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MicroRNAs that target Ca^{2+} transporters are involved in vascular smooth muscle cell calcification

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The role of microRNAs (miRNAs) in vascular calcification is currently unclear. To examine how miRNAs are involved in vascular smooth muscle cell (VSMC) calcification, we explored the alteration of miRNAs in VSMC calcification *in vitro* and *in vivo*. Klotho homozygous mutant mice (*kl/kl*) display vascular calcification and have perturbations of calcium handling. We therefore hypothesized that the calcium perturbations in VSMCs could be mediated by miRNAs. Using an miRNA array analysis, we demonstrated that miRNAs are aberrantly expressed in the aortic media of 3-week-old *kl/kl* mice compared with wild-type (WT) mice. The expression levels of miR-135a*, miR-762, miR-714, and miR-712* in the aortic media of *kl/kl* mice were significantly higher than in WT mice. We used quantitative real-time reverse transcriptase polymerase chain reaction to further confirm that these miRNAs were increased in the aortic media of *kl/kl* mice and in cultured VSMCs treated with high phosphate and calcium. A search of the miRNA database indicated that the Ca^{2+} efflux proteins NCX1, PMCA1, and NCKX4 frequently appeared as potential targets of these miRNAs. The transfection of miRNA mimics into cultured VSMCs reduced the protein levels of each potential target. Conversely, miRNA inhibitors reduced phosphate and calcium-induced VSMC calcification. Furthermore, these inhibitors decreased the intracellular Ca^{2+} concentration in cultured VSMCs after treatment with phosphate and calcium. Our results suggest that increased expression of miR-135a*, miR-762, miR-714, and miR-712* in VSMCs may be involved in VSMC calcification by disrupting Ca^{2+} efflux proteins.
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Vascular calcification is a common complication in patients with chronic kidney disease (CKD), and the extent of vascular calcification is a predictor of subsequent vascular mortality.[1] A fraction of patients with CKD are deficient in Klotho, suggesting that the lack of Klotho may be a key factor to control calcification of the vasculature.[2] Klotho functions as a co-receptor of fibroblast growth factor (FGF)-23 with FGFR.[3,4] Klotho is expressed in the renal distal convoluted tubules and parathyroid cells. Klotho mediates the role of FGF-23 in the control of phosphate (Pi) and calcium (Ca) concentration.[5] Klotho homozygous mutant mice (*kl/kl*) display premature aging and vascular calcification due to hyperphosphatemia, presenting the symptoms of CKD-associated bone and mineral disorder.[6]

Although the mechanisms of vascular calcification are not fully understood, abnormalities in mineral metabolism are considered to be important risk factors. Epidemiological studies have highlighted the impact of dysregulated mineral metabolism and have linked elevated Pi and Ca to accelerated vascular calcification.[7] *In-vitro* studies using human vascular smooth muscle cells (VSMCs) have provided mechanistic insights into the role of Pi and Ca in the initiation and progression of calcification.[8] However, the contribution of dysregulated Pi and Ca to vascular calcification is still not completely understood at the molecular level.

MicroRNAs (miRNAs) are endogenous, noncoding single-stranded RNAs of ~22 nucleotides that are considered to be a novel class of gene regulators.[9] The biological roles of only a small fraction of identified miRNAs have been elucidated to date. A growing body of evidence suggests that miRNAs are important regulators of cell growth, differentiation, and apoptosis,[10] but only a small number of the hundreds of identified miRNAs have been characterized.[11,12] miRNAs play important roles not only in normal development and physiologic conditions but also in pathogenic processes.[13] Investigations of their role in cardiovascular biology are in

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the early stages.[14] Most research in this area focuses on the relationship between miRNAs and proliferative vascular diseases, such as neointimal lesion formation.[15]

Although miRNAs are expressed in the cardiovascular system, the roles of these miRNAs in vascular diseases are still unclear. In this study, we used *kl/kl* mice to examine whether miRNAs are involved in VSMC calcification. We also tested whether miRNAs have any potential regulatory effects on Pi- and Ca-induced calcification of VSMCs *in vitro* and *in vivo*. We hypothesized that a specific group of miRNAs play important roles in vascular calcification.

