identified as a Ca^{2+}-activated chloride channel (CaCC) in 2008[5−7]. During the past decade, TMEM16A has been demonstrated to be associated with a wide variety of physiological and pathological functions. Especially, the pivotal roles of TMEM16A in numerous types of cancers are extensively determined. It has been reported that TMEM16A is highly expressed and promotes cancer cell migration in many human tumors including breast cancer, lung cancer, prostate cancer and gastric cancer[8−10]. Modulation of TMEM16A expression has been shown to be a promising novel strategy for cancer therapy by controlling cancer cell migration and invasion[8−10]. However, contrary to the extensive research into the effects of TMEM16A on cancer cells, data on its involvement in the migration of non-tumor cells are rarely reported.

Recently, we showed that TMEM16A expression in the basilar artery (BA) is significantly decreased in both 2-kidney 2-clip and angiotensin II (AngII)-induced hypertensive rodents, and this is negatively correlated with hypertension-induced cerebrovascular remodeling[11,12]. Conversely, overexpression of TMEM16A significantly protects the basilar artery against remodeling following AngII infusion[12]. Given that VSMC migration is one of the important determinants of vascular remodeling, we assumed that TMEM16A may have an inhibitory effect on migration in basilar artery smooth muscle cell (BASMC), unlike its effect observed in cancer cells. Thereby, this study aims to explore the functional significance of TMEM16A to BASMCs migration, the underlying signal mechanisms and their relation to basilar artery remodeling in AngII-induced hypertension.

2. Materials and methods

2.1. Reagents

Antibodies to MLC20 (ab76092), p-MLC20 (ab79935), MLCK (ab76092), p-MLCK (ab200809), TMEM16A (ab53213) were from Abcam Biotechnology Inc (Abcam, Cambridge, UK). Antibodies to integrin β1 (sc-374429), FAK (sc-558), p-FAK (sc-11766), ROCK1 (sc-17794), ROCK2 (sc-100425) and α-tubulin (sc-5286) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies to p-ROCK1 (C00666H, SAB) were from Signalway Antibody (SAB, China). Antibodies to p-ROCK2 (GTX122651) were from GeneTex Biotechnology (GeneTex, USA). Antibodies to p-MYPT1 (04−773) and RhoA (05−778) were from Millipore (Millipore Biotechnology Inc., USA). Antibody to β-actin (BM0627) was from Boster biological technology (Boster, Nanjing, China). Antibody to GAPDH (822051) was from Vazyme Biotechnology (Vazyme, Nanjing, China). Antibodies to integrin β3 (4702 S), MYPT1 (2634 S) and secondary antibodies for WB (anti-mouse IgG: 7076 S; anti-rabbit IgG: 7074 S) were from CST (Cell Signaling Technology, USA). Cy3-conjugated secondary antibodies for IF (anti-mouse IgG: A0521; anti-rabbit IgG: A0516) were from Beyotime Biotechnology (Beyotime, Nanjing, China). Dulbecco's modified Eagle's medium (DMEM)/F-12 and fetal bovine serum (FBS) were obtained from GIBCO (Life Tech, Grand Island, NY, USA). Angiotensin II (AngII) and DAPI were obtained from Sigma−Aldrich (Merck, Germany). Other chemicals, if not indicated, were all from Sigma−Aldrich.

2.2. Vascular smooth muscle-specific TMEM16A transgenic mice

The vascular smooth muscle-specific TMEM16A transgenic mice (SM^Tg) were produced by Cyagen (Cyagen Biosciences Inc., Suzhou, CN) as previously reported[12] and bred in house. SM^Tg and its corresponding control mice (SM^Cre) were genotyped by PCR on tail DNA using specific primers as previously described[12]. The genetic background of all the mice used in this study was C57BL/6 J.

2.3. Animal model

All animal experiment procedures were performed in accordance with the policies of the Sun Yat-sen University Animal Care and Use Committee, and conformed to the "Guide for the Care and Use of Laboratory Animals" of the National Institute of Health (NIH Publications No. 8023, revised 1978). Animal studies were reported in compliance with the ARRIVE guidelines. All animals were maintained with standard food and water in pathogen-free, humidity- and temperature-controlled room in the animal research facility at Sun Yat-sen University with a 12-h light/dark cycle.

The AngII-induced hypertensive model was created as we previously described[12,13]. Briefly, male TMEM16A over-expression mice and their littermate controls at 8−10 weeks were randomly divided into two groups (AngII treatment or not). The animal number used in the experiments are shown in the corresponding figure legends (5 in each group). After anesthetized with intraperitoneal injection of 60 mg/mL pentobarbital sodium, an Alzet osmotic pump (model 1004, Alzet Durect Corp., Cupertino, CA, USA) filled with AngII in saline solution was implanted subcutaneously on the back of the mouse. AngII was continuously delivered at a dose of 1.5 mg/kg per day for 4 weeks. Control mice were implanted with osmotic pump by infusion of saline solution only. The animals recovered within 2 h after the operation, then were moved to a clean cage and fed a standard rodent diet with water *ad libitum*. Systolic blood pressure (SBP) was measured by noninvasive, tail-cuff method (tail-cuff plethysmography, BP-98 A, Softron, Tokyo, Janpan) in conscious mice every week. The tissues were collected 4 weeks post AngII implantation, after the mice were sacrificed by overdose of pentobarbital intraperitoneally.

2.4. Tissue preparation and immunofluorescence staining

Mice were sacrificed by overdose of pentobarbital intraperitoneally and perfusion-fixed at a constant pressure (100 mmHg) *via* the left ventricle with oxygenated Krebs solution (pH 7.4, 37 °C) containing heparin (100 U/kg), followed by 4 °C fixative solution containing 4% freshly depolymerized paraformaldehyde in 0.1 mol/L phosphate buffer for 10 min. The brains were carefully removed and sections (8 μm) of freshly frozen basilar arteries were prepared.

Immunofluorescence staining was carried out as described previously[12,14]. Briefly, three frozen sections randomly selected in each mouse were blocked with blocking solution (Beyotime, China) containing 0.3% Triton X-100 for 1 h at room temperature (RT), and then incubated with primary antibody overnight at 4 °C. After that, the samples were washed 3 times in PBS and labeled with specific Cy3-conjugated secondary antibody for 1 h at room temperature. Subsequently, the samples were incubated with DAPI for 5 min to locate the nuclei. All the images were captured by Zeiss microscopy (Axio Imager Z1, Jena, Germany).

2.5. Western blotting

Tissues or cells were homogenized in RIPA lysis buffer (Beyotime, China) containing protease inhibitor cocktail (Merk, Germany). Then, the lysate was centrifuged at $12,000 \times g$ for 15 min to remove cell debris. The supernatant was collected and the protein concentration was determined using BCA protein assay kit (Pierce, Thermo Fisher Scientific, Waltham, MA, USA). Subsequently, protein was separated with SDS-PAGE and transformed to PVDF membranes. After blocked with 5% milk for 1 h, the membranes were incubated with primary antibodies at 4 °C overnight. Then they were incubated with secondary antibodies for 1 h at room temperature. Signals was detected using ECL Western blotting substrate (Pierce, Thermo Fisher Scientific, Waltham, MA, USA) and quantified with the 1-D gel image system (Bio-Red, Hercules, CA, USA).

2.6. Cell culture

The basilar artery smooth muscle cells (BASMCs) were isolated and cultured as we previously reported[11−13]. Briefly, the basilar artery was isolated from male C57BL/6 mice (8−10 weeks old) after they had been anesthetized with intraperitoneal injection of 60 mg/mL pentobarbital sodium and immersed into cold Kreb's buffer. After the connective tissue and endothelium was removed, the BA was cut into small pieces about 0.5 mm long and placed into DMEM/F-12 containing 20% FBS. Then, the tissue segments were cultured at 37 °C in a 5% CO_2 humidified incubator. Thereafter, the BASMCs migrated from the tissue pieces were passaged and confirmed by the positive response to antibody against α-smooth muscle actin (Sigma, Germany). Cells of 6−10 generations were used for the experiments after randomly divided into different treatment groups according to the individual experimental design.

2.7. Cell migration assay

For the wound-healing assay, BASMCs were incubated in a 6-well dish and a scratch lesion was created in a cross shape on confluent BASMC cultures with a 10 μL pipette tip. After stimulation with AngII for 24 h, three randomly selected fields at the lesion border were examined using living cells workstations (Zeiss, Oberkochen, Germany). The migrated distance for cells was measured from gap closure between 0 and 24 h after stimulation[13].

