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Circ_0008285 silencing suppresses angiotensin II-induced vascular smooth muscle cell apoptosis in thoracic aortic aneurysm via miR-150-5p/BASP1 axis

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Abstract

Background: Vascular smooth muscle cells (VSMCs) are the predominant cell type of the aortic middle layer, the abnormal number or function of which has been demonstrated to have a role in thoracic aortic aneurysm (TAA). Here, this study aimed to identify the function of circ_0008285 in VSMC apoptosis.

Methods: Human VSMCs were treated with angiotensin II (Ang II) for functional experiments. Cell counting kit-8, 5-ethynyl-2′-deoxyuridine (EdU), and flow cytometry were applied for function analysis. The interaction between miR-150-5p and circ_0008285 or brain acid-soluble protein 1 (BASP1) was also evaluated by dual-luciferase reporter assay and RNA immunoprecipitation assay. Exosomes were isolated by the commercial kit.

Results: A highly expressed circ_0008285 was observed in the aortic tissues of TAA patients and Ang-II-induced VSMCs. Circ_0008285 deficiency dramatically reversed Ang-II-induced proliferation arrest and apoptosis promotion in VSMCs. Circ_0008285 functionally targeted miR-150-5p. MiR-150-5p inhibition attenuated the inhibitory effects of circ_0008285 silencing on Ang-II-evoked apoptosis in VSMCs. BASP1 was verified to be a target of miR-150-5p, and was proved to attenuate miR-150-5p-triggered apoptosis arrest in Ang-II-stimulated VSMCs. Additionally, extracellular circ_0008285 was packaged into exosomes, which could be transferred into the recipient cells.

Conclusion: Circ_0008285 silencing could suppress Ang-II-induced VSMCs apoptosis via miR-150-5p/BASP1 axis, adding further understanding of the pathogenesis of TAA.

KEYWORDS
BASP1, circ_0008285, miR-150-5p, thoracic aortic aneurysm, VSMC

INTRODUCTION

Thoracic aortic aneurysm (TAA) is the most common pathology of the thoracic aorta, resulting from the enlargement or dilation in the weakened area in the wall of a blood vessel. TAA is a rare event with an incidence of 5.9 cases per 100 000 persons annually; however, it has an extremely high mortality upon rupture.[1–3] Vascular smooth muscle cells (VSMCs) are the major component of the aortic middle layer, the abnormal number or function of which can lead to structural reconstruction of vascular wall, weaken elasticity and strengthen frigidity.[4] TAA is pathologically characterized by the disruption of VSMCs and destruction/remodeling of the vascular extracellular matrix.[5,6] Therefore, an in depth investigation of the molecular mechanism underlying VSMC apoptosis may provide new targets for TAA prevention.

Circular RNAs (circRNAs) are molecules with a circular configuration which lack 5′-3′ polarity and poly A tail.[7] CircRNAs processes sequence conservation,[8] and have been

reported to be extensively involved in regulating numerous biological processes.[9] Recently, circRNAs have been proposed to be involved in the pathogenesis and development of many diseases, such as cancer,[10] nervous system disease,[11] and cardiovascular disease.[12] Moreover, some studies have manifested that circRNAs participate in the progression of aortic aneurysm. For example, Yang et al. showed that circCCDC66 promotes the formation of abdominal aortic aneurysm (AAA) by inducing apoptosis in aortic VSMCs via upregulating CCDC66 expression.[13] CircCBFB has also been demonstrated to suppress VSMC apoptosis via upregulating LYPD3 and GRIA4 through sequestering miR-28-5p.[14] Circ_0008285 is derived from its parental gene CDYL in chr6: 4 891 946–4 892 613 with a length of 667 bp. It has been observed to be elevated in aortic samples of AAA.[15] However, the action of circ_0008285 in TAA remains unclear.

The renin-angiotensin system has been revealed to be involved in TAA.[16] Herein, this study used Ang II to stimulate VSMCs to mimic the injury of VSMCs during TAA in vitro, and then investigated the action of circ_0008285 on VSMC apoptosis. In addition, the mechanisms underlying the potential promoting role of circ_0008285 in VSMC loss were also studied.

METHODS

Tissues collection

Aortic tissues were obtained from TAA patients undergoing elective aortic surgery ($n = 27$). Normal control artery tissues were collected from brain-dead but heart beating organ donors ($n = 24$). The excised segments were stored at −80°C until use. All the participants had provided written informed consent before sample collection, and this study was approved by the Ethics Committee of Central China Fuwai Hospital of Zhengzhou University. The clinicopathological features of all participants are displayed in Table 1.

Cell culture

Human VSMCs were obtained from Procell, and then cultured in Dulbecco's modified Eagle's medium (DMEM: Procell) supplemented with 10% fetal bovine serum (FBS: Procell) and 1% penicillin/streptomycin (Solarbio) with 5% CO_2 at 37°C.

TABLE 1 Clinicopathological features of all participants.

Parameters	Control ($n = 24$)	TAA ($n = 27$)
Gender (male/female)	15/9	14/13
Age (years)	59.5 ± 7.2	61.2 ± 8.5
BMI	24.7 ± 1.9	26.8 ± 2.4
Current smoking (male/female)	7/0	16/0
Thrombus volume (cm^3)	NA	8.8 ± 1.5
Aneurysm neck length (cm)	NA	1.0 ± 0.3
Maximum aneurysm diameter (cm)	NA	5.9 ± 0.8

Abbreviation: TAA, thoracic aortic aneurysm.

Cell transfection and treatment

Transient transfection was conducted using lipofectamine 2000 (Invitrogen) with 100 nM circ_0008285-specific siRNA (si-circ_0008285), 50 ng pcDNA 3.1-BASP1 overexpression plasmid (BASP1) (Geneseed), 100 nM miR-150-5p mimic (miR-150-5p) or inhibitor (anti-miR-150-5p) (Ribobio) and equal content negative control (si-NC, pcDNA, miR-NC, or anti-miR-NC).