MATERIALS AND METHODS

Animals

Klotho mice were purchased from CLEA Japan, and the heterozygous (HT) offspring were crossed to produce homozygous mutant (*kl/kl*) animals. All of the experiments using animals in this study were performed in accordance with the guidelines of the Animal Studies Committee of Wakayama Medical University.

Cell Culture and Induction of Calcification

Aortas were isolated from wild-type (WT) and *kl/kl* mice after euthanasia by cervical dislocation. The aortic media was isolated using Dumont hand-crafted tweezers under stereomicroscope in iced PBS. Aortic VSMCs from 7-week-old WT mice were obtained with enzymatic dissociation with collagenase (Worthington) and lyophilized crystalline enzyme dispase I (Sanko Junyaku, Japan) as previously described[11] and were cultured in DMEM containing 10% FBS. The medium was changed every 2 days. Using $CaCl_2$ and NaH_2PO_4, graded concentrations of ionic Pi and Ca were added to DMEM containing 10% FBS to produce four *in-vitro* conditions as described previously: control medium (1.0 mM Pi + 1.8 mM Ca), high Pi medium (2.0 mM Pi + 1.8 mM Ca), high Ca medium (1 mM Pi + 2.7 mM Ca), and high Ca + Pi medium (2 mM Pi + 2.7 mM Ca). VSMCs were incubated for 7 days in these culture media for all of the experiments.[16]

Determination of Calcification

The calcification of VSMCs was identified by von Kossa staining as described elsewhere.[17] For quantification of Ca deposition, VSMCs were decalcified with 0.6 M HCl for 24 h, and the calcium content in the supernatant was determined using a calcium colorimetric assay kit (BioVision, Mountain View, CA, USA).[18]

Fluo4 Staining

Intracellular calcium concentrations were determined using the Fluo4 NW kit (Invitrogen) as described previously.[19] VSMCs on fibronectin-coated glass coverslips were washed in Hanks' balanced salt solution (HBSS; Invitrogen) and resuspended in HBSS supplemented with 25 mM HEPES buffer (Invitrogen) containing Fluo4 NW (Invitrogen) at 37 °C for 30 min and subsequently at room temperature for an additional 30 min. The cells were stimulated with high Ca and/or high Pi medium and were visualized at time increments of 0.2 s. The average autofluorescence levels were calculated for 12 cells at each time point in three separate experiments with a Zeiss LSM 700 microscope system. The standard equation $[Ca^{2+}]i = Kdx(F–Fmin)/(Fmax–F)$ was used to calculate the experimental values of $[Ca^{2+}]$.

Knockdown and Overexpression of miRNAs in Cultured VSMCs

The miRNA mimics and inhibitors (Qiagen, Valencia, CA, USA) were transfected according to an established protocol.[11] Briefly, subconfluent cultured VSMCs were transfected using a transfection reagent (Qiagen) according to the manufacturer's instructions. miRNA mimics and inhibitors were added directly to the transfection complexes at a final concentration of 50 nmol/l. The medium was replaced with regular culture medium 4 h after transfection, and the cells were cultured for another 72 h. For miRNA mimics, Syn-has-miR-1 miScript miRNA and AllStars Negative control siRNA (Qiagen) were used as positive and negative controls, respectively. miScript Inhibitor Negative Control (Qiagen) was used as the negative control for miRNA inhibitors.

Microarray Analysis

Total RNA was isolated from the aortic media of 3-week-old WT and *kl/kl* mice using a miRNeasy kit (Qiagen). Five hundred nanograms of total RNA were labeled with Hy5 and hybridized to 3D-Gene (TORAY). The slides were scanned on a ScanArray Express HT (Perkin-Elmer). The acquired images were analyzed in TORAY.

