Exosomal miR-151-3p Induces Calcium Deposition in Vascular Smooth Muscle Cells by Inhibiting Atg5

Li Chen (lichenxuhui23@163.com)
Shanghai Xuhui Central Hospital https://orcid.org/0000-0002-4415-8649
Rongrong Zhang
Shanghai Xuhui Central Hospital
Jinyin Li
Shanghai Xuhui Central Hospital
Yiping Gao
Shanghai Xuhui Central Hospital
Shilong Mao
Shanghai Xuhui Central Hospital

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Abstract

Background: Calcium deposition in vascular smooth muscle cells (VSMCs) can lead to the rigidity of the vasculature and an increase of risk in cardiac events. This study aimed to explore the role of exosomal microRNA-151-3p (miR-151-3p) in the regulation of VSMC calcification.

Methods: A cellular calcification model was established using the mouse primary aortic VSMCs by β-glycerophosphate treatment. The calcium deposition was evaluated by Alizarin Red staining. The expression of miR-151-3p in exosomes was evaluated by qRT-PCR. The relationship between miR-151-3p and Atg5 was determined by bioinformatics analysis and dual-luciferase gene reporter assay. The exosome derived from mouse VSMCs transfected with miR-151-3p mimics/inhibitor were isolated and used to stimulate VSMCs. The expression of Atg5, α-SMA, OPN, Runx2 and BMP2 was evaluated by western blot. An animal model was established to investigate the role of miR-151-3p in exosomes.

Results: MiR-151-3p was significantly upregulated in the exosomes of VSMCs treated with β-glycerophosphate. Exosomes derived from calcific VSMCs increased the calcium deposition of general VSMCs without any treatment. Exosomes derived from miR-151-3p mimics transfected VSMCs increased the expression of Runx2 and BMP2, while reduced the expression of α-SMA and OPN in general VSMCs. and exosomes derived from miR-151-3p inhibitor transfected VSMCs reversed these effects *in vitro*. Meanwhile, miR-151-3p served as a ceRNA of Atg5 by directly binding to the 3'UTR of Atg5. Moreover, the expression of α-SMA, OPN, Runx2 and BMP2 *in vivo* was consistent with the results in VSMCs *in vitro*.

Conclusion: Our study revealed that miR-151-3p in VSMCs-derived exosomes might induce calcium deposition through regulating Atg5 expression, suggesting that miR-151-3p might be a potential biomarker for vascular calcification.

Introduction

Certain chronic stresses and other metabolic or cardiovascular diseases may cause the abnormal deposition of calcium deposition in blood vessels, and those stresses are always from atherosclerosis, chronic kidney disease and diabetes(1, 2). β-glycerophosphate was widely used to induce the cellular calcification model *in vitro*(3). It has been reported that calcium deposition occurred in vascular smooth muscle cells (VSMCs) can lead to the rigidity of the vasculature, and also increasing the risk of cardiac events(4). Therefore, studying the pathogenesis of β-glycerophosphate induced cellular calcification contributes to develop potential therapeutic strategy for vascular calcification.

MicroRNAs (miRNAs) are a class of non-coding RNAs with approximately 22 nucleotides in length, and more studies have indicated that miRNAs play important functions in eukaryotic cells at post-transcriptional level(5). A large number of miRNAs have been identified to participate in the metabolic process of calcium deposition, and potentially acted as efficient predictors for vascular calcification. For example, miR-134-5p induces calcium deposition by inhibiting the expression of histone deacetylase 5 in VSMCs(6). MiR-135a has been demonstrated to suppress the calcification in senescent VSMCs by

modulating KLF4/STAT3 signaling pathway(7). Increasing evidences indicate the secretion of exosomes is identified as a feature of 'synthetic' VSMCs and the secreted exosomes are crucial mediators during vascular repair processes as well as pathological vascular thrombosis and calcification(8). Recently, exosomes derived from VSMCs have been identified to may mediate cell-to-cell crosstalk and regulate the proatherogenic phenotypes of VSMCs through miRNAs(9). However, there are some exosomal miRNAs derived from VSMCs during the regulation of proatherogenic phenotypes of VSMCs remains unclear. This study aimed to investigate the role of exosomal miR-151-3p in the VSMC calcification.

Autophagy, an evolutionarily conserved cell pathway, has been identified to play an essential role in maintaining the cellular functions(10). Stimulating evidences indicate that the incubation of autophagy is a potential protective phenomenon against high Pi-induced calcium deposition in VSMCs(11). Autophagy-related gene 5 (ATG5) is required for the initiation of auto-phagosome formation(12). It has been reported that a series of miRNAs play regulatory roles during autophagy in human diseases through targeting Atg5. For example, miR-153-3p promotes gefitinib-sensitivity in non-small cell lung cancer by inhibiting ATG5-mediated autophagy(13). MiR-20a inhibits hypoxia-induced autophagy by targeting ATG5 in colorectal cancer(14). Although the effects of various miRNAs in VSMC calcification have been well studied(15), the underlying regulatory axis of miRNAs and Atg5 have not been elucidated.

In the present study, we found that the expression of exosomal miR151-3p was significantly upregulated in the cellular calcification model and animal model. Moreover, our results demonstrated the exosomes derived miR-151-3p inhibitor transfected VSMCs could efficiently reduce the expression of calcification-related genes both *in vitro* and *in vivo*. Mechanically, the bioinformatic analysis and luciferase reporter assay determined that Atg5 was a target of miR-151-3p. Moreover, the reduction of Atg5 in calcific VSMCs and mouse model with soft tissue calcification further confirmed the potential role of VSMCs-derived exosomal miR-151-3p/Atg5 axis in the regulation of VSMC calcification.

