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YAP1 up-regulation inhibits apoptosis of aortic dissection vascular smooth muscle cells

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Abstract. – OBJECTIVE: Elevated apoptosis of vascular smooth muscle cell (VSMC) is correlated with the occurrence of aortic dissection (AD). Yes-associated protein 1 (YAP1) is the major effector in Hippo-YAP signal pathway, which facilitates cell proliferation and suppressing apoptosis. Several studies have been performed regarding the relationship between YAP1 and AD pathogenesis. This study established the AD rat model to investigate possible roles of YAP1 in regulating VSMC apoptosis and AD pathogenesis.

MATERIALS AND METHODS: Cell apoptosis and YAP1 expression were compared between AD vascular tissues and normal rats. In vitro studies with rat thoracic VSMCs were divided into control, cyclic stretch, cyclic stretch + pIRES2-blank and cyclic stretch + pIRES2-YAP1 groups. Cell apoptosis rate, YAP1 and survivin expressions were measured. AD rats were divided into model, Ad-NC injection, and Ad-YAP1 injection group for the detection of AD formation rate, expressions of YPA1 and survivin, and VSMCs apoptosis.

RESULTS: Compared to control group, vascular cell apoptosis was increased, and YAP1 expression was reduced in AD rats. Cyclic stretch significantly induced VSMCs apoptosis. The over-expression of YAP1 up-regulated survivin and impeded the cell apoptosis induced by cyclic stretch. The treatment with Ad-YAP1 up-regulated the levels of YAP1 and survivin in AD model rat vascular tissues, and decreased apoptosis and AD formation rate/AD diameter/length.

CONCLUSIONS: YAP1 played a critical role in affecting VSMC apoptosis and AD pathogenesis. Up-regulation of YAP1 decreased VSMC apoptosis and AD formation.

Key Words:
YAP1, Hippo-YAP, VSMC, Aortic dissection, Cell apoptosis.

Introduction

Aortic dissection (AD) is featured as atrophy of middle layer of aorta wall with ruptured endo-membrane of vascular, through which blood flows into middle layer of aorta to rupture it and form hematoma or pseudo-cavity, eventually leading to dual cavity of aorta or even complete rupture[1,2]. Vascular smooth muscle cell (VSMC) is the major component in middle layer of aorta, and plays a significant role in maintaining wall structure, mediating vascular dilation/constriction function, and ensuring the vessel tension or elasticity. Abnormal number or function of VSMCs can cause the deregulation of blood pressure, structural re-construction of vascular wall, weaken elasticity and enhance frigidity. Once the aorta wall is damaged, VSMCs transform from maturely differentiated constriction phenotype toward synthetic phenotype, leading to the synthesis of collagen, over-activation of matrix meatalloproteinase (MMPs), destruction of extracellular matrix (ECM) homeostasis. AD pathogenesis is eventually caused due to the collagen deposition and elastin degradation, with dysfunctions of elastic lamina layer of aorta wall. Multiple studies revealed the critical role of VSMCs apoptosis in aorta middle layer in AD pathogenesis[3,4].

Yes-associated protein (YAP) is a type of pluripotent intracellular junction protein functioned as a transcriptional co-activator, and is the major effector in the downstream of Hippo-YAP signal pathway. Its expression level and functional activity were regulated by phosphorylation activity of upstream kinase cascade[5]. Increasing evidence showed that YAP1 belonged to the family of tumor suppressor proteins, as it can translocate from cytoplasm to nucleus, in which it bound with multiple nuclear transcription factor such as transcriptional enhancer associate domain transcription factors (TEADs), and facilitated transcription and expression of multiple target genes, leading to the loss of cellular contact-dependent inhibition or malignant transformation of cells via the suppression of cell apoptosis[6,7]. Accumulative studies showed that the

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up-regulation of YPA1 was closely correlated with myocardial hypertrophy[8], occurrence, clinical stage, metastasis, chemo-/radio-therapy sensitivity and prognosis of multiple tumors[9-11]. Recent papers indicated that the important role of YAP1 in mediating VSMCs proliferation, migration[12], apoptosis[13] and phenotype transition[14], was correlated with occurrence of aneurysm[15]. However, the role of YAP1 in AD pathogenesis has not been widely studied. This study thus generated a rat AD model, by using which possible regulatory roles of YAP1 in VSMC apoptosis of aorta wall and AD pathogenesis were investigated.