VSMCs were treated with increasing doses of Ang II (0, 5, 10, and 15 μM) (Solarbio) for 48 h to mimic the injury of VSMCs during TAA in vitro. The 10 μM Ang II was selected as the final concentration for functional analysis.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNAs were isolated from cells using TRIzol reagent (Invitrogen) in accordance with the manufacturer's protocols. Treatment with 5 U/μg RNase R (Solarbio) or mock was implemented at 37°C for 20 min to determine the stability of circRNA. Then cDNAs were synthesized using PrimeScript RT Master Mix kit (TaKaRa) and SYBR Premix Ex Taq II (TaKaRa) was applied for qRT-PCR. The amplification parameters were: 95°C for 10 min, 40 cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 1 min. Fold changes were calculated by $2^{-\Delta\Delta Ct}$ method and GAPDH or U6 acted as an endogenous control. The specific primers used are listed in Table 2.

Cell counting kit-8 (CCK-8) assay

VSMCs were reacted with 10 μL CCK-8 reagent (Beyotime) per well of 24-well plates according to the protocol, followed

TABLE 2 The specific primers used for qRT-PCR.

Name	Primers for qRT-PCR (5′-3′)	
hsa_circ_0008285	Forward	ACCCACTAGTGCCTCAGGTG
	Reverse	TGTCGTCCTCGCTGTCATAG
BASP1	Forward	CAACTGGCTCCTCGCTCC
	Reverse	TGAGCTTGCCTCCCATCTTG
miR-150-5p	Forward	GTATGAGTCTCCCAACCCTTGTA
	Reverse	CTCAACTGGTGTCGTGGAG
GAPDH	Forward	AAGGCTGTGGGCAAGGTCATC
	Reverse	GCGTCAAAGGTGGAGGAGTGG
U6	Forward	CTCGCTTCGGCAGCACATA
	Reverse	CGAATTTGCGTGTCATCCT

Abbreviation: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; qRT-PCR, quantitative reverse transcription-polymerase chain reaction.

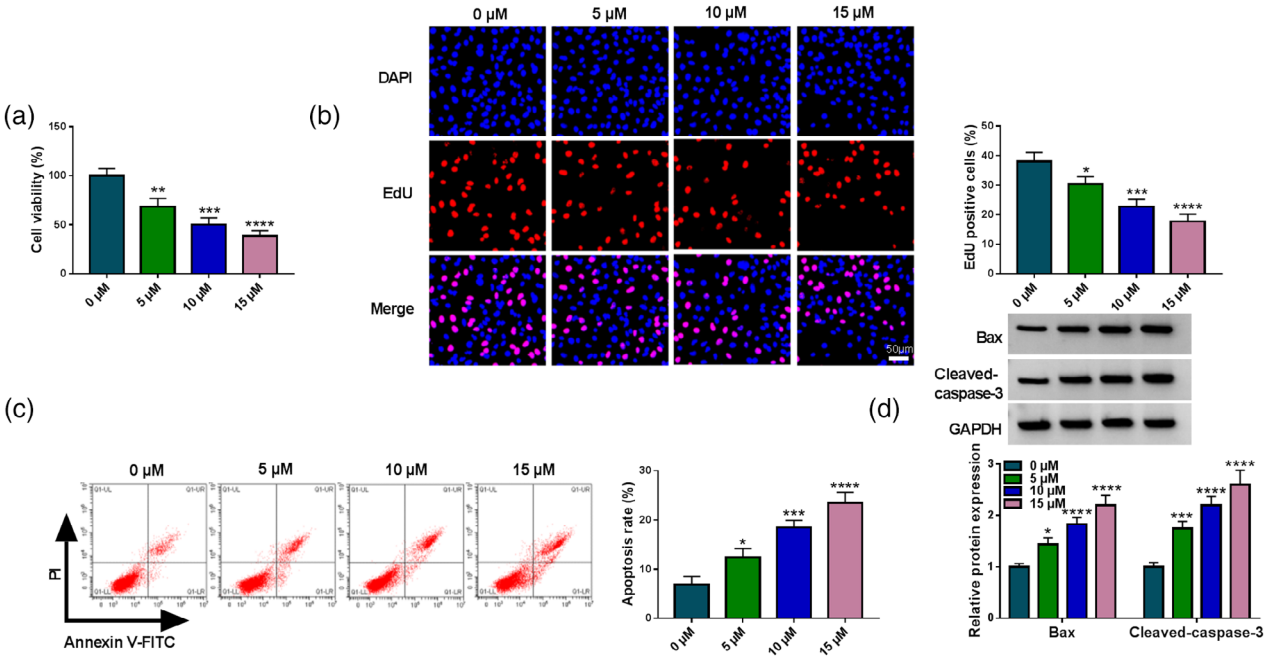

FIGURE 1 Ang-II treatment induces apoptosis of vascular smooth muscle cells (VSMCs). (a–d) VSMCs were exposed to different concentrations of Ang-II (0, 5, 10, and 15 μM) for 48 h. (a, b) Cell proliferation analysis was conducted using cell counting kit-8 (CCK-8) and 5-ethynyl-2′-deoxyuridine (EdU) assays. (c) Flow cytometry for cell apoptosis. (d) Western blotting analysis was performed to determine the levels of Bax and cleaved-caspase-3 in cells. $n = 3$. $^*p < 0.05$.

by assaying the absorbance at 490 nm with a microplate reader (Tecan, NanoQuant).

5-ethynyl-2′-deoxyuridine (EdU) assay

VSMCs were inoculated into a 96-well plate overnight and then incubated with 500 μL of 50 μmol/L EdU medium (RiboBio) for 2 h, followed by mixing with Apollo staining for 30 min and subsequent DAPI reaction fluid for 30 min in the dark. The EdU-positive cells were later observed.

Flow cytometry

Annexin V-FITC/PI staining kit (BestBio) was employed to determine cell apoptosis of VSMCs following the protocol. Finally, cell apoptosis was assessed using flow cytometry.

Western blotting

Radioimmunoprecipitation assay (RIPA: Beyotime) buffer was used to extract the protein which were then boiled and run on 10% SDS-PAGE, and subsequently transformed into the nitrocellulose membranes. The membranes were then incubated with primary antibodies (Abcam) which included cleaved-caspase-3 (ab2302, 1:1000), Bax (ab32503, 1:1000), CD63 (ab68418, 1:2000), CD9 (ab223052, 1:2000), BASP1 (ab214322, 1:1000), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (ab181602, 1:10 000) at 4°C overnight, and then interacted with secondary antibodies conjugated by HRP at 37°C for 2 h. Finally, proteins were visualized using an ECL reagent (Beyotime).

