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ORIGINAL ARTICLE

The protective effects of long non-coding RNA-ANCR on arterial calcification

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Abstract

Introduction Arterial calcification is a major factor for cardiovascular events and is characterized by vascular smooth muscle cells (VSMCs) transformed into osteoblast-like cells. Long non-coding RNAs (lncRNA) were recognized as important regulators of diverse biological processes. Previous studies have demonstrated that lncRNAs could regulate the proliferation and apoptosis of VSMCs. LncRNA-ANCR (Anti-differentiation ncRNA) is an essential mediator governing the differentiation of human osteoblast. However, it is unclear whether ANCR could regulate the osteoblastic differentiation of VSMCs. In this study, we determined the effect of ANCR on VSMCs differentiation and arterial calcification.

Materials and Methods Both cellular and mouse model of arterial calcification were, respectively, established to investigate the role of ANCR in the mechanism of arterial calcification. ANCR overexpressing lentivirus were used to investigate the effects of ANCR on the expression of bone proteins and autophagy-related molecules.

Results ANCR could inhibit β-glycerophosphate (β-GP)-induced VSMCs osteoblastic differentiation and mineralization due to decreased expressions of Runt-related transcription factor 2, bone morphogenetic protein-2, and formation of mineralized nodule, and attenuate high calcitriol-induced mice model of arterial calcification. Furthermore, ANCR could significantly increase LC3 and autophagy protein 5 expression in β-GP-stimulated VSMCs, and the effect could be inhibited by 3-methyladenine, a pharmacological inhibitor of autophagy.

Conclusion ANCR may inhibit the osteoblastic differentiation of VSMCs and attenuate mice arterial calcification through activating autophagy.

Keywords Arterial calcification · Vascular smooth muscle cells · Osteoblastic differentiation · ANCR · Autophagy

Introduction

Arterial calcification is prevalent in the aged and patients with diabetes, hypertension and chronic kidney disease. Arterial calcification contributes to a number of clinical complications including cardiovascular and cerebrovascular diseases. Being similar to bone formation, arterial calcification is an active process of osteoblastic differentiation of vascular smooth muscle cells (VSMCs), characterized by significant expression of bone proteins such as Runt-related transcription factor 2 (Runx2) and bone morphogenetic protein-2 (BMP-2) [1, 2]. Till now, the definite mechanism of arterial calcification remains to be elucidated.

Long non-coding RNAs (lncRNAs) are a class of transcribed RNA molecules with a length of over 200 nucleotides which serve as key regulators in a series of physiological and pathophysiological processes such as cell proliferation and differentiation [3, 4]. Anti-differentiation noncoding RNA (ANCR) is a single 855-base pair lncRNA which recently displayed inhibitory effects on human osteoblast differentiation by reducing the expression of Runx2 [5]. It remains unknown whether ANCR is involved in the process of osteoblastic differentiation of

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VSMCs. The current study aimed to investigate the roles of ANCR in the mechanism of osteoblastic differentiation of VSMCs using the cellular and mouse model of arterial calcification.

Materials and methods

Animals

Six-week male C57BL/6 mice were purchased from Pengyue experimental animal breeding Co., Ltd (Jinan, Shandong, China) and housed at a controlled temperature in a 12 h light–12 h dark cycle with free access to food and water. The experiment was approved by the Institutional Animal Care and Use Committee of the Affiliated Hospital of Jining Medical University and performed in accordance with the ARRIVE guidelines and National Institutes of Health guide for the care and use of laboratory animals.

Reagents

Beta-glycerophosphate (β-GP) and Alizarin Red S were purchased from Sigma (MO, USA). Polybrene, Enhanced Infection Solution and Von Kossa Staining kit were purchased from Genmed (Shanghai, China). Fetal bovine serum and DMEM/F12 (1:1) were purchased from Gibco (NY, USA). All-in-One First-Strand cDNA Synthesis Kit and All-in-One™ qPCR Mix were purchased from GeneCopoeia (Guangzhou, China). Calcitriol was purchased from Source leaf organism (Shanghai, China). Triton X-100 was purchased from Genview (TX, USA). Mouse anti-Runx2 antibody, mouse anti-BMP-2 antibody, Alexa-555-conjugated donkey anti-mouse IgG, and 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Abcam (MA, USA). Normal goat serum was purchased from Zhongshan Golden Bridge Company (Beijing, China). Rapamycin, 3MA and calcium diagnostic kit were purchased from Sigma (MO, USA). Anti-Atg5 was purchased from Abgent (San Diego, USA). Anti-LC3I/II was purchased from Cell Signaling Technology (MA, USA).

Cell model of arterial calcification

The cell model of arterial calcification was established as previously described [6]. Mouse VSMCs of passages 3–8 were used in this study. VSMCs were cultured in 6-well plates (5×10^5/well) in DMEM/F12 (1:1) complete medium (containing L-glutamine, 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin) and 10 mmol/L β-GP. On days 3, 6, 9 and 12 after the stimulus, the cells were harvested for the detection of Runx2 and BMP-2 and ANCR using quantitative PCR.

VSMCs infected by lentiviral vector

The ANCR overexpressing lentivirus (Lv-ANCR, 5×10^8 TU/mL) and negative control lentivirus (Lv-NC) were constructed by Shanghai GeneChem Co., Ltd (Shanghai, China). VSMCs were seeded in 6-well plates at 5×10^5/well in DMEM/F12 (1:1) complete medium. After 12 h, VSMCs were infected by Lv-ANCR or Lv-NC at 100 multiplicity of infection with Eri. S and Polybrene conditions (5 μg/mL). After 48–72 h post-infection, the over-expression of ANCR was examined using fluorescent microscopy and quantitative real-time PCR (qPCR).