Boyden chamber assays were carried out in Transwell filters with 8 μm pores (Millipore Billerica, MA, USA) according to the manufacturer's instructions[13]. Cultured BASMCs were harvested using trypsin−EDTA and re-suspended in serum-free medium. A total of 600 μL serum-free medium with or without 67 nmol/L AngII was added in the lower chamber. The upper chamber was added with 5×10^4 cells in 100 μL medium. After 24 h, the membranes to which the cells migrated were fixed with methanol for 15 min and stained with crystal violet staining solution. The membranes were washed with PBS and non-migrated cells were wiped gently with cotton-swab. For every individual assay, to ensure the reliability of singe values, the migrated cells per group were repeated in duplicates and quantified by measurement of the light absorbance of crystal violet by microplate reader at 570 nm. The averaged value of the duplicates was used for analysis. Representative photographs were taken under $100 \times$ magnifications. To exclude the unwanted sources of variation between individual experiments, the data in Boyden chamber assay were normalized to control.

2.8. siRNA transfection

Gene silencing with gene-specific small interfering RNA (siRNA) was performed according to the method described previously[12]. The sequences of TMEM16A siRNA (5′-CUGCUCAAGUUU-GUGAACUTT-3′), ROCK2 siRNA (5′-GAGCAACAUGGAAAU AGAUAUGACA-3′), and WNK1 siRNA (5′-GGU-GUCGGCAAAUCCUUAATT-3′) were synthesized by Invitrogen

(Life Technologies, USA). Integrinβ3 siRNA (sc-35677) was purchased from Santa Cruz. BASMCs were transfected with siRNA in serum-free medium using Hiperfect Transfection Reagent (Qiagen, Valencia, CA, USA). After incubation for 6 h, fresh medium containing 20% FBS was added to BASMCs for another 42 h.

2.9. Adenoviral infection

TMEM16A containing adenovirus (Ad-TMEM16A) were designed and produced by Sunbio Medical Biotechnology (Shanghai, China). The cDNA of TMEM16A plasmid was a kind gift from Dr. Jan LY (University of California, San Francisco, USA). Adenovirus was infected according to the protocol previously described[12]. Briefly, BASMCs were infected with Ad-TMEM16A when the cells reached 50% confluence. Following incubation for 4 h, the medium was removed and fresh medium containing 20% FBS was added to the cells for another 44 h.

2.10. Statistical analyses

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology. Blinded data analysis was performed using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA). All data are expressed as mean $\pm$ standard error of mean (SEM), and the n value represents the number of independent experiments on different batches of cells or different mice. Statistical significance was determined by Student's t test or ANOVA followed by the Bonferroni multiple comparison test. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. AngII-induced BASMC migration was inhibited by TMEM16A

BASMC migration was tested upon AngII stimulation. As shown in Fig. 1, using the scratch wound-healing assay, AngII increased the migration of WT BASMC in a concentration-dependent manner (Fig. 1A, Supporting information Fig. S1A for the representative images). The dose−effect curve indicated an ED_{50} at 67.13 nmol/L (Fig. 1B). Thereby AngII at 67 nmol/L concentration was used for subsequent experiments. To explore the influence of TMEM16A on BASMC migration, TMEM16A siRNA or TMEM16A-expressing adenovirus was used to knockdown or overexpress TMEM16A. The expression of TMEM16A was markedly increased after transfection of BASMCs with TMEM16A adenovirus for 48 h. On the other hand, transfection with TMEM16A siRNA for 48 h reduced TMEM16A protein level (Fig. S1B and S1C). Results in Fig. 1C−F show that overexpression or knockdown of TMEM16A expression did not affect cell migration at the basal condition. However, AngII-induced BASMCs migration was significantly inhibited by TMEM16A overexpression with 100 MOI adenovirus transfection. Conversely, silence of TMEM16A by 20 nmol/L siRNA further facilitated BASMCs migration following AngII stimulation.

Transwell assays were also performed simultaneously. Here also, AngII significantly increased the migration of WT BASMCs. As in the wound-healing assays, migration was not increased by AngII in TMEM16A overexpressed BASMCs; whereas transfection

with TMEM16A siRNA further increased the migration (Fig. 1G−J). These results indicate that TMEM16A was closely related to BASMCs migration.

3.2. TMEM16A inhibits AngII-induced MLC20 phosphorylation in BASMCs

Focal adhesion formation in the cell front and contraction in the cell rear are the two key steps for cell migration[3]. To further understand the signal mechanism underlying the effects of TMEM16A on BASMCs migration, we firstly examined whether TMEM16A affects the cell contraction signals, in which myosin light chain (MLC) phosphorylation is critical to trigger the acto-myosin interaction. Fig. 2A and B shows the time course of MLC20 phosphorylation in AngII-treated BASMCs. Treatment with AngII increased MLC20 phosphorylation in cultured BASMCs, which started 0.5 min after AngII administration with a peak at 2 min and later gradually reduced to the baseline level within 16 min. Since MLC20 phosphorylation reached its maximal level after 2 min of AngII treatment, this time point was chosen for the subsequent experiments. As shown in Fig. 2C−F, consistent with the effects on BASMCs migration, phosphorylation of MLC20 in response to AngII was attenuated significantly in TMEM16A-overexpressing cells (Fig. 2C and D) and further increased in TMEM16A siRNA-treated cells (Fig. 2E and F) compared with that in non-TMEM16A transfected groups. These data suggest that inhibition of MLC20 phosphorylation might be one of the explanations for the effect of TMEM16A on BASMCs migration.

3.3. MLCP but not MLCK contributes to the inhibition of TMEM16A on MLC20 phosphorylation

The extent of MLC20 phosphorylation is determined by the balance of the activities of myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP), two key enzymes that mediate the phosphorylation and dephosphorylation of MLC, respectively[15]. In order to identify which pathway (MLCP and/or MLCK) contributes to the inhibition of TMEM16A on MLC20 phosphorylation, the protein expression and phosphorylation level of these enzymes were examined in primary cultured BASMCs by Western blot. The results show that AngII evoked an increase in phosphorylation of myosin phosphatase target subunit 1 (MYPT1), a regulatory subunit of MLCP, at both Thr696 (p-Thr696) and Thr850 (p-Thr850), but only p-Thr696 was markedly reduced by overexpression of TMEM16A (Fig. 3A and B, Supporting information Fig. S2 for p-Thr850). On the contrary, p-Thr696 of MYPT1 was further increased after knocking down the expression of TMEM16A by TMEM16A siRNA transfection (Fig. 3C and D). The total expression of MYPT1 was neither altered by AngII nor by TMEM16A gene modification. Because phosphorylation of MYPT1 will lead to a decreased activity of MLCP, these results indicate that TMEM16A might reduce MLC20 phosphorylation by inhibiting MLCP inactivation from phosphorylation of MYPT1 subunit on Thr696 but not Thr850 site.

