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Original Article

Adenovirus-expressing miR-153-3p alleviates aortic calcification in a rat model with chronic kidney disease

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Abstract: Background: Patients with chronic kidney disease (CKD) have abnormal calcification in vascular tissue that is a risk factor for cardiovascular disease. However, the specific molecular mechanisms for vascular calcification remain largely unknown. The present study aimed to determine the differentially expressed miRs and the underlying molecular mechanisms of miR-153-3p in vascular calcification induced by adenine. Methods: Differentially expressed miRs were screened using a microarray chip in the thoracic aorta. miRs and mRNA expression were measured by RT-qPCR. Protein expression was performed by western blotting analysis. Aortic calcification was confirmed by Von Kossa staining. The targeted genes were predicted by a bioinformatics algorithm and confirmed by a dual luciferase reporter assay. Results: Our results revealed that the expression of miR-153-3p was significantly down-regulated in the thoracic aorta from adenine-fed rats compared with that of the control group. Transfection of miR-153-3p into the thoracic aorta markedly suppressed adenine-induced aortic calcification and significantly decreased the mRNA expression of ALP, OC, OSX, SOST and Runx2. Further studies indicated that Runx2 was a direct target gene of miR-153-3p, which was verified by dual luciferase reporter assay. Conclusion: These results suggest that increased vascular miR-153-3p expression attenuates adenine-induced aortic calcification via inhibiting osteogenic trans-differentiation in the thoracic aorta.

Keywords: miR-153-3p, aortic calcification, Runx2, osteogenic trans-differentiation

Introduction

Vascular calcification is a frequently occurring atherosclerosis-related complication in patients with chronic kidney disease (CKD), which is a known cardiovascular risk factor [1]. The biochemical abnormalities, including parathyroid hormone (PTH), calcium, phosphorus, fibroblast growth factor-23 (FGF-23) and 1, 25-dihydroxyvitamin D, in CKD patients are associated with bone abnormalities and extraskeletal calcification [2]. Previous study indicates that extraskeletal calcification affects multiple organs, including the myocardium and arteries [3]. Epidemiological studies have suggested an association of arterial calcification and CKD, arterial calcification appearing in 30-65% of patients with CKD (stage 3-5) and 50-80% of patients with stage CKD5D [4, 5]. Using different calcification models, the pathogenesis of vascular calcification has emerged. For example, dietary adenine supplementation induced severe hyperparathyroidism can accelerate bone abnormalities and metastatic calcification in rats [6]. Accumulating evidence has suggested that osteogenic trans-differentiation in vascular smooth muscle cells (VSMCs) plays a crucial role in vascular calcification [7, 8], which appears to be initiated by the release of vesicular structures from VSMCs, and mineralization within vesicular structures is promoted by osteogenesis-related genes such as osterix (OSX), osteocalcin (OC), sclerostin (SOST), alkaline phosphatase (ALP) and runt-related transcription factor 2 (Runx2) [7, 9]. These studies have provided abundant information about the pathogenesis of vascular calcification [7-9], however, the precise mechanisms have yet to be fully characterized.

Recently, a class of small non-coding RNA (18-25 nucleotides) is known as microRNA (miR)

that mediates post-translational regulatory mechanism [10]. The miRs induce specific mRNA degradation or block translation by binding to the 3'-untranslated regions (3'-UTRs) of the targeting mRNA [11]. In vascular biology, dysregulated miRs have found to contribute to various pathological conditions, including vascular senescence, atherosclerosis, vascular neointimal formation and carotid artery restenosis [12-15]. In addition, specific miRs, miR-29, -32, -133b and -211, also play critical roles in the vascular calcification [16-18]. Interestingly, miR-133a modulates osteogenic differentiation of VSMCs by targeting Runx2 [19]. However, miR-mediated Runx2 expression in arterial calcification needs to be further characterized.

In the present study, we had identified that Runx2 was a direct target of miR-153-3p using on-line prediction algorithms. Therefore, we elucidated the relationships between Runx2 and miR-153-3p during arterial calcification, and the effects of exogenous miR-153-3p for regulation of calcification in vivo were also investigated.