To investigate the effects of ANCR on osteoblastic differentiation of VSMCs, VSMCs were divided into the following 4 groups: control group, β-GP group, β-GP plus Lv-ANCR group and β-GP plus Lv-NC group. Each group of VSMCs was cultured in 6-well plates (5×10^5/well) in DMEM/F12 (1:1) complete medium, respectively. After a culture of 12 h, the groups of β-GP, β-GP plus Lv-ANCR and β-GP plus Lv-NC were, respectively, treated with 10 mmol/L β-GP for 96 h. After the treatment, cells were harvested for the mRNA of Runx2 and BMP-2. After a culture of 21 days, Alizarin Red S staining was performed to detect the formation of mineralized nodules.

To investigate the role of autophagy in the mechanism of β-GP-induced osteoblastic differentiation of VSMCs, 10 μmol/L rapamycin and 5 mmol/L 3MA were, respectively ,added into the culture of VSMCs. After 30 min, β-GP was added to induce osteoblastic differentiation of VSMCs. VSMCs with and without β-GP were used as the controls.

qPCR

The qPCR was performed as previously described [7]. In detail, the total RNA was extracted by TRIzol (Life Technologies Inc., NY, USA). The cDNA was synthesized from 1 μg total RNA using an All-in-One™ First-Strand cDNA Synthesis Kit. A total of 20 μL of the reaction system consists of 2 μL cDNA template, 2 × All-in-One® qPCR Mix 10 μL, Rnase-free water 3.8 μL, 2 μL forward/reverse primer (0.2 μmol/L) and 0.2 μL of ROX. Amplification and detection were performed in an ABI 7500 as follows: 95 °C for 10 min and then 40 cycles of 95 °C for 10 s, 60 °C for 20 s, and 72 °C for 30 s. GAPDH was used as the inner control. The expression of mRNA was analyzed by a semi-quantitative method using $2^{-\Delta\Delta Ct}$.

Measurement of mineralized matrix formation

After the incubation, cells were fixed in 95% ethanol for 10 min at room temperature, washed with 2 mL PBS three times, and then stained with 1% Alizarin Red S for 1 h at 37 °C. After the staining, cell preparations were washed three times with PBS to eliminate nonspecific staining. The formation of mineralized nodules was observed using bright field in Axio Observer A1 inverted fluorescence microscope (Zeiss, Jena, Germany). The Alizarin Red S-positive area in each well was measured using Image J (version 6.0, Media Cybernetics, Bethesda, MD).

Western blot analysis

Cytoplasmic protein and nuclear protein extracts of cultured cell were prepared using nuclear and cytoplasmic extraction lysate (Beyotime, Nantong, China). Equal amounts of protein were submitted to SDS-PAGE and transferred onto 0.2 μm PVDF membranes to be stained with LC3I, LC3II and Agt5 antibodies. The reaction was visualized with chemiluminescence. The results were shown by the gray value between target protein and β-actin.

Mouse model of arterial calcification

To establish the animal model of arterial calcification, the mice were subcutaneously injected with olive oil-dissolved calcitriol at a dose of 500,000 IU/kg/day for a total of 7 days. The mice injected with the same volume of olive oil only were used as control. Seven days after the last injection, the mice were anesthetized with 10% chloral hydrate intraperitoneally, and perfused with 4% paraformaldehyde in PBS through the heart. The thoracic aortas were dissected and placed under a high power optical microscope to remove perivascular tissue. The thoracic aortas were collected and put in 4% paraformaldehyde for 4 h and to 30% sucrose overnight for further immunostaining and pathological analysis.

To investigate the effects of ANCR on the mouse model of arterial calcification, the mice were randomly divided into the following 4 groups ($n = 5$, each group): control group, Calcitriol group, Calcitriol plus Lv-ANCR group, and Calcitriol + Lv-NC group. The Calcitriol + Lv-ANCR and Calcitriol + Lv-NC groups were injected with 100 μL of lentivirus via their tail veins at the first day of calcitriol treatment. Injections of lentivirus were administered once a week over a period of 2 weeks. The final lentivirus concentration achieved in each mouse at the end of the treatment window was 1×10^8 TU/mL. After 4 weeks, the mice were anesthetized with 10% chloral hydrate intraperitoneally and the aorta was surgically dissected. Each thoracic aorta was fixed with 4% paraformaldehyde and 30% sucrose, dehydrated and

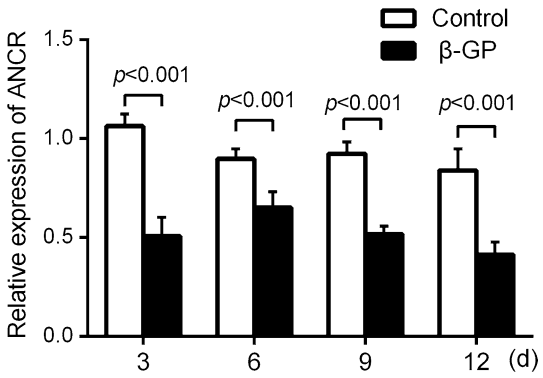


Fig. 2 Dynamic expression of ANCR in vitro. As shown, qPCR analysis of ANCR level in β-GP-stimulated VSMCs was significantly decreased from 3 to 12 days compared with the control. The data shown were based on three independent experiments

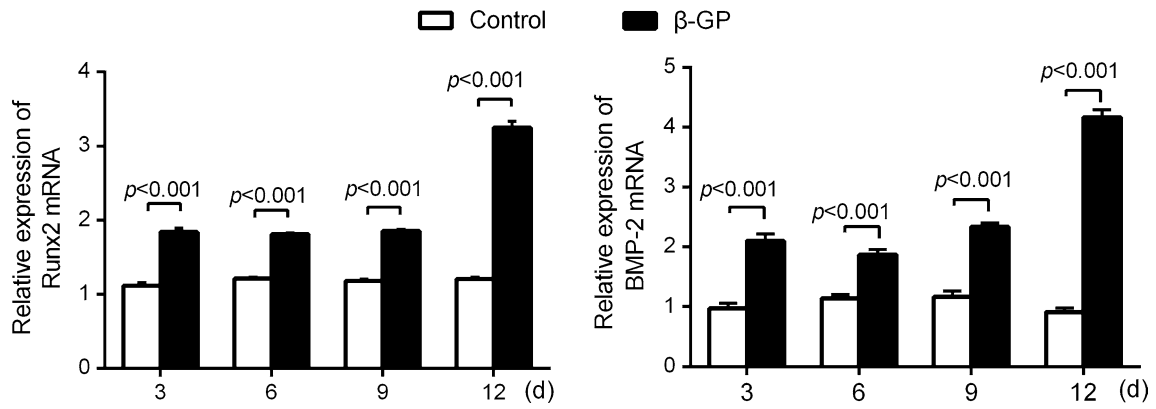


Fig. 1 Dynamic expression of Runx2 and BMP-2 in vitro. As shown, qPCR analysis of Runx2 and BMP-2 mRNA levels in β-GP-stimulated VSMCs were significantly increased from 3 to 12 days compared with the control. The data shown were based on three independent experiments

frozen for Alizarin Red S, Von Kossa staining and immunofluorescence staining.

