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Original Research

Cyclic stretch induces VEGFA alternative splicing via Serine/Arginine-Rich Splicing Factor 1

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Abstract: Abnormal proliferation of vascular smooth muscle cells (VSMCs) induced by high cyclic stretch is crucial in the vascular remodeling during hypertension. Vascular endothelial growth factor A (VEGFA) alternative splicing plays important roles in the pathological process of vascular diseases and remodeling. However, the roles of VEGFA isoforms in modulating VSMC functions in response to cyclic stretch remain unclear. We hypothesize that high cyclic stretch may induce VEGFA alternative splicing via Serine/arginine-rich splicing factor 1 (SRSF1) which subsequently induce VSMC proliferation. In the present research, hypertensive rat model was established using the abdominal aortic constriction method. In comparison with sham-operated group, immunohistology staining showed translocation of SRSF1 into nuclei in hypertensive rat thoracic aorta, and RT-PCR detected a shift of VEGFA expression pattern, including the increased expression of VEGFA120 and VEGFA164, but not VEGFA188.Then VSMCs were subjected to cyclic stretch *in vitro* using a Flexercell strain unit. VEGFA ELISA assay showed 15% cyclic stretch increased the secretion of VEGFA which significantly increased proliferation of VSMCs. Western blot and immunofluorescence detected accumulation of SRSF1 in nuclei after 15% cyclic stretch application. Furthermore, SRSF1-specific siRNA transfection reversed the VEGFA secretion induced by pathological high cyclic stretch. Our present results suggested that pathologically high cyclic stretch induces the shuttling of SRSF1 which results in the secretive pattern splicing of VEGFA and finally contributes to the proliferation of VSMCs.

Key words: Hypertension; Cyclic stretch; Vascular endothelial growth factor A; Vascular smooth muscle cells; Serine/arginine-rich splicing factor 1.

Introduction

Hypertension, characterized as abnormally escalatory blood pressure, is a major risk factor of stroke, heart failure and coronary artery disease (1). Many evidences proved that proliferation and migration of VSMCs are of great importance in vascular remodeling during hypertension (2).

Clinical investigations showed that the arterial wall is subjected to cyclic stretch caused by the pulsatile blood pressure. Normally, the magnitude of cyclic stretch is about 5%-10%. While during hypertension, a significant higher cyclic stretch above 15% is observed (3). VSMCs localized in the media portion of artery wall are mainly exposed to this mechanical stretch stimulation (4). Our previous work revealed that pathologically high cyclic stretch induced proliferation of VSMCs which play important roles in vascular remodeling during hypertension (5). An increasing number of studies have demonstrated that cyclic stretch modulates VSMC phenotypic transformation, proliferation, migration and apoptosis through various signaling pathways (6). In particular, cyclic stretch induces the expression of many growth factors/cytokines, such as: vascular endothelial growth factor (VEGF) (7), platelet-derived growth factor (PDGF) (8), angiotensin II (Ang II) and transforming growth factor-β (TGF-β) (9). VEGFA is the most potent angiogenesis inducer in VEGF family (10). Rat VEGFA mRNA are transcribed from 8 different exons, and as a result of alternative splicing, three prevalent VEGFA isoforms, i.e. VEGFA120, VEGFA164 and VEGFA188, are generated. The human versions are one residue longer and called VEGFA121, VEGFA165 and VEGFA189, respectively (11, 12). Those isoforms can be distinguished according to the absence or presence of exon 6 and 7. VEGFA188 contains all 8 exons; VEGFA164 lacks the exon 6; VEGFA120 lacks exon 6 and 7. This divergence in molecular structure results in a varied cellular association ability and thus lead to a different localization and function of VEGFA isoforms. VEGFA120 is freely secreted, while VEGFA188 is strongly associated with extracellular matrix or on the surface of the cell, and 50%-70% of VEGFA164 remains bounded and the others are secreted (13, 14).

Alternative splicing is an important regulatory step in mRNA metabolism. Through different combinations of exons, it enables a single gene to produce multiple transcripts that results in isoforms with different molecular weight and biology functions (15). Many studies have demonstrated the crucial role of alternative splicing in cardiovascular diseases. Works conducted by Sek Won Kong *et al*. found the association of Sarcomere genes splicing and heart failure (16). Joy Cogan *et al*. focused on the different isoforms of bone morphogenic protein receptor 2 (BMPR2) and detected their roles in heritable pulmonary arterial hypertension (17). Our previous work also identified that pathological cyclic stretch can induce the alternative splicing of large conductance calcium and voltage-activated potassium (BK) channels and regulate VSMC differentiation (18).

However, the effect of high cyclic stretch on the alternative splicing of VEGFA in VSMCs, and the underlying mechanisms remain to be investigated.