Dual-luciferase reporter assay

The fragments of circ_0008285 or BASP1 3′UTR comprising the binding sites of miR-150-5p and the site-directed mutagenesis in putative binding site were cloned into pmirGLO luciferase reporters to construct wild-type (WT) or mutated (MUT) luciferase reporter vector (WT-circ_0008285 or BASP1 3′UTR or MUT-circ_0008285 or BASP1 3′UTR). Later on, 100 ng of constructed pmirGLO luciferase vector with 20 pmol of miR-150-5p or miR-NC were cotransfected into VSMCs, followed by the detection of luciferase activity 48 h later.

Radioimmunoprecipitation assay (RIP) assay

VSMCs were homogenized in complete RNA lysis buffer (Millipore), followed by interacting with the magnetic beads and 5 μg Ago2 or a negative control IgG antibody for 4 h at 37°C. After washing, the RNA immunoprecipitation was purified for qRT-PCR.

Exosome (exo) isolation

Exosomes were isolated from culture media of VSMCs using ExoQuick exosome precipitation solution

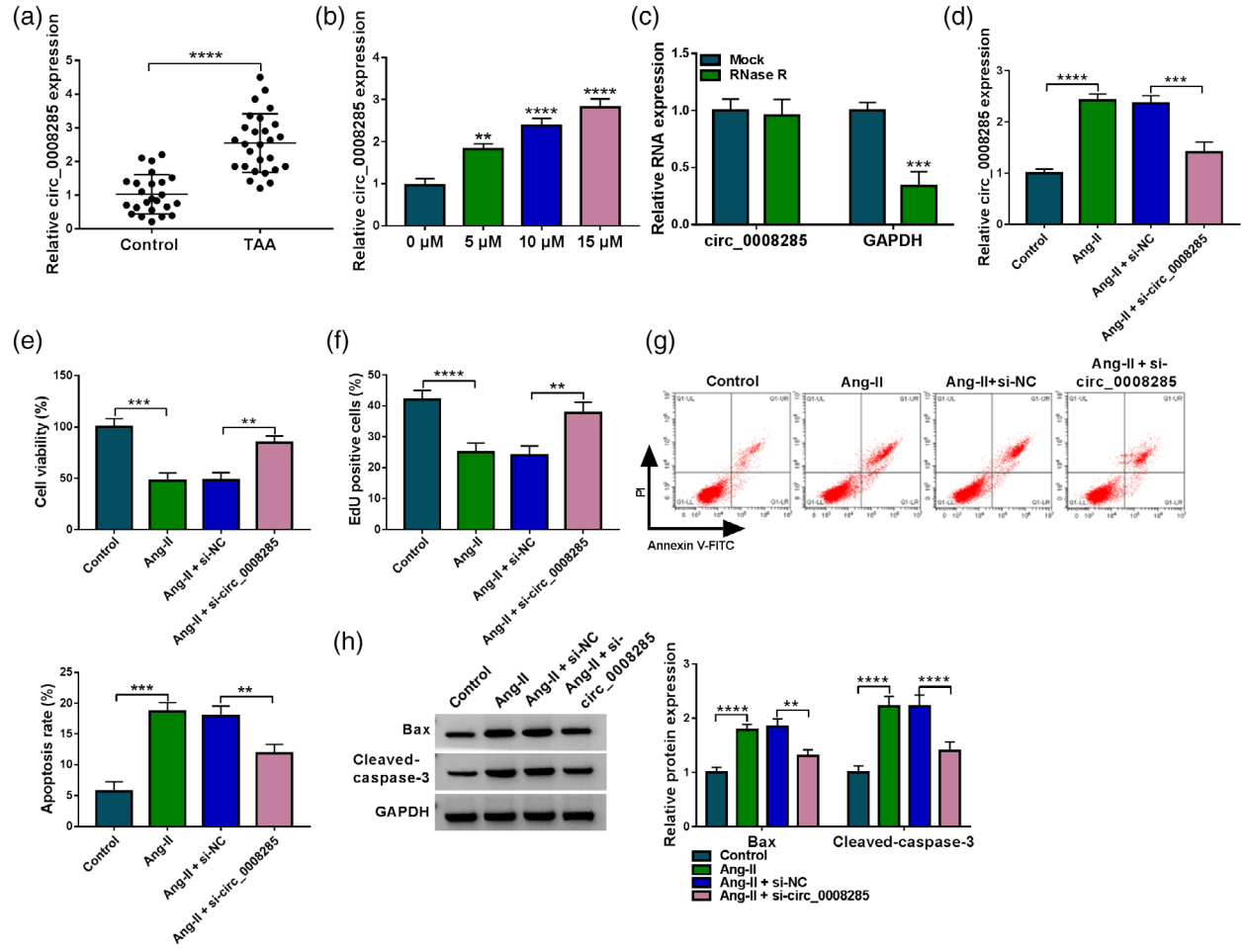

FIGURE 2 Circ_0008285 silencing attenuates Ang-II-induced apoptosis of vascular smooth muscle cells (VSMCs). (a) Circ_0008285 expression in aortic tissues of thoracic aortic aneurysm (TAA) patients ($n = 27$) and normal donors ($n = 24$). (b) Circ_0008285 level in VSMCs exposed to Ang-II for 48 h. (c) Stability analysis of circ_0008285 by RNase R. (d–h) VSMCs were transfected with si-circ_0008285 or si-NC and then stimulated with 10 μM Ang-II for 48 h. (d) The level of circ_0008285. (e, f) Cell proliferation analysis. (g) Cell apoptosis detection. (h) Levels of Bax and cleaved-caspase-3. $n = 3$. $^*p < 0.05$.

