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ARTICLE

Hyaluronan negatively regulates vascular calcification involving BMP2 signaling

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Abstract

Vascular calcification is a highly regulated biological process similar to bone formation involving osteogenic differentiation of vascular smooth muscle cells (VSMCs). Hyaluronan (HA), a major structural component of the extracellular matrix in cartilage, has been shown to inhibit osteoblast differentiation. However, whether HA affects osteogenic differentiation and calcification of VSMCs remains unclear. In the present study, we used in vitro and ex vivo models of vascular calcification to investigate the role of HA in vascular calcification. Both high and low molecular weight HA treatment significantly reduced calcification of rat VSMCs in a dose-dependent manner, as detected by alizarin red staining and calcium content assay. Ex vivo study further confirmed the inhibitory effect of HA on vascular calcification. Similarly, HA treatment decreased ALP activity and expression of bone-related molecules including Runx2, BMP2 and Msx2. By contrast, inhibition of HA synthesis by 4-methylumbelliferone (4MU) promoted calcification of rat VSMCs. In addition, adenovirus-mediated overexpression of HA synthase 2 (HAS2), a major HA synthase in VSMCs, also inhibited calcification of VSMCs, whereas CRISPR/Cas9-mediated HAS2 knockout promoted calcification of rat A10 cells. Furthermore, we found that BMP2 signaling was inhibited in VSMCs after HA treatment. Recombinant BMP2 enhanced high calcium and phosphate-induced VSMC calcification, which can be blocked by HA treatment. Taken together, these findings suggest that HA inhibits vascular calcification involving BMP2 signaling.

Introduction

Vascular calcification is a very common pathologic condition in chronic kidney diseases [1, 2]. Vascular calcification contributes to hemodynamic imbalance and decrease of vessel wall elasticity, thus increasing cardiovascular morbidity and mortality among patients with chronic kidney diseases. In the past, vascular calcification

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was regarded as a passive and degenerative process with precipitation of calcium and phosphate in arteries. Accumulating studies have shown that vascular calcification is a highly regulated biological process involving osteogenic differentiation of vascular smooth muscle cells (VSMCs) similar to bone formation, characterized by the upregulation of bone-related proteins including Runx2 and BMP2, and downregulation of contractile proteins including SMA and SM22α [3, 4]. Elevated calcium and phosphate levels are important factors promoting vascular calcification in chronic kidney disease [5–7]. However, the precise molecular mechanisms underlying vascular calcification are not fully understood.

Extracellular matrix (ECM) plays important roles in a variety of cellular physiological process including cell differentiation, survival and proliferation. A number of studies have demonstrated that ECM molecules including matrix Gla protein, osteoprotegerin and decorin are important regulators of vascular calcification [8–11]. Hyaluronan (HA), a liner polysaccharide consisting of repeated disaccharide units of D-glucuronate and *N*-acetyl-D-gluco-samine, is a main structural component of the ECM. HA synthase 2 (HAS2) is the major HA synthase responsible for HA synthesis in cultured VSMCs [12, 13]. Conditional knockout (KO) of HAS2 study reveals that HAS2-mediated production of HA is required for bone formation and chondrocyte maturation [14]. HA is known to participate in the regulation of many cellular activities including cell proliferation, cell migration and osteoclastogenesis [15–17]. Recent studies have demonstrated that HA promotes the embryonic stem cell differentiation toward a smooth muscle cell lineage, and inhibits osteoblast differentiation [18, 19], but another study suggests that HA prmotes osteoblast differentiation [20]. However, it is unclear whether HA regulates osteogenic differentiation of VSMCs and vascular calcification.

Bone morphogenetic protein-2 (BMP2), a member of the transforming growth factor beta (TGF-β) superfamily, has been identified as one of the most important molecules to regulate bone formation and osteoblast differentiation [21]. It has been reported that BMP2 expression is increased in VSMCs during vascular calcification [22]. Both in vitro and in vivo studies have demonstrated that BMP2 promotes vascular calcification [23, 24]. However, the link between HA and BMP2 during vascular calcification remains elusive. In this study, we used in vitro and ex vivo models of vascular calcification to investigate the role of HA in vascular calcification and the link between HA and BMP2 signaling during vascular calcification.

Materials and methods

Cell culture

All animal experiments were performed in accordance with the US National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee at Southern Medical University. VSMCs were isolated from aortic arteries of Sprague-Dawley (SD) rats (200–220 g) using explant method as described previously [11]. Rat VSMC line A10 was purchased from the American Type Culture Collection (Manassas, USA). All cell culture reagents were purchased from Life Technology Company, USA. VSMCs were grown in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml of penicillin and 100 mg/ml of streptomycin at 37 °C with 5% CO_2. Cells between passages five and eight were used for all the experiments. Calcifying medium (CM; DMEM supplemented with 10 mM beta-glycerophosphate (BGP) and 3 mM $CaCl_2$) was used to induce VSMC calcification [25]. To investigate the role of HA in vascular calcification, HA (R&D System, USA) was used to treat cells in CM for 6 days or 10 days at different concentrations (50, 100 or 200 μg/ml). In all, 0.25 μM of 4-methylumbelliferone (4MU, Sigma), a HA synthesis inhibitor, was used to treat VSMCs in some experiments. VSMCs were treated with recombinant human BMP2 at 100 ng/ml in the presence of CM for 10 days. All in vitro experiments were performed independently three times.

Aortic ring organ culture

Thoracic aortas were isolated from SD rats and cut into segments of 0.5 cm in length. To induce arterial calcification, aortic segments were incubated in CM for 8 or 10 days at 37 °C in a humidified 5% CO_2 incubator. HA (200 μg/ml) was used to treat aortic segments in CM for 8 days or 10 days. The organ culture experiment was performed independently four times.

Cell transfection

Cells were transfected with adenovirus encoding HAS2 (Ad/HAS2) or control virus (Ad/GFP) as described previously [11, 26]. Cells were seeded in 35 mm dishes at 4×10^5 cells/dish and grown in DMEM supplemented with 10% FBS. When cells reached 80% confluence, Ad/HAS2 or Ad/GFP were used to infect cells at an optimal multiplicity of infection (MOI = 10) and the transfection efficiency reaches 70%. Transfected cells were harvested at indicated time points for further analysis.

CRISPR/Cas9-mediated HAS2 KO in A10 cells

Knockdown of HAS2 in A10 cells was performed by CRISPR/Cas9-guided genome editing. Two of 20 nucleotide sgRNA sequences: gRNA1 5′-ATGCAACTAAATACA CGGCT-3′ (23263–23282) and gRNA2 5′-CCTTGATCTA TGCGTGCTAC-3′ (23884–23903) were designed using sgRNA CRISPR design tool online (http://crispr.mit.edu) and cloned into the pSpCas9(BB)-2A-Puro (PX459) plasmid (Addgene, catalog no. 62988). After cloning, plasmids were purified and verified by sequencing. Rat A10 VSMCs were seeded into six-well plates at 70% confluence and transfected with PX459 plasmid encoding a target specific sgRNA using Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions. Puromycin was then added to select the transfected cells after 24h transfection. KO efficiency for HAS2 was assessed by western blot.

