Original Article

5-aza-2'-Deoxycytidine, a DNA Methyltransferase Inhibitor, Facilitates the Inorganic Phosphorus-Induced Mineralization of Vascular Smooth Muscle Cells

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Aim: Vascular calcification, an independent risk factor for cardiovascular disease in patients with chronic kidney disease (CKD), refers to the mineralization of vascular smooth muscle cells (VSMCs) caused by phenotypic changes toward osteoblast-like cells. DNA methylation, mediated by DNA methyltransferases (DNMTs), plays an important role in the differentiation of osteoblasts. We herein assessed the effects of a DNMT inhibitor on phenotypic changes in VSMCs and the development of vascular calcification.

Methods: The effects of 5-aza-2'-deoxycytidine (5-aza-dC), a DNMT inhibitor, on human aortic smooth muscle cells (HASMCs) were evaluated. The expression and DNA methylation status of osteogenic genes were determined using RT-qPCR and bisulfite sequencing, respectively. Mineralization of HASMCs was induced by high concentrations of inorganic phosphate (Pi), as confirmed by quantitation of the calcium levels and von Kossa staining. Moreover, we examined the effects of the suppression of DNMT1 and/or alkaline phosphatase (ALP) on the mineralization of HASMCs.

Results: 5-aza-dC increased the expression and activity of ALP and reduced the DNA methylation levels of the ALP promoter region in the HASMCs. In addition, both treatment with 5-aza-dC and downregulation of the DNMT1 expression promoted the Pi-induced mineralization of HASMCs. Moreover, both treatment with phosphonoformic acid (PFA), a sodium-dependent phosphate transporter inhibitor, and suppression of the ALP expression inhibited the 5-aza-dC-promoted mineralization of HASMCs.

Conclusions: The present study showed that DNMT inhibitors facilitate the Pi-induced development of vascular calcification via the upregulation of the ALP expression along with a reduction in the DNA methylation level of the ALP promoter region.

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Key words: Vascular calcification, Vascular smooth muscle cells, Alkaline phosphatase, 5-aza-2'-deoxycytidine, DNA methylation

Introduction

Vascular calcification, which refers to the deposition of hydroxyapatite in cardiovascular tissue, decreases the elasticity and compliance of the aorta and increases the risk of cardiovascular disease (CVD)¹. Vascular

calcification is commonly observed in the setting of atherosclerosis and diabetes and is a major problem in patients with chronic kidney disease (CKD)². Recently, several studies have indicated that vascular calcification is an independent predictor of subsequent cardiovascular mortality in patients on dialysis (CKD stage 5D)^{3, 4}. In addition, hyperphosphatemia has been implicated as a risk factor for vascular calcification in patients with CKD, and the serum phosphorus levels are correlated with cardiovascular events and mortality in this population^{5, 6}. Therefore, the prevention and treatment of vascular calcification is directly linked to improvements in the quality of life among patients

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with CKD.

Recent studies suggest that vascular calcification is an active cell-mediated process similar to osteogenesis⁷⁻⁹. Osteogenic markers have been observed to be expressed in regions of arterial calcification in uremic mice fed a high-phosphate diet^{10, 11}. It has also been reported that hyperphosphatemia induces a phenotypic change in vascular smooth muscle cells (VSMCs) toward osteoblast-like cells followed by the development of vascular calcification⁹. Moreover, bone morphogenetic protein-2 (BMP-2), considered to be a reliable marker of progressive atherosclerosis in patients with CKD¹², promotes the inorganic phosphate (Pi)-induced osteoblast differentiation of VSMCs and exacerbates vascular calcification¹³. Therefore, it is important to understand the mechanisms underlying VSMC differentiation, as controlling this process should provide a useful approach to preventing and/or treating vascular calcification.

DNA methylation, a form of epigenetic modification, is known to be associated with diverse genetic events, including the gene expression, chromatin modification, genome imprinting and gene silencing¹⁴⁻¹⁸. In humans and other mammals, DNA methylation predominantly occurs at CpG di-nucleotides, and the level of methylation within the promoter region of genes is negatively correlated with the transcriptional activity. DNA methylation is mediated by a number of DNA methyltransferases (DNMTs), such as the maintenance enzyme DNMT1^{19, 20}. DNA methylation influences the development and/or progression of several diseases, including cancer, and plays an important role in the osteoblastic differentiation of stem cells^{18, 21-24}. Several studies have shown that the expression levels of several osteogenic genes are correlated with the state of DNA methylation during osteoblast differentiation²⁵. Moreover, DNMT inhibitors, such as 5-azacytidine and 5-aza-2'-deoxycytidine (5-aza-dC), have been reported to facilitate the differentiation of mesenchymal stem cells (MSCs) to osteoblasts via upregulation of the osteogenic gene expression^{26, 27}. Therefore, we hypothesized that DNMT inhibitors also affect the differentiation of VSMCs, resulting in the development of vascular calcification. The aim of this study was to determine the effects of 5-aza-dC, a representative DNMT inhibitor, on the differentiation of VSMCs and development of vascular calcification.

