EXPERIMENTAL STUDY

Identification of Circ-FNDC3B, an Overexpressed circRNA in Abdominal Aortic Aneurysm, as a Regulator of Vascular Smooth Muscle Cells

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Summary
Circular RNAs (circRNAs) have been implicated in the dysfunction of vascular smooth muscle cells (VSMCs), which is linked with the development of abdominal aortic aneurysm (AAA). Herein, we explored the precise action of circRNA fibronectin type III domain containing 3B (circ-FNDC3B) in VSMC injury triggered by angiotensin II (Ang-II).

Circ-FNDC3B, microRNA (miR)-143-3p, and a disintegrin and metalloproteinase 10 (ADAM10) were quantified by quantitative real-time polymerase chain reaction or western blot assay. Ribonuclease R and subcellular localization assays were applied to characterize circ-FNDC3B. Cell viability, apoptosis, and proliferation were assessed by the Cell Counting Kit-8 assay, flow cytometry, and 5-Ethynyl-2’-Deoxyuridine assay, respectively. The levels of tumor necrosis factor alpha, interleukin-6, superoxide dismutase, and malonaldehyde were estimated by enzyme-linked immunosorbent assay. Direct relationship miR-143-3p and circ-FNDC3B or ADAM 10 was verified by dual-luciferase reporter and RNA immunoprecipitation assays.

Circ-FNDC3B was highly expressed in AAA tissues and Ang-II-treated VSMCs. Knocking down circ-FNDC3B alleviated Ang-II-induced VSMC injury. Mechanistically, circ-FNDC3B directly targeted miR-143-3p, and miR-143-3p was a downstream mediator of circ-FNDC3B in regulating cell injury induced by Ang-II. ADAM10 was directly targeted and inhibited by miR-143-3p. MiR-143-3p-mediated inhibition of ADAM10 relieved Ang-II-induced VSMC injury. Furthermore, circ-FNDC3B acted as a competing endogenous RNA for miR-143-3p to modulate ADAM10 expression.

Our findings suggested that circ-FNDC3B silencing ameliorated cytotoxicity triggered by Ang-II in VSMCs at least partially depending on the regulation of the miR-143-3p/ADAM10 axis.

Key words: Angiotensin II, ceRNA, miR-143-3p, ADAM10

Abdominal aortic aneurysm (AAA) is a common degenerative vascular pathology that is serious and life-threatening.1,2 Despite the development of ultrasonography screening programs, AAA is still an important cause of death in adults, especially in men over 65 years.3 The pathogenesis of AAA formation was complex and elusive, and dysfunction of vascular smooth muscle cells (VSMCs) has been recognized to be closely linked with the development of AAA.4 Knowing the mechanisms of VSMC dysfunction will provide a novel opportunity to design better preventive and therapeutic interventions against AAA.

As a new type of noncoding RNAs, circular RNAs (circRNAs) form by head-to-tail splicing of exons and introns in mammalian cells.5 Evidence is emerging that circRNAs operate as post-transcriptional modulators via competing for binding to shared microRNAs (miRNAs), highlighting their competing endogenous RNA (ceRNA) function.6 Several recent reports have discovered the essential regulation of circRNA-mediated ceRNA networks in human diseases, including AAA.6-8 Yang et al. uncovered that circRNA coiled-coil domain containing 66 worked as a modulator of VSMC apoptosis and growth through miR-342-3p-dependent regulation of CCDC66.9 Yue et al. underscored the repressive activity of circRNA core-binding factor subunit beta in VSMC apoptosis by binding to miR-28-5p to up-regulate LY6/PLAUR domain containing 3 and glutamate ionotropic receptor AMPA type subunit 4.10 CircRNA fibronectin type III domain containing 3B (circ-FNDC3B, circBase ID: hsa_circ_0006156), generated by the back-splicing of exons 5 and 6 of FNDC3B mRNA, was established as a potent onco-

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genic driver of papillary thyroid cancer and gastric cancer. Overexpression of circ-FNDC3B was also found in aortic tissues of patients with AAA. However, relatively little is known about the precise critical role of circ-FNDC3B in the development of AAA.

MiRNAs can regulate gene expression posttranscriptionally, and deregulated miRNAs have been reported in the development of AAA. MiR-143-3p was underexpressed in abdominal aortic tissues of patients with AAA using miRNA qPCR array analysis. However, the biological function of miR-143-3p in AAA development remains to be elucidated. Moreover, it is still unclear whether the action of circ-FNDC3B in the development of AAA is mediated by miR-143-3p.