Materials And Methods

Ethics statement

All Kunming mice (6-week-old, female, weight 180-220 g) were purchased from Shanghai Sippr-Bk Laboratory Animals (Shanghai, China) and maintained under a standard environment. This study was approved by the animal Ethics Committee of Shanghai Xuhui District Central Hospital, and all experiments were performed according to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH publication no. 85–23, revised 1996).

Cell isolation, culture and construction of calcification model

The mouse primary vascular smooth muscle cells (VSMCs) were isolated from Kunming mice as previously described(16). The VSMC phenotype was confirmed by western blot using the specific antibody against α-smooth muscle actin (α-SMA). VSMCs were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, and 1% penicillin-streptomycin at 37°C with 5% CO_2. To induce calcification, VSMCs at passage 3 were treated with 10 mM β-glycerophosphate (Sigma, St Louis, MO, USA) for total six days as previously reported(6). After washing with PBS for twice, the quantification of calcium deposition was evaluated by using Alizarin Red staining.

Cell transfection

MiR-151-3p mimics/inhibitor and the corresponding negative controls (miR-NC and inhibitor NC) were purchased from GenePharma Co. Ltd. (Shanghai, PR, China). 50 nM of mimics/inhibitor, and negative controls were transfected into mouse primary VSMCs by using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. After transfection for 48 h, the transfection efficiency was confirmed by qRT-PCR. Then the exosomes were extracted and used for the subsequent experiments. miR-NC: 5'-ACAAAGUUCUGUGAUGCACUGA-3', inhibitor NC: 5'-ACAAAGUUCUGUGAUGCACUGA-3', miR mimics: 5'-UCGAGGAGCUCACAGUCUAGUAU-3', miR inhibitor: 5'-ACTAGACTGTGAGCTCCTCGA-3.

The isolation and characterization of exosomes

The isolation of exosomes from VSMCs, transfected VSMCs or calcific VSMCs was performed by using the Total Exosome Isolation Reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. The protein concentration of the exosome was determined by using a BCA protein assay kit (Thermo Fisher Scientific). The exosomes were characterized by detection of exosome-specific biomarkers (including CD63, HSP70 and Tsg101) using western blot, and the morphology of the exosomes was evaluated by using transmission electron microscopy (TEM).

Co-culture of exosomes and VSMCs

To explore the effect of exosomes on the calcium deposition in VSMCs, the general VSMCs were treated with 10 μg/ml of exosomes extracted from calcific VSMCs for 24 h. To further determine the role of miR-151-3p, the exosomes were isolated from miR-151-3p mimics/ inhibitor and corresponding negative controls transfected with VSMCs, and 10 μg/ml of these exosomes were used to stimulate calcific VSMCs for 24 h. Finally, the calcium deposition was evaluated.

Quantification of calcium deposition

The quantification of calcium deposition was performed by using Alizarin Red staining as previously described(17). In brief, VSMCs received different treatments were fixed with 4 % paraformaldehyde at 4 °C for 10 min and then stained with 2 % Alizarin Red solution (sodium alizarinsulfonate; Sigma) at room temperature, pH 4.2 for 10 min. After washing with PBS for twice, the stained cells were imaged using a microscope. In addition, the semi-quantitative analysis of calcification was evaluated as the OD_{570} value by using a spectrophotometer.

QRT-PCR

Total RNA from exosomes was extracted by using a Total Exosome RNA kit (Invitrogen, Carlsbad, CA, USA). And that from VSMCs or artery samples of mice was extracted by using TRIzol reagent (Invitrogen). The total RNA was reversely transcribed into cDNA using SuperScript™ First-Strand Synthesis kit (ThermoFisher Scientific). Then quantitative reverse transcription-polymerase chain reactions (qRT-PCRs) were performed on a 7500 fast PCR detection system using the QuantiTect SYBR Green PCR Kit (Qiagen). The relative expression levels of target genes were analyzed by using $2^{-\Delta\Delta CT}$ method, with GAPDH and U6 as the internal controls, respectively. The primers used for qRT-PCR were as follows: miR-151-3p forward: 5'-GGATGCTAGACTGAAGCTCCT-3', reverse: 5'-CAGTGCGTGTCGTGGAGT-3'; U6 forward: 5'-CTCGCTTCGGCAGCACA-3', reverse: 5'-AACGCTTCACGAATTTGCGT-3'; GAPDH forward: 5'-TGTTCGTCATGGGTGTGAAC-3', reverse: 5'-ATGGCATGGACTGTGGTCAT-3'.

Western blot

Total protein of exosomes, VSMCs and artery samples of mice was extracted by using RIPA lysis buffer. Approximately Equal amounts of protein were separated by 10% SDS-PAGE and transferred into PVDF membranes. Then the membranes were incubated with the specific primary antibody against α-SMA (ab5694, 1:1000, Abcam), Runx2 (#8486, 1:1000, Cell Signaling Technology), OPN (ab8448, 1:1000, Abcam), BMP-2 (ab14933, 1:1000, Abcam), CD63 (ab134045, 1:1000, Abcam), HSP70 (#BM4335; 1:1000, Boster Biological Technology, Wuhan, China), Tsg101 (ab125011, 1:1000, Abcam), Atg5 (#9980, 1:1000, Cell Signaling Technology), p62 (#16177, 1:1000, Cell Signaling Technology), LC3B/A (#4108, 1:1000, Cell Signaling Technology), and the internal reference GAPDH (ab9485, Abcam, 1: 1000) at 4 °C overnight. Then the membranes were exposed to horseradish peroxidase (HRP)-conjugated secondary antibody for 2 h at room temperature. The protein bands were visualized by the enhanced chemiluminescence detection kit (ECL, thermo scientific, USA), and the relative grey values of targets were determined by using Image JO software.