Histology analysis of calcified lesion and calcium content

The frozen tissues were cut into 8 μm-thick sections for subsequent analysis. The slices were dyed with 1% Alizarin Red S solution for 30 min. Calcification sections were shown in an orange-red color in light microscope. Von Kossa staining was performed according to the Von Kossa staining kit instructions. The sections were stained with Reagent C for 1 h under 60 W of light at room temperature. Positive results were shown in a black color in light microscope. Imaging of stained sections was performed using Observer Axio A1 inverted microscope (Zeiss, Jena, Germany). The Alizarin Red S and Von Kossa staining-positive area to total area of thoracic aorta sections was measured using Image J (version 6.0) (Media Cybernetics, Bethesda, MD), respectively.

The level of calcification was quantified using the O-cresolphthalein complexone method as previously described [8]. Thoracic aortas were lyophilized and decalcified with 0.6 mmol/L HCl at 37 °C for 48 h. Calcium released from the lyophilized tissues was determined colorimetrically by the calcium diagnostic kit. Total protein was quantified using the Bradford protein assay. The calcium content was normalized to protein content and expressed as micrograms of calcium per milligram of protein.

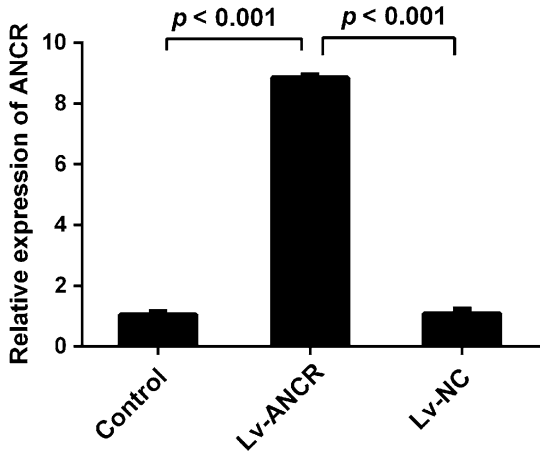


Fig. 3 Effects of Lv-ANCR on ANCR expression. As shown, qPCR analysis of ANCR level in Lv-ANCR-intervened VSMCs was significantly increased compared with the control and Lv-NC. The data shown were based on three independent experiments

Immunofluorescent staining

The frozen sections were blocked in 0.1 M PBS containing 0.3% Triton X-100 and 10% normal goat serum. Afterward, the sections were incubated with mouse anti-Runx2 and mouse anti-BMP-2 at 4 °C overnight. After three times of washing with PBS for 30 min, donkey anti-mouse secondary antibodies conjugated with Alexa Fluor-555 were added for incubation for 1 h at room temperature. After three times of washing, the sections were stained with DAPI (1:25 diluted in 0.1 M PBS) for 10 min. The stained sections were captured with Observer Z1 confocal laser-scanning microscope (Zeiss, Jena, Germany) under the same setting. Using the software of Image J, semi-quantitative analysis of the positive staining of Runx2 and BMP-2 in the area of whole

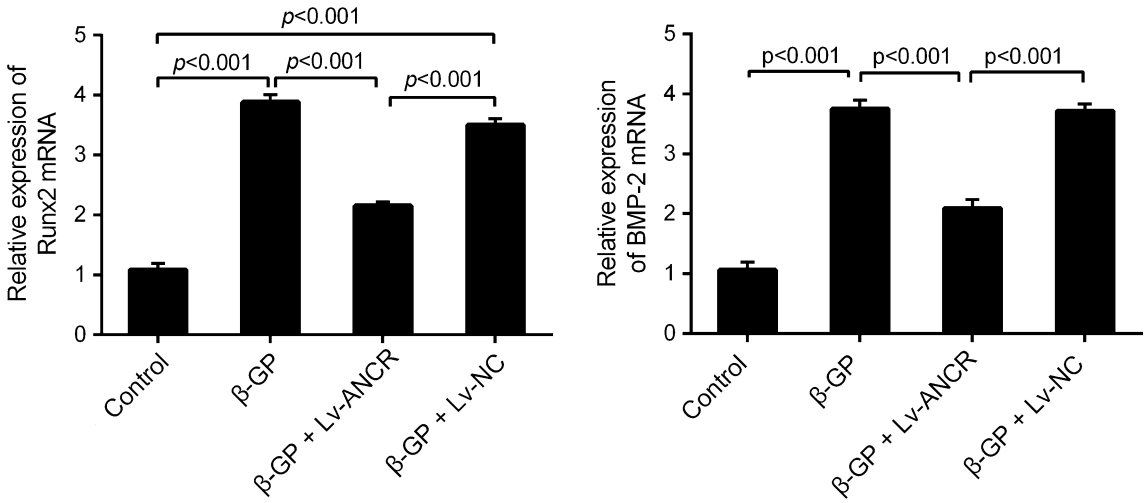


Fig. 4 Effects of Lv-ANCR on the expression of Runx2 and BMP-2 in vitro. As shown, qPCR analysis of Runx2 and BMP-2 mRNA level in β-GP and β-GP + Lv-NC-intervened VSMCs indicated significantly increased level compared with the control. The expressions of Runx2 and BMP-2 in β-GP + Lv-ANCR-intervened VSMCs were significantly decreased compared with β-GP and β-GP + Lv-NC. The data shown were based on three independent experiments