Here, we showed that circ-FNDC3B modulated angiotensin II (Ang-II)-triggered cytotoxicity in human aortic VSMCs by influencing cell growth, inflammation, and oxidative stress. Furthermore, we identified that circ-FNDC3B functioned as a ceRNA for miR-143-3p to induce a disintegrin and metalloproteinase 10 (ADAM10), a strong contributor in AAA growth of mice model. These findings provided novel insights into the causal mechanisms of AAA development.

Methods

Human aorta tissue samples: Study subjects were 25 consecutive patients with AAA who underwent open surgery and 21 controls who underwent coronary artery bypass graft from May 2017 and February 2019 at The Second Affiliated Hospital of Soochow University. The clinical characteristics of these subjects are shown in Table I. The aortic specimens were obtained from these subjects with written informed consent to quantify the expression of circ-FNDC3B, miR-143-3p, and ADAM10 and to isolate primary VSMCs. The study protocol was approved by the Ethics Committee of The Second Affiliated Hospital of Soochow University.

Isolation of primary VSMCs: Primary VSMCs were isolated from human aortic tissue samples as reported elsewhere. After the connective tissues of the adventitia and inner membrane were removed, the blood vessels were cut longitudinally, and the smooth muscles were cut into a piece of 1-2 mm³, followed by the incubation with collagenase type 2 (Gibco, Uppsala, Sweden). After the digested endothelial cells were washed by PBS, the isolated cells were maintained in 15% FBS DMEM/F12 (Gibco). The isolated VSMCs were used to measure circ-FNDC3B, miR-143-3p, and ADAM10 expression levels.

Table I. Characteristics of 25 Patients with AAA and 21 Controls in This Study

| Parameters                              | Control group (n = 21) | AAA group (n = 25) |
|-----------------------------------------|-----------------------|-------------------|
| Gender (male/female)                    | 13/8                  | 18/7              |
| Age (years)                             | 56.9 ± 7.6            | 59.9 ± 8.3        |
| BMI                                     | 24.2 ± 2.2            | 27.9 ± 3.1        |
| Current smoking (male/female)           | 5/0                   | 12/0              |
| Maximum aneurysm diameter (cm)          | NA                    | 6.2 ± 0.6         |
| Thrombus volume (cm³)                   | NA                    | 9.1 ± 2.1         |
| Aneurysm neck length (cm)               | NA                    | 0.9 ± 0.2         |

Cell culture and Ang-II treatment: Human aortic VSMCs (#PCS-100-012) supplied from the American Type Culture Collection (ATCC, Manassas, VA, USA) were propagated using the VSMC Growth Kit (ATCC) in a 37°C incubator in the presence of 5% CO₂.

For Ang-II treatment, VSMCs were plated at 5 × 10⁶ cells per 60-mm dish and subsequently incubated for 48 hours in the culture medium with or without Ang-II (Sigma-Aldrich, Saint-Aubin, France) at 5, 10, or 15 μM.

Plasmids: Human circ-FNDC3B (hsa_circ_0006156) sequence and the corresponding scrambled control, human ADAM10 (accession no. AF009615.1) encoding sequence and a nontarget control sequence, circ-FNDC3B fragment harboring the miR-143-3p pairing sites or mismatched seed sequence, and ADAM10 3’UTR and its mutation in the miR-143-3p pairing region were synthesized by BGI (Shenzhen, China). Circ-FNDC3B sequence and the corresponding control were individually subcloned into the pCD5-ciR vector (Geneseed, Guangzhou, China) with EcoRI and BamHI sites to create circ-FNDC3B overexpression plasmid and pCD5-ciR negative control. ADAM 10 encoding sequence and the nontarget control sequence were individually subcloned into the pcDNA3.1 vector (Life Technologies, Abingdon, UK) opened with NotI and XhoI sites to produce ADAM10 overexpression plasmid and pcDNA negative control. Circ-FNDC3B fragment, ADAM10 3’UTR, and mutated 3’UTR were individually inserted with XhoI and NotI sites into the 3’UTR region of Renilla luciferase reporter gene in the psiCHECK-2 vector (Promega, Mannheim, Germany) to construct luciferase reporter plasmids.

