Hyperhomocysteinemia induces vascular calcification by activating the transcription factor RUNX2 via Krüppel-like factor 4 up-regulation in mice

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Running title: Hhcy induces VC by activating RUNX2 via KLF4 up-regulation

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ABSTRACT
One of the main characteristics of atherosclerosis is vascular calcification (VC), which is linked to adverse cardiovascular events. Increased homocysteine (Hcy), a feature of hyperhomocysteinemia (Hhcy), is correlated with advanced vascular calcification and phenotypic switching of vascular smooth muscle cells (VSMCs). Oxidative stress and high phosphate levels also induce VSMC calcification, suggesting that the Krüppel-like factor 4 (KLF4) signaling pathway may also contribute to vascular calcification. In this study, we investigated this possibility and the role and mechanisms of Hcy in vascular calcification. We found that in atherosclerotic apolipoprotein E–deficient (ApoE−/−) mice, Hcy significantly increases vascular calcification in vivo, as well as VSMC calcification in vitro. Of note, the Hcy-induced VSMC calcification was correlated with elevated KLF4 levels. Hcy promoted KLF4 expression in calcified atherosclerotic lesions in vivo and in calcified VSMCs in vitro. shRNA-mediated KLF4 knockdown blocked the Hcy-induced up-regulation of runt-related transcription factor 2 (RUNX2) and VSMC calcification. RUNX2 inhibition abolished Hcy-induced VSMC calcification. Using ChIP analysis, we demonstrate that KLF4 interacts with RUNX2, an interaction promoted by Hcy stimulation. Our experiments also revealed that the KLF4 knockdown attenuates Hcy-induced RUNX2 transactivity, indicating that KLF4 is important in modulating RUNX2 transactivity.
These findings support a role of Hcy in regulating vascular calcification through a KLF4–RUNX2 interaction and indicate that Hcy-induced, enhanced RUNX2 transactivity increases VSMC calcification. These insights reveal possible opportunities for developing interventions that prevent or manage vascular calcification.

**INTRODUCTION**

Atherosclerosis increases the occurrence of peripheral vascular disease, cerebral infarction, and coronary heart disease. Among its characteristics, arterial calcification is the most common feature (1-7). Abnormal deposition of hydroxyapatite mineral in the arteries leads to arterial wall thickening and hardening, vascular lumen narrowing and ischemia or necrosis of tissues or organs (1,8). Intimal calcification and medial calcification are two types of vascular calcification, they share many common mechanisms, but they have their unique set of risk factors, cellular process and clinical presentations. The mechanisms of vascular calcification (VC) in atherosclerosis are complex and not yet fully clear (5). Cardiovascular calcification is now recognized as an active process, which provides a potential opportunity for effective therapeutic targeting(6). Many reports indicated that osteogenic differentiation of VSMC in response to various local stimuli is a key mechanism in the development of vascular calcification (9,10).

Hyperhomocysteinemia (Hhcy) is closely linked to atherosclerosis and is one of the risk factors of atherosclerosis (11,12). We and others have reported that Hhcy promotes atherosclerosis via promoting macrophage apoptosis (13,14), inhibiting endothelial cell growth (15), and promoting inflammatory monocyte differentiation (16). Studies have shown that Hcy significantly increases oxidative stress via upregulation of NOX4 and NOX2 in endothelial cells(17); and Hcy significantly upregulates the expression of NOX4 in adventitial fibroblasts in vitro(18). Earlier studies found that Hcy was linked to increased calcification of blood vessels, possibly by strengthening of lipid peroxidation (19,20). Hcy also induced calcium deposition and alkaline phosphatase activity in mesenchymal stem cells (21). However, the mechanism underlying Hcy-promoted phenotypic switch of VSMC in atherosclerotic vascular calcification remains unknown.

Krüppel-like factor 4 (KLF4) is a zinc finger transcription factor family member required in mammalian embryonic development and various diseases. KLF4 promotes phenotypic switching of VSMC (4,22,23). Furthermore, activation of KLF4 in VSMC by phosphate (24) and or oxidized phospholipids (25) promotes phenotypic switch of VSMC. In addition, KLF4 also mediates H2O2-induced injury in human cardiomyocyte (26) and oxidative stress-induced apoptosis in follicular granulosa cell (27). As oxidative stress and high phosphate also induce VSMC calcification (28-30), we hypothesized that activating KLF4 signaling pathway may contribute to vascular calcification.