Quantitative Real-Time Reverse Transcriptase Polymerase Chain Reaction (*q*RT–PCR)

Briefly, *q*RT–PCR was performed using the TaqMan MicroRNA reverse transcription kit according to the manufacturer's protocol. SnoRNA202 was used as an endogenous control for normalization. Each reaction was performed in triplicate. The threshold cycle (Ct) was set in the exponential phase, and the relative gene expression was calculated by comparing cycles with each target PCR. The relative expression levels were subsequently calculated using the $2^{-\Delta\Delta Ct}$ method as described previously.[11] For mRNA analysis, the mRNA level of each gene was normalized to the *GAPDH* level of each sample. The gene-specific *q*RT–PCR primers are shown in Supplementary Table 2.

Western Blot Analysis

Aortic media and cultured VSMCs were homogenized in ice-cold buffer containing 100 mmol/l NaCl, 1% Triton X-100, 10% glycerol, 50 mmol/l HEPES, pH 7.4, and 1 mmol/l EDTA with protease inhibitors. After electrophoresis and transfer to nitrocellulose membranes, the membranes were blocked overnight with 5% nonfat milk and were incubated with NCX1 (1:2000, Santa Cruz), NCKX4 (1:2000), or PMCA (1:2000,

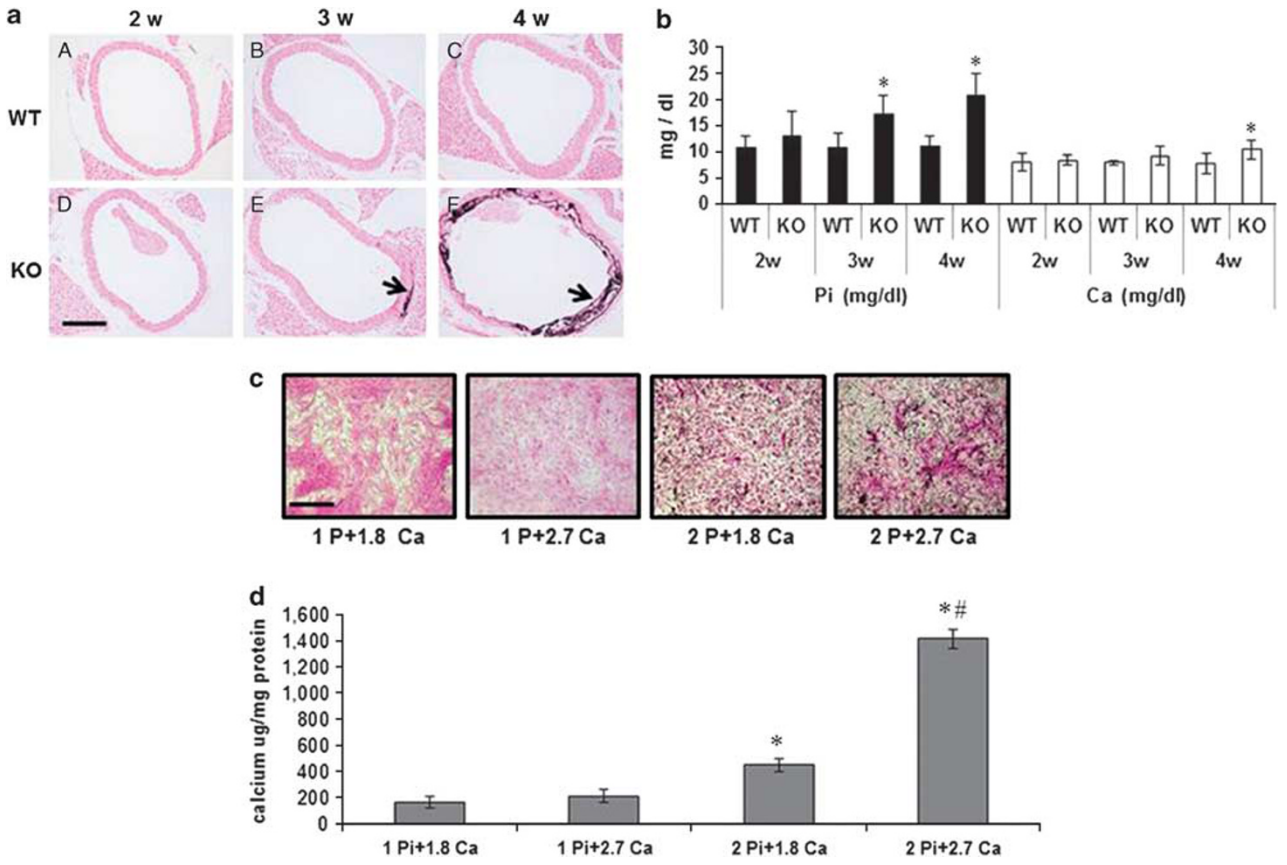

Figure 1 Phosphate and calcium have a synergistic effect on the induction of vascular calcification *in vivo* and *in vitro*. (**a**) Von Kossa staining of coronal sections of aortas from WT and *kl/kl* mice (arrows). Note that aortic medial calcification begins at 3 weeks in *kl/kl* mice. Scale bar: 200 μm. (**b**) Serum concentration of phosphate (Pi) and calcium (Ca) in WT and *kl/kl* mice. The data are presented as the means ± s.d. *$P<0.05$ when compared with WT mice of the corresponding age ($n=10$) by unpaired *t*-test. (**c**) Von Kossa staining of cultured VSMCs from WT mice treated with 1 mM Pi + 1.8 mM Ca (1 Pi + 1.8 Ca), 1 mM Pi + 2.7 mM Ca (1 Pi + 2.7 Ca), 2 mM Pi + 1 mM Ca (2 Pi + 1 Ca), or 2 mM Pi + 2.7 mM Ca (2 Pi + 2.7 Ca) for 7 days. Scale bar: 100 μm. The representative pictures are from three independent experiments. (**d**) Ca content of cultured VSMCs treated with 1 mM Pi + 1.8 mM Ca, 1 mM Pi + 2.7 mM Ca, 2 mM Pi + 1 mM Ca, or 2 mM Pi + 2.7 mM Ca for 7 days. *$P<0.05$ *vs* 1 mM Pi + 1.8 mM Ca, and $P<0.05$ *vs* 1 mM Pi + 2.7 mM Ca by one-way ANOVA followed by Student–Newman–Keul's test. The experiments were repeated in triplicate and the data are presented as the mean ± s.d.