Methods

Cell Culture

Human aortic smooth muscle cells (HASMCs; Lonza, Walkersville, MD, USA) were routinely cul-

tured in SmGM-2 medium (Lonza). For the *in vitro* calcification assay, HASMCs (at passages 8-10) were pretreated with 5-aza-dC (Sigma, St. Louis, MO, USA) for two days in SmGM-2 medium followed by treatment with a pathological concentration of Pi in calcification medium. To prepare the calcification medium, a mixed solution of Na₂HPO₄ and NaH₂PO₄ (pH7.4) was added to DMEM containing 10% FBS to a final concentration of 3.0 mM. The HASMCs were also treated with phosphonoformic acid (PFA; Sigma), a sodium-dependent phosphate transporter inhibitor, during culture in the calcification medium. For other experiments, the HASMCs were cultured in SmGM-2 medium.

siRNA Transfection

HASMCs were transfected with 10 nM small interfering RNAs (siRNAs; Dharmacon, Chicago, IL, USA) specific for alkaline phosphatase (ALP; siGENOME SMARTpool, Human ALPL) or the control (Ctrl; ON-TARGETplus Non-Targeting Pool) using Lipofectamine RNAiMax (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Following pretreatment with 5-aza-dC, the HASMCs were simultaneously treated with the siRNAs at the start of Pi treatment.

Preparation of DNMT1 shRNA Lentivirus

The short hairpin RNA (shRNA) oligonucleotides designed for this experiment contained sense strands of human DNMT1, followed by short spacers (CTC-GAG), the reverse complements of the sense strands and five thymidines as an RNA polymerase III transcriptional stop signal. The sequences of the oligonucleotides used to generate the DNMT1 and control (Ctrl) shRNA are shown in **Table 1**. The oligonucleotides were annealed and cloned into the AgeI and EcoRI sites of the pLKO.1-hygro vector (Addgene, Cambridge, MA, USA). HEK293FT cells were transfected with both aforementioned vectors using the ViraPower Lentiviral Expression System (Lifetechnologies) and cultured at 37°C for 24 hours. After changing the medium, the cells were further incubated at 37°C for 24 hours, and the virus supernatant was collected and enriched for infection. HASMCs were plated on 100-mm dishes 24 hours before infection, and the medium was replaced with the enriched viral supernatant containing 8 µg/mL of polybrene. After two days, transformants were selected via culture in medium containing 50 µg/mL of hygromycin B.

Western Blot Assay

HASMCs were washed with cold phosphate-buff-

Table 1. Oligonucleotides used to generate shRNA

Target	Oligonucleotide sequence (5' to 3')
DNMT1 shRNA#1	
Forward	5'- CCGGTGGTCCGCATGGGCTATCAGTCTCGAG ACTGATAGCCCATGCGGACCATT'TTTTG -3'
Reverse	5'- AATTCAAAAATGGTCCGCATGGGCTATCAGT CTCGAGACTGATAGCCCATGCGGACCA -3'
DNMT1 shRNA#2	
Forward	5'- CCGGCGAGAAGAATATCGAACTCTTCTCGAG AAGAGTTCGATATTCTTCTCGTTT'TTG -3'
Reverse	5'-AATTCAAAAACGAGAAGAATATCGAACTCTT CTCGAGAAGAGTTCGATATTCTTCTCG -3'
Control (Ctrl) shRNA	
Forward	5'- CCGGCAACAAGATGAAGAGCACCAACTCGAG TTGGTGCTCTTCATCTTGTGTTT'TTG -3'
Reverse	5'- AATTCAAAACAACAAGATGAAGAGCACCAA CTCGAGTTGGTGCTCTTCATCTTGT'TG -3'