The present study investigated the function of Hcy in the regulation of vascular calcification in vitro and in vivo. In addition, we determined the role of KLF4 in mediating Hcy-induced VSMC calcification and explored the interplay between KLF4 and RUNX2 in calcification.
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development. We found that Hcy enhanced atherosclerotic vascular calcification in vivo; and promoted calcification of VSMC in vitro. Furthermore, activation of KLF4 upregulated RUNX2 and its transactivity, leading to VSMC calcification. Our findings add to the current understanding of vascular calcification mechanisms, present new fields of research and new strategies for prevention and treatment vascular calcification.

RESULTS

Vascular calcification in ApoE<sup>−/−</sup> mice stimulated by methionine diet in vivo.

To examine the effects of Hhcy on vascular calcification in vivo, homocysteinemia was administrate to mice. Compared with ApoE<sup>−/−</sup> mice fed control diet, methionine diet-fed mice showed a significant increase in Hcy after 30 weeks (Figure. 1A). The plasma Hcy level was 5.964 ± 0.24 mmol/L in control ApoE<sup>−/−</sup> mice; while increased to 18.76 ± 1.25 mmol/L in the methionine-fed ApoE<sup>−/−</sup> mice. These results demonstrate the effects of methionine-triggered Hhcy on vascular calcification in the atherosclerotic mice. It was observed that Hhcy increased vascular calcification in mice carotid arteries in vivo (Figure. 1B, C). Carotid arteries with Alizarin Red staining displayed increased calcification in methionine-treated ApoE<sup>−/−</sup> mice (Figure. 1B, C). Moreover, high calcium level in the carotid arteries revealed that Hhcy remarkably promoted calcification (Figure. 1D). These results suggest that Hhcy increases vascular calcification in atherosclerosis in vivo.

Hcy induces VSMC calcification.

To define whether Hcy increases vascular calcification in vitro, we treated A7r5 VSMC with different concentrations of Hcy in the osteogenic medium. Alizarin Red staining showed that Hcy increased VSMC calcification in vitro (Figure. 2A). The increases in calcium content and Alkaline Phosphate activity in a dose-dependent manner further confirmed the effect of Hcy on VSMC calcification in vitro (Figure. 2B, C). Results confirmed that Hcy suppressed the level of smooth muscle marker genes alpha smooth muscle actin (α-SMA) and smooth muscle myosin heavy chain (SM MHC), but elevated the level of bone-associated markers, RUNX2 and osteopontin (OPN) (Figure. 2D, E), suggesting that Hcy promotes osteogenic differentiation of VSMC.

Hcy induces expression of KLF4.

To understand the mechanisms of Hcy-induced VSMC calcification, we determined the effects of Hcy on the expression of KLF4, a key regulator for the phenotypic switch of VSMC, and the expression of RUNX2, the master osteogenic regulator for VSMC calcification. We found increased KLF4 in the carotid artery sections from mice fed on methionine diet (Figure. 3A, B). Similarly, increased RUNX2 was observed in the carotid artery sections from mice fed on methionine diet (Figure. 3A, C). Western blot and qPCR analyses demonstrated that methionine diet increased KLF4 expression and RUNX2 level in the carotid artery (Figure. 3D, E). Therefore, increased expression of KLF4 and RUNX2 may contribute to Hcy-triggered vascular calcification.

Knockdown of KLF4 expression attenuates Hcy-induced VSMC calcification.

Having shown that Hhcy increased
expression of KLF4 in vivo, we determined whether KLF4 inhibition can reverse the calcification caused by Hcy in vitro. We designed in vitro studies in which KLF4 expression was knocked down by specific shRNA. Hcy-induced calcification was blocked in the KLF4 knockdown cells (Figure 4A, B). Knockdown of KLF4 blocked RUNX2 expression (Figure 4C), supporting the role of KLF4 in mediating Hcy-induced RUNX2 upregulation and VSMC calcification. The results presented here demonstrate that Hcy activates KLF4 thereby upregulating RUNX2 and VSMC calcification.

**Hcy-induced KLF4 expression promotes VSMC calcification via RUNX2.**

To examine whether Hcy-induced increase in RUNX2 is critical for calcification of VSMC, we used shRNA to knock-down the expression of RUNX2 in VSMC. The results showed that VSMC calcification was abolished in VSMC with RUNX2 knockdown, compared to the control group (Figure 5Aa). The difference in calcium content between the groups indicated that RUNX2 knock-down inhibited Hcy-induced VSMC calcification (Figure 5Ab). Notably, Hcy-induced KLF4 expression occurred similarly in RUNX2 knockdown VSMC and in control cells (Figure 5Ac), suggesting that RUNX2 is a downstream effector of Hcy-induced KLF4 expression. In addition, Alizarin Red staining (shScr, Figure 5Ba) and quantitative calcium measurement (shScr, Figure 5Bb) showed that overexpression of RUNX2 induced VSMC calcification in control VSMC. The amount of KLF4 was not affected by RUNX2 overexpression (shScr, Figure 5Bc). In contrast, RUNX2-induced VSMC calcification was eliminated following KLF4 knockdown (shKLF4, Figure 5Ba, b), but this knockdown did not alter RUNX2 expression (Figure 5Bc). Furthermore, overexpression of RUNX2 increased the expression of osteogenic marker genes OPN, osteocalcin (OC) (Figure 5C, D). These datasets demonstrated that inhibition of KLF4 or RUNX2 blocks Hcy-induced VSMC calcification, indicating an interaction between RUNX2 and KLF4 in Hcy-induced VSMC calcification.

