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Original Article

LncRNA H19/Runx2 axis promotes VSMCs transition via MAPK pathway

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Abstract: Arterial calcification (AC) is mainly caused by osteoblast phenotypic transition of vascular smooth muscle cells (VSMCs). Long noncoding RNA H19 (lncRNA H19) has attracted increasingly attention because of their transcriptional regulation crucial potency. We reported that lncRNA H19 expression is up-regulated after VSMCs transition. Thus, we aim to study the role of H19 and the molecular mechanisms in VSMCs transition. To determine the expression of H19 in calcified VSMCs, we induced VSMCs calcification with 10 mM β-glycerophosphate. By qPCR and Western Blot analysis, we found that the expression of lncRNA H19, Runx2 and OSX were all highly increased in calcified VSMCs compared with normal VSMCs, while the expression of VSMCs differentiation markers, SM22-α and α-SMA, were significantly decreased. SiRNA study showed that knockdown of lncRNA H19 can decrease VSMCs calcification and Runx2 expression. We further validated that lncRNA H19 promoted VSMCs calcification via the p38 MAPK and ERK1/2 signal transduction pathways. As a conclusion, the present study showed that lncRNA H19/Runx2 axis promotes VSMCs transition via MAPK pathway. This finding not only reveal a novel function of lncRNA H19, but also provides a new opinion on the role of lncRNA H19 which participant in the Runx2 regulatory pathway in AC and can be a new indication for the diagnosis and treatment of AC at an early time.

Keywords: Arterial calcification, VSMCs, lncRNA H19, RUNX2, MAPK

Introduction

Arterial calcification (AC), namely aberrant calcium deposition in vessel walls, is a distinguishing feature of many diseases such as atherosclerosis, diabetes mellitus and chronic kidney disease with a high burden of morbidity and mortality [1, 2]. More specifically, it's still a well-known predictive risk factor of subsequent myocardial infarction [3-6]. AC has now been recognized as an active and regulated process reminiscent of physiological bone formation [3], rather than simply passive calcium deposition [7, 8]. Subsequent studies demonstrate that vascular smooth muscle cells (VSMCs) are the main cell type involved in AC, the transformation of VSMCs into osteoblast-like cells cause a kind of cellular adaptations and damage then promote calcification finally [9, 10]. This phenotypic transition is characterized by the increased expression of alkaline phosphatase (ALP), runt-related transcription factor 2 (RUNX2), osteocalcin, osteopontin (OPN) and the formation of mineralized bone-like structures, and decreased the expression of several differentiation marker genes of VSMCs, including smooth muscle α-actin (α-SMA) and smooth muscle 22α (SMA-22α) [11-13]. However, the potential molecular mechanisms on how VSMCs transition occurred in AC is still unclear.

Runx2, a master transcription factor that belongs to the runt-domain gene family, is expressed by osteoblasts. Studies have shown that Runx2 is a key regulator which is essential for osteoblast differentiation, many signal pathways focus on Runx2 to regulate osteoblast dif-

ferentiation [14]. What's more, the level of Runx2 is a decisive factor in the occurrence of osteoblastic differentiation [15, 16], Runx2-null mice lacks osteoblasts and unable to form bone [15, 17]. Besides that, overexpression of Runx2 in vascular cells seems to be associated with AC [18, 19]. What's more, Runx2 is also an important osteogenic transcription factor in VSMCs calcification both in mouse models and human patients [20, 21]. The expression of Runx2 has been identified not only in calcified human atherosclerotic lesions but also in calcifying aortic smooth muscle cells in mice, while never in normal vessels [19, 22]. Recent studies showed that Runx2 in VSMCs is also critical in the formation of atherosclerotic calcification in mice [20]. Runx2 deficiency in VSMCs can inhibit vascular calcification in vivo [20]. As for the pivotal role of Runx2 in VSMCs calcification, to identify the factors which regulate transcription may lead to novel medical therapies to prevent or even treat AC.

Long noncoding RNA H19 (lncRNA H19) has aroused increasingly attention because of their important potency in transcriptional regulation. The expression of H19 is strongly induced during embryogenesis and downregulated after birth, except in adult heart and skeletal muscle [23]. Kim et al. found highly expression of H19 in the balloon-injured carotid artery. Notably, common polymorphisms of H19 have been identified to be associated with the risk and severity of coronary artery disease [24]. Recent study indicates that H19 is highly expressed in calcified aortic valve and associated with indexes of disease activity [25]. Moreover, Prominent H19 expression is detected in both ECs and VSMCs of prenatal rabbit aorta [26]. However, as the most important cell type that involved in AC, whether VSMCs is regulated by H19 is still undefined.