Cell transfection: Circ-FNDC3B-siRNA (si-circ-FNDC3 B), ADAM10-siRNA (si-ADAM10), and the corresponding control siRNAs (si-NC and si-con), control miRNA mimic (miR-NC) and miR-143-3p mimic, and control miRNA inhibitor (anti-miR-NC) and miR-143-3p inhibitor (anti-miR-143-3p) were provided by Ribobio (Guangzhou, China), and their details are shown in Supplemental Table. VSMCs were seeded at 5 × 10⁶ cells per 60-mm dish 18 hours before transfection using Lipofectamine 2000 (Life Technologies) with 100 nM siRNA, 30 nM miRNA mimic, 50 nM miRNA inhibitor, or 200 ng of plasmid as per the accompanying recommendation. Cells were harvested at 24 hours thereafter and subjected to Ang-II treatment.

Preparation of total RNA and protein: For the preparation of RNA and protein from tissue samples and cultured cells, we used the RNA STAT-60 reagent based on the guidance of the manufacturer (Amsbio, Lake Forest, CA,
USA). RNA purity and quantity were gauged spectrophotometrically, and protein concentration was evaluated using the DC-Assay Kit as recommended by the manufacturer (Bio-Rad, Dalian, China) with PrimeScript RT Reagent Kit (TaKaRa) and subjected to qRT-PCR using SYBR Green Mix (TaKaRa) and specific primers (described in Supplemental Table). For miR-143-3p analysis, matured miRNAs (500 ng) were used for cDNA preparation with TaKaRa RT Reagent Kit and stem-loop RT primer (TaKaRa), and qRT-PCR was done using TaqMan miRNA Assays (Life Technologies). The Chromo4 PCR System with Cell quest software (BD Biosciences) was used for all qRT-PCR experiments. Using Assays (Life Technologies). The Chromo4 PCR System with Cell quest software (BD Biosciences, Oxford, UK). Apoptotic cells were scored using the FACS-Vantage (FITC)-labeled Annexin V using an Assay Kit as per the manufacturer's recommendation (BD Biosciences, Cambridge, UK). Whereafter, lysates were incubated with antibody against Argonaute 2 (Ago2, ab186733, 1:50 dilution) or IgG isotype control (ab172730, 1:100 dilution; all from Abcam) loading buffer. Anti-mouse (ab6789, 1:10,000 dilution; Abcam) or anti-rabbit (ab6721, 1:10,000 dilution; Abcam) immunoglobulin-antibody labeled by horseradish peroxidase was used as secondary antibody. The LAS-4000 Mini Image Analyzer (Fuji Film, Minamiashigara, Japan) was used for data analysis after the application of enhanced chemiluminescence (GE Healthcare, Little Chalfont, UK).

Subcellular localization assay: For the preparation of cytoplasmic and nuclear RNAs from Ang-II-treated VSMCs, we applied the Cytoplasmic & Nuclear RNA Purification Kit as recommended by the manufacturer (Norgen Biotek, Thorold, ON, Canada). GAPDH and U6 served as cytoplasmic and nuclear controls, respectively.

Cell viability, apoptosis, and proliferation assays: The viability of VSMCs after various treatments was estimated using the Cell Counting Kit-8 (CCK-8) colorimetric assay based on the guidance of the manufacturer (MedChemExpress, Shanghai, China). The Infinite M200 reader (Tecan, Männedorf, Switzerland) was used for absorbance measurement at 450 nm. The apoptosis of treated VSMCs was assessed by flow cytometry after double staining with propidium iodide (PI) and fluorescein isothiocyanate (FITC)-labeled Annexin V using an Assay Kit as per the manufacturing recommendation (BD Biosciences, Oxford, UK). Apoptotic cells were scored using the FACS-Vantage System with Cell quest software (BD Biosciences). Assessment of proliferation of cultured VSMCs was conducted by 5-Ethynyl-2'-Deoxyuridine (EDU) assay using EDU DNA Cell Proliferation Kit (RiboBio) and 4',6-diamidino-2-phenyldirole (DAPI) for staining of nuclei as described. A fluorescent microscope (Olympus, Tokyo, Japan) was applied to photograph and analyze the stained cells.

Enzyme-linked immunosorbent assay (ELISA): The production levels of tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), superoxide dismutase (SOD), and malondialdehyde (MDA) in cultured VSMCs were estimated by routine ELISA technique with Human TNF-α ELISA Kit (Life Technologies), Human IL-6 ELISA Kit (Abcam), Human SOD ELISA Kit (Abcam), and Human MDA ELISA Kit (Abbkine, Wuhan, China), respectively.