Materials and Methods

Major Reagent and Materials

Dulbecco's Modified Eagle Medium (DMEM) was purchased from Biological industries (Cromwell, CT, USA). Fetal bovines serum (FBS) was purchased from Gibco (Gaithersburg, MD, USA). RNA extraction kit PureLink RNA MiNi Kit was purchased from Ambion (Waltham, MA, USA). QuantiTech SYBR Green RT-PCR Kit was purchased from Qiagen (Hilden, Germany). Rabbit anti-YAP1, anti-survivin and anti-β-actin were purchased from Abcam (Cambridge, MA, USA). Horse radish peroxidase (HRP) conjugated secondary antibody was purchased from Jackson ImmunoResearch (West Grove, PA, USA). Beta-amino propionitrile (BAPN) was purchased from Sigma-Aldrich (Carlsbad, CA, USA). TUNEL apoptosis assay kit was purchased from Sangon (Shanghai, China). Annexin V/PI apoptosis kit was purchased from BD Bioscience (Franklin Lakes, NJ, USA). RiboFECT™ CP transfection kit was purchased from RiboBio (Guangzhou, China). PIRES2-EGFP-blank empty plasmid and YAP1 over-expression plasmid pIRES2-EGFP-YAP1 were purchased from Yantuo Biotech (Shanghai, China). Adenovirus vector carrying CDS domain of YAP1 gene Ad-YAP1 and negative control Ad-NC were provided by Jikai Bio (Shanghai, China). Elastic silicon culture plate and stretch force loading system were purchased from Flexcell (Burlington, NC, USA).

AD Rat Model Generation and YAP1 Over-Expression

A total of 30 male SD rats (3 weeks age, body weight 55±1.3 g) were purchased from Silaike Laboratory Animal Center (Shanghai, China). Animals were randomly divided into two groups. Control group (N=8) received normal diet feeding for 6 weeks. AD group (N=12) were fed with the same diet containing 0.25% BAPN for 6 weeks. During 6-week experiment, AD formation rate was observed between two groups and AD diameter was recorded.

At the end of experiment, aorta middle layer tissues were collected at the rupturing site of aorta. For control group, middle layer tissues were collected from left subclavian artery. All sample tissues were tested for cell apoptosis, Caspase-3 activity, gene and protein expression.

In YAP1 interference study, rat AD model was generated as above mentioned and animals were further divided into three groups (N=15 each). AD model group received tail vein injection of 50 μL saline once a week for 6 consecutive weeks. In Ad-NC group, 50 μL Ad-NC virus (viral titer=1×10^{10} PFU/mL) was injected once a week for 6 consecutive weeks. A third Ad-YAP1 group received tail vein injection of 50 μL Ad-YAP1 adenovirus (viral titer=1×10^{10} PFU/mL) once a week for 6 consecutive weeks.

Rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of Taihe Hospital, Hubei University of Medicine.

TUNEL Assay for Cardiomyocytes Apoptosis

Middle layer tissues of rat aorta were prepared for paraffin sections, and were tested for cell apoptosis following manual instruction of TUNEL kit. Five fields were randomly selected from each slide. The number of apoptotic cells in 100 cells was counted in each field to calculate cell apoptotic rate.