Mitogen-activated protein kinase (MAPKs) such as ERK1/2 and p38 MAPK are widely known as the main molecules responsible for initiating the signaling pathway involved in VSMCs migration, proliferation, and growth [27, 28]. Previous studies indicated that activation of MAPKs promote VSMCs proliferation in atherogenesis [29], and also showed that MAPKs pathways play a crucial role in modulating the VSMCs phenotype transition [30, 31]. Substantial evidence showed that H19 can promote the migration and invasion of colon cancer cells and CD133+ cancer stem cells via MAPK signaling pathway [32, 33]. Taken together, it may be possible that H19 can induces VSMCs transition through MAPKs pathway.

In this report we show that H19 expression is up-regulated after VSMCs transition, and also recovered the molecular mechanisms. What's more, we also detected the expression of p38 and p44/42 after H19 was knockdown in VSMCs.

Materials and methods

Cell culture

Vascular Smooth Muscle Cells (VSMCs) were purchased from the IBCB (Shanghai, China). The cells were culture at 37°C in a humidified incubator with 5% CO_2 and 95% air, and maintained in DMEM (Hyclone, South Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) and 1% penicillin/streptomycin (100 U/mL/100 mg/mL) (Beyotime, Beijing, China). Cells were used between passages P2-P5.

Induction of calcification

VSMCs were routinely sub cultured in growth medium (DMEM containing 10% FBS supplemented with 100 U/mL penicillin, 0.1 mg/mL streptomycin). At 80% confluence, the cells were switched to calcification medium (DMEM containing 15% FBS, and supplemented with 10 mM β-glycerophosphate, 100 U/mL penicillin and 0.1 mg/mL streptomycin) for up to 14 days. The medium was replaced every 2 days. For time course experiments, the first day of culture in calcification medium was defined as Day 0.

Transfection

For siH19 transfection, gently removed the culture medium, then cover the VSMCs with transfection cocktails. To prepare transfection cocktails for each well of 24-well plates, mixed 125 pmol of siRNA (with a stock solution of 5 μM) with 150 μl of OPTI-MEM by gentle pipetting. In parallel, diluted 6.25 μl of Lipofectamine 2000 in 150 μl of OPTI-MEM. After incubation at room temperature for 5 mins, combined the two tubes. After incubation at room tempera-

ture for 25 min, gently added the resulting cocktail (300 μl) to the VSMCs. After incubation for 6-10 hours in a tissue culture incubator, the cocktail was gently replaced with fresh DMEM. 48 hours after transfection, RNA and protein were extracted for analysis.

RNA extraction and qPCR

Total RNAs were extracted using Pure Link RNA Mini Kit (Ambion, catalog number 12183018A). cDNA was synthesized using Prime Script RT Reagent Kit (TAKARA, RR037A) in a 20 μl reaction containing 0.5-1 μg of total RNA. Real-time quantitative PCR was performed in a 15 μl reaction containing 0.5-1 μl of cDNA using iQSYBR Green (Bio-Rad) in a Bio-Rad iCycler. PCR was performed by initial denaturation at 95°C for 5 min, followed by 40 cycles of 30 sec at 95°C, 30 sec at 60°C, and 30 sec at 72°C. Specificity was verified by melting curve analysis and agarose gel electrophoresis. The threshold cycle (Ct) values of each sample were used in the post-PCR data analysis. Gene expression levels were normalized against GAPDH. The primers used were as follows: lncRNA H19 (forward: 5-TACAACCACTGCACTACCTG-3, reverse: 5-TGACTCCTGTGTTCCTGTTA-3); GAPDH (forward: 5-ACCACAGTCCATGCCATCAC-3, reverse: 5-TCCACCACCCTGTTGCTGTA-3); RUNX2 (forward: 5-TGTCCATCTCCAGCCGTGTC-3, reverse: 5-TCTGTCTGTGCCTTCTTGGTTC-3); OSX (forward: 5-ATACACTGACCTTTCAGCCCC-3, reverse: 5-CAGACGGGTAAGTAGGCAGC-3); SM22-α (forward: 5-TGGTGGAGTGGATCGTAATGC-3, reverse: 5-AGAGGTCAACGGTCTGGAACA-3); α-SMA (forward: 5-CATCCGACCTTGCTAACGGA-3, reverse: 5-GTCCAGAGCGACATAGCACA-3); si-1 (GAAGATGATGCTAAGAAGCACCA); si-2 (AAGATGATGCTAAGAAGCACCAT).

