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Hypomethylation of the LncRNA H19 promoter accelerates osteogenic differentiation of vascular smooth muscle cells by activating the Erk1/2 pathways

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Abstract

Objective: Vascular calcification is a common chronic kidney disease complication. This study aimed to investigate the function of long non-coding RNA (LncRNA) H19 in vascular calcification to explore new therapeutic strategies.

Methods: We induced osteogenic differentiation and calcification of vascular smooth muscle cells (VSMCs) using β-glycerophosphate. Then, we detected the LncRNA H19 promoter methylation status and Erk1/2 pathways using methylation-specific polymerase chain reaction and western blotting, respectively.

Results: Compared with the control group, high phosphorus levels induced VSMC calcification, accompanied by increases in LncRNA H19 and the osteogenic marker Runx2 and reduction of the contractile phenotype marker SM22a. LncRNA H19 knockdown inhibited osteogenic differentiation and calcification of VSMCs. However, the suppressed role of VSMC calcification caused by shRNA H19 was partially reversed by simultaneous activation of the Erk1/2 pathways. Mechanically, we found that the methylation rate of CpG islands in the LncRNA H19 promoter region was significantly lower in the high-phosphorus group, and the hypomethylation state elevated LncRNA H19 levels, which in turn regulated phosphorylated Erk1/2 expression.

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Conclusions: LncRNA H19 promoted osteogenic differentiation and calcification of VSMCs by regulating the Erk1/2 pathways. Additionally, hypomethylation of LncRNA H19 promoter CpG islands upregulated LncRNA H19 levels and subsequently activated Erk1/2 phosphorylation.

Keywords
Long non-coding RNA H19, DNA methylation, Erk1/2 pathway, osteogenic differentiation, vascular calcification, chronic kidney disease

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Introduction

Vascular calcification, especially medial calcification, is a distinguishing feature of chronic kidney disease (CKD)[1,2] and has a high morbidity and mortality burden. It is characterized by the trans-differentiation of vascular smooth muscle cells (VSMCs) into osteogenic-like cells with high expression of osteogenic markers such as runt-related transcription factor 2 (Runx2) and loss of smooth muscle markers such as smooth muscle protein 22α (SM22α). This process results in deposition of hydroxyapatite minerals in the arterial wall[3,4] and is regulated by multiple signaling pathways, such as the Erk1/2 pathways. Therefore, the vascular calcification process is similar to that of bone formation. However, the specific mechanism remains poorly understood.

Long non-coding RNAs (lncRNAs), defined as endogenous cellular RNAs with a length exceeding 200 nucleotides,[5] have been found to serve as important regulators for fine-tuning gene expression and cellular pathophysiological processes under different stimuli through competing endogenous RNA networks[6,7] and signaling pathways.[8] Moreover, some LncRNAs are involved in the control of gene imprinting and are imprinted, showing parent-of-origin allelic expression. LncRNA H19, an imprinted lncRNA, can contribute to the progression of various disease processes including atherosclerosis,[9] bronchopulmonary dysplasia,[10] and cancer.[11] Our previous research results showed that LncRNA H19 was upregulated and promoted osteogenic differentiation of VSMCs in CKD-associated vascular calcification.[12] Nevertheless, the mechanism underlying LncRNA H19 dysregulation remains unclear.

Gene imprinting is regulated by DNA methylation. DNA methylation, one of the most stable epigenetic modifications, is a process whereby DNA methyltransferases add methyl groups to cytosines, which often occurs at CpG islands in the promoter region to block transcription and downregulate the target genes.[13] Recently, hypomethylation of the LncRNA H19 promoter, accompanied by high LncRNA H19 expression levels, was discovered in mineralized aortic valves[14] and in cases of atherosclerotic calcification.[15] However, whether the aberrant expression of LncRNA H19 during vascular calcification in CKD is caused by dysregulation of LncRNA H19 promoter methylation remains unclear.

In the current study, we sought to explore the epigenetic mechanism of LncRNA H19 expression and its function in CKD-associated vascular calcification. We found that the notably increased LncRNA H19 expression was caused by

hypomethylation of the LncRNA H19 promoter, which promoted induction of VSMC calcification by high phosphorus levels. Moreover, we demonstrated that LncRNA H19 promotes the osteogenic fate of VSMCs by fine-tuning Erk1/2 pathway expression. Thus, a novel epigenetic mechanism consisting of lncRNAs regulating CKD-associated vascular calcification was identified, which will provide new insight into potential therapies for vascular calcification.