Rat Aorta VSMC Separation, Culture and Grouping

SD rats were sacrificed by cervical dislocation, and were immersed in 75% ethanol for 3-5 min. The chest cavity was opened under sterile condition. Thoracic aorta was completely removed to separate outer vascular mesenchyme tissues. Vessels were longitudinally dissected, and inner membrane was gently removed by sterile forceps. The middle membrane was scratched by compression of forceps, and was removed by clapping after rupture. Tissues were ruptured and added into culture flask by sterile dropping. After evenly paving on the flask wall, cells were cultured in a vertically placed flask in 5% CO_2 incubator under 37°C. 1 h later, the culture flask was placed horizontally, with addition of complete culture medium for co-

vering the bottom. Cells were incubated for 3 days, with medium changed every 3 days. Cells were passed at the ratio of 1:4. Cells at 5-6 generation were obtained for further experiments.

Rat thoracic aorta VSMCs at 5th generation were seeded into 6-well plate and were divided into three groups: control group, pIRES2-EGFP-blank empty plasmid transfection group, and pIRES2-EGFP-YAP1 plasmid transfection group. In cell transfection, 100 μl 1× riboFECT™CP buffer was used to dilute 3 μl pIRES2-EGFP-blank or pIRES2-EGFP-YAP1 plasmid. After gentle mixture and incubation at room temperature for 5 min, 10 μl riboFECT™ CP Reagent was added for mixture and incubated at room temperature for 0-15 min. riboFECT™ CP mixture was added into culture medium for gentle mixture and 72 h of continuous incubation. Then, cells were collected for further assays.

Cyclic Stretch Loading for Induction of CSMC Apoptosis

Rat thoracic aorta VSMCs were inoculated into elastic silicon rubber culture plate. Flexcell cyclic stretch loading system was used to treat VSMCs, with the tension strain rate at 18%, frequency at 60 per min. After 48 h of stretch loading, cells were collected for assays of apoptosis, gene and protein expression.

qRT-PCR for Gene Expression

QuantiTest SYBR Green RT-PCR Kit was used to test gene expression by one-step qRT-PCR. RNA was extracted by PureLink RNA MiNi Kit as the template. In a 20 μL qRT-PCR system, there were 10 μL 2×QuantiTect SYBR Green RT-PCR Master Mix, 1.0 μL of forward and reverse primer (0.5 μm/L), 2 μg template RNA, 0.5 μL QuantiTect RT Mix, and ddH_2O. Reverse transcription conditions were: 50°C for 30 min. PCR conditions were: 95°C pre-denature for 15 min, followed by 40 cycles each containing 94°C denature for 15 s, 60°C annealing for 30 s, and 72°C elongation for 30 s. Gene expression was examined on ABI ViiA™ 7 Real-time fluorescent quantitative PCR cycler.

Western Blot for Protein Expression

SDS lysis buffer was used by 5 min boiling. After protein quantification by BCA method, 50 μg samples were separated in 8-10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) separation gel plus 5% condensing gel, and were then transferred to polyvinylidene-difluoride (PVDF membrane, which was blocked in 5% defatted milk powder at room temperature incubation. Primary antibodies (YAP1 at 1:3000, Survivin at 1:3000, β-actin at 1:8000) were added for incubation at 4°C overnight. After phosphate buffered saline tween (PBST) rinsing for three times, HRP conjugated secondary antibody (1:10000 dilution) was added for 60 min incubation. The membrane was rinsed in PBST and incubated using enhanced chemiluminescence (ECL) method for detecting protein expression.

Flow Cytometry for Cell Apoptosis

Cells were digested in trypsin and were collected by centrifugation after being rinsed in PBS. After re-suspending in 500 μL 1× Binding Buffer, 5 μL Annexin V-FITC and 5 μL PI dyes were sequentially added. FC500 MCL/MPL flow cytometry (Beckman Coulter, Brea, CA, USA) was used for measuring cell apoptosis.

Statistical Analysis

SPSS 18.0 software (SPSS Inc., Chicago, IL, USA) was used for data analysis. Measurement data were presented as mean±standard deviation (SD). Enumeration data were presented as percentage and were tested by x^2 test. Student *t*-test was used to compare measurement data between groups. Statistical significance was considered when $p<0.05$.