ered saline (PBS) and lysed with ice-cold lysis buffer containing 2% sodium dodecyl sulfate (SDS), 10% glycerol, and 60 mM Tris HCl (pH 6.8). In addition, the nuclear fraction of the HASMCs was prepared using a ProteoExtract Subcellular Proteome Extraction Kit (Merck Millipore, Darmstadt, Germany) and ProteoExtract Protein Precipitation Kit (Merck Millipore) according to the manufacturer's instructions. Following measurement of the protein levels using a BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA) and reduction with 0.1M dithiothreitol (DTT), the same amounts of cell lysates or nuclear fraction containing 0.01% bromophenol blue (BPB) were electrophoresed on SDS PAGE gel. The proteins were electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane (EMD Millipore, Billerica, MA, USA). The membrane was washed with Tris-buffered saline containing 0.05% Tween20 (TBS-T) and then blocked with TBS-T containing 5% non-fat dry milk (Megmilk Snow Brand Company, Tokyo, Japan) or 3% bovine serum albumin (Sigma) for one hour. After blocking, the membrane was incubated overnight at 4°C with antibodies against DNMT1 (Sigma), heat shock protein 90 α / β (HSP 90 α / β ; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), cleaved-poly (ADP-ribose) polymerase (cleaved-PARP; Cell Signaling Technology, Danvers, MA, USA), histone H3 (Cell Signaling) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EMD Millipore). The membrane was then washed with TBS-T and incubated with an appropriate secondary anti-IgG antibody conjugated to horseradish peroxidase (Cell Signaling Technology). Immunodetection was performed

using the ECL Western blotting substrate (Pierce Biotechnology) and Amersham Hyperfilm ECL (GE Healthcare, Little Chalfont, UK).

RT-qPCR

Total RNA isolated from cultured HASMCs with RNazol RT (Molecular Research Center, Cincinnati, OH, USA) was reverse transcribed using the ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO, Osaka, Japan). Real-time PCR was performed using a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) with a KAPA SYBR FAST qPCR Kit (Kapa Biosystems, Woburn, MA, USA), according to the manufacturer's instructions. The sequences of the forward and reverse primers are described in **Table 2**. Each sample was analyzed in duplicate using the standard curve method according to the manufacturer's protocol, and the data were normalized to the corresponding values of GAPDH.

Alkaline Phosphatase (ALP) Activity Assay

The activity of intracellular ALP was measured with a SensoLyte pNPP Alkaline Phosphatase Assay Kit (AnaSpec, Fremont, CA, USA), according to the manufacturer's instructions. The measurements were compared to those of an alkaline phosphatase standard and normalized using the total protein levels obtained with the BCA protein assay kit (Pierce Biotechnology).

Bisulfite Sequencing

Total genomic DNA was isolated from cultured

Table 2. Primer sequences for RT-qPCR and bisulfite sequences

Target		Oligonucleotide sequence (5' to 3')
For RT-qPCR		
ALP	Forward	5'- GGGACTGGTACTCAGACAACG -3'
	Reverse	5'- CCCATGATCACGTCAATGTC -3'
Runx2	Forward	5'- TTACTTACACCCCGCCAGTC -3'
	Reverse	5'- TATGGAGTGCTGCTGGTCTG -3'
Msx2	Forward	5'- CTGGTGAAGCCCTTCGAGAC -3'
	Reverse	5'- AGGGCTCATATGTCTTGCGC -3'
BMP-2	Forward	5'- CTGCGGTCTCCTAAAGGTCG -3'
	Reverse	5'- CAACTCGAACTCGCTCAGGA -3'
OPN	Forward	5'- GTTTCGACAGCCTGACATCC -3'
	Reverse	5'- CATTCAACTCCTCGCTTTCC -3'
Pit-1	Forward	5'- TGCCTAATGGGAACCTTGTC -3'
	Reverse	5'- TCTTTGTACAGGCCGGAATC -3'
SM22 α	Forward	5'- AAGAATGATGGGCACTACCG -3'
	Reverse	5'- ATGACATGCTTTCCCTCCTG -3'
α -SMA	Forward	5'- TCCAGCGTTCTGAGCACCCG -3'
	Reverse	5'- GCTGCTTCACAGGATTCCCGTCT -3'
DNMT1	Forward	5'- ACATAACCAGGTTGAGCTCGG -3'
	Reverse	5'- TGAGGATGGGCTGGTACTGT -3'
DNMT3a	Forward	5'- CTCGCGATTCTCGAGTCCA -3'
	Reverse	5'- ATACCGGGAAGGTTACCCCA -3'
DNMT3b	Forward	5'- CTGTATGAGGGTACAGGCCG -3'
	Reverse	5'- CCAGGAACCGTGAGATGTCC -3'
GAPDH	Forward	5'- CTTTGTGCGTCGCCAGCCGAG -3'
	Reverse	5'- ACCAGGCGCCCAATACGACC -3'
For bisulfite sequence		
ALP Promoter	Forward	5'- GGGAGTGGTTATTTTATTAGAGTTGG -3'
	Reverse	5'- AAAACCCTTCCCTCTTCTCTC -3'