The serine/arginine-rich (SR) proteins are a family of highly conserved proteins and crucially regulate the metabolism of mRNA. Twelve numbers of human SR proteins share similar molecular structures, with mRNA recognition motifs and serine/arginine repeats. Recent studies have revealed its various roles in different aspects of mRNA regulation, such as alternative splicing, stability, and translation (19). Serine/arginine-rich splicing factor 1(SRSF1), one of the SR proteins that can shuttle between nucleus and cytoplasm, is highly expressed in VSMCs and can promote the proliferation of VSMCs by regulating alternative splicing events (20).

Therefore, using the hypertensive animal model and the *in vitro* cyclic stretch application system, the present study examined the effect of cyclic stretch on VEGFA alternative splicing, and sought to detect the potential role of SRSF1 in this process. Understanding of the mechanobiological effects of cyclic stretch on VEGFA alternative splicing will help to identify the molecular and mechanical bases of vascular pathologies during hypertension, as well as the clinical complications.

Materials and Methods

Hypertensive rat model

The experimental protocols are in accordance with the Animal Management Rules of China (Documentation 55, 2001, Ministry of Health, China), and approved by the Animal Research Committee of Shanghai Jiao Tong University.

Twelve male Sprague-Dawley (SD) rats (180–200g) were randomly divided into two groups. The hypertensive rat group was established by using the abdominal aortic constriction method (21). Briefly, the rats were anesthetized with isofluraneat inhalation and treated under sterile conditions. The banding site was upper the renal artery crotch and the constriction was conducted by tying a 3-0 silk suture ligature against a 21-gauge needle. Then the needle was removed to result in a 0.9 mm constriction in diameter. Sham-operated rats were treated equally without constriction. One week after the surgery, blood pressure was measured through a catheter that was introduced into one of the carotid arteries. The thoracic aortas from both the hypertensive rats and the sham-operated rats were surgically removed for further examinations.

Cell culture

VSMCs were obtained from the media portion of rat thoracic aorta by an explant technique. Briefly, the media portion of thoracic aorta was surgically isolated and cut into 1 mm^2 pieces, planted onto 25 cm^2 culture flasks in Dulbec's modified Eagle's medium (DMEM, Gibco) containing 10% fetal bovine serum (FBS, Gibco), 100 U/ml penciling and 100 μg/ml streptomycin, and incubated at 37°C with 5% CO_2. Primary cultured VSMCs were identified by immunofluorescent staining using antibody against smooth muscle-specific α-actin (αSMA) (1:100, DAKO). VSMCs between passages 4 and 8, with cell populations of more than 95 % purity, were used in all experiments.

Application of cyclic stretch

VSMCs were seeded into 6-well Flexercell plates (Flexercell International, USA) coated with gelatin (Sigma, 0.1%) at a density of 2×10^5 cells per well (9.32 cm^2). After achieving 70% confluence, the cells were starved with DMEM for 24 h before the application of cyclic stretch. The following loading conditions were used: stretch magnitude of 5% and 15% respectively, at 1.0 Hz frequency for 1 h. VSMCs cultured under the same condition without the application of cyclic stretch were used as static control.

Real-Time PCR

After removing the adventitia and 30-second snap-frozen in liquid nitrogen, the tissue was pulverized by tissue grinder, and lysed in TRIzol reagent (Invitrogen). Total RNA from VSMCs was also extracted by using TRIzol reagent according to the manufacturer's instructions. The RNA concentration and purity were examined spectrophotometrically (Beijing Tiangen Co., Ltd). RevertAid First Strand cDNA kit (Thermo Scientific) was used for the synthesis of cDNA. SYBR Premix Ex Taq (TakaRa) and Applied Biosystems (Thermo Scientific) were used for the real-time (RT)-PCR analysis. The specific sequences of primers used in this study were showed in Table 1. Target gene expression level was determined by using rat GAPDH as reference in each sample.

VEGFA ELISA Assay

The protein level of VEGFA secreted from VSMCs was detected by using the Rat VEGFA ELISA Kit (Neo-Bioscience, China). Culture medium from VSMCs was collected immediately after the application of cyclic

Table 1. Primer sequence.

Primer name		Sequence
pan-VEGFA	Forward:	TCA TGC GGA TCA AAC CTC AC
	Reverse:	CTT TGG TCT GCA TTC ACA TCT
VEGFA120	Forward:	GCA CAT AGG AGA GAT GAG CT
	Reverse:	GGC TTG TCA CAT TTT TCT GGC
VEGFA164	Forward:	GCA CAT AGG AGA GAT GAG CT
	Reverse:	GCT CAC AGT GAT TTT CTG GC
VEGFA188	Forward:	CAG TTC GAG GAA AGG GAA AG
	Reverse:	CAG TGA ACG CTC CAG GAT TT
GAPDH	Forward:	TGA AGG GTG GGG CCA AAA
	Reverse:	GCT GAC AAT CTT GAG GGA GT

stretch. Experimental procedures were according to the manufacturer's instructions, and the absorbance at 450 nm was measured in ELISA plate reader (Bio-Rad 680).