**KLF4 regulates RUNX2 transcription.**

We found that KLF4 knockdown inhibited RUNX2 expression (Figure 4C), indicating that RUNX2 is modulated by KLF4 transcription factor. Using the JASPAR dataset, we identified three putative KLF4 binding sites in the 5' flanking region of the RUNX2 gene: +1897/1888, +1640/1649 and -555/546, based on the KLF4 sequence outlined in Figure 6A (“+” refers that the binding site is on the sense strand, and “-” refers the binding site on the antisense strand). The sequence is shown in Figure 6A. To assess whether KLF4 interacts with RUNX2 gene, an anti-KLF4 antibody was used for chromatin immunoprecipitation (ChIP) assay. The RUNX2 promoter segment containing the putative KLF4 binding site was amplified by PCR using the selected primer site1-2, +1897/1649 and site3, -555/546. Figure 6B revealed that KLF4 overexpression in VSMC increased KLF4 binding to the RUNX2 promoter at the upstream site 3 of the TSS, while in the other putative site, no significant KLF4 binding was observed between +1897/1649. We then generated a plasmid construct (pGL3-RUNX2-Luc) containing the upstream region of the RUNX2 promoter fused to the luciferase reporter gene.
KLF4 overexpression elevated the luciferase activity in VSMC transfected with RUNX2 FL-Luc (Figure. 6C). However, deletion of the region between -555 to -546 bp containing the predicted KLF4 binding site 3 did not increase luciferase activity (Figure. 6C), indicating that this region is required for KLF4 binding and RUNX2 transcription. Alizarin red staining demonstrated that overexpression of KLF4 triggered VSMC calcification in control VSMC (Figure. 6D). Furthermore, overexpression of KLF4 was sufficient to promote RUNX2 expression in VSMC (Figure. 6E, F). Thus, increased expression of KLF4, induced by Hcy or mediated by a viral infection, upregulates the expression of RUNX2 in calcified VSMC.

**DISCUSSION**

In this study, we showed that Hcy induced VSMC calcification in atherosclerosis mice in vivo and in VSMC in vitro. It was also found that activation of KLF4 by Hcy in the vasculature is key to atherosclerosis vascular calcification. We provide evidence that binding of KLF4 to RUNX2 promoter contributes to high expression of RUNX2 and VSMC calcification. Because KLF4 is a master transcription factor, these new findings have exciting implications for treatment as well as prevention of atherosclerosis vascular calcification through therapies targeting KLF4 and signaling.

Emerging studies have linked atherosclerosis and vascular calcification with Hhcy(31,32). Hcy was found to be independently associated with intracranial arterial calcification (31). A previous report showed that Hhcy induced by supplementation promotes atherosclerosis progression in ApoE⁻/⁻ mice(33). However, whether methionine induced Hhcy can cause atherosclerosis vascular calcification in vivo is not clear. In vivo, we identified that increased Hcy enhanced vascular calcification during atherosclerosis in mice (Figure.1). Using cultured VSMC, we found that high expression of Hcy induced VSMC calcification. Although it was previously known to be a passive process characterized by calcium deposition, vascular calcification is now recognized as a tightly modulated dynamic process driven by osteochondrogenic differentiation of vascular cells(3). Based on the analysis of gene expression of the osteogenic factors and markers, it was herein observed that Hcy promoted vascular calcification and osteogenic differentiation of VSMC in vitro. Moreover, these effects were dose-dependent (Figure 2). These findings are in line with prior reports that osteogenic differentiation of VSMC plays a key role in vascular calcification associated with atherosclerosis.

Our studies determined a new role of Hcy in promoting VSMC calcification via KLF4 pathway. KLF4 is a master transcription factor associated with many biological processes (34). Emerging studies demonstrate that KLF4 participates in many contexts during phenotypic switching of SMCs (22,25,35,36). For instance, KLF4 inhibits the level of SMC differentiation-associated genes e.g. SM22, SM-myosin heavy chain, and SM α–actin following exposure to oxidized phospholipids or PDGF-BB (37,38).