Determination of calcification

Alizarin red staining was used to detect cell and arterial calcification at indicated time points. For cell culture, VSMCs grown in 35 mm dishes were fixed in 4% formaldehyde for 10 min and incubated with 2% alizarin red (pH 4.2) for 5 min at room temperature. To remove the excess dye, cells were washed with deionized water. Alizarin red dye was eluted with 10% formic acid and quantified by spectrophotometry. For aortic ring culture, aortic segments were fixed in 4% paraformaldehyde (PFA) and embedded in paraffin. Aortic samples were cut into 6 μm in thickness. Sections were deparaffinized followed by staining with 2% alizarin red. Images were taken using an inverted microscope. Calcium content was measured using o-cresolphthalein complexone method as previously described [27]. VSMCs were washed with phosphate-buffered saline (PBS) and incubated with 0.6 N HCl for 24 h. The aortic segments were dried and weighed, followed by incubation in 0.6 N HCl for decalcification. Protein concentration was quantified using BCA^{TM} protein assay (Pierce, USA). Calcium content was expressed as μg/mg protein or μg/mg dry tissue.

ALP activity assay

Alkaline phosphatase (ALP) activity was assessed as previously described [11, 25]. Briefly, VSMCs were harvested with 0.1% Triton X-100 in PBS at indicated time points. Protein samples were then mixed with para-nitrophenylphosphate (p-NPP) (180 μl) substrate and incubated for 15 min at 37 °C. After the reaction was stopped by NaOH, absorbance was then measured at 405 nm. Protein concentration was measured using BCA^{TM} protein assay (Pierce, USA). ALP activity was expressed as nmol/ml p-nitrophenol converted per microgram of protein per minute.

HA ELISA

HA levels in the media of cell culture were determined using HA Elisa kit (SBJ-R0037, Nanjing SenBeiJia Biological Technology, China) according to the manufacturer's instructions. A HA standard curve generated was linear between 22.5 and 270 ng/l of the HA standard supplied by the manufacturer. HA levels were normalized to control.

Quantitative real-time PCR

Total RNA was extracted from cells using TRIzol regents (Invitrogen, USA) according to the manufacturer's instructions. Reverse transcription kit (Takara Company, Japan) was used to synthesize complementary DNA. Quantitative real-time PCR (qPCR) was performed using SYBR Green mixture (Takara Company, Japan) in a StepOne Plus real-time PCR system (Applied Biosystems, USA). PCR primers were as follows: β-actin (forward): 5′-TGTCACCAACTGGGACGA TA-3′, β-actin (reverse): 5′-GGGGTGTTGAAGGTCTCAA A-3′; Runx2 (forward): 5′-GCCGGGAATGATGAGAA CTA-3′, Runx2 (reverse): 5′-GGACCGTCCACTGTCACT TT-3′; BMP2 (forward): 5′-GTTTGGCCTGAAGCAGAGA C-3′, BMP2 (reverse): 5′-CTCGATGGCTTCTTCGT GAT-3′; Msx2 (forward): 5′-CCTCGGTCAAGTCGGA AAAT-3′, Msx2 (reverse): 5′-ACTGTTTCTGGCGGAAC TTG-3′. Value was normalized to β-actin and relative gene expression was determined using comparative Ct method.

Western blot analysis

Total protein was extracted from cultured VSMCs and protein concentration was determined by a BCA protein assay kit. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Millipore, USA). The membranes were then blocked with 5% nonfat milk for 1 h and incubated with primary antibodies including BMP2 (1:2000, Abcam, USA), Runx2 (1:2000, Abcam, USA), p-Smad1/5/8 (1:1000, Cell Signaling Technology, USA), Smad1 (1:2000, ABclonal Biotechnology, USA), HAS2 (1:1000, Abcam, USA), Akt, p-Akt, extracellular signal-regulated kinase (ERK), phosphorylated extracellular signal-regulated kinase (p-ERK) (1:1000, Cell Signaling Technology, USA), β-actin (1:3000, Cell Signaling Technology, USA) overnight at 4 °C. Then, the membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies (EarthOx Life Sciences, USA) for 1 h. Protein signals were detected by Lumazone Chemiluminescent Substrate (Millipore, USA). Protein expression levels were quantified by densitometry and normalized to β-actin expression.