Western blot analysis

VSMCs in 24-well plates were dissociated with 0.25% trypsin. Cell pellets were collected by centrifugation at 1000 rpm for 5 min. Cell pellets were homogenized in 2x SDS-sample buffer (100 μl/well), then heating at 100°C for 5 min, with appropriate vortex. Then loaded homogenized samples onto 12% SDS gel (5 μl/well), followed by western blotting analysis. Bands on Western blot gels were quantified using ImageJ. GAPDH was used as a loading control.

Alizarin red S staining and quantification

We use 4% paraformaldehyde to fix the cultured cells and then stained them with 0.1% pH=4.2 Alizarin red S (Sigma-Aldrich, Saint Louis, MO) for 20 mins. For quantitative assessment of the degree of mineralization, the stain was eluted by 100 mM cetylpyridinium chloride (Sigma-Aldrich) for 1 h.

Alkaline phosphatase (ALP) staining and activity

ALP activity was test by a commercially kit (Cat. No. A059-2; Nanjing Jiancheng Bioengineering Institute). We lysed cell layers with buffer on ice for 30 mins and then centrifuged at 6.2 g for 10 min at 4°C. Then, the protein concentrations were quantified using a BCA assay kit. The final results were normalized with total protein concentration.

Statistical analysis

All experiments were conducted at least three times. SPSS 25.0 software (IBM Corp., Armonk, NY, USA) and GraphPad Prism 8 (San Diego, CA, USA) were used for the statistical analyses. Data were expressed as mean ± standard error of mean (SEM). Two-tailed Student's t tests (or as otherwise indicated) were used to compare means between groups. $P< 0.05$ were considered as significant differences.

Results

LncRNA H19 promotes VSMCs calcification which induced by β-glycerophosphate

Previous studies have shown that β-glycerophosphate can induced VSMCs calcification [34]. Studies also showed that as an osteoblast marker, ALP is thought to be essential for VSMCs calcification [35]. So we cultured VSMCs from 0 to 21 days in 10 mM β-glycerophosphate or control. Accumulated matrix mineral deposition was proved by Alizarin Red S staining. The mineralization nodes of VSMCs cultured with β-glycerophosphate medium for 7, 14 and 21 days were significantly increased in a time-dependent manner, as shown in **Figure 1A**. In addition, ALP activity of VSMCs was also upregulated more than 4 folds after cultured with β-glycerophosphate medium for