Methods

Materials

We purchased β-glycerophosphate (β-GP, catalog no. 50020-100G) from Sigma Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). We purchased 5-azacytidine (5-Aza) from Medchemexpress (Shanghai, China, HY-10586) and the MEK inhibitor (PD0325901) from CSN pharm (CSN12886, Guangzhou, China). Reagents for calcification assays, such as 0.2% Alizarin red S (pH 8.3) were purchased from Solarbio Science & Technology Co., Ltd. (Beijing, China), and the calcium and alkaline phosphatase (ALP) assay kits were purchased from LEAGENE (Beijing Leagene Biotechnology Co. Ltd., Beijing, China). The reagents used to implement quantitative polymerase chain reaction (PCR), such as TRIzol for RNA extraction, were purchased from Invitrogen (Thermo Fisher Scientific, Inc.). The PrimeScript RT reagent kit was purchased from TaKaRa (Beijing, China), and the mRNA/lncRNA quantitative reverse transcription (RT-q)PCR starter kit was obtained from Ribo (Guangzhou, China). The materials used for methylation-specific PCR, including a PCR Genomic DNA Kit (DP304), DNA bisulfite (EM101), and a methylation-specific PCR kit (EM171207), were obtained from Tiangen Biotech Co. Ltd. (Beijing, China). Antibodies against Runx2 (ET1612-47) and Erk1/2 (ET1601-29) were purchased from Huabio Technology (Hangzhou, China). The antibody against phosphorylated Erk1/2 (p-Erk1/2) was purchased from Cell Signaling Technology (#4370, Beijing, China). The antibody against SM22α was purchased from Abcam (Cat. No. Ab14106; Cambridge, UK). The antibody against GADPH that was used as an internal loading control was purchased from Bioworld (Ap0063, Guangzhou, China).

For the RNA interference experiments, recombinant lentivirus vectors harboring a short-hairpin RNA sequence (5′-GCAAGU GAUAGGAGGCCUUTT-3′, shRNA H19) targeting LncRNA H19 and a scrambled shRNA (5′-UUCUCCGAACGUGUCAC GUTT-3′, shRNA-NC) were obtained from NoVo Bio Company (Guangzhou, China).

Cell culture, groups, and treatments

The experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of The Fourth Affiliated Hospital of Hebei Medical University (No. 2020ky189). Twenty adult male Sprague-Dawley rats (age, 8 weeks; weight, 200–250 g) were purchased from the Hebei Medical University Laboratory Animal Center (Shijiazhuang, China). The rats were allowed free access to standard rat chow and tap water and maintained under controlled conditions (22 ± 1°C and 40%–60% humidity) with a 12-hour dark/light cycle. The rats were anesthetized with 400 'mg/kg chloral hydrate and intraperitoneally euthanized with 300 mg/kg sodium pentobarbital (three times the anesthetic dose). Rats were considered deceased after breathing had stopped, cardiac arrest was

confirmed, the righting reflex disappeared, and the pupils were dilated. The cause of death for all rats was cardiac arrest. Primary VSMCs were extracted from the thoracic aorta using a previously described method.[16] The experiment was usually completed within 30 minutes, and no peritonitis was observed.

We obtained the thoracic aorta under aseptic conditions. Then, the thoracic aorta was cut into 1- to 2-mm^2 pieces after any residual blood was washed off. To eliminate differences between individuals, the removed thoracic aortic tissue was cut into pieces in a petri dish and mixed evenly. Then, the tissue pieces were cultured in dishes with DMEM supplemented with 10% FBS, 100 μg/mL streptomycin, and 100 U/mL penicillin (60462ES76, China Gaad Company, Shanghai, China) at 37°C in a 95% air and 5% CO_2 atmosphere. The cells that migrated from tissue pieces were collected when they reached confluence. Third through fifth generation VSMCs were used for all experiments. For calcification, confluent VSMCs were incubated in DMEM containing 10% FBS and 10 mmol/L β-GP for 2 to 7 days. The medium was replaced every 3 days. For time-course experiments, the first day of culture in calcification medium was defined as day 0.

To investigate the effect of high phosphorus concentrations on VSMC calcification and LncRNA H19, VSMCs were divided into a normal control group and a high phosphorus group (VSMCs were incubated in DMEM containing 10% FBS and 10 mmol/L β-GP). To identify the effect of LncRNA H19 on phenotype switching of VSMCs, VSMCs were divided into four groups: the normal control group, high phosphorus group, high phosphorus with shRNA-NC group, and high phosphorus with shRNA H19 group. To investigate whether increases in LncRNA H19 were caused by DNA demethylation, VSMCs were randomly divided into three groups: the normal group, high phosphorus group, and high phosphorus with 10 μM 5-Aza group (5-Aza, as a DNA demethylating agent, was added to high-phosphorus medium for 2 days). To detect whether the Erk1/2 pathways participated in VSMC calcification, VSMCs were randomly divided into four groups: the normal control group, high phosphorus group, high phosphorus with dimethyl sulfoxide (DMSO) control group (DMSO is a solvent that dissolves PD0325901), and high phosphorus with PD0325901 group (PD0325901, which can inhibit Erk1/2 phosphorylation, was added to the high-phosphorus medium at a final concentration of 10 μM for 2 days). To investigate whether LncRNA H19 promoter hypomethylation activated Erk1/2 phosphorylation, VSMCs were randomly divided into three groups: the high phosphorus group, high phosphorus with shRNA H19 group, and high phosphorus with both shRNA H19 and 5-Aza group. To detect whether LncRNA H19 promoted VSMC calcification through the Erk1/2 pathways, VSMCs were randomly divided into three groups: the high phosphorus group, high phosphorus with shRNA H19 group, and high phosphorus plus both shRNA H19 and Ro 67-7476 group (Ro 67-7476, which can activate Erk1/2 phosphorylation, was added to the high-phosphorus medium at a final concentration of 2 μM for 2 days).

Cell transfection

VSMCs were transiently transfected with shRNA H19 or shRNA-NC in accordance with the manufacturer's instructions to silence LncRNA H19 expression. After 8 hours of transfection, the VSMCs were exposed to high-phosphorus or control medium for 48 hours. Then, the cells were harvested for further experiments.