Luciferase reporter assay

The putative binding site between miR-151-3p and Atg5 was predicted by using StarBase (http://starbase.sysu.edu.cn/index.php). The wild type (WT) and mutant type (MUT) of Atg5 fragments

containing the putative miR-151-3p binding site were synthesized by Sangon Biotech (Shanghai, China) and cloned into pmirGLO luciferase reporter vector (OBIO, Shanghai, China) to generate the recombinant reporter vectors Atg5 WT and Atg5 MUT. VSMCs were co-transfected with Atg5 WT/MUT and miR-151-3p mimics/inhibitor by using Lipofectamine 2000 reagent. After transfection for 48 h, the relative luciferase activity was measured by using dual luciferase reporter system (Promega), and normalized against *Renilla* luciferase activity.

Animal model

The animal model with soft tissue calcification was constructed by using Vitamin D3 as previously described(18). A total of 30 female Kunming mice were divided into five groups: control group (without any treatment), Vitamin D3 group, Vitamin D3 + Exosome group (the exosomes were extracted from general VSMCs), Vitamin D3 + miR-151-3p mimic Exosome group (the exosomes were extracted from miR-151-3p mimics transfected VSMCs), and Vitamin D3 + miR-151-3p inhibitor Exosome group (the exosomes were extracted from miR-151-3p inhibitor transfected VSMCs). Six mice in each group. The mice in Vitamin D3 group received a dose of 500 000 IU/kg body weight Vitamin D3 (Sigma, St Louis, MO, USA) through subcutaneous injection, and mice in control group received equal amount of saline, followed by three days of maintaining to induce soft tissue calcification as previously reported. On the day 4, 15 μl of exosomes (500 μg/mL) derived from general VSCMs, or miR-151-3p mimics/inhibitor transfected VSMCs were intravenously injected into mice by the jugular vein. Mice in control group and Vitamin D3 group received the equal amount of PBS. After 7 days, the mice were sacrificed by cervical dislocation, and the artery samples were dissected from the mice for the subsequent western blot and qRT-PCR analysis.

Statistical analysis

All data were presented as mean ± standard deviation (SD) from more than three biological replicates. Statistical analysis was performed by using SPSS 18.0 software. The difference between two groups was tested by Student's t-test, and the difference among multiple groups was determined by one-way analysis of variance. $P < 0.05$ was defined as the significant threshold.

Results

establishment of the mouse VSMC calcification model *in vitro*

Firstly, we established a cellular calcium deposition model by using the mouse primary VSMCs as introduced the materials and methods section. The results of Alizarin Red staining indicated that β-glycerophosphate (β-GP) treatment significantly increased the calcium deposition in VSMCs compared

with that in VSMCs without any treatment ($p < 0.01$, Fig. 1A). Meanwhile, we found that β-glycerophosphate treatment significantly elevated the expression of Runx2 ($p < 0.01$) and BMP-2 ($p < 0.05$), while reduced the expression of α-SMA ($p < 0.01$) and OPN ($p < 0.01$) in VSMCs compared with that in VSMCs without any treatment (Fig. 1B). These results suggest that the mouse VSMC calcification model is successfully established, and could be used for the subsequent experiments.

The characterization of exosomes from VSMCs

To explore the effect of exosomes on calcium deposition in VSMCs, we then extracted the exosomes from general VSMCs and calcific VSMCs. The results of TEM showed that the sizes of exosomes in general VSMCs and calcific VSMCs were both about 100 nm (Fig. 1C), which conformed to the shape of the exosomes in previous studies(19). Meanwhile, the expression of exosome surface makers including CD63, HSP70 and Tsg101 in exosomes was detected by western blot. The results indicated that three exosome surface makers were all expressed in exosomes, but the expression of CD63, HSP70 and Tsg101 in calcific VSMCs was obviously reduced compared with that in general VSMCs (Fig. 1D). These results indicate that the exosomes were successfully extracted.

Exosomes derived from miR-151-3p overexpressing VSMCs exacerbated calcium deposition in the calcification model

To explore the function of exosomes derived from the calcification model in the regulation of calcium deposition in VSMCs, we isolated the exosomes from calcific VSMCs and treated with general VSMCs. The results of Alizarin Red staining showed that calcific VSMCs-derived exosomes significantly enhanced the calcium deposition in general VSMCs compared with that in VSMCs without any treatment ($p < 0.01$, Fig. 2A). Meanwhile, the expression of α-SMA and OPN was significantly downregulated (α-SMA, $p < 0.01$; OPN, $p < 0.01$), while the expression of Runx2 and BMP-2 was upregulated (Runx2, $p < 0.01$; BMP-2, $p < 0.05$) in VSMCs treated with calcific VSMCs-derived exosomes compared with that in VSMCs without any treatment (Fig. 2B). Then we detected the expression of miR-151-3p in exosomes of general VSMCs and calcific VSMCs, and the results showed that miR-151-3p was significantly upregulated in exosomes of calcific VSMCs compared with that in general VSMCs ($p < 0.01$, Fig. 2C). To further determine the role of miR-151-3p, miR-151-3p mimics/inhibitor were transfected into VSMCs, and the exosomes were isolated. The results of qRT-PCR assay indicated that the expression of miR-151-3p was significantly increased in the exosomes of mimics-transfected VSMCs than that in miR-NC transfected VSMCs ($p < 0.01$), and reduced in the exosomes of inhibitor-transfected VSMCs than that in inhibitor NC transfected VSMCs ($p < 0.01$, Fig. 2D). Subsequently, the exosomes of miR-151-3p mimics/inhibitor transfected VSMCs were isolated and stimulate calcific VSMCs. The results of Alizarin Red staining indicated that miR-151-5p mimics-exosomes further promoted the calcium deposition in calcific VSMCs compared with miR-NC-exosomes ($p < 0.01$), and miR-151-5p inhibitor-exosomes attenuated the calcium deposition in calcific VSMCs compared with inhibitor NC-exosomes ($p < 0.01$, Fig. 2E). The expression of α-SMA, OPN, Runx2

and BMP-2 was consistent of the results of Alizarin Red staining assay (Fig. 2F). These results suggest that exosomal miR-151-3p plays an important role in calcium deposition in calcification model.

