The Role of Blood-derived Exosomal hsa-miR-130a-5p from Abdominal Aortic Aneurysm patients in Human Aortic Smooth Muscle Cells

Jung-Hyun Kim

The Graduate School Yonsei University

Graduate Program in Biomedical Engineering

The Role of Blood-derived Exosomal hsa-miR-130a-5p from Abdominal Aortic Aneurysm patients in Human Aortic Smooth Muscle Cells

Directed by Professor Donghoon Choi

A Dissertation submitted
to the Graduate Program in Biomedical Engineering
and the Graduate School of Yonsei University
in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

Jung-Hyun Kim

December 2022

This certifies that the Doctoral Dissertation of Jung-Hyun Kim is approved.

Thesis Supervisor: Donghoon Choi

Thesis Committee Member #1: Sang-Hak Lee

Thesis Committee Member #2: Jung-Sun Kim

Thesis Committee Member #3: Chan Joo Lee

Thesis Committee Member #4: Seung-Jun Lee

The Graduate School
Yonsei University

December 2022

ACKNOWLEDGEMENTS

I would like to thank my dissertation advisor professor Dr. Donghoon Choi, who has been an invaluable teacher and mentor. He gift for conceptualization, he enduring encouragement, and he practical advice have been an inestimable source of support for me during this process. I would also like to thank Dr. Seung-Jun Lee for their advice and feedback. Their varied perspectives have helped me to strengthen my work.

Thank you to Dr. Subin Jung who shared this journey with me. And thank you to Dr. Sang-Hak Lee, Dr. Jung-Sun Kim and Dr. Chan Joo Lee for your feedback and comments. I am grateful to Soyoung Cho, Heejung Lim, Juyeon Lim, Seul-Gi Lee, Jung-Jae Lee Gi-Bbeum Lim, Hyunju Lee, and Suyeon Jin who helped me a lot during my lab life.

And lastly, I sincerely thank my family for patiently waiting for me to obtain my degree for a long time.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

The Role of Blood-derived Exosomal has-miR-130a-5p from Abdominal Aortic Aneurysm patients in Human Aortic Smooth Muscle Cells

Jung-Hyun Kim

Graduate Program in Biomedical Engineering

The Graduate School, Yonsei University

(Directed by Professor Donghoon Choi)

Abdominal aortic aneurysm (AAA) is a perilous condition involving pathological dilation of the aortic wall. AAA is also a disease known to be associated with the proliferation and apoptosis of aorta smooth muscle cells (AoSMC).

Therefore, due to the asymptomatic course and rupture of this disease with dangerous consequences, the biomarker identification for early diagnosis is of great important for

clinical benefit. Abnormal phenotypic switch, migration, and proliferation of AoSMCs are hallmarks for pathogenesis of AAA.

This study, we identified miR-130a-5p as a critical regulator controlling human AoSMC phenotypic switch and migration to investigate whether miR-130a-5p affects human AoSMC functions and development of AAA. Using miRNA sequencing of blood-derived exosomes from 7 AAA and 2 controls, we identified significantly downregulated hsa-miR-130a-5p within blood-derived exosomes from AAA blood.

Ectopic expression of hsa-miR-130a-5p evidently promoted AoSMC differentiation and expression of contractile markers, such as a-SMA, SM22a, MYH11 and CNN1. hsa-miR-130a-5p potently inhibited PDGF-BB induced AoSMC phenotypic switch and migration. We further identified myocardin(MYOCD), SP-1 and TCF21 as downstream targets of hsa-miR- 134-5p in human AoSMCs and proved them to be mediators in AoSMC phenotypic switch and progression of AAA. The study results revealed that hsa-miR-130a-5p was a novel regulator in vascular remodeling and pathological progress of AAA via targeting TCF21/MYOCD expression.

In conclusion, targeting hsa-miR-130a-5p or its downstream molecules in AoSMCs might develop new avenues in clinical treatment of AAA.

Key words: abdominal aortic aneurysm (AAA), exosomal microRNA, vascular smooth muscle cell (VSMC), Phenotypic switch, Platelet-Derived Growth Factor-BB(PDGF-BB), Transcription factor 21(TCF21), myocardin(MYOCD)

The Role of Blood-derived Exosomal hsa-miR-130a-5p from Abdominal Aortic Aneurysm patients in Human Aortic Smooth Muscle Cells

Jung-Hyun Kim

Graduate Program in Biomedical Engineering

The Graduate School, Yonsei University

(Directed by Professor Donghoon Choi)

I. INTRODUCTION

Abdominal aortic aneurysm (AAA) is a pathological enlargement of the subrenal aorta close to the aortic bifurcation and is an important cause of death in the elderly. Abdominal aortic aneurysm (AAA) is a common condition involving dilatation of the aorta, greater than 5 mm in diameter, and occurs below the renal arteries [1]. Smoking is a major risk factor for enlarged vascular degeneration [2], and other risk factors include age, male, low HDL cholesterol level and genetic susceptibility [3, 4]. Immune inflammatory response,

increased biomechanical stress, extracellular matrix degradation, proteolytic degeneration of elastin and collagen in the aortic wall, genetic factors and vascular smooth muscle cell (VSMC) dysfunction are suggested to be important factors in AAA formation [5, 6]. A variety of clinical and laboratory studies have provided insight into the pathogenesis of AAA, but the underlying mechanism remains to be elucidated. Therefore, more studies and experiments are needed to explore ways to prevent early AAA formation and progression.

Extracellular vesicles are membrane-enclosed nanoscale particles released from essentially all prokaryotic and eukaryotic cells that carry proteins, lipids, RNA and DNA [7]. The population of extracellular vesicles transported in biological fluids, tissues, and conditioned media of cultured cells is heterogeneous with respect to size, shape, and composition. The four major subclasses of extracellular vesicles appear to arise from distinct biogenesis pathways and can be roughly distinguished according to their size : exosomes (50–150 nm), microvesicles (100–1,000 nm), large oncosomes (1,000–10,000 nm) and apoptotic bodies (100–5,000 nm), but are difficult to distinguish from high-density and low-density lipoproteins, chylomicrons, protein aggregates and cell debris [8]. To date, microvesicles have been detected in blood (plasma and serum), bronchoalveolar lavage fluid, urine, bile, ascites, breast milk and cerebrospinal fluid [9-14]. These circulating vesicles can be taken up by recipient cells allowing intercellular communication regardless of the distance between cells. Exosome-mediated RNA delivery is considered an effective method for cell signaling and exosomal RNA will certainly affect the biological processes of recipient cells [9-14].

In recent years, exosomes have received a lot of attention because they play an important role in the pathogenesis of cardiovascular diseases, and exosomes have been identified as a new cell-cell communication pathway and have received considerable attention as a medium for transmitting pathological information [15-19]. Exosomes participate in a wide range of physiological and pathological processes by selectively packaging and delivering cargo (proteins, nucleic acids, lipids, and metabolites) to adjacent or long-distance recipient cells. MicroRNAs (miRNAs), endogenous 18–24 nucleotide noncoding RNAs, are known to influence VSMC functions including proliferation, apoptosis, differentiation, synthesis and secretion, and have emerged as key regulators of cardiovascular physiology and disease [20, 21]. miRNAs of 21-23 nucleotides in length can specifically associate with mRNA and inversely regulate the expression of genes [22]. Gene expression is regulated by miRNAs at the post-transcriptional level, and miRNAs control mRNA stability or translational repression through base pairing with the 3' untranslated region of the target RNA [23]. miRNAs are found not only inside cells but also in biological fluids such as serum, plasma, urine and saliva, and can be delivered to other tissues via vesicular and lipoprotein transport [24, 25].

Arterial injury stimulates the production of growth factors such as platelet-derived growth factor (PDGF) by platelets and various cellular components associated with the vessel wall. Growth factor production induces phenotypic changes in vascular smooth muscle cells (VSMCs) through a transition from a quiescent "contractile" (differentiated) state to a proliferative "synthetic" (dedifferentiated) state, these changes characterized by

cell proliferation, migration and secretion of matrix. [26]. Dedifferentiated VSMCs exhibited high viability in proliferation, migration and synthesis, as well as reduced expression of differentiation markers a-SMA, SM22a, MYH11 and CNN1 [27, 28]. Phenotype switching is a pivotal factor contributing to the development of aortic aneurysm and dissection [29].

We analyzed miRNA profiles in the blood of AAA patients and applied miRNA-mRNA interactions to predict molecular pathways that could explain potential targets for therapeutic approaches to control AAA progression.Then, through miRNA sequencing screening, miR-130a-5p was identified as a key molecule to inhibit aortic detachment development, and the effect of hsa-miR-130a-5p on AoSMC phenotypic conversion and migration was further investigated. We also investigated potential molecular mechanisms.

In this study, provided important new insights into the importance and functional complexity of miRNAs for regulating AoSMC function, vascular remodeling and development of macrovascular disease.

II. MATERIALS AND METHODS

1. Study Subjects

From June 2021 to March 2022, blood samples were collected from 7 AAA patients at the Cardiology Department of Severance Hospital, and blood from two control groups was collected. All the subjects have signed the informed consent form. The tests using human blood specimens were approved by the Institutional Review Board from the Department of Cardiology of Severance Hospital(4-2019-1047).

2. Exosome Isolation

Transfer entire plasma/serum sample to 1ml per each of 1.5ml tubes. Centrifuge the sample at 3,000 x g for 15 minutes to remove cells and cell debris. Transfer 0.2 volume (almost 200ul) Exo2D™ (EV isolation kit for RNA ananlysis(for plasma/serum) code No. EP-RU20)-L Reagent A buffer to a new 1.5ml tube. Incubate the Exo2D™-L Reagent A buffer at 37℃ for 15 minutes. Transfer supernatant to each incubated 1.5ml tube. Mix by vortexing. Rotating for 30 minutes at 4℃. Centrifuge the sample at 3,000 x g for 30 minutes at 4℃. The phase appears as white pellet. The remainder should be eliminated. Resuspend the homogenized samples using 100ul of PBS , aliquoted to avoid freeze and thaw cycles, and stored at −80°C.

3. Exosomal miRNA Characterization

Adapter trimming

Raw reads were pre-processed to eliminate adapter sequences. Adapters in the raw reads were trimmed using cutadapt program. If a sequence was matched to more than the first 5 bp of 3' adapter for read 1 or 5’ adapter for read 2, it was regarded as an adapter sequence, and then trimmed from the each read, respectively. Trimmed reads, whose length is longer than 18 bp, were selected for mapping reliability. Then, the remaining reads are classified into non-adapter reads, whose adapter sequences were not sequenced.

Clustering

To minimize the sequence redundancy for computational efficiency, trimmed reads were clustered by a sequence. A unique cluster consists of the reads whose sequences and length are same.

Ribosomal RNA filtering

In order to eliminate rRNA, reads were excluded that aligned to the 45S pre-rRNA and mitochondrial rRNA of *Homo sapiens*.

Identification of known miRNA reads

Sequence alignment and detection of known and novel microRNAs were performed using miRDeep2 software algorithm. rRNA-filtered reads were aligned to the matured and precursor miRNAs of *Homo sapiens* obtained from miRBase v22.1 using miRDeep2

quantifier module. The miRDeep2 algorithm is based on the miRNA biogenesis model; it aligns reads to potential hairpin structures to check whether their mapping context is consistent with Dicer processing, and assigns scores representing the probability that hairpins are true miRNA precursors.

