**MiR-143 Mediates the TLR2/NF-κB Pathway to Attenuate AngII-induced Damage to VSMCs**

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Abdominal aortic aneurysm (AAA) is a perilous vascular disease with inflammatory response as its main feature. It is known that the expression of miR-143 is down-regulated in the human aortic aneurysm. In this study, we investigated the effect of miR-143 on AngII-induced VSMCs to learn the potential mechanisms of miR-143 on AAA at the cellular level. The experimental results showed that the expressions of IL-1β, MCP-1, MMP9/13, TLR2, and NF-κB p65 and the percentage of TUNEL-positive cells in AngII-VSMCs were increased significantly compared with the control group. miR-143 had the opposite result. When the expression of miR-143 was up-regulated, the expression of IL-1β, MCP-1, and MMP9/13 and the percentage of TUNEL-positive cells in AngII-VSMCs was suppressed. With the transfection of miR-143 over-expression plasmid, IL-1β, MCP-1, and MMP9/13 and the percentage of TUNEL-positive cells were reversed, compared to the AngII group and the AngII+oe-TLR2+miR-143 mimic group. In AngII-induced mouse VSMC, the up-regulation of the miR-143 expression could inactivate the TLR2/NF-κB pathway, thereby alleviating inflammatory response, ECM degradation, and cell apoptosis.

**Key words:** miR-143, abdominal aortic aneurysm (AAA), inflammation, TLR2/NF-κB pathway, vascular smooth muscle cell (VSMC).

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Abdominal aortic aneurysm (AAA), a common chronic vascular disease, is the most challenging of aneurysms owing to its high morbidity rate (LI et al. 2020). It is characterized by a cascade of pathological features including inflammation (PESHKOVA et al. 2016), smooth muscle cell (SMC) growth disorder and apoptosis surge (YUE et al. 2020), and extracellular matrix (ECM) degradation (KUMAR et al. 2019; WANG et al. 2018b). Currently, the main treatment method is surgical operation, replacing the damaged blood vessel with a new one (LIEBERG et al. 2018). However, since AAA patients usually have other complex conditions, surgical intervention is often difficult and risky. Therefore, it is quite necessary to develop novel treatment strategies that are low-risk and highly effective.

MicroRNA (miRNA), a small non-coding RNA molecule, is a key regulator of post-translational gene modification (BOREK et al. 2019). The dysregulation of miRNAs is strongly associated with the formation and development of AAA (KUMAR et al. 2019). Previous studies have demonstrated that some miRNAs could affect the development of AAA, such as miR-21, miR-155, and miR-712, which are involved in the degradation of ECM and the apoptosis of SMCs (IYER et al. 2017; KIM et al. 2014; ZHANG et al. 2018). A recent clinical survey showed that the expression of miR-143 is significantly lower in AAA patients than in healthy individuals (ELIA et al. 2009). Researchers have examined the expression of multiple miRNAs in unruptured human cerebral aneurysms, of which the expression of miR-143-5p was...
The most downregulated (BEKELIS et al. 2016). CORDES et al. revealed that miR-143 and miR-145 were downregulated in less differentiated SMCs and injured or atherosclerotic vessels (CORDES et al. 2009). miR-143 may provide a key link with the target genes of SMC function. Nevertheless, the exact role of miR-143 in AAA is still uncertain.

The aortic wall is thinned in AAA, caused by the inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and monocyte chemotactic protein (MCP-1) and matrix metallo-proteinases like MMP-1, MMP-2, MMP-9 and MMP-13 released by macrophages. Risk of vascular rupture is exacerbated correspondingly (CIAVARELLA et al. 2015; RABKIN 2017; SHI et al. 2020). Under the stimulation of inflammatory factors, vascular smooth muscle cells (VSMCs) release chemokines that play a key role in vascular inflammation (REN et al. 2014). The expression of Toll-like receptor 2 (TLR2) in the blood of AAA patients was noticeably higher than that of healthy individuals. It suggested that TLR2 may exert a role in the local and systemic inflammatory response to AAA (JABLONSKA et al. 2020). SHI et al. has noted that miR-144-5p promoted AAA formation by inhibiting the signal transduction of the downstream pathway of TLR2 and OLR1 (SHI et al. 2020). After abnormal activation of the downstream receptor protein nuclear factor-κB (NF-κB) of TLR2, the consequent upregulation of inflammatory mediators and MMPs becomes the major culprit in the inflammatory response and ECM degradation. In addition, NF-κB is also involved in the regulation of apoptosis (MA et al. 2018). It was proposed that the inactivation of TLR2 could reduce the expression of TLR2, thereby alleviating chronic inflammation in AAA vascular tissue (YAN et al. 2015). However, this theory needs further exploration about whether miR-143 acts on the TLR2/NF-κB pathway or participates in the development of AAA.