Calcification staining

The extent of VSMC mineralization was evaluated via Alizarin red S staining. The VSMCs were washed twice with phosphate-buffered saline, fixed with 95% ethanol for 30 minutes at room temperature, and then stained with 0.2% Alizarin red S (batch number C0148S, China Beyotime, Shanghai, China) for 40 minutes. Subsequently, the cells were washed with phosphate-buffered saline to eliminate nonspecific staining. Next, an LH50A inverted phase contrast microscope (Olympus Corporation, Tokyo, Japan) was used to capture images to record the incidence of induced calcification.

Quantification of calcium deposition and ALP activity

For precise biochemical calcium measurements, calcium deposited in the extracellular matrix was treated with 0.6 M HCl for 24 hours at 37°C, and the calcium content in the supernatant was determined using a Calcium Colorimetric Assay Kit in accordance with the manufacturer's instructions. In addition, ALP activity was measured using an ALP assay kit, and the results were normalized to the total protein concentration as described in the manufacturer's protocol.

RT-qPCR analysis

RT-qPCR was used to analyze the mRNA expression of LncRNA H19, Runx2, SM22α, and GADPH in VSMCs. Total RNA was extracted from VSMCs using TRIzol and quantified at an ultraviolet absorbance of 260 to 280 nm. cDNA synthesis was performed via reverse transcription reactions using the PrimeScript RT reagent kit or the mRNA/lncRNA RT-qPCR Starter kit. The designed primers are shown in Table 1. Amplification reactions were carried out using a Real-Time PCR System (LightCycler 96, Roche, Shanghai, China), and SYBR green was used as the fluorescence dye. Relative expression analysis was performed using the $2^{-\triangle\triangle CT}$ method.

Methylation-specific PCR

Methylation-specific PCR was used to determine whether the increase in LncRNA H19 in VSMCs exposed to high phosphorus levels was related to changes in its promoter methylation status. First, we used MethPrimer2.0 software (http://www.urogene.org/methprimer/) to predict CpG islands with a span of at least 200 base pairs, GC content > 50%, and CpG frequency >0.6[17] and then designed methylation primers. In this experiment, two primers, one for methylated DNA and the other for unmethylated DNA, were used to amplify the DNA sequences and detect the extent of LncRNA H19 promoter methylation. The primer sequences are shown in Table 2.

DNA was extracted from VSMCs in accordance with the manufacturer's

Table 1. Primers for rat H19, Runx2, SM22α, and GAPDH.

Gene	Forward primer	Reverse primer
LncRNA H19	TACAACCACTGGACTACCTG	TGACTCCTGTGTTCCTGTTA
Runx2	CCGCACGACAACCGCACCAT	CGCTCCGGCCCACAAATCTC
SM22α	TGGTGGAGTGGATCGTAATGC	AGAGGTCAACGGTCTGGAACA
GAPDH	CAAGGTCATCCATGACAACTTTG	GTCCACCACCCTGTTGCTGTAG

LncRNA H19, long non-coding RNA H19; Runx2, runt-related transcription factor 2; SM22α, smooth muscle protein 22α; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Table 2. Methylation-specific polymerase chain reaction primers of rat H19.

Gene		Forward primer	Reverse primer
Region 1	M	GAACGGGGAGTTAGATATTTATTTC	ACTACTTCTTCTCAACGACCTCG
	U	ATGGGGAGTTAGATATTTATTTTGG	ACTACTTCTTCTCAACAACCTCACT
Region 2	M	TTGGTGTATTGTATGTTTTAATCGT	CTAACCTAATCTCCAATCCGAA
	U	TTTGGTGTATTGTATGTTTTAATTGT	AAACCTAACCTAATCTCCAATCCA

M: methylated; U: unmethylated.

instructions for the Genomic DNA Kit. DNA was treated with bisulfite to convert unmethylated cytosine to uracil, and then the DNA was detected using a methylation-specific PCR kit according to the manufacturer's instructions.

Western blotting analysis

Western blotting experiments were performed as previously described.[18] The primary antibodies used were as follows: rabbit anti-Runx2 antibody (1:1000), rabbit anti-Erk1/2-antibody (1:2000), rabbit anti-p-Erk1/2 antibody (1:1000), and rabbit anti-SM22α antibody (1:2000). GADPH expression was used as an internal loading control (1:5000). The optical density of immunoreactive bands was analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

All experiments were conducted at least three times. SPSS 23.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism 8 (San Diego, CA, USA) software were used for the statistical analyses. Data are expressed as the mean ± standard deviation. Unpaired, two-tailed Student's *t*-tests or one-way analysis of variance were performed to evaluate the statistical significance between two groups or among multiple sample groups. The association of LncRNA H19 and Runx2 at the RNA expression level was evaluated using a two-tailed Pearson's correlation analysis. A value of $p < 0.05$ indicated a significant difference.

Results

LncRNA H19 was upregulated and associated with the osteoblastic differentiation of VSMCs in a high-phosphorus environment

Consistent with our previous study,[12] we found that high phosphorus levels induced VSMC calcification. The calcified nodes of VSMCs cultured in high β-GP medium for 2, 4, and 7 days were significantly increased in a time-dependent manner, as shown in Figure 1a. In addition, the calcium content and ALP activity of VSMCs were also upregulated more than 3-fold after culturing in 10 mM β-GP medium for 7 days ($p < 0.05$) (Figure 1b and 1c).