Novel miRNAs prediction

The reference genome of *Homo sapiens*, release hg19, was retrieved from RefSeq. The reference genome was indexed and rRNA-filtered reads were mapped to it using Bowtie (1.1.2). Novel microRNAs were predicted from the mature, star and loop sequence according to the RNAfold algorithm using miRDeep2. The RNAfold function uses the nearest-neighbor thermodynamic model to predict the minimum free-energy secondary structure of an RNA sequence.

Proportion of miRNA and other RNA categories

Uniquely clustered reads are then sequentially aligned to reference genome, miRBase v22.1 and non-coding RNA database RNAcentral release 14.0 [6] to identify known miRNAs and other types of RNA for classification.

4. Cell Culture

Human primary Aorta smooth muscle cells (AoSMCs) were purchased from Lonza (CC-257) and were maintained in SmBM (Lonza, CC-3181) containing 5% fetal bovine serum (FBS), respectively. Recombinant human PDGF-BB was purchased from Sigma-Aldrich (P3201). The cells were treated with 20 ng/mL PDGF-BB under starvation conditions. For starvation conditions, cells were maintained in Dulbecco's modified egles medium (DMEM, SH30243.01) containing 0.5% FBS for 24 h.

5. Quantitative real-time PCR (qRT-PCR)

Cells were harvested an total RNA was extracted by using a Ribospin RNA Extraction Kit (GeneAll, Seoul, Korea) according to the manufacturer's instruction. The integrity of the extracted RNA was analyzed with a NanoDrop-2000/2000c (Thermo Fisher, Long Beach, NY, USA). Next, 1 μg of RNA was used for cDNA synthesis using an iScript™ cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA). RT-qPCR was performed with a SYBR Green dye system on a StepOnePlus™ real-time PCR machine (Thermo Fisher, Long Beach, NY, USA) using a standard protocol. The primer sequences are shows in Table 1.

Table 1. Primer sequences for qRT-PCR

Gene	Primer Sequence (5' - 3')
β-actin	F CACCATTGGCAATGAGCGGTTC
	R AGGTCTTTGCGGATGTCCACGT
a-SMA	F CATCACCATCGGGAATCAACGC
	R CTTAGAAGCATTTGCGGTGGAC
SM22 alpha	F AGTGCAGTCCAAAATCGAGAAG
	R CTTGCTCAGAATCACGCCAT
MYH11	F GGTCACGGTTGGGAAAGATGA
	R GGGCAGGTGTTTATAGGGGTT
CNN1	F CTGTCAGCCGAGGTTAAGAAC
	R GAGGCCGTCCATGAAGTTGTT
MYOCD	F GTCGAGTCCAACAGTTCCGGA
	R CTCACTGTCGGTGGCATAGTG
SMAD3	F CCATCTCCTACTACGAGCTGAA
	R CACTGCTGCATTCCTGTTGAC
SMAD4	F CCAGGATCAGTAGGTGGAAT
	R GTCTAAAGGTTGTGGGTCTG
NF-κB	F ATGTGGAGATCATTGAGCAGC
	R CCTGGTCCTGTGTAGCCATT
TCF21	F TCCTGGCTAACGACAAATACGA
	R TTTCCCGGCCACCATAAAGG
SP-1	F ACCAGCAGCAGCAACACCAC
	R GTCTGGAGGCCTGGCATGGA
IGF-1	F TCGCATCTCTTCTATCTGGCCCTGT
	R GCAGTACATCTCCAGCCTCCTCAGA
VEGF-A	F ACTGGACCCTGGCTTTACTG
	R TCTGCTCTCCTTCTGTCGTG

6. miRNA mimics and miRNA Inhibitor oligonucleotides

Chemically modified double-stranded RNAs designed to mimic the endogenous mature hsa-miR-130a-5p (5'-UUCACAUUGUGCUACUGUCUGC-3'), hsa-miR-1290 (5'-UGGAUUUUUGGAUCAGGGA-3') were purchased from Sigma Aldrich (St. Louis, USA). Antisense inhibitor RNAs (hsa-miR-130a-5 Inhibitor and hsa-miR-1290 Inhibitor) and negative control miRNA were purchased from Sigma Aldrich (St. Louis, USA) (anti-hsa-130a-5p and anti-hsa-1290). The miRNA mimics and miRNA Inhibitor oligonucleotides were transfected at 10 nM, respectively, using RNAi Max (Invitrogen, 13778150, Carlsbad, California, CA, USA) according to the manufacturer's protocol.

7. In Vitro Scratch Wound Assay

AoSMCs transfected with indicated miRNAs were plated in 24-well plates and two scratch wounds were generated with a 200 uL disposable pipette tip. After this, PDGF-BB was treated for 12 hours. Scratch wounds were photographed over 12 h with a Nikon inverted microscope (Nikon, Tokyo, Japan) with an attached digital camera and their widths were quantitated with ImageJ software. Distance of migration was calculated by subtracting the width measured at a given time from the width initially measured.

8. miRNA target prediction

We used the miR Base Target database to find putative miR18a-5p targets (www.mirDB.org). Candidate target genes were determined by qRT-PCR.

9. Statistical Analysis

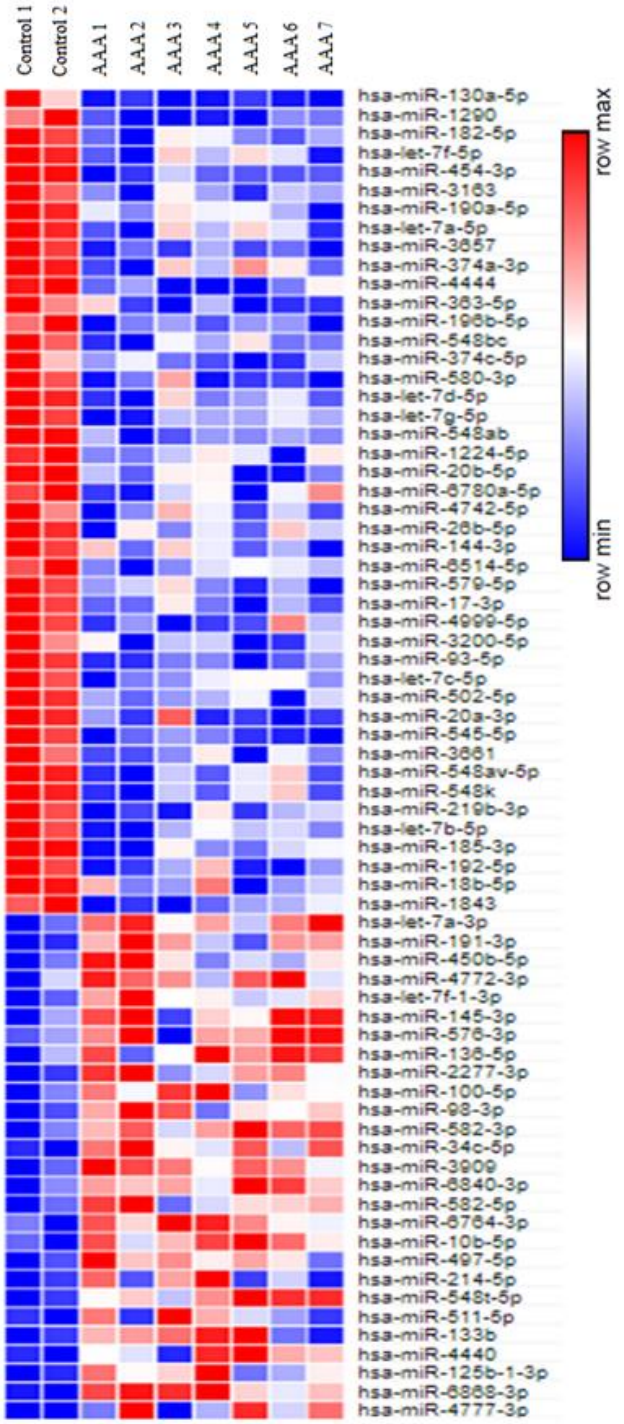
For each of the assays, three experiments were performed in triplicate, and the results were presented as the average with standard error. Statistical analyses were performed by an analysis of variance followed by Student's t test using Prism 7.04 software (GraphPAD Software Inc., San Diego, CA, USA). p values lower than 0.05 is considered as statistically significant.

III. RESULTS

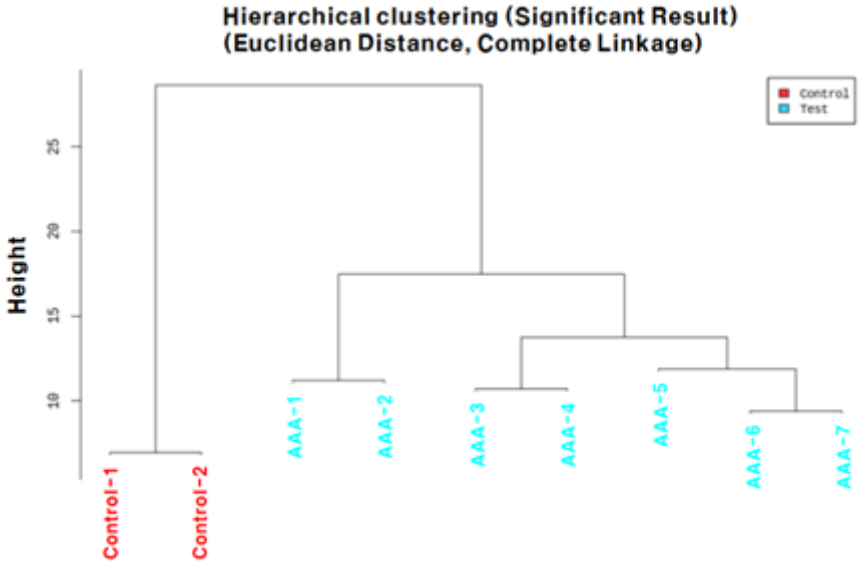
1. Differentiated Expression, Functional Enrichment for miRNA Signature in AAA Patients