Materials and methods

Cell culture

Human embryonic kidney (HEK) 293 T cells (American Type Culture Collection, ATCC, Manassas, USA) were incubated in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and supplemented with 10% FBS, 100 μg/mL streptomycin and 100 IU/mL penicillin (all of them from Sigma-Aldrich).

Animal treatment

The experiment was approved by the Ethics Committee of Chinese PLA General Hospital and was performed in accordance with its guidelines. A total of 36 male Sprague-Dawley (SD) rats (ten-week-old; 200-220 g) were purchased from the Animal Center of General Hospital of the Chinese People's Liberation Army (PLA) (Beijing, China). Adenine-induced aortic calcification in a rat model with chronic kidney disease (CKD) was prepared as described previously [6, 7]. The rats were housed under controlled temperature (23±2°C) and humidity (55±5%) with an artificial 12-h light/dark cycle and were given free access to food and tap water. The rats were randomly divided into 3 groups (n = 12 in each group): control group fed with normal diet; adeninie group fed with 0.75% adenine (cat.no: A8626; Sigma, St Louis, MO, USA) diet combined with ad-scramble injection; ad-miR-153-3p group fed with 0.75% adenine combined with adenovirus (ad)-expressed miR-153-3p. At the end of the 4 weeks in vivo experiments, all animals were sacrificed by intraperitoneal injection of sodium pentobarbital (2%; 150 mg/kg; cat.no: P3761; Sigma). Blood samples were collected by heart puncture into EDTA/acid-free tubes and centrifuged at 1, 500 g for 10 min at 4°C for collecting serum.

Biochemical markers in serum

Blood urea nitrogen (BUN; cat.no: C013-2), creatinine (Cre; cat.no: C011-1), calcium (Ca; cat.no: C004-3) and phosphorus (P, cat.no: A060-1) kits were purchased from Nanjing Jiancheng Biology Engineering Institute, Nanjing, China; Parathyroid hormone (PTH; cat.no: E-EL-M0709c) was purchased from Elabscience Biotechnology Co., Ltd, Wuhan, China and measured using ELISA reader (MD SpectraMax M5; Molecular Devices, LLC, Sunnyvale, CA, USA) according to the manufacturer's protocol.

Thoracic aorta Ca content

The thoracic aorta were incinerated with muffle furnace (Thermo Fisher Scientific, Inc.) at 800°C for 12 hours, and 10 mg of bone ash was then dissolved in 1 ml of 37% HCl and diluted with Millin-Q water. The calcium content was determined by the kit (cat.no: C004-3; Nanjing Jiancheng Biology Engineering Institute, Nanjing, China).

Von Kossa staining

Vascular calcification was assessed by Von Kossa's method, as described previously [7]. Von Kossa (Calcium Staining) kit was purchased from Abcam (cat.no: ab150687) and visualized under a microscope (Leica DM 2500; Leica Microsystems GmbH, Wetzlar, Germany).

Adenoviruses-mediated miR-153-3p expression

Recombinant adenoviruses for expression of miR-153-3p or control scrambled short hairpin RNA (ad-scramble) were generated using the

Table 1. Primers were used to RT-qPCR of miRNA and mRNA

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
miR-153-3p	GCCGGGCTTGCATAGTCACAA	TGGTGTCGTGGAGTCG
U6	CGCTTCGGCAGCACATATACTAA	TATGGAACGCTTCACGAATTTGC
ALP	CACCCACGTCGATTGCATCT	TAGCCACGTTGGTGTTGAGC
OC	CCGCTCTCAGGGGCAGAC	AGGGGATGCTGCCAGGACTAAT
OSX	GCCCTGCCACACCAACA	AGGAAATAAGCTTGAGAAGCAGAA A
SOST	ATCACATCCGCCCCAACTT	CCT TTCACTTCTCTTCGGAAGGT
Runx2	GGACGAGGCAAGAGTTTCAC	CCAGAGGCAGAAGTCAGAGG
GAPDH	GCACCGTCAAGCTGAGAAC	TGGTGAAGACGCCAGTGGA

BLOCK-iT adenoviral RNAi expression system (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Viruses were diluted in PBS and administered at a dose of 10^7 plaque-forming units per well in 12-well plates, 10^9 plaque-forming units per rat via tail-vein injection every other day.