To observe whether LncRNA H19 was associated with VSMC calcification induced by high phosphorus levels, we detected the RNA expression of LncRNA H19. The results showed that compared with the control group, LncRNA H19 expression was highly increased in VSMCs subjected to high phosphorus levels (Figure 1d) in a time-dependent manner. These results implied that LncRNA H19 participated in the calcification of VSMCs, which might accelerate the formation of vascular calcification.

Expression of VSMC marker and osteogenic differentiation genes was examined by RT-qPCR and western blotting to clarify

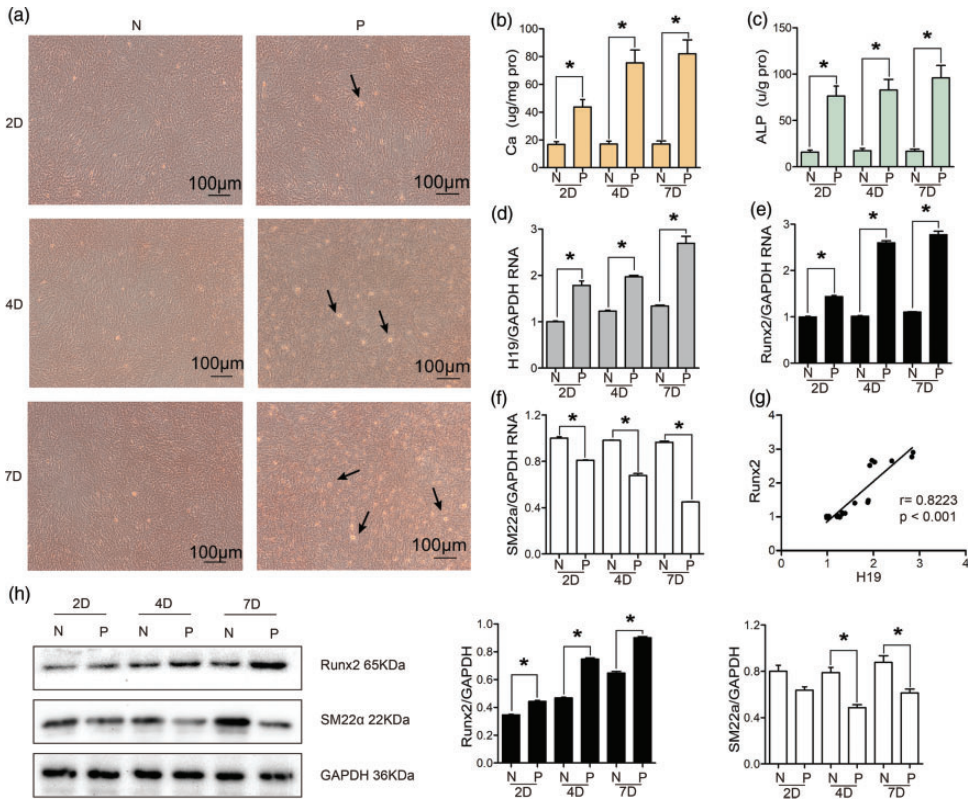

Figure 1. LncRNA H19 was up-regulated in a time-dependent manner and associated with Runx2 expression in high phosphorus-induced vascular smooth muscle cells (VSMCs). (a) Alizarin Red S staining of VSMCs treated in the normal group (N) and high phosphorus group (10 mmol/L β-glycerophosphate (β-GP), P) for 2 days (2D), 4 days (4D), and 7 days (7D). (b) Calcium content of VSMCs cultured with 10 mM β-GP for different durations compared with that in the normal group. *$p < 0.05$. (c) Alkaline phosphatase activity of VSMCs cultured with 10 mM β-GP for different durations compared with that in the normal group. *$p < 0.05$. (d–f) Expression levels of LncRNA H19, Runx2, and SM22α in high phosphorus-induced VSMCs at 2 days, 4 days, and 7 days compared with that in the normal group via quantitative reverse transcription polymerase chain reaction. *$p < 0.05$. (g) Correlation between the mRNA levels of LncRNA H19 and Runx2 expression in high phosphorus-induced VSMCs and (h) Western blot images and quantification data of Runx2 and SM22α protein levels in β-GP-induced VSMCs at indicated time points compared with that in the normal group. *$p < 0.05$. The arrows indicate calcified nodules.

the phenotypic transition of VSMCs after culturing with high phosphorus levels. After 7 days of incubation with high-phosphorus medium, Runx2 mRNA expression was significantly increased ($p < 0.05$) (Figure 1e). However, SM22α expression, a VSMC marker gene, was significantly decreased ($p < 0.05$) by more than 50% after 7 days of exposure to high-phosphorus medium (Figure 1f). Furthermore, western blotting analysis showed that the protein level of Runx2 was upregulated and the expression of SM22α was downregulated (Figure 1h). Moreover, we analyzed the correlation between LncRNA H19 and Runx2 at the RNA expression level to determine whether LncRNA H19 was associated with trans-

differentiation of VSMCs. The results showed a significant and positive correlation ($p < 0.001$) (Figure 1g). Collectively, these findings indicated that LncRNA H19 was upregulated and involved in osteoblastic differentiation and calcification of VSMCs under a high-phosphorus environment.