In contrast to MLCP phosphorylation, analysis of MLCK phosphorylation revealed that its level, although was also increased by AngII, was not affected by TMEM16A overexpression (Fig. 3E and F), suggesting that MLCK might not be responsible for the regulation of MLC20 phosphorylation by
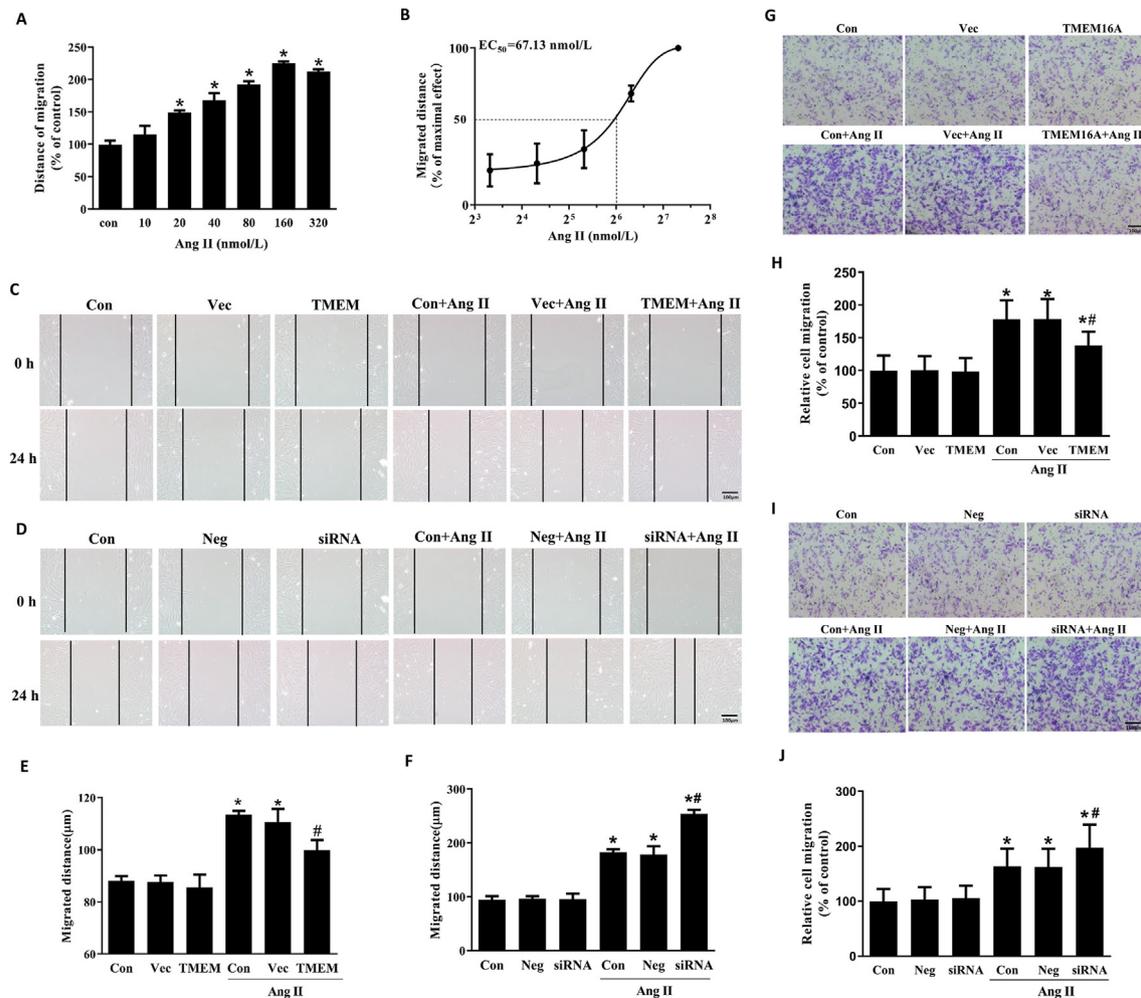
Figure 1 Effect of TMEM16A on AngII-induced migration in BASMCs. (A) Bar graph illustrating the effect of AngII on BASMCs migration by scratch test. BASMCs incubated in a 6-well dish were scratched by a pipette tip and stimulated with AngII (10, 20, 40, 80, 160, and 320 nmol/L, respectively) in serum-free medium for 24 h ($n = 5$ for each group, $*P < 0.05$ vs. Con, data were normalized to control). (B) Concentration−response curve of BASMCs migration in response to AngII. Results are expressed as percent increase in cell migration as compared to untreated control ($n = 5$). AngII-induced migration was increased in a concentration-dependent manner. The $EC_{50} = 67.13$ nmol/L. (C)−(F) Effects of TMEM16A overexpression ($n = 5$ for each group) or downregulation ($n = 6$ for each group) on AngII-induced BASMCs migration by scratch test ($*P < 0.05$ vs. Con; $^{\#}P < 0.05$ vs. AngII-treated group, one-way ANOVA). The migrated distance for cells was measured from gap closure between 0 and 24 h after stimulation. (G)−(J) Effects of TMEM16A overexpression or downregulation on AngII-induced BASMCs migration by Transwell assay ($n = 5$ for each group, $*P < 0.05$ vs. Con; $^{\#}P < 0.05$ vs. AngII-treated group, one-way ANOVA, data are normalized to control). After BASMCs were transiently transfected with Ad-TMEM16A or TMEM16A siRNA for 48 h, they underwent the scratch or Transwell assay and were stimulated with 67 nmol/L AngII for another 24 h. Values are mean ± SEM. The images were pictured under microscope (100 ×). Con (control), Vec (vector of TMEM16A), TMEM (TMEM16A overexpression), siRNA (TMEM16A siRNA).

TMEM16A. Instead, TMEM16A might reduce BASMCs migration by increasing MLCP-mediated MLC20 dephosphorylation.

3.4. RhoA/ROCK2 is required for the regulation of TMEM16A on AngII-induced BASMCs migration

It has been reported that Rho/Rho kinase plays an important role in MYPT1 phosphorylation and leads to inhibition of MLCP[16]. Thereby, the activation of RhoA, ROCK1 and ROCK2 in BASMCs was analyzed by Western blot. As shown in Fig. 4, although the total expression of RhoA was not altered, AngII induced a clear increase in the expression of GTP-RhoA, the activated manner of RhoA, an effect which was significantly suppressed in TMEM16A overexpressed BASMCs. By contrast, transfection with TMEM16A siRNA further facilitated RhoA activation by AngII (Fig. 4A−D).

Consistent with the RhoA activation, we found that ROCK2, the downstream kinase of RhoA, exhibited the same pattern of results. AngII-induced ROCK2 phosphorylation was remarkably reduced by TMEM16A overexpression and facilitated by TMEM16A knockdown (Fig. 4E−H). However, alteration in AngII-induced ROCK1 phosphorylation was not observed in either TMEM16A-expressing adenovirus or TMEM16A siRNA transfected cells (Supporting Information Fig. S3). These results suggest that TMEM16A might affect AngII-induced BASMC migration through RhoA/ROCK2 pathway.
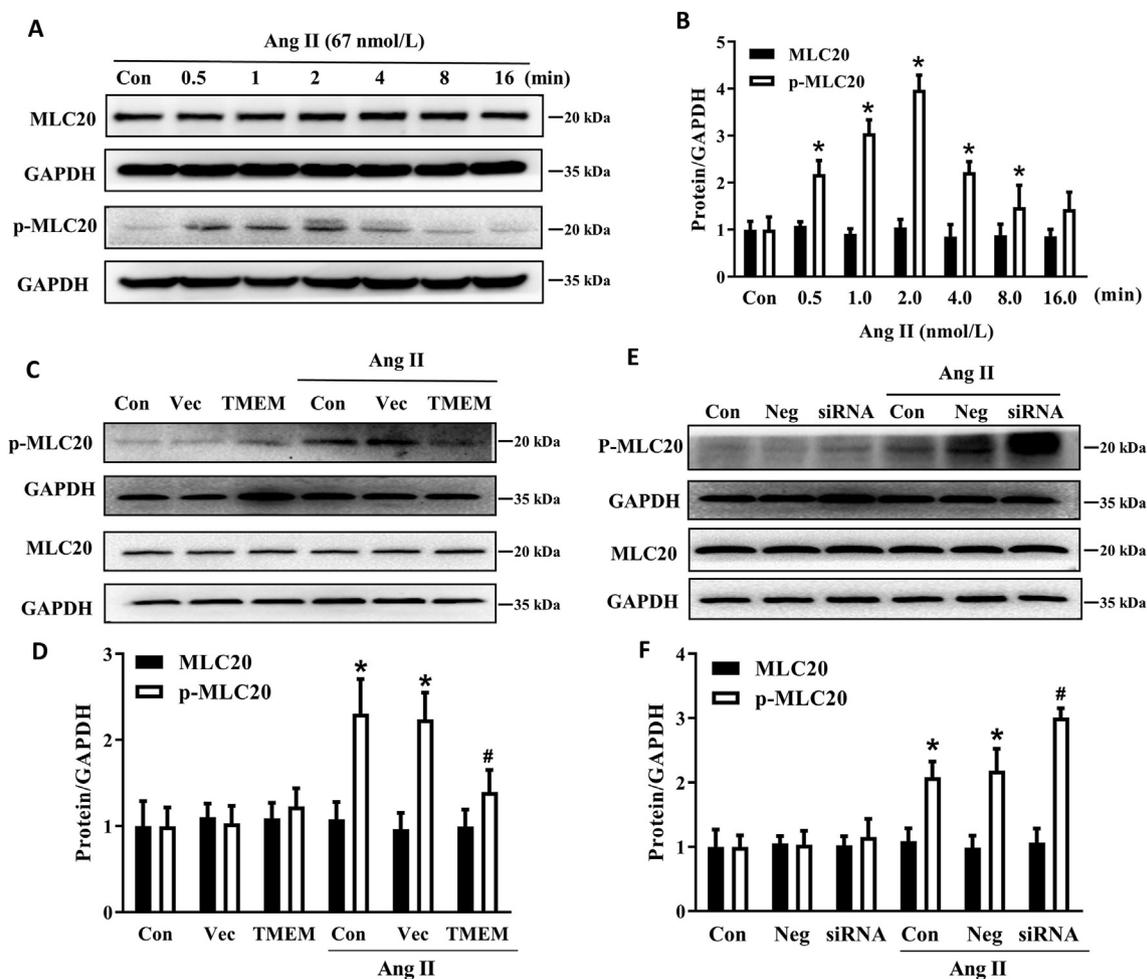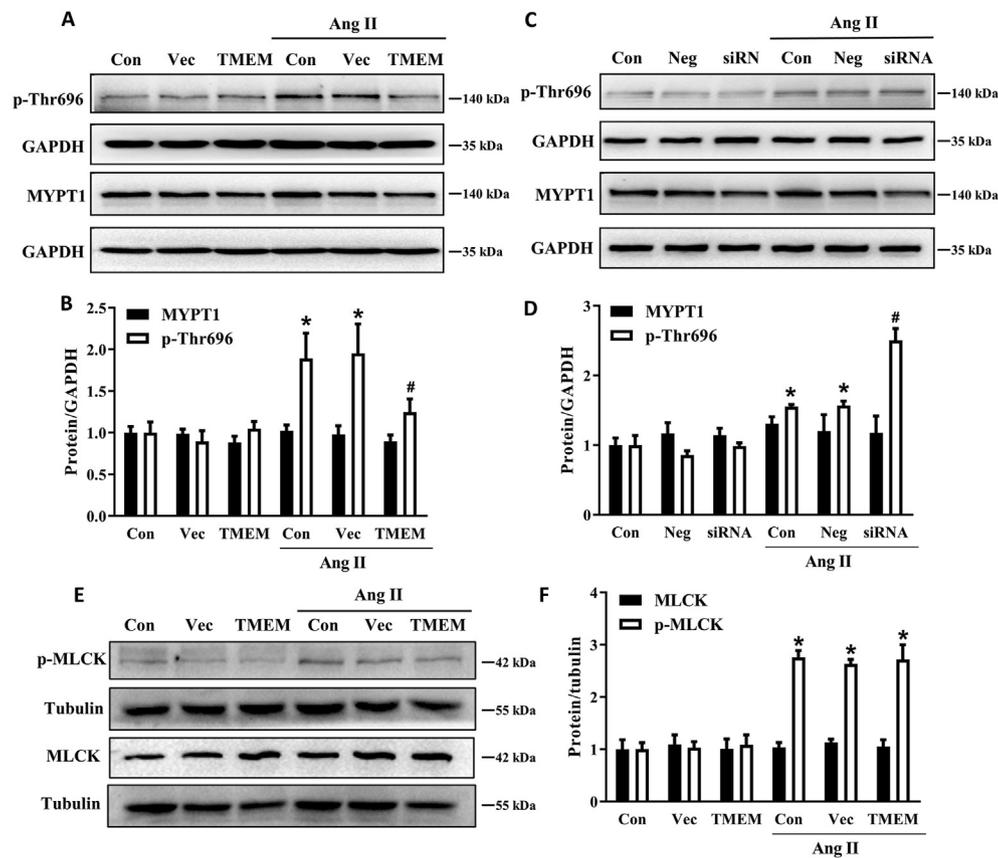
Figure 2 Effects of TMEM16A overexpression and knockdown on phosphorylation of MLC20 induced by AngII in mice BASMCs. (A) and (B) The time course of MLC20 phosphorylation at Ser19 site in BASMCs stimulated by AngII (67 nmol/L) for 0.5−16 min. Representative images of Western blot and bar charts of densitometric analysis are shown ($n = 5$ for each group; *$P < 0.05$ *vs.* Con, one-way ANOVA). (C) and (D) TMEM16A overexpression ameliorated AngII-induced MLC20 phosphorylation in mice BASMCs ($n = 6$ for each group; *$P < 0.05$ *vs.* Con, #$P < 0.05$ *vs.* AngII-treated group, one-way ANOVA). (E) and (F) TMEM16A knockdown promoted AngII-induced increase in p-MLC20 level in mice BASMCs ($n = 6$ for each group; *$P < 0.05$ *vs.* Con, #$P < 0.05$ *vs.* AngII-treated group, one-way ANOVA). After transfection with Ad-TMEM16A or TMEM16A siRNA for 48 h, BASMCs were stimulated by 67 nmol/L AngII for 2 min. Representative images of Western blot analysis and bar charts of densitometric analysis are shown. Values are mean ± SEM.