We found that increased expression of KLF4 was associated with vascular calcification in the Hcy-induced atherosclerosis arteries (Figure 3). Furthermore, inhibition of KLF4 expression attenuated VSMC calcification in vitro,
confirming the essential role of KLF4 activation in mediating Hcy-induced vascular calcification (Figure 4). Notably, constitutively active KLF4 was able to induce calcification of VSMC (Figure 6), implying that KLF4 activation has profound effects on vascular calcification.

This study has revealed an important regulatory mechanism of RUNX2 transcription by KLF4, which underlies the Hcy-induced osteogenic differentiation and calcification of VSMC. Stimulation of KLF4 has been shown to promote bone formation and cell differentiation by interacting with RUNX2, thereby slightly inhibiting RUNX2 expression (35). In contrast, we found that high expression of KLF4 induced by Hcy increased RUNX2 expression in VSMC (Figure 6), thus promoting VSMC calcification. Therefore, KLF4 may be differentially regulated and have different functions depending on the cell types, cellular environment and disease status. Our results reveal an important role of KLF4 activation in promoting osteogenic differentiation of VSMC, which contributes to increased vascular calcification in atherosclerosis. Furthermore, this study identified the link between RUNX2 and KLF4 in VSMC, which played important roles in Hcy-induced VSMC calcification. Hence, Hcy-potentiated the binding of KLF4 to RUNX2 promoter thereby upregulating RUNX2 transcription and promoting VSMC calcification.

CONCLUSION

Our study has revealed an important role of Hcy in promoting VSMC osteogenic differentiation and vascular calcification in atherosclerosis. Results further show that Hcy upregulates KLF4 signal, and KLF4 in turn regulates RUNX2 transcription, leading to VSMC calcification. Further studies using SMC-specific KLF4 knockout mice are warranted to determine the role of KLF4 in regulating Hcy-induced atherosclerotic vascular calcification in vivo. Nonetheless, a key molecular mechanism in which Hcy-promoted upregulating RUNX2 and VSMC calcification via KLF4 has been presented. This offers new targets for vascular calcification management.

EXPERIMENTAL PROCEDURES

Experimental animals

All animal experimental protocols were approved by the Ethics Committee of Animal Experiments of the Health Science Centre of Ningxia Medical University and were carried out in conformity with the recommended guidelines. Eight-week-old ApoE^{-/-} mice of C57BL/6J genetic origin were purchased from the Animal Centre of Peking University Health Science Centre (Beijing, China). ApoE^{-/-} mice is a well-established atherosclerosis model (39). Nine mice were assigned to each group, including male and female, and were fed with following diet (KeAoXieLi, Beijing) for 30 weeks: control group, a standard rodent maintenance diet -93 purified diet (AIN-93G) as recommended by the American Institute of Nutrition; and hypermethionine group (HM group), a chow diet plus methionine-containing diet (17g/kg) (13,14). Random selection and blinding methods were used for the selection of experimental animal.

Tissue processing

After feeding for 30 weeks, mice were anesthetized with isoflurane (catalog number 55, China), inhaled at a concentration of 2%, which
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was maintain at the concentration of 1.5% after which 2 ml of peripheral blood was obtained from the iliac vein. The blood was immediately collected into microtubes containing cooled EDTA, followed by centrifugation at 12,000 rpm and 4 °C for 15 minutes, then kept at -80 °C for experimentation. Serum Hcy was measured as previously described (14). The carotid artery tissue from mice was washed with sterile PBS, fixed with paraformaldehyde (15 min; 4% PFA), and then embedded in paraffin. Sections cut into 4mm thickness were subjected to Alizarin Red staining, Hematoxylin & Eosin (H&E) and immunohistological analysis. Carotid tissue was also used for western blot and quantitative polymerase chain reaction detection tests.

**Artery calcification**

Calcium deposits in the carotid artery was detected by Alizarin Red (Sigma Aldrich) staining (40,41). Paraffin sections were gradually deparaffinized in Histoclear, and incubated in Alizarin Red solution for 5 min after washing with distilled water. The sections were dehydrated and cover slipped, examined by microscopy and then photographed. The % of positive-stained surface area in each section was determined using ImageJ software (NIH Bethesda, MD). Calcium content in carotid arteries was quantified as reported in a prior study (40,41). Carotid artery specimen was lyophilized to obtain dry weight and incubated with 0.6 mmol/L HCl at 37°C for 48 h. Calcium was examined by Arsenazo III method with calcium diagnostic kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The content of carotid artery calcium is represented as millimolar/gram standardized to the dry weight of the tissues.