Nuclear protein extraction and western blotting

Nuclear protein was separated according to the instructions of a Nuclear Extraction Kit (Affymetrix) after the application of cyclic stretch. Equal amount of protein was subjected to electrophoretic separation with 10% SDS-PAGE gel and transferred to PVDF membranes (Millipore, USA). Western blotting was performed by using antibodies directed against SRSF1 (1:1000, Proteintech), LaminA/C (1:1000, Cell Signaling Technology) respectively. After incubation with alkaline phosphatase-conjugated secondary antibodies (Jackson Immunoresearch), the signals were detected by BCIP/NBT (KPL, USA) and quantified by Image Studio software (C-DiGit) using LaminA/C as loading control.

In virto analysis of VSMC proliferation

VSMC proliferation was detected with BrdU Labeling and Detection Kit (Roche) according to the manufacturer's instructions. Briefly, the cells were seeded in 96-well plates for 24 h, followed by 24-h serum starvation to synchronize the cells. Then the cells were incubated with 1 ng/ml VEGFA164 (Beyotime Biotechnology, China) for 18 h in the presence of BrdU. The signals were detected with an ELISA plate reader (Bio-Rad 680).

RNA interference

For small interference (siRNA) fragments screening in 6-well plates, VSMCs were planted at a density of 1.0×10^5 per well and grown in 10% FBS/DMEM. After 24 h, the cells were transfected with 100 nM target small interfering RNA (siRNA) mixed with 5 μl Lipofectamine™2000 (Invitrogen) and 0.5 ml opti-MEM media (Gibco) according to the manufacturer's instructions. All siRNA fragments showed in Table 2. were designed and synthesized by GenePharma Biological Company (Shanghai, P.R. China).

For RNA interference in Flexercell plates, the initial density of cells was 1.5×10^5 per well and the complete culture medium was replaced by DMEM (1% FBS) to synchronize the VSMCs before cyclic stretch application.

Hematoxylin and eosin (HE) staining

The thoracic aortas obtained from different groups were paraffin-sectioned into 5 μm slides and the procedures for HE staining were as follows. Briefly, after rehydration, the sections were stained with hematoxylin for 5 min, treated with hydrochloric acid alcohol for 5 s and ammonia water for 5 min. Then the sections were counterstained with eosin for 5 s and dehydrated in ethanol before observing with a microscope (Olympus IX71).

Immunofluorescence staining

After the loading of cyclic stretch, VSMCs seeded on Flexercell plates were fixed with 4% paraformaldehyde for 20 min at room temperature, treated with 0.5% Triton X-100 for 5 min and then blocked with 10% goat serum in PBS (0.01M), and incubated with SRSF1 antibody (1:50, Proteintech) at 4°C overnight. After washing the membrane 3 times in PBS, VSMCs were stained with Alexa Fluor 488-conjugated secondary antibody (1:1000, Cell Signaling Technology) and DAPI at room temperature for 2 h and detected with an laser confocal microscope (Olympus, LV1000).

To detect the distribution of SRSF1, thoracic aortas of experimental animals were surgically removed and fixed with 4% paraformaldehyde for 24 h at 4°C. After 24-h dehydration in 30% buffered sucrose solution, the vessel was frozen-sectioned to 6-μm slides. The sections were rehydrated, and antigen retrieval was performed by incubation of the slides for 15 min at 90-100℃ in 10 mM citrate buffer (PH 6.0). Then the sections were treated with 0.5% Triton X-100 for 30 min, blocked with 10% goat serum in PBS (0.01M) for 1 h before incubating with primary antibody to SRSF1 (1:50, Proteintech) and αSMA (1:100, DAKO) at 4°C overnight. After rinsing in PBS, the slides were incubated with Alexa Fluor 594-conjugated goat anti-rabbit secondary antibody; and Alexa Fluor 488-conjugated goat anti-mouse secondary antibody (1:1000, Cell Signaling Technology) and DAPI at room temperature for 2 h, and observed with a fluorescence microscope (Olympus IX71).

Fluorescence quantification

Image pro plus (version 6.0.0.260) was used in the fluorescence quantification experiment. To quantify the distribution of SRSF1, the colocalization coefficient m_2 between red signal and blue signal was measured, which suggested the proportion of red pixels colocalized with blue pixels (DAPI). The mean density of red signal in the nucleus was calculated according to this formula: mean density = IOD (Integrated optical density) / Area (pixels).

Table 2. SiRNA fragments.