To further confirm that ROCK2 mediated AngII-induced BASMC migration, ROCK2 siRNA was transfected to primary cultured BASMCs, then its downstream signals MYPT1 and MLC20 phosphorylation were examined. As shown in Fig. 4I−K, knocking down the expression of ROCK2 protein did not affect MYPT1 and MLC20 phosphorylation directly under control conditions, but did clearly decrease AngII-induced phosphorylation of these proteins. More importantly, the increase in AngII-induced MYPT1 and MLC20 phosphorylation by TMEM16A siRNA transfection was greatly reversed in ROCK2 knockdown cells. Note that moreover, the potentiated migration induced by AngII in TMEM16A siRNA transfected BASMCs was also accordingly abolished by silencing of ROCK2 (Fig. 4L and M). These results suggest that RhoA/ROCK2 was required for TMEM16A regulation of MLC20 phosphorylation and BASMCs migration. A decreased TMEM16A expression was associated with an up-regulated RhoA/ROCK2 activation which in turn increased MLC20 activity by promoting MLCP inactivation *via* its

subunit MYPT1 phosphorylation, and finally facilitated BASMC migration. Overexpression of TMEM16A produced the opposite effects by inhibiting the RhoA/ROCK2/MLCP/MLC20 pathway.

3.5. Integrinβ3/FAK signal contributes to the effect of TMEM16A on BASMC migration

After demonstrating that RhoA/ROCK2/MLCP/MLC20 signaling pathway was critical in TMEM16A-regulated BASMC migration, we next explored whether regulation of integrin signal, the key molecule that mediates focal adhesion formation of the mobile cell[3], contributed to the effect of TMEM16A. Results in Fig. 5 show that AngII caused a significant increase in the expression of integrinβ3 but not integrinβ1 (Supporting Information Fig. S4 for integrinβ1 expression), accompanied by an elevated downstream signal focal adhesion kinase (FAK) phosphorylation. Compared with the non-transfected controls, AngII further increased integrinβ3 expression and FAK phosphorylation in cells

Figure 3 Effects of TMEM16A overexpression and knockdown on myosin phosphatase target subunit 1 (MYPT1) and myosin light chain kinase (MLCK) phosphorylation induced by AngII in mice BASMCs. (A) and (B) TMEM16A overexpression ameliorated AngII induced phosphorylation of MYPT1 at Thr696 site in mice BASMCs ($n = 6$ for each group; *$P < 0.05$ *vs*. Con, #$P < 0.05$ *vs*. AngII-treated group, one-way ANOVA). (C) and (D) TMEM16A knockdown promoted the phosphorylation of MYPT1 at Thr696 site in AngII treated BASMCs ($n = 5$ for each group; *$P < 0.05$ *vs*. Con, #$P < 0.05$ *vs*. AngII-treated group, one-way ANOVA). (E) and (F). TMEM16A overexpression did not influence the phosphorylation of MLCK in mice BASMCs ($n = 6$ for each group, *$P < 0.05$ *vs*. Con, one-way ANOVA). After transiently transfected with Ad-TMEM16A or TMEM16A siRNA for 48 h, BASMCs were stimulated by 67 nmol/L AngII for 2 min. Values are mean ± SEM.

transfected with TMEM16A siRNA. By contrast, these alterations induced by AngII were significantly inhibited by TMEM16A overexpression (Fig. 5A−H).

To further confirm that the increased FAK activation in TMEM16A knockdown cells was dependent on integrinβ3 signaling pathway, FAK phosphorylation was examined after the BASMCs were transfected with integrinβ3 siRNA. The results show that there was no difference in the total FAK expression between groups; however, AngII-induced FAK phosphorylation was significantly reduced by silencing of integrinβ3. Moreover, knockdown of TMEM16A increased the phosphorylation level of FAK in response to AngII, whereas this effect was not observed in BASMCs with integrinβ3 siRNA co-transfection (Fig. 5I and J), suggesting that TMEM16A inhibited FAK phosphorylation induced by AngII in an integrinβ3-dependent pathway.

The actin cytoskeleton undergoes dynamic assembly and disassembly during cell crawling, which regulate lamellipodia formation[17]. Integrin-mediated focal adhesions link to the actin filaments in the lamellipodia and allow cells to crawl on the ECM during migration. We next examined the effects of TMEM16A on filamentous actin (F-actin) expression induced by AngII in BASMCs. As shown in Fig. 5K and L, compared with control cells, in BASMCs treated with AngII, F-actin expression

was significantly increased as indicated by fluorescence staining of Alex Fluor™ 488-phalloidin with a changed shape of the cell and more lamellipodia formation. TMEM16A overexpression markedly suppressed these effects and rearranged the actin structures into parallel arrays. Conversely, silencing of TMEM16A showed the opposite results (Fig. 5K and L). These findings suggest that integrinβ3/FAK/F-actin signaling pathway was responsible for the inhibitory effect of TMEM16A on BASMC migration.