In this study, we will investigate the function of miR-143 and the relationship between miR-143 and the TLR2/NF-κB pathway in damaged VSMCs. Our findings may provide some novel insights into the molecular mechanism of miR-143 in AAA.

Materials and Methods

This study was approved by the animal experiment ethics committee of the Second Affiliated Hospital of Fujian Medical University and conducted in strict accordance with the national institutes of health guidelines for the care and use of experimental animals.

Cells culture and angiotensin II (AngII) induction

Mouse primary VSMCs were retrieved from Wellbio Co., Ltd (Changsha, China). They were cultured in Dulbecco’s modified Eagle medium (DMEM, Corning), containing 10% fetal bovine serum (FBS, HyClone), 1% penicillin-streptomycin solution, and 5% CO₂ for about 3-7 days at 37°. The cells were treated as previously described (LI et al. 2019). Next, the VSMCs of 4-8 passages were selected and induced with AngII (10⁻⁷ M, Sigma-Aldrich) for 24 h. Before the induction, the VSMCs were pretreated with PD98059 (10⁻⁸ M, Tocris) and apocynin (10⁻⁶ M, Santa Cruz) for 1 h.

Cell transfection

Lipofectamine™ 2000 Transfection Reagent (Invitrogen, UK) was utilized to transfect the VSMCs with miR-143 mimics, TLR2 overexpressed plasmid or TLR2 siRNA according to the manufacturer’s protocol. The mimics and plasmids used for transfection were all purchased from Honorgene (Changsha, China).

ELISA

After the supernatants of the VSMCs were isolated, the concentrations of IL-1β and MCP-1 were measured by ELISA kit (cat. CSB-E12986B and cat. CSB-E04655H, Huamei Biotechnology, Wuhan, China) following the manufacturer’s procedure. Absorbance was tested using a microplate reader (Bio-Rad Laboratories, Inc.) at 450 nm.

Real-time quantitative reverse transcriptase-polymerase chain reaction (RT-qPCR)

Total RNA was extracted from transfected VSMCs using TRIzol (Invitrogen, Waltham, MA, USA) and reverse transcribed to cDNA by M-MuLV reverse transcriptase (Promega Corporation, Madison, WI, USA) according to the manufacturer’s recommendation. The absorbance value of the extracted RNA was measured at 260 nm and 280 nm by ultraviolet spectrophotometer, and calculated its concentration and purity. RNA concentration (ng/µl) = A260 x dilution factor x 40, concentration in the range of 100 ng/µl - 200 ng/µl, RNA purity = OD260/OD280, ratio range between 1.8-2.0. miRNA amplification was carried out using the GenoExplorer miRNA qRT-PCR kit (Genosensor Corporation, Tempe, AZ, USA). The RT-qPCR was conducted by an Applied Biosystems Prism 7900HT Fast Real-Time PCR system (Applied Biosystems, Thermo Fisher Scientific, Inc.) and SYBR-Green (Applied Biosystems, Foster City, CA). Relative mRNA quantification was determined with respect to the mean value of GAPDH. The relative expression levels of the miRNAs were evaluated with the 2⁻ΔΔCt method. The primer sequences of miR-143 and other mRNAs (Sangong, Shanghai, China) were as in Table 1.
Western blot