LncRNA H19 knockdown inhibited VSMC calcification and osteoblastic differentiation induced by high phosphorus levels

To confirm the role of LncRNA H19 in the VSMC calcification process, we designed shRNA oligonucleotides targeting LncRNA H19 to knockdown its expression and used a scrambled shRNA as the control. Figure 2a shows the transfection efficiency of shRNA H19 in VSMCs. Compared with the control, LncRNA H19 knockdown greatly reduced high phosphorus-induced VSMC calcification, as indicated by the Alizarin Red S staining assay, calcium content, and ALP activity (Figure 2b–2d).

We detected the RNA and protein levels of different markers to clarify the effect of LncRNA H19 on the phenotypic transition of VSMCs. As indicated by RT-qPCR and western blot assays, Runx2 expression was significantly decreased ($p < 0.05$) after LncRNA H19 was silenced in high-phosphorus medium (Figure 2e and 2g), whereas expression of the VSMC genetic marker SM22α was increased (Figure 2f and 2g). Specifically, treatment with shRNA H19 attenuated the trans-differentiation of osteogenesis in high phosphorus-induced

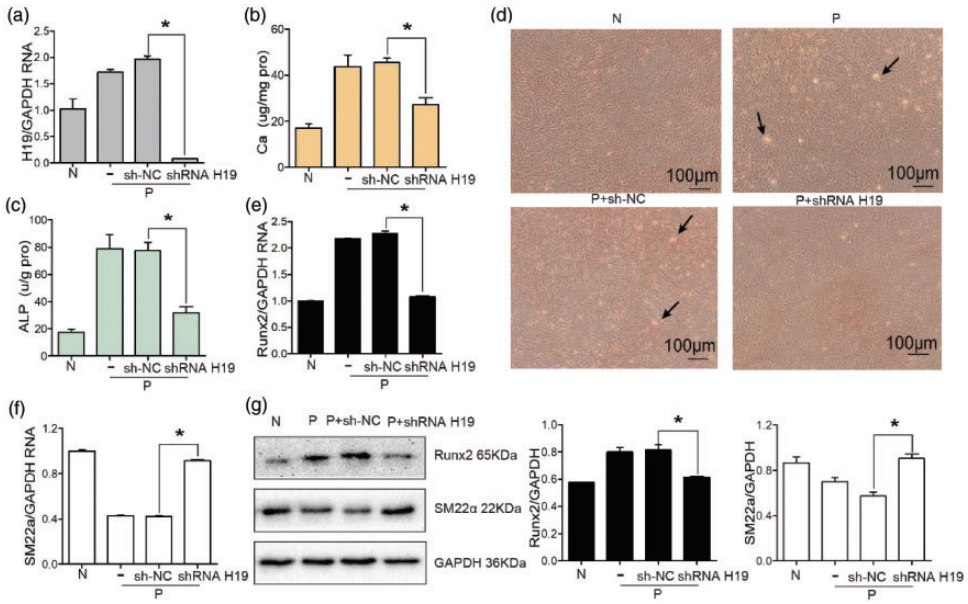

Figure 2. LncRNA H19 accelerated β-glycerophosphate (β-GP)-induced vascular smooth muscle cell (VSMC) osteoblastic differentiation and calcification. (a) Transfection efficiency of shRNA H19 in VSMCs. (b) Calcium content, (c) alkaline phosphatase activity, and (d) Alizarin Red S staining in VSMCs infected with shRNA H19 compared with that in control VSMCs. (e–f) Quantitative reverse transcription polymerase chain reaction analysis of Runx2 and SM22α in shRNA H19-infected VSMCs compared with that in control VSMCs. *$p < 0.05$ and (g) Western blotting images and quantification data of Runx2 and SM22α protein levels in different groups. *$p < 0.05$. The arrows indicate calcified nodules.

VSMCs. Overall, these results demonstrated that LncRNA H19 plays a crucial role in the calcification and osteoblastic differentiation of VSMCs.

LncRNA H19 upregulation is related to hypomethylation of its promoter region in a high-phosphorus environment

As mentioned above, alteration in promoter DNA methylation is an important regulator of LncRNA H19 transcription in osteogenesis-related diseases.[19] Thus, we investigated whether LncRNA H19 upregulation was regulated by aberrant DNA methylation in its promoter region during high β-GP-induced VSMC calcification. Then, we cultured VSMCs with 5-Aza, a DNA demethylating agent that can inhibit the methylation state of a gene. As expected, LncRNA H19 expression was further increased after treatment with 5-Aza in high-phosphorus medium (Figure 3a). To further explore the DNA methylation status of the LncRNA H19 promoter region during VSMC calcification, a methylation-specific PCR test was carried out. We first found two CpG islands, named region 1 and region 2, in the LncRNA H19 promoter region using MethPrimer2.0 software (Figure 3b). Then, the methylation-specific PCR results showed that the methylation rate of CpG sites in LncRNA H19 promoter region 1 was significantly lower in the high-phosphorus group ($p < 0.05$), whereas the methylation rate of LncRNA H19 in region 2 did not significantly change. However, the methylation rate of the two regions was further reduced in cells treated with both β-GP and 5-Aza (Figure 3c). Thus, we considered that the upregulation of LncRNA H19 in VSMCs induced by high phosphorus levels was associated with hypomethylation of its promoter region.