HASMCs using a NucleoSpin tissue kit (Macherey-Nagel, Düren, Germany). Bisulfite conversion of isolated total genomic DNA was then performed using a MethylEasy Xceed Rapid DNA Bisulphite Modification Kit (Human Genetic Signatures, North Ryde, NSW, Australia). In order to identify the methylation patterns of the genes, primers were designed to amplify the ALP promoter region. The sequences of the primers are described in **Table 2**. PCR was carried out using TaKaRa EpiTaq HS (Takara Bio, Shiga, Japan). The PCR products were purified using Wizard SV Gel and PCR Clean-Up Systems (Promega) and subsequently cloned into the T-Vector pMD20 (Takara Bio). The recombinant plasmid was then transformed into *Escherichia coli* strain DH5 α . Positive clones were isolated for sequencing. The methylation patterns of the genes were evaluated using the QUMA software program (<http://quma.cdb.riken.jp/top/index.html>).

Evaluation of HASMC Calcification

For quantitation of the calcium levels, the cells were decalcified with 0.6 M HCl for 24 hours. The calcium level in the HCl supernatant was determined according to the methyl xylene blue (MXB) method using the Wako Calcium E Test (Wako Pure Chemical Industries, Osaka, Japan). Following decalcification, the cells were washed with PBS and solubilized with 0.1 M NaOH/0.1% SDS. The protein levels were measured using the BCA protein assay kit. The calcium levels of the cell layers were normalized to the protein levels.

Deposition of hydroxyapatite was visualized using von Kossa staining. Briefly, after fixing with 4% paraformaldehyde, hydroxyapatite deposits were stained with 5% silver nitrate followed by 5% sodium thiosulfate. Deposition of hydroxyapatite was visualized as dark brown spots under a microscope.

Fig. 1. Treatment of HASMCs with 5-aza-dC increased the expression of genes related to osteogenesis.

Human aortic smooth muscle cells (HASMCs) were treated with 5-aza-2-deoxycytidine (5-aza-dC) for 48 hours. A, The nuclear and cytoplasmic fractions were isolated from the HASMCs and subjected to a Western blot analysis to determine the expression of DNA methyltransferase-1 (DNMT1) with heat shock protein 90α/β (Hsp90α/β) and histone H3. B, Total RNA was extracted from the HASMCs, and RT-qPCR was performed. The data (mean ± SD) are expressed as the ratio of each mRNA/*GAPDH* mRNA relative to the control (without 5-aza-dC), which was assigned a value of 1.0; * $p < 0.05$ vs the control. ($n = 3$ each). The open and closed bars indicate DMSO- and 5-aza-dC-treated HASMCs, respectively. C, The ALP activity was measured using an AnaSpec pNPP Alkaline Phosphatase Kit. The values [International Unit (IU)/g protein] are expressed as the mean ± SD; * $p < 0.05$ ($n = 3$ each).

Statistical Analysis

One-way ANOVA using Tukey-Kramer's post-hoc test was used to compare multiple groups. The unpaired t -test was performed for the statistical analysis of two groups. All results are expressed as the mean ± standard deviation (SD), and p values of < 0.05 were considered to be statistically significant.

Results

5-aza-dC Increases the Expression of Genes Related to Osteogenesis in HASMCs

Because the level of DNMT1 is reduced by 5-aza-dC treatment in several types of cells²⁸⁻³⁰, we explored the effects of 5-aza-dC on the protein levels of DNMT1 in HASMCs. As shown in **Fig. 1A**, Hsp 90α/β and histone H3 served as cytoplasmic and nuclear markers, respectively. 5-aza-dC reduced the DNMT1 expression in the nuclear fraction of the HASMCs. Furthermore, it has been reported that the osteogenic gene expression is increased by DNMT inhibitors in MSCs and bone marrow stromal cells^{26, 27}. Therefore, we evaluated the effects of 5-aza-dC on the expression of osteogenic genes in HASMCs. The RT-qPCR results showed that the expression of ALP, a marker of early osteoblast differentiation^{31, 32}, was increased by 5-aza-dC pretreatment in the HASMCs (**Fig. 1B**). 5-aza-dC also increased the expression of BMP2, msh homeobox 2 (*Mx2*) and type III sodium-dependent phosphate cotransporter (*Pit-1*), which are known to play

important roles in osteoblast mineralization, in the HASMCs. In contrast, treatment with 5-aza-dC did not alter the expression of runt-related transcription factor 2/Core binding factor a-1 (*Runx2/Cbfa-1*) or osteopontin (*OPN*), an osteogenic master transcription factor and osteoblast marker, respectively, in the HASMCs. Additionally, 5-aza-dC increased the expression of smooth muscle protein 22-α (*SM22α*) and α-smooth muscle actin (*α-SMA*), smooth muscle (SM) lineage genes, and significantly increased the ALP activity in the HASMCs (**Fig. 1C**). These results suggest that 5-aza-dC pretreatment partly converts the phenotype of HASMCs toward that of osteoblast-like cells.