3.6. Smooth muscle specific TMEM16A overexpression inhibits BASMC migration in vivo during hypertension induced cerebrovascular remodeling

Our recent study has demonstrated that overexpression of TMEM16A was associated with reduced cerebrovascular remodeling during hypertension[12]. Given that VSMC migration is an important contributor to vascular remodeling, we hypothesized that inhibition of BASMC migration might account for the reduced cerebrovascular remodeling by TMEM16A overexpression. To confirm this postulation, the role of TMEM16A in migrating signal expression in basilar artery *in vivo* was examined during hypertension-induced cerebrovascular remodeling. AngII-
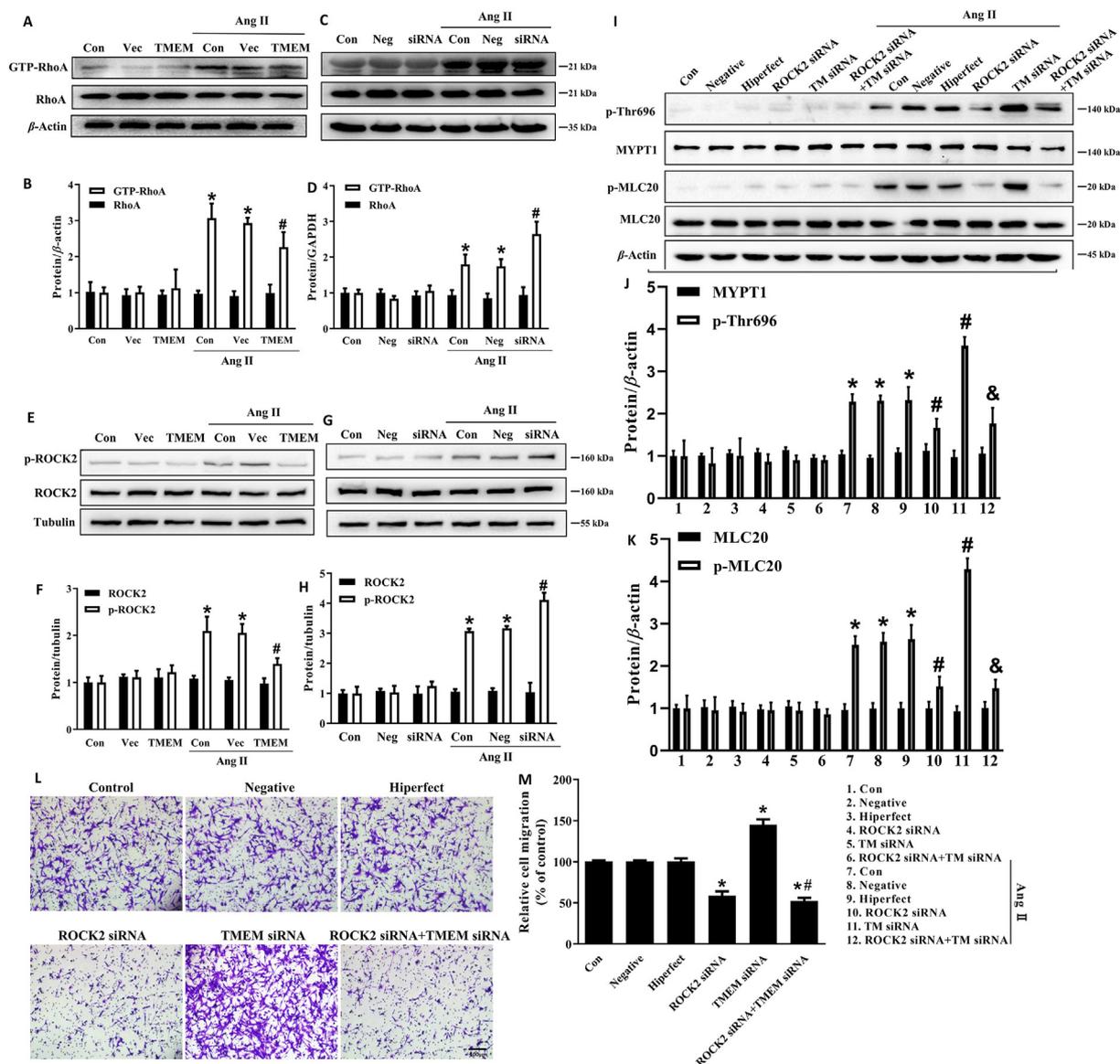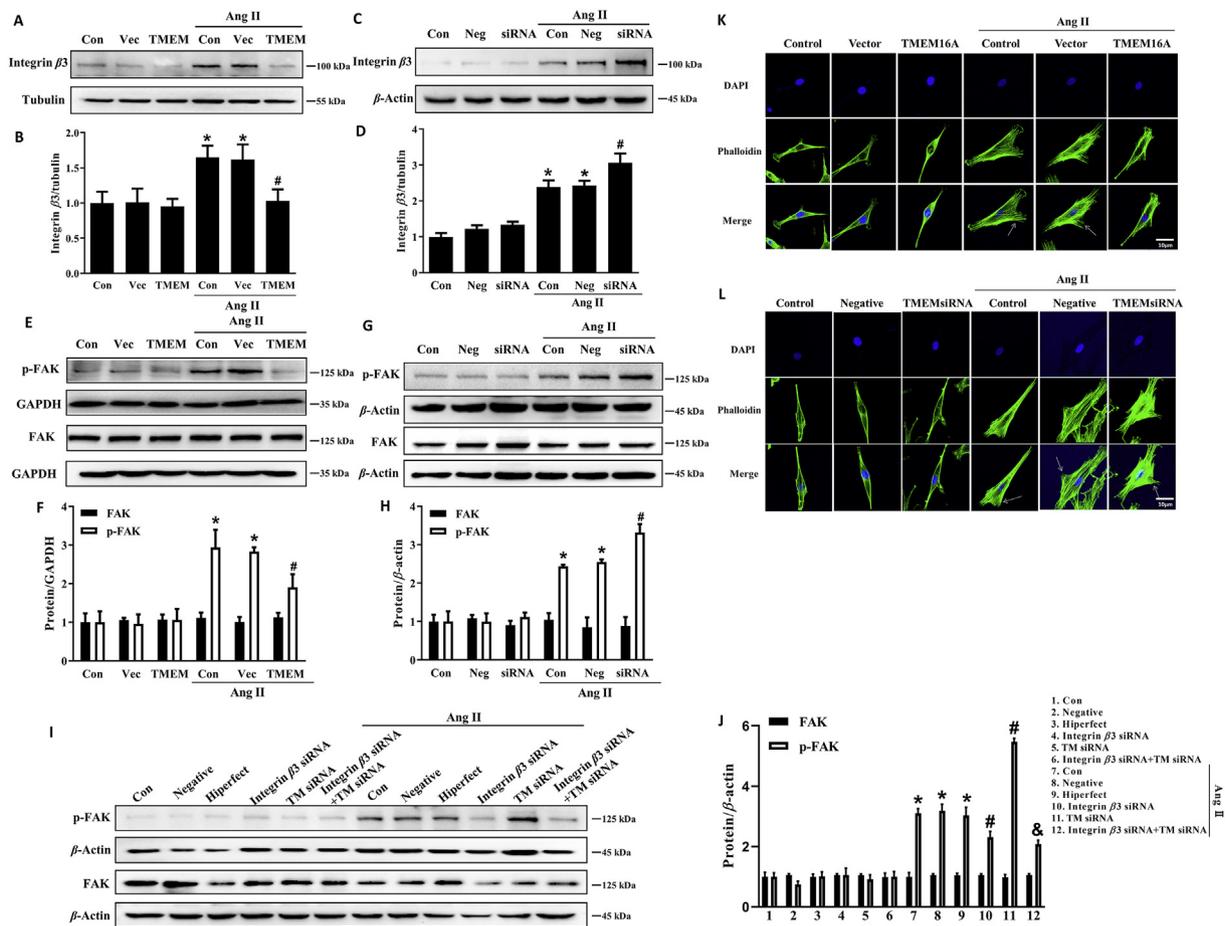
Figure 4 Effects of TMEM16A on the activation of RhoA/ROCK2 signaling pathway induced by AngII in mice BASMCs. (A)−(D) TMEM16A overexpression ameliorated, whereas TMEM16A knockdown promoted, AngII-induced RhoA activation in BASMCs ($n = 5$ for each group; *$P < 0.05$ vs. Con, #$P < 0.05$ vs. AngII-treated group, one-way ANOVA). (E)−(H) TMEM16A overexpression ameliorated, whereas TMEM16A knockdown promoted, AngII-induced phosphorylation of ROCK2 at Ser 1366 site in BASMCs ($n = 6$ for each group; *$P < 0.05$ vs. Con, #$P < 0.05$ vs. AngII-treated group, one-way ANOVA). (I) Representative Western blot images of MYPT1 and MLC20 phosphorylation in ROCK2 siRNA and TMEM16A siRNA co-transfected BASMCs. (J) and (K) AngII-induced phosphorylation of MYPT1 at Thr696 site (J) and MLC20 (K) in TMEM16A knockdown BASMCs was abolished by ROCK2 silencing ($n = 6$ for each group, *$P < 0.05$ vs. Con, #$P < 0.05$ vs. AngII-treated control groups, &$P < 0.05$ vs. AngII+TM-siRNA group, one-way ANOVA). (L) and (M) AngII-induced cell migration in TMEM16A knockdown BASMCs was inhibited by ROCK2 deletion. Representative images of Transwell assay and statistical analysis were shown ($n = 5$ for each group, *$P < 0.05$ vs. Con, #$P < 0.05$ vs. TMEM-siRNA group, one-way ANOVA). Values are mean ± SEM.

induced hypertension was created in vascular smooth muscle specific TMEM16A knock-in mice (SM[Tg]) and their control littermates expressing TAGLN-Cre but without TMEM16A transgene (SM[Cre]). The images of genotype identification[12] were shown in Supporting Information Fig. S5. Western blot showed that TMEM16A was steadily expressed at a high level in the primary cultured BASMCs of SM[Tg] from passages 2−8 (Fig. 6A and B). Four weeks after AngII implantation, Transwell assay revealed that migration of freshly isolated BASMCs from AngII-

infused SM[Cre] mice was significantly increased compared with that from normotensive controls. This effect was markedly reduced in BASMCs from AngII-treated TMEM16A-SM[Tg] mice (Fig. 6C and D). The results suggest that BASMC migration was increased during AngII-induced hypertension, which could be suppressed by TMEM16A overexpression.