miRs expression profiling

Total RNA in thoracic aorta was extracted by TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.). miRs were isolated from total RNA using the miRNA isolation kit (Invitrogen). Denaturing agarose gel electrophoresis was performed using 1% formaldehyde electrophoresis reagent. miRs were labeled with Hy3 or Hy5 fluorescence using the miRCURY™ Array Power Labeling Kit (Exiqon) to obtain the fluorescent probe that can be hybridized with the chip. The labeled probe was hybridized with the miRCURY™ chip under the standard condition using the MAUI hybridization system. The fluorescence intensity of the chip was scanned with the Agilent chip scanner and analyzed using Agilent feature extraction software (version 12). The differentially expressed miRs were screened based on the fold change ≥ 2, $P < 0.05$ and FDR < 0.05. Finally, the differentially expressed miRs in thoracic aorta were displayed by hierarchical clustering analysis between the control group and adenine group.

Transfection with miR-153-3p mimics and miR-Con and Dual-luciferase reporter gene assay

The sequences of the miR-153-3p mimics (5'-UUGCAUAGUCACAAAAGUGAUC-3') and miR-Con (5'-CCAUGCCAAUCGCGGCUCAGCA-3') were synthesized by RiboBio (Guangzhou, China). The HEK 293 T cells were transfected using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) at a final concentration of 50 nM. At 48 h post-transfection, the cells were harvested for analysis. The wild-type (WT: 5'-UCCUGCUUCUCCCUUUAUGCAAA-3') and mutant-type (MUT: 5'-UCCUGCUUCUCCCUUUAguagcA-3') 3'-UTR of Runx2 were PCR synthesized, and the fragments were subcloned into the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, Beijing, China). The potential binding site between miR-153-3p and Runx2 were obtained using online predict software (miRanda-mirSVR; http://www.microrna.org), miRDB (http://www.mirdb.org/miRDB/) and TargetScan (http://www.targetscan.org/). HEK-293T cells were transfected with luciferase reporter vectors containing the WT and MUT of Runx2-3'-UTR (0.5 μg). MiR-153-3p mimics or miR-Con was co-transfected at 50 nM. The luciferase activity was measured using the Dual Luciferase Reporter® Assay System (cat.no: E1960; Promega, USA) on a Luminoskan™ Ascent Microplate Luminometer (Thermo Fisher Scientific, Waltham, MA, USA).

Real-time quantitative PCR (RT-qPCR)

Total RNA was extracted by TRIzol (Invitrogen) according to the manufacturer's protocol. The cDNA was synthesized by reverse transcription reactions with 2 μg of total RNA using moloney murine leukemia virus reverse transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Both mRNA and miRNA levels were determined using the TaqMan quantitative real-time PCR (qRT-PCR) ELISA (Thermo Fisher Scientific, Inc.) on an ABI 7500 Real-Time PCR System (Applied Biosystems, San Mateo County, CA, USA). The relative expression levels of miR and mRNA were calculated using the $2^{-\Delta\Delta Cq}$ method [20] and normalized to the internal control U6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), respectively. The primers were synthesized by Sangon Biotech (Shanghai, China) as shown in **Table 1**.