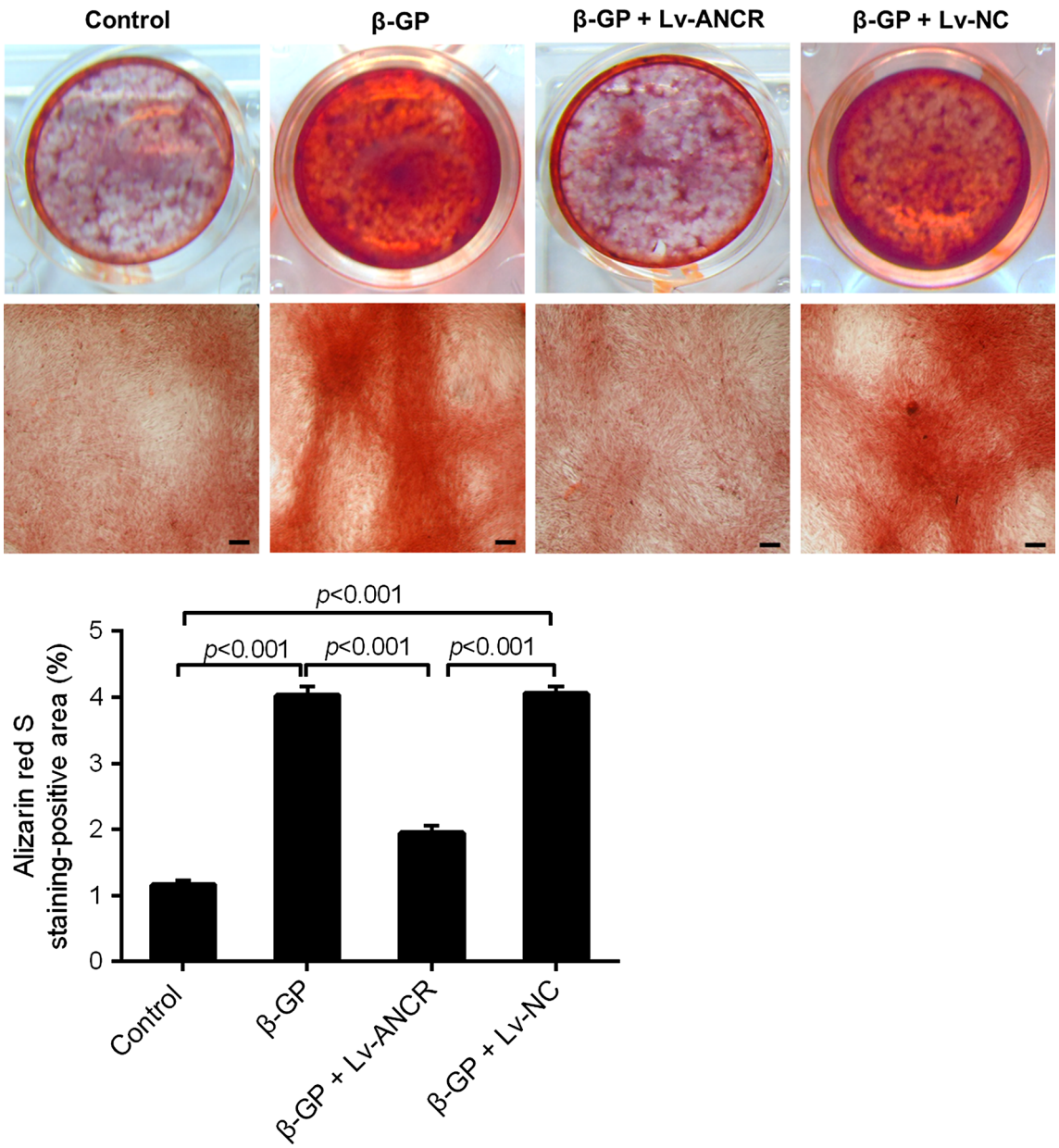


Fig. 5 Effects of Lv-ANCR on Alizarin Red S staining in vitro. As shown, there were significantly more mineralized nodule formation in β-GP and β-GP + Lv-NC-intervened VSMCs than in the control. However, compared with β-GP and β-GP + Lv-NC, the mineralized nodule formation of Alizarin Red S staining in β-GP + Lv-ANCR-intervened VSMCs was significantly decreased. The data shown were based on three independent experiments

artery was performed. In detail, Image J software was used to open the image and convert the image to 8 bit. From Image J's menu bar, Analyze/Set Scale was used to convert the pixel size of the image to the actual physical size, the positive area was drawn with the tool and the area was measured. Image/Adjust/Threshold was chosen to measure the entire arterial area.

Statistics

Normally distributed data are shown as mean ± SD. The difference among different groups was determined by Student's *t* test or one-factor analysis of variance (one-way ANOVA). A two-sided *p* value of < 0.05 was considered to be significant. The analysis was performed using GraphPad Prism 7.0.