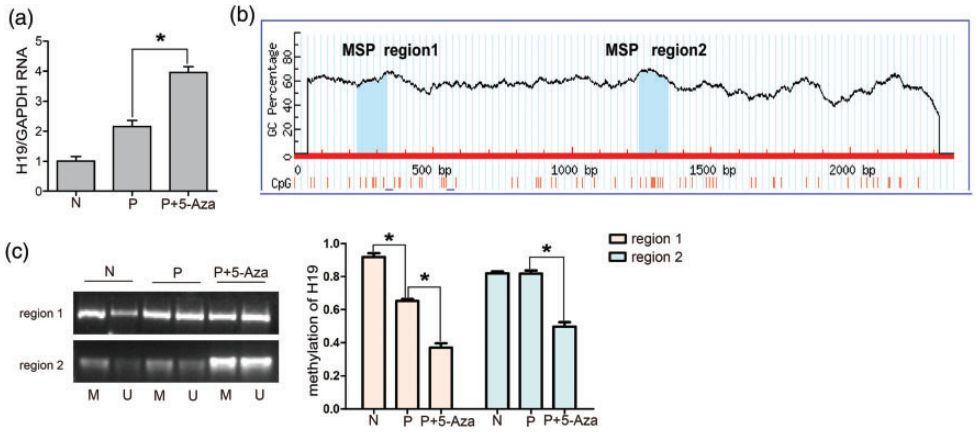

Figure 3. Upregulation of LncRNA H19 in vascular smooth muscle cells (VSMCs) induced by high phosphorus levels was related to the hypomethylation of its promoter region. After cell cycle synchronization, VSMCs were randomly divided into three groups: the normal group (N), high phosphorus group (P), and high phosphorus with 10 μM 5-azacytidine (5-Aza) for 48 hours group (P+5-Aza). (a) Quantitative reverse transcription polymerase chain reaction showed the different expression levels of LncRNA H19 in VSMCs cultured with different media. *$p < 0.05$. (b) Schematic illustration of the CpG islands in the LncRNA H19 promoter region (c) methylation status of the LncRNA H19 promoter region in VSMCs determined by methylation-specific polymerase chain reaction under different stimulation conditions. *$p < 0.05$. M: methylated; U: unmethylated.

LncRNA H19 activated the Erk1/2 pathways in VSMCs exposed to high phosphorus levels

We performed an intensive literature search and discovered that LncRNA H19 could regulate the development of various diseases through the Erk1/2 signaling pathways.[20,21] However, determination of whether the Erk1/2 pathways are activated by LncRNA H19 in VSMCs under high-phosphorus medium required further confirmation. First, we found that after 7 days of culture with 10 mM β-GP, p-Erk1/2 expression was obviously increased (Figure 4a) in a time-dependent manner, suggesting that the Erk1/2 pathways were activated in calcified VSMCs and these pathways might be associated with LncRNA H19. Second, we transfected shRNA H19 into VSMCs. As illustrated in Figure 4b, the elevated protein expression level of p-Erk1/2 induced by high phosphorus levels was partially inhibited after depressing LncRNA H19 in VSMCs, suggesting that LncRNA H19 promotes Erk1/2 pathway activation in a high-phosphorus environment. Finally, we knocked down LncRNA H19 and performed simultaneous 5-Aza treatment and found that DNA demethylation could partially reverse the decreased p-Erk1/2 levels caused by shRNA H19 (Figure 4c). Overall, these results suggested that hypomethylation of LncRNA H19 promoter CpG islands upregulated LncRNA H19 levels and in turn enhanced Erk1/2 phosphorylation.

LncRNA H19 regulates VSMC calcification via the Erk1/2 pathway

Furthermore, we treated the VSMCs with an Erk1/2 pathway inhibitor (PD0325901) to observe whether the Erk1/2 pathways regulate calcification and phenotypic