Name		Sequence
SRSF1-174	sense:	GCAUCUACGUGGGUAACCUTT
	anti-sense:	AGGUUACCCACGUAGAUGCTT
SRSF1-351	sense:	GCUACGAUUACGACGGCUATT
	anti-sense:	UAGCCGUCGUAAUCGUAGCTT
SRSF1-547	sense:	GCGUGAAGCAGGUGAUGUATT
	anti-sense:	UACAUCACCUGCUUCACGCTT
Negative Control	sense:	UUCUCCGAACGUGUCACGUTT
	anti-sense:	ACGUGACACGUUCGGAGAATT

Statistical Analysis

All values were expressed as mean ± *SD*. The *Student t*-test was used for the comparing between two groups, and p-value < 0.05 was accepted as statistically significant.

Results

Vascular remodeling, expression of VEGFA isoforms, and the translocation of SRSF1 in thoracic aorta of hypertensive rat

To visualize the existence of vascular remodeling of thoracic aorta induced by hypertension, HE staining was used to detect the tunica media of thoracic aorta after 1-week constriction. In comparison with sham-operated group, the media portion of rat thoracic aorta was remarkably thickened (Figure 1A).

RT-PCR was performed to detect the expression level of different isoforms of VEGFA. Compared with the control group, the mRNA level of total-VEGFA (pan-VEGFA), and the secreted isoforms including VEGFA120 and VEGFA164 were significantly increased after the surgery, while there was no significant change in the mRNA level of VEGFA188 which binds to cell surface and extracellular matrix (Figure 1B).

To further explore the molecules that might be responsible for the VEGFA expression pattern change, Ingenuity Pathways Analysis (IPA, QIAGEN Redwood City, http://www.ingenuity.com/products/ipa) was used for the prediction. IPA software is used to model, analyze, and understand the complex biological and chemical systems at the core of life science research. Figure 1C showed that up to 4 SR proteins-SRSF1, SRSF3, SRSF5, and SRSF6-were potential associated with VEGFA, and SRSF1 was in the center of the predicted network (Figure 1C).

Immunofluorescence was performed to detect the location of SRSF1 (red) with α-SMA (green) to visualize cytoplasm and DAPI (blue) to visualize nuclei. The images revealed that in normal aorta (sham control), SRSF1 was co-localized with both αSMA and DAPI, which resulted in a predominant red signal (SRSF1) in the merge. Whereas, in aorta from 1-week hypertensive rat model, the immunofluorescence signal of SRSF1 co-localized with DAPI was significantly increased and SRSF1 signal in cytoplasm was attenuated, which led to a remarkable increase of green signal (α-SMA) showed in cytoplasm of the merged photo (Figure 1D). This co-localization indicated the shuttling of SRSF1 from cytoplasm to nucleus in response to hypertension. To more convincingly show this, a quantification experiment was carried out, and the results certificated the translocation of SRSF1 and increased mean density of red signal in the nucleus (Figure 1E).

Based on those results, our assumption is that the nuclear translocation of SRSF1 may play an important role in regulating the VEGFA alternative splicing during the pressure-overload induced vascular remodeling.