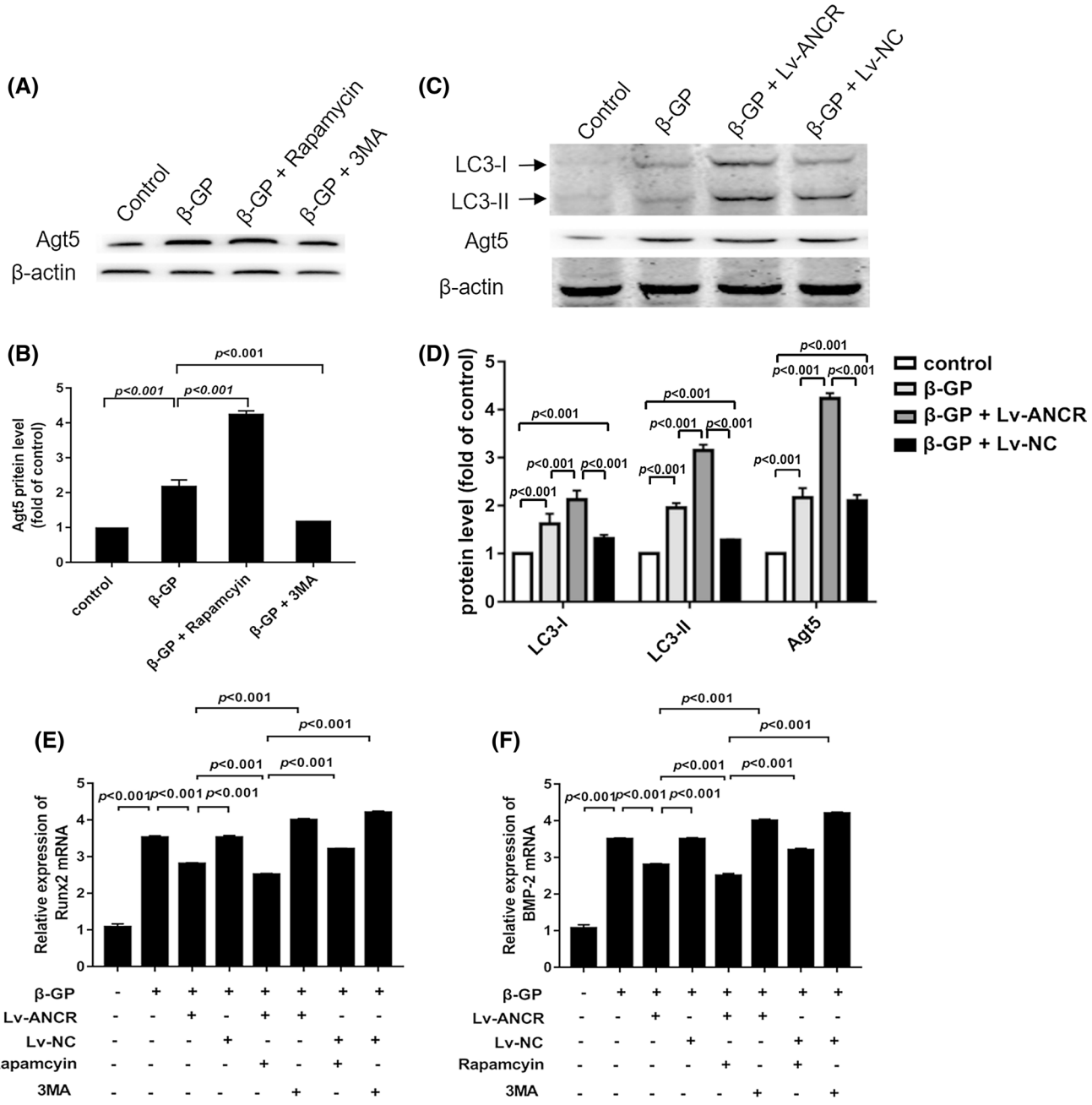


Fig. 6 Overexpression of ANCR reduces the expression of Runx2 and BMP-2 via increasing LC3I/II and Agt5. **a**, **b** Western blotting analysis of Agt5 protein level in β-GP-stimulated VSMCs showed significantly increased level than the control. Rapamycin significantly increased the Agt5 protein level compared with β-GP-stimulated VSMCs. However, 3MA significantly inhibited the Agt5 protein level compared with β-GP-stimulated VSMCs. **c**, **d** The expressions of LC3I/II and Agt5 in β-GP and β-GP + Lv-NC-intervened VSMCs were significantly increased compared to the control. Compared with β-GP and β-GP + Lv-NC, LC3I/II and Agt5 protein levels in β-GP + Lv-ANCR-intervened VSMCs were significantly decreased. **e**, **f** The qPCR analysis of Runx2 and BMP-2 mRNA level in β-GP + Lv-ANCR + Rapamycin-intervened VSMCs showed significantly decreased level than β-GP + Lv-ANCR. 3MA significantly increased the Runx2 and BMP-2 mRNA level compared with β-GP + Lv-ANCR. The results were similar to β-GP + Lv-NC. The data shown were based on three independent experiments