5-aza-dC Reduces the DNA Methylation Level of the ALP Promoter Region in HASMCs

5-aza-dC is a typical inhibitor of DNMT1 that is widely used to reduce the levels of DNA methylation. A recent study suggested the important role of DNA methylation in the regulation of the ALP expression in human osteoblast lineage cells³³. In order to examine whether reductions in the DNA methylation levels are accompanied by the upregulation of the ALP expression in HASMCs treated with 5-aza-dC, the DNA methylation status of the ALP promoter region was determined using bisulfite sequencing. The results showed that the methylation levels were reduced from 86.7% to 55.6% in the HASMCs treated with 5-aza-dC (**Fig. 2A**). Notably, demethylation of CpGs was

Fig. 2. Treatment with 5-aza-dC reduced the DNA methylation level of the ALP promoter region in the HASMCs.

HASMCs were treated with 5-aza-dC for 48 hours. Total genomic DNA was extracted from the HASMCs, and the ALP promoter region from nucleotide positions -982 to -594 (relative to the transcriptional start site) was subjected to bisulfite sequencing. Five positive clones were sequenced. A, Each row represents one clone with one circle symbolizing one CpG. The closed circles indicate methylated CpG, and the open circles indicate unmethylated CpG. B, Percentage of individual methylated CpGs (closed circles in A) in DMSO-treated (solid line) or 5-aza-dC-treated HASMCs (dashed line). The position relative to the transcription start site is indicated.

observed at several sites, with the exception of the sixth and seventh sites of the ALP promoter region (Fig. 2B).

5-aza-dC Facilitates the Mineralization of HASMCs Under High-Phosphorus Conditions

We next examined the effects of 5-aza-dC on the mineralization of HASMCs treated with or without

excessive Pi stimulation. Similar to the results shown in Fig. 1B, 5-aza-dC pretreatment increased the ALP expression in the HASMCs under both normal and high-phosphorus conditions (Fig. 3A). Moreover, the calcium levels in the HASMCs were increased by excessive Pi stimulation; this increase was significantly enhanced by 5-aza-dC pretreatment (Fig. 3B). Pi-induced hydroxyapatite deposition was also signifi-

Fig. 3. Treatment of HASMCs with 5-aza-dC promoted Pi-induced vascular calcification.

HASMCs were pretreated with 5-aza-dC, after which vascular calcification was induced with 3.0 mM of Pi. A, After 24 hours, total RNA was extracted from the HASMCs and analyzed using RT-qPCR. The data (mean $\pm$ SD) are expressed as the ratio of *ALP* mRNA/*GAPDH* mRNA relative to the control (Pi-/DMSO), which was assigned a value of 1.0; * $p < 0.05$. ($n = 3$ each). The open and closed bars indicate DMSO- and 5-aza-dC-treated HASMCs, respectively. B, After 14 days, the calcium levels of the cultures were determined according to the MXB method and normalized to the total cellular protein levels. The values are expressed as the mean $\pm$ SD; * $p < 0.05$ ($n = 3$ each). C, Mineralization of HASMCs was confirmed using von Kossa staining and visualized as dark brown spots under a microscope. Scale bar: 100 μ m. D, Total protein was extracted from the HASMCs and subjected to a Western blot analysis with antibodies against cleaved PARP and GAPDH. Total protein from HASMCs treated with staurosporine (1 μ g/mL) for three hours served as a positive control.

cantly increased by 5-aza-dC pretreatment (**Fig. 3C**). Although apoptosis is known to be a key regulator of vascular calcification^{34, 35}, we were unable to determine the expression of cleaved PARP, a marker of apoptosis, in the cells exhibiting Pi-induced vascular calcification with or without 5-aza-dC pretreatment (**Fig. 3D**). Furthermore, because Pi uptake via Pit-1 plays an important role in the development of Pi-induced vascular calcification³⁶⁻³⁸, we assessed the effects of PFA, a Pit-1 inhibitor, on the 5-aza-dC-promoted mineralization of HASMCs. PFA blocked Pi-

induced calcium deposition in the HASMCs treated with or without 5-aza-dC pretreatment, as confirmed by the quantitation of calcium deposition and von Kossa staining (**Fig. 4A, B**).