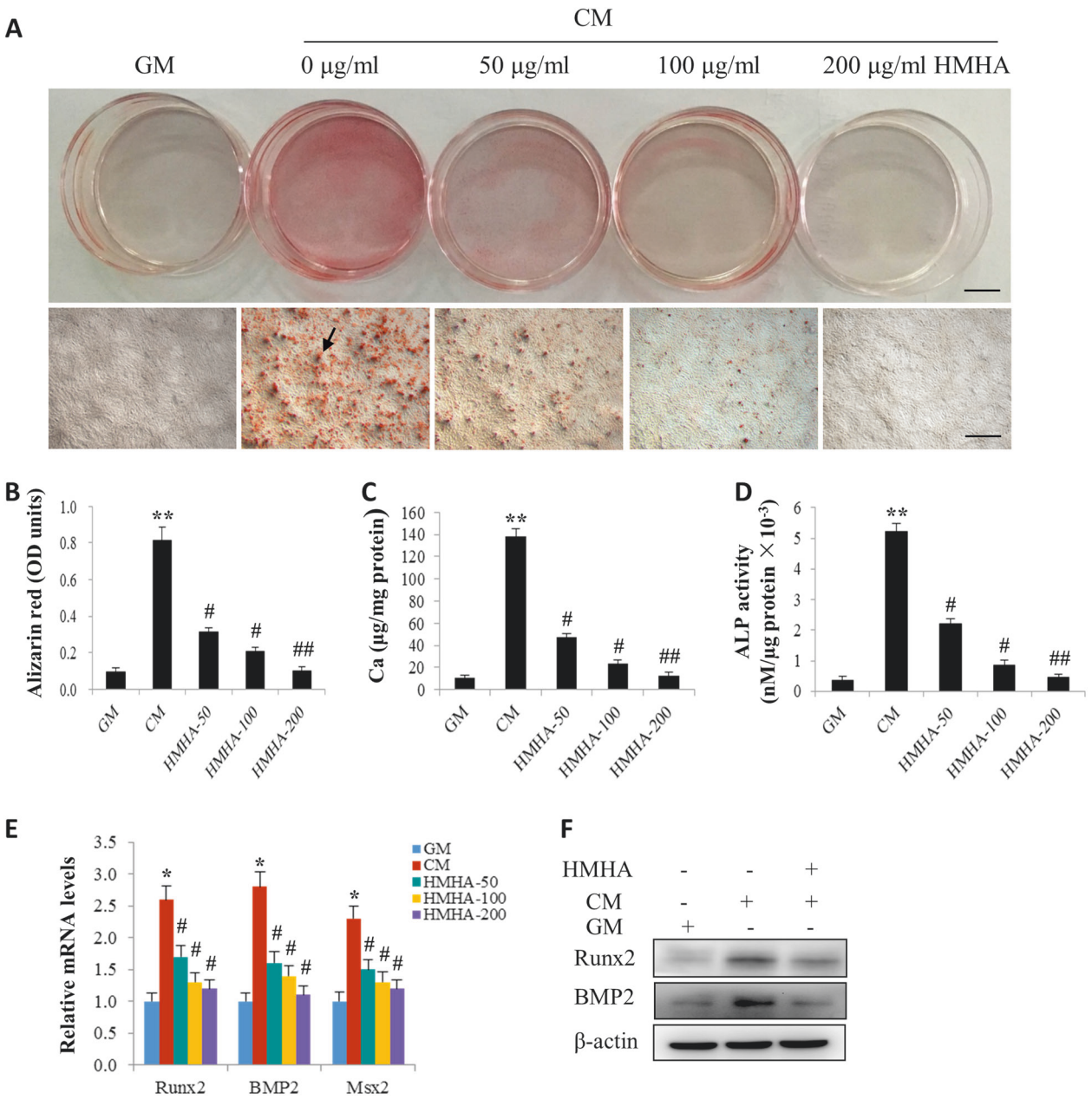

Fig. 1 Effect of High molecular weight-hyaluronan on rat VSMC calcification. Confluent rat VSMCs were cultured in growth medium (GM), calcifying medium (CM) or CM supplemented with high molecular weight-hyaluronan (HMHA) for 10 days ($n = 3$). **a** Mineral deposition in VSMCs was detected by alizarin red staining at day 10. Scale bar = 200 μm. **b** Alizarin red dye was eluted with 10 % formic acid and quantified by a microplate reader. **c** Calcium content was measured at day 10. **d** ALP activity was assessed by spectrophotometry at day 6. **e** Runx2, BMP2, and Msx2 mRNA levels were determined by qPCR at day 6. **f** The protein expression of Runx2 and BMP2 was analyzed by western blot at day 6. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. GM; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. CM

Immunocytochemistry

Rat VSMCs were cultured in chamber slides, fixed in 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 for 15 min at room temperature. After being blocked with 10% goat serum in PBS at room temperature for 1 h, cells were incubated with antibody for p-Smad1/5/8 in 5% bovine serum albumin solution overnight at 4 °C. Cells were washed and then incubated with Alexa Fluor-594-conjugated goat anti-rabbit secondary antibody for 1 h at room temperature. The nuclei were counterstained in blue with 4, 6-diamidino-2-phenylindole (DAPI, Molecular Probes). The images were visualized and photographed with a fluorescent microscope (LEICA, DM4000B).

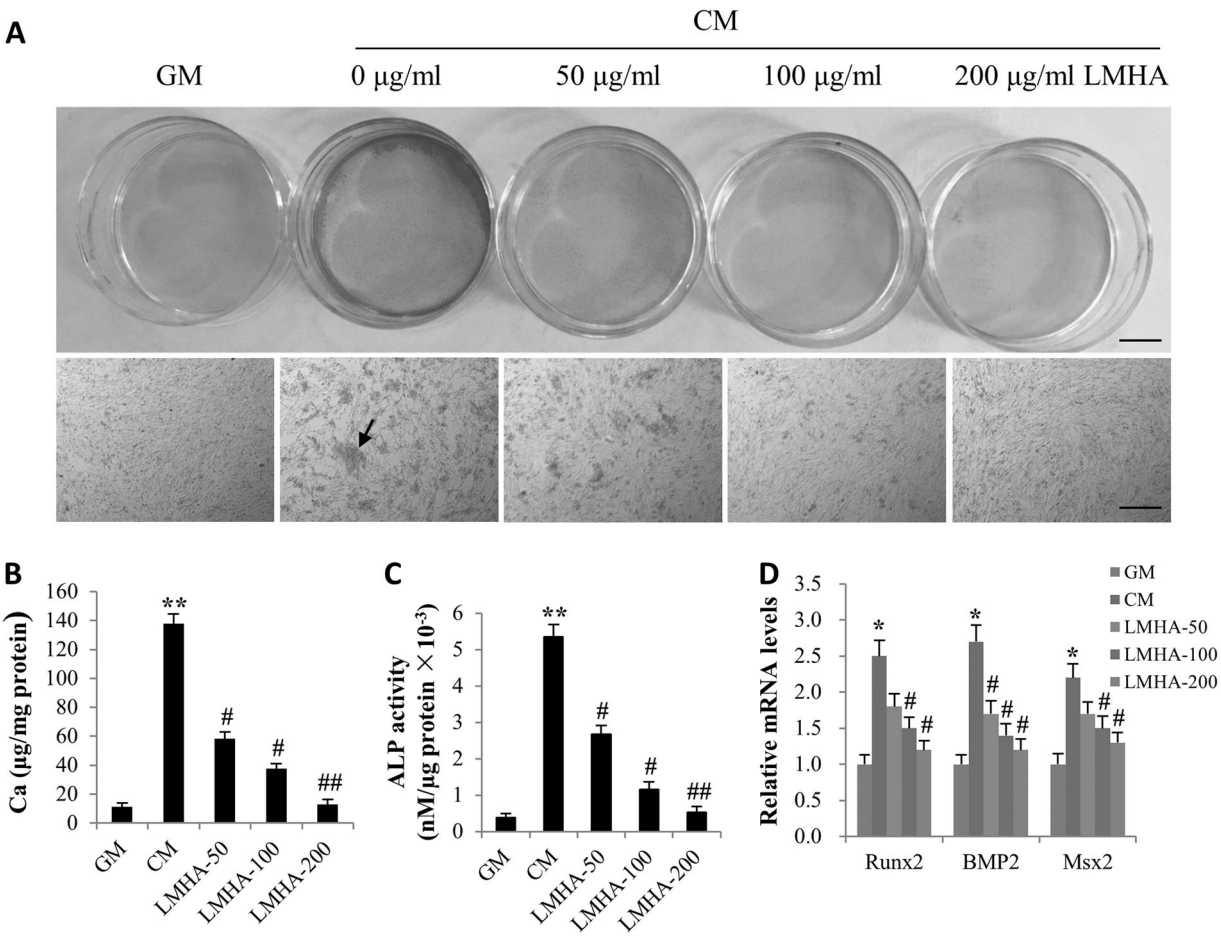

Fig. 2 Effect of low molecular weight-hyaluronan on rat VSMC calcification. Confluent rat VSMCs were cultured in growth medium (GM), calcifying medium (CM) or CM supplemented with low molecular weight-hyaluronan (LMHA) for 10 days ($n = 3$). **a** Mineral deposition in VSMCs was detected by alizarin red staining at day 10. Scale bar = 200 μm. **b** Calcium content was measured at day 10. **c** ALP activity was assessed by spectrophotometry at day 6. **d** Runx2, BMP2, and Msx2 mRNA levels were determined by qPCR at day 6. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. GM; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. CM