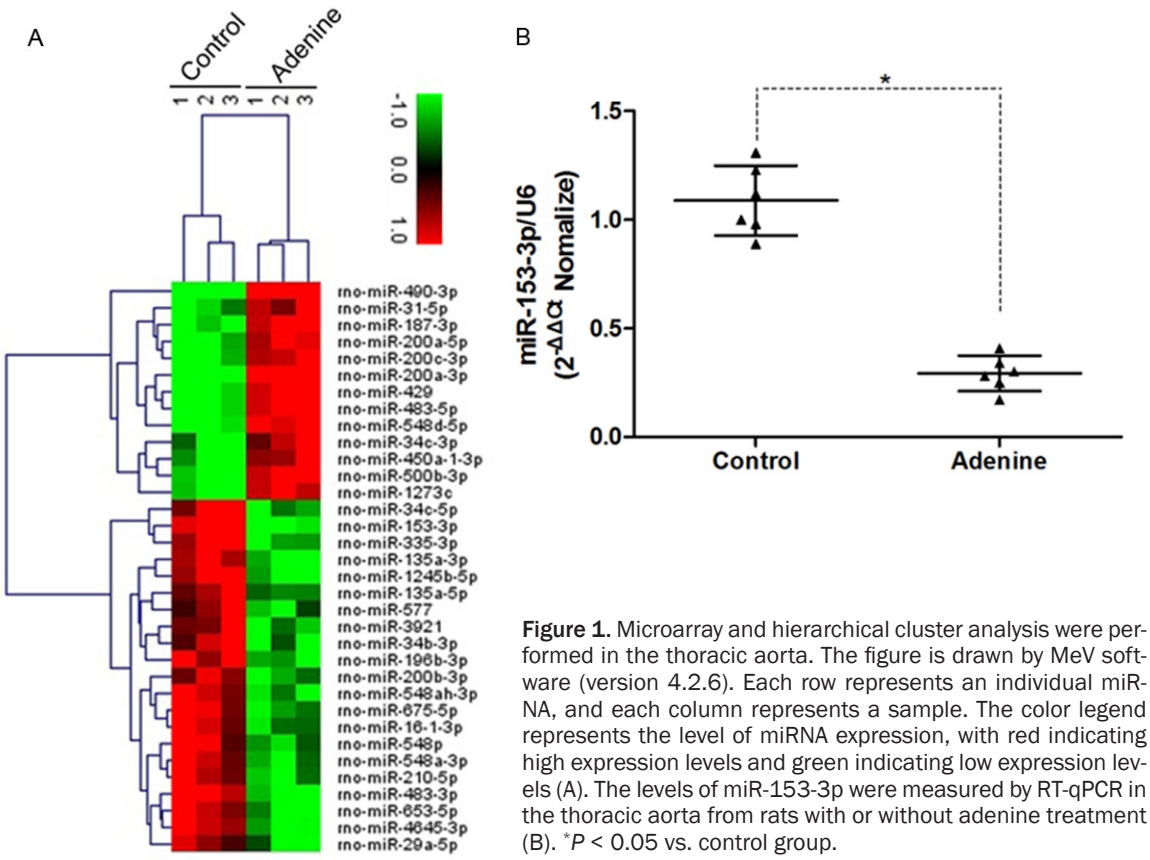

Figure 1. Microarray and hierarchical cluster analysis were performed in the thoracic aorta. The figure is drawn by MeV software (version 4.2.6). Each row represents an individual miRNA, and each column represents a sample. The color legend represents the level of miRNA expression, with red indicating high expression levels and green indicating low expression levels (A). The levels of miR-153-3p were measured by RT-qPCR in the thoracic aorta from rats with or without adenine treatment (B). $^{*}P < 0.05$ vs. control group.

Western blotting

Proteins were extracted with RIPA buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 0.1% Tryton X-100; 0.25% Na-deoxycholate; 0.1 M EDTA and 1% SDS) containing protease inhibitor cocktail. The concentration was measured using a BCA protein assay kit (Beyotime Institute of Biotechnology, Haimen, China). 50 μg of protein for each sample was separated on a 10% SDS-PAGE gel and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA). After blocking with 5% non-fat dry milk at room temperature for 2 h, the membranes were incubated with the primary antibody of Runx2 (cat.no: sc-390715; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at at room temperature for 2 h. GAPDH (cat.no: sc-137179; Santa Cruz Biotechnology) signals were used to correct for unequal loading. Following three washes with TBST, the membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (cat.no: sc-516102; dilution: 1:10,000; Santa Cruz Biotechnology) at room temperature for 2 h and visualized by chemiluminescence (Thermo Fisher Scientific, Inc.). Signals were analyzed with Quantity One software version 4.5 (Bio Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis

Data were presented as the mean ± standard deviation (SD) for each group. All statistical analyses were performed using PRISM version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). Inter-group differences were analyzed by one-way analysis of variance, followed by a post hoc Tukey test for multiple comparisons. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Aberrantly miRs expression in thoracic aorta

miR differential expression in the thoracic aorta of rats following normal diet or dietary adenine supplementation was detected. Based on the fold change ≥ 2, p-value < 0.05 and FDR ≤ 0.05, 34 miRs were differentially expressed,

Table 2. Minerals and biomarker in serum

	Control	Adenine	Ad-miR-153-3p
BUN (mg/dL)	8.95±1.23	145.37±36.78*	152.61±42.53*
Cre (mg/dL)	0.52±0.05	3.71±0.43*	3.55±0.54*
Serum Ca (mg/dL)	10.34±0.45	9.08±0.51*	10.61±0.64$
Serum P (mg/dL)	7.13±0.71	8.92±0.95*	7.33±0.59$
Serum PTH (pg/mL)	105±18	296±67*	134±72$

BUN, blood urea nitrogen; Cre, creatinine; Ca, calcium; P, phosphorus; PTH, parathyroid hormone. *$P < 0.05$ compared with control group; $$P < 0.05$ compared with adenine group.