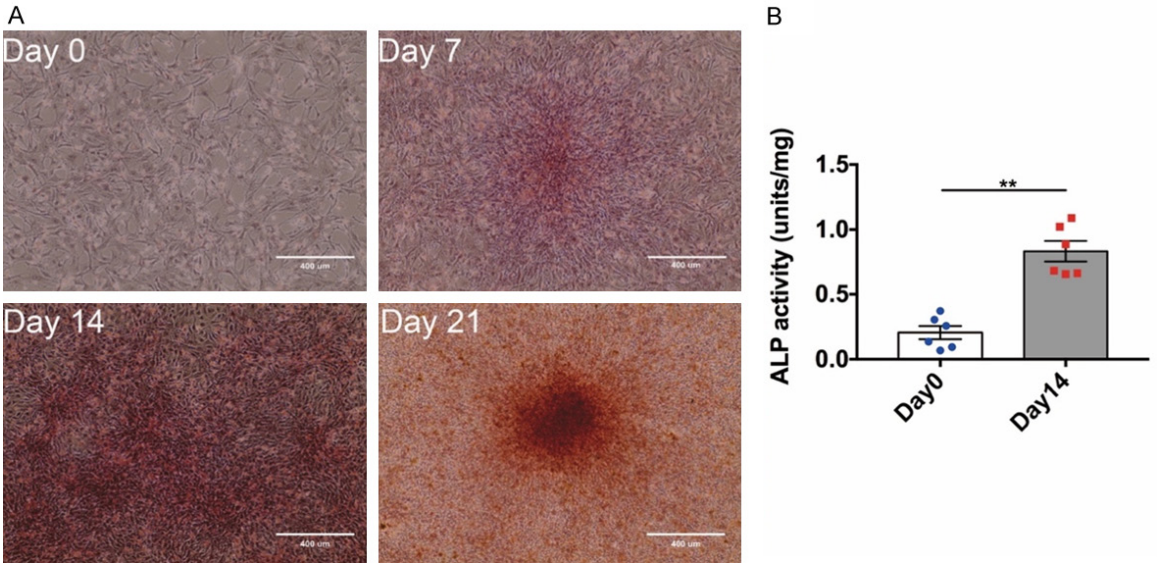

Figure 1. 10 mM β-glycerophosphate can induced VSMCs to form calcification. A. Alizarin Red S staining of VSMCs cultured with 10 mM β-glycerophosphate medium for 7, 14 and 21 days. B. ALP activity of VSMCs after cultured with 10 mM β-glycerophosphate medium for 14 days compare with 0 day. All experiments were performed in triplicate with three technical replicates. **P<0.01.

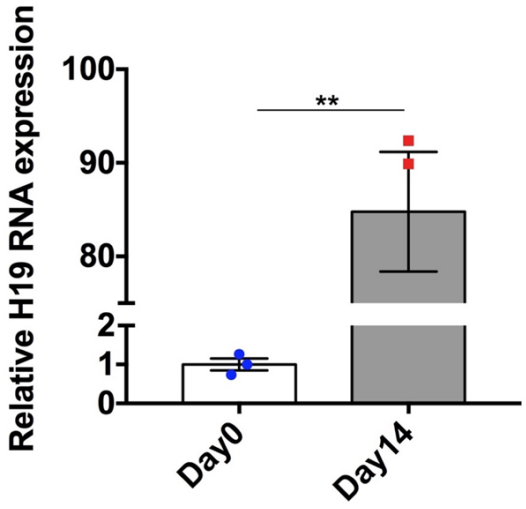

Figure 2. The expression of lncRNA H19 was highly increased in calcified VSMCs, compared with the normal VSMCs. This figure showed the qPCR analysis of lncRNA H19. **P<0.01.

14 days which means extensive calcification (**Figure 1B**). These results indicate that β-glycerophosphate can enhance the activity of ALP, osteocalcin secretion and calcium content. After cultured with 10 mM β-glycerophosphate for 14 days, VSMCs can be induced to form calcification.

To determine whether lncRNA H19 is associated with β-glycerophosphate-induced VSMCs calcification, we then detected the RNA expression of H19 in VSMCs. We concluded that compared with the normal VSMCs, the expression of lncRNA H19 was highly increased about 85-fold in calcified VSMCs (**Figure 2**). These results indicated that lncRNA H19 might promote the calcification of VSMCs, which could accelerate the formation of AC.

To clarify the phenotypic switch of VSMCs after cultured with β-glycerophosphate, expression of VSMCs osteogenic gene and differentiation gene was examined by qPCR and Western Blotting. We demonstrated that after incubated with β-glycerophosphate medium for 14 days, the expression of Runx2 and OSX mRNA increased 13.0-fold and 36.8-fold, respectively (**Figure 3A**). However, the expression of VSMCs differentiation marker genes, including SM22-α and α-SMA, were significantly decreased by 91 or 96% after exposed to β-glycerophosphate for 14 days as assessed by PCR (**Figure 3B**). Furthermore, the Western Blot analysis showed up regulation of the protein levels of Runx2 and OSX in calcified VSMCs relative to those of the normal VSMCs. Moreover, the expression of α-SMA protein was downregulated (**Figure 3C**, **3D**).