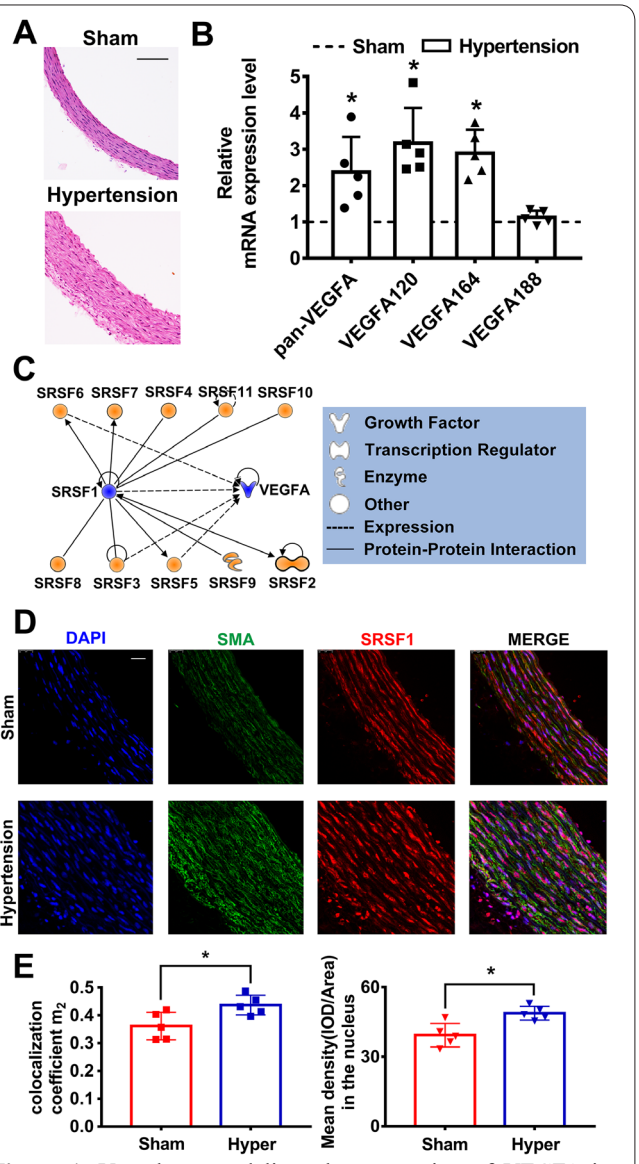

Figure 1. Vascular remodeling, the expression of VEGFA isoforms, and the translocation of SRSF1in the thoracic aorta of hypertensive rat after the constriction surgery for 1 week. **(A)** HE staining revealed that the vascular wall was significantly thickened in hypertension group compared with the sham-operated group (Bar=100 μm). **(B)** The mRNA level of pan-VEGFA, VEGFA120, and VEGFA164 were significantly increased in hypertension group (n=5). **(C)** Ingenuity Pathways Analysis predicated the regulatory networks between VEGFA and SRSF families. **(D)** Immunohistofluorescence study revealed more complete co-localization of SRSF1 with DAPI, the nuclear marker, in hypertension group compared with the control group (Bar=25 μm). **(E)** The fluorescence quantification experiment carried out with Image pro plus showed a significant higher AOI and colocalization coefficient in the hypertension group compared with sham group. Values were presented as the mean ± *SD*, **P*<0.05 vs. control.

High cyclic stretch induced VEGFA alternative splicing in VSMCs

To evaluate the effect of hypertensive cyclic stretch on VEGFA alternative splicing in VSMCs, different magnitudes of cyclic stretch were applied *in vitro* and the expression of VEGFA isoforms were examined. Figure 2A showed that in comparison with 5% cyclic stretch (mimics the normotension), 15% cyclic stretch (mimics the hypertension) significantly increased pan-VEGFA, VEGFA120, VEGFA164 and VEGFA188 expression at mRNA level. ELISA revealed an increased secretion level of VEGFA protein in the supernatant from VSMCs after 15% cyclic stretch application (Figure 2B). Interestingly, there was no significant difference between 5% and 15% cyclic stretch groups when it came to the amount of VEGFA accumulated in the

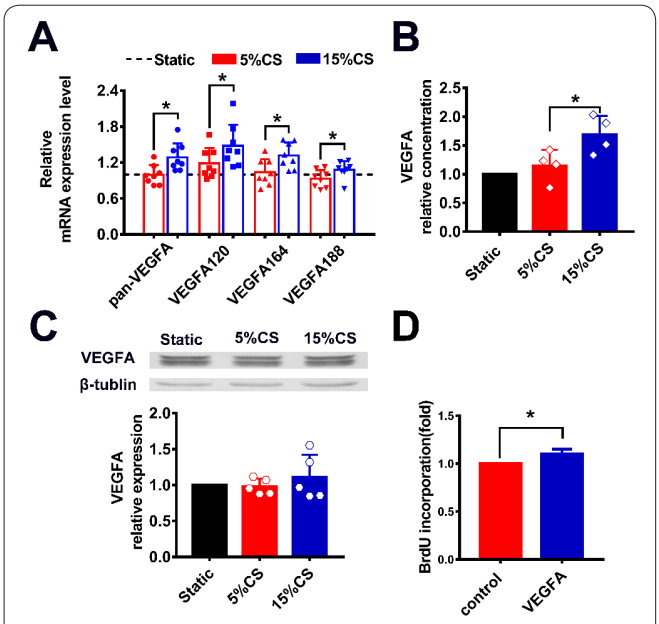

Figure 2. High cyclic stretch induced VEGFA alternative splicing in VSMCs. **(A)** In comparison with 5% cyclic stretch, 15% cyclic stretch significantly increased expression of pan-VEGFA, VEGFA120, VEGFA164 and VEGFA188 at mRNA level which was detected with RT-PCR (n=8). **(B)** The secretion level of VEGFA protein was increased in the supernatant from VSMCs subjected to the 15% cyclic stretch detected with ELISA (n=4). **(C)** 15% cyclic stretch revealed no significant effect on the amount of VEGFA accumulated in the cytoplasm compared with 5% cyclic stretch (n=5). **(D)** Incubation with 1 ng/ml VEGFA for 18 h significantly increased the proliferation of VSMCs (n=4). Values were presented as the mean ± *SD*, **P*<0.05 vs. control.

VSMCs which was detected by western blot (Figure 2C). Previous work of our lab revealed that high cyclic stretch enhanced proliferation of VSMCs (5). To verify if secreted VEGFA can increase the VSMC proliferation, we incubated VSMCs with 1 ng/ml VEGFA for 18 h. Figure 2D showed VEGFA significantly increased the proliferation of VSMCs detected with BrdU. These results indicate that the increased secretion of VEGFA may be responsible for the VSMC proliferation induced by pathological high cyclic stretch.