Abcam) antibodies. The NCKX4 antibody was obtained by immunizing a rabbit with the NCKX4 peptide CLRIMITNKFGPRTRLRMAS conjugated with KLH. β-Actin antibodies (Invitrogen) were used as a control for normalization.

Statistical Analysis

All experiments were performed at least in triplicate for each treatment and were repeated in three independent experiments. The data represent the mean ± s.d. and were analyzed by Student's *t*-test and a one-way ANOVA with a Student–Newman–Keuls test (SPSS, 13.0). The data were considered to be statistically significant when $P<0.05$.

RESULTS

Pi and Ca have a Synergistic Effect on Inducing VSMC Calcification *In Vivo* and *In Vitro*

To study the mechanisms of Pi- and Ca-induced calcification, we compared the aortas of *kl/kl* mice with those of WT mice. At 3 weeks, medial calcification was observed in the aortic media of KO mice, and this calcification gradually progressed in severity with age; conversely, no calcification was observed in WT aortas (Figure 1a).

Serum Pi and Ca concentrations increased in *kl/kl* mice in a time-dependent manner, whereas no change was observed in WT mice (Figure 1b). To further determine which ion was responsible for the vascular calcification, cultured WT VSMCs were exposed to high Pi (2 mM Pi with 1.8 mM Ca), high Ca (1 mM Pi with 2.7 mM Ca), or high Pi and Ca (2 mM Pi with 2.7 mM Ca) for 7 days. Von Kossa staining showed significant calcification in cultured VSMCs treated with high Pi and Ca medium (Figure 1c). The cultured VSMCs treated with high Ca alone did not increase in Ca content, whereas the VSMCs treated with high Pi alone showed a 2.7-fold increase in Ca content (Figure 1d). The VSMCs treated with high Pi and Ca showed a significant increase in Ca content (Figure 1d). From these results, we conclude that high Pi and Ca together have a synergistic effect on VSMC calcification compared with high Pi alone.