Bioinformatics and dual-luciferase reporter assay: Analyses for the targeted miRNAs of circ-FNDC3B and miRNA-binding sites in the 3'UTR region were conducted using the computer algorithm ENCORI (http://starbase.sys.u.edu.cn/). Approximately 5 × 10⁵ VSMCs in 24-well culture dishes were co-transfected with 500 ng of luciferase reporter plasmid (described above) and 50 nM of miRNA mimic. The cells were harvested at 24 hours thereafter, and dual-luciferase reporter assay was done as recommended by the manufacturer (Promega).

RNA immunoprecipitation (RIP) assay: Lysates were obtained by lysing VSMCs (4°C, 1 hour) in RIPA buffer (Life Technologies) as per the accompanying instructions. Whereafter, lysates were incubated with antibody against Argonaute 2 (Ago2, ab186733, 1:50 dilution) or IgG isotype control (ab172730, 1:100 dilution; all from Abcam) and protein A/G magnetic beads (MedChemExpress) overnight at 4°C. We extracted total RNA from the beads for quantification of circ-FNDC3B, miR-143-3p, and ADAM10 mRNA.

Statistical analysis: Data were analyzed using a two-tailed Student’s t-test or one-way analysis of variance with Tukey-Kramer multiple comparison test. The mean ± standard deviation of at least three independent experiments performed in quintuplicate was presented. P < 0.05 was regarded as significant (*P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001). Expression correlations among circ-FNDC3B, miR-143-3p, and ADAM10 in AAA tissues and the correlation between circ-FNDC3B expression and AAA diameter were evaluated by the Pearson’s correlation coefficient.

Results

Circ-FNDC3B is up-regulated in AAA tissues and Ang-II-treated VSMCs: We firstly used qRT-PCR to examine the expression of circ-FNDC3B in the AAA aorta tissues. Circ-FNDC3B expression was higher in AAA tissues than in normal aorta tissues (Figure 1A). We also isolated the VSMCs from the aorta AAA tissues and assayed for circ-FNDC3B expression. Remarkably, VSMCs under AAA exhibited higher levels of circ-FNDC3B than the normal controls (Supplemental Figure 1A). Interestingly, in VSMCs, Ang-II induced circ-FNDC3B expression in a dose-dependent manner (Figure 1B and Supplemental Figure 2). To evaluate the stability of circ-FNDC3B, we per-
Figure 1. Circ-FNDC3B is overexpressed in AAA tissues and Ang-II-treated VSMCs. A: qRT-PCR analysis of circ-FNDC3B in a panel of AAA aorta tissues (n = 25) and normal aorta tissues (n = 21). B: Relative circ-FNDC3B expression by qRT-PCR assay in VSMCs treated with (0, 5, 10, 15 μM) of Ang-II for 48 hours. n = 3 independent biological replicates. C: RNase R assay in Ang-II-treated VSMCs. n = 3 independent biological replicates. D: Subcellular localization assay in Ang-II-treated VSMCs. n = 3 independent biological replicates. **P < 0.01 or ****P < 0.0001.

formed RNase R assays. We observed that the treatment of RNase R markedly reduced the level of the corresponding linear FNDC3B, whereas circ-FNDC3B was refractory to RNase R (Figure 1C). We then adopted subcellular localization assays to examine the localization of circ-FNDC3B in Ang-II-treated VSMCs. As shown in Figure 1D, circ-FNDC3B was mainly present in the cytoplasm. Additionally, circ-FNDC3B expression was positively correlated with the diameter of AAA in these patients (Table II and Supplemental Figure 3). These data together suggested that up-regulated circ-FNDC3B might be correlated with the development of AAA.