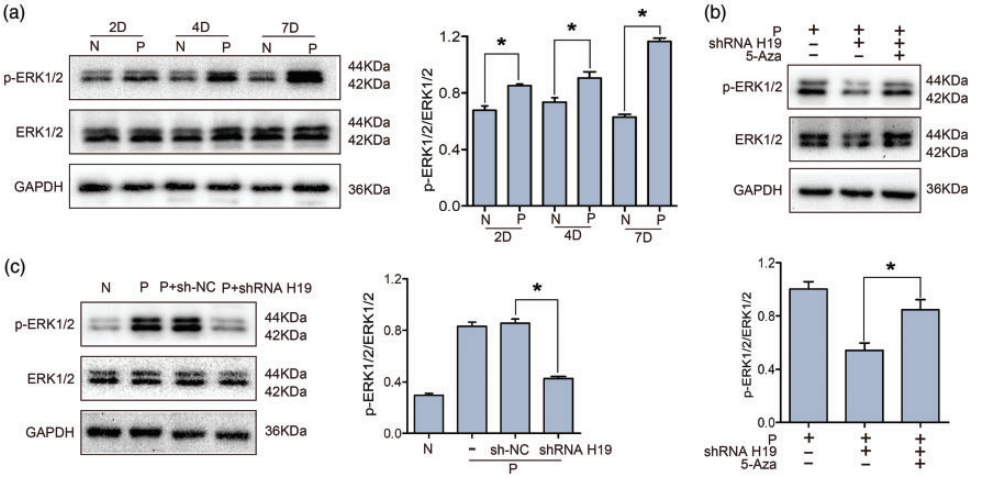

Figure 4. LncRNA H19 activated the Erk1/2 pathway in β-glycerophosphate (β-GP)-induced vascular smooth muscle cells (VSMCs). (a) Western blot images and quantification data of p-Erk1/2 and Erk1/2 protein levels in β-GP-induced VSMCs on days 2, 4, and 7 compared with those in the normal group. (b) Western blotting images and quantification data of p-Erk1/2 and Erk1/2 protein levels in shRNA H19-infected VSMCs induced by β-GP and (c) Western blotting images and quantification data of p-Erk1/2 and Erk1/2 protein levels in the high phosphorus group (P), high phosphorus with shRNA H19 group (P+shRNA H19), and high phosphorus with both shRNA H19 and 5-azacytidine (5-Aza) group (P+shRNA H19 + 5-Aza). *$p < 0.05$.

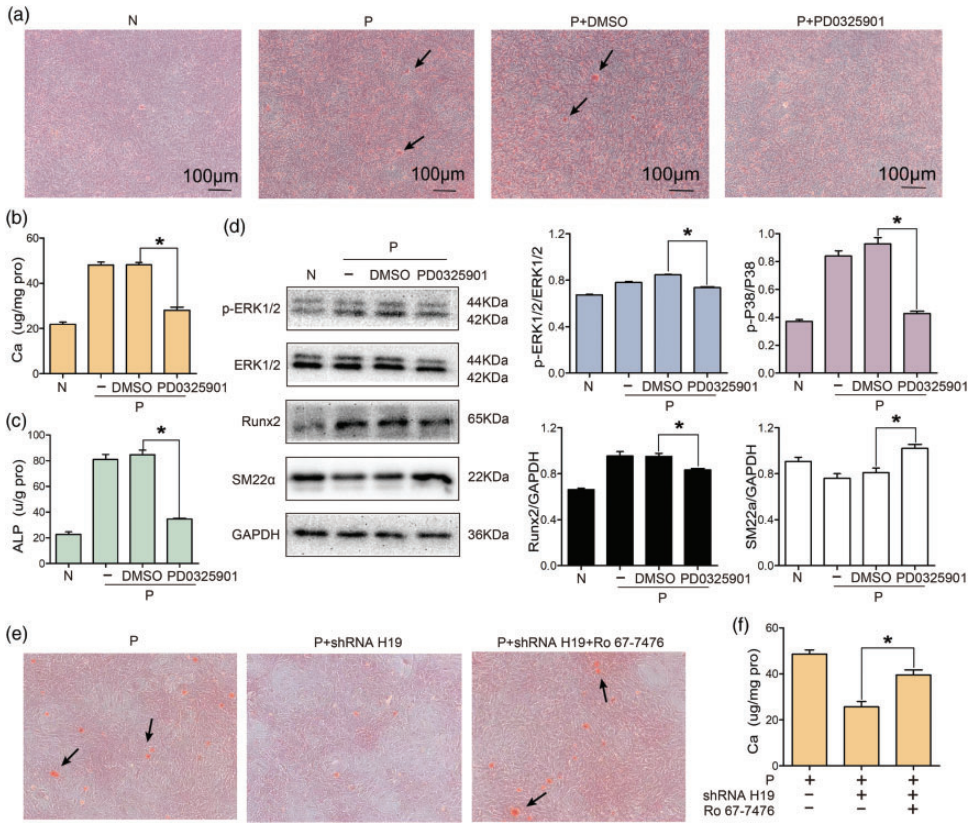

Figure 5. LncRNA H19 regulated vascular smooth muscle cell (VSMC) calcification via the Erk1/2 pathways in a high-phosphorus environment. After cell cycle synchronization, VSMCs were first randomly divided into four groups: the normal group (N), β-glycerophosphate group (P), dimethyl sulfoxide control group (P+DMSO), and PD0325901 for 48 hours group (P+PD0325901). (a) Alizarin Red S staining, (b) calcium content, and (c) alkaline phosphatase (ALP) activity in VSMCs treated with different stimuli. (d) Western blotting images and quantification data of p-Erk1/2 and Erk1/2 protein levels in VSMCs treated with different stimuli. For the rescue experiment, VSMCs were randomly divided into three groups: the high phosphorus group (P), the high phosphorus with shRNA H19 group (P+shRNA H19), and the high phosphorus plus both shRNA H19 and Ro 67-7476 group (P+shRNAH19+Ro 67-7476). (e) Alizarin Red S staining and (f) calcium content in VSMCs in the above groups. *$p < 0.05$. The arrows indicate calcified nodules.

transition in VSMCs. The results in Figure 5a–c indicated that inhibition of Erk1/2 phosphorylation greatly reduced the high phosphorus-induced VSMC calcification presented by the Alizarin Red S staining assay, calcium content, and ALP activity. Meanwhile, the protein expression of the osteogenic marker gene Runx2 was significantly decreased and expression of the VSMC marker gene SM22α was increased (Figure 5d) after inhibiting the Erk1/2 pathways in high phosphorus-stimulated VSMCs. Thus, these outcomes showed that the Erk1/2 pathways facilitate calcification and osteoblastic differentiation of VSMCs.