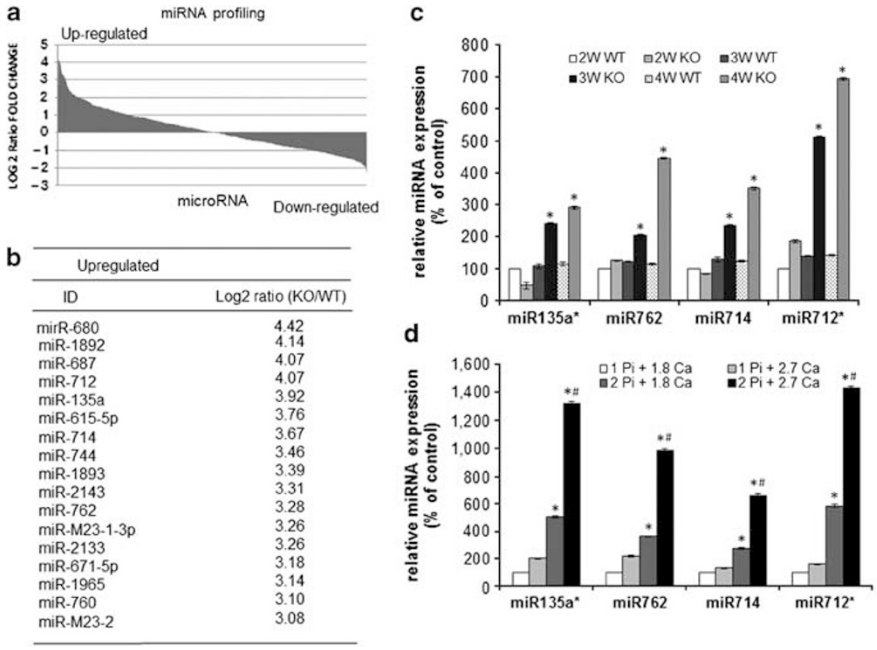

Figure 2 Specific miRNAs are expressed in the aortic media of *kl/kl* mice and in cultured VSMCs exposed to high concentration of Pi and Ca. (**a**) The miRNA expression profile in the aortic media of 3-week-old *kl/kl* mice ($n=10$) determined by Exiqon mercury LNA microRNA array. Data are expressed as the $\log_2$-fold change compared with WT mice ($n=10$). (**b**) List of the top 17 upregulated miRNAs. (**c**) Expression of miR-135a*, miR-762, miR-714, and miR-712* in the aortic media of 2- (2W), 3- (3W), and 4-week-old (4W) *kl/kl* mice ($n=7$ for each age) and their WT controls ($n=9$ for each age), as determined by qRT–PCR. $^{*}P<0.05$ compared with WT mice of the same age by one-way ANOVA followed by Student–Newman–Keul's test. (**d**) Expression of miR-135a*, miR-762, miR-714, and miR-712*, as determined by qRT–PCR in cultured VSMCs treated with the same conditions as in Figure 1c. $^{*}P<0.05$ *vs* 1 mM Pi + 1.8 mM Ca, $P<0.05$ *vs* 1 mM Pi + 2.7 mM Ca, and $^{\S}P<0.05$ *vs* 2 mM Pi + 1.8 mM Ca by one-way ANOVA followed by Student–Newman–Keul's test. The experiments were repeated in triplicate, and the data are presented as the mean ± s.d.

Many miRNAs Are Expressed in the Aortic Media of *kl/kl* Mice

To further study the biological functions of miRNAs in the aortic calcification that occur in *kl/kl* mice, we performed a miRNA array analysis using the aortic media from 3-week-old WT and *kl/kl* mice. We found that 17 miRNAs were upregulated more than threefold ($\log_2$ ratio) in *kl/kl* mice compared with WT mice (Figure 2a and b). The downregulated miRNAs in *kl/kl* mice are presented in the Supplementary data (Supplementary Table 1). It is therefore possible that the expression of many species of miRNAs is altered in the pathogenesis of VSMC calcification.