Results

Significantly Decreased YAP1 Expression in AD Model rat Vascular Middle Membrane

In control group, no rat was found to present AD formation, whilst 5 out of 12 rats in model group showed AD formation (41.67% formation rate). Among those 5 AD rats, 2 of them appeared lesion at ascending aorta, 1 in aorta arch and 1 in proximal descending aorta. In model group, AD diameter was 6.31±1.27 mm and length was 9.24±2.36 mm. TUNEL assay showed significantly higher apoptotic rate in vascular middle membrane layer of AD model rats compared to that of control rats (Figure 1A). Spectrometry showed significantly higher Caspase-3 activity in AD model rats compared to that in control rats (Figure 1B). The qRT-PCR results showed that, compared to control rats, YAP1 mRNA level in vascular middle membrane of AD rats was significantly decreased (Figure 1C). Western blot assay also showed the expression of YAP1 protein in va-

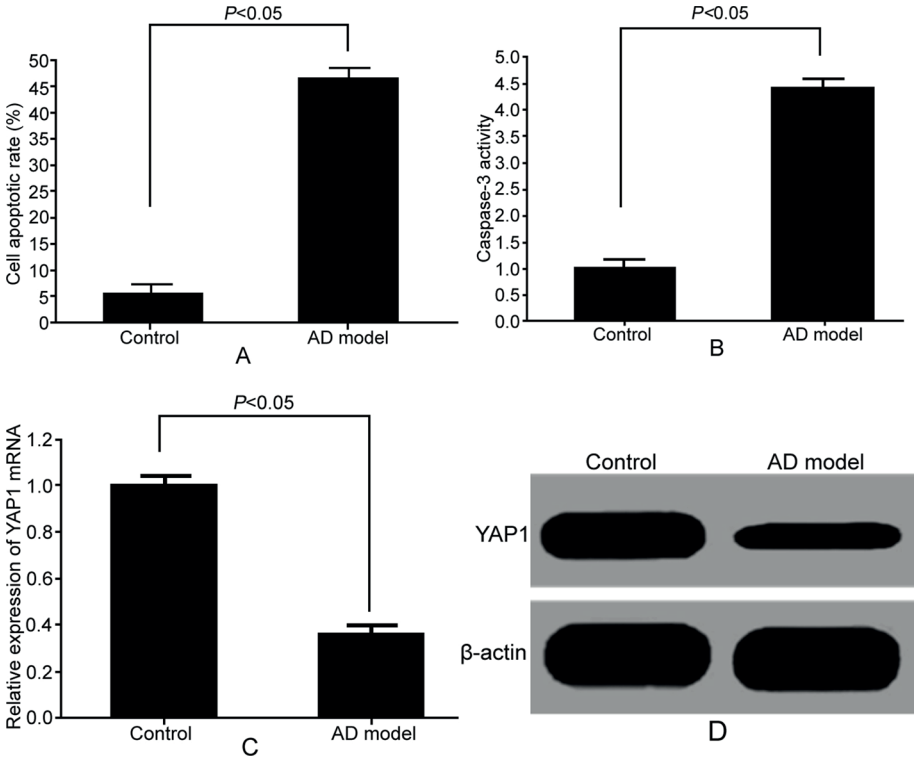

Figure 1. Significantly decreased YAP1 expression in vascular middle membrane of AD rat. ***(A)*** TUNEL assay for apoptosis in vascular middle membrane; ***(B)*** spectrometry for Caspase-3 activity in vascular middle membrane; ***(C)*** qRT-PCR for YAP1 mRNA expression in vascular middle membrane; ***(D)*** Western blot for YAP1 protein expression in vascular middle membrane.

scular middle membrane layer of AD model rats was significantly decreased compared to that in control group (Figure 1D). These results indicated that the down-regulation of YAP1 in Hippo-YAP signal pathway might be related with the aggravation of cell apoptosis and AD pathogenesis.