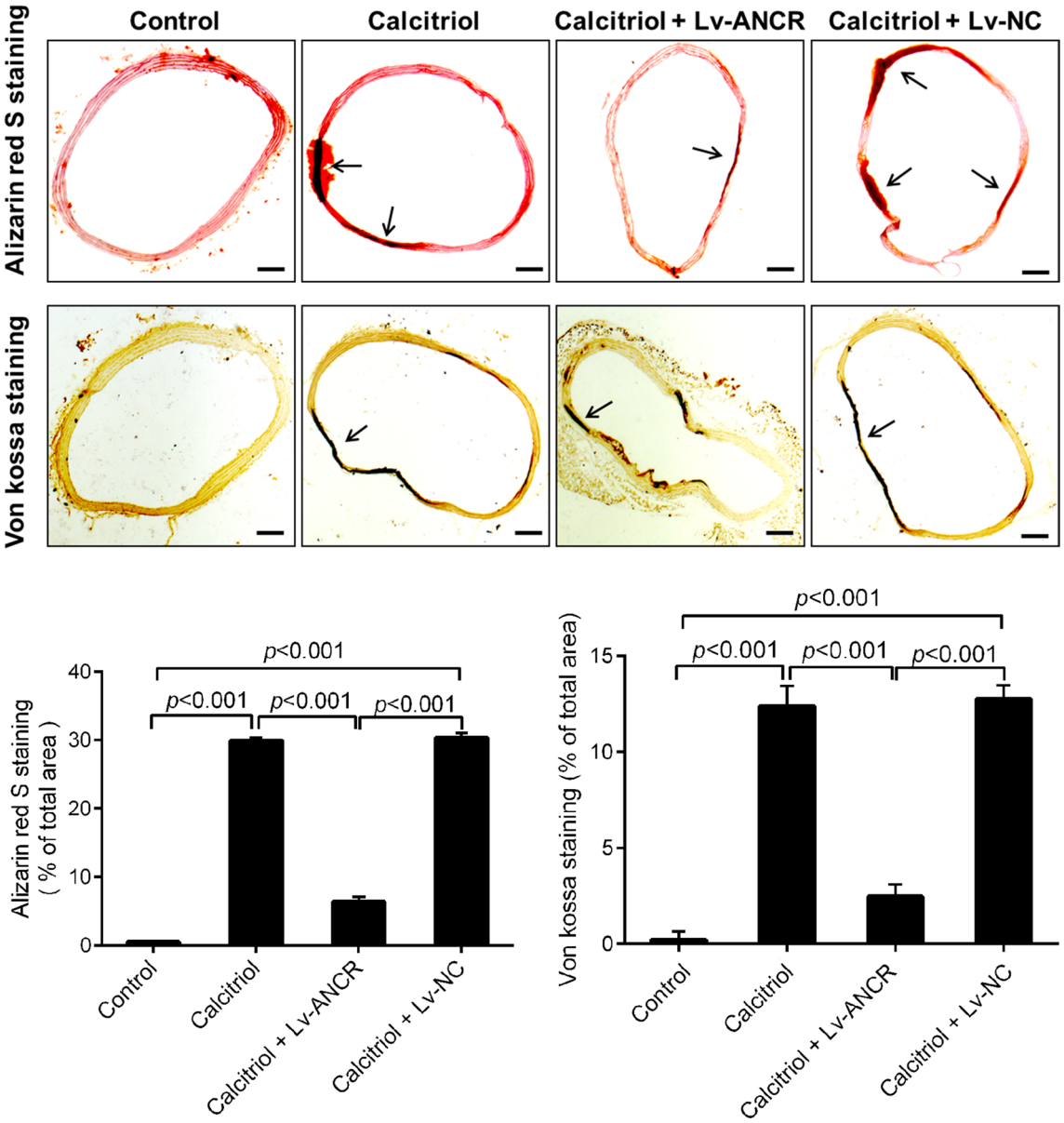


Fig. 7 Alizarin Red S and Von Kossa staining. As shown, there were more calcification nodule formations in Calcitriol and Calcitriol + Lv-NC than the control groups. However, compared with the Calcitriol and Calcitriol + Lv-NC, the calcification nodule formation with Alizarin Red S and Von Kossa staining in + Lv-ANCR was significantly decreased. The data shown were based on three independent experiments

Results

Overexpression of ANCR inhibited the osteoblastic differentiation of VSMCs

VSMCs were treated with 10 mM β-GP to induce osteoblastic differentiation. There was a significantly higher mRNA expression of Runx2 and BMP-2 in the β-GP group compared with the control group from 3 to 12 days (Fig. 1). There was a significantly less ANCR expression in the β-GP group than the control group from 3 to 12 days (Fig. 2).

To determine the effect of ANCR on osteoblastic differentiation of VSMCs, Lv-ANCR and Lv-NC were applied to infect VSMCs. Quantitative PCR analysis showed significantly increased expression of ANCR in the Lv-ANCR group (Fig. 3). There was a significantly increased mRNA expression of Runx2 and BMP-2 in the β-GP + Lv-ANCR group than the β-GP group and the β-GP + Lv-NC group (Fig. 4). As shown in Fig. 5, Alizarin Red S staining data

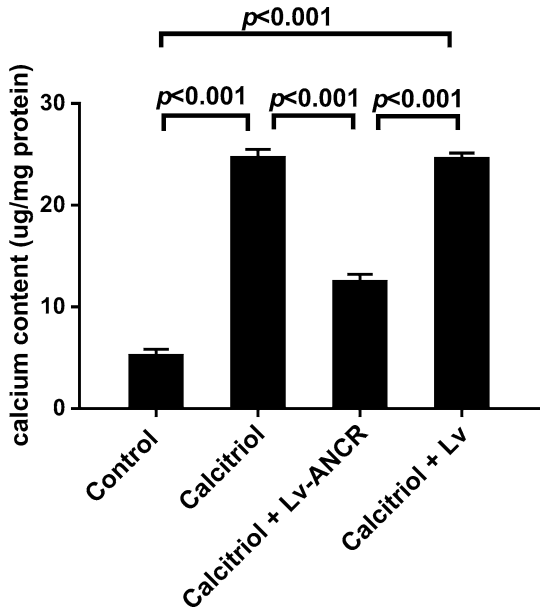


Fig. 8 The calcium content assay. As shown, there was significantly less calcium content in the thoracic aortas of the Calcitriol + Lv-ANCR group than those in the Calcitriol and Calcitriol + Lv-NC groups. The data shown were based on three independent experiments

showed that β-GP significantly increased the formation of mineralized nodules in the β-GP group and β-GP + Lv-NC group compared with the control. The formation of mineralized nodules was reduced in β-the GP + Lv-ANCR group compared with the β-GP group and β-GP + Lv-NC group.

ANCR promoted the expression of LC3 and Agt5 in β-GP-induced VSMCs

Western blot analysis was performed to evaluate the effect of ANCR on autophagy and autophagy-specific markers (LC3I/LC3II) and Agt5 in β-GP-induced VSMCs. Our data showed that compared with the control group, the expressions of LC3I/LC3II and Agt5 were increased in the β-GP group. Compared with the β-GP group and β-GP + Lv-NC group, the β-GP + Lv-ANCR group exhibited further increased expression of both LC3I/LC3II and Agt5 (Fig. 6). Furthermore, quantitative PCR showed that rapamycin decreased the expression of Runx2 and BMP-2 compared with the β-GP + Lv-ANCR group. However, the effect of ANCR-induced autophagy was inhibited by 3MA, and showed significant increased expression of Runx2 and BMP-2 (Fig. 6).