Statistical analysis

All data are expressed as mean ± SD from three independent experiments and analyzed using the software package SPSS 17.0. Statistical differences between two groups were analyzed by Student's *t*-test and differences between more than two groups were compared by one-way analysis of variance. A value of $P < 0.05$ was considered statistically significant.

Results

High molecular weight HA inhibits calcification of rat VSMCs

To investigate the effect of HA on calcification of rat VSMCs, CM was used to stimulate VSMC calcification and rat VSMCs were treated with 50, 100 or 200 μg/ml of high molecular weight HA (HMHA). Calcium deposition was observed in CM-treated rat VSMCs, but not in growth medium (GM)-treated cells, as indicated by alizarin red staining. HMHA reduced mineral deposition in a dose-dependent manner in the presence of CM at day 10 (Figs. 1a, b). Calcium content assay further confirmed that calcium content was reduced by 66% ($P < 0.05$), 83% ($P < 0.05$), 91% ($P < 0.01$) in 50, 100, 200 μg/ml of HMHA-treated cells, respectively, compared with CM-treated cells (Fig. 1c). Next, we examined the effect of HMHA on osteogenic differentiation of rat VSMCs. The activity of ALP, an early osteogenic differentiation marker, was significantly decreased in HMHA-treated cells at day 6, compared with cells treated without HMHA in CM (Fig. 1d). Additionally, qPCR analysis showed that HMHA reduced mRNA expression of bone-related molecules including Runx2, BMP2, and Msx2 in VSMCs at day 6 (Fig. 1e).

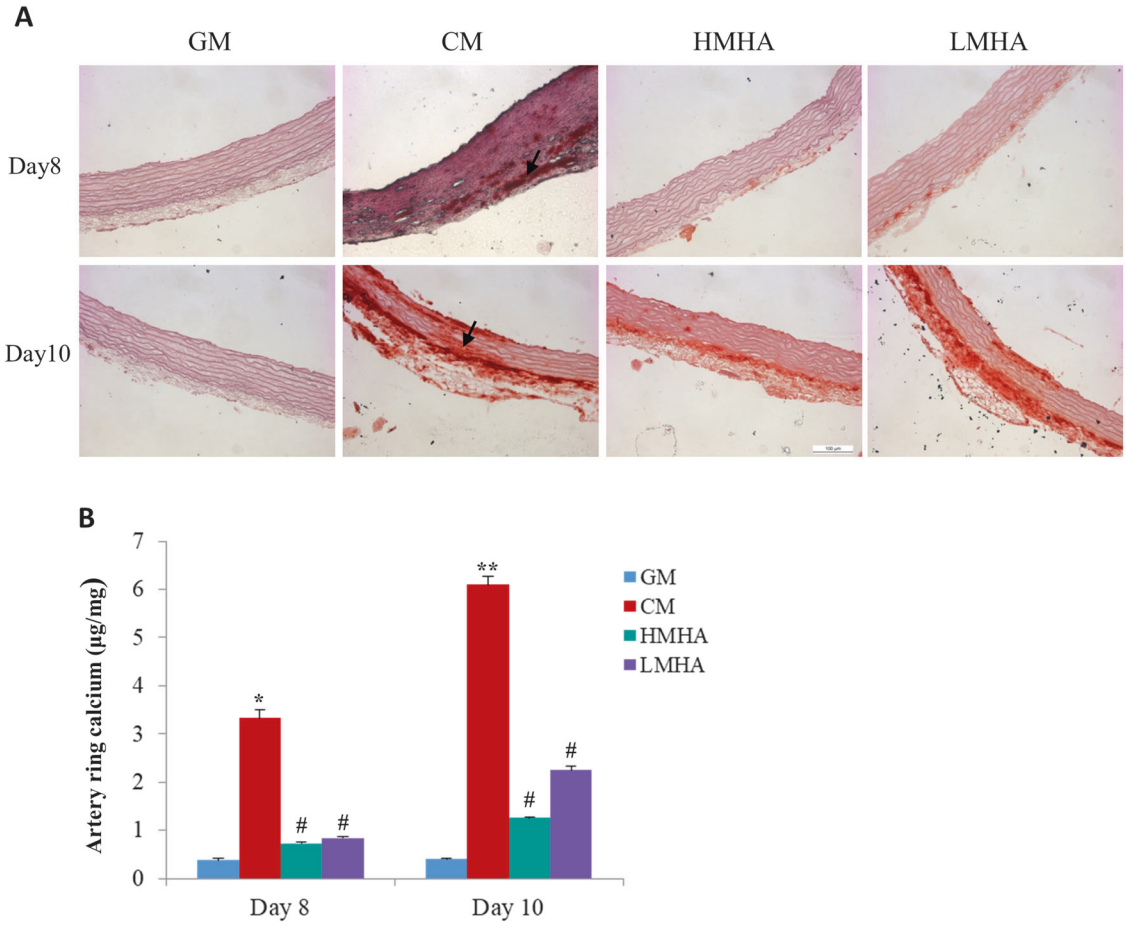

Fig. 3 Effect of hyaluronan on calcification of rat aortic ring. Rat aortic ring were cultured in growth medium (GM), calcifying medium (CM) or CM supplemented with high molecular weight-hyaluronan (HMHA) or low molecular weight-hyaluronan (LMHA) for 10 days. **a** Mineral deposition in aortas was detected by alizarin red staining at day 8 and 10 ($n = 4$). Scale bar = 100 μm. **b** Calcium content was measured at day 8 and 10. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. GM; $^{\#}P < 0.05$ vs. CM

Western blot analysis confirmed the downregulation of Runx2 and BMP2 protein expression by HMHA treatment (Fig. 1f).