YAP1 Over-Expression Significantly Suppressed Cyclic Stretch Induced VSMCs Apoptosis

Flow cytometry results unraveled that, compared to control group, VSMCs apoptosis was significantly elevated in rats with cyclic stretch loading (Figure 2A). qRT-PCR results showed that cyclic stretch loading significantly suppressed YAP1 and survivin mRNA expression in VSMCs (Figure 2B). Western blot results also confirmed in protein level that, compared to control group, YAP1 and survivin protein expressions were significantly down-regulated in rats with cyclic stretch loading (Figure 2C). The transfection of pIRES2-EGFP-YAP1 remarkably up-regulated mRNA and protein expressions of YAP1 and survivin in VSMC cells (Figure 2B-C), and weakened VSMCs apoptosis caused by cyclic stretch (Figure 2A).

YAP1 Adenovirus Injection Significantly Weakened Cell Apoptosis and Decreased AD Formation Rate

Compared to AD model group and Ad-NC injection group, significantly elevated protein expression of YAP1 and survivin were found in Ad-YAP1 injection group (Figure 3A), with reducing apoptosis rate in AD vascular middle membrane (Figure 3B). Moreover, Ad-YAP1 injection decreased Ad risk in model rats, and decreased AD diameter and length (Table I).

Table I. AD formation rat, length and diameter among three groups of rats.

Group	AD formation rate (%)	AD diameter (mm)	AD length (mm)
AD model	43.1	6.21±1.13	9.25±1.77
Ad-NC injection	42.3	6.35±1.22	9.11±1.64
Ad-YAP1 injection	28.5*#	3.72±0.43*#	6.42±1.16*#

Note: *, $p<0.05$ compared to AD model group; #, $p<0.05$ compared to Ad-N injection group.

Discussion

Hippo-YAP represented a kind of kinase regulatory signal pathway composing of multiple upstream tumor suppressor factors and downstream oncogenic factors, and played important roles in maintaining cell contact-dependent inhibitor, regulating cell proliferation/apoptosis homeostasis, and mediating tissue development or organ size[5]. Abnormal transduction of Hippo-YAP signal pathway was closely correlated with organ hyperplasia[16,17], cell number decrease or organ atrophy, and occurrence of various diseases including tumors[18,19]. YAP1 gene located on human chromosome 11q13, and encoded a protein with 65KD molecular weight[6]. In Hippo-YAP signal pathway, YAP1 expression level and functional activity were mainly modulated by phosphorylation of various upstream tumor suppressor proteins such as mammalian Ste20-like protein kinase 1/2 (MST kinase 1/2), large tumor suppressor (Lats) kinase 1/2, and Mps One binder 1 (Mobl)[20]. As a transcriptional co-activator without inherent DNA binding domain, YAP1, can specifically recognize and bind with nuclear transcriptional factor containing PPXY sequence via WW structural specific domain, thus modulating gene transcription and expression. During the process from cytoplasm to nucleus, YAP1 protein can bind with TEA domain family (TEAD), p73, IAP family (including Bric2 and Brits) and ErbB4 to facilitate transcription and expression of downstream genes including cyclin E, beta-catenin, AXL receptor tyrosine kinase, and connective tissue growth factor (CTGF), thus facilitating cell proliferation, inhibiting cell apoptosis, depriving contact dependent inhibition loss and promoting cell malignant transformation[6,20]. Previous studies illustrated that the up-regulation of YAP1 was related with occurrence of myocardial hypertrophy[8], thyroid cancer[9], lung cancer[11] and pancreatic carcinoma[10]. Recent investigations also found the important role of YAP1 in mediating VSMCs proliferation, migration[12], apoptosis[13] and phenotype transition[14], and was correlated with aneurysm occurrence[15].