Collectively, these results indicate that β-glycerophosphate can promote the expression of osteogenic gene Runx2 and VSMCs calcifica-

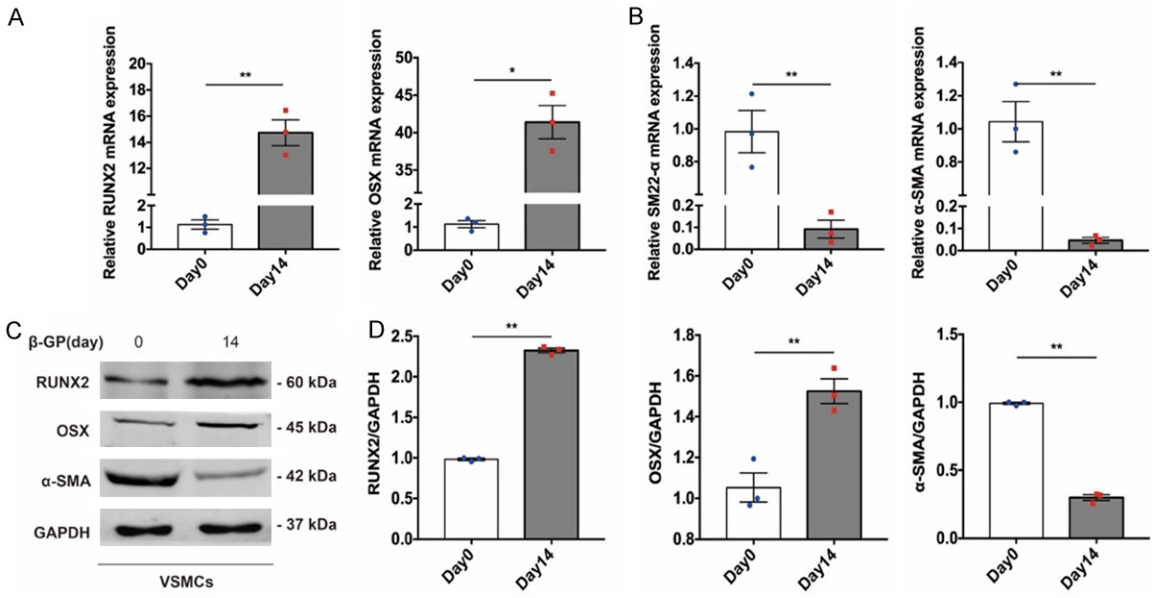

Figure 3. Expression of VSMCs osteogenic gene as well as differentiation gene was examined to clarify the phenotypic switch of VSMCs after cultured with β-glycerophosphate. A, B. qPCR analysis of Runx2, OSX, SM22-α and α-SMA expression in the VSMCs. C, D. Western blotting analysis of Runx2, OSX and α-SMA expression levels. **P<0.01.

tion, and that H19 may play an important role in mediating Runx2 expression in VSMCs.

Knockdown of lncRNA H19 decreased VSMCs calcification and Runx2 expression

To further investigate whether the knockdown of lncRNA H19 using siRNA could decrease the calcification of VSMCs and the role of lncRNA H19 in β-glycerophosphate induced calcification, we then knocked down lncRNA H19 in VSMCs by H19 siRNA and used β-glycerophosphate to induce calcification. As shown in **Figure 4A**, lncRNA H19 knockdown greatly reduced β-glycerophosphate-induced VSMCs calcification, as indicated by Alizarin Red S staining assay, calcium deposition and ALP calcium content in VSMCs compared with that transfected using scramble siRNA (**Figure 4B**, **4C**).

In order to determine the effect of lncRNA H19 on the phenotypic switch of VSMCs, we then detected the RNA and protein levels of calcification and differentiation markers in β-glycerophosphate induced VSMCs calcification. Additionally, as indicated by qPCR (**Figure 4D**) and Western Blot (**Figure 4E**), expression of osteogenic marker gene Runx2 and OSX significantly decreased after lncRNA H19 was knockdown. As expected, knockdown of lncRNA H19 and incubation with the β-glycerophosphate medium increased the expression of α-SMA comparing with the cells transfected with scramble siRNA (**Figure 4D**, **4E**). After all, treatment of calcified VSMCs with siH19 attenuated the expression of osteogenic-related mRNA/proteins and VSMCs calcification. These results demonstrated that lncRNA H19 plays a crucial role on mediating the phenotypic switch of VSMCs into osteogenic cells and AC via a Runx2 dependent pathway.

LncRNA H19 promoted VSMCs calcification via the p38 MAPK and ERK1/2 signal transduction pathways

Although we have found that lncRNA H19 could promote VSMCs calcification, but the molecular mechanism is still unclear. Studies have showed that MAPK signaling pathway was involved in regulating the function of VSMCs, suggesting that this signaling pathway maybe the key factor which induced the transition of VSMCs [36, 37].