Next, the expression of signal molecules involved in cell migration was detected in the basilar artery (BA) in vivo. As shown in Fig. 6E and F, consistent with the in vitro results in Figs.

Figure 5 Effects of TMEM16A overexpression and knockdown on the activation of integrinβ3/FAK signaling pathway induced by AngII in mice BASMCs. (A)−(D) TMEM16A overexpression attenuated, whereas TMEM16A knockdown increased, integrinβ3 expression induced by AngII in mice BASMCs ($n = 6$ for each group; *$P < 0.05$ *vs.* Con, #$P < 0.05$ *vs.* AngII-treated group, one-way ANOVA). (E)−(H) TMEM16A overexpression attenuated, whereas TMEM16A knockdown increased, p-FAK expression induced by AngII in mice BASMCs ($n = 6$ for each group; *$P < 0.05$ *vs.* Con, #$P < 0.05$ *vs.* AngII-treated group, one-way ANOVA). (I)−(J) Effects of integrinβ3 deletion along with TMEM16A knockdown on the activation of FAK kinase. The increased FAK phosphorylation induced by AngII in TMEM16A knockdown BASMCs was reversed by integrinβ3 deletion ($n = 6$ for each group, *$P < 0.05$ *vs.* Con, #$P < 0.05$ *vs.* AngII-treated control groups, &$P < 0.05$ *vs.* AngII+TM-siRNA group). (K)−(L) Effects of TMEM16A overexpression and knockdown on filamentous actin (F-actin) expression induced by AngII in mice BASMCs. F-actin was examined with fluorescence staining of Alex Fluor™ 488-phalloidin. Green fluorescence indicated the expression of F-actin in BASMCs; blue fluorescence indicates the nucleus of BASMCs under confocal microscope (600 ×, $n = 5$ for each group). Values are mean ± SEM.

2−5, immunofluorescence staining of the BA sections showed that the increased phosphorylation of MYPT1 and MLC20 observed in AngII-treated SM^Cre mice was markedly suppressed by TMEM16A overexpression. In addition, we also assessed the activation of integrinβ3/FAK signal in the BA of SM^Cre and SM^Tg hypertensive mice. As shown in Fig. 6G and H, both integrinβ3 expression and FAK phosphorylation were increased in the BA sections of AngII-treated SM^Cre, whereas AngII failed to increase these proteins expression in the BA of SM^Tg. These findings together provide convincing evidence that BASMC migration was activated during AngII-induced hypertension, in accordance with the induction of basilar artery remodeling[12]; overexpression of TMEM16A inhibited this migration *via* RhoA/ROCK2/MLCP/MLC20 and integrinβ3/FAK signaling pathways, which might contribute to the protection of basilar artery remodeling in SM^Tg mice.

3.7. TMEM16A inhibits migration-related signal via decreasing WNK1 phosphorylation

To further understand the association between TMEM16A Cl⁻ channel and VSMC migration, we propose that a Cl⁻-sensitive kinase might be activated by alterations in intracellular Cl⁻ concentration ($[Cl^-]_i$), subsequently leading to the activation of the migration-related signaling cascade. We therefore examined whether WNK1 (with-no-lysine (K)-1), a serine/threonine kinase which was shown to be regulated by $[Cl^-]_i$[18], mediated the inhibitory effect of TMEM16A on the migration-related signaling pathway. As shown in Fig. 7A and B, phospho-WNK1 was upregulated in response to AngII stimulation, which was significantly suppressed by overexpression of TMEM16A. The total WNK1 expression was comparable in each group. These data suggest that TMEM16A inhibits WNK1 activation.
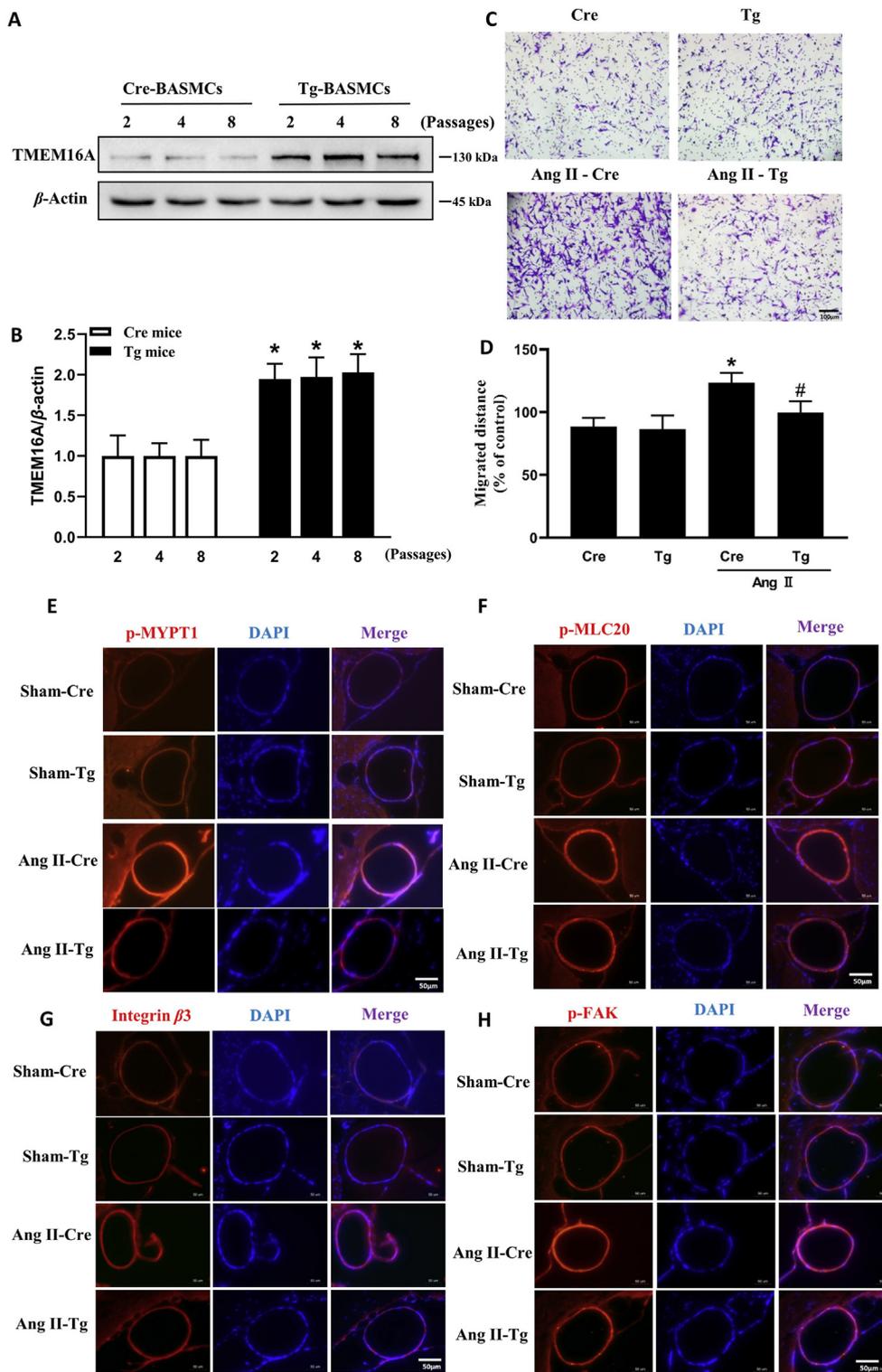
Figure 6 Inhibition of BASMCs migration contributed to the alleviated basilar artery remodeling in TMEM16A overexpressed hypertensive mice. (A) and (B) TMEM16A was steadily expressed at a high level in cultured BASMCs of 2, 4, and 8 passages isolated from smooth muscle specific TMEM16A transgenic mice (Tg) ($n = 5$ for each group; $*P < 0.05$ *vs.* Cre mice, *t*-test). (C)−(D) Migration of BASMCs isolated from TMEM16A-Tg or Cre littermates with or without AngII infusion by transwell assay ($n = 6$ for each group; $*P < 0.05$ *vs.* Con, $^{\#}P < 0.05$ *vs.* AngII-cre group, one-way ANOVA, 100 ×). (E)−(F) Immunofluorescent staining for phosphorylated MYPT1 and MLC20 in basilar artery sections from sham and hypertensive TMEM16A-Tg or Cre mice. The bar is 50 μm ($n = 5$ for each group). (G)−(H) Immunofluorescent staining for integrinβ3 and p-FAK in BA sections from TMEM16A-Tg and Cre mice with or without AngII infusion. Scale bar is 50 μm ($n = 5$ for each group). Values are mean ± SEM.
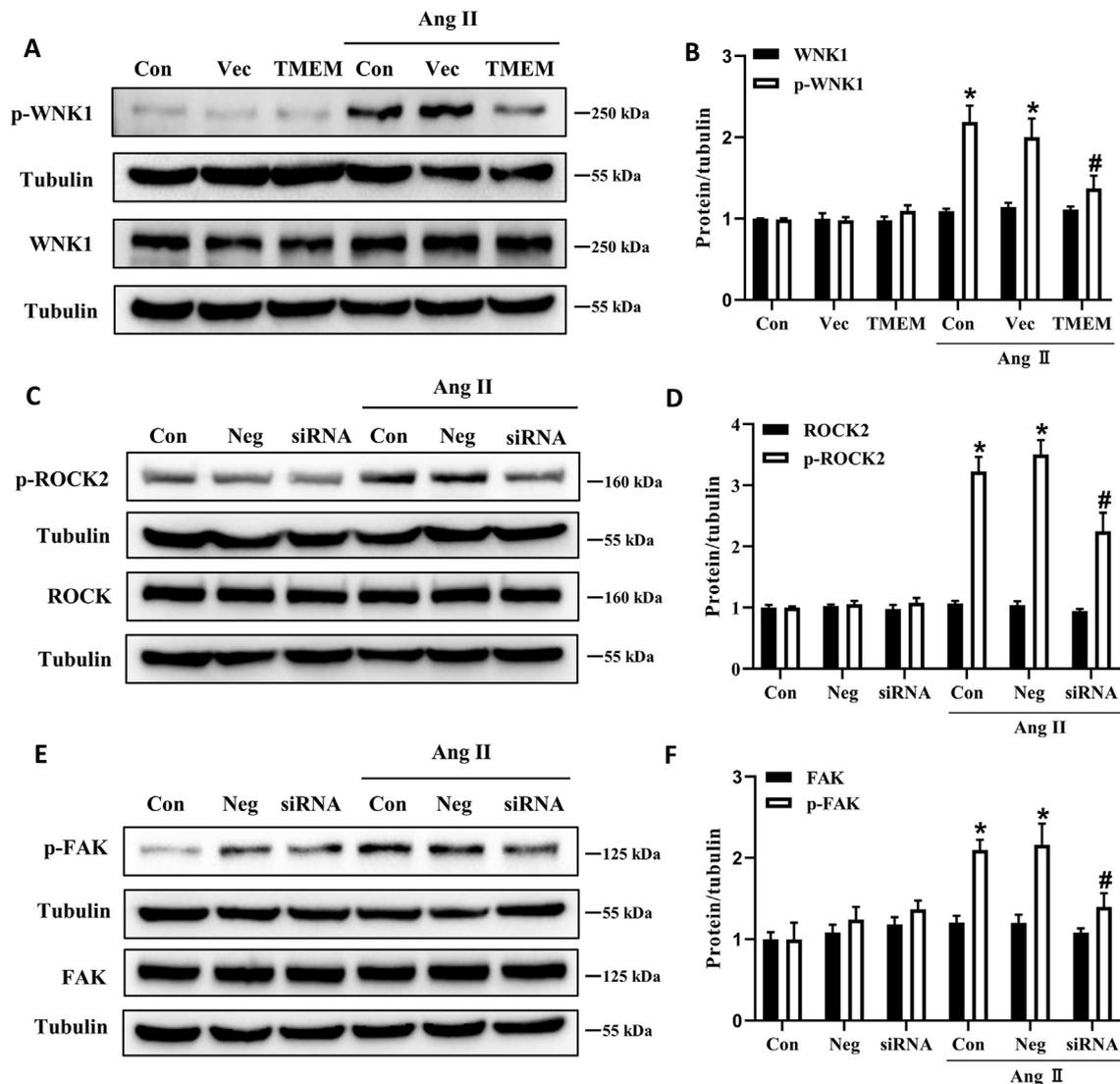
Figure 7 TMEM16A inhibited ROCK2 and FAK activation by decreasing WNK1 phosphorylation in BASMCs. (A) and (B) The effect of TMEM16A on AngII (67 nmol/L, 2 min)-induced WNK1 activation. Overexpression of TMEM16A downregulated the increase in AngII-induced WNK1 phosphorylation ($n = 6$, *$P < 0.05$ *vs*. Con, #$P < 0.05$ *vs*. AngII). (C)−(D) The effect of WNK1 on AngII-induced ROCK2 activation. Silencing of WNK1 downregulated AngII-induced increase in the phosphorylation of ROCK2 ($n = 6$, *$P < 0.05$ *vs*. Con, #$P < 0.05$ *vs*. AngII). (E)−(F) The effect of WNK1 on AngII-induced FAK activation. Silencing of WNK1 downregulated AngII-induced increase in the phosphorylation of FAK ($n = 6$, *$P < 0.05$ *vs*. Con, #$P < 0.05$ *vs*. AngII). Values are mean ± SEM.