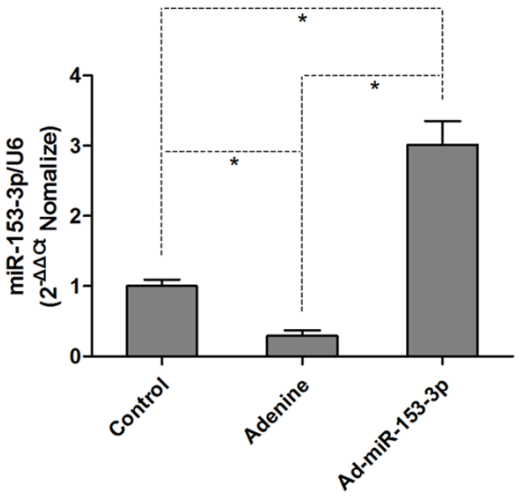

Figure 2. Adenovirus-mediated increased miR-153-3p expression. After ad-miR-153-3p or ad-scramble transfetion, the levels of miR-153-3p were measured by RT-qPCR in the thoracic aorta from rats with or without adenine treatment. *$P < 0.05$.

among which 13 miRs were up-regulated and 21 miRs were down-regulated. Based on the differential expression of miRs, a hierarchical clustering analysis was conducted (**Figure 1A**). Moreover, we found that the expression of miR-153-3p showed the lowest level of all the miRs. Subsequently, the differentially expressed miR-153-3p was further validated by qRT-PCR, and the result indicated that miR-153-3p was significantly decreased by dietary adenine supplementation compared with control group (**Figure 1B**).

Overexpressed miR-153-3p by ad-miR-153-3p improved arterial calcification in vivo

Rats fed adenine for 4 weeks showed significant increase in serum BUN and Cre levels, which confirmed the establishment of a CKD model (**Table 2**). Both serum P and PTH significantly increased in adenine-fed rats. However, the serum Ca was significantly reduced in adenine-fed rats compared with control group. Overexpressed miR-153-3p could reverse adenine-induced mineral metabolic disturbance in CKD rats (**Table 2**). To investigate the effects of miR-153-3p on arterial calcification in vivo, we infected adenine-fed male SD rats with ad-miR-153-3p or control scrambled adenovirus (ad-scramble) via tail-vein injection and found that the levels of miR-153-3p were significantly increased in the thoracic aorta of these rats in ad-miR-153-3p group compared with adenine group (**Figure 2**). These results suggest that adenovirus-mediated gene transfer occurs in thoracic aorta. As predicted, adenine-fed rats showed a remarkable increased in calcium deposition in thoracic aorta compared with control, whereas arterial calcification was markedly attenuated by miR-153-3p transfection (**Figure 3A**). In addition, Ca content was significantly increased in adenine group as compared to control group, but overexpression of miR-153-3p in adenine-fed rat could significantly reverse adenine-induced calcium deposition in thoracic aorta (**Figure 3B**).

Ad-miR-153-3p inhibited adenine-induced osteogenic trans-differentiation in thoracic aorta

To confirm whether miR-153-3p regulates adenine-induced osteogenic trans-differentiation in thoracic aorta, we examined the expression of osteogenic markers (ALP, OC, OSX, SOST and Runx2) using RT-qPCR and western blotting following ad-miR-153-3p injection. Adenine significantly increased the mRNA expression of ALP, OC, OSX, SOST and Runx2 (**Figure 4A-E**) and the protein expression of Runx2 (**Figure 4F** and **4G**). However, ad-miR-153-3p injection significantly attenuated the induction of osteogenic markers in thoracic aorta.