Low molecular weight HA inhibits calcification of rat VSMCs

To further verify the effect of HA on VSMC calcification, we also used low molecular weight HA (LMHA) to treat rat VSMCs. Alizarin red staining showed that LMHA reduced calcium deposition in a dose-dependent manner in the presence of CM at day 10 (Fig. 2a). LMHA-treated cells showed less levels of calcium content than CM-treated cells (Fig. 2b). In addition, we found that LMHA inhibited osteogenic differentiation of rat VSMCs, as indicated by the reduction of ALP activity and downregulation of Runx2, BMP2 and Msx2 mRNA expression in VSMCs at day 6 (Figs. 2c, d).

HA attenuates calcification of rat aortic ring

To determine the effect of HA on vascular calcification ex vivo, we used HMHA and LMHA to treat rat aortic ring. Alizarin red staining revealed that both HMHA and LMHA reduced CM-induced calcium deposition in aortic arteries at day 8 and day 10 (Fig. 3a). Quantification of calcium content analysis further confirmed that HMHA reduced calcium content by 78% and 80% ($P < 0.05$) in aortic arteries at day 8 and day 10, respectively (Fig. 3b). Similarly, LMHA reduced calcium content by 75% and 63% ($P < 0.05$) in aortic arteries at day 8 and day 10, respectively (Fig. 3b).

Inhibition of HA synthesis promotes calcification of rat VSMCs

Next, we examined the effect of 4MU, a competitive inhibitor of HA biosynthesis, on VSMC calcification. Alizarin

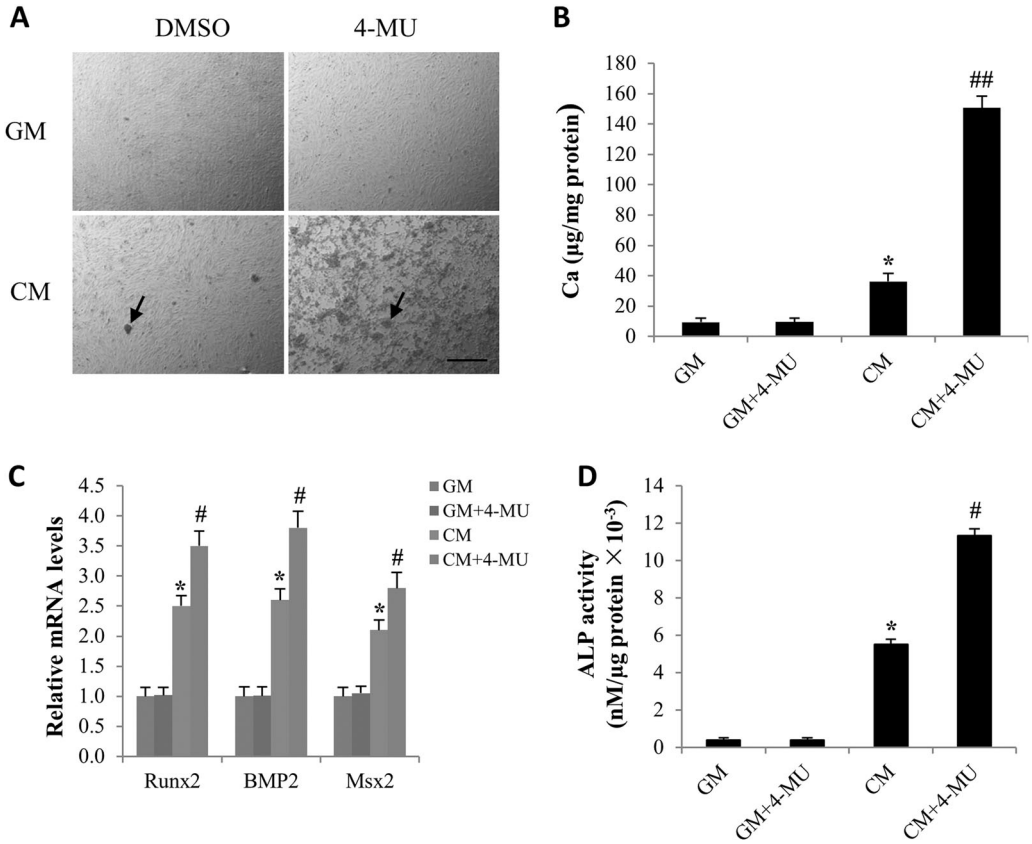

Fig. 4 Effect of 4MU on rat VSMC calcification. Confluent rat VSMCs were cultured in growth medium (GM), calcifying medium (CM) or CM supplemented with 4MU for 7 days ($n = 3$). **a** Mineral deposition in VSMCs was detected by alizarin red staining. Scale bar = 200 μm. **b** Calcium content was measured. **c** Runx2, BMP2, and Msx2 mRNA levels were determined by qPCR. **d** ALP activity was assessed by spectrophotometry. $^{*}P < 0.05$ vs. GM; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. CM

red staining showed that 4MU treatment promoted calcium deposition in VSMCs at day 6 (Fig. 4a). The levels of calcium content were significantly increased by 4.15-fold ($P < 0.01$) in 4MU-treated cells compared with cells treated without 4MU in the presence of CM at day 6 (Fig. 4b). Furthermore, we found that 4MU treatment upregulated the mRNA expression of Runx2 by 1.4-fold, BMP2 by 1.46-fold and Msx2 by 1.33-fold ($P < 0.05$) in VSMCs and increased ALP activity by 2-fold ($P < 0.05$) at day 6 (Figs. 4c, d).

HAS2 overexpression inhibits calcification of rat VSMCs

As HAS2 is the main HAS isoform responsible for HA biosynthesis in cultured vascular SMCs [12, 13], we used adenovirus encoding HAS2 to investigate the role of HAS2 in VSMC calcification. Western blot analysis showed increased levels of HAS2 protein in Ad/HAS2-infected cells compared with control Ad/GFP-infected cells and enzyme-linked immunosorbent assay (ELISA) showed elevated production of HA in Ad/HAS2-infected cells versus controls (Fig. 5a). Mineral deposition was reduced by 89% ($P < 0.05$) in Ad/HAS2-infected cells compared with controls, which was confirmed by calcium content assay (Figs. 5b, c). Moreover, HAS2 overexpression significantly reduced ALP activity by 90% ($P < 0.05$) in rat VSMCs (Fig. 5d). These data suggest a key role of HAS2 in regulating osteogenic differentiation and calcification of VSMCs.

KO of HAS2 accelerates calcification of rat VSMCs

To further validate the role of HAS2 in VSMC calcification, we performed KO of HAS2 in rat VSMC line A10 using CRISPR/Cas9-guided genome editing. Western blot analysis confirmed decreased levels of HAS2 protein in HAS2 KO cells compared with control cells (Fig. 6a). KO of HAS2 accelerated mineral deposition in VSMCs (Fig. 6b) and obviously increased the levels of calcium deposition by 3.9-fold ($P < 0.05$, Fig. 6c). Similarly, ALP activity was also increased by 2.9-fold ($P < 0.05$) in HAS2 KO cells compared with controls (Fig. 6d).