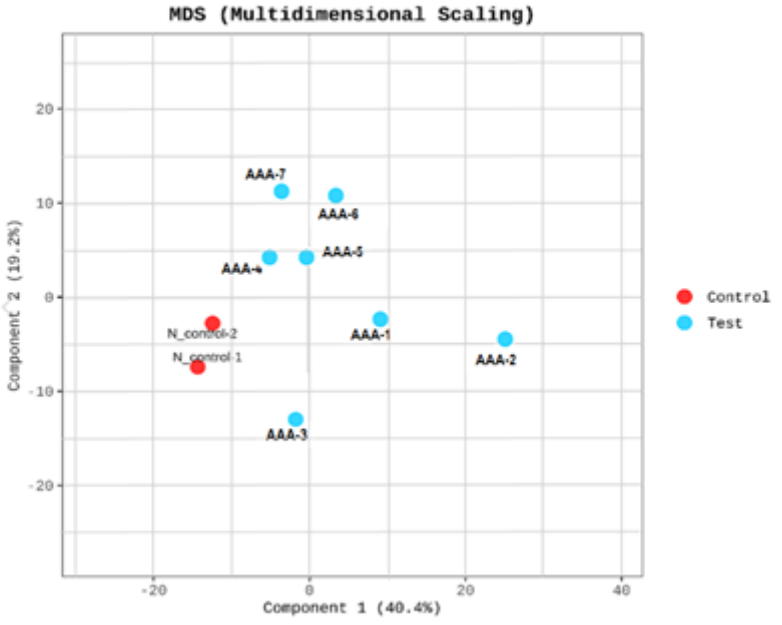
In miRNA sequencing profiles, the hierarchical cluster of miRNA sequence revealed that exosomes from the blood of AAA patients had significantly different miRNA expression signatures from exosomes from the blood of normal subjects. A total of 142 microRNAs were found to be differentially expressed between AAA and controls. Among them, 71 miRNAs showing significant differences were selected and a heat map was created (Fig. 1a) Hierarchical clustering and multidimensional scaling were conducted based on fragments per kilobase of transcripts per million mapped reads values (fold change > 2; $p < 0.05$), and the miRNA expression pattern in exosomes from the blood was distinct from that of the controls. Multidimensional scaling visualized differences in gene expression between the two groups (Fig. 1b). Overall, 44 miRNAs, including hsa-miR-130a-5p, hsa-miR-1290, hsa-miR-454-3p, hsa-miR-4444, and hsa-miR-196b-5p showed marked downregulation, and 27 miRNAs (such as hsa-miR-125b-1-3p, hsa-miR-4440, hsa-miR-497-5p, hsa-miR-6840-3p, and hsa-miR-3909) exhibited evident upregulation in AAA(Fig. 1c).

a



b





c

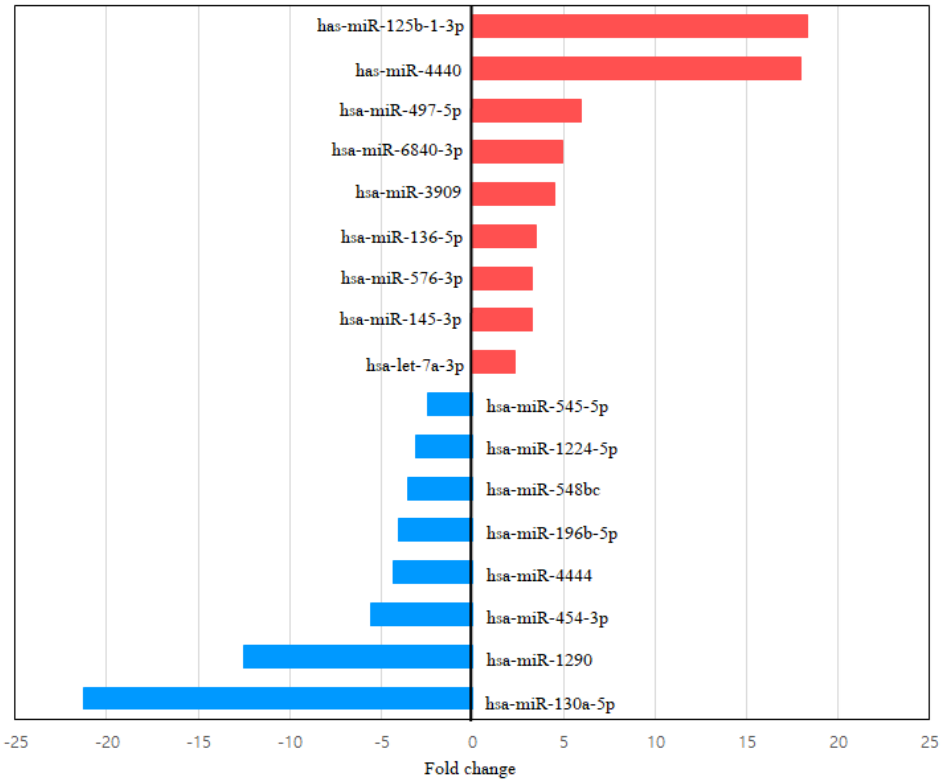


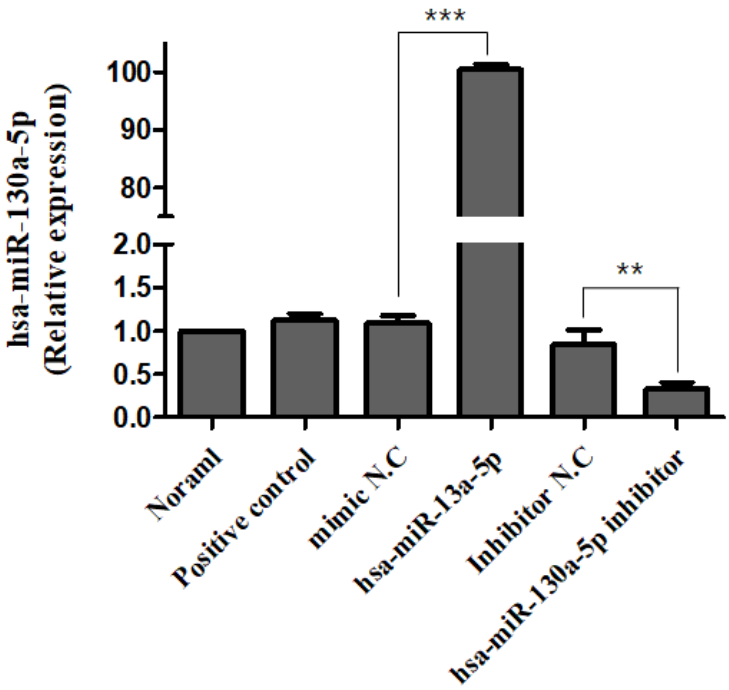
Figure 1. Differentially Expressed miRNAs

(a) The hierarchical cluster profiles of miRNA microarrays differentiate human Abdominal Aortic Aneurysm patients blood from normal blood (Control: n=2, AAA: n=7). Downregulated and upregulated miRNAs are shown in blue and red, respectively. (b) Hierarchical clustering and multidimensional scaling of data from AAA patients blood and controls blood. (c) Differentially expressed miRNAs and the corresponding fold changes.

2. Changes in miRNA expression level when miRNA was transfected in human AoSMC

To investigate the effects of hsa-miR-130a-5p and hsa-miR-1290 on human AoSMCs, negative control miRNAs, hsa-miR-130a-5p, hsa-miR-1290 mimic and hsa-miR-130a-5p, hsa-miR-1290 inhibitor were transfected into AoSMCs respectively. As shown in Fig. 2A, RT-PCR analysis revealed that hsa-miR-130a-5p expression was significantly increased in AoSMCs after hsa-miR-130a-5p mimic transfection compared to cells treated with miRNA mimic negative control. Conversely, hsa-miR-130a-5p expression was significantly decreased in AoSMCs treated by hsa-miR-130a-5p inhibitor transfection compared to AoSMCs with miRNA inhibitor control(Fig. 2a). And the same was also shown in hsa-miR-1290. RT-PCR analysis revealed that hsa-miR-1290 expression was significantly increased in AoSMCs after hsa-miR-1290 mimic transfection compared to cells treated with miRNA mimic negative control(Fig. 2b).

a



b

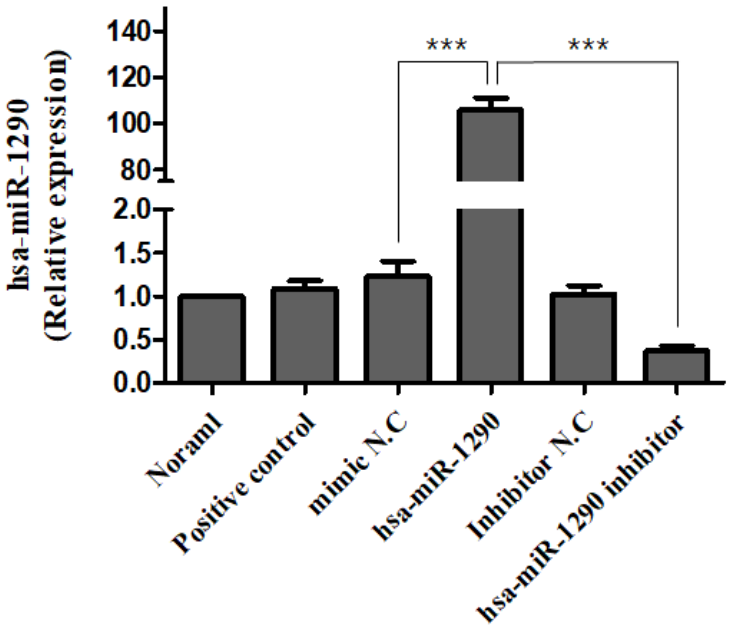


Figure 2. Changes in miRNA expression level when miRNA was transfected in human AoSMC

(a) Real-time PCR analysis revealed that hsa-miR-130a-5p mimics and inhibitor are effectively transfected into AoSMCs. (b) Real-time PCR analysis revealed that hsa-miR-1290 mimics and inhibitor are effectively transfected into AoSMCs. To compare the expression of hsa-miR-130a-5p and hsa-miR-1290 in different transfection cells, the expression of hsa-miR-130a-5p and hsa-miR-1290 in negative control cells was set as 1. Values are mean ±SEM. *, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.001$; ns, not significant

3. hsa-miRNA increases expression of phenotypic markers and suppresses expression of phenotypic switch in AoSMC *in vitro*

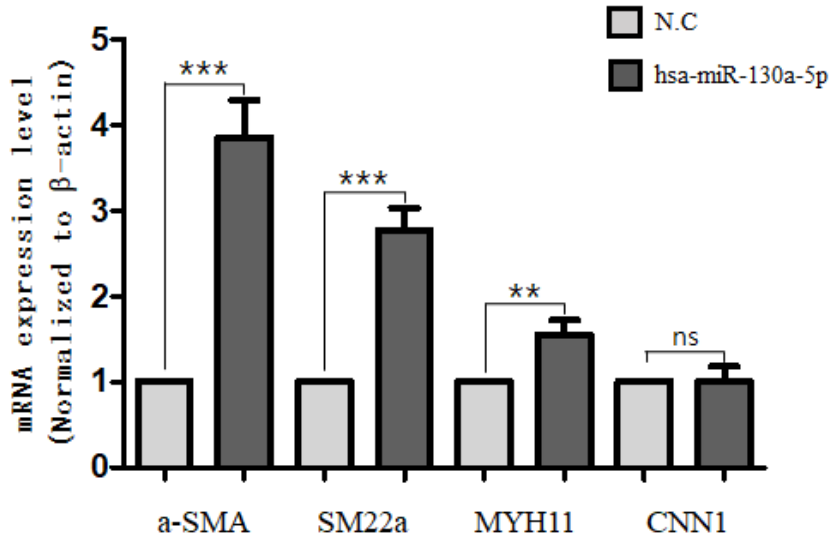
The target genes of AAA-related miRNA-hsa-miR-130a-5p were enriched in vascular development and platelet activation. These biological processes were consistent with the pathogenesis of AAA[32]. We then investigated the effect of hsa-miR-130a-5p on AoSMCs, the most pivotal cells in vascular pathophysiological response of aortic wall. With transfection of hsa-miR-130a-5p mimic (10 nM), we found a morphology change of AoSMCs.