Next, we examined whether WNK1 was involved in the regulation of migration-related signal molecules expression in BASMCs. WNK1 siRNA transfection was used (Supporting Information Fig. S6). The results show that AngII-induced increase in the phosphorylation of ROCK2 and FAK, the two critical molecules in migration-related signaling pathways, was abruptly suppressed in cells transfected with WNK1 siRNA (Fig. 7C−F), suggesting WNK1 increased the activation of ROCK2 and FAK. Taken together, these results suggest WNK1 might serve as the intermediate link between TMEM16A and migration-related signaling activation.

4. Discussion

The novel findings of the present study are (1) BASMC migration induced by AngII was suppressed by TMEM16A; (2) Both RhoA/

ROCK2/MLCP/MLC20 and integrinβ3/FAK signaling pathways were involved in the molecular mechanisms of TMEM16A inhibition on BASMC migration; (3) The inhibitory effect of TMEM16A on BASMC migration contributed to the reduction of cerebrovascular remodeling during hypertension. (4) TMEM16A inhibited AngII-induced BASMC migration *via* regulation of WNK-1. The schematic drawing of the study is shown in Fig. 8.

Cell migration is involved in various important physiological and pathophysiological processes including angiogenesis, tumor metastases, vascular remodeling and atherosclerosis. In the past two decades, numerous ion channels and transporters, such as voltage-gated Na^+ channels (Na_V), Ca^{2+}-sensitive K^+ channels (K_{Ca}) and transient receptor potential channels, have been shown to be required for cell migration in various cell types[19]. Recently, TMEM16A, the molecular component of Ca^{2+}-activated Cl^- channel (CaCC), is demonstrated to play a critical role in tumor
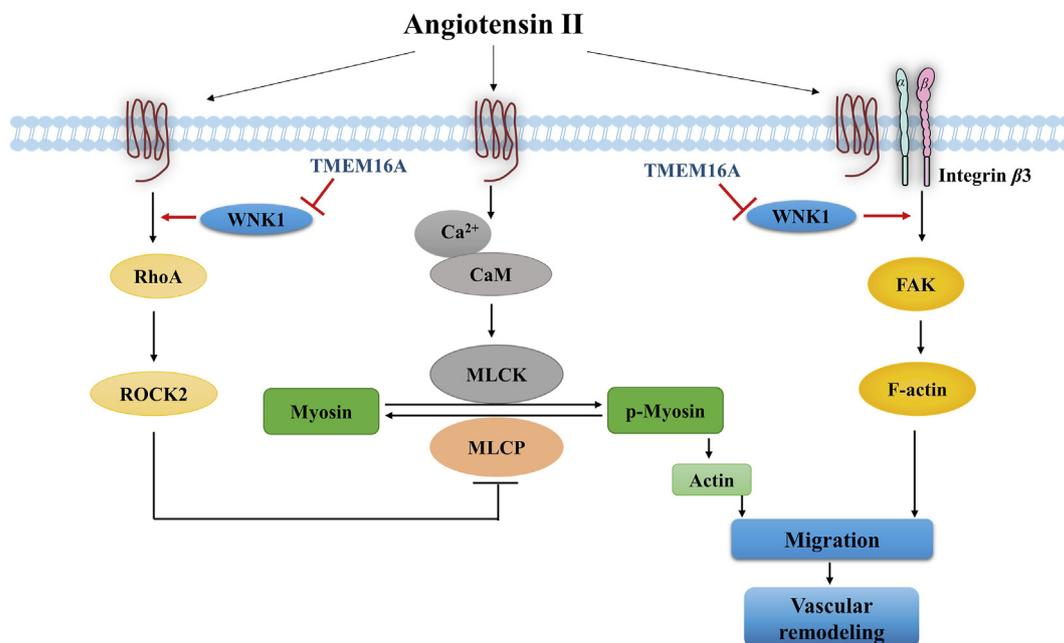
Figure 8 Schematic overview of vascular smooth muscle cell migration regulated by TMEM16A. By suppressing WNK1 activation, TMEM16A inhibited AngII-induced BASMCs migration and vascular remodeling *via* the RhoA/ROCK2/MLCP/MLC20 and integrinβ3/FAK signaling pathways.