ANCR attenuated mice arterial calcification induced by high calcitriol

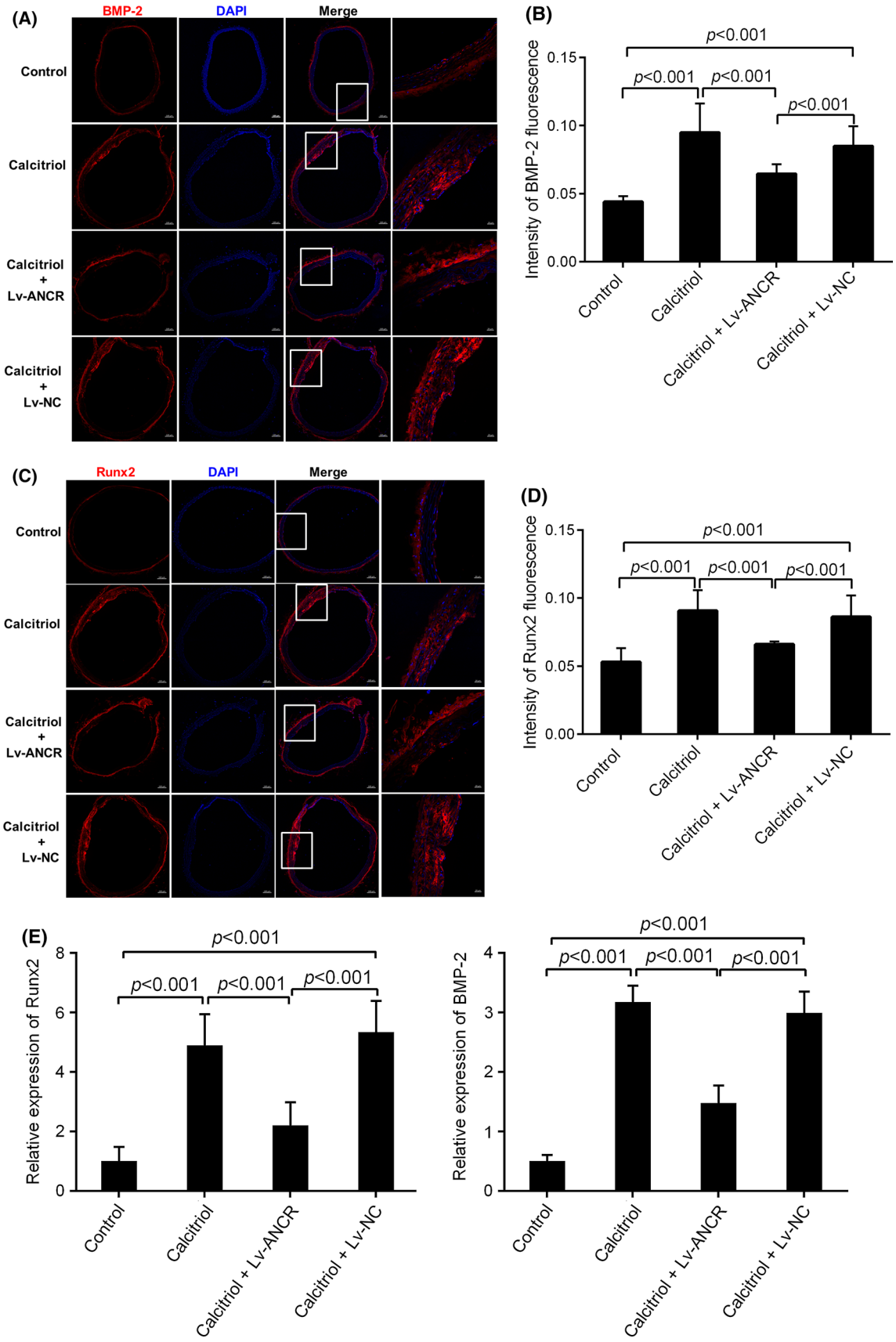
To determine whether ANCR inhibited vascular calcification in vivo, mice were treated with high doses of calcitriol to induce arterial medial calcification. Calcification of thoracic aortas was visualized by Alizarin Red S and Von Kossa staining. The staining was enhanced in the arteries of mice in the Calcitriol group and Calcitriol + Lv-NC group compared with the control group. Quantitative analysis of Alizarin Red S and Von Kossa staining demonstrated a significant increase. Compared with the Calcitriol group and Calcitriol + Lv-NC group, the staining was attenuated and quantitative analysis of Alizarin Red S and Von Kossa staining was significantly decreased in the Calcitriol + Lv-ANCR group (Fig. 7). The calcium content analysis showed that there was significantly reduced calcium in the Calcitriol + Lv-ANCR group (Fig. 8). Both the immunofluorescence and qPCR analysis showed that the expressions of BMP-2 and Runx2 were significantly decreased in the Lv-ANCR group compared with the Calcitriol group and Calcitriol + Lv-NC group (Fig. 9).

Fig. 9 Effects of LV-ANCR on Runx2 and BMP-2 expression in vivo. ▶ **a–d** Immunofluorescent staining analysis of the expression of Runx2 and BMP-2 in Calcitriol and Calcitriol + Lv-NC were significantly increased than that in the control. Compared with Calcitriol and Calcitriol + Lv-NC groups, the Runx2 and BMP-2 protein level in Calcitriol + Lv-ANCR was significantly decreased. **e** The mRNA expression of Runx2 and BMP-2 in the thoracic aorta was detected using quantitative PCR. Being consistent with the immunofluorescent staining analysis, there was significantly reduced mRNA expression of Runx2 and BMP-2 in the thoracic aorta of Calcitriol + Lv-ANCR-treated mouse than that of the control. The data shown were based on three independent experiments

Discussion

The current concepts view that arterial calcification is an active regulation process similar to osteogenesis, which is regulated by some hormones and cytokines [9-11]. LncRNAs were recognized as important regulators of diverse biological processes such as transcriptional regulation, cell proliferation and differentiation [3, 12, 13]. Previous studies reported that lncRNAs played important roles in the development of VSMCs. Lnc-ANRIL could influence VSMCs proliferation and apoptosis [14, 15]. Lnc-p21 suppressed VSMCs proliferation and induced VSMCs apoptosis [16]. Lnc-GAS5-knockdown accelerated VSMCs proliferation and decreased VSMCs apoptosis [17]. Lnc-TUG1 promoted VSMCs proliferation and inhibited VSMCs apoptosis [4]. In this study, our results demonstrated that ANCR plays a protective role in vascular calcification in mice.