We detected the expression of contractile markers in AoSMCs transfected with negative control (NC) or hsa-miR-130a-5p mimic and hsa-miR-1290 mimic. hsa-miR-130a-5p overexpression significantly increased α-SMA, SM22α, and MYH11 expression, while CNN1 was not significant(Fig. 3a). We also hsa-miR-1290 overexpression significantly increased α-SMA expression, while SM22α, MYH11 and CNN1 was not significant. (Figure 3b). The difference in the expression level of contractile markers was greater in hsa-miR-130a-5p than in has-miR-1290. Therefore, in subsequent experiments were conducted with hsa-miR-130a-5p.

PDGF-BB is involved in VSMC differentiation, vascular remodeling, and aortic aneurysm model construction [30]. Therefore, PDGF-BB was used in a pathological VSMC models. First, when AoSMC was treated with PDGF-BB, the expression level of

hsa-miR-130a-5p was first confirmed (Fig. 4b). Whether hsa-miR-130a-5p repressed PDGF-BB-induced downregulation of contractile markers was measured by RT-PCR. As shown in Figures 4c, overexpression of hsa-miR-130a-5p significantly increased the mRNA expression of α-SMA, SM22α and MYH11 in both quiescent and PDGF-BB stimulated conditions. These findings supported the viewpoint that hsa-miR-130a-5p was a novel regulator for phenotypic switch of AoSMCs.

a



b

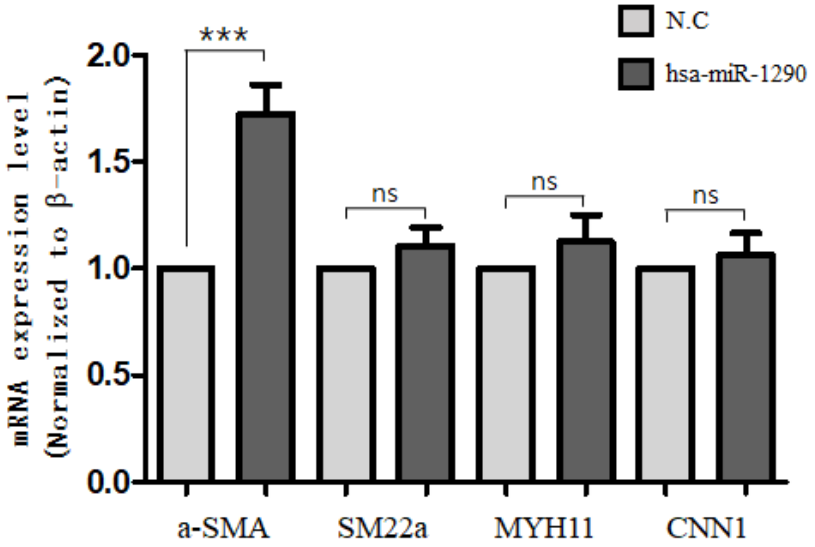


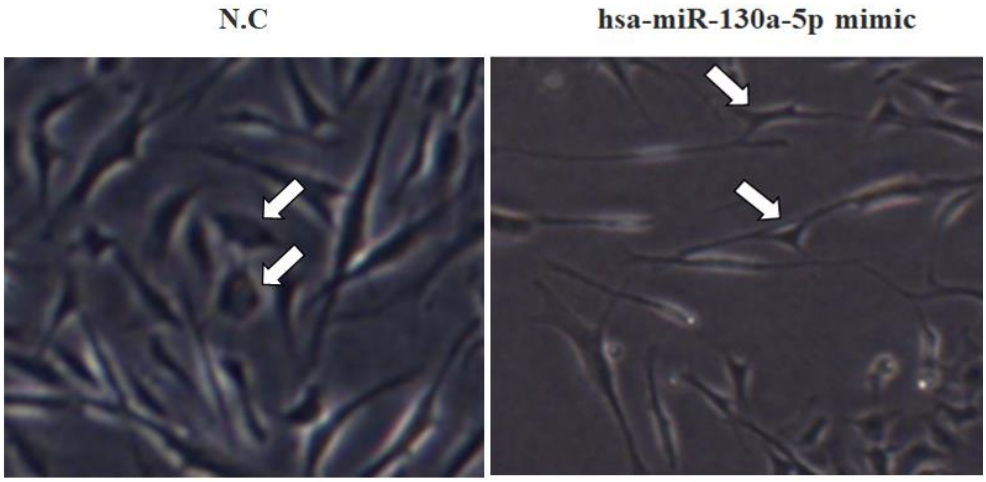
Figure 3. hsa-miRNA increases the expression of phenotypic markers in AoSMC in vitro

(a) mRNA level of contractile genes in AoSMCs transfected with NC or hsa-miR-130a-5p.

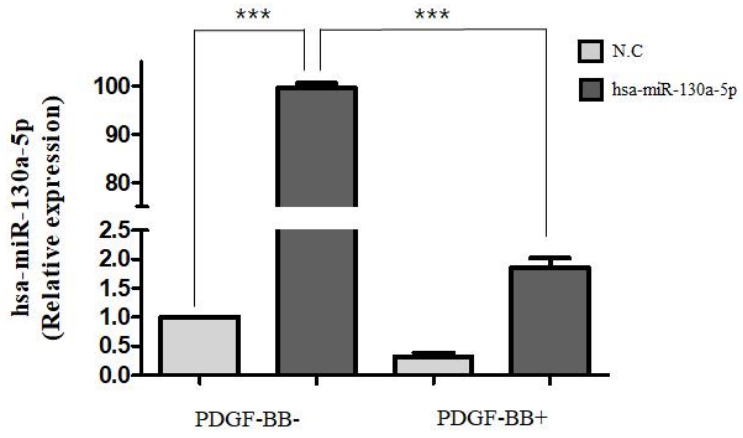
(b) mRNA level of contractile genes in AoSMCs transfected with NC or hsa-miR-1290.

Values are mean ±SEM. *, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.001$; ns, not significant