The VSMCs were lysed using lysis buffer containing a 1-fold protease inhibitor cocktail on ice. Cellular proteins were loaded onto S.D.S-PAGE (10% w/v) and transferred to PVDF membranes (Millipore, Billerica, MA, USA). Then the membranes were blocked with dried 5% non-fat milk for 1 h at room temperature. Samples were probed with the primary antibodies overnight at 4°C. The primary antibodies applied as follows: Rabbit anti-IL-1β antibody (1:1000, cat. 16806-1-AP), Rabbit anti-MCP-1 antibody (1:1000, cat. 25542-1-AP), Rabbit anti-MMP9 antibody (1:1000, cat. 10375-2-AP), Rabbit anti-MMP13 antibody (1:2000, cat. 18165-1-AP), Mouse anti-TLR2 antibody (1:1000, cat. 66645-1-Ig), and Rabbit anti-NF-κB p65 antibody (1:5000, cat. 10745-1-AP). Subsequently, HRP-goat anti-mouse IgG (1:5000, cat. SA00001-1) or HRP-goat anti-rabbit IgG (1:6000, cat. SA00001-2) was used as the secondary antibody. The membranes were then incubated for 90 min at room temperature. GAPDH (1:4000, cat. 10494-1-AP) was used as an internal control. All antibodies were purchased from Proteintech (USA). Protein concentrations were quantified with the Bio-Rad Protein Assay kit (Hercules, CA, USA). Image Quant software version 5.2 (GE Healthcare Life Sciences, Chalfont, UK) was performed to quantify band intensity.

TUNEL

For the visualization of VSMCs apoptosis was adopted One Step TUNEL Apoptosis Assay Kit (Beyotime, Shanghai, China) following manufacturer’s recommendation. The apoptotic cells were observed by fluorescence microscopy. The percentage of TUNEL-positive cells in total number of cells in 5 random fields was counted as the relative cell apoptosis rate for each sample.

Statistical analysis

The study data were all conducted in at least triplicate. All data were presented as the mean ± standard deviation (SD). Analyses were performed by use of GraphPad Prism 8.0 (GraphPad Software, Inc., San Diego, CA, USA). Comparisons of 2 independent groups were analyzed through the two-tailed Student’s t-test, p<0.05 was considered to be statistically significant.

Results

Up-regulation of miR-143 inhibited the expression of inflammatory mediators in AngII-VSMCs

The AngII-induced VSMC damage model has often been used to simulate the pathological conditions of AAA in vitro (Li et al. 2019). After induction by AngII, the expression of inflammatory factors IL-1β and MCP-1 increased significantly in the VSMCs of the AngII group compared with the control group (Fig. 1A, B), while the expression of miR-143 was markedly decreased in in the AngII group (Fig. 1C). This implied that miR-143 was low-expressed in the AAA cell model. Then, miR-143 was overexpressed with miR-143 mimic (Fig. 1D) to investigate the effect of miR-143 on the expression of inflammatory factors IL-1β and MCP-1. From Figure 1E, when miR-143 was overexpressed, the expression of IL-1β and MCP-1 upregulated by AngII was reversed. This was consistent with the expression of the two at the protein level (Fig. 1F). The above results indicated that in the AngII-VSMCs, up-regulation of miR-143 could abate their inflammatory response.

Up-regulation of miR-143 suppressed the expression of MMP-9 and MMP-13 in AngII-VSMCs

The degradation of ECM in AAA is one of the main reasons for accelerating vascular rupture. To further understand the effect of overexpressed miR-143 on
Fig. 1 A-G. Up-regulation of miR-143 inhibited the expression of inflammatory mediators in AngII-VSMCs. A, B – the concentrations of IL-1β and MCP-1 in cell supernatant were determined by ELISA kit. C, D – q-PCR was used to detect the expression of miR-143. E – the expression of IL-1β and MCP-1 in mRNA. F, G – Western Blot was applied to measure the expression of IL-1β and MCP-1 in protein. All data are presented as means ±SD with three independent experiments; * p<0.05 vs. control group, # p<0.05 vs. AngII group.
this physiological process, the expression of MMP9 and MMP13 in VSMCs was examined respectively. In Figure 2A, the expression of MMP9 was augmented overtly with the AngII treatment compared with the control group, while in the miR-143 mimic + AngII group it was obviously lower than that in the AngII group. By detecting the expression at the protein level, we also obtained consistent results with those of mRNA (Fig. 2B, 2C). Similarly, although there was a highly expressed MMP13 in the AngII group, overexpressed miR-143 still downregulated MMP13 expression in AngII-VSMCs as shown in Figure 2D. It exhibited similar effects at the protein level (Fig. 2E, 2F). These data suggested that miR-143 could attenuate the degradation of ECM in AngII-VSMCs.