Next, we used the bioinformatics program miRanda-mirSVR to identify which potential target genes of these miRNAs could contribute to VSMC calcification. Intriguingly, we found that genes encoding calcium transport proteins, such as sodium/calcium exchange member 1 (NCX1), plasma membrane calcium pump isoform 1 (PMCA1), and sodium/potassium/calcium exchange member 4 (NCKX4), frequently appeared in the databases as the target genes of most highly upregulated miRNAs, including miR-135a*, miR-762, miR-714, and miR-712*. Therefore, we proposed that NCX1, PMCA1, and NCKX4 are the potential targets of miR-135a* and miR-762, miR-714, and miR-712* (Table 1).

Table 1 MicroRNAs and their potential target genes

ID of microRNA	Potential target gene (miRanda-mirSVR)
Mmu-miR712*	NCKX4
Mmu-miR135a*	NCX1
Mmu-miR714	PMCA1
Mmu-miR762	NCX1

We focused on miR-135a*, miR-762, miR-714, and miR-712* and their association with VSMC calcification.

In agreement with the results from the microarray analysis, miR-135a*, miR-762, miR-714, and miR-712* were highly upregulated in the aortic media of 3- and 4-week-old *kl/kl* mice; alternatively, no significant increase was observed in the expression of these miRNAs in WT mice (Figure 2c). Interestingly, in ~10% of Klotho HT mice 6 weeks of age or older, minor aortic calcification was observed, which was caused by high Pi concentration (19.01 mg/dl in average). The expression of miR-135a*, miR-762, miR-714, and miR-712* was significantly increased in the aortic media with calcifications from 7-week-old HT mice compared with those without calcifications (Supplementary Figure 4). These