In this work, we successfully generated BAPN-induced AD rat model in order to study the role of YAP1 in AD pathogenesis. Previously, Yuan et al[4] showed significantly higher VSMCs apopto-

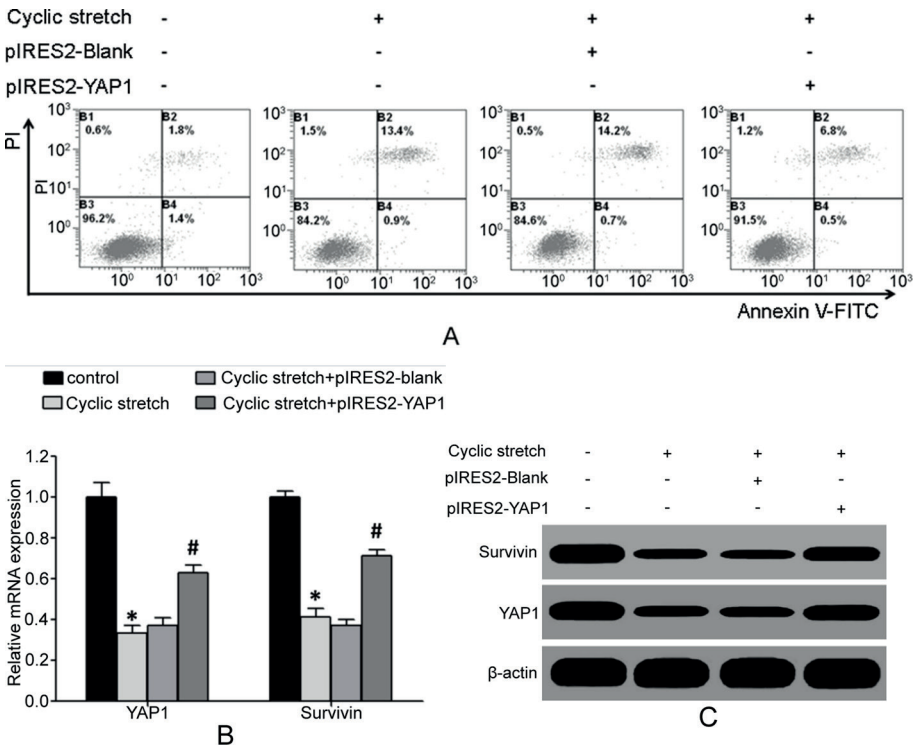

Figure 2. Over-expression of YAP1 significantly weakened VSMCs apoptosis induced by cyclic stretch. ***(A)*** Flow cytometry for VSMC apoptosis; ***(B)*** qRT-PCR for YAP1 and survivin mRNA expression in VSMC; ***(C)*** Western blot for YAP1 and survivin protein expression in VSMCs. *, $p<0.05$ comparing between cyclic stretch group and control group; #, $p<0.05$ comparing cyclic stretch + pIRES2-YAP1 and cyclic stretch + pIRES2-blank group.

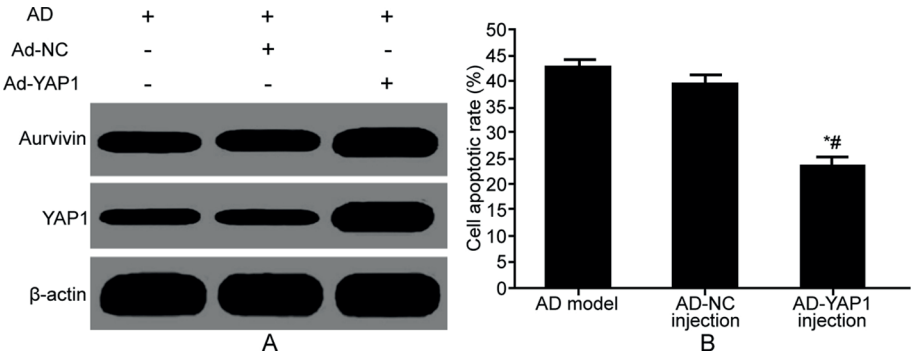

Figure 3. Adenovirus-vectored YAP1 injection inhibited cell apoptosis and decreased AD formation rate. ***(A)*** Western blot for YAP1 and survivin protein expressions in vascular middle membrane; ***(B)*** TUNEL assay for vascular middle membrane cell apoptosis. *, $p<0.05$ compared to AD model group; #, $p<0.05$ compared to Ad-N injection group.