Runx2 is a potential target gene for miR-153-3p

To investigate the underlying molecular pathways associated with miR-153-3p, on-line prediction algorithms revealed that the 3'-UTR of Runx2 contained one conserved target site of miR-153-3p. The putative binding sites of miR-

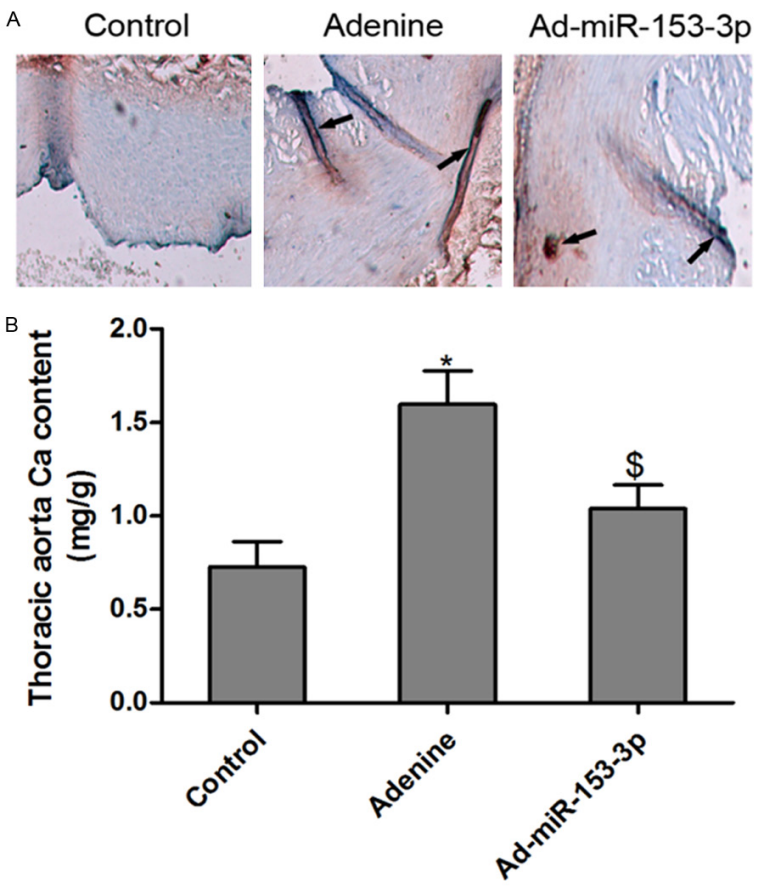

Figure 3. Overexpressed miR-153-3p attenuated vascular calcification in the thoracic aorta. After ad-miR-153-3p or ad-scramble transfetion, Von Kossa staining for aortic sections were performed in rats with or without adenine treatment (A). After ad-miR-153-3p or ad-scramble transfetion, the Ca content in the thoracic aorta was measured in mice with or without adenine supplementation (B). $^{*}P < 0.05$ vs. control group; $^{\$}P < 0.05$ vs. adenine group.

153-3p on the 3'-UTR of Runx2 was highlighted as shown in **Figure 5A**. To confirm this, either the wild-type (WT) sequence of Runx2 or its mutant-type (MUT) sequence was transfected into the luciferase-reporter plasmids, and then the reporters were co-transfected with miR-153-3p mimics into HEK 293 T cells. The results demonstrated that the luciferase enzyme activity was significantly inhibited by miR-153-3p mimics compared with control group in the presence of WT 3'-UTR of Runx2. However, transfected with miR-153-3p mimics or corresponding NC, the luciferase enzyme activity had no significant difference in the reporter vector containing MUT 3'-UTR of Runx2 (**Figure 5B**). These findings suggest that Runx2 is a direct target gene of miR-153-3p. Based on above results, we conclude that overexpressed miR-153-3p can improve adenine-induced arterial calcification, and the underlying mechanism was mediated, at least partially, through the inhibition of Runx2 expression.

Discussion

The present study screened the differentially expressed miRs using a miRNA microarray in the thoracic aorta of rats with adenine-induced CKD. We focused on a down-regulated miR, miR-153-3p, which was validated the same expression tendency by microarray and RT-qPCR. In addition, we found a negative relationship between miR-153-3p and Runx2 expression. Luciferase reporter assay showed that Runx2 was a direct target of miR-153-3p. Furthermore,adenovirus-expressed miR-153-3p transfer occurred in the thoracic aorta, and over-expression of exogenous miR-153-3p could inhibit adenine-induced arterial calcification via down-regulating Runx2 and others osteogenic markers such as ALP, OC, OSX and SOST.