Silencing of circ-FNDC3B alleviates cell injury induced by Ang-II: To examine the biological effect of circ-FNDC3B on VSMC cell functional properties, we performed “phenocopy” silencing by siRNA targeting circ-FNDC3B (si-circ-FNDC3B) in Ang-II-treated cells. Transient transfection of si-circ-FNDC3B significantly reduced circ-FNDC3B expression in Ang-II-treated VSMCs compared with the si-NC control (Figure 2A). Knocking down circ-FNDC3B markedly rescued Ang-II-mediated cell viability defect (Figure 2B). Moreover, silencing endogenous circ-FNDC3B suppressed the pro-inflammatory cytokines IL-6 and TNF-α production and cell apoptosis, which were induced by Ang-II (Figure 2C and D). In addition, circ-FNDC3B loss-of-function strikingly abolished Ang-II-mediated suppression on cell proliferation (Figure 2E). Western blot data showed that Ang-II increased pro-apoptotic protein Bax level and inhibited anti-apoptotic protein Bcl-2 expression in VSMCs, and these effects were counteracted by circ-FNDC3B loss-of-function (Figure 2F). Silencing endogenous circ-FNDC3B remarkably abrogated Ang-II-mediated SOD level inhibition and MDA content enhancement in VSMCs (Figure 2G and H). Additionally, circ-FNDC3B depletion did not affect cell viability and proliferation in VSMCs treated without Ang-II (Supplemental Figure 4). Taken together, these data implied that circ-FNDC3B silencing relieved Ang-II-triggered cell injury by regulating cell proliferation, apoptosis, inflammation, and oxidative stress.

Circ-FNDC3B directly targets miR-143-3p: To understand how circ-FNDC3B modulated Ang-II-triggered cell injury, we considered its downstream miRNAs that potentially bound to circ-FNDC3B. The computer algorithm ENCORI predicted that circ-FNDC3B harbored a putative pairing sequence for miR-143-3p (Figure 3A). To test this possibility, we attached circ-FNDC3B fragment encompassing the miR-143-3p pairing sites or miss-matched seed sequence into the 3’UTR of Renilla luciferase reporter gene and compared reporter activities in VSMCs. Addition of the circ-FNDC3B fragment harboring the seed sequence to the luciferase reporter strongly suppressed expression in VSMCs, and the repression was rescued by the mutation in the seed sites (Figure 3B). To validate the
endogenous correlation between circ-FNDC3B and miR-143-3p in VSMCs, we conducted RIP experiments and found that the enrichment levels of circ-FNDC3B and miR-143-3p were simultaneously augmented by Ago2 antibody (Figure 3C). The results of qRT-PCR also revealed the underexpression of miR-143-3p in AAA tissues, VSMCs isolated from the AAA aorta tissues, and Ang-II-treated VSMCs (Figure 3D, E, and Supplemental Figure 1B). Intriguingly, we found a strong negative correlation between miR-143-3p and circ-FNDC3B levels in the AAA.
gonucleotide of mature miR-143-3p (anti-miR-143-3p) in this, we knocked down miR-143-3p using antisense oli-
gi-II-evoked cell damage by miR-143-3p. To address the issue of miR-143-3p mediated by circ-FNDC3B, we decided to examine whether circ-FNDC3B could modulate cell injury induced by Ang-II: MiR-143-3p is a mediator of circ-FNDC3B in modulating expression by binding to miR-143-3p. Notably, overexpression of circ-FNDC3B augmented miR-143-3p level (Figure 3H). All these results established that circ-FNDC3B regulated miR-143-3p expression, and silencing endogenous circ-FNDC3 B augmented miR-143-3p expression plasmid transfection (Figure 3G) inhibited miR-143-3p, and the sites of target mutation (MUT-circ-FNDC3B) or mutation (WT-circ-FNDC3B) luciferase reporters with miR-143-3p mimic or mimic control. n = 3 independent biological replicates. C: RIP experiments in VSMCs using antibody against Ago2 or isotype IgG. n = 3 independent biological replicates. D: qRT-PCR analysis of miR-143-3p in a panel of AAA aorta tissues (n = 25) and normal aorta tissues (n = 21). E: Relative miR-143-3p level in VSMCs treated with (0, 5, 10, 15 μM) of Ang-II for 48 hours. n = 3 independent biological replicates. F: The Pearson’s correlation coefficient for the correlation between miR-143-3p and circ-FNDC3B levels in AAA aorta tissues. G: qRT-PCR analysis of circ-FNDC3B in control- or Ang-II-treated VSMCs transfected with or without pCD5-ciR control plasmid or circ-FNDC3B overexpression plasmid. n = 3 independent biological replicates. H: The expression level of miR-143-3p by qRT-PCR analysis in control- or Ang-II-treated VSMCs transfected with or without si-NC, si-circ-FNDC3B, pCD5-ciR control plasmid, or circ-FNDC3B overexpression plasmid. n = 3 independent biological replicates. **P < 0.01, ***P < 0.001, or ****P < 0.0001.