Atg5 was a target of miR-151-3p

To explore the mechanism of miR-151-3p, the potential targets of miR-151-3p were predicted by using Starbase and the prediction showed that there was a putative binding site between miR-151-3p and Atg5 (Fig. 3A), suggesting that Atg5 might be a target of miR-151-3p. Then luciferase reporter assays were performed and the results indicated that miR-151-3p mimics significantly reduced the relative luciferase activity of Atg5 WT compared with miR-NC ($p < 0.01$), and miR-151-3p inhibitor markedly increased the relative luciferase activity of Atg5 WT compared with inhibitor NC ($p < 0.01$, Fig. 3B). Meanwhile, miR-151-3p mimics significantly reduced the expression of Atg5 compared with miR-NC in VSMCs (mRNA level, $p < 0,01$; protein level, $p < 0.01$), and miR-151-3p inhibitor increased the expression of Atg5 compared with inhibitor NC in VSMCs (mRNA level, $p < 0,01$; protein level, $p < 0.05$; Fig. 3C and D). In addition, the expression of Atg5, p62 and LC3 in the exosomes of general VSMCs and calcific VSMCs was evaluated by western blot, and the results showed that β-GP treatment significantly increased the expression of p62 ($p < 0.01$), while reduced the expression of Atg5 ($p < 0.05$) and the ratio of LC3II/I ($p < 0.05$) in VSMCs (Fig. 3E). These results suggest that Atg5 is a target of miR-151-3p.

Exosomes derived from miR-151-3p inhibitor transfected VSMCs attenuated the medial artery calcification *in vivo*

To further confirm the effect of miR-151-3p in artery calcification, an animal model with soft tissue calcification was used, and the exosomes derived from general VSMCs, miR-151-3p mimics transfected VSMCs and miR-151-3p inhibitor transfected VSMCs were administrated into mice. . The results of western blot indicated that the protein levels of α-SMA, OPN, LC3 and ATg5 were downregulated in the aortas from Vitamin D3-treated mice, and miR-151-3p mimics-exosomes further enhanced the effect of Vitamin D3, but increased in the aortas from Vitamin D3 + miR-151-3p inhibitor-exosomes treated mice; meanwhile, the protein levels of Runx2, BMP-2, and p62 were upregulated in the aortas from Vitamin D3-treated mice, and miR-151-3p mimics-exosomes further enhanced the effect of Vitamin D3, but reduced in the aortas from Vitamin D3 + miR-151-3p inhibitor-exosomes treated mice (all $p < 0.05$, Fig. 4A). In addition, Vitamin D3 treatment significantly increased the expression of miR-151-3p compared with control group ($p < 0.01$), and miR-151-3p mimics-exosomes further enhanced the effect of Vitamin D3 ($p < 0.01$), but miR-151-3p inhibitor-exosomes significantly reversed the effect of Vitamin D3 on miR-151-3p expression ($p < 0.01$, Fig. 4C). These results indicate that miR-151-3p regulates medial artery calcification through modulating Atg5 *in vivo*.

Discussion

Vascular calcification is a complex pathological process, which is associated with the diseases including atherosclerosis, type 2 diabetes mellitus and brain arterial stiffness, and so on(20-22). Although recent advances in our knowledge, the specific mechanisms involved in vascular calcification have yet well studied. In this study, we used the cellular calcification model *in vitro* and the mouse model *in vivo* to explore the regulatory role of miR-151-3p and Atg5 in vascular calcification. Our study revealed that miR-151-3p in exosomes derived VSMCs could aggravate vascular calcification by downregulating Atg5 expression, which contributed our understanding of the pathogenesis in vascular calcification.

Our study found that miR-151-3p was significantly upregulated in β-glycerophosphate treated VSMCs and Vitamin D3 induced mouse model. Exosomes derived from miR-151-3p inhibitor transfected VSMCs attenuated the medial artery calcification both *in vitro* and *in vivo*. Previous studies indicated that miR-151-3p played essential roles in human diseases. MiR-151-3p inhibits proliferation and invasion of colon cancer cells by targeting Close Homolog of L1(23). MiR-151-3p has been identified to be a computational unpredictable miRNA to target Stat3 and suppresses innate IL-6 production by using a MicroRNA in vivo precipitation approach(24). MiR-151-3p regulates slow muscle gene expression by targeting ATP2a2 in skeletal muscle cells(25). In addition, miR-151-3p has also been demonstrated to inhibit the migration of human breast cancer cells by targeting TWIST1(26). Although these reports confirmed the important roles of miR-151-3p, there were no relevant reports in vascular calcification. In this study, we demonstrated that overexpression of miR151-3p could exacerbate the calcium deposition of VSMCs, and downregulation of miR-151-3p attenuated the calcium deposition of VSMCs. Taken together, we provided that VSMCs-derived exosomes might be a promising therapeutic strategy for vascular calcification.