**Immunohistochemical staining**

Immunohistochemical staining was performed to determine protein expression in paraffinized sections. Carotid paraffin section was deparaffinized in Histoclear reagent, washed with distilled water and treated with avidin/biotin blocking kit to inhibit endogenous biotin activity according to the manufacturer’s protocol. The sections were incubated with 0.3% hydrogen peroxide to quench endogenous peroxidase activity followed by blocking with 10% goat serum 1 hour at room temperature (RT). Next, specimens were incubated with primary antibody KLF4 (Abcam, ab215036), RUNX2 (Abcam, ab23981) at 4°C overnight. The sections were incubated with diluted biotinylated secondary antibody (Cell Signalling, 7075) for 1 hour at 37 °C, then treated with horseradish peroxidase-labeled streptavidin solution for 20 min at 37°C. Lastly, the sections were stained with diaminobenzidine (DAB kit, ZSGB-BIO, Beijing, China) and counterstained with hematoxylin.

**VSMC Culture and transfection**

Rat aortic smooth muscle cells (A7r5) used for in vitro experiments were bought from the American Type Culture Collection (ATCC, USA). The cell cultured at 37°C in a 5% CO₂ humidified atmosphere and grin-grown to reach the desired confluence. The growth medium contained Dulbecco’s Modified Eagle’s Medium (DMEM, High glucose, Hyclone) together with 10% fetal bovine serum (FBS, Gibco, MA, USA), 100 mg/mL streptomycin and 100 unit/mL penicillin. The media was changed once a day. To prepare a calcification model in vitro, 10 mmol/L β-sodium glycerophosphate was added to the growth medium and cultured for 3 weeks (9,41). To
Hhcy induces VC by activating RUNX2 via KLF4 up-regulation determine the role of Hcy in vitro, 50, 100 and 200 μmol/L Hcy (Sigma Aldrich) was added to the growth medium for 3 weeks (14). For shRNA knockdown, the specific shRNA against RUNX2 (5’-AAGCTTGATGACTCTAACCACCTAG-3’), KLF4 (5’-GACCGAGGAGTTCAACGATCT-3’) and control (5’-TTCTCCGAACGTGTCACGT-3’) were designed by Shanghai genePharma Co., Ltd. (Shanghai, China) and packaged into viral vector, purified and transfected with 293T cells with helper vectors. After 6 h of transfection, the cells were replaced with fresh medium and cultured for 72 h. The supernatant of the cells enriched in lentiviral particles was collected and the virus was measured. The VSMCs were infected with the lentivirus particles at a multiplicity of infection of 50. After virus transduction for 48 hours, the VSMCs were passaged at a ratio of 1:10, and cultured in a medium containing 2μg/ml puromycin for 1 week. For RUNX2 and KLF4 overexpression, lentiviral vector containing their cDNAs was obtained from Shanghai genePharma Co., Ltd. (Shanghai, China). The recombinant lentivirus is packaged into 293T cells and collected, then the VSMCs were infected with lentivirus particles.

**In Vitro Calcification**

For calcification staining in vitro, the cells were cultured in a medium containing 10 mmol/L β-sodium glycerophosphate with or without Hcy for 3 weeks. After washing with PBS, cell samples were fixed with paraformaldehyde for 45 minutes at 4 °C, and then treated with Alizarin Red staining (Sigma Aldrich) for 5 minutes at room temperature, washed with double-distilled water, dehydrated and cover slipped. The section were examined by microscopy and photographed. ImageJ software (NIH Bethesda, MD) was used to calculate the percentage of positive-stained surface area in each section. Determination of calcium content in cells requires decalcification with 0.6 mmol / L HCl at 37 °C for 48 hours (40, 41). The calcium content was examined by Arsenazo III method with calcium diagnostic kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), following the manufacturer’s protocols. For ALP activity, cell lysates were treated with Alkaline Phosphatase Assay Kit (Abcam, ab83369) following the manufacturer’s guidelines after which absorbance was read at OD 405 nm.

**Western blot analysis**

Vascular tissue or cells were lysed with lysis buffer, then centrifuged at 12000 rpm, 4 °C for 30 minutes to extract total protein from the lysate (KeyGEN BioTECH, JiangSu, China). Protein concentration was carried out using BCA protein assay kit (KeyGEN BioTECH, JiangSu, China). About 40μg protein samples were resolved by 10% SDS-PAGE gel, and electrophoretically transferred to a nitrocellulose membrane. 5% skim milk was used to treat the membrane for 1 hour at RT, and the blot was incubated with following primary antibody overnight at 4 °C; anti-SMα-actin (Abcam, ab5694, 1:2000), anti-SM MHC (Abcam; ab53219, 1:1000), anti-RUNX2 (Abcam, ab23981, 1:1000), anti-KLF4 (Abcam, ab215036, 1:1000), anti-OPN (Abcam, ab214050, 1:1000), anti-OC (Abcam, ab93876, 1:1000) and anti-GAPDH (Cell Signaling, 97166, 1:2000) antibodies for normalization. The blot membrane was washed with TBST and incubated with HRP-conjugated antibody for 1 hour at RT, then detected by ECL test.
**Reverse-Transcriptase Quantitative PCR (RT-qPCR)**