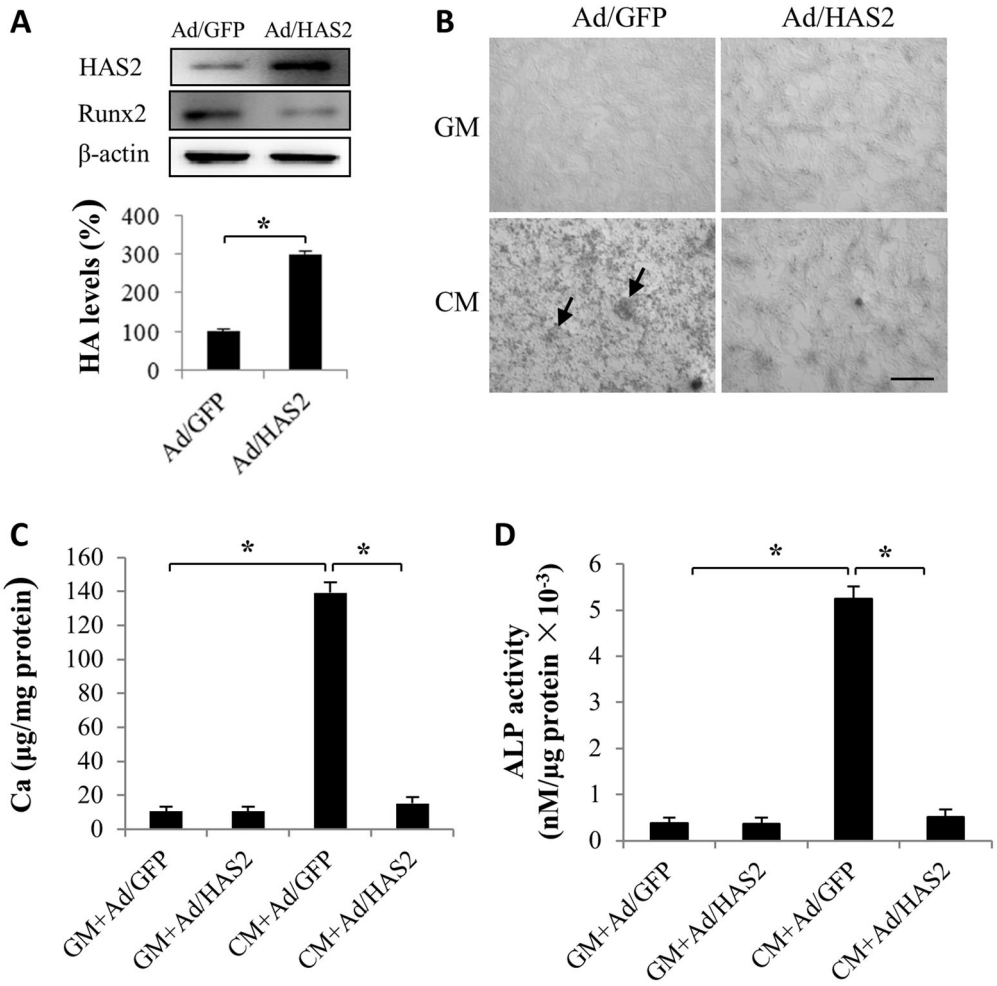

Fig. 5 Effect of HAS2 overexpression on rat VSMC calcification. Rat VSMCs were transfected with Ad/GFP or Ad/HAS2 ($n = 3$). **a** The protein expression of HAS2 was analyzed by western blot and the levels of HA were assessed by Elisa at day 3. **b** Mineral deposition in VSMCs was detected by alizarin red staining at day 10. Scale bar = 200 μm. **c** Calcium content was measured at day 10. **d** ALP activity was assessed by spectrophotometry at day 6. $*P < 0.01$

HA regulates BMP2 signaling in rat VSMCs

Previous studies have demonstrated that BMP2 is an important positive regulator of vascular calcification [24, 28]. Therefore, we then investigated the effect of HA on BMP2 signaling in VSMCs. Western blot analysis revealed that BMP2 and downstream signaling molecules p-Smad1/5/8 were highly expressed in CM-treated cells compared with controls, and both HMHA and LMHA treatment can downregulate protein expression of BMP2 and inhibit phosphorylation of Smad1/5/8 (Fig. 7a). In contrast, 4MU treatment upregulated BMP2 protein expression and potentiated phosphorylation of Smad1/5/8 (Fig. 7b). Immunocytochemistry analysis confirmed that CM treatment stimulated phosphorylation of Smad1/5/8, and 4MU treatment potentiated phosphorylation of Smad1/5/8, but this effect was inhibited by HMHA treatment (Fig. 7c). In addition, we examined the effect of HA on the activation of p-Smad1/5/8 in VSMCs induced by recombinant BMP2. Immunocytochemistry analysis showed that HA treatment partially reduced the phosphorylation of Smad1/5/8 induced by recombinant BMP2 (Supplementary Fig. 1). Taken together, these results suggest a potential role of BMP2 signaling in regulation of VSMC calcification inhibition by HA.

BMP2 signaling is required for VSMC calcification inhibition by HA

To further determine whether BMP2 signaling is required for VSMC calcification inhibition by HA treatment, recombinant BMP2 protein was used to treat cells in CM for 10 days. We found that recombinant BMP2 protein treatment enhanced CM-induced mineral deposition in VSMCs, which can be blocked by both HMHA and LMHA treatment. More mineral deposition was detected in BMP2

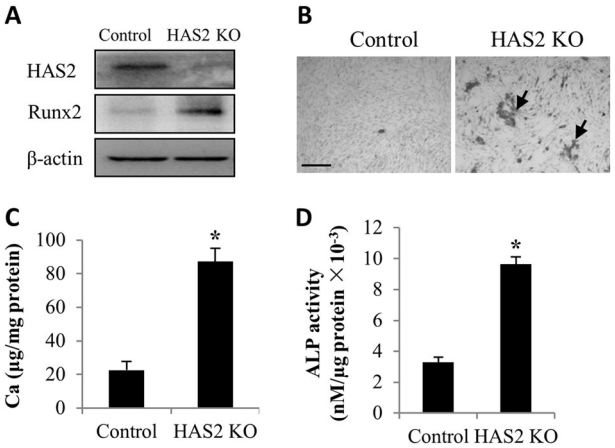

Fig. 6 Effect of HAS2 knockout on VSMC calcification. Confluent rat A10 VSMCs were transfected with PX459 plasmid encoding a target specific sgRNA using Lipofectamine 3000 ($n = 3$). **a** The protein expression of HAS2 was analyzed by western blot. **b** Mineral deposition in VSMCs was detected by alizarin red staining at day 10. Scale bar = 200 μm. **c** Calcium content was measured at day 10. **d** ALP activity was assessed by spectrophotometry at day 6. $^*P < 0.05$ vs. control