Up-regulation of miR-143 counteracted the apoptosis in AngII-VSMCs

Further, we continued to explore the effect of miR-143 on the apoptosis of AngII-VSMCs using TUNEL experiments. From Figure 3A, it was observed that there were more apoptotic VSMCs in the AngII group than in the control group, while they were eased back notably in the miR-143 mimic + AngII group. Equally, in Figure 3B, the percentage of TUNEL-positive cells was multiplied significantly in the AngII group compared with the control group. The percentage in the miR-143 mimic + AngII group was markedly inferior to that in the AngII group, which showed no statistical difference from the control group. All these indicated that overexpression of miR-143 in AngII-VSMCs could slow down apoptosis.

Fig. 2. A-F. Up-regulation of miR-143 suppressed the expression of MMP-9 and MMP-13 in AngII-VSMCs. A – the expression of MMP-9 in mRNA level. B, C – the expression of MMP-9 in protein level. D – the expression of MMP-13 in mRNA level. E, F – the expression of MMP-13 in protein level. All data were presented as means ±SD with three independent experiments; * p<0.05 vs. control group, # p<0.05, vs. AngII group.
Up-regulation of miR-143 hindered the activation of the TLR2/NF-κB pathway in AngII-VSMCs

The TLR2/NF-κB pathway is a classical inflammation-related signal transduction pathway. Next, we compared the expression of TLR2 and NF-κB p65 between the AngII group and the control group. At the molecular level (Fig. 4A), the expression of both showed a jump in the AngII group, which was compatible with their expression at the protein level (Fig. 4B, 4C). This illustrated that AngII triggers the TLR2/NF-κB pathway in VSMCs. Subsequently, the AngII-VSMCs were treated with miR-143 mimic. It was perceived that the overexpression of miR-143 in AngII-VSMCs effectively reduced the expression of not only TLR2 protein, but also NF-κB p65 (Fig. 4D, 4E). These above revealed that up-regulation of miR-143 in AngII-VSMCs could inactivate the TLR2/NF-κB pathway.

Up-regulation of TLR2 contributed to inflammation via activating the TLR2/NF-κB pathway in AngII-VSMCs

To investigate whether a correlation exists between inflammatory response in AngII-VSMCs and the TLR2/NF-κB pathway, the effects of TLR2 overexpression and low-expression on inflammatory factors in AngII-VSMCs were surveyed. From Figure 5A and 5B, when TLR2 was overexpressed in AngII-VSMCs, the expression of IL-1β and MCP-1 was vastly upregulated compared with both the control and AngII groups. As the low-expressed TLR2, IL-1β expression, and MCP-1 expression in the AngII + si-TLR2 group were notably lower than in the AngII group, even, the expression of MCP-1 was nearly as much as the control group. Similarly, in Figure 5C and 5D, the expression of MMP9 and MMP13 in the AngII-oe-TLR2 group was the highest compared with other groups. By contrast, the expression of both in the

Fig. 3. A-B. Up-regulation of miR-143 counteracted apoptosis in AngII-VSMCs. A – the level of apoptosis was analyzed by TUNEL assay (magnification x 200, scale bar = 50 μm). B – the percentage of TUNEL positive cells. All data were presented as means ±SD with three independent experiments; * p<0.05 vs. control group, # p<0.05 vs. AngII group.
AngII + si-TLR2 group was significantly lower than that in the AngII group. In addition, by TUNEL analysis (Fig. 5E, 5F), the up-regulation of TLR2 expression in AngII-VSMCs made the TUNEL-positive cells more abundant compared with the AngII group. With a low-expressed TLR2, the percentage of TUNEL-positive cells in the AngII + si-TLR2 group was not statistically different from that in the control group. Therefore, it was inferred that the overexpression of TLR2 in AngII-VSMCs could enhance cellular inflammation and accelerate the degradation of ECM and cell apoptosis.

MiR-143 inactivated the TLR2/NF-κB pathway through diminishing TLR2 expression in AngII-VSMCs. After the upregulation of miR-143, the interaction between overexpressed miR-143 and TLR2 on AngII-VSMCs was observed. The expression of IL-1ß and MCP-1 was known to be up-regulated by AngII + oe-TLR2, while it was extremely down-regulated in the AngII + oe-TLR2 + miR-143 mimic group, even well below that in the AngII group (Fig. 6A, 6B). Correspondingly, the expression of MMP9 and MMP13 in the AngII + oe-TLR2 + miR-143 mimic group was also greatly reduced compared with the AngII + oe-TLR2 and AngII groups, especially MMP13 (Fig. 6C, 6D). In terms of apoptosis, TUNEL-positive cells in AngII + oe-TLR2 + miR-143 mimic group were enormously sparser than those in the AngII + oe-TLR2 and AngII groups as Figure 6E shows, which was fit with the quantitative results in Figure 6F. These denoted that miR-143 overexpression could alleviate inflammation in AngII-VSMCs and related ECM degradation and apoptosis by blocking the activation of TLR2.