Total RNA was obtained from vascular tissues or cells using Total RNA kit (Omega bio-Tek, CA, USA) according to the manufactures’ instructions. The RNA was used to synthesize cDNA by PrimeScript Master Mix kit (Takara, Tokyo). The mRNA level of SMα-actin, SM MHC, RUNX2, KLF4, OPN, OC and GAPDH were detected by real-time quantitative PCR using the SYBR Select Master Mix (Invitrogen, Carlsbad, CA, USA) on a q5 Multicolour Real-time PCR Detection System (Bio-Rad, Hercules, CA, USA). GAPDH mRNA levels were used for normalization. The formula $2^{-\Delta\Delta CT}$ was used to calculate mRNA relative expression. Primers used for RT-qPCR in table1 were obtained from Sangon Biotech (Shanghai) Co., Ltd.

**Promoter construction and luciferase reporter assays**

To detect the transactivity of RUNX2 following KLF4 stimulation, bioinformatics methods were used to analyze and predict possible KLF4 binding sites in the promoter region of RUNX2. The RUNX2 promoter fragment was cloned from the genomic DNA by PCR using the designed primers, and the fragment was inserted into the luciferase reporter plasmid (pGL3-basic), then the pGL3-Runx-Luc (RUNX2 FL-Luc) was constructed. The mutant RUNX2 luciferase reporter plasmid (RUNX2 site3-Luc) was generated by deleting the putative KLF4-binding site (TGGGTGGGGA) at position -546/-555, then the fragment was inserted into the luciferase reporter plasmid (pGL3-basic). A control plasmid encoding the Renilla luciferase was co-transfected into the cells with RUNX2 luciferase reporter plasmid for normalizing the transfection efficiency. After transfection with luciferase reporter plasmid for 6 hours, the medium of the cells was changed and after which cells were transfected with KLF4 overexpression plasmid for 24 hours. The luciferase activity was determined by Dual-Luciferase assay kit (Promega) (9). Primers used for generating the luciferase reporter in table 2 and lentiviruses were obtained from Hanbio Biotech. Co., Ltd. (Shanghai).

**Chromatin immunoprecipitation (ChIP) assays**

ChIP assays were conducted according to the established protocol using anti-KLF4 (Abcam, ab106629) and anti-rabbit IgG (Santa Cruz). Briefly, VSMCs were cross-linked with 0.75% formaldehyde for 10 min at RT and quenched with 125 mM glycine. Cells were lysed in RIPA (Santa Cruz) buffer and sonicated to fragment the DNA to a size of 200-1000 bp. The cell lysates were incubated with Dynabeads (Santa Cruz) and vortexed at 4 °C for 1 hour, then precipitated by centrifugation. The supernatant was collected and 10 μl (1%) was taken as input. Anti-KLF4 (Abcam, ab106629) and anti-rabbit IgG (Santa Cruz) antibodies were added to the supernatant and co-cultured with Dynabeads overnight at 4 °C and were precipitated by centrifugation. Immunoprecipitated DNA fragments were reverse cross-linked and DNA binding was quantified as the percentage of input using qPCR. Primers used for ChIP assays were obtained from Sangon Biotech (Shanghai) Co., Ltd. in table3.

**Statistical analysis**

All experimental results were analyzed by
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GraphPad Prism 7.0 (USA), and presented as mean ± standard deviation or median. In vitro data are from at least three independent experiments. All data were tested for normality and equal variance. Student's t-test was used for compare differences between two groups, and one-way or two-way ANOVA was used for multiple comparisons followed by a Student-Newman-Keuls test. Mann-Whitney test was used for data not normally distributed. $P<0.05$ was set as the threshold for significant.

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Competing of interests:

The authors declare that they have no conflicts of interest.

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**The abbreviations used are:** Hcy, Homocysteine, Hhcy, Hyperhomocysteinemia, VSMC, vascular smooth muscle cells, KLF4, krüppel-like factor 4, RUNX2, runt-related transcription factor 2, OPN, osteopontin, OC, osteocalcin, α-SMA, alpha smooth muscle actin, SM MHC, smooth muscle myosin heavy chain, GAPDH, glyceraldehyde-3-phosphate dehydrogenase. OE, overexpression.