cell migration. It has been shown that TMEM16A expression is up-regulated in most types of cancers, which contributes to the increased tumor cell migration and invasion[8−10]. Knockdown of TMEM16A has been demonstrated to inhibit cancer cell migration in many cancers such as gastric cancer, lung cancer, pancreatic cancer and hepatocellular carcinoma[8−10,20,21]. However, in contrast to the extensive research into the effects of TMEM16A on tumor cells, its functional significance in the migration of other cell types are still sparse. Here, we provided a novel finding that TMEM16A plays an important role in VSMC migration. However, different to its migration-promoting effect on cancer cells, overexpression of TMEM16A was shown to suppress AngII-induced BASMC migration, whereas inhibition of TMEM16A expression promoted this process. Therefore, it seems that TMEM16A promotes or inhibits cell migration *via* cell type-dependent mechanisms.

A question to answer is how TMEM16A regulates BASMC migration. In general, VSMC migration can be described as a continuous cycle of protrusion of the lamellipodium in the cell front and retraction of the rear part of the cell[3,19]. In this process, the critical roles of integrin-mediated focal adhesion formation and Rho/Rho kinase modulated cell contraction have been well recognized. Therefore, in our present study, we put much effort to explore whether TMEM16A has an effect on these two signal pathways. Firstly, we found that while both integrinβ3 and β1 were elevated in response to AngII, only integrinβ3 was associated with the inhibitory effect of TMEM16A on BASMC migration. Thereby, it seems that although other integins in the integrin family likely play important functions, integrinβ3 is of great importance in smooth muscle cell migration. In line with our results, the previous reports by pharmacological inhibition of α5β1 and αvβ3 revealed the similar findings[22,23]. Although both α5β1 and αvβ3 inhibitors reduce atherosclerosis formation, only αvβ3 inhibition reduced the incidence of fibrous cap formation, a

process driven by smooth muscle cell migration[22]. In contrast, inhibition of α5β1 might be related to blunted plaque inflammation without reducing smooth muscle incorporation[23].

At focal adhesion, extracellular domains of integrins connect with ECM, and the intracellular tails of integrin link to the actin filaments in the lamellipodia and allow cells to crawl on the ECM during migration[1,3]. Integrin-induced FAK activation is able to promote actin polymerization and facilitate cell migration[24,25]. Conversely, inhibition of FAK activity is associated with reduced VSMC migration[4]. In line with these studies, here we showed that in the same manner as integrinβ3 regulation, overexpression of TMEM16A significantly reduced FAK phosphorylation induced by AngII. Simultaneously, F-actin expression and lamellipodium formation were also accordingly inhibited in TMEM16A over-expressed BASMCs with closer to normal cell morphology. Our findings thereby provide convincing data that influencing integrinβ3/FAK signaling and thereby lamellipodium formation is one of the rational explanations for the inhibition of TMEM16A on BASMC migration.

On the other hand, it has been suggested that Rho and Rho kinase play an important role in smooth muscle cell locomotion[26,27]. RhoA-mediated contraction may promote the retraction of the cell rear during migration[28]. By inhibiting MLCP *via* phosphorylation of its regulatory subunit MYPT1, Rho/Rho kinase triggers MLC activity and leads to cell contraction[16,26]. MLC phosphorylation can also be catalyzed by MLCK which is activated by Ca^{2+}/calmodulin in smooth muscle cells[15]. A balance between phosphorylation/dephosphorylation of the MLC, catalyzed respectively by MLCK and MLCP, is a key event in the regulation of smooth muscle contraction and migration. In the present study, we found that AngII treatment induced a significant increase in p-MLCK expression, indicating a possible contribution of MLCK to AngII-induced MLC20 phosphorylation; also consistent with the previous reports that MLCK is necessary for

migration of various cell types including SMC[26,29]. However, interestingly, TMEM16A overexpression had no effect on the p-MLCK level despite it did clearly reduce MLC20 phosphorylation, indicating that TMEM16A might not suppress MLC20 activation and BASMC migration by regulating MLCK. In contrast to p-MLCK expression, overexpression of TMEM16A significantly reduced the MLCP regulatory subunit MYPT1 phosphorylation, as well as its upstream kinase RhoA/ROCK2 activation. Thereby, a decreased MYPT1 phosphorylation by RhoA/ROCK2 in TMEM16A overexpressed BASMCs might preserve the activity of MLCP, which in turn lead to a reduced p-MLC20 level by dephosphorylating this protein. The involvement of RhoA/ROCK2/MLCP/MLC20 pathway in BASMC migration were also supported by the reversal experiment of ROCK2 deletion, in which the enhanced p-MLC20 level and cell migration in TMEM16A knockdown BASMCs were abrogated by silencing of ROCK2.

It is well known that VSMC migration is related to many cardiovascular diseases such as atherosclerosis, neointima formation and vascular remodeling. It is reasonable to postulate that the inhibitory effect of TMEM16A on BASMC migration might affect the vascular function *in vivo*. In our recently published article, we have demonstrated that TMEM16A expression is down-regulated in basilar artery of AngII-induced hypertensive mice accompanying by increased vascular remodeling[12]. Overexpression of TMEM16A in smooth muscle cells significantly suppressed the hypertension-induced cerebrovascular remodeling, which has been explained by a reduced ECM deposition[12]. Given the well-known impact of SMC migration on ECM deposition and its critical contribution to vascular remodeling, we assumed that inhibition of BASMC migration may play an important role in the improved vascular remodeling in TMEM16A overexpressed mice. Accordingly, in line with the *in vitro* study in primary cultured BASMCs, our *in vivo* results in the AngII-induced hypertensive mice showed that the expressions of migration-related signal molecules were significantly lower in basilar artery from smooth muscle-specific TMEM16A transgenic mice than that in AngII-Cre mice, further strengthened the idea that TMEM16A suppressed VSMC migration and contributed to vascular remodeling *via* RhoA/ROCK and integrinβ3 mediated signaling pathways.

One issue that needs to be addressed is how TMEM16A contributes to migration-related signal activation. Recently, it has been demonstrated that Cl^- can serve as an intracellular messenger, and a wide range of kinases and proteins in many signaling pathways are sensitive to intracellular Cl^-. Given that TMEM16A is a Cl^- channel, we propose that alteration in intracellular Cl^- concentration *via* TMEM16A might activate some Cl^--sensitive kinases, subsequently leading to the activation of migration-related signaling cascade. In our recent studies, we have demonstrated that WNK1 [with-no-lysine (K)-1], a member of WNK serine/threonine kinase family, is a Cl^--sensitive kinase and mediates both the proliferation and ECM-related signaling pathways in vascular smooth muscle cells[12,18]. Therefore, it is possible that the signal molecules in cell migration machinery are also regulated by TMEM16A through WNK-1. In line with our speculation, the experiments showed that WNK-1 activation by AngII increased ROCK2 and FAK phosphorylation in BASMCs, while TMEM16A inhibited this pathway by decreasing WNK-1 phosphorylation. These results suggested that WNK-1 may serve as the mediator between TMEM16A chloride channel and migration-related signaling activation. However, whether other mechanisms are also involved in this process deserves further exploration.

It is also noteworthy that under the baseline condition (*e.g.*, in the absence of AngII stimulation) the alteration of TMEM16A expression level may have a minor role in BASMC migration, while TMEM16A may exert a more prominent effect on BASMC migration under pathological conditions (*e.g.*, when AngII stimulates). There is still much to learn concerning the relevance of TMEM16A and VSMC migration. Elucidating the exact association between TMEM16A and migration-related signal molecules is worthy of further study in the future.

5.   Conclusions

In summary, our data indicate that TMEM16A exerted an inhibitory effect on BASMC migration, which was associated with reduced cerebrovascular remodeling during AngII-induced hypertension. This finding suggests that modulation of TMEM16A expression and/or activity might be a novel strategy to prevent hypertension-induced vascular remodeling. Our study may also open up potential therapeutic opportunities in other VSMC migration-related diseases, such as arteriosclerosis.

Acknowledgments

Author contributions

Yongyuan Guan and Yanhua Du designed the study, drafted and edited the manuscript; Huaqing Zheng and Xiaolong Li performed most of the experiments; Other colleagues assisted with the experimental operation or data collection.

Conflicts of interest

All the authors declared no competing interests.

Appendix A.   Supporting information

Supporting data to this article can be found online at https://doi.org/10.1016/j.apsb.2021.04.013.

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