Discussion

MiR-143 is highly expressed in normal VSMCs and plays a key role in the phenotypic transformation of VSMCs. However, miR-143 is decreased in most cardiovascular events (Rangrez et al. 2011), AAA in-

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**Figure 4.** A-E. Up-regulation of miR-143 hindered the activation of the TLR2/NF-κB pathway in AngII-VSMCs. A – the expression of TLR2 and NF-κB subunit p65 in mRNA. B, C – the expression of TLR2 and NF-κB subunit p65 in protein. D, E – the expression of TLR2 and NF-κB subunit p65 was measured by Western Blot in setting of overexpressed miR-143. All data were presented as means ±SD with three independent experiments; * p<0.05 vs. control group, # p<0.05 vs. AngII group.
Fig. 5. A-F. Up-regulation of TLR2 contributed to inflammation via activating the TLR2/NF-κB pathway in AngII-VSMCs. A, B – the protein expression of IL-1β and MCP-1. C, D – the protein expression of MMP9 and MMP13. E, F – TUNEL assay for VSMC apoptosis (magnification x 200, scale bar = 50 μm). All data were presented as means ±SD with three independent experiments; * p<0.05 vs. control group, # p<0.05 vs. AngII group.
Fig. 6. A-F. MiR-143 inactivated the TLR2/NF-κB pathway through diminishing TLR2 expression in AngII-VSMCs. A, B – the protein expression of IL-1β and MCP-1. C, D – the protein expression of MMP9 and MMP13. E, F – TUNEL assay for VSMC apoptosis (magnification x 200, scale bar = 50 μm). All data were presented as means ± SD with three independent experiments; * p<0.05 vs. control group, # p<0.05 vs. AngII group, & p<0.05 vs. AngII+oe-TLR2 group.
cluded. The formation of AAA is often accompanied by the occurrence of vascular inflammation (Sun et al. 2019). Among various inflammation-inducing factors, cytokines are the key mediators of inflammation (Wang et al. 2018a). The abnormal expression of miRNAs affecting translation is a principal consideration of anabolic inflammation and the aberrant remodeling of ECM in AAA cells (Pahl et al. 2012). According to a recent study, IFI16 can modulate the Caspase-1/IL-1/M CPIP1 pathway to impair VSMCs, implicating in the pathogenesis of AAA (Xue et al. 2020). Akshaya et al. has found that IL-1β-induced formation of the neutrophil extracellular trap is relevant with the germination of AAA (Meher et al. 2018). The results of this study were consistent with their's, the inflammatory factors IL-1β and MCP-1 were overtly upregulated in AngII-induced VSMCs, while the expression of miR-143 was downregulated. After the expression of miR-143 was elevated by experimental intervention, the transcription and translation levels of IL-1β and MCP-1 were significantly downregulated, which implied that miR-143 might play an important role in inhibiting the inflammatory response of AAA.

Although both miR-143-5p and miR-143-3p are developed from pre-miR-143, the base sequences of the two are different due to the different splicing sites. As a result, the downstream targeted regulatory genes of the two are also different (Jiang a et al. 2013). According to different regulatory mechanisms and results. For example, in gastric cancer, since the direct targeting relationship of miR-143-5p to COX-2, miR-143-5p showed a stronger tumor suppressive effect than miR-143-3p (Wu et al. 2013). In unruptured brain aneurysms, miR-145 was also significantly downregulated, as was miR-143. Bekelis et al. 2016). As another molecular link regulating vascular smooth muscle cell phenotypes, miR-145 inhibited oxidative stress and inflammatory responses in VSMCs and inhibited AAA progression by down-regulating downstream target genes (e.g., EGR1) (Lin et al. 2020). Interestingly, miR-143 and miR-145 are co-transcribed from the same gene. Therefore, they have been shown to be directly associated with a number of important cardiovascular diseases.