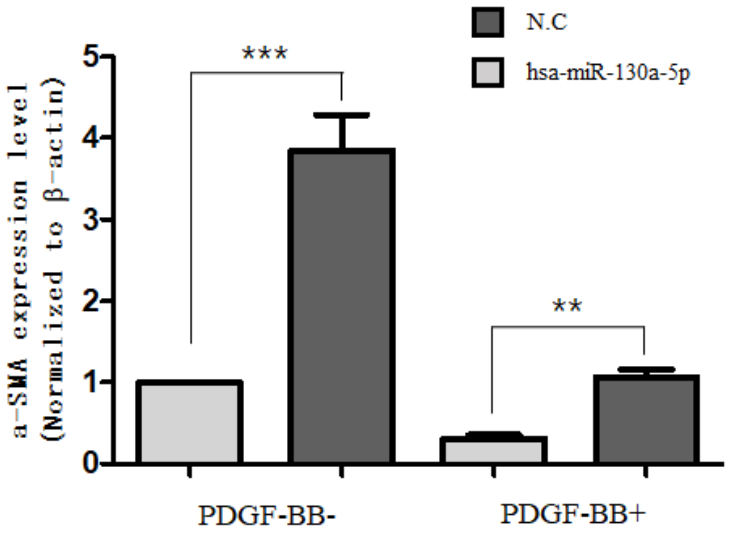
a

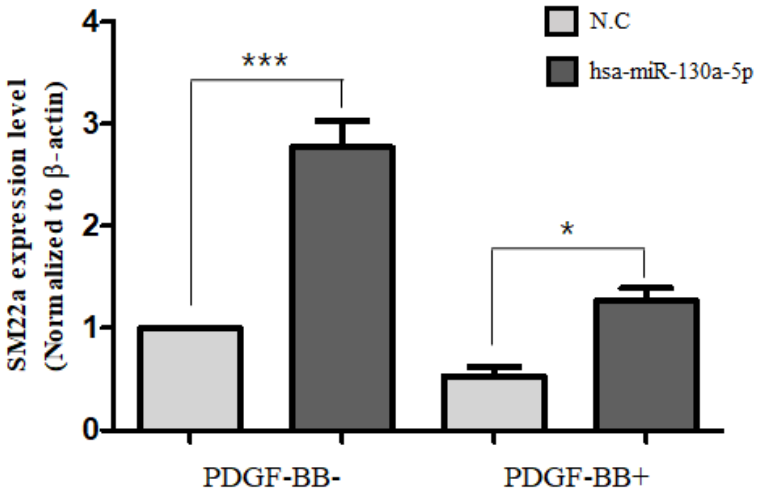


b

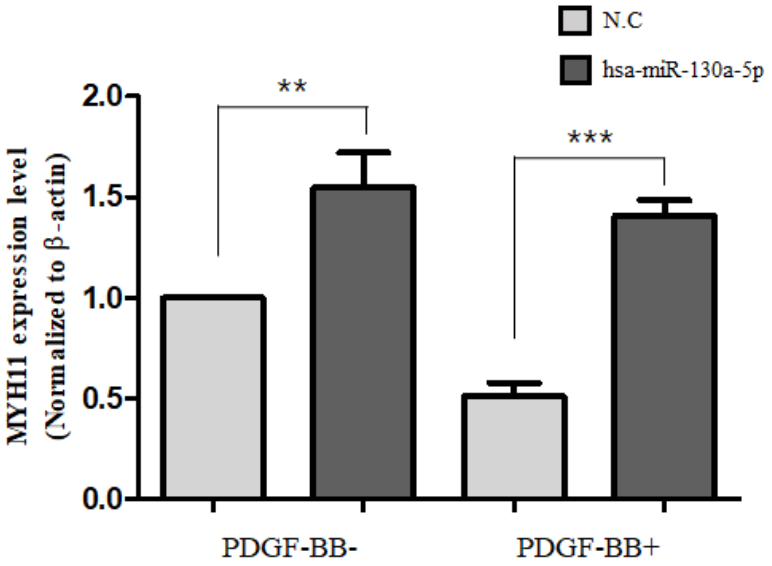


c





d



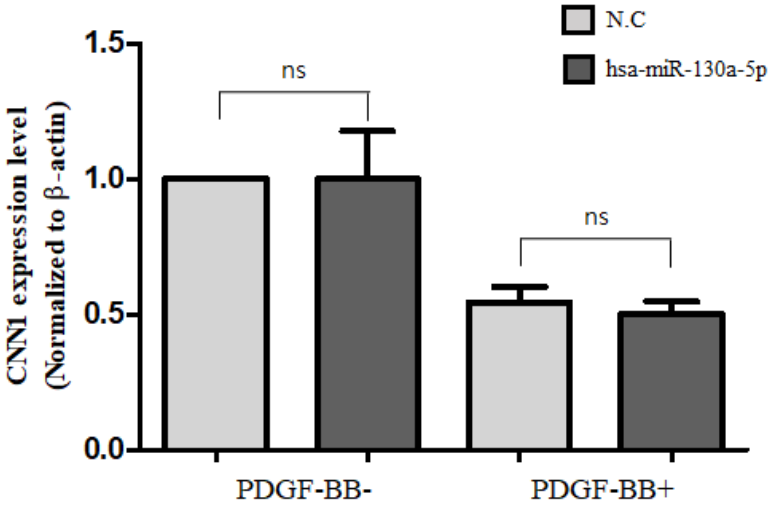


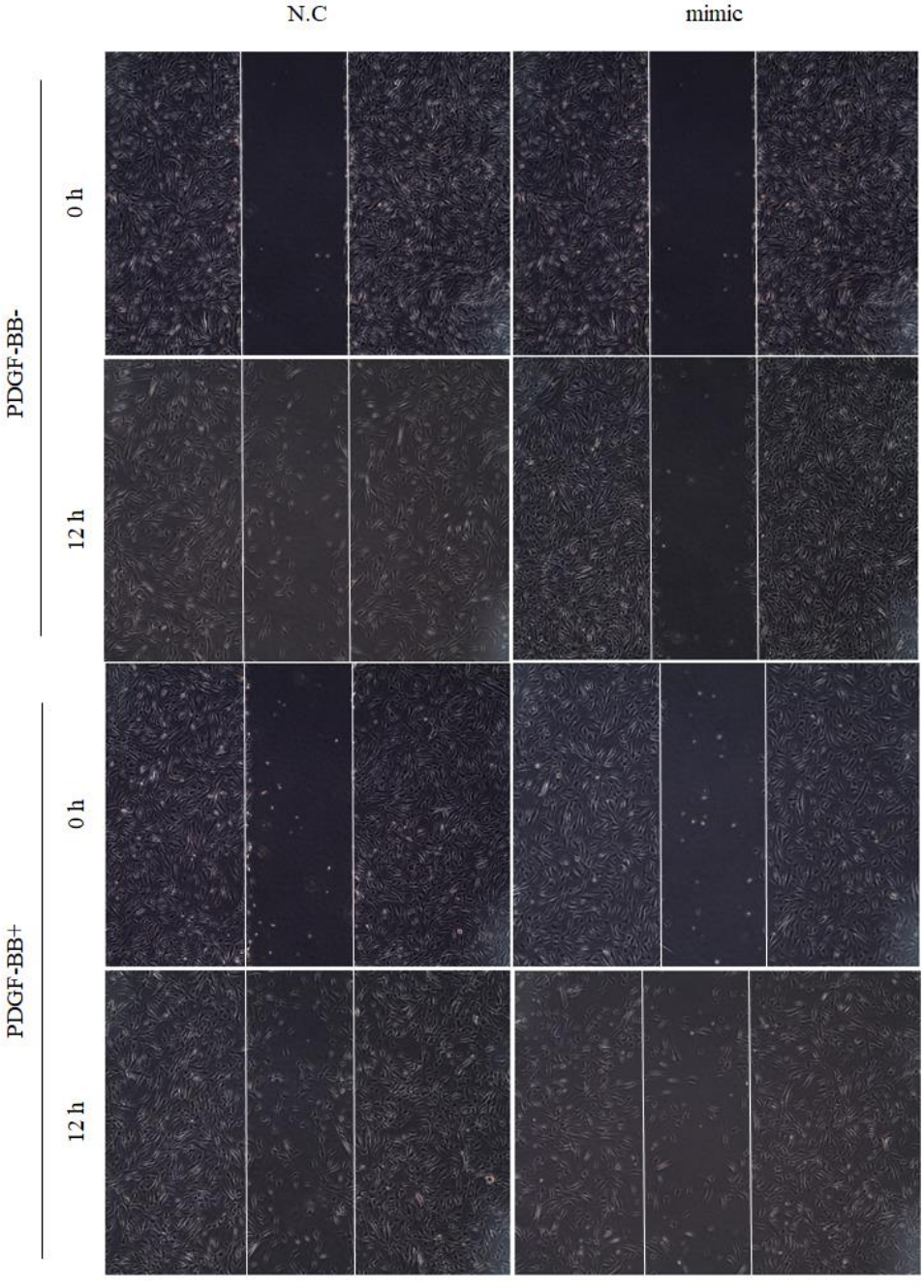
Figure 4. miR-134-5p Promoted AoSMC Phenotypic Switch and Enhanced the Expression of AoSMC Contractile Genes

(a) Morphology changes of AoSMCs transfected with hsa-miR-134-5p. (b) The quantitative real-time PCR verification of microRNA expression for PDGF-BB treat in AoSMC. (c, d) mRNA level of contractile genes in AoSMCs transfected with N.C or hsa-miR-134-5p. Values are mean ±SEM. *, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.001$; ns, not significant

4. hsa-miR-134-5p is a Novel Regulator of AoSMC Migration

Concerning the pathological mechanism of AAA, AoSMC dedifferentiation is accompanied with increased migration potential. To corroborate the role of hsa-miR-130a-5p in AoSMC migration, AoSMCs were transfected with NC and hsa-miR-130a-5p mimic. We assessed the migration potential of AoSMCs transfected with N.C or hsa-miR-130a-5p mimic by scratch-wound healing assay. Follow-up observations were conducted for 12 h. As presented in Figures 5a and 5b, hsa-miR-130a-5p mimic markedly inhibited the migration of AoSMCs in contrast to the N.C group with or without PDGF-BB induction.

a



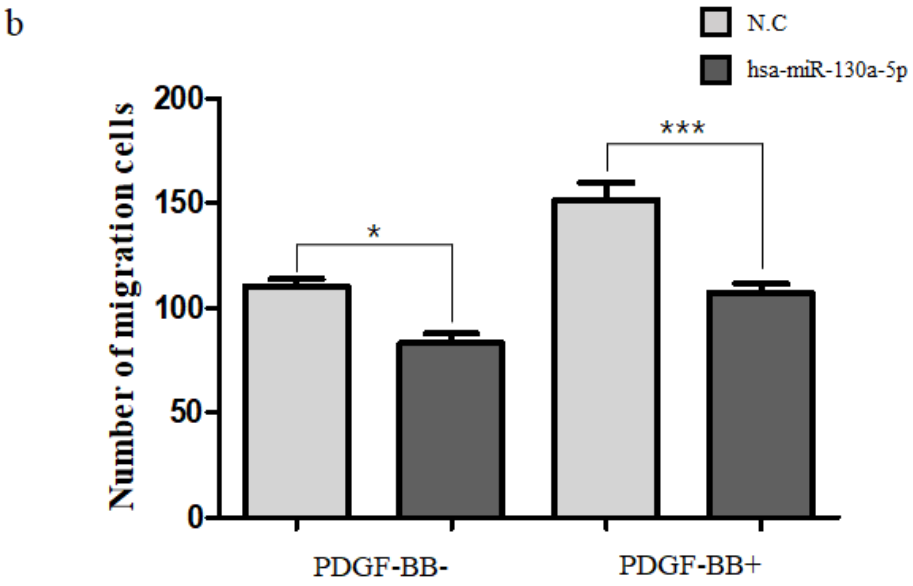


Figure 5. Changes in cell migration after transfection with hsa-miR-130a-5p or negative control

(a) Representative pictures of scratch-wound assay in different groups with or without PDGF-BB treatment. (b) Quantification of migrated cells in different groups. Values are mean ±SEM. *, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.001$; ns, not significant

5. hsa-miR-130a-5p is a novel regulator of matrix metalloproteinase excretion

We demonstrated that matrix metalloproteinases ADAMTS8 and ADAMTS18 were significantly downregulated by hsa-miR-130a-5p overexpression. However, no alteration was observed in MMP2, MMP9, MMP12 and ADAMTS4 expression (Fig. 6). Of note, ADAMTS family members were newly found metalloproteinases that were implicated in the progression of thoracic aortic aneurysm and AAA. [31].

Collectively, these results revealed that hsa-miR-130a-5p is a novel regulator for matrix metalloproteinase excretion AoSMC migration in pathogenesis of AAA.

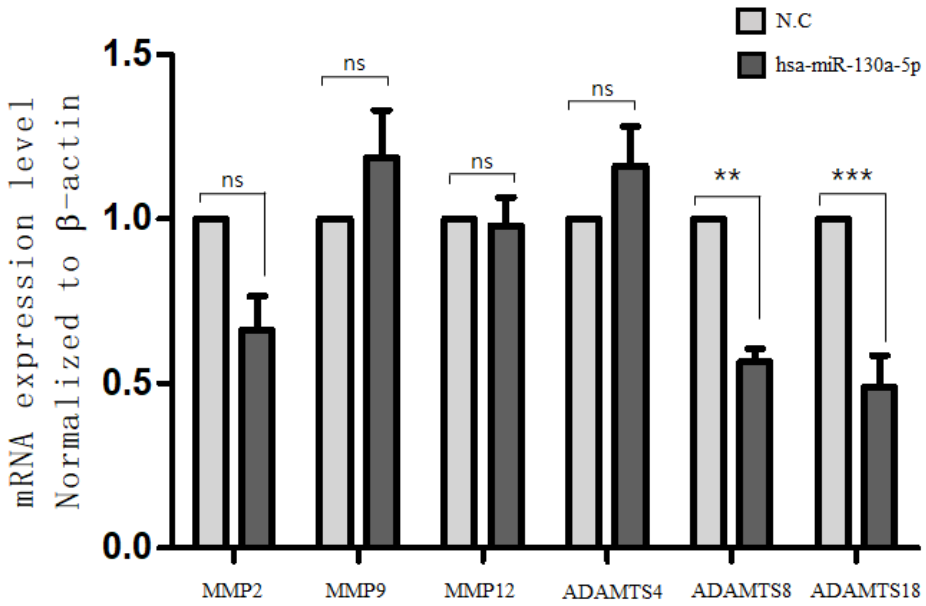


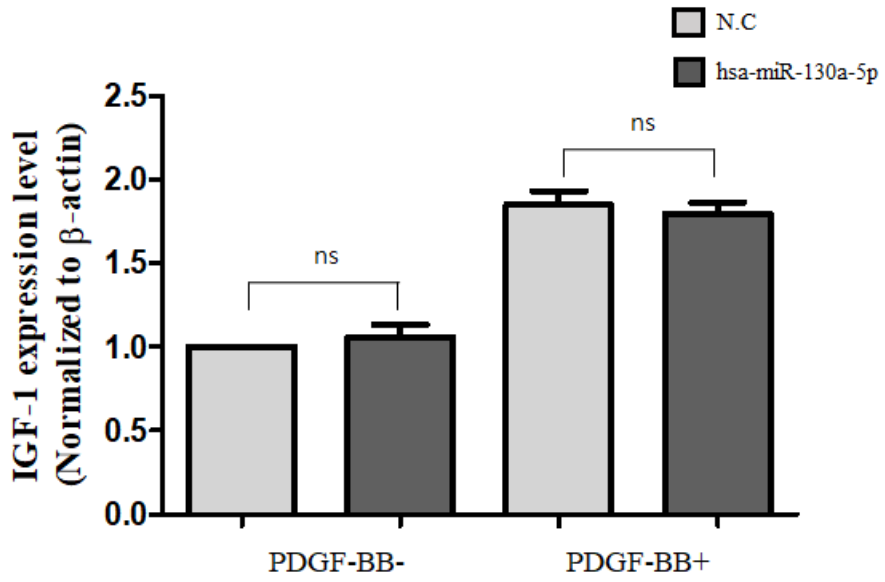
Figure 6. Matrix metalloproteinase excretion