Previous studies have indicated that OPG was an inhibitor in VSMC calcification, while Runx2 and α-SMA were up- and downregulated in obviously calcified VSMCs, respectively(27). In addition, Su et al. reported that oxidized low-density lipoprotein obviously enhanced the expression of BMP-2 in arterial endothelial cells(28), and the expression of BMP-2 was also significantly upregulated during VSMC calcification(29). Our study confirmed the results in previous studies, and VSMC calcification model both *in vitro* and *in vivo* exhibited a significant reduction of α-SMA and OPN, and an obvious increase of Runx2 and BMP-2. Moreover, exosomes derived from miR-151-3p mimics transfected VSMCs promoted these effects, while exosomes derived from miR-151-3p inhibitor transfected VSMCs reversed these effects. These results further confirmed the role of miR-151-3p in VSMC calcification.

It has been reported that miRNAs can act as ceRNAs to regulate the cellular processes such as proliferation, differentiation, migration and invasion of different types of eukaryotic cells by directly binding to the 3'UTR of their target mRNAs(5, 30, 31). MiR-34b/c inhibits aldosterone-induced VSMC calcification by modulating the SATB2/Runx2 signaling pathway(32). In this study, we identified that Atg5 was a target of miR-151-3p, and luciferase reporter assay determined their relationship. Although previous studies reported that autophagy is a potential protective phenomenon to protect against VSMC calcification(33, 34), the role of Atg5 in VSMC calcification remains unclear. Our study determined that Atg5 was significantly downregulated during VSMC calcification both *in vitro* and *in vivo*. In addition, there were several targets of miR-151-3p have been identified such as Anxa2(35), NRAS(36), and tumor

protein p53(37). This study focused on the regulation of miR151-3p and Atg5 in VSMC calcification, other potential regulatory axis should be explored in the subsequent experiments. Previous studies indicated that p62 is an autophagic substrate which can interact with LC3 to recognize ubiquitinated protein aggregates and then transport them to the autophagosome for degradation(38). The expression of p62 and LC3 further determined the role of miR151-3p in the regulation of VSMC calcification.

Conclusion

In summary, we demonstrated that VSMCs-derived exosomes might aggravate the calcium deposition *in vitro* and *in vivo* by downregulating Atg5, providing a potential therapeutic strategy for vascular calcification.

Declarations

Ethical Approval and Consent to participate

This study was approved by the animal Ethics Committee of Shanghai Xuhui District Central Hospital, and all experiments were performed according to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH publication no. 85–23, revised 1996).

Consent to publish

Not applicable.

Availability of supporting data

The data that support the findings of this study are available on request from the corresponding author.

The data are not publicly available due to their containing information that could compromise the privacy of research participants.

Competing interests

All other authors have no conflicts of interest.

We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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Authors' contributions

Li Chen: study concepts, literature research, clinical studies, data analysis, experimental studies, manuscript writing and review; Rongrong Zhang: study design, literature research, experimental studies and manuscript editing; Jinyin Li: definition of intellectual content, clinical studies, data acquisition and statistical analysis; Yiping Gao: data acquisition, manuscript preparation and data analysis; Shilong Mao: data acquisition and statistical analysis.