Many miRs are differentially expressed in the progression of vascular calcification, such as miR-29b-3p, miR-32, miR-34b/c, miR-125b, miR-133b, miR-135a, miR-204, miR-211 and miR-223 [8, 16, 18, 21-25]. However, the majority of miRs are reported for the first time in vascular calcification. The present study detected the differential expression of miR-34 and miR-135 homologues involving in arterial calcification, which are consistent with previous studies [21, 25]. miR-34b/c and miR-135a as potential osteogenic differentiation suppressors can inhibit calcification in different cell model by down-regulating osteogenic markers [21, 25]. In the present study, we found that miR-34b-3p, miR-34c-5p and miR-135a-3p were markedly decreased in the thoracic aorta of rats following adenine-induced vascular calcification. These results suggest that miRs in

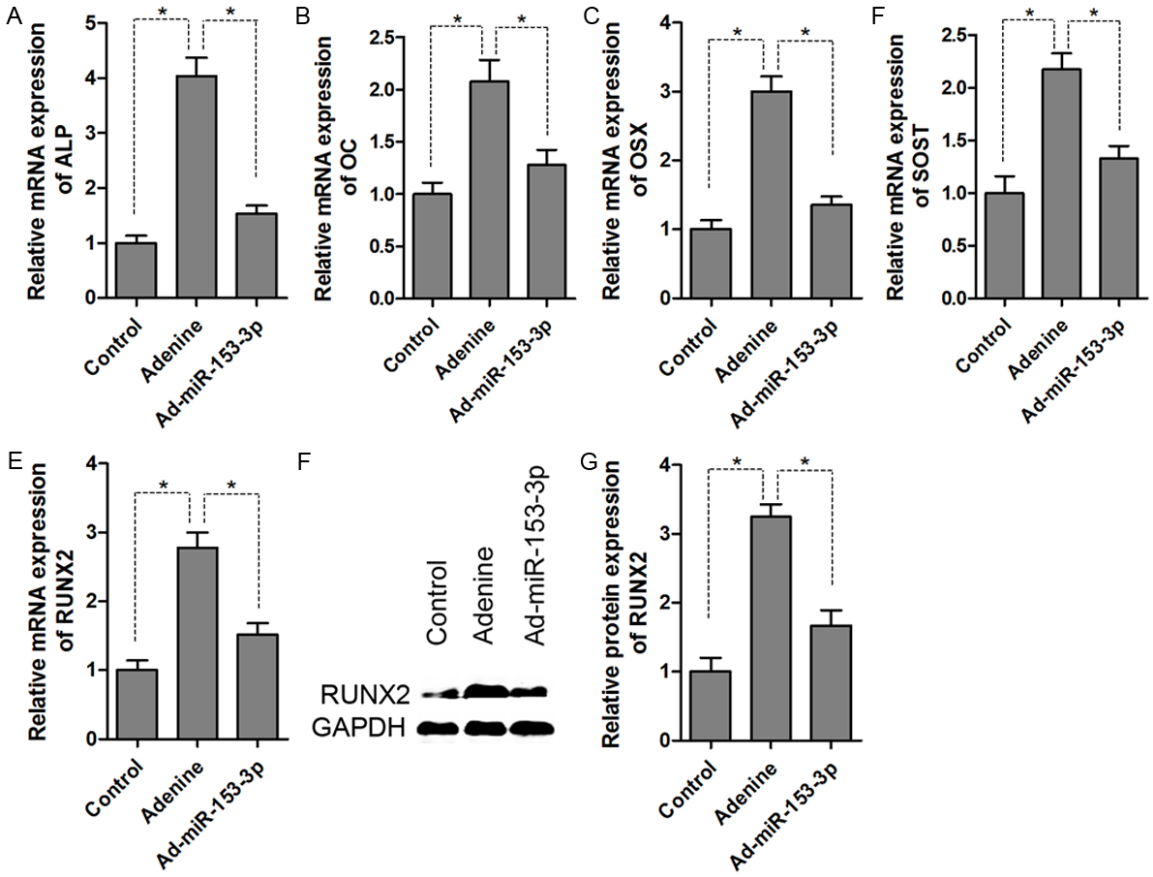

Figure 4. Overexpressed miR-153-3p inhibited adenine-induced osteogenic trans-differentiation. After ad-miR-153-3p or ad-scramble transfetion, the mRNA expression of ALP (A), OC (B), OSX (C), SOST (D) and Runx2 (E) was measured using RT-qPCR, and the protein expression of Runx2 was measured by western blotting (F and G). $^{*}P < 0.05$.