Matrix metalloproteinase expression in human AoSMCs transfected with N.C or hsa-miR-134-5p mimic were detected using quantitative real-time PCR (n = 6). Values are mean ±SEM. *, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.001$; ns, not significant

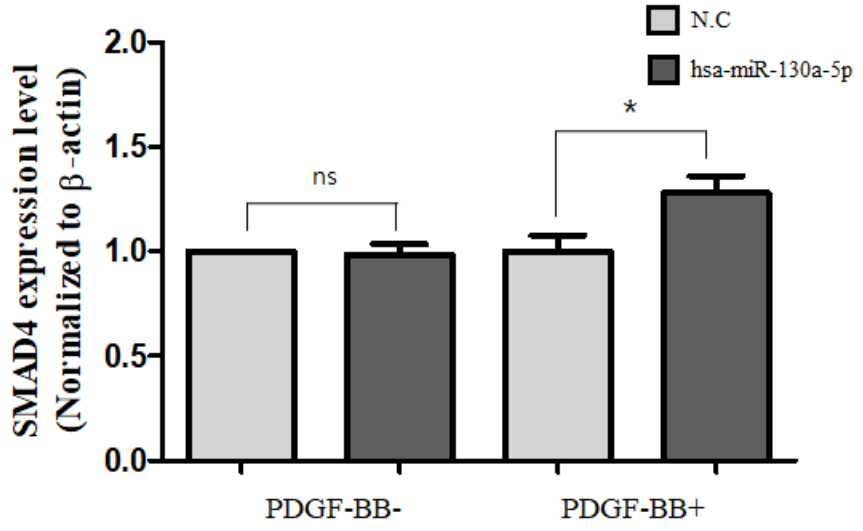
6. TCF21, SP-1 and SMAD3 is a target gene of hsa-miR-130a-5p in AoSMC

We used bioinformatics tools to identify candidate hsa-miR-130a-5p target genes. Considering that hsa-miR-130a-5p is involved in VSMC differentiation, we hypothesized that downstream hsa-miR-130a-5p target genes might be involved in differentiation. Therefore, we selected six genes (TCF21, SP-1, smad3/4, IGF1 and VEGF-A). These candidate genes have been reported to relate to differentiation [32]. According to the quantitative real-time PCR data, TCF21, SP-1 and SMAD3 exhibited a striking downregulation after hsa-miR-134-5p overexpression(Fig. 7c, d, e), while IGF-1, SMAD4 and VEGF-A had no discrimination (Fig. 7a, b, f).

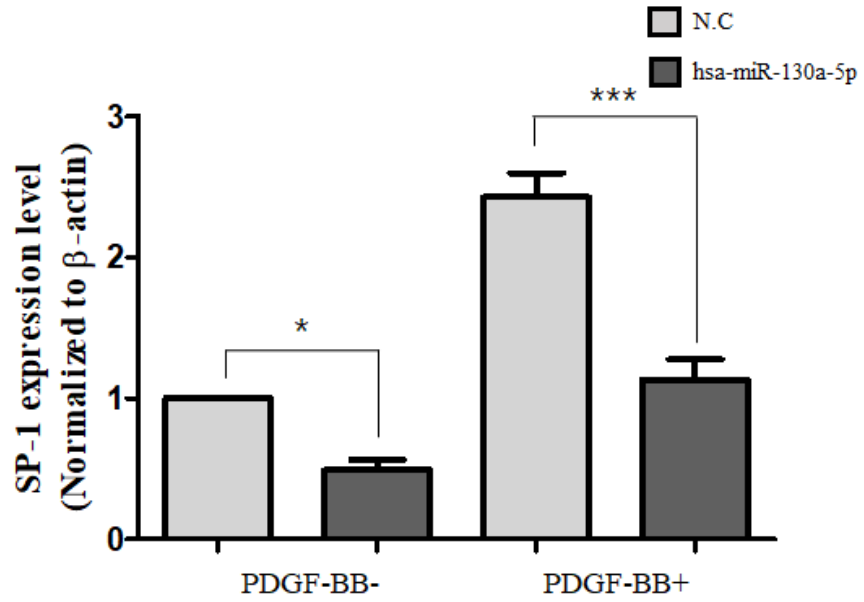
a



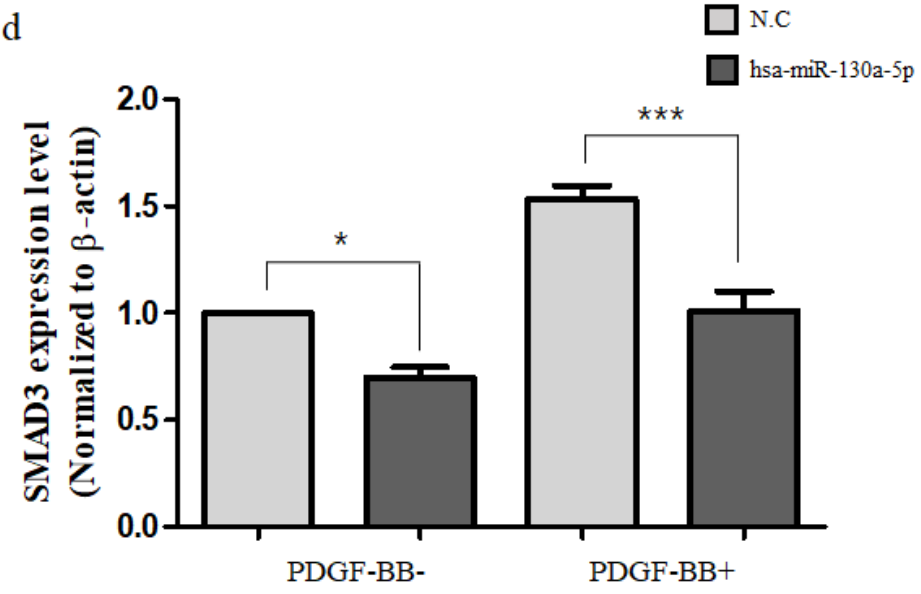
b



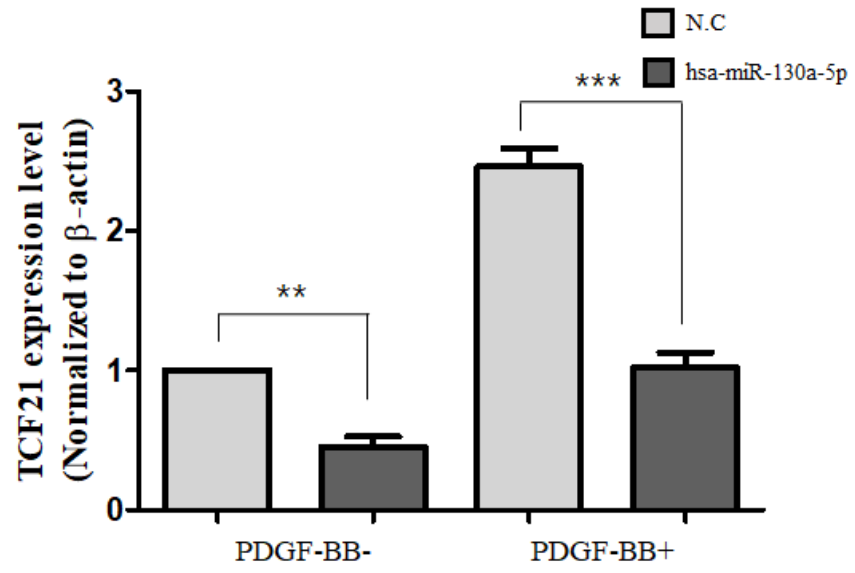
c



d



e



f

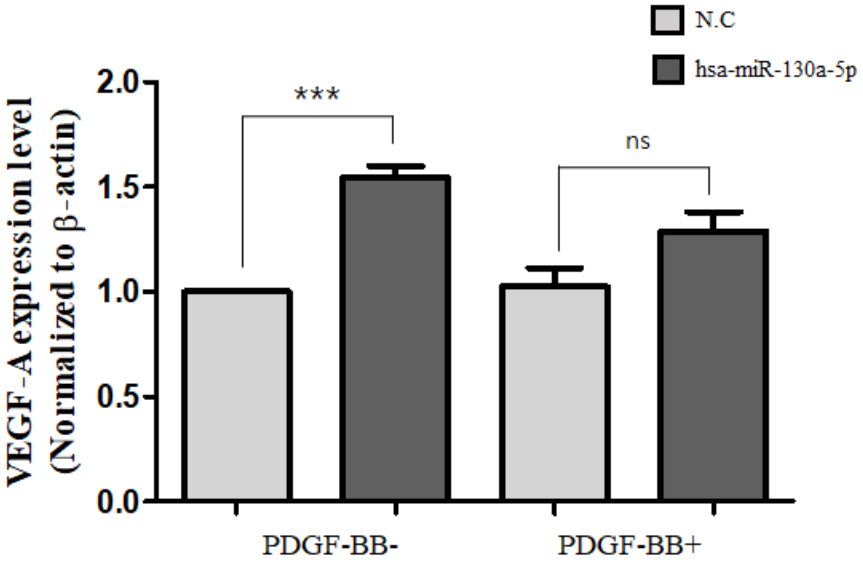


Figure 7. Measurement of target gene expression level of hsa-miR-130a-5p

Quantitative real-time PCR analysis for target gene expression in PDGF-BB treated and untreated groups in AoSMC transfected with hsa-miR-134-5p mimics (normalized to N.C group). (a) IGF-1, (b) SMAD4, (c) SP-1, (d) SMAD3, (e) TCF21 and (f) VEGF-A. Values are mean ±SEM. *, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.001$; ns, not significant

7. TCF21 suppresses the expression of MYOCD in AoSMC

We next investigated whether TCF21 affects the expression of MYOCD. We hypothesized that TCF21 preferentially colocalizes at SRF target loci, independently repressing the expression of SMC contractile state genes or regulating the binding of MYOCD-SRF. Therefore, to determine the effect of TCF21 on MYOCD expression, we perturbed TCF21 expression in HCASMC and measured MYOCD expression at the mRNA level(Fig. 8).

As a result, it was confirmed that the MYOCD expression level increased in the group transfected with hsa-miR-130a-5p, in which TCF21 expression decreased, than in the N.C two groups, in which TCF21 expression increased.