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Figures

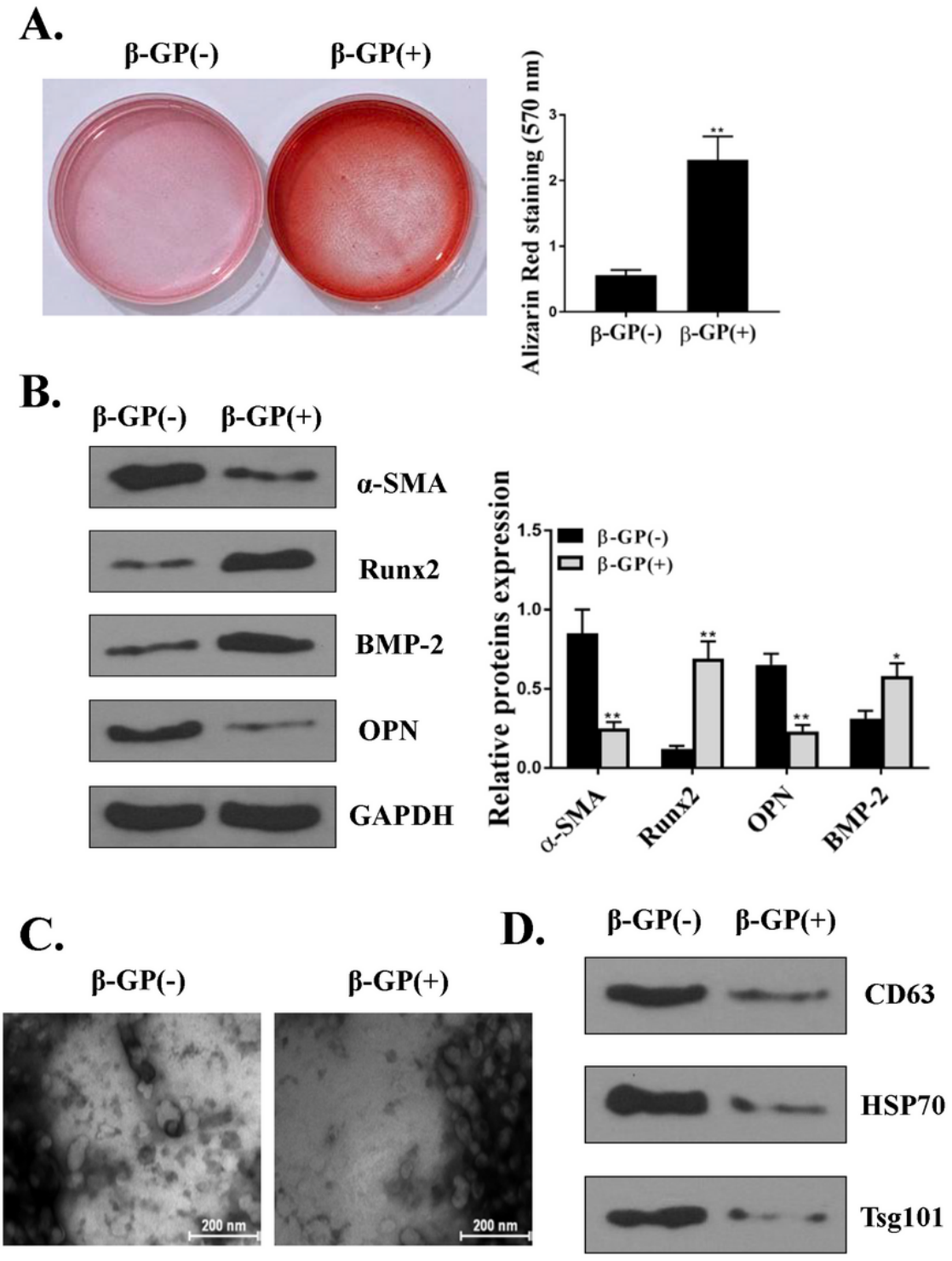

Figure 1

The establishment of the mouse VSMC calcification model and the characterization of exosomes. Mouse primary VSMCs were treated with or without 10 mM β-glycerophosphate (GP) for six days. (A) The calcium deposition in VSMCs was evaluated by Alizarin Red staining. (B) The expression of α-SMA, Runx2 and OPN and BMP-2 in general VSMCs and calcific VSMCs was evaluated by western blot. (C) The morphology and sizes of exosomes derived from general VSMCs and calcific VSMCs were observed by

TEM. Scale bar = 200 nm. (D) The expression of surface makers including CD63, HSP70 and Tsg101 in exosomes was evaluated by western blot. β-GP (-) indicates that general VSMCs treated without β-glycerophosphate, and β-GP (+) indicates that general VSMCs treated with β-glycerophosphate (calcific VSMCs). * $p < 0.05$, ** $p < 0.01$.