together with HMHA-treated cells than HMHA-treated cells in the presence of CM, suggesting inhibitory effect of HA on VSMC calcification can be reversed by BMP2 treatment (Fig. 8a). Similarly, BMP2 treatment significantly increased the levels of calcium content, and this effect was prevented by HA treatment. The levels of calcium content were increased in BMP2 together with HMHA-treated cells compared with HMHA-treated cells in the presence of CM (Fig. 8b). Additionally, ALP activity in rat VSMCs was enhanced by BMP2 treatment in the presence of CM, which can be blocked by HA treatment (Fig. 8c). These results suggest that BMP2 signaling plays an important role in regulation of VSMC calcification by HA. Given that Akt and ERK signaling pathways play important roles in vascular calcification [29, 30], we examined the effect of HA on Akt and ERK signaling pathways using Ad/HAS2 and 4MU. Western blot analysis showed both HAS2 overexpression and 4MU have no significant effect on the phosphorylated levels of Akt and ERK (Supplementary Fig. 2), suggesting that HA does not affect on Akt and ERK signaling pathways.

Discussion

Vascular calcification is now considered as an active gene-regulated process resembling osteogenesis [31, 32]. Therefore, it is very important to decipher the molecular mechanisms underlying vascular calcification, so that therapeutic strategies can be designed to prevent and treat vascular calcification. In this study, we demonstrate that both HMHA and LMHA treatment reduces calcification of rat VSMCs and arterial calcification. Similarly, HA treatment prevents osteogenic differentiation of VSMCs. By contrast, inhibition of HA synthesis by 4MU promotes calcification of rat VSMCs. In addition, HAS2 overexpression inhibits calcification of VSMCs, whereas HAS2 KO promotes calcification of rat A10 cells. Furthermore, BMP2 signaling is required for the inhibitory effect of HA on VSMC calcification. To our knowledge, this is the first report to show that HA inhibits vascular calcification involving BMP2 signaling.

A growing evidence has demonstrated the importance of glycosaminoglycan chains for the regulation of mineralization [33, 34]. Previous studies have shown that oversulfated chondroitin sulfate promotes osteoblast differentiation and mineralization [35] and decorin glycosaminoglycan chain regulates human VSMC calcification [11]. HA is a glycosaminoglycan polymer component of the ECM. An animal study has revealed that inhibition of HA synthesis by 4MU accelerates atherosclerosis in ApoE$^{-/-}$ mice [36]. HA has been shown to inhibit osteoblast differentiation [18] and the calcification-mitigating role of the HA grafts on the biomaterial has been demonstrated [34]. In this study, we find that HMHA inhibits osteogenic differentiation and calcification of rat VSMCs and aterial calcification. The molecular mass of HA ranges from 10^5 to 10^7 Da and HA function depends on the length of the polysaccharide chains. Different forms of HA have distinct effects on cellular function. For instance, HMHA inhibits VSMC cycle progression, whereas LMHA stimulates cell cycle progression [15]. Therefore, we also investigated the effect of LMHA on vascular calcification in this study. Interestingly, LMHA also reduces calcification of rat VSMCs and aterial calcification, suggesting that the molecular mass of HA is not the major determinant affecting vascular calcification under the conditions of high calcium and phosphate. These findings further confirmed the inhibitory effect of HA on vascular calcification.

HAS2 is the major enzyme responsible for HA synthesis in VSMCs [12] and has been shown to regulate atherosclerosis [37]. In this study, we show that overexpression of HAS2 inhibits calcification of rat VSMCs, whereas KO of HAS2 accelerates calcification of rat VSMCs, suggesting that HAS2 plays important role in vascular calcification. Previous studies have demonstrated that BMP2 regulates osteoblast differentiation through osteogenic transcription factors including Runx2 and Msx2 [38, 39]. Several studies have revealed that BMP2 positively regulates vascular calcification [23, 24]. Inhibition of BMP signaling reduces vascular calcification [40] and HA has been shown to inhibit BMP2-induced osteoblast differentiation [18]. Therefore, we investigated whether HA negatively regulates vascular calcification involving BMP2 signaling. We find that HA treatment downregulates protein expression of