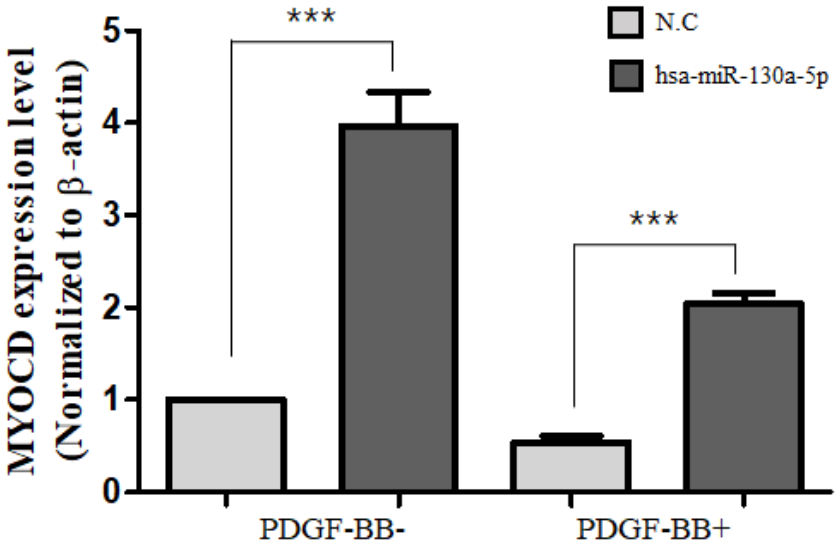


Figure. 8 TCF21 suppresses the expression of MYOCD in AoSMC

Quantitative real-time PCR analysis for MYOCD expression in PDGF-BB treated and untreated groups in AoSMC transfected with hsa-miR-134-5p mimics (normalized to N.C group). Values are mean ±SEM. *, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.001$; ns, not significant

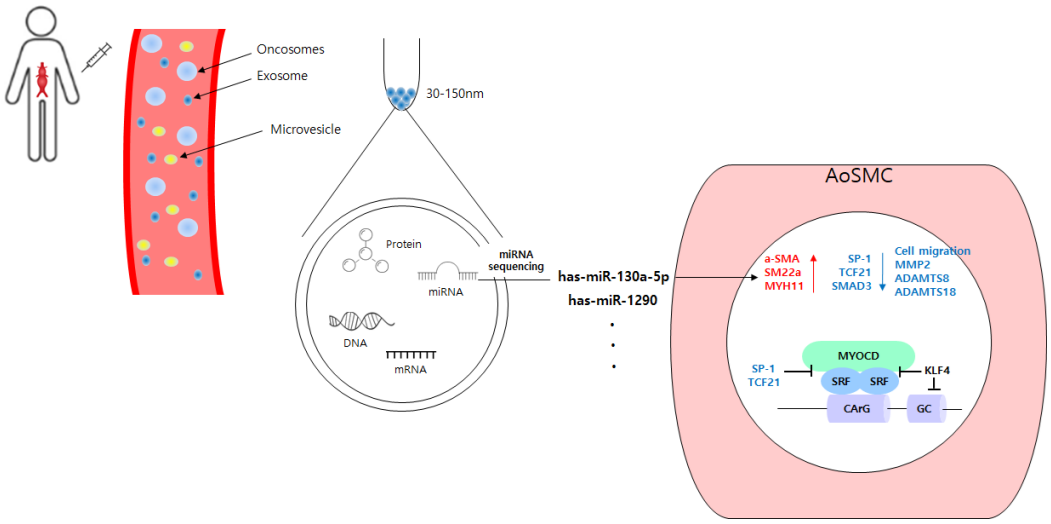


Figure 9. Summury

Using miRNA sequencing of blood-derived exosomes from 7 AAA and 2 controls, we identified significantly downregulated hsa-miR-130a-5p within blood-derived exosomes from AAA blood. Overexpression of hsa-miR-130a-5p evidently promoted AoSMC differentiation and expression of contractile markers, such as a-SMA, SM22a and MYH11. hsa-miR-130a-5p potently inhibited PDGF-BB induced AoSMC phenotypic switch and migration. According to the quantitative real-time PCR data, TCF21, SP-1 and SMAD3 exhibited a striking downregulation after hsa-miR-134-5p overexpression, while IGF-1, SMAD4 and VEGF-A had no discrimination.

IV. DISCUSSION

The major finding of this study are as follows: First, Through Blood-derived exosomal miRNA profiling, it was confirmed that the expression levels of hsa-miR-130a-5p and hsa-1290 were different between AAA patients and control groups. Second, I confirmed that SP-1 and TCF21 plays an important role in AoSMC-foam cell formation by showing that hsa-miR-130a-5p overexpression is does not prevent MYOCD from binding to SRF through decrease SP-1 expression. Third, hsa-miR-130a-5p has a potential vascular protective effect by modulation SP-1 and MYOCD signal pathway in PDGF-BB-induced Phenotypic Switch of AoSMC.

Despite essential progress in acknowledgment of pathogenesis of abdominal aortic aneurysm, the molecular mechanism underlying the phenotypic switch of human AoSMCs remains unclear. The transport of biological mediators by extracellular vesicles (EVs) has received substantial attention and emerged as a key messenger in intercellular communication [7, 33, 34]. In particular, exosomes are known to be mediators of intercellular communication in vascular diseases, including atherosclerosis and pulmonary artery hypertension [35]. Circulating exosomes may transfer miRNAs for intercellular communication and regulate target gene expression and the function of the recipient cell [36]. Therefore, we used miRNA sequencing to compare the miRNA profiles of blood-

derived exosomes from healthy controls and patients with AAA. Our study found that hsa-miR-130a-5p was markedly down-regulated in AAA exosomes compared to control exosomes. So we ultimately chose hsa-miR-130a-5p for subsequent experiments.

We also identified a set of blood-derived exosomal miRNA in the AAA group with 76 downregulated (e.g., hsa-miR-130a-5p and hsa-miR-1290) and 67 upregulated (e.g., hsa-miR-125b-1-3p) miRNAs in AAA patients. As a result, we identified miR-130a-5p as a human VSMC phenotypic switch, a novel regulator of vascular remodeling and AAA development. The data revealed that miR-130a-5p expression is obviously decreased in AAA patients. Functional analysis demonstrated that miR-134-5p promotes AoSMC phenotypic switch, proliferation and migration by increasing MYOCD via targeting SP-1 and TCF21. The detailed molecular mechanism needs to be clarified in a future study.

Platelet-derived growth factor-BB (PDGF-BB) is a effective mitogen for AoSMCs, acting on the PDGF-beta receptor in AoSMCs and promoting the proliferation and migration of AoSMCs[36]. Additionally, PDGF-BB was among the first factors identified to promote the phenotypic transformation of AoSMCs and has been widely used in in vitro experiments as a model for generating dedifferentiated cells from AoSMCs. PDGF-BB exerts its pro-SMC dedifferentiation effects mainly through the Ras/Raf/MEK/ERK [37], NF-κB [38] and PI3K/Akt [39] pathways. This leads to transcription of dedifferentiated genes under the control of SRF, as well as suppression of SMC-specific markers. Most importantly, PDGF-BB prevents the binding of the SRF/MYOCD complex to the

promoters of differentiation-promoting genes, inducing KLF4, which controls the transcriptional program of SMC-specific genes[32].

The phenotypic switch of VSMCs in vivo is often influenced by the integration of a range of extracellular signals, signalling pathways, and transcription factors. The main extracellular signals (e.g. lipids, retinoic acid, inflammatory mediators, growth factors, reactive oxygen species) activate signalling pathways that converge on transcription factors (e.g. KLF4, NF-κB, SP-1, and TCF21) to regulate the transdifferentiation of VSMCs into various cell types such as macrophage-like cells, foam cells, osteochondrocytes, mesenchymal stem cell-like cells and myofibroblast-like cells in disease states[32].

Mechanistically, we authenticated transcription factor SP-1 and TCF21 as pivotal targets of miR-130a-5p in AoSMCs. The TCF21 gene also contains one of 27 SNPs associated with increased risk of coronary artery disease[40]. The protein encoded by SP-1 is involved in many cellular processes, including cell differentiation, cell growth, apoptosis, immune responses, response to DNA damage, and chromatin remodeling[41]. Consistent with these studies, we found that TCF21 silence significantly inhibited human AoSMC proliferation. SP-1 deficiency obviously facilitated the expression of AoSMC differentiation markers and inhibited AoSMC migration. In addition, downregulation of TCF21 and SP-1 in AoSMC resulted in increased MYOCD expression.

In this study, we evaluated the impact of miR-130a-5p on its target key factors related to vascular pathophysiology and found that miR-130a-5p did not notably interfere with the

expression of SMAD4, IGF-1 and VEGF-A, whereas TCF21 and SP-1 was confirmed as a mediator in the effect of miR-130a-5p on VSMC phenotypic switch. This process might occur in VSMC through a mechanism that needs to be further investigated.

V. CONCLUSION

Using miRNA sequencing of blood-derived exosomes from 7 AAA and 2 controls, we identified significantly downregulated hsa-miR-130a-5p within blood-derived exosomes from AAA blood. Overexpression of hsa-miR-130a-5p evidently promoted AoSMC differentiation and expression of contractile markers, such as a-SMA, SM22a and MYH11. hsa-miR-130a-5p potently inhibited PDGF-BB induced AoSMC phenotypic switch and migration. Based on the above, blood-derived exosomal miRNA may represent a novel potential therapeutic target for AAA.

REFERENCES

1. Sakalihasan N, Limet R and Defawe OD: Abdominal aortic aneurysm. *Lancet.* 2005: 365:1577–1589
2. Lindholt J., Heegaard NH., Vammen S, Fasting H, Henneberg E., Heickendorff L. Smoking, but not Lipids, Lipoprotein (a) and Antibodies Against Oxidised LDL, is Correlated to the Expansion of Abdominal Aortic Aneurysms. *Eur J Vasc Endovasc Surg*. 2001; 21: 51–56.
3. Golledge J, Muller J, Daugherty A, Norman P. Abdominal aortic aneurysm: Pathogenesis and implications for management. *Arterioscler Thromb Vasc Biol.* 2006; 26: 2605–2613.
4. Nordon IM, Hinchliffe RJ, Loftus IM, Thompson MM. Pathophysiology and epidemiology of abdominal aortic aneurysms. *Nat Rev Cardiol*. 2011; 8: 92–102.
5. Iyer V, Rowbotham S, Biros E, Bingley J, Golledge J. A systematic review investigating the association of microRNAs with human abdominal aortic aneurysms. *Atherosclerosis.* 2017: 78–89. 10.1016
6. Folkesson M, Kazi M, Zhu C, Silveira A, Hemdahl A-L, Hamsten A, et al. Presence of NGAL/MMP-9 complexes in human abdominal aortic aneurysms. *Thromb Haemost*. 2007; 98: 427–433.

7. Killian O'Brien, Koen Breyne, Stefano Ughetto, Louise C. Laurent & Xandra O. Breakefield RNA delivery by extracellular vesicles in mammalian cells and its applications *Nature Reviews Molecular Cell Biology* volume 21, 2020: 585–606

8. Mathieu, M., Martin-Jaular, L., Lavieu, G. & Théry, C. Specificities of secretion and uptake of exosomes and other extracellular vesicles for cell-to-cell communication. *Nat. Cell Biol.* 2019: 21, 9-17

9. Saman S, Kim W, Raya M, Visnick Y, Miro S, Saman S, Jackson B, McKee AC, Alvarez VE, Lee NCY: Exosome-associated Tau Is Secreted in Tauopathy Models and Is Selectively Phosphorylated in Cerebrospinal Fluid in Early Alzheimer Disease. *J Biol Chem.* 2012: 287 (6): 3842-3849. 10.1074

10. Ben-Dov IZ, Brown M, Whalen VM, Tuschl T: Profiling Urine Cell and Exosome Microrna Using a Barcoded Small Rna Deep Sequencing Approach. *Am J Kidney Dis.* 2011: 57 (4): A24-A24.