**Table 1 Lists of primers used for RT-qPCR**

| Name     | Species       | Sequence                        |
|----------|---------------|---------------------------------|
| RUNX2    | Rattus norvegicus | Forward: CCACCACTCACTACCACACG   |
|          |               | Reverse: GGACGCTGAGAAGTACCAT    |
| RUNX2    | Mus musculus  | Forward: TAGCGGCAGAATGGATGAGTC  |
|          |               | Reverse: AAACCCAGTTATGACTGCCCC  |
| KLF4     | Rattus norvegicus | Forward: GAGACCGAGAGTTCAACGA   |
|          |               | Reverse: GGAAGACGAGAGATGAGCTG   |
| KLF4     | Mus musculus  | Forward: TGGCCATCGAGCTACTATTAC  |
|          |               | Reverse: CATGTCAGACTGCCAGTG     |
| GAPDH    | Rattus norvegicus | Forward: AGACTGCGAGTGTTGCTT    |
|          |               | Reverse: CTCTGTGCTGAGCTGCTC    |
| GAPDH    | Mus musculus  | Forward: CAGTTGCTCTGCGACATT    |
|          |               | Reverse: TATGGGGGCTGGAGTGGAA    |
| SMα-actin| Rattus norvegicus | Forward: CCACCAACCCCCAAAGAAGA  |
|          |               | Reverse: GGGAAAGAAGAGGATGCA    |
| SM MHC   | Rattus norvegicus | Forward: CCAAGTTCTCCAAGGGTGA  |
|          |               | Reverse: CACAGAAGAGGCCGAGTAG   |
| OPN      | Rattus norvegicus | Forward: AGACTGGCCAGTGTTGCTT |
|          |               | Reverse: CTCTGTGCTGAGCTGCTC    |
| OC       | Rattus norvegicus | Forward: ATTTGTGACAGCTAGCGGAC |
|          |               | Reverse: TCGAGTCTGAGAGTAGG     |

**Table 2 Lists of primers used for construction of luciferase reporter**

| Name       | Species       | Sequence                        |
|------------|---------------|---------------------------------|
| RUNX2 FL-Luc | Rattus norvegicus | Forward: CTCGAGACATCTCATCTGGAAGT |
|            |               | Reverse: AAGCTTTCATCCTTCTTCTTCTTCTG |
| RUNX2 site3-Luc | Rattus norvegicus | Forward: cGtGAAGAGGGAGAGAGAGAGA |
|            |               | Reverse: CaCaCGTTCTCTCTCTG     |
**Table 3 Lists of primers used for ChIP assays**

| Name          | Species             | Sequence                                      |
|---------------|---------------------|-----------------------------------------------|
| KLF4-Site 1-2 | Rattus norvegicus   | Forward:CTGTCTGGCGACCCTATTGA                  |
|               |                     | Reverse:GTTGCCAGATCACAACGTG                 |
| KLF4-Site 3   | Rattus norvegicus   | Forward:GGCAAGGAGTTTGCAAGCAGACC             |
|               |                     | Reverse:GCAGTGCGGCACATCTTGGGAT              |

**FIGURES**

**Figure 1.** Methionine diet increases Hhcy thereby accelerating vascular calcification in ApoE<sup>-/-</sup> mice. Hhcy increases vascular calcification in atherosclerosis mice. ApoE<sup>-/-</sup> mice were fed on control diet and methionine diet for 30 weeks (n = 9 mice/group). The results are expressed as the ratio of positive staining area to the total area, and bar values stands for means ± SD (*P* < 0.01). A) Quantification of plasma homocysteine level. B) Determination of vascular calcification in the carotid artery. Paraffinized carotid artery sections stained with Alizarin Red. The scale of the image is shown in the lower right corner, scale bar: 500 μm. C) Total area of calcification quantified in the carotid artery using NIH ImageJ software. D) Determination of vascular calcification in the carotid artery based on the calcium content with Arsenazo III method.
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Figure 2. Hcy induces VSMC calcification. VSMC lines grown in osteogenic growth medium with or without Hcy for 3 weeks. VSMC were serum starved for 24 hours and then treated with different concentrations of Hcy. 

A) Level of VSMC calcification in vitro by Alizarin Red staining. Lower image shows the high magnification, scale bar: 1 mm.

B) Quantification of calcium content by Arsenazo III method. Data are normalized to the total protein (n = 3, *P < 0.01).

C) ALP activity quantified by a colorimetric analysis (n = 3, *P < 0.01).

D) Western blot results of α-SMA, SM MHC, RUNX2 and OPN in VSMC. GAPDH served as the loading control.