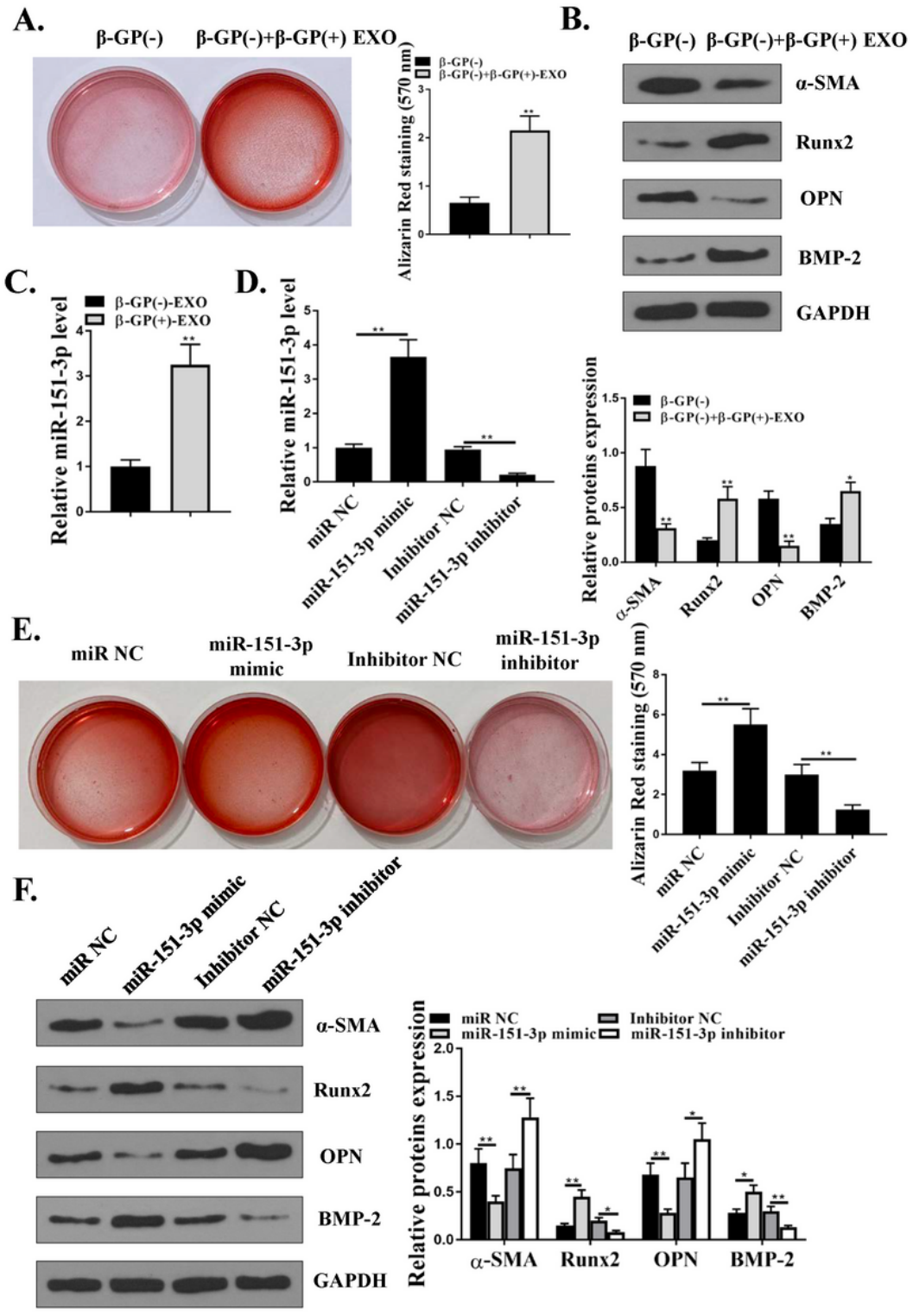

Figure 2

Exosomes derived from miR-151-3p overexpressing VSMCs exacerbated calcium deposition in the calcification model. (A and B) The exosomes were isolated from calcific VSMCs and general VSMCs were treated with 10 μg/ml of the isolated exosomes for 24 h. (A) The calcium deposition in general VSMCs was evaluated by Alizarin Red staining. (B) The expression of α-SMA, Runx2 and OPN and BMP-2 in general VSMCs was evaluated by western blot. (C) The expression of miR-151-3p in exosomes isolated from general VSMCs and calcific VSMCs was evaluated by qRT-PCR. (D) General VSMCs were transfected with miR-151-3p mimics/inhibitor and corresponding negative controls (miR-NC and inhibitor NC), and the exosomes were isolated. The expression of miR-151-3p in the exosomes was evaluated by qRT-PCR. (E and F) Calcific VSMCs were treated with 10 μg/ml of exosomes isolated from transfected VSMCs for 24 h. (E) The calcium deposition in calcific VSMCs was evaluated by Alizarin Red staining. (F) The expression of α-SMA, Runx2 and OPN and BMP-2 in calcific VSMCs was evaluated by western blot. β-GP (-) indicates that general VSMCs treated without β-glycerophosphate, and β-GP (+) indicates that general VSMCs treated with β-glycerophosphate (calcific VSMCs). * $p < 0.05$, ** $p < 0.01$.

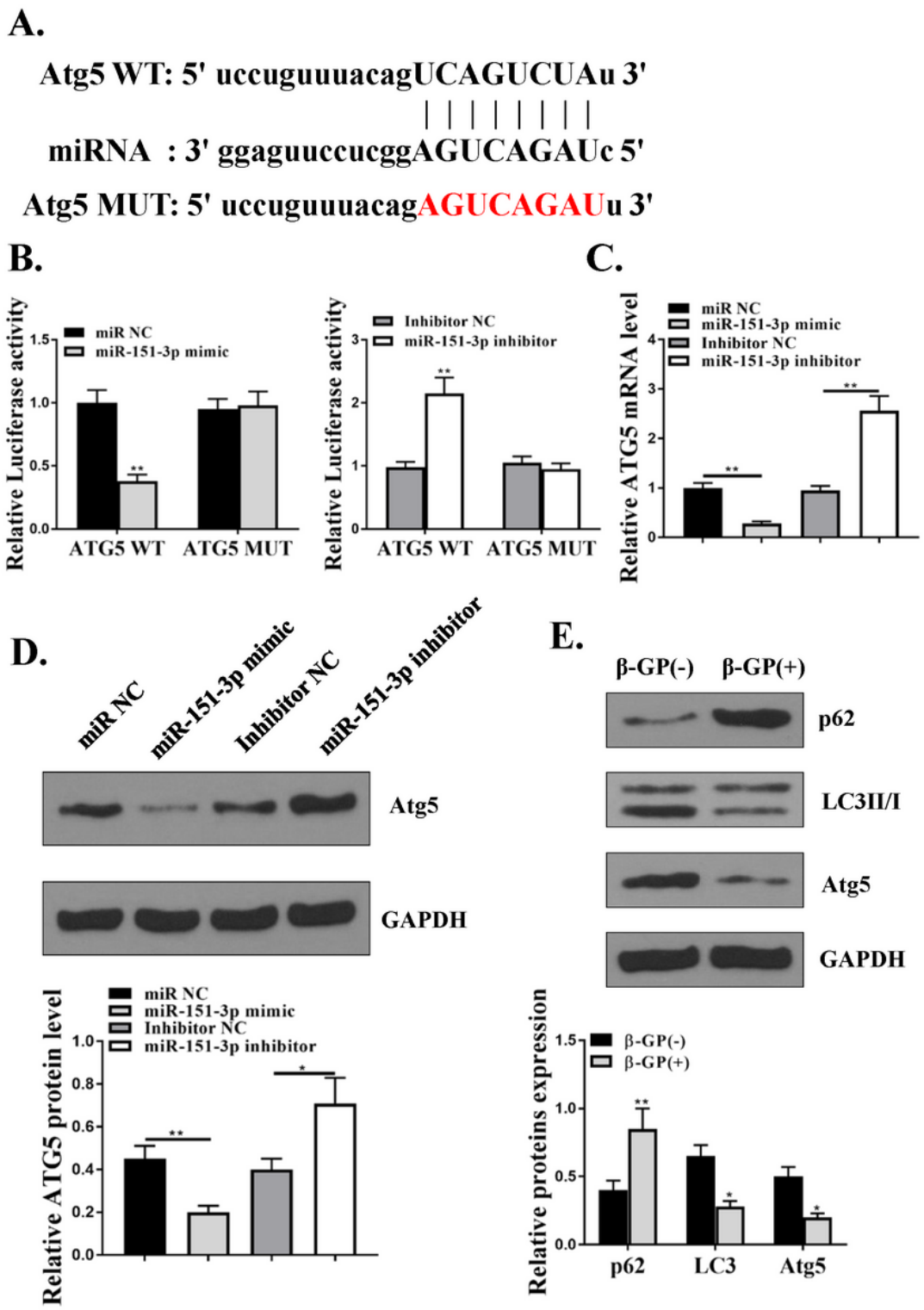

Figure 3

Atg5 was a target of miR-151-3p. (A) The putative binding site between miR-151-3p and Atg5 was predicted by StarBase. (B) The relative luciferase activity of Atg5 WT or Atg5 MUT was evaluated by dual luciferase reporter assay. (C and D) General VSMCs were transfected with miR-151-3p mimics/inhibitor and corresponding negative controls. The expression of Atg5 was evaluated by qRT-PCR (C) and western blot (D). (E) Mouse primary VSMCs were treated with or without 10 mM β-glycerophosphate (GP) for six