**Table II.** Correlation Between Circ-FNDC3B Expression and AAA Diameter

| Characteristics         | Circ-FNDC3B expression |  |  | P value |
|-------------------------|------------------------|---|---|---------|
| Maximum aneurysm diameter | Low (n = 12) | High (n = 13) |  |  |  |
| ≤ 5 cm                  | 11                     | 8  | 3  | 0.0472* |
| > 5 cm                  | 14                     | 4  | 10 |  |  |

*aP < 0.05 by Chi-square test.

Figure 3. Circ-FNDC3B directly targets miR-143-3p. A: Sequence of miR-143-3p, circ-FNDC3B fragment showing putative pairing sites for miR-143-3p, and the sites of target mutagenesis. B: Dual-luciferase reporter assays in VSMCs co-transfected with circ-FNDC3B wild-type (WT-circ-FNDC3B) or mutant (MUT-circ-FNDC3B) luciferase reporters with miR-143-3p mimic or mimic control. n = 3 independent biological replicates. 

circ-FNDC3B-silencing VSMCs under Ang-II treatment, which expressed high level of miR-143-3p (Figure 4A). Remarkably, reduced level of miR-143-3p abolished si-circ-FNDC3B-mediated cell viability enhancement (Figure 4B), inflammation repression (Figure 4C), and apoptosis suppression (Figure 4D), as well as proliferation promotion (Figure 4E) in Ang-II-treated VSMCs. Western blot data also confirmed the reverse effect of miR-143-3p down-regulation on si-circ-FNDC3B-mediated apoptosis suppression in Ang-II-treated VSMCs, as presented by the alteration of Bax and Bcl-2 levels (Figure 4F). Furthermore, reduced expression of miR-143-3p markedly counteracted si-circ-FNDC3B-mediated SOD increase and MDA decrease in Ang-II-treated VSMCs (Figure 4G and H). Together, these results suggested that the alleviative effect of circ-FNDC3B silencing on Ang-II-induced cyto-
ROLE OF CIRC-FNDC3B IN VSMCs

Figure 4. Circ-FNDC3B silencing ameliorates Ang-II-evoked cell damage by up-regulating miR-143-3p. VSMCs were transfected with or without si-circ-FNDC3B, si-NC, si-circ-FNDC3B + anti-miR-NC, or si-circ-FNDC3B + anti-miR-143-3p and then treated with or without 10 μM of Ang-II for 48 hours. A: The expression of miR-143-3p in treated VSMCs by qRT-PCR analysis. B: CCK-8 assay for viability of treated VSMCs. C: The production levels of IL-6 and TNF-α in treated VSMCs by ELISA assay. D: Apoptosis of treated VSMCs by flow cytometry. E: EDU assay for proliferation of treated VSMCs. F: Western blot showing the levels of Bax and Bcl-2 in treated VSMCs. G and H: SOD and MDA levels in treated VSMCs using the assay kits. n = 3 independent biological replicates. *P < 0.05, **P < 0.01, ***P < 0.001, or ****P < 0.0001.

toxicity might be in part due to up-regulation of miR-143-3p.

ADAM10 is directly targeted and suppressed by miR-143-3p: To further understand the role of miR-143-3p, we used computer algorithm ENCORI to help identify its molecular targets and found that the 3'UTR of ADAM10 harbored a binding region for miR-143-3p (Figure 5A). To establish the direct relationship between miR-143-3p and ADAM10, we generated ADAM10 3'UTR wild-type or mutant-type reporter constructs and tested them by luciferase activity. Co-transfection of the wild-type reporter and miR-143-3p mimic into the VSMCs produced lower luciferase activity than cells co-transfected with control mimic (Figure 5B). However, site-directed mutation markedly abolished the suppression of miR-143-3p on reporter gene expression (Figure 5B), demonstrating that ADAM10 was targeted by miR-143-3p. The enrichment levels of miR-143-3p and ADAM10 were synchronously elevated by Ago2 antibody as detected by RIP assays (Figure 5C). Moreover, qRT-PCR and western blot analyses showed that ADAM10 mRNA and protein levels were clearly up-regulated in the AAA aorta tissues and VSMCs isolated from the AAA aorta tissues compared with the normal controls (Figure 5D, E, and Supplemental Figure 1C). Consistently, in VSMCs, Ang-II-induced the expression of ADAM10 protein in a dose-dependent manner (Figure 5F). We then undertook to examine the expression correlation between ADAM10 and miR-143-3p in AAA tissues and observed that ADAM10 mRNA negatively correlated with miR-143-3p (Figure 5G). In support of these results, we decided to evaluate whether miR-143-3p regulated ADAM10 expression in Ang-II-treated VSMCs. The effectiveness of miR-143-3p mimic and anti-miR-143-3p in increasing and reducing miR-143-3p expression, respectively, was confirmed by qRT-PCR (Figure 5H). As would be expected, ADAM10 protein expression was strikingly repressed by miR-143-3p overexpression and augmented as a result of miR-143-3p depletion in Ang-II-treated VSMCs (Figure 5I). All these results strongly established the notion that ADAM10 was a direct target of miR-143-3p.