11. Blanc L, De Gassart A, Geminard C, Bette-Bobillo P, Vidal M: Exosome release by reticulocytes - An integral part of the red blood cell differentiation system. *Blood Cell Mol Dis*. 2005: 35 (1): 21-26.

12. Alge JL, Janech M, Schwacke J, Arthur J, Costa LJ: Proteomic Analysis of Plasma Exosome-Associated Proteins Reveals That Differences In Kappa: Lambda Ratios Predict Severe Acute Graft-Versus-Host Disease Early After Allogeneic Hematopoietic Stem Cell Transplantation. *Blood*. 2010: 116 (21): 547-547.

13. Paredes PT, Esser J, Admyre C, Nord M, Rahman QK, Lukic A, Radmark O, Gronneberg R, Grunewald J, Eklund A: Bronchoalveolar lavage fluid exosomes contribute to cytokine and leukotriene production in allergic asthma. *Allergy*. 2012: 67 (7): 911-919.

14. Palanisamy V, Sharma S, Deshpande A, Zhou H, Gimzewski J, Wong DT: Nanostructural and Transcriptomic Analyses of Human Saliva Derived Exosome. *PLoS One*. 2010: 5 (1): e8577-10.1371

15. Charla E, Mercer J, Maffia P, Nicklin SA. Extracellular vesicle signalling in atherosclerosis. *Cell Signal.* 2020: 75:109751.

16. Hughes VA, Fiatarone MA, Ferrara CM, McNamara JR, Charnley JM, Evans WJ. Lipoprotein response to exercise training and a low-fat diet in older subjects with glucose intolerance. *Am J Clin Nutr.* 1994: 59(4):820-6.

17. Gai C, Carpanetto A, Deregibus MC, Camussi G. Extracellular vesicle-mediated modulation of angiogenesis. *Histol Histopathol.* 2016;31:379–91.

18. Kapustin AN, Chatrou ML, Drozdov I, Zheng Y, Davidson SM, Soong D, et al. Vascular smooth muscle cell calcification is mediated by regulated exosome secretion. *Circ Res.* 2015 10;116(8):1312-23.

19. Vajen T, Benedikter BJ, Heinzmann ACA, Vasina EM, Henskens Y, Parsons M, et al. Platelet extracellular vesicles induce a pro-inflammatory smooth muscle cell phenotype. *J Extracell Vesicles.* 2017;6:1322454.

20. Piccoli M.T. Gupta S.K. Thum T. Noncoding RNAs as Regulators of Cardiomyocyte Proliferation and Death. *J Mol Cell Cardiol*. 2015; 89: 59-67

21. Philippen L.E. Dirkx E. da Costa-Martins P.A. De Windt L.J. Non-coding RNA in Control of Gene Regulatory Programs in Cardiac Development and Disease. *J Mol Cell Cardiol*. 2015: 89: 51-58

22. Vishnoi A and Rani S: miRNA Biogenesis and Regulation of Diseases: An Overview. *Methods Mol Biol*. 1509:1–10. 2017.

23. Olena AF, Patton JG. Genomic organization of microRNAs. *JCell Physiol*. 2014; 222: 540–545.

24. Schöler N, Langer C, Döhner H, Buske C, Kuchenbauer F. Serum microRNAs as a novel class of biomarkers: a comprehensive review of the literature. *Exp Hematol*. 2010;38: 1126–1130.

25. Kosaka N, Iguchi H, Yoshioka Y, Takeshita F, Matsuki Y, Ochiya T. Secretory mechanisms and intercellular transfer of microRNAs in living cells. *J Biol Chem*. 2010;285: 17442–17452.

26. Marmur, J.D.; Poon, M.; Rossikhina, M.; Taubman, M.B. Induction of PDGF-responsive genes in vascular smooth muscle. Implications for the early response to vessel injury. *Circulation* 1992, 86, III53–III60.

27. A.Y. Rangrez, Z.A. Massy, V. Metzinger-Le Meuth, L. Metzinger miR-143 and miR-145: molecular keys to switch the phenotype of vascular smooth muscle cells. *Circ Cardiovasc Genet*, 4 2011: pp. 197-205

28. B.N. Davis-Dusenbery, C. Wu, A. Hata Micromanaging vascular smooth muscle cell differentiation and phenotypic modulation. Arterioscler. Thromb. *Vasc. Biol.*, 31 2011: pp. 2370-2377

29. E. Ignatieva, D. Kostina, O. Irtyuga, V. Uspensky, A. Golovkin, N. Gavriliuk, O. Moiseeva, A. Kostareva, A. Malashicheva Mechanisms of Smooth Muscle Cell Differentiation Are Distinctly Altered in Thoracic Aortic Aneurysms Associated with Bicuspid or Tricuspid Aortic Valves Front. *Physiol*., 2017: 8. p. 536

30. Elaine W. Raines PDGF and cardiovascular disease Cytokine & Growth Factor Reviews 2004: 15. 237–254

31. Farrell, K.; Simmers, P.; Mahajan, G.; Boytard, L.; Camardo, A.; Joshi, J.; Ramamurthi, A.; Pinet, F.; Kothapalli, C.R. Alterations in Phenotype and Gene Expression of Adult Human Aneurysmal Smooth Muscle Cells by Exogenous Nitric Oxide. *Exp. Cell Res.* 2019: *384*, 111589

32. Feng Zhang, Xiaoqing Guo, Yuanpeng Xia & Ling Mao An update on the phenotypic switching of vascular smooth muscle cells in the pathogenesis of atherosclerosis Cellular and Molecular Life Sciences 2022: Article number: 6

33. Maacha, S.; Bhat, A.A.; Jimenez, L.; Raza, A.; Haris, M.; Uddin, S.; Grivel, J.C. Extracellular vesicles-mediated intercellular communication: Roles in the tumor microenvironment and anti-cancer drug resistance. *Mol. Cancer* 2019: *18*, 55.

34. Sullivan, R.; Maresh, G.; Zhang, X.; Salomon, C.; Hooper, J.; Margolin, D.; Li, L. The Emerging Roles of Extracellular Vesicles As Communication Vehicles within

the Tumor Microenvironment and Beyond. *Front. Endocrinol. (Lausanne)* 2017: *8*, 194.

35. Hergenreider, E.; Heydt, S.; Treguer, K.; Boettger, T.; Horrevoets, A.J.; Zeiher, A.M.; Scheffer, M.P.; Frangakis, A.S.; Yin, X.; Mayr, M.; et al. Atheroprotective communication between endothelial cells and smooth muscle cells through miRNAs. *Nat. Cell Biol*. 2012: 14, 249–256.

36. X. Chen, H. Liang, J. Zhang, K. Zen, C.-Y. Zhang, Secreted microRNAs: A new form of intercellular communication. *Trends Cell Biol.* 2012: 22, 125–132.

37. Lee CK, Lee HM, Kim HJ, Park HJ, Won KJ, Roh HY et al Syk contributes to PDGF-BB-mediated migration of rat aortic smooth muscle cells via MAPK pathways. *Cardiovasc Res* 2007: 74:159–168

38. Lu QB, Wan MY, Wang PY, Zhang CX, Xu DY, Liao X et al Chicoric acid prevents PDGF-BB-induced VSMC dedifferentiation, proliferation and migration by suppressing ROS/NFkappaB/mTOR/P70S6K signaling cascade. *Redox Biol* 2018: 14:656–668

39. Wang H, Zhong B, Geng Y, Hao J, Jin Q, Zhang Y et al TIPE2 inhibits PDGF-BB-induced phenotype switching in airway smooth muscle cells through the PI3K/Akt signaling pathway. *Respir Res* 2021: 22:238

40. Mega JL, Stitziel NO, Smith JG, Chasman DI, Caulfield MJ, Devlin JJ, et al. Genetic risk, coronary heart disease events, and the clinical benefit of statin therapy:

an analysis of primary and secondary prevention trials. *Lancet.* 2015: 385 (9984): 2264–71

41. Tang Y, Yu S, Liu Y, Zhang J, Han L, Xu Z. MicroRNA-124 controls human vascular smooth muscle cell phenotypic switch via Sp1. *Am J Physiol Heart Circ Physiol* 2017: 313:H641–H649.

ABSTRACT (IN KOREAN)

인간 대동맥 평활근 세포에서 복부 대동맥류 환자의 혈액 유래 Exosomal has-miR-130a-5p 의 역할

<지도 교수 최동훈>

연세대학교 대학원 생체공학협동과정

김정현

복부대동맥류는 복부 대동맥 벽의 병리학적 확장과 관련된 위험한 상태를 말한다. 또한 복부대동맥류는 대동맥 평활근 세포(AoSMC)의 증식 및 세포사멸과 관련이 있는 것으로 알려진 질병이다.

따라서 무증상 경과와 위험한 결과를 초래하는 파열로 인해 조기 진단을 위한 바이오마커 식별은 임상적 이점에 매우 중요하다. AoSMC 의 비정상적인 표현형 전환, 이동 및 증식은 AAA 의 병인에 대한 특징으로 잘 알려져 있다.

이 연구에서는 hsa-miR-130a-5p 가 인간 혈관 평활근 세포의 표현형 전환 및 이동을 제어하는 중요한 조절기로 hsa-miR-130a-5p 가 인간 인간 혈관 평활근 세포의 기능 및 AAA 발달에 영향을 미치는지 여부를 조사하였다. 7 명의 복부대동맥류환자 와 2 명의 대조군으로부터 혈액 유래 엑소좀의 miRNA 시퀀싱을 사용하여, 우리는 복부대동맥류환자 혈액으로부터의 혈액 유래 엑소좀 내에서 상당히 하향 조절된 hsa-miR-130a-5p 를 확인하였다.

hsa-miR-130a-5p 의 이소성 발현은 분명히 혈관 평활근 세포의 분화 및 a-SMA, SM22a, MYH11 및 CNN1 과 같은 수축 마커의 발현을 촉진했습니다. hsa-miR-130a-5p 는 혈소판 유래 성장인자-BB 유도 혈관 평활근 세포의 표현형 전환 및 이동을 강력하게 억제하는 것을 확인 하였다. 우리는 인간 혈관 평활근 세포에서 hsa-miR-134-5p 의 하위 표적으로서 myocardin(MYOCD), SP-1 및 TCF21 을 추가로 확인하고 이들이 혈관 평활근 세포의 표현형 전환 및 복부대동맥류의 진행에서의 중재자임을 입증했습니다. 연구 결과는 hsa-miR-130a-5p 가 TCF21/MYOCD 발현 표적화를 통해 AAA 의 혈관 리모델링 및 병리학적 진행에서 새로운 조절인자임을 밝혔다.

결론적으로, 혈관 평활근 세포에서 hsa-miR-130a-5p 또는 그 발현이 줄어드는 분자를 표적으로 삼는 것은 복부대동맥류의 임상 치료에서 새로운 길을 개발할 수 있을 것으로 기대된다.

주제어: 복부대동맥류, 엑소조멀 마이크로알엔에이, 혈관평활근세포, 표현형 전환, 혈소판유래성장인자-BB, 전사인자 21, 마이오카딘