days. The expression of p62, LC3II/I, and Atg5 was evaluated by western blot. β-GP (-) indicates that general VSMCs treated without β-glycerophosphate, and β-GP (+) indicates that general VSMCs treated with β-glycerophosphate (calcific VSMCs). * $p < 0.05$, ** $p < 0.01$.

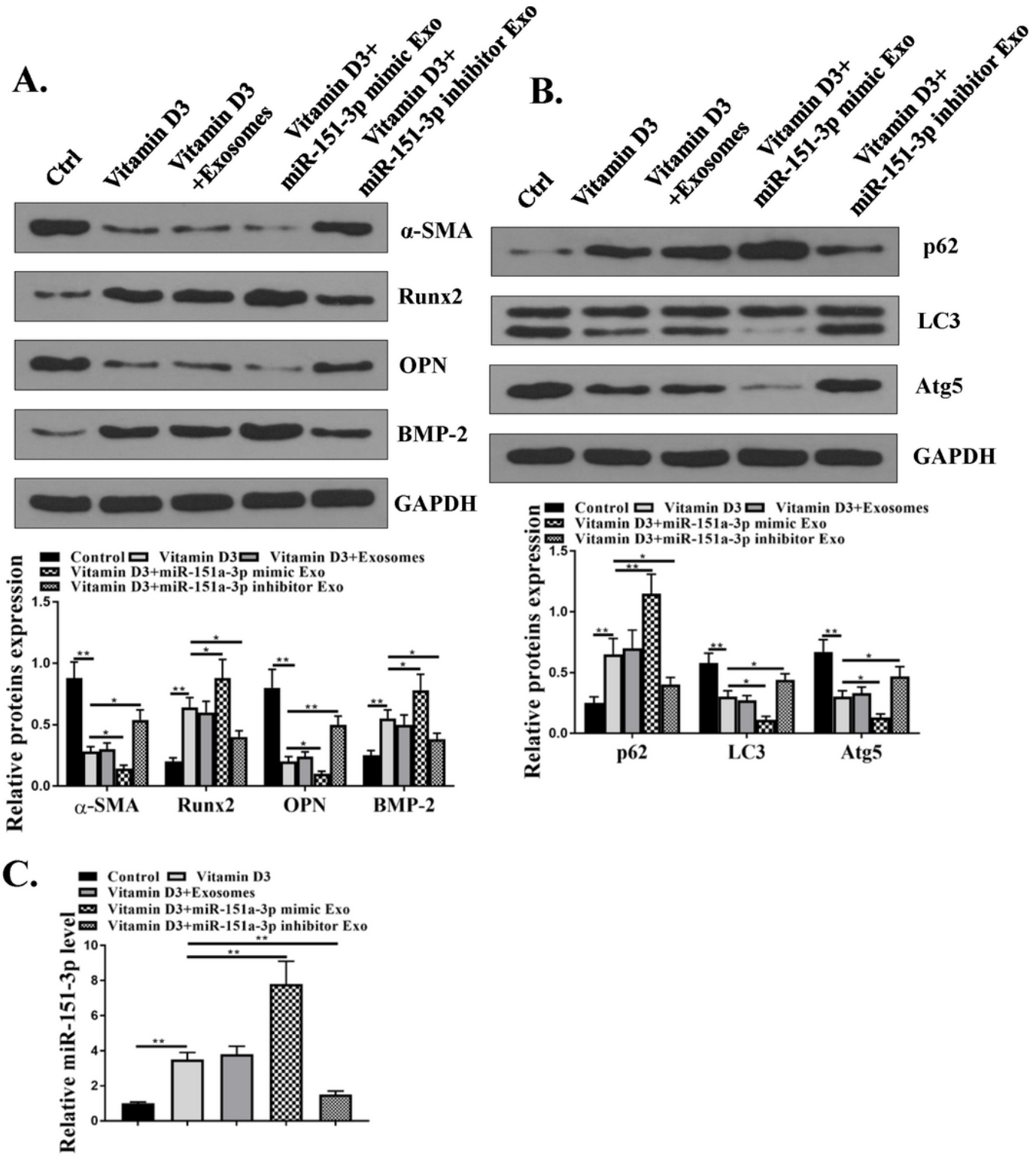

Figure 4

Exosomes derived from miR-151-3p inhibitor transfected VSMCs attenuated the medial artery calcification by targeting Atg5 in vivo. The animal model with soft tissue calcification was constructed,

following by the injection with different exosomes derived from general VSMCs, miR-1511-3p mimics transfected VSMCs, and miR-151-3p inhibitor transfected VSMCs. (A) The expression of α-SMA, Runx2 and OPN and BMP-2 in the aortas was evaluated by western blot. (B) The expression of p62, LC3 and Atg5 in the aortas was evaluated by western blot. (C) The expression of miR-151-3p in the aortas was evaluated by qRT-PCR. * $p < 0.05$, ** $p < 0.01$.