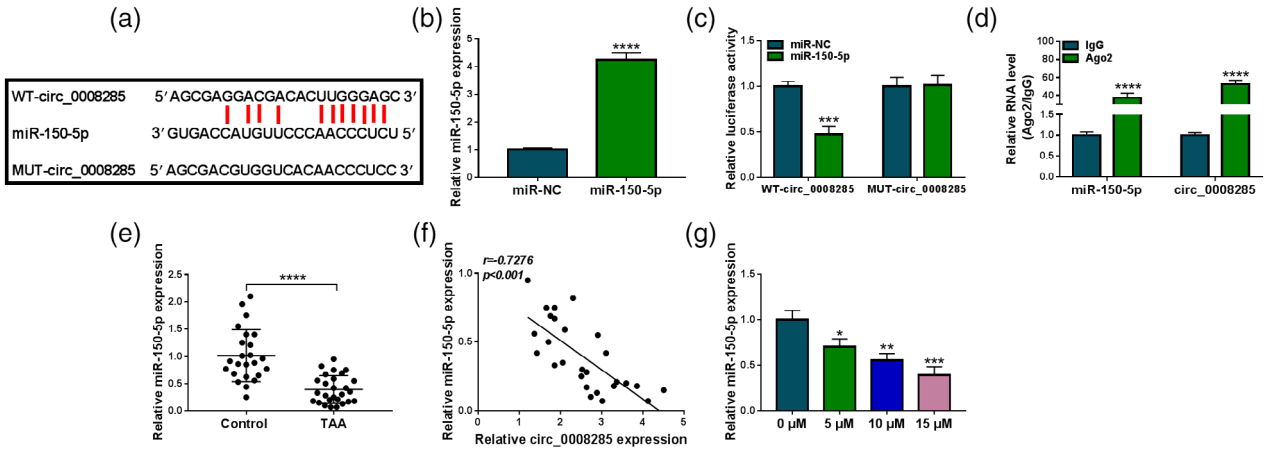

FIGURE 3 Circ_0008285 targets miR-150-5p. (a) The putative conserved target site of miR-150-5p on circ_0008285. (b) The transfection efficiency validation. (c, d) The binding analyses between circ_0008285 and miR-150-5p. (e) Abundances of miR-150-5p in aortic tissues of thoracic aortic aneurysm (TAA) patients ($n = 27$) and normal donors ($n = 24$). (f) Correlation analysis in aortic tissues. (g) MiR-150-5p expression in vascular smooth muscle cells (VSMCs) treated with Ang-II. $n = 3$. $^*p < 0.05$.

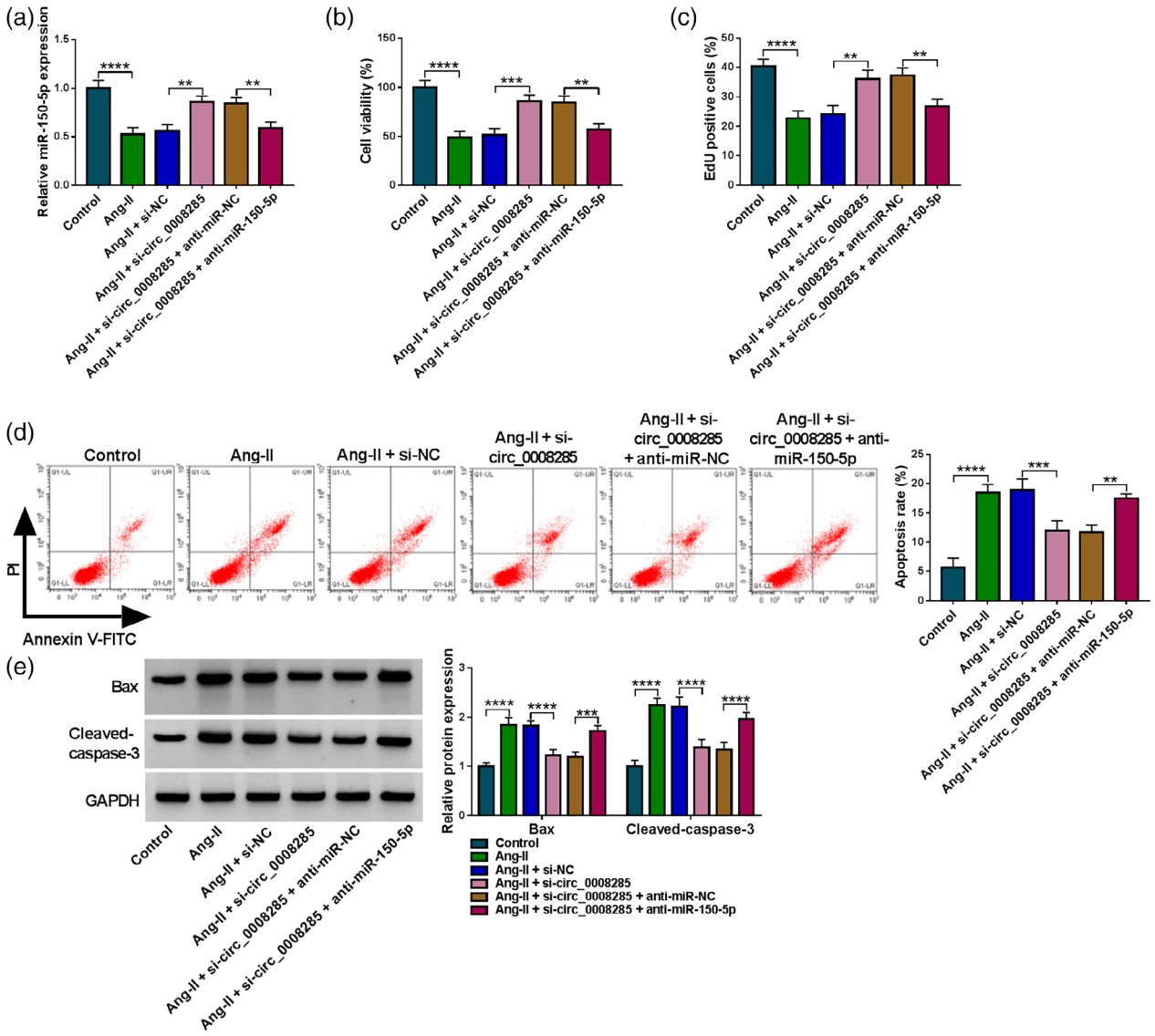

FIGURE 4 Circ_0008285 silencing attenuates Ang-II-induced apoptosis of vascular smooth muscle cells (VSMCs) by miR-150-5p. (a–e) VSMCs were cotransfected with si-circ_0008285 and anti-miR-150-5p, and then exposed to 10 μM Ang-II for 48 h. (a) MiR-150-5p expression measurement. (b, c) Cell proliferation analysis. (d) Detection of cell apoptosis. (e) Contents of Bax and cleaved-caspase-3. $n = 3$. $^{*}p < 0.05$.