First, we found that after cultured with β-glycerophosphate for 14 days, the expression of p-p38 and p-p44/42 (the key factors of MAPK signaling pathway) increased (**Figure 5A**), suggesting that lncRNA H19 might activate the MAPKs pathway in VSMCs.

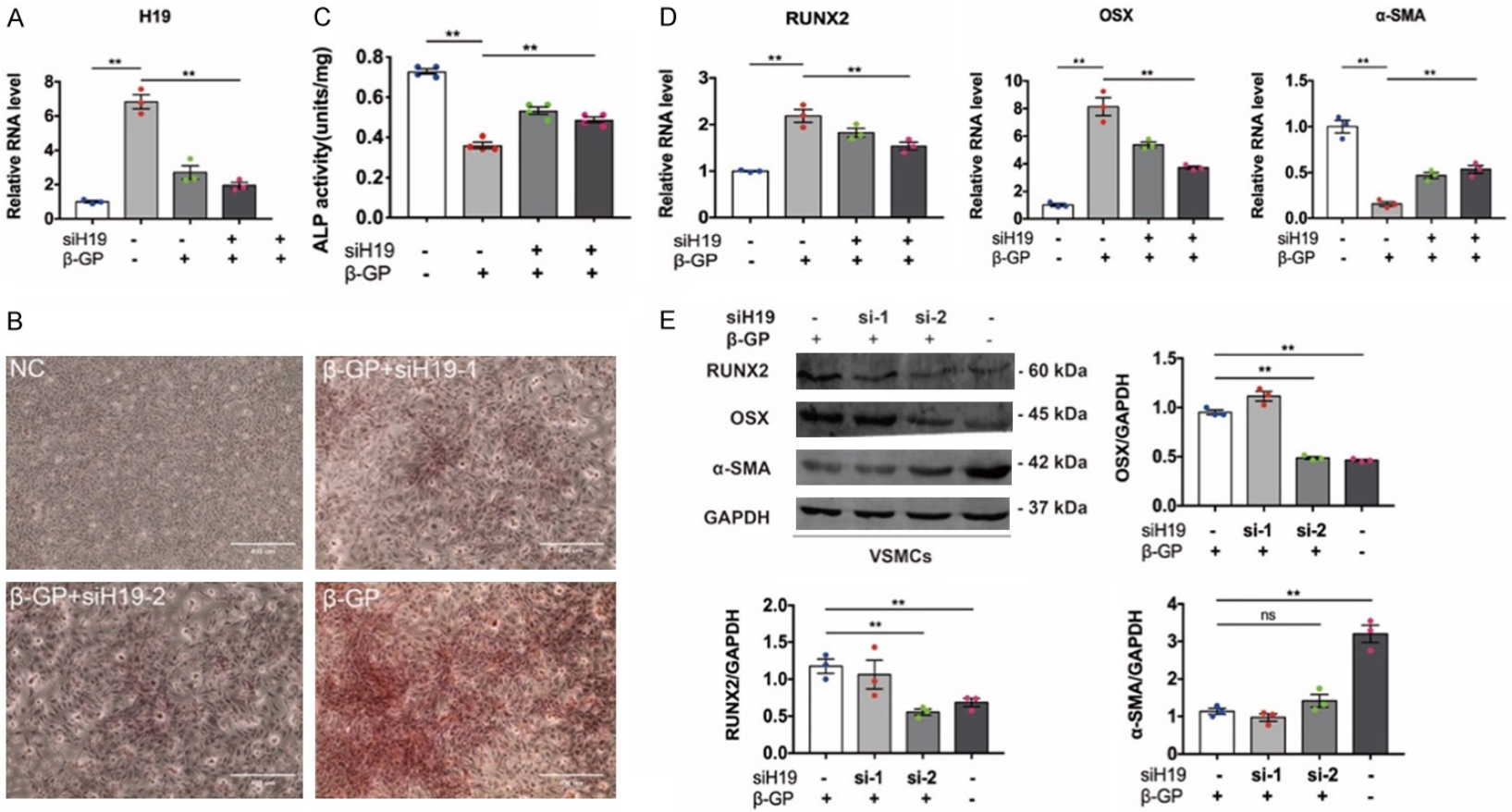

Figure 4. Knockdown of lncRNA H19 decreased VSMCs calcification and Runx2 expression. A. lncRNA H19 knocked down in VSMCs by H19 siRNA and used β-glycerophosphate to induce calcification. B, C. Alizarin Red S staining assay and ALP calcium content in VSMCs compared with that transfected with scramble siRNA. D and E. qPCR and Western Blot analysis of the expression of osteogenic marker gene Runx2, OSX and differentiation marker α-SMA in response to β-glycerophosphate stimulation after knockdown of lncRNA H19. **P<0.01.