ANCR is a single 855-base-pair lncRNA that is downregulated during the differentiation of progenitor cells [12]. In epidermal tissues, ANCR excludes the expression of differentiated genes to maintain the undifferentiated state of progenitor cells [12]. Recent studies indicated that ANCR is


(A)
BMP-2
DAPI
Merge
Control
Calcitriol
Calcitriol + Lv-ANCR
Calcitriol + Lv-NC
(B)
Intensity of BMP-2 fluorescence
p<0.001
p<0.001
p<0.001
p<0.001
0.00
0.05
0.10
0.15
Control
Calcitriol
Calcitriol + Lv-ANCR
Calcitriol + Lv-NC
(C)
Runx2
DAPI
Merge
Control
Calcitriol
Calcitriol + Lv-ANCR
Calcitriol + Lv-NC
(D)
Intensity of Runx2 fluorescence
p<0.001
p<0.001
p<0.001
p<0.001
0.00
0.05
0.10
0.15
Control
Calcitriol
Calcitriol + Lv-ANCR
Calcitriol + Lv-NC
(E)
Relative expression of Runx2
p<0.001
p<0.001
p<0.001
p<0.001
0
2
4
6
8
Control
Calcitriol
Calcitriol + Lv-ANCR
Calcitriol + Lv-NC
Relative expression of BMP-2
p<0.001
p<0.001
p<0.001
p<0.001
0
1
2
3
4
Control
Calcitriol
Calcitriol + Lv-ANCR
Calcitriol + Lv-NC

an essential mediator governing osteoblast differentiation of hFOB1.19 cell [5]. In this study, quantitative PCR analysis showed that the expression of ANCR significantly decreased from 3 to 12 days, suggesting that ANCR is inhibited during the process of arterial calcification. To explore the effect of ANCR, we used ANCR overexpression lentiviral vector to infect VSMCs. Our data showed that ANCR expression was significantly upregulated after infection with ANCR lentiviral vector, suggesting that lentivirus infection was effective. Arterial calcification resembles bone formation and many bone-related markers are expressed in calcified arteries, such as Runx2 and BMP-2. Runx2 belongs to the runt-related transcription factor family and is required for osteoblast differentiation. Many studies showed that the expression of Runx2 is increased significantly in calcified arteries [18-21]. BMP-2 belongs to bone morphogenetic protein family and is essential for differentiation of osteoblastic cells. BMP-2 is considered as a mediator of vascular calcification and is highly expressed in arterial calcification [21-23]. In this study, we observed that ANCR overexpression could significantly downregulate the expression of Runx2 and BMP-2 in β-GP-stimulated VSMCs. The Alizarin Red S staining confirmed that ANCR overexpression could significantly reduce the formation of mineralized nodule in β-GP-stimulated VSMCs. Our data demonstrated that ANCR can attenuate the osteoblastic differentiation of VSMCs.

Autophagy is a process in which cells use lysosomes to degrade or recycle their damaged or unnecessary cellular components and exist in calcified blood vessels. Autophagy can improve the pathological process of arterial calcification [24, 25]. Recent studies have shown that autophagy inhibits arterial calcification [26, 27]. Autophagy may be an endogenous protective mechanism counteracting phosphate-induced arterial calcification by reducing matrix vesicle release [27]. In this study, we explored the role of autophagy in β-GP-induced osteoblastic differentiation of VSMCs and whether ANCR acts its roles via modulating autophagy in this process. Our data showed that the expression of autophagy makers, LC3I/LC3II and Agt5, were significantly increased in β-GP-induced VSMCs compared with control VSMCs. This is consistent with the findings of a previous study showing that high Pi can promote autophagy in VSMCs and that autophagy plays a protective role by counteracting phosphate-induced vascular calcification [27]. Furthermore, we also found that expression of LC3-I/LC3-II and Agt5 in the β-GP + lv-ANCR group was further increased compared with the β-GP group. Rapamycin, a pharmacological inducer of autophagy, could further reduce Runx2 and BMP-2 expressions compared with ANCR alone. As a pharmacological inhibitor of autophagy, 3MA increased the expression of Runx2 and BMP-2 significantly. This suggested that ANCR could enhance the autophagy in β-GP-induced VSMCs to attenuate calcification. Enhanced autophagy plays a protective role in arterial calcification, but excessive autophagy also leads to apoptosis [28]. A recent study found that indoxyl sulfate activates the autophagy pathway by downregulating the expression of SET domain containing lysine methyltransferase 7/9 inducing osteoblast differentiation and matrix mineralization of VSMCs [29]. We hypothesize that the reason for this difference may be that autophagy may be regulated by multiple signaling pathways in the osteogenic differentiation of VSMCs.

In the in vivo experiments, arterial calcification was induced by subcutaneous injection of calcitriol, followed by Alizarin Red S staining and Von Kossa staining to detect the calcification of mice thoracic aorta. Our data showed that calcitriol could significantly increase thoracic aorta calcification. To investigate the role of ANCR in arterial calcification, ANCR overexpression lentiviral vector was applied via tail vein injection. Alizarin Red S staining and Von Kossa staining results showed that upregulated expression of ANCR attenuated vascular calcification. These results demonstrate that ANCR can inhibit mice arterial calcification. Our immunofluorescence results showed that the expressions of Runx2 and BMP-2 are increased significantly in calcified artery. ANCR, an important regulator of osteoblast differentiation, can inhibit Runx2 expression and human osteoblast differentiation [5]. Being consistent with the results of in vitro experiment that ANCR could decrease the expression of Runx2 and BMP-2 of β-GP-stimulated VSMCs, upregulated expression of ANCR reduced the expression of Runx2 and BMP-2 in mice calcified aorta.

In conclusion, ANCR may attenuate arterial calcification through activating autophagy to inhibit osteogenic differentiation of VSMCs. ANCR might play a protective role in vascular calcification, which represents a new mechanism for the treatment and prevention of vascular calcification.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval All applicable international, national, and institutional guidelines for the care and use of animals were followed.

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