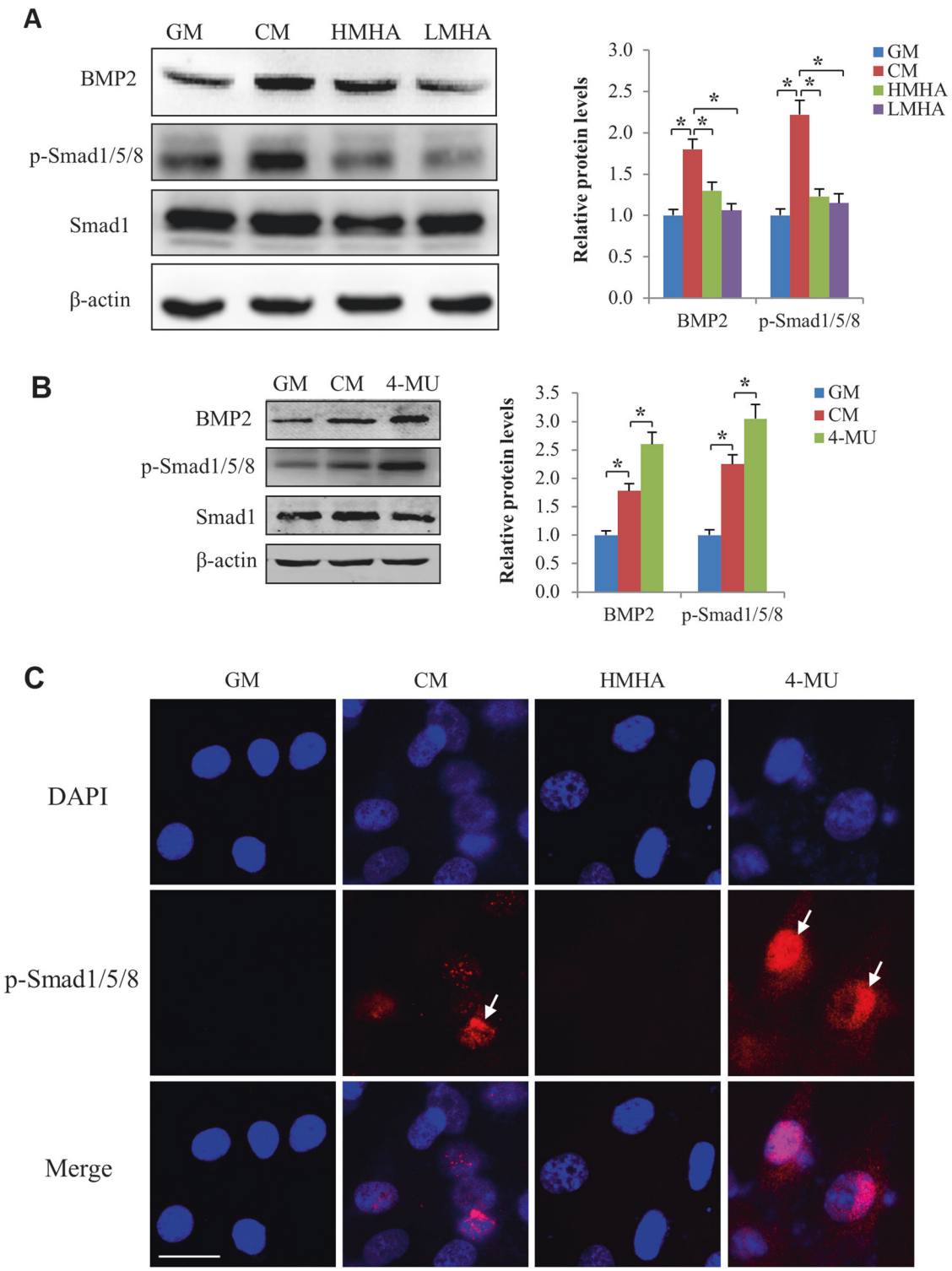

Fig. 7 Effect of HA on BMP2 signaling in rat VSMCs. Confluent rat VSMCs were cultured in growth medium (GM), calcifying medium (CM) or CM supplemented with HMHA, LMHA or 4MU for 6 days ($n = 3$). **a**, **b** The protein expression of BMP2, Smad1 and p-Smad1/5/8 was analyzed by western blot. **c** The levels of p-Smad1/5/8 were analyzed by immunostaining. $^*P < 0.05$

BMP2 and inhibits phosphorylation of Smad1/5/8. Conversely, inhibition of HA synthesis by 4MU upregulates BMP2 protein expression and potentiates phosphorylation of Smad1/5/8. Importantly, inhibitory effect of HA on VSMC calcification can be reversed by BMP2 treatment and BMP2-induced VSMC calcification was inhibited by HA treatment, suggesting the key role of BMP2 in HA-regulated vascular calcification.

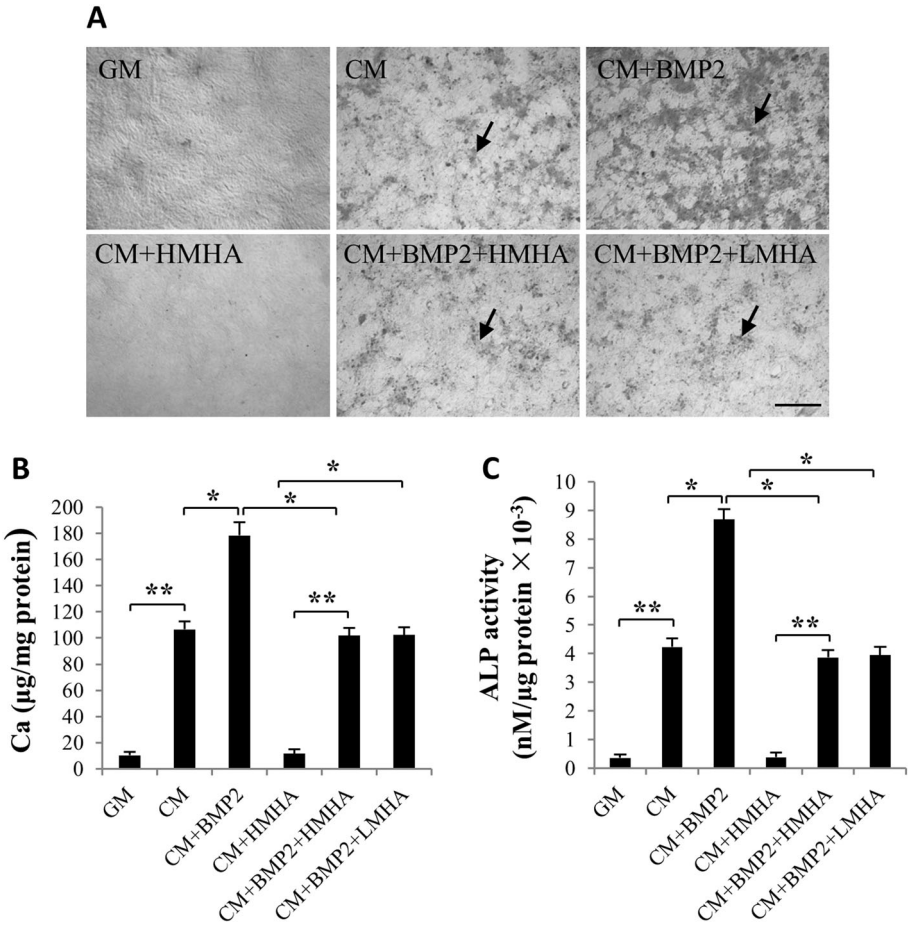

Fig. 8 Effect of BMP2 on rat VSMC calcification. Confluent rat VSMCs were cultured in growth medium (GM), calcifying medium (CM) or CM supplemented with BMP2, HMHA, BMP2 with HMHA, or BMP2 with LMHA for 10 days ($n = 3$). **a** Mineral deposition in VSMCs was detected by alizarin red staining at day 10. Scale bar = 200 μm. **b** Calcium content was measured at day 10. **c** ALP activity was assessed by spectrophotometry at day 6. $^{*}P < 0.05$, $^{**}P < 0.01$

In addition, ECM not only acts as a major site for mineral deposition, but actively regulates vascular calcification. Abnormal assembly and/or remodeling of the vascular ECM may initiate ectopic calcification [41]. Previous studies have revealed that Elastin, a major elastic lamina protein produced by VSMCs, acts as a mineral nucleator to initiate vascular calcification [42, 43]. Given that HA is a main structural component of the ECM, we can not rule out the possibility that HA may directly affect the minerals' predisposition to precipitate into ECM.

In conclusion, this study identifies HA as a novel regulator of vascular calcification and HA negatively regulates vascular calcification involving BMP2 signaling, suggesting that HA may act as a novel potential therapeutic target for the prevention and treatment of vascular calcification. Further studies are necessary to determine whether HA regulates vascular calcification in vivo using HAS2 conditional KO mouse model.

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Compliance with ethical standards

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

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