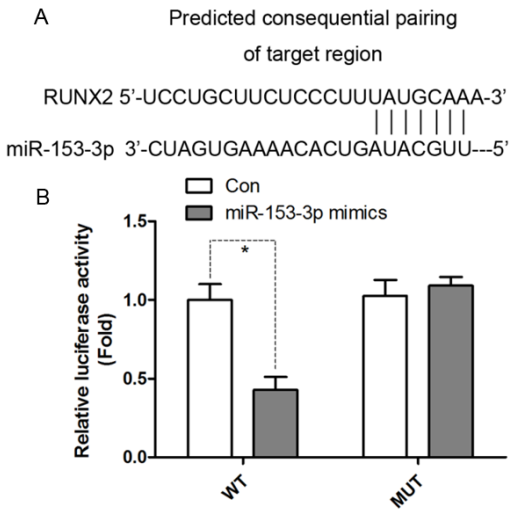

Figure 5. Runx2 was a direct target gene of miR-153-3p. Schematic representation of the putative miR-153-3p binding site in the 3'UTR of Runx2 was predicted by the on-line database (A). The HEK293T cells were co-transfected with the WT and MUT of Runx2-3'-UTR and miR-153-3p mimics or miR-Con, and the luciferase activity assay was performed (B). $^{*}P < 0.05$.

miR-34 and miR-135 families may play a vital role in vascular calcification. More importantly, we found that miR-153-3p was more susceptible to adenine in CKD rats, and miR-153-3p was significantly decreased in the thoracic aorta from adenine-fed rats compared with control group. To determine the role of miR-153-3p in vascular calcification in vivo, we developed an adenine-induced CKD-associated arterial calcification model. The present study showed that in vivo ad-miR-153-3p transfection prevented adenine-induced arterial calcification. More importantly, delivery of ad-miR-153-3p into the thoracic aorta significantly inhibited arterial the mRNA and protein expression and prevented osteogenic trans-differentiation. Previous study shows that overexpression of miR-153 inhibits stretch stress-enhanced VSMC proliferation and may be available therapeutic target for mechanical stretch-induced neointimal hyperplasia in vein grafts [26]. These results suggest that miR-153-3p can protect blood vessels and its function.

As a typical pathway, Runx2 signaling pathway has been discussed well in vascular calcification [21, 25]. For its positive role, Runx2 is a master determinant of osteoblast differentiation, and its haplo-insufficiency causes a skeletal dysplasia [27]. However, Runx2 may be "a double-blade sword" on its biological function. In the progression of vascular calcification, which has been recognized to occur as a consequence of the processes similar to bone formation, up-regulation of Runx2 plays an essential role in Ca deposition [28]. Runx2 as a directly transcriptional target can be regulated by WNT/β-catenin signaling in VSMCs [28]. Interestingly, several calcification-related miRs have been verified regulating Runx2 expression, including miR-32, miR-34b/c and miR-133a [16, 19, 25]. Here, we assessed the effect of ad-miR-153-3p on Runx2 signaling in vivo and discovered that overexpression of miR-153-3p in the thoracic aorta suppressed Runx2 expression, indicating a protective role for the miR-153-3p/Runx2 axis in adenine-induced arterial calcification.

Taken together, our findings demonstrate that miR-153-3p targeted to suppress Runx2 expression and osteogenic trans-differentiation improves pathological vascular calcification in the thoracic aorta. MiR-153-3p/Runx2 signaling pathway may be a treatment option for vascular calcification. VSMCs play a key role in vascular osteogenic trans-differentiation [24]. However, whether miR-153-3p can inhibit VSMCs osteogenic phenotype in vitro needs to be confirmed with further studies.

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Disclosure of conflict of interest

None.

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