(System Biosciences) following the manufacturer's protocol. The exosomes were then dissolved in ice-cold PBS for further analysis. The structures of exosomes were identified by electron microscopy, and exosome marker protein CD9 and CD63 were determined by western blotting.

Statistical analysis

All data are presented as the mean ± SD. The normal distribution and similarity of variance was tested, and analysis of variance followed by post hoc testing was performed for the comparisons of multiple groups. The comparisons between two groups were conducted by *t*-test, and, if not normally distributed, by Mann-Whitney test. Pearson's correlation was applied for correlation analysis. *p*-values < 0.05 were considered statistically significant ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$). Unless otherwise noted, *n* was the number of individual experiments.

RESULTS

Ang II treatment induces apoptosis of VSMCs

VSMCs were exposed to different concentrations of Ang II (0, 5, 10, and 15 μM) for 48 h. It was proved that Ang II treatment dose-dependently reduced cell viability rate and DNA synthesis activity in VSMCs (Figure 1a,b). Meanwhile, the apoptosis of VSMCs was found to be dose-dependently

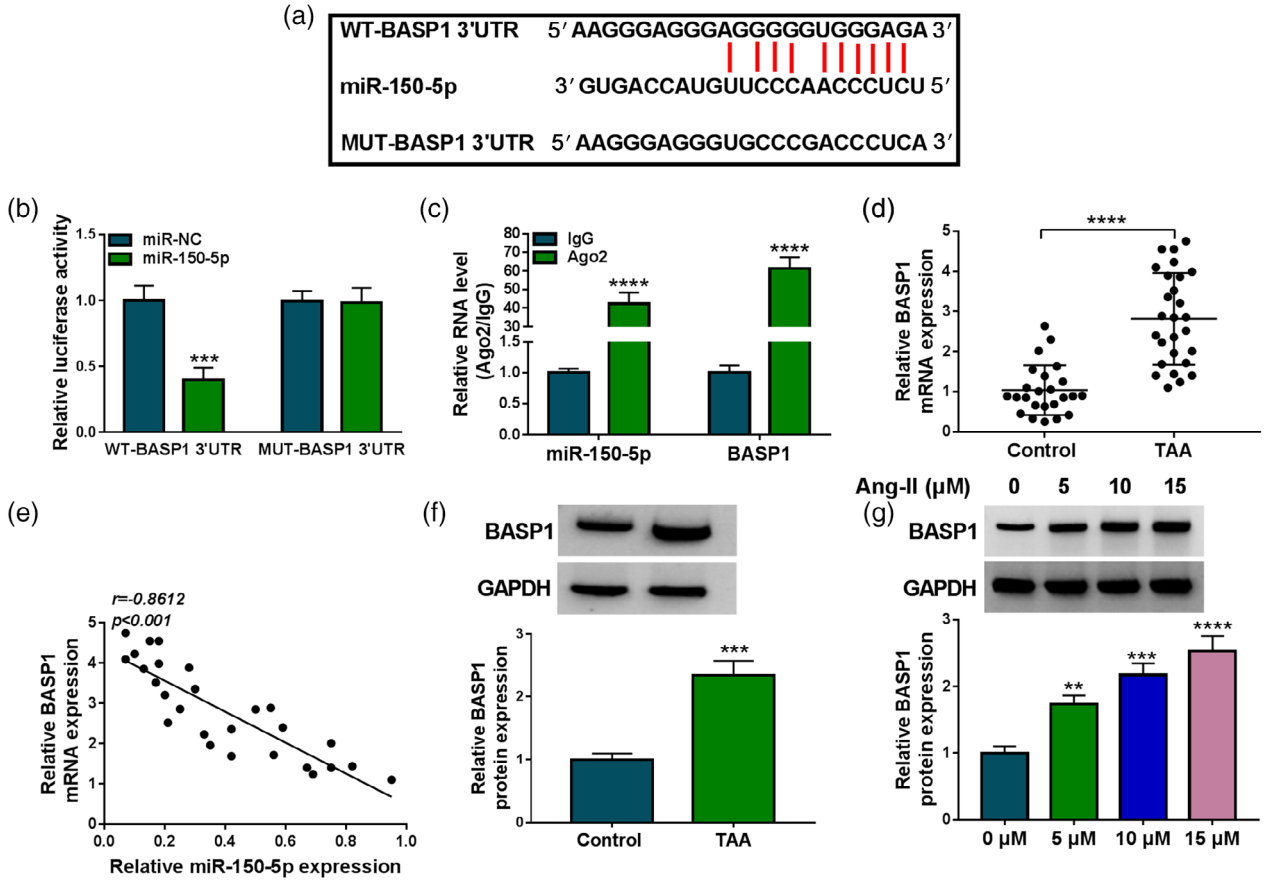

FIGURE 5 Brain acid-soluble protein 1 (BASP1) is targeted by mR-150-5p. (a) The putative binding site of BASP1 on miR-150-5p. (b, c) The interaction analysis of BASP1 and miR-150-5p. (d) BASP1 mRNA expression in aortic tissues of thoracic aortic aneurysm (TAA) patients ($n = 27$) and normal donors ($n = 24$). (e) Correlation detection in aortic tissues. (f) BASP1 protein level expression in aortic tissues of TAA patients ($n = 27$) and normal donors ($n = 24$). (g) BASP1 level in vascular smooth muscle cells (VSMCs) exposed to Ang-II for 48 h, $n = 3$. $^*p < 0.05$.

elevated by Ang II, and the increases of Bax (an apoptosis promoter) and cleaved-caspase-3 (indicator for apoptosis) in VSMCs caused by Ang II also confirmed it (Figure 1c,d). Therefore, Ang-II-stimulated VSMCs were used for subsequent analyses.