Lastly, a rescue experiment was performed to determine whether LncRNA H19 promoted VSMC calcification through the Erk1/2 pathways. We knocked down

LncRNA H19 with simultaneous activation of the Erk1/2 pathways by Ro 67-7476 and found that activating the Erk1/2 pathways could partially reverse the attenuated VSMC calcification caused by shRNA H19 (Figure 5e and 5f). Thus, the above data demonstrated that LncRNA H19 regulates VSMC calcification via the Erk1/2 pathways in a high-phosphorus environment.

Discussion

When calcium phosphate crystals are abnormally deposited on vessel walls, vascular calcification occurs, resulting in arterial hardening and stenosis. This condition is commonly observed in patients with CKD. Recent studies have shown that vascular calcification is a process of active cell regulation rather than pure passive calcium deposition.[22] The potential mechanisms of VSMC calcification are involved in apoptosis, matrix vesicle release, conversion of the VSMC phenotype into osteoblast-like cells characterized by the loss of contractile markers, such as SM22α and α-smooth muscle actin, and high expression of osteogenic profile markers, including Runx2, osteocalcin, osterix, and BMP2.[23,24] In accordance with these studies, we used 10 mmol/L β-GP to induce VSMC calcification and found that SM22α expression was decreased, whereas expression of Runx2 and ALP activity were increased. These results indicated that β-GP induced VSMC calcification and conversion of VSMCs into an osteoblast-like cell phenotype.

Currently, numerous studies have demonstrated that lncRNAs play integral roles in regulating vascular calcification. LncRNA H19, a regulatory lncRNA, has been shown to play an important role in regulation of osteoblast proliferation and differentiation, indicating its potential association with bone-associated diseases.[25,26] In the present study, we found that LncRNA H19 was significantly upregulated. Additionally, we examined different time points and found that LncRNA H19 expression was increased in a time-dependent manner in a high-phosphorus environment. Moreover, our results showed that LncRNA H19 knockdown could inhibit Runx2 expression levels and attenuate VSMC calcification, suggesting that LncRNA H19 plays a vital role in vascular calcification and osteogenic differentiation. However, the mechanisms underlying LncRNA H19 upregulation during vascular calcification remain unknown.

Epigenetic modifications, including DNA methylation, histone modifications, and acetylation, have been recently found to be important and crucial for regulation of gene expression in various chronic diseases.[27,28] Aberrant methylation of the promoter region can also result in abnormal LncRNA H19 expression, which is linked to several human diseases.[14] Dai et al. found that hypomethylation of the H19 promoter contributed to S-adenosylhomocysteine hydrolase deficiency-induced Runx2-dependent atherosclerotic calcification.[15] However, whether epigenetic upregulation of H19 also participates in the regulation of high phosphorus-induced calcification is not clear. In our study, we performed methylation-specific PCR and found that the methylation rate of the LncRNA H19 promoter region in the normal group was higher than that under 10 mmol/L β-GP treatment. Moreover, 5-Aza markedly decreased the methylation level of the LncRNA H19 promoter and increased the LncRNA H19 expression level. Taken together, our results indicate that hypomethylation of CpG islands in the promoter increases LncRNA H19 expression during high phosphorus-induced VSMC calcification.

In addition, the exact pathway involving LncRNA H19 that regulates osteogenic differentiation and calcification of VSMCs

needs to be further explored. Erk1 and 2 are widely known as the main molecules responsible for initiating the signaling pathway involved in VSMC proliferation and differentiation.[29–31] A previous study found that the mechanism by which LncRNA H19 promotes vascular calcification might be related to the Erk1/2 pathways, but it did not confirm this process.[32] In the present study, we found that after 7 days of culture with β-GP, p-Erk1/2 expression was increased. In contrast, when LncRNA H19 was knocked down, p-Erk1/2 expression was decreased. While DNA demethylation could partially reverse the decreased p-Erk1/2 levels caused by shRNA H19, we showed, for the first time, that hypomethylation of the promoter upregulated LncRNA H19 levels and in turn enhanced Erk1/2 phosphorylation in VSMC calcification. Furthermore, inhibition of the Erk1/2 pathways decreased Runx2 expression, accompanied by reduction of hyperphosphorus-induced calcification. Finally, we performed a rescue experiment to confirm that LncRNA H19 promotes VSMC calcification through the Erk1/2 pathways. In the future, we will further verify these results using in vivo experiments for better clinical transformation.

In conclusion, our results were the first to confirm that LncRNA H19 promotes vascular calcification by regulating the Erk1/2 pathways and indicated that hypomethylation of the promoter upregulated LncRNA H19 levels and in turn enhanced Erk1/2 phosphorylation during VSMC calcification induced by high phosphorus levels. These findings provide new insights into therapeutic strategies for vascular calcification in patients with CKD.

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Author contributions

YL and JS conceived and designed the experiments. TX, MJ, DX, and SL performed the experiments and analyzed the data. TX and JJ wrote the manuscript. All authors read and approved the final manuscript.

Declaration of conflicting interests

The authors declare that there is no conflict of interest.

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