High cyclic stretch induced the nuclear accumulation of SRSF1

To confirm the effect of high stretch on SRSF1 translocation that was suggested in the hypertensive rat model, western blot and immunofluorescence staining were used to detect the expression of SRSF1 after cyclic stretch application. Figure 3A showed that a 15% cyclic stretch increased the expression of SRSF1 in nuclear extraction referenced with nuclear protein LaminA/C while the amount of SRSF1 in cytoplasm remained unchanged. Furthermore, immunofluorescence staining also revealed that 15% cyclic stretch significantly increased the accumulation of SRSF1 in nuclei which were localized with DAPI (Figure 3B).

Based on those results, further experiments were carried out to identify the specific role of SRSF1 in VEGFA alternative splicing.

The expression of VEGFA isoforms induced by high cyclic stretch was reversed by siRNA transfection

Three siRNA fragments target to SRSF1 were desi-

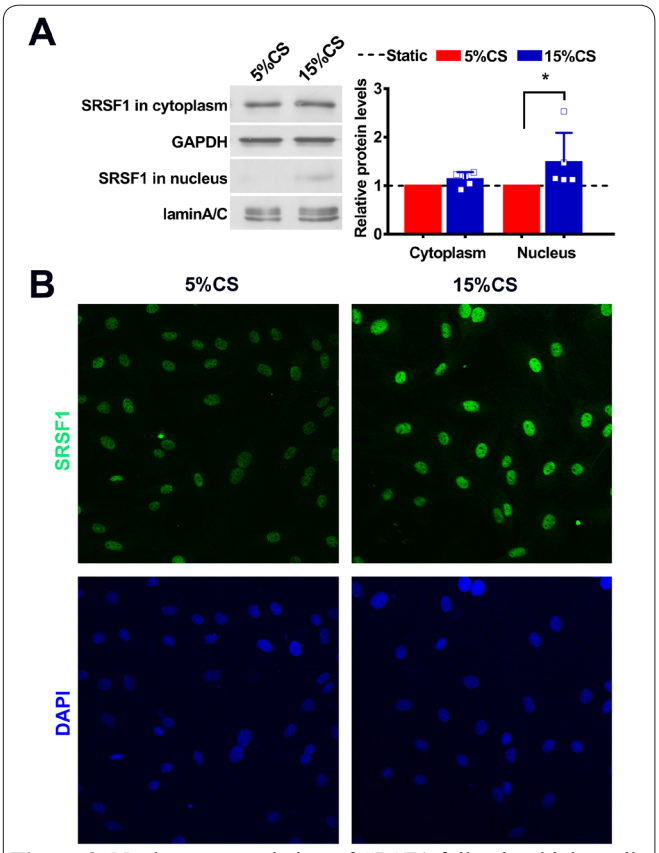

Figure 3. Nuclear accumulation of SRSF1 following high cyclic stretch. **(A)** Western blot analysis showed that 15% cyclic stretch significantly increased the amount of SRSF1 in the nuclei compared with 5% cyclic stretch but the amount of SRSF1 in cytoplasm didn't change. LaminA/C was used as nuclear protein loading control and GAPDH was used as cytoplasmic protein loading control. (n=5). **(B)** Immunofluorescence staining showed a higher nuclear fluorescence intensity after the application of 15% cyclic stretch compared with 5% cyclic stretch (Bar=50 μm). Values were presented as the mean ± *SD*, **P*<0.05 vs. control.

gned and synthesized, after detecting the expression of SRSF1 after transfection, SRSF1-351 was identified as the most effective fragment and used for the following siRNA interference (Figure 4A).

Our results indicated that, after SRSF1 siRNA interference, 15% cyclic stretch failed to induce the expression of different VEGFA mRNA. For VEGFA120, transfecting VSMCs with SRSF1 specific siRNA completely reversed the effect of 15% cyclic stretch. For other isoforms, 15% cyclic can't induce the expression of those isoforms after transfection, which was compared with 5% group (Figure 4B). ELISA was then used for the detection of secreted VEGFA protein, and the secretion of VEGFA from VSMCs induced by 15% cyclic stretch was abolished by SRSF1 siRNA interference (Figure 4C).

Our results showed that SRSF1 specific interference modulates the alternative splicing and secretion of VEGFA, and suggested the important role of SRSF1 in the high cyclic stretch induced VEGFA expression pattern change.