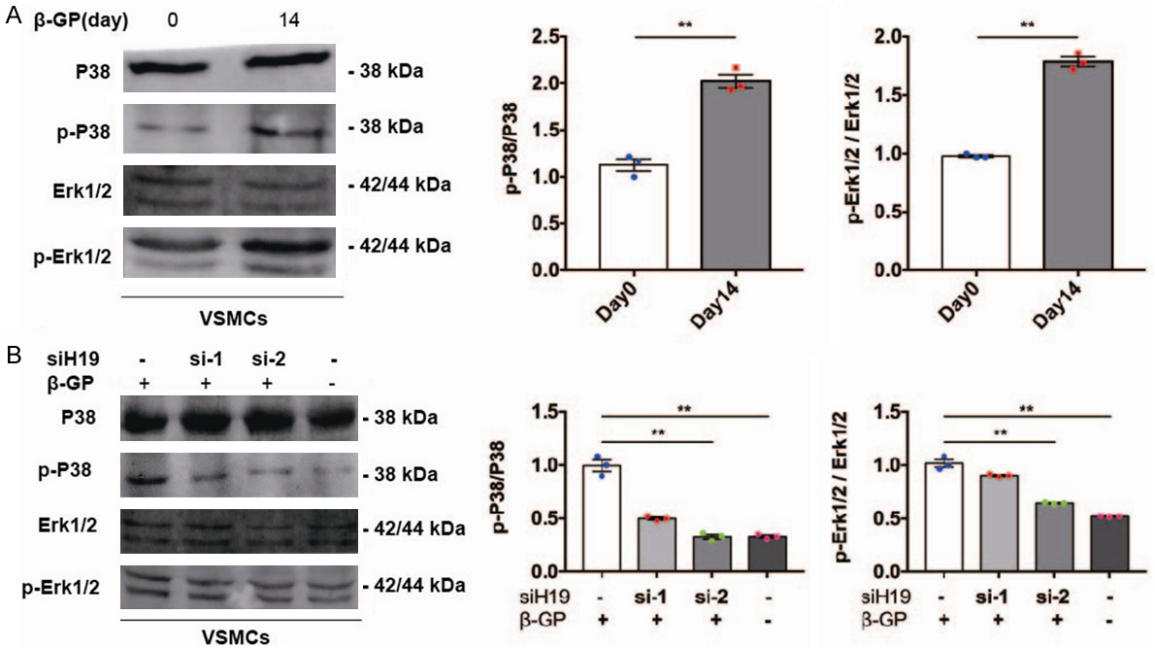

Figure 5. LncRNA H19 promoted VSMCs calcification via the p38 MAPK and ERK1/2 signal transduction pathways. A. Western Blot analysis of the expression of p-p38 and p-p44/42 after cultured with β-glycerophosphate for 14 days. B. Western Blot analysis of the expression of p-p38 and p-p44/42 with H19 siRNA were transfected into rat VSMCs after stimulation with 10 nM β-glycerophosphate for 14 days. **P<0.01.

To further clarify whether lncRNA H19 attenuated VSMCs calcification via the p38 MAPK and ERK1/2 signal transduction pathways, H19 siRNA were transfected into VSMCs. We evaluated the expression of p-p38 and p-p44/42 with Western Blot after VSMCs has been stimulated with 10 nM β-glycerophosphate for 14 days. As shown in **Figure 5B**, the results showed that the expression of p-p38 and p-p44/42 protein levels were decreased in VSMCs after knockdown of lncRNA H19. Thus, the data suggested that lncRNA H19 promoted protein translation of p-p38 and p-p44/42 in β-glycerophosphate-induced VSMCs calcification.