Silencing endogenous ADAM10 relieves cell injury induced by Ang-II: To elucidate the detailed effect of ADAM10 on Ang-II-triggered cytotoxicity in VSMCs, we performed loss-of-function experiments with si-ADAM10 designed to prevent up-regulation of ADAM10 protein in
Ang-II-treated VSMCs. The effectiveness of si-ADAM10 in reducing ADAM10 protein expression was verified by western blot (Figure 6A). Strikingly, knockdown of ADAM10 protein abrogated Ang-II-mediated anti-viability (Figure 6B), pro-inflammation (Figure 6C), pro-apoptosis (Figure 6D), and anti-proliferation (Figure 6E) effects in VSMCs. Additionally, silencing endogenous ADAM10 significantly reversed the impact of Ang-II on Bax, Bcl-2, SOD, and MDA production levels in VSMCs (Figure 6F-H). Taken together, these findings suggested that Ang-II-induced cell injury might be due to enhanced expression of ADAM10.
Knockdown of ADAM10 protein alleviates cell injury induced by Ang-II: Having demonstrated inhibition of ADAM10 by miR-143-3p, we wanted to evaluate whether inhibition of ADAM10 was responsible for miR-143-3p-mediated regulation in Ang-II-evoked cell injury. An overexpression plasmid for ADAM10 was used to up-regulate ADAM10 in miR-143-3p-overexpressing VSMCs under Ang-II, and its effectiveness was confirmed by western blot (Figure 7A). As expected, elevated expression of ADAM10 markedly abrogated miR-143-3p overexpression-mediated cell viability promotion (Figure 7B), inflammation reduction (Figure 7C), apoptosis suppression (Figure 7D), and proliferation promotion (Figure 7E) in Ang-II-treated VSMCs. Additionally, miR-143-3p overexpression led to a clear increase in the levels of Bcl-2 and SOD, as well as a strong reduction in the levels of Bax and MDA in Ang-II-treated VSMCs, and these effects were abrogated by elevated expression of ADAM10 (Figure 7F-H). All these results indicated that miR-143-3p overexpression alleviated Ang-II-induced cell damage by inhibiting ADAM10.

Circ-FNDC3B controls ADAM10 expression by targeting miR-143-3p: On the basis of the preceding observations, we also investigated whether circ-FNDC3B could modulate ADAM10 expression by the ceRNA function. Notably, in Ang-II-treated VSMCs, silencing endogenous circ-FNDC3B led to a clear decrease in the levels of ADAM10 mRNA and protein, and this effect was abrogated by miR-143-3p knockdown (Figure 8A and B). All these data strongly pointed to the ceRNA function of circ-FNDC3B on regulating ADAM10 expression through miR-143-3p.

Discussion

CircRNA deregulation is a crucial component of human tumorigenic processes through both oncogenic and tumor-suppressive effects of circRNAs.21) Of these, circ-FNDC3B is overexpressed in papillary thyroid cancer and gastric cancer and has been causally linked to oncogenesis.11,12) Up-regulation of circ-FNDC3B has also been discovered in AAA tumors,6) yet its functional action underlying AAA development is poorly understood. These results reported here showed that circ-FNDC3B was highly expressed in Ang-II-treated VSMCs and knocking down circ-FNDC3B protected VSMCs from Ang-II-triggered cyto-
Figure 7. MiR-143-3p-mediated inhibition of ADAM10 relieves Ang-II-triggered cell injury. Western blot analysis of ADAM10 protein (A), CCK-8 assay of cell viability (B), IL-6 and TNF-α production by ELISA assay (C), flow cytometry for cell apoptosis (D), EDU assay of cell proliferation (E), Bax and Bcl-2 levels by western blot (F), and the production levels of SOD and MDA using the assay kits (G and H) in control- or Ang-II-treated VSMCs transfected with or without miR-143-3p mimic, miR-NC mimic, miR-143-3p mimic + ADAM10 overexpression plasmid (ADAM10), or miR-143-3p mimic + pcDNA control plasmid. n = 3 independent biological replicates. *P < 0.05, **P < 0.01, ***P < 0.001, or ****P < 0.0001.