The ratio of different VEGFA variants referenced with pan-VEGFA after siRNA transfection

To more convincingly show the important role of SRSF1 in the alternative splicing induced by 15% cyclic stretch, the ratio of different variants referenced with

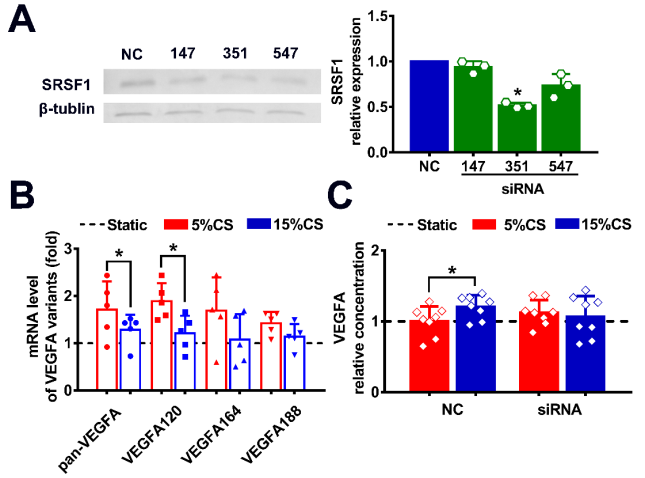

Figure 4. The expression of VEGFA isoforms induced by high cyclic stretch was reversed by specific SRSF1 siRNA transfection. VSMCs were transfected with nonspecific control (NC) siRNA or a siRNA specific for SRSF1 for 48 hours and then subjected to cyclic stretch. **(A)** siRNA-351 was the most effective SRSF1 siRNA fragment (n=3). **(B)** After siRNA interference, 15% cyclic stretch resulted in a significant decreased mRNA level of pan-VEGFA and VEGFA120 compared with 5% cyclic stretch (n=5). **(C)** After siRNA interference, no significant difference of VEGFA concentration in the supernatant was detected between 5% group and 15% group (n=8). Values were presented as the mean ± *SD*, **P*<0.05 vs. control.

pan-VEGFA was analyzed. The results showed that compared with the NC group, siRNA interference resulted in a significant decrease of the ratio of VEGFA120 and VEGFA164 referenced with pan-VEGFA under 15% cyclic stretch application, while no significant change was observed under 5% cyclic stretch. Those results certificated the important role of SRSF1 in the alternative splicing induced by 15% cyclic stretch from a different perspective.

Discussion

Present study showed that the expression pattern changes of VEGFA isoforms were associated with the nuclear translocation of SRSF1 in hypertensive rat thoracic aorta. We further demonstrated that high cyclic stretch induced the expression and translocation of SRSF1 in the nuclei which may result in, at least in part, the expression pattern changes of VEGFA that subsequently increases its secretion and plays important role in modulating proliferation of VSMCs.

Numerous researchers have identified high cyclic stretch as a crucial factor that disturbs the homeostasis of VSMCs. For example, experiments identified the thrombin generation resulted from cyclic stretch in the rat VSMCs and the potential role of integrin related pathway in this pathological process (22). Our previous work also demonstrated that cyclic stretch stimulated the secretion of TGF-β1 from VSMCs and resulted in the dedifferentiation of VSMCs via silent information regulator 6 (SIRT6) (9). These results indicated that cyclic stretch can induce the secretion of various cytokines from VSMCs and then modulate cell function in an autocrine or paracrine way.

VEGFA, the prototype of VEGF family, is a key inducer of angiogenesis in most tumors. Swaathi Jayaraman *et al.* discovered that VEGFA is responsible for the Cbp/p300–interacting transactivator with Glu/Asp–rich carboxy-terminal domain-2 (CITED2) silencing induced inhibition of tumor angiogenesis, and explored the role of CITED2 as a potential therapeutic target for breast cancer (23). In vascular system, VEGFA mediated the crosstalk between human adipocytes and VSMCs which might play an important role in obesity-related vascular diseases (24). Fan *et al.* also found that the autocrine signaling of VEGFA regulated by cysteine-rich motor neuron 1 (Crim1) is of great importance in the regulation of retinal vascular stability (25).

Those works exploited the biological roles of VEGFA in different pathological processes. To gain further insight into the significant roles of VEGFA, many studies have focused on the specific VEGFA isoforms. In human, RT-PCR and automated laser fluorescence fragment analysis showed that VEGFA121 and VEGFA165 are predominant isoforms in breast and ovarian cancer cell lines while VEGFA189 is less abundant (26). Rabbit corneal angiogenesis assay carried out by Zhang *et al*. identified VEGFA121 as the isoform with the most potent angiogenic activity among the three variants (27). Yuan *et al*. proved that the expression level of VEGFA189 mRNA was an important survival indicator for patients with non-small-cell lung cancer (28). These investigations underlined the different biological property of VEGFA variants and the necessity for the understanding of underlying mechanisms.

Our present work identified the increased expression of VEGFA120 and VEGFA164 in both the *in vivo* hypertensive group and the *in vitro* high-cyclic-stretch group. This led us to focus on the mechanism that was responsible for the generation of different isoforms of VEGFA mRNA.