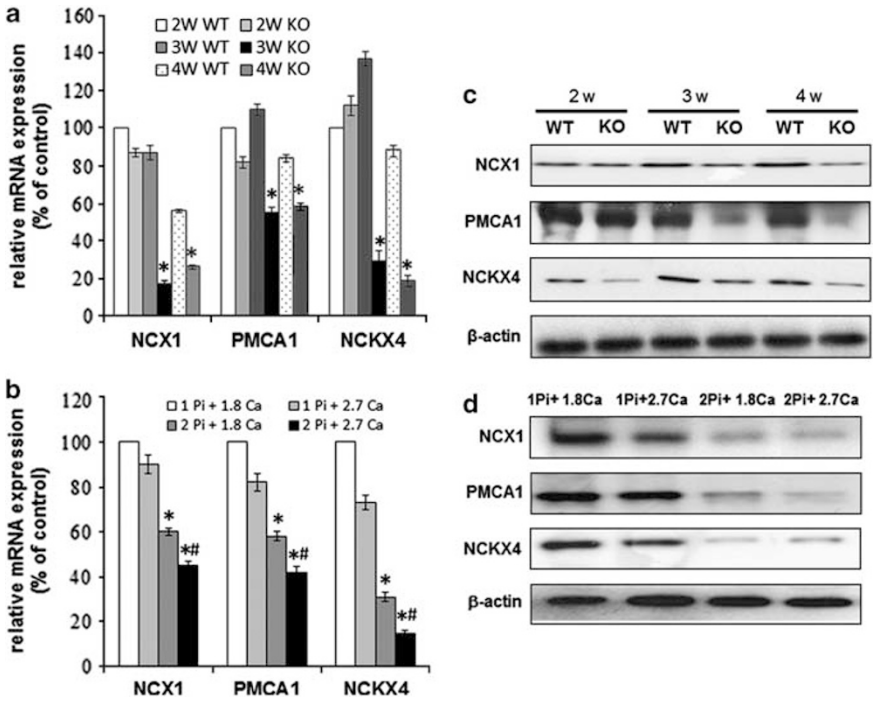

Figure 3 The mRNA and protein expression levels of the potential targets, NCX1, PMCA1, and NCKX4, decrease in the aortic media of *kl/kl* mice and in cultured VSMCs exposed to high concentrations of Pi and Ca. (**a**) Relative expression of NCX1, PMCA1, and NCKX4 mRNAs in the aortic media of 2W, 3W, and 4W-old *kl/kl* mice compared with WT mice as determined by qRT–PCR ($n = 7$ for each age), and their WT controls ($n = 9$ for each age), as determined by qRT–PCR. *$P < 0.05$ compared with WT mice of the same age by one-way ANOVA followed by Student–Newman–Keul's test. (**b**) The mRNA levels of NCX1, PMCA1, and NCKX4, as determined by qRT–PCR, in cultured VSMCs treated with the same conditions as in Figure 1c. *$P < 0.05$ *vs* 1 mM Pi + 1.8 mM Ca, and $P < 0.05$ *vs* 1 mM Pi + 2.7 mM Ca by one-way ANOVA followed by Student–Newman–Keul's test. The experiments were repeated in triplicate, and the data are presented as the mean ± s.d. (**c**) Western blot analysis of NCX1, PMCA1, and NCKX4 expression in the aortic media of 2W, 3W, and 4W-old WT and *kl/kl* mice. β-Actin was used as a loading control. The experiments were repeated in quadruplicate. (**d**) Western blot analysis of NCX1, PMCA1, and NCKX4 expression in cultured VSMCs treated with the same conditions as in Figure 1c. β-Actin was used as a loading control. The experiments were repeated in triplicate.

results reinforce our hypothesis that the miRNAs are involved in vascular calcification.

To further examine whether the upregulation of these four miRNAs is induced by high Pi and/or Ca, we measured the miRNA expression in cultured VSMCs treated with high Pi and/or Ca by qRT–PCR. At day 7 after treatment, the expression levels of miR-135a*, miR-762, miR-714, and miR-712* were elevated in cultured VSMCs treated with high Pi and Ca together whereas no significant changes were detected in those with high Ca alone (Figure 2d). We used VSMCs harvested at day 7 after the treatment of high Pi and/or Ca because the VSMCs began to die after day 7, although the levels of the miRNAs increased in a time-dependent manner (data not shown). These results suggest that the overexpression of miR-135a*, miR-762, miR-714, and miR-712* can be attributed to calcification induced by high Pi and Ca.

NCX1, PMCA1, and NCKX4 may be the Targets of miR-135a*, miR-762 and miR-714, and miR-712*, Respectively

To examine the expression of NCX1, PMCA1, and NCKX4, we evaluated their mRNA and protein levels *in vivo* and *in vitro*. Their expression levels were downregulated at both the mRNA (Figure 3a) and protein levels (Figure 3c; Supplementary Figure 1A) in the aortic media from 3- and 4-week-old *kl/kl* mice compared with WT mice. There was no significant difference between 2-week-old *kl/kl* and WT mice.

In cultured VSMCs, NCX1, PMCA1, and NCKX4 were decreased after treatment with high Pi alone and with high Pi and Ca at both the mRNA (Figure 3b) and protein levels (Figure 3d; Supplementary Figure 1B). However, high Ca alone did not induce a change in the expression of these genes. These results suggest that the expression of NCX1, PMCA1, and NCKX4 may be inhibited by miR-135a*, miR-762, miR-714, and miR-712*.

Overexpression of miR-135a* and miR-762, miR-714, and miR-712* Decreases the Protein Levels of NCX1, PMCA1, and NCKX4 in Cultured VSMCs

To further explore whether NCX1, PMCA1, and NCKX4 are target genes of miR-135a* and miR-762, miR-714, and miR-712*, miRNA mimics were transfected into the cultured VSMCs. The forced expression of an individual miRNA(s), such as miR-135a* and miR-762, miR-714, or miR-712*