Circ_0008285 silencing attenuates Ang-II-induced apoptosis of VSMCs

Circ_0008285 expression was higher in aortic tissues obtained from TAA patients than those in normal artery tissues (Figure 2a). Importantly, the level of circ_0008285 was dose-dependently increased by Ang II in VSMCs (Figure 2b). Moreover, we found that RNase R could rapidly degrade linear GAPDH rather than circ_0008285 in VSMCs (Figure 2c). Thereafter, the circ_0008285 siRNA was designed and transfected into VSMCs, then cells were stimulated with 10 μM Ang II for 48 h. The knockdown efficiency was first validated with declined circ_0008285 in Ang-II-challenged VSMCs (Figure 2d). Functionally, circ_0008285 knockdown reversed Ang-II-triggered VSMC proliferation arrest (Figure 2e,f) and VSMC apoptosis promotion in VSMCs (Figure 2g). In addition, both the increases in Bax and cleaved-caspase-3 caused by Ang II in VSMCs were reduced after circ_0008285 knockdown (Figure 2h). Taken together, circ_0008285 knockdown relieved Ang-II-induced apoptosis of VSMCs.

Circ_0008285 targets miR-150-5p

Subsequently, we probed the molecular mechanisms underlying the function of circ_0008285. The starBase database predicted that circ_0008285 possesses the putative conserved target site of miR-150-5p (Figure 3a). MiR-150-5p mimic was first validated to elevate miR-150-5p level in VSMCs (Figure 3b). Then cotransfection of miR-150-5p markedly reduced the luciferase activity of wild-type circ_0008285 vector but not the mutant type in VSMCs (Figure 3c). Furthermore, RIP assay manifested that circ_0008285 and miR-150-5p were significantly pulled down by Ago antibody relative to the control IgG antibody in VSMCs (Figure 3d). In addition, miR-150-5p level was

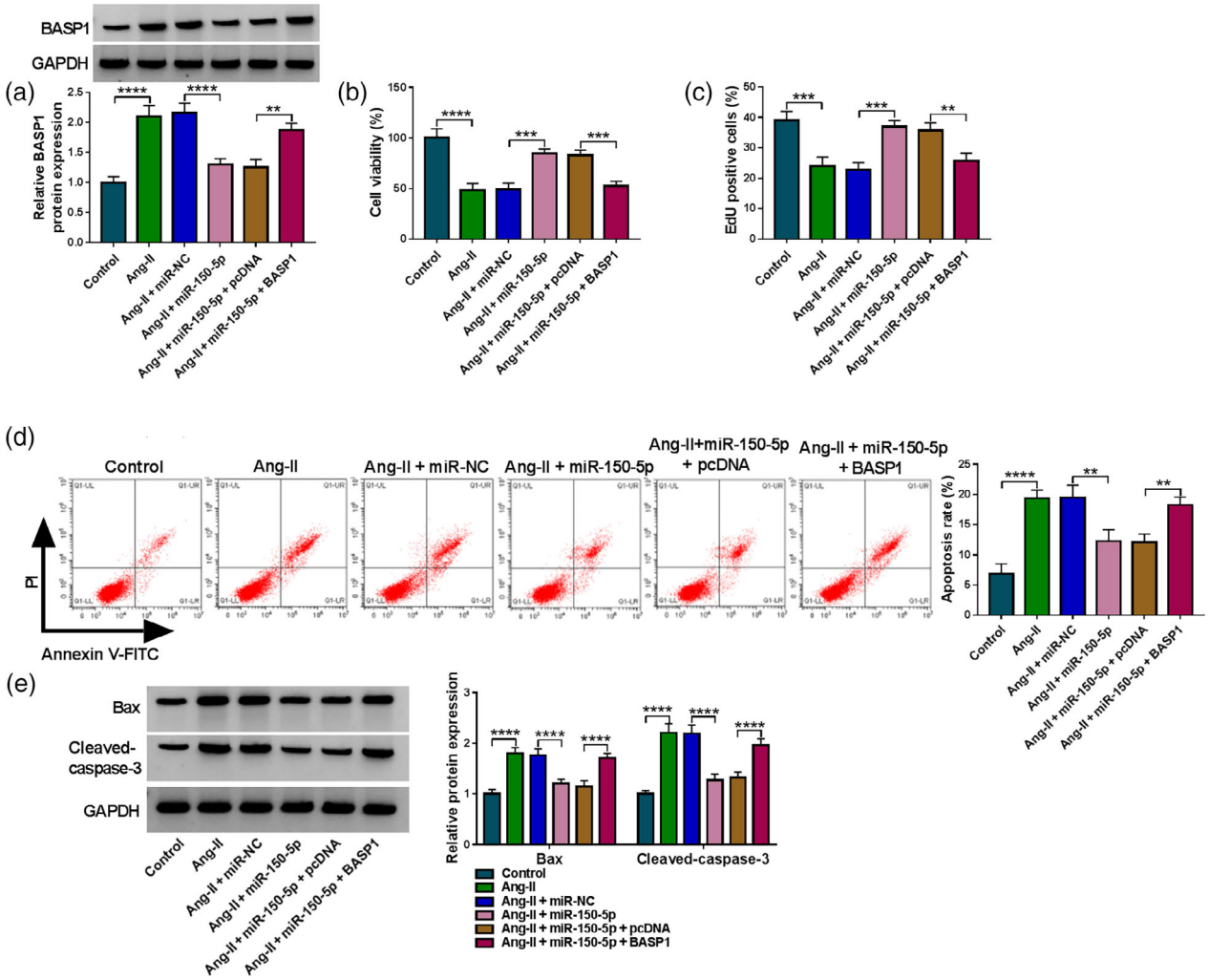

FIGURE 6 MiR-150-5p suppresses Ang-II-induced apoptosis in vascular smooth muscle cells (VSMCs) by regulating brain acid-soluble protein 1 (BASP1). (a–e) VSMCs were cotransfected with miR-150-5p and BASP1, and then treated with 10 μM Ang-II for 48 h. (a) BASP1 expression in VSMCs. (b, c) Measurement of cell proliferation and (d) apoptosis. (e) Levels of Bax and cleaved-caspase-3 in cells. $n = 3$. $^*p < 0.05$.