Toxicity. Furthermore, we provided a novel molecular explanation for circ-FNDC3B-mediated regulation. Such analysis was hampered at present by the lack of in vivo experiments using AAA animal models.

The ceRNA function of circRNAs in human carcinogenesis has been proposed to sponge miRNAs.\(^22\) Our data indicated that circ-FNDC3B was mainly present in the cytoplasm, which provided the possibility for the endogenous relationship between circ-FNDC3B and miRNAs. By combining target prediction and expression modulation analysis, we first demonstrated that circ-FNDC3B directly targeted miR-143-3p, a down-regulated miRNA in AAA tissues.\(^15\) MiR-143-3p has been established as an anti-tumor factor in multiple cancers, such as pancreatic ductal adenocarcinoma, osteosarcoma, and nasal squamous cell carcinoma.\(^23-25\) Previous works also uncovered that several other circRNAs, such as circ_0003998 and circRNA casein kinase 1 gamma 3, acted as potent miR-143-3p sponges in human carcinogenesis.\(^26,27\)

ADAM10, an important transmembrane sheddase, plays a critical role in the development of human cancer when overexpressed.\(^28\) Targeting AMAD10 has been proposed as a promising anti-tumor strategy.\(^29,30\) Moreover, elevated expression of ADAM10 was discovered to be linked to the formation of aortic aneurysm.\(^17,31,32\) Our results first found that ADAM10 was directly targeted and suppressed by miR-143-3p. Furthermore, miR-143-3p-mediated suppression of ADAM10 alleviated cell damage evoked by Ang-II. Consistently, Jiao et al. uncovered the repressive effect of miR-103a on AAA growth of mice models depending on the regulation of ADAM10.\(^16\) Importantly, we first pointed to the ceRNA function of circ-FNDC3B on modulating ADAM10 expression through miR-143-3p. Unsatisfactorily, the Ang-II-induced cytotoxicity in human aortic VSMCs cannot accurately mimic the pathological characteristics of VSMCs involved in AAA development, which limited the investigation for the effect of the ceRNA network in AAA. With these findings in vi-
ROLE OF CIRC-FNDC3B IN VSMCs

Figure 8. Circ-FNDC3B modulates ADAM10 expression through miR-143-3p. VSMCs were transfected with or without si-NC, si-circ-FNDC3B, si-circ-FNDC3B + anti-miR-NC, or si-circ-FNDC3B + anti-miR-143-3p and then treated with 10 μM of Ang-II or control for 48 hours. A: qRT-PCR analysis of ADAM10 mRNA in treated VSMCs. n = 3 independent biological replicates. B: Western blot showing ADAM10 protein level in treated VSMCs. n = 3 independent biological replicates. **P < 0.01, ***P < 0.001, or ****P < 0.0001.

Figure 9. Schematic model of the circ-FNDC3B/miR-143-3p/ADAM10 ceRNA network in AAA development. In AAA tissues, circ-FNDC3B was up-regulated, and up-regulated circ-FNDC3B inhibited miR-143-3p expression. Then, down-regulated miR-143-3p resulted in increased level of ADAM10 protein. Finally, enhanced expression of ADAM10 destroyed VSMC viability and promoted inflammation and oxidative stress, thereby enhancing the development of AAA.

required to determine how the novel ceRNA network regulates AAA development in various experimental models.

To conclude, we showed that knocking down circ-FNDC3B ameliorated cytotoxicity triggered by Ang-II in VSMCs. We also outlined a novel ceRNA network in regulating Ang-II-evoked VSMC damage, providing a new principle for understanding the mechanisms of VSMC dysfunction, with potential implications for AAA development.

Disclosure

Conflicts of interest: The authors declare that they have no conflicts of interest.

Availability of data and materials: Please contact the correspondence author for the data request.

Ethics approval and consent to participate: Written informed consent was obtained from patients with approval by the Institutional Review Board of The Second Affiliated Hospital of Soochow University.

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30. Supplemetal Files

Supplemental Files

- Supplemental Table
- Supplemental Figures 1-4
- Please see supplemental files at https://doi.org/10.1536/ihj.21-186.