Alternative splicing is of great importance in mRNA metabolism and strikingly expand the coding capacity of genome (15), which enables the cells to react quickly and efficiently under different stimulus (29, 30). Based on the bioinformatic analysis, our present works, both *in vivo* and *in vitro*, revealed the nuclear translocation of SRSF1 which was associated with the expression pattern change of VEGFA. These results were consistent with the role of SR proteins as a key regulator of alternative splicing identified in other cells or tissues. Studies carried out by Lopezmejia IC *et al*. focused on the roles of SRSF1 and SRSF6 in LaminA gene associated alternative splicing events (31). The shuttling of these SR proteins between nucleus and cytoplasm was also marked as the activation of alternative splicing. By immunofluorescence, Sanford JR *et al*. certificated the progressive translocation of SR proteins during the development of nematode *Ascarislumbricoides* which correlated with the startup of pre-mRNA splicing (32).

Thus, to explore the possible role of SRSF1 in high cyclic stretch induced VEGFA alternative splicing, the expression of SRSF1 in VSMCs was specifically inhibited by siRNA interference *in vitro*. After the transfection, the results (Fig. 4B and Fig. 5) showed that the inhibition of SRSF1 decreased the mRNA level of different VEGFA variants and reversed the effect of 15% cyclic stretch. And the secretion of VEGFA was also reversed. Those results revealed the important role of SRSF1 in

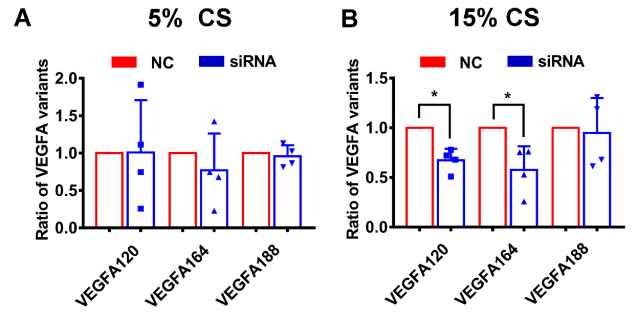

Figure 5. VSMCs were transfected with nonspecific control (NC) siRNA or a siRNA specific for SRSF1 for 48 hours and then subjected to cyclic stretch. **(A)** the ratio of different VEGFA variants referenced with pan-VEGFA in the condition of 5% cyclic stretch; **(B)** the ratio of different VEGFA variants referenced with pan-VEGFA in the condition of 15% cyclic stretch (n=4).

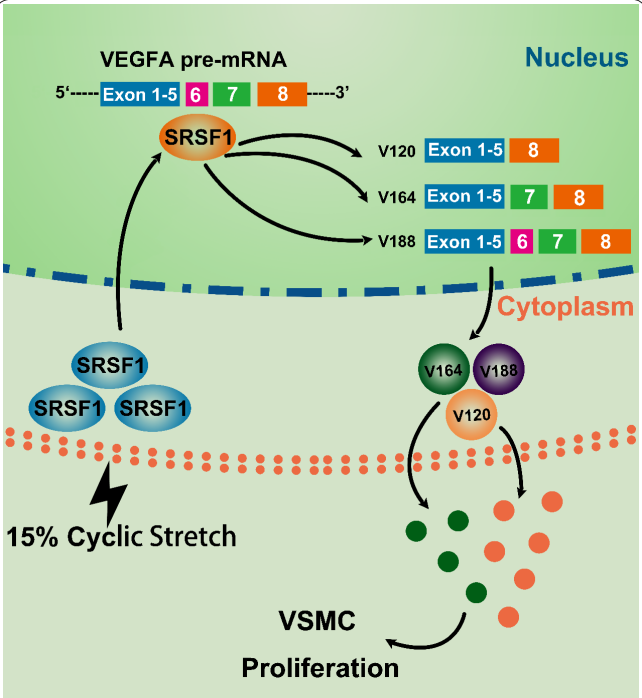

Figure 6. Schematic diagram of the possible roles of 15% cyclic stretch in SRSF1 nuclear translocation and VEGFA alternative splicing in VSMCs proliferation.

high cyclic stretch induced VEGFA alternative splicing. However, how cyclic stretch influences the subcellular distribution of SRSF1 and the role of other SR proteins in the VEGFA alternative splicing induced by hypertension remained unclear. Further studies are still needed to understand the underlying mechanisms.

To draw a conclusion, in hypertension, pathological increased cyclic stretch may induce the nuclear translocation of SRSF1, which induces the alternative splicing of VEGFA and subsequently enhances the secretion of VEGFA into the extracellular space and then induces the abnormal proliferation of VSMCs (Figure 6).

In this study, we investigated the role of SRSF1 in the regulation of VEGFA during pathological cyclic stretch induced vascular remodeling. Those results may be valuable for the understanding of mechanobiology mechanisms during hypertension.

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Interest conflict

The authors confirm that there are no conflicts of the interest.

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