Downregulation of the DNMT1 Expression Promotes Pi-Induced Vascular Calcification

Next, we examined whether downregulation of the DNMT1 expression also enhances the ALP expression and mineralization of HASMCs using shRNA. The RT-qPCR results showed that each shRNA against

Fig. 4. PFA inhibited Pi-induced vascular calcification with or without 5-aza-dC pretreatment.

HASMCs were pretreated with 5-aza-dC, after which vascular calcification was induced with 3.0 mM of Pi and 100 µM of PFA for 14 days. A, The calcium levels of the cultures were determined according to the MXB method and normalized to the total cellular protein levels. The values are expressed as the mean $\pm$ SD; * p < 0.05 (n = 3 each); N.S., not significant. B, Mineralization of HASMCs was confirmed using von Kossa staining and visualized as dark brown spots under a microscope. Scale bar: 100 µm.

DNMT1 reduced the expression of DNMT1, but not DNMT3a or 3b, in the HASMCs (**Fig. 5A**). Similar to the results shown in **Fig. 1B**, downregulation of the DNMT1 expression increased the expression of ALP in the HASMCs (**Fig. 5B**). Moreover, the Pi-induced mineralization of HASMCs was significantly promoted by the downregulation of the DNMT1 expression (**Fig. 5C, D**). These data suggest that downregulation of the DNMT1 expression promotes Pi-induced vascular calcification.

Suppression of the ALP Expression inhibits Pi-Induced Vascular Calcification Following 5-aza-dC Pretreatment

Because the ALP expression levels affect the development of Pi-induced vascular calcification^{39, 40}, we assessed whether upregulation of the ALP expression plays a significant role in 5-aza-dC-promoted vascular calcification using siRNA. The RT-qPCR results showed that transfection of ALP siRNA reduced the ALP expression in the HASMCs treated with or without 5-aza-dC pretreatment (**Fig. 6A**). Next, we induced the mineralization of HASMCs using Pi stimulation and 5-aza-dC pretreatment under conditions of knockdown of the ALP expression. Quantitation of the calcium levels and von Kossa staining showed that suppression of the ALP expression inhibited the 5-aza-dC-promoted mineralization of HASMCs (**Fig. 6B, C**). In addition, the degree of Pi-induced vascular calcification was significantly reduced by the knockdown of the ALP expression without 5-aza-dC pretreatment. These results suggest that upregulation of the ALP expression plays an important role in the progression of Pi-induced vascular calcification.

Discussion

The purpose of this study was to determine the effects of DNMT inhibitors on the incidence of vascular calcification. The results demonstrated the effects of 5-aza-dC on VSMCs, which play a central role in the development of vascular calcification. Our findings showed that 5-aza-dC increases the expression and activity of ALP in HASMCs and reduces the DNA methylation level of the ALP promoter region. We further showed that excessive Pi stimulation induces the mineralization of HASMCs, which is promoted by 5-aza-dC pretreatment. Moreover, downregulation of the DNMT1 expression using shRNA enhanced the ALP expression and Pi-induced mineralization of the HASMCs. Finally, both PFA treatment and suppression of the ALP expression by siRNA transfection inhibited the 5-aza-dC-promoted mineralization of the HASMCs. These results suggest that 5-aza-dC enhances the mineralization of HASMCs with Pi stimulation via upregulation of the ALP expression and reductions in the DNA methylation level of the ALP promoter region.

A recent study reported that 5-aza-dC increases the expression of osteogenic genes in MSCs²⁷. Similarly, our data showed that 5-aza-dC increases the expression of ALP, BMP-2 and Msx2 in HASMCs. However, the expression of Runx2 or OPN was not altered by 5-aza-dC treatment in the present study,

Fig. 5. Downregulation of the DNMT1 expression promoted Pi-induced vascular calcification in the HASMCs.

HASMCs were infected with DNMT1 shRNA#1, #2 or Control (Ctrl) shRNA. A, Total RNA was extracted from the cells and analyzed using RT-qPCR. The data (mean $\pm$ SD) are expressed as the ratio of each mRNA/*GAPDH* mRNA relative to the control (Ctrl shRNA), which was assigned a value of 1.0; * p < 0.05. (n = 3 each); N.S., not significant. B, Vascular calcification was induced in these cells with 3.0 mM of Pi for four days. The calcium levels of the cultures were determined according to the MXB method and normalized to the total cellular protein levels. The values are expressed as the mean $\pm$ SD; * p < 0.05 (n = 3 each). C, Mineralization of HASMCs was confirmed using von Kossa staining and visualized as dark brown spots under a microscope. Scale bar: 100 μ m.