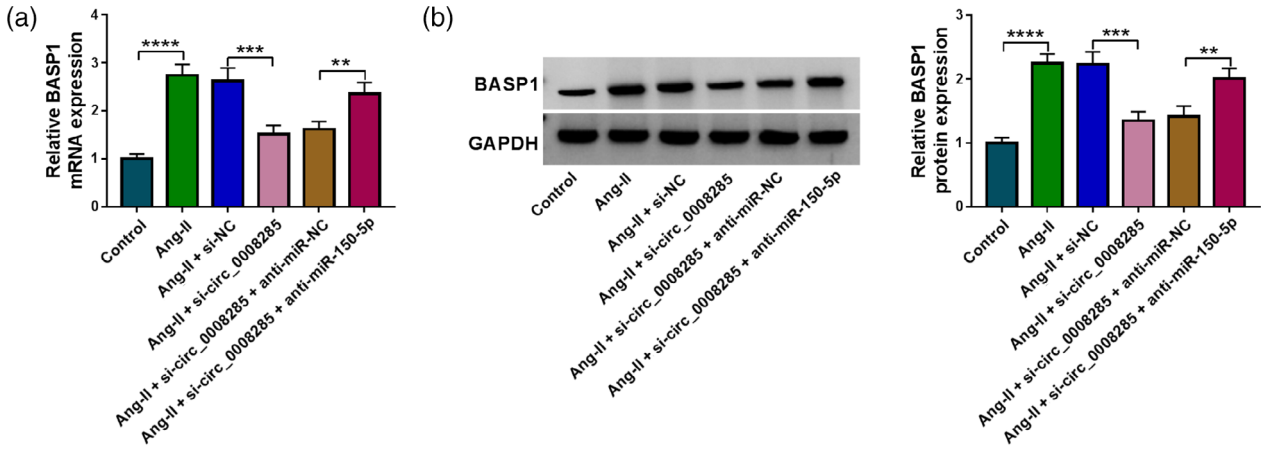

FIGURE 7 Circ_0008285/miR-150-5p axis can regulate brain acid-soluble protein 1 (BASP1) expression. (a, b) BASP1 expression in vascular smooth muscle cells (VSMCs) transfected with si-NC, si-circ_0008285, si-circ_0008285 + anti-miR-NC, si-circ_0008285 + anti-miR-150-5p under Ang-II treatment. $n = 3$. $^*p < 0.05$.

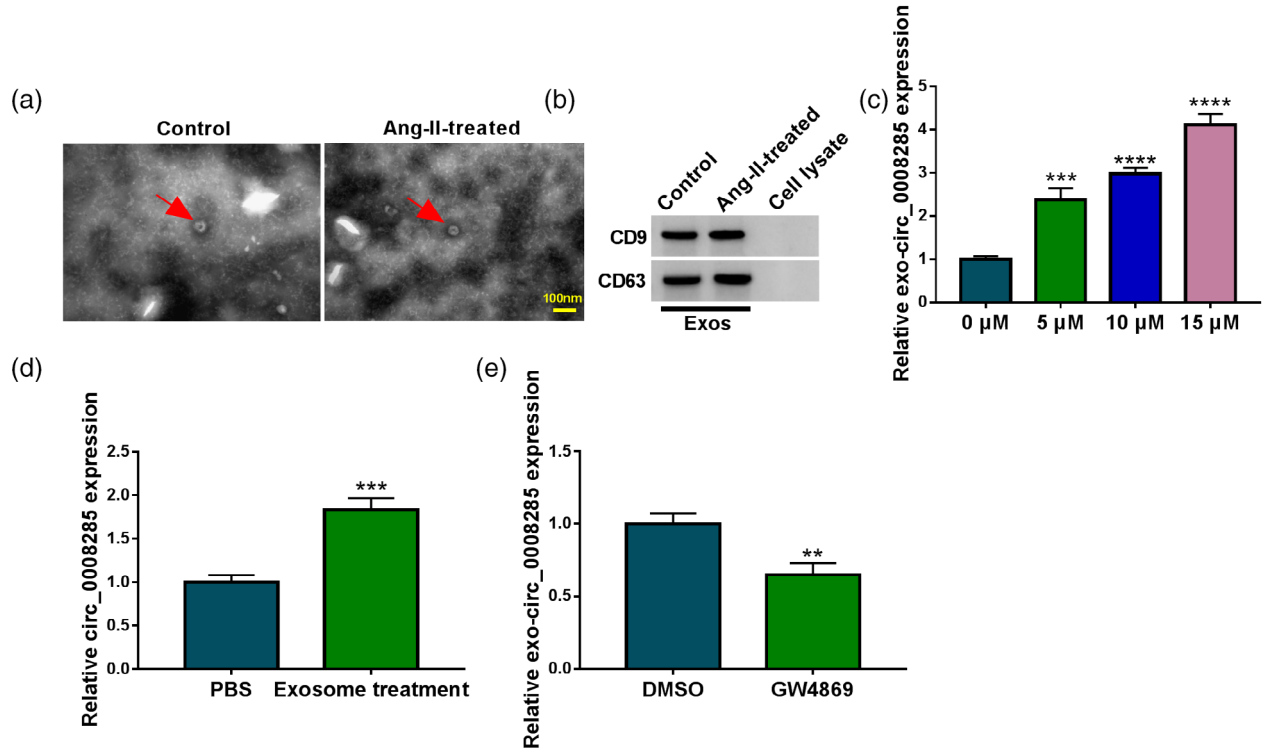

FIGURE 8 Circ_0008285 is secreted by exosomes in vascular smooth muscle cells (VSMCs). (a) Purified exosomes derived from VSMCs treated with or without Ang-II captured by transmission electron microscopy (TEM). (b) Exosomal marker CD9 and CD63. (c) Circ_0008285 expression in exosomes. (d) Circ_0008285 level in VSMCs after incubation with exosomes. (e) Circ_0008285 expression in exosomes treated with dimethyl sulfoxide (DMSO) and GW4869. $n = 3$. $^*p < 0.05$.

discovered to be decreased in the aortic tissues obtained from TAA patients (Figure 3e), and an inverse correlation was shown between miR-150-5p and circ_0008285 expression in aortic tissues (Figure 3f). In addition, miR-150-5p was dose-dependently downregulated by Ang II in VSMCs (Figure 3g). Altogether, miR-150-5p was targeted by circ_0008285.