E) qPCR analysis of α-SMA, SM MHC, RUNX2 and OPN in VSMC. VSMC genes expression at control conditions (first bar in each group) is defined as 1 (n = 3, *P < 0.01 relative to control conditions). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
Figure 3. Methionine diet increased KLF4 expression. ApoE⁻/⁻ mice were fed on control diet and methionine diet for 30 weeks, (n = 9 mice/group) (*P<0.01). A) Immunohistochemistry analysis of KLF4 (a, c) and RUNX2 (b, d) in carotid artery paraffinized sections. Higher-magnification images are shown to the right of each image (a1, b1, c1 and d1), the scale of the image is provided on the lower right corner (a, a1), scale bar: a, 500μm; a1, 100μm. B, C) Measurement of KLF4 and RUNX2 expression in carotid artery paraffinized sections. The results are expressed as the ratio of positive staining area to total area by ImageJ software, and bar values stands for means ± SD (*P<0.01). D) Western blot analysis of KLF4 and RUNX2 in carotid artery tissues in the two groups. GAPDH was used as the loading control. E) qPCR analysis of RUNX2 and KLF4 expression in carotid artery tissues in two groups. The expression of RUNX2 and KLF4 in the first bar in each group is defined as 1, (n=3, *P<0.01 relative to control conditions).
Figure 4. Inhibition of KLF4 attenuates Hcy-induced VSMC calcification. VSMC were infected with lentivirus particles contain control (shScr) or KLF4 specific shRNA (shKLF4) and then exposed to Hcy in osteogenic media for 3 weeks. A) VSMC calcification measurement using Alizarin red staining. B) Measurement of calcium content (n = 3, *P<0.01). C) RUNX2 and KLF4 protein expression.
Hhcy induces VC by activating RUNX2 via KLF4 up-regulation

Figure 5. Hcy-induced KLF4 activation enhances VSMC calcification through RUNX2. A) RUNX2 knockout blocked Hcy-induced VSMC calcification. VSMCs from infected with lentivirus particles containing control (Control) or RUNX2 specific shRNA (RUNX2 KO) were cultured in osteogenic medium for 21 days. a) Alizarin red staining for calcification. b) Calcium content in different experimental groups (n = 3, *P<0.01). c) Western blot results of RUNX2 and KLF4.

B) Knockdown of KLF4 inhibited RUNX2-induced VSMC calcification. Stably selected VSMCs with control (shScr) or KLF4 shRNA (shKLF4) were infected with the control lentivirus (Control) or RUNX2 protein (RUNX2 OE) and cultured for 21 days in osteogenic medium. a) Alizarin red staining for calcification. b) Calcium content in different experimental groups. (n = 3, *P<0.01). c) Western blot analysis of RUNX2 and KLF4 expression.

C) qPCR analysis of RUNX2, OC and OPN in VSMC with RUNX2 overexpression. The expression of each gene in VSMC (the first column in each group) was defined as 1 under control conditions (n=3, *P<0.01 compared to control conditions). D) Western blot analysis of RUNX2, OC and OPN expression in VSMC with RUNX2 overexpression.
**Figure 6.** KLF4 regulates RUNX2 transcription. A) Description of the predicted KLF4 binding site in rat RUNX2 gene from +1897 to +1888 bp (site 1), +1640 to +1649 bp (site 2), and -555 to -546 bp (site 3). B) VSMCs treated with or without KLF4 overexpression were analyzed by ChIP assay using anti-KLF4. KLF4 binding site from +1897 to +1649 bp (site 1-2), and -555 to -546 bp (site 3). KLF4 promoter enrichment analysis by qPCR. IgG was used as the control isotype. C) Transfection of the luciferase reporter gene with the VSMC and RUNX2 promoter region (RUNX2 FL-Luc) or Site3 deletion (RUNX2 site3-Luc) and co-transfection with a control plasma expressing Renilla luciferase for 6 hours, followed by infection with LV-KLF4 (KLF4 OE) or control lentivirus (LV-GFP) (Control) for 24 hours. Luciferase activity was measured and normalized to the activity of Renilla luciferase. D) VSMCs were transduced with LV-KLF4 (KLF4 OE)
or control lentivirus (LV-GFP) (Control) and cultured for 21 days in osteogenic medium. Alizarin red staining for calcification. 

E) qPCR analysis of KLF4 and RUNX2 in VSMCs with KLF4 overexpression. The expression of each gene in VSMC (the first column in each group) was defined as 1 under control conditions (n=3, *P< 0.01 relative to control conditions). 

F) Western blot analysis of KLF4 and RUNX2 expression in VSMCs with KLF4 overexpression.
Hyperhomocysteinemia induces vascular calcification by activating the transcription factor RUNX2 via Krüppel-like factor 4 up-regulation in mice
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