suggesting that 5-aza-dC induces a phenotypic change from HASMCs to osteoblast-like cells, but not osteoblast cells themselves. It has been reported that the upregulation of ALP promotes the mineralization of nonosteogenic cells in the presence of β -glycerophosphate³⁹. Our previous study also showed that an increased ALP expression contributes to the promotion of vascular calcification in the presence of trichostatin A (TSA), a representative histone deacetylase (HDAC) inhibitor⁴⁰. Accordingly, in the present study, the increased ALP expression induced by 5-aza-dC promoted the mineralization of HASMCs, while suppression of the ALP expression inhibited the development of Pi-induced vascular calcification with or

without 5-aza-dC pretreatment. Moreover, there were no statistical differences in calcium deposition between the cells treated with or without 5-aza-dC pretreatment under conditions of a suppressed ALP expression, indicating that ALP plays an important role in 5-aza-dC-promoted vascular calcification under high-phosphorus conditions.

Moreover, our data also showed that 5-aza-dC increases the expression of SM lineage genes, such as SM22 α and α -SMA. Although the relationship between SM lineage genes and vascular calcification is controversial, it has been reported that the expression of myocardin, an upstream gene of SM22 α and α -SMA, has no effect on the development of Pi-induced vascu-

lar calcification⁴¹). Therefore, the upregulation of SM lineage genes was not associated with the 5-aza-dC-promoted mineralization of HASMCs in the present study. Additional studies are needed to further understand the effects of SM lineage genes on the incidence of vascular calcification.

A recent study reported that 5-aza-dC increases the expression of ALP in osteoblast lineage cells³³. 5-aza-dC is also known to reduce the expression of DNMT1 in mammalian cells. In the present study, 5-aza-dC decreased the DNMT1 expression and increased the ALP expression in HASMCs. In addition, downregulation of the DNMT1 expression using shRNA also increased the ALP expression in HASMCs, indicating the possibility that the upregulation of the ALP expression induced by 5-aza-dC treatment is associated with a decline in the DNMT1 expression. DNA methylation occurs at CpG dinucleotides and is regulated by a number of DNMTs, including the maintenance enzyme DNMT1 and *de novo* methyltransferases DNMT3a/3b^{19, 20}. Therefore, DNMT inhibitors reduce the DNA methylation levels. Our present study showed that 5-aza-dC reduces the DNA methylation level of the ALP promoter region. In most cases, DNA methylation patterns are inversely correlated with the expression levels of several genes, including ALP^{33, 42}. In addition, we confirmed that DNA methylation of the ALP promoter region reduced the promoter activity (**Supplemental Fig. 1**). These data suggest that 5-aza-dC decreases the DNA methylation level of the ALP promoter region followed by upregulation of its expression and that the ALP expression is regulated by DNMTs in VSMCs.

In this study, we also confirmed that 5-aza-dC increases the expression of Pit-1 in HASMCs. Recent studies have shown that an excessive Pi uptake via Pit-1 plays a key role in the development of Pi-induced vascular calcification³⁶, while suppression of Pit-1 using siRNA and PFA inhibits Pi-induced vascular calcification^{37, 38}. Consistent with these reports, PFA treatment prevented the Pi-induced mineralization of HASMCs treated with or without 5-aza-dC pretreatment in the present study. Therefore, an excessive Pi uptake via Pit-1 may play an important role in the formation of 5-aza-dC-promoted vascular calcification under high phosphorus conditions. Further studies are required to understand the effects of DNMT inhibitors on the Pi uptake via Pit-1 in HASMCs.

Recently, several studies have demonstrated that epigenetic modifications, such as DNA methylation and histone acetylation, are associated with the development of several diseases, including cancer and psychiatric disorders^{21-24, 43-46}. Therefore, modulating such

Fig. 6. Suppression of the ALP expression inhibited Pi-induced vascular calcification in the HASMCs pretreated with 5-aza-dC.