Circ_0008285 silencing attenuates Ang-II-evoked apoptosis of VSMCs by miR-150-5p

Next, we explored whether circ_0008285 regulated VSMC apoptosis through miR-150-5p. The miR-150-5p inhibitor was transfected into circ_0008285-downregulated VSMCs, and then cells were exposed to Ang II (Figure 4a). Thereafter, miR-150-5p lack attenuated circ_0008285 knockdown-evoked antiproliferation (Figure 4b,c) and proapoptosis (Figure 4d,e) actions in VSMCs under Ang II stimulation. Overall, we determined that the circ_0008285/miR-150-5p axis could regulate Ang-II-evoked apoptosis of VSMCs.

BASP1 is targeted by miR-150-5p

Next, the underlying mechanism by which circ_0008285/miR-150-5p axis induces apoptosis in VSMCs was dissected. Based on the prediction of starBase, miR-150-5p has the putative binding site of BASP1 (Figure 5a). Then the binding of them was validated by decreased luciferase activity in WT-BASP1 3′UTR group with miR-150-5p mimic as well as the enrichment of miR-150-5p and BASP1 in AGO2 immunoprecipitates (Figure 5c). BASP1 mRNA was increased in aortic tissues obtained from TAA patients (Figure 5d) and was inversely correlated with miR-150-5p expression (Figure 5e). Similarly, an increased BASP1 protein was also observed in the aortic tissues obtained of TAA patients (Figure 5f). In addition, Ang II treatment dose-dependently elevated BASP1 expression in VSMCs (Figure 5g). Thus, we verified that miR-150-5p targeted BASP1.

MiR-150-5p suppresses Ang-II-evoked apoptosis in VSMCs by regulating BASP1

After Ang II exposure, we observed that the introduction of BASP1 vector rescued miR-150-5p mimic-induced decrease of BASP1 in VSMCs under Ang II treatment (Figure 6a). Functionally, miR-150-5p re-expression suppressed Ang-II-evoked VSMC proliferation arrest, which was reversed by BASP1 overexpression (Figure 6b,c). Moreover, it was proved that the reduction of apoptotic VSMCs under Ang II exposure caused by miR-150-5p re-expression were attenuated by ascended BASP1 (Figure 6d,e). Thus, the miR-150-5p/BASP1 axis was responsible for Ang-II-induced apoptosis in VSMCs.

Circ_0008285/miR-150-5p axis can regulate BASP1 expression

As shown in Figure 7a,b, circ_0008285 deficiency caused a decrease in the BASP1 level, which was reversed by lack of miR-150-5p in Ang-II-induced VSMCs (Figure 7a,b), indicating the circ_0008285/miR-150-5p/BASP1 axis in VSMCs.

Circ_0008285 is packaged into exosomes in VSMCs

Previous studies showed that exosomes mediate intercellular communication and are ideal particles for the transfer of genes and drugs.[17,18] Therefore, the origin of circ_0008285 was investigated. We isolated exosomes from VSMCs with or without Ang II treatment. Transmission electron microscopy (TEM) data exhibited a round shape with a double layer membrane of exosomes (Figure 8a), which expressed CD9 and CD63 (Figure 8b). Circ_0008285 was then discovered in abundance in exosomes with the increasing doses of Ang II (Figure 8c). Thereafter, untreated VSMCs were incubated with exosomes isolated from Ang-II-stimulated VSMCs, and we observed an increase of circ_0008285 in cells (Figure 8d). Moreover, treatment of exosome secretion inhibitor GW4869 in VSMCs led to the decreased exosome quantity and subsequent circ_0008285 expression reduction (Figure 8e). Collectively, extracellular circ_0008285 was packaged into exosomes, which could be transferred into the recipient cells.

DISCUSSION

TAA has been reported to be related to the loss of VSMCs.[19,20] This study showed a highly expressed circ_0008285 in aortic tissues of TAA patients and Ang-II-induced VSMCs. Functionally, knockdown of circ_0008285 attenuated Ang-II-evoked proliferation arrest and apoptosis enhancement. CircRNAs can competitively bind to the miRNAs to alleviate the degeneration of the gene expression.[21,22] Differential expression of miRNAs has been previously reported in an aneurysmal sample and found to be associated with SMC apoptosis, inflammatory response, and ECM degradation in the aneurysm site.[23–25] In our study, we were able to confirm that circ_0008285 directly binds to miR-150-5p. MiR-150-5p was discovered to be decreased in patients with abdominal aortic dilation.[26] Thus, we assumed that miR-150-5p might be implicated in TAA. Subsequently, a decline of miR-150-5p in aortic tissues of TAA patients and Ang-II-induced VSMCs was manifested in our study, and re-expression of miR-150-5p improved the survival of Ang-II-challenged VSMCs. Importantly, miR-150-5p inhibition could abolish the action of circ_0008285 siRNA on Ang-II-evoked apoptosis of VSMCs. Subsequently, we also confirmed that miR-150-5p directly targeted BASP1, which has previously been identified to be able to modulate diverse cell biological behaviors, including apoptosis, proliferation, and differentiation.[27,28] Moreover, Tian et al. showed that LINC00473 upregulated BASP1 expression via sponging miR-212-5p, thus accelerating aneurysm formation by inducing VSMC apoptosis.[29] In the current study, an increased BASP1 expression was observed in aortic tissues and Ang-II-induced VSMCs; furthermore, BASP1 overexpression abated the protection of miR-212-5p on VSMC survival under Ang II treatment.

Additionally, our study also showed that extracellular circ_0008285 was secreted by exosomes in VSMCs. Exosomes can deliver complex biofunctional cargoes to target cells or extracellular microenvironment.[30,31] Exosomes have been previously investigated as delivery vehicles for loading therapeutic/diagnostic agents.[32,33] Currently, exosomes loaded with therapeutic circRNA have been reported that may be able to enable personalized treatment in diseases.[34,35]

In conclusion, our study first demonstrated that circ_0008285 silencing could suppress Ang-II-induced VSMCs apoptosis via the miR-150-5p/BASP1 axis, suggesting a further understanding of the action on the pathogenesis of TAA. Moreover, circ_0008285 was packaged into exosomes, indicating a new exosome-based therapeutic strategy in TAA.

AUTHOR CONTRIBUTIONS

Leilei Zhang: Designed and supervised the study, conducted the experiments and drafted the manuscript. Ziniu Zhao and Xiaoqiang Quan: Collected and analyzed the data. Zhouliang Xie and Jian Zhao: Contributed the methodology and edited the manuscript.

CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

The data used to support the findings of this study are available from the corresponding author upon request.

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