In the vasculature, MMPs could affect the migration, proliferation, and apoptosis of vascular smooth muscle cells, endothelial cells, and inflammatory cells, thereby producing effects on the intima, atherosclerosis, and the formation of aneurysms, which have been confirmed in previous studies (Cai & Wang 2017). In AAA and other diseases, the unusual augment in MMPs is a fundamental marker of ECM destruction. Upregulation of MMP-9 has also been identified as a critical event occurring during aneurysm growth. It has been stated that administrating AngII to VSMCs caused a high-expression of MCPIP1 and led to the up-regulation of MMP-2 and MMP-9 accordingly, but the silence of MCPIP1 reversed above (Xue et al. 2019). The polymorphism of MMP-13 may change its transcriptional activity and protein level, which is also a possible cause of AAA (Makrygiannis et al. 2019). Audrey et al. has found that among a series of increased MMPs in patients with PET-positive AAA (usually symptomatic and at high risk of rupture), the increase of MMP1 and MMP13 was particularly evident. They have advanced that there might be a synergistic degradation between MMP1 and MMP13, accelerating aneurysm wall thinning and eventual rupture (Courtois et al. 2013). In this study, we found that the expression of MMP-9 and MMP-13 was significantly inhibited after overexpression of miR-143 in AngII-VSMCs. That is to say, in AAA, miR-143 has the potential to retard the degradation of ECM, thereupon the risk of aneurysm wall rupture was reduced.

At the pathological level, apoptosis of VSMCs could facilitate the development of AAA by reducing the level of elastin (Greenway et al. 2020). In a new study, miR-7 inhibited by CDR1as increased Ckap4 expression to promote proliferation and hinder the apoptosis of VSMCs, resulting in VSMC remodeling and progression of AAA (Zhao et al. 2020). Through the TUNEL experiment, in the present study, the apoptosis of VSMCs was effectively improved when the expression of miR-143 was up-regulated in VSMCs due to AngII induction. According to our data, miR-143 might choke apoptosis of damaged VSMCs by inhibiting the activation of the TLR2/NF-κB pathway. Similar situations have been found in other AAA-related miRNAs. For example, one study has displayed that targeting P53 with miRNA-504 inhibited VSMC apoptosis, which exerts a certain role in preventing the formation of aneurysms (Cao et al. 2017). In another study, miR-15a-5p could negatively regulate SM C apoptosis by targeting Cdkn2B (Ga o et al. 2017). These studies all have provided new biological targets for the treatment of AAA with miRNAs.

The TLR2/NF-κB pathway is highly associated with inflammation in a variety of cancers and diseases. Through animal experiments, Wang et al. has demonstrated that miR-143 prevented Cr(VI)-induced carcinogenesis by counteracting the expression of IL-6, HIF-1α, and NF-κB p65 (Wang et al. 2019). According to a novel study, in AAA, the protein AEBP1 upregulated pro-inflammatory factors and MMPs by activating the NF-κB pathway (Ren et al. 2020). These are signs that AAA inflammation is sensitive to the activation of the NF-κB pathway. In the current study, we perceived that TLR2 expression was inhibited due to an overexpression of miR-143, so that the expression of the receptor of TLR2, NF-κB, was also hampered, thus leading to the inactivation of the TLR2/NF-κB pathway. The targeted relationship between miR-143 and TLR2 has been
verified in keratinocytes (Xia et al. 2016). Combined with the experimental results of this study, we speculate that miR-143 could inactivate the TLR2/NF-κB pathway to attenuate inflammation, ECM degradation, and apoptosis in damaged VSMCs. Therefore, we conjecture that miR-143 may act the role of an ‘anti-onco’ miRNA and result in anti-inflammatory factors in the progression of AAA.

Conclusion
In conclusion, the up-regulation of miR-143 expression could effectively inactivate the TLR2/NF-κB pathway in AngII-induced VSMCs, thereby alleviating VSMC inflammation, ECM degradation, and cell apoptosis. This finding may provide an effective biomarker for targeted therapy of AAA.

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Research concept and design: Z.W., F.L.; Collection and/or assembly of data: Z.W., F.L.; Data analysis and interpretation: Z.W., Z.C.; Writing the article: Z.W., G.L.; Critical revision of the article: G.L.; Final approval of article: Z.C., F.L.

Conflict of Interest
The authors declare no conflict of interest.

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