tic rate in AD patient vascular tissues compared to control group. Durdu et al[3] found significantly elevated expression of pro-apoptotic factor Bax and enhanced apoptosis of VSMCs in AD patients, compared to healthy control group. The apoptosis and phenotype transformation of vascular smooth muscle cells was likewise correlated with AD formation[21]. In this study, AD model rats presented significantly higher apoptotic rate of VSMCs in vascular middle membrane layer than those in control rats, suggesting that abnormally elevated cell apoptosis was correlated with AD pathogenesis, as similar with Yuan et al[4] and Durdu et al[3]. Test results showed significantly lower YAP1 expression in vascular tissues of AD model rats compared to control group, indicating possible relationship between YAP1 down-regulation and enhanced VSMC apoptosis or AD pathogenesis. Li et al[15] reported significantly suppressed YAP expression in blood vessels of patients with ascending aorta aneurysm, accompanied with potentiated VSMCs apoptosis compared to healthy control group. Jiang et al[13] studied AD model mice and found significantly enhanced VSMC apoptosis during AD pathogenesis, whilst YAP expression was decreased. In this paper, YAP1 down-regulation might be related with enhanced VSMCs apoptosis or AD occurrence, as similar with Li et al[15] and Jiang et al[13] findings. A study showed that cyclic stretch as a result of hemodynamic change played a pivotal role in inducing VSMCs apoptosis and facilitating AD pathogenesis[22]. Exogenous cyclic stretch loading effectively mimicked internal stretch by blood flow, and facilitated VSMCs apoptosis, which was regarded as a common *in vitro* model to induce VSMCs apoptosis[22]. This *in vitro* study treated rat thoracic aorta VSMCs with cyclic stretch stimulus, and showed significantly elevated VSMCs apoptosis, as consistent with Jia et al[22]. Survivin, also named as human baculoviral inhibitor of apoptosis repeat-containing 5 (BIRC5), was the most potent anti-apoptotic factor in inhibitor of apoptosis protein (IAPs) family, and minimized cell apoptosis via suppressing apoptotic executing protein Caspase-3 and Caspase-7 activity[23]. Various researches illuminated the role of survivin in mediating VSMCs survival, proliferation and apoptosis, which was correlated with pulmonary hypertension[24] and atherosclerosis (AS) occurrence[25]. Fruitful evidence shed light on that anti-apoptotic factor survivin was the key target gene downstream of YAP[26-28]. This work showed that cyclic stretch loading induced VSMCs apoptosis and decreased YAP1 or survivin expression at the same time, indicating that YAP1 down-regulation induced survivin suppression might be one facilitating factor for mechanic stretch induced VSMCs apoptosis, which has also been confirmed by Jiang et al[13]. Further *in vitro* study showed that over-expression of YAP1 significantly decreased VSMCs apoptosis induced by stretch, and *in vivo* assay exhibited that over-expression of YAP1 by virus vector significantly decreased apoptosis of middle membrane cell in AD model rats, and suppressed AD formation rate, diameter or length, suggesting that both YAP1 up-regulation and VSMCs apoptotic suppression impeded the AD formation and, therefore, decreased AD occurrence. Jia et al[22] observed that knockout of CHOP gene significantly decreased mouse VSMCs apoptosis or AD formation, whilst Liu et al[29] found that ursodeoxycholic acid (UDCA) played a role in protecting VSMCs from apoptosis and in decreasing AD appearance. All these studies directly illustrated that VSMCs apoptosis

exerted favorable function in AD formation, and the decrease of VSMCs apoptosis could suppress AD occurrence. In contrast with previous findings, we achieved similar goals via over-expressing YAP1 gene. This study revealed the critical role of YAP1 down-regulation in AD occurrence, and YAP1 up-regulation could reduce VSMC apoptosis or AD formation, all of which have not been reported yet.

Conclusions

This study shed light on the function of YAP1 in regulating VSMCs apoptosis and AD occurrence through animal model and *in vitro* cultured VSMCs, suggesting YAP1 as a potential target in the therapy of AD in the future.

Acknowledgments

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Conflict of interest

The authors declare no conflicts of interest.

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