Discussion

The present study has, for the first time, demonstrated that lncRNA H19 can promote VSMCs calcification. Although we have made significant progress on the treatment of AC, there is still no effective method to prevent it. Recently, increasing studies have indicated that lncRNAs are associated with lots of human disease and cellular development [38, 39], and also have pivotal roles in the cardiovascular system [40]. In particular, lncRNA H19 is associated with heart dysfunction, for example in coronary artery disease [24], and aortic valve calcification [25]. In the present study, we found that lncRNA H19 was highly expressed in VSMCs calcification. Knockdown of lncRNA H19 could decrease VSMCs calcification, indicating that H19 may play a critical role in mediating Runx2 expression in VSMCs, suggesting that lncRNA H19 might be used as a novel biomarker on the diagnosis of AC.

Recent studies have shown that lncRNA H19 can accelerate VSMCs proliferation and may be crucial in the regulation of atherosclerosis. Lv J et al. reported the assumption that lncRNA H19 accelerates VSMCs proliferation and that lncRNA H19/miR-675/PTEN is the signaling pathway in restenosis [41]. Pan J-X found that lncRNA H19 expressed highly in serum of atherosclerosis patients and might also promote proliferation at the same time reduce apoptosis of VSMCs, suggesting that lncRNA H19 could regulate atherosclerosis via MAPK pathway [40]. However, whether calcified VSMCs are regulated by lncRNA H19 has not been investigated. Our finding expressed that lncRNA H19 is upregulated in the VSMCs calcification for the first time. We also confirmed that β-gly-

cerophosphate can induced VSMCs calcification after cultured for 14 days in vitro which is consistent with Hao J et al. [34]. Thus, it is evident that lncRNA H19 might play an important role in the process of VSMCs calcification induced by β-glycerophosphate.

The phenotypic transition of VSMCs from contractile/differentiated to synthetic/proliferative phenotypes appears to be an early event in AC. More and more studies accepted that the mechanism of VSMCs calcification is some kind of like bone formation. Previous studies have concluded that Runx2 is an important part in osteogenic trans-differentiation and play a critical role in VSMCs calcification [21, 42]. Furthermore, lncRNA H19 and Runx2 expression were upregulated in β-glycerophosphate-induced VSMCs. However, after treatment with siRNA of lncRNA h19, the overall effects on calcified VSMCs were abrogated. Therefore, it is conceivable that lncRNA H19 is crucial in VSMCs calcification by regulating the expression of Runx2.

These results showed that lncRNA H19 promoted the osteogenic trans-differentiation and the calcification of VSMCs. However, the exact pathway by was uncertain. As reported, Yang W et al. showed long non-coding RNA H19 promotes the migration and invasion of colon cancer cells via MAPK signaling pathway [32]. Ding K et al. found that downregulation of H19 may induce OS and reverse chemotherapy resistance of CD133+ cancer stem cells [33]. In this research, we further focus on whether MAPK pathway is also involved in β-glycerophosphate-induced VSMCs calcification. We showed that lncRNA H19 overexpression significantly upregulated the expression of p-p38 and p-ERK1/2, while inhibition of lncRNA H19 led to the opposite effect. Therefore, we suggest that lncRNA H19 might promote β-glycerophosphate-induced VSMCs calcification via the MAPK pathway in a Runx2-dependent manner.

In summary, we further confirmed that β-glycerophosphate contributes to VSMCs calcification. We also found that lncRNA H19 was upregulated in calcified VSMCs and that up-regulation of lncRNA H19 may be associated with the pathogenesis of AC. Moreover, our study provides insights into the role of lncRNA H19 as a participant of Runx2 regulatory pathway in AC and as a novel indicator for early diagnosis and treatment of AC. Further studies are required to illuminate other potential mechanisms through which lncRNA H19 participates in the biological functions of calcified VSMCs in AC.

Acknowledgements

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Disclosure of conflict of interest

None.

Abbreviations

AC, arterial calcification; ALP, alkaline phosphatase; FBS, fetal bovine serum; LncRNA H19, long noncoding RNA H19; MAPKs, Mitogen-activated protein kinase; OPN, osteopontin; RUNX2, runt-related transcription factor 2; SM22α, smooth muscle 22α; SM-α, smooth muscle α-actin; VSMCs, vascular smooth muscle cells.

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