HASMCs were pretreated with 5-aza-dC, after which vascular calcification was induced via excessive Pi stimulation with or without knockdown of the *ALP* expression. **A**, After one day, total RNA was extracted from the HASMCs and analyzed using RT-qPCR. The data (mean $\pm$ SD) are expressed as the ratio of *ALP* mRNA/*GAPDH* mRNA relative to the control (Pi-/DMSO/*Ctrl* siRNA), which was assigned a value of 1.0; * p < 0.05 (n = 3 each); N.S., not significant. **B**, After 10 days, the calcium levels of the cultures were determined according to the MXB method and normalized to the total cellular protein levels. The values are expressed as the mean $\pm$ SD; * p < 0.05 (n = 3 each); N.S., not significant. **C**, Mineralization of HASMCs was confirmed using von Kossa staining and visualized as dark brown spots under a microscope. Scale bar: 100 μ m.

modifications using DNMT and/or HDAC inhibitors may serve as a new approach to therapy for these diseases. Moreover, DNA methylation and renal fibrosis, a common pathology in patients with CKD⁴⁷⁾, have been shown to be related. The injection of indoxyl sulfate induces renal fibrosis and DNA hypermethylation of the *klotho* gene and decreases the *klotho* expression in the renal tubules in mice⁴⁸⁾. Moreover, 5-azacytidine ameliorates experimental renal fibrosis⁴⁹⁾, thus indicating the possibility that DNMT inhibitors may serve as therapeutic agents against CKD. However, considering that DNMT inhibitors are systemically administered in patients with CKD, it is important to determine the effects on representative complications, such as vascular calcification. In the present study, treatment with 5-aza-dC exacerbated vascular calcification at high, but not normal, phosphorus levels. Previous studies have reported that the serum phosphorus levels are elevated in patients with advanced (stage 4/5), but not early (stage 1-3), CKD^{50, 51)}. Therefore, DNMT inhibitors may not affect the risk of cardiovascular mortality in early-stage CKD patients, while increasing the risk of cardiovascular mortality in the setting of advanced-stage CKD. Accordingly, treatment with DNMT inhibitors is considered useful in patients with early-stage CKD, although it may not be beneficial in those with advanced-stage CKD.

An association between epigenetic modification and the incidence of CVD has been established based on findings that genomic DNA hypomethylation occurs in advanced atherosclerotic lesions in human patients as well as in mice deficient in apolipoprotein E⁵²⁾. Moreover, one study found that TSA exacerbates atherosclerosis in low-density lipoprotein receptor (LDLR)-deficient mice⁵³⁾. In contrast, other reports have demonstrated the protective effects of HDAC inhibitors both *in vitro* and *in vivo* in a mouse model of neointimal thickening^{54, 55)}. Although the reasons for these discrepancies remain unknown, epigenetic modification may play a beneficial or adverse role in several types of CVD. Moreover, although little is known about the association between epigenetic modification and vascular calcification, Takemura *et al.* demonstrated that the downregulation of sirtuin-1 (SIRT1), a member of the HDAC family, due to cellular aging and/or siRNA transfection promotes Pi-induced vascular calcification⁵⁶⁾. In addition, our previous report showed that an HDAC inhibitor promoted the development of Pi-induced vascular calcification via the upregulation of the ALP expression⁴⁰⁾. Furthermore, our present study showed, for the first time, that both 5-aza-dC and downregulation of the DNMT1 expression enhance Pi-induced vascular cal-

cification, suggesting that epigenetic modification induced by the inhibition of DNMTs and/or HDACs plays an important role in the development and progression of vascular calcification. Further studies evaluating epigenetic modifications, HDACs, and DNMTs may lead to the development of novel therapies for vascular calcification.

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Supplemental Methods

Plasmid Construction

We prepared ALP promoter specific-methylated luciferase plasmids according to the methods of Tomikawa (J Biol chem, 2006; 281:12163-12169). Briefly, a GLuc-ON promoter Reporter Clone (GeneCopoeia, Rockville, MD, USA), including the ALP promoter region (from - 1085 to + 260 relative to the transcriptional start site), was digested with Bgl II and Hind III, releasing two DNA fragments (the ALP promoter region and a promoterless pEZX-PG04 luciferase vector). The ALP promoter region was methylated via SssI methylase (New England Biolabs, Beverly, MA, USA) and ligated with the promoterless luciferase vector (pEZX-PG04-meALP). As a control, the unmethylated ALP promoter region was also ligated with the

vector (pEZX-PG04-umALP). The methylation status of the ALP promoter region was confirmed via digestion with Msp I (methylation non-sensitive restriction enzyme: TaKaRa) and Hpa II (methylation-sensitive restriction enzyme: TaKaRa).

Transfection and Promoter Luciferase Assay

HEK293FT cells were replated on 24-well plates at 1.6×10^5 cells/well one day prior to transfection. All plasmids were transfected into these cells using FuGENE HD Transfection Reagent (Promega). After 48 hours, the luciferase activity was determined using a Secrete-Pair Dual Luminescence Assay Kit (GeneCopoeia), according to the manufacturer's instructions, and the promoter activity was normalized to the corresponding SEAP activity.

Supplemental Fig. 1.