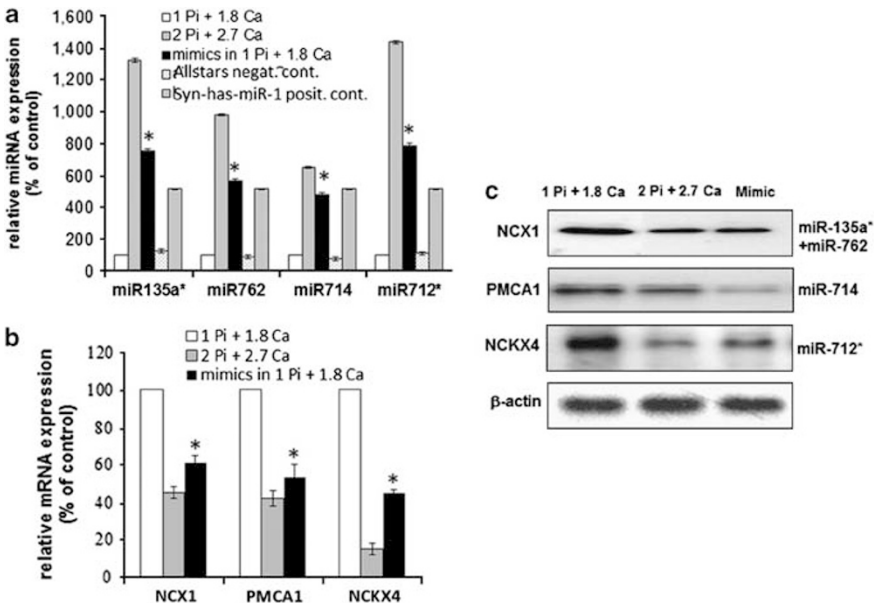

Figure 4 miRNA mimics suppress the expression of their potential target genes in cultured VSMCs. (**a**) The effects of miRNA mimics on the expressions of miR-135a*, miR-762, miR-714, and miR-712*, as determined by qRT–PCR. Cultured VSMCs were exposed to 1 mM Pi + 1.8 mM Ca or 2 mM Pi + 2.8 mM Ca without treatment with the miRNA mimics, or to 1 mM Pi + 1.8 mM Ca after treatment with the miRNA mimics. Allstar negative control siRNA (Qiagen) was used as a negative control, and Syn-has-miR-1 miScript miRNA mimic was used as a positive control to confirm that the conditions remained optimal. $^*P<0.05$ compared with cultured VSMCs treated with 1 mM Pi + 1.8 mM Ca without miRNA mimics by one-way ANOVA followed by Student–Newman–Keul's test. The experiments were repeated in triplicate, and the data are presented as the mean ± s.d. (**b**) The effects of miRNA mimics on the expression levels of NCX1, PMCA1, and NCKX4 in cultured VSMCs as determined by qRT–PCR. $^*P<0.05$ compared with cultured VSMCs treated with 1 mM Pi + 1.8 mM Ca without miRNA mimics by one-way ANOVA followed by Student–Newman–Keul's test. The experiments were repeated in triplicate, and the data are presented as the mean ± s.d. (**c**) The effects of miRNA mimics on the expression levels of NCX1, PMCA1, and NCKX4 in cultured VSMCs, as determined by western blot analysis. β-Actin was used as a loading control. The experiments were repeated in triplicate.

(Figure 4a), decreased the mRNA levels of NCX1, PMCA1, and NCKX4 (Figure 4b). The forced expression of these miRNAs also suppressed the protein expression of NCX1, PMCA1, and NCKX4 (Figure 4c; Supplementary Figure 2A).

Inhibition of miRNAs Restores the Protein Levels of Calcium pumps and Reduces the Degree of Pi- and Ca-induced VSMC Calcification

To further confirm the relationship between overexpressed miRNAs and Pi- and Ca-induced calcification in cultured VSMCs, we applied miRNA inhibitors to knockdown the overexpressed miRNAs. Inhibitors of miR-135*, miR-762, miR-714, and miR-712* suppressed the individual miRNA levels, whereas the negative control had no effect on the miRNA levels (Figure 5a). In addition, the miRNA inhibitors restored the protein levels of NCX1, PMCA1, and NCKX4 in the cultured VSMCs treated with high Pi and Ca (Figure 5c; Supplementary Figure 2B), although a significant restoration at the mRNA level was observed only for NCX1 (Figure 5b). These results reinforce the possibility that NCX1, PMCA1, and NCKX4 are the targets of miR-135a* and miR-762, miR-714, and miR-712*, respectively.

To further examine whether the miRNA inhibitors reduced the degree of VSMC calcification, calcification levels were determined on the basis of Ca content and von Kossa staining. Cultured VSMCs treated with all of the miRNA inhibitors showed a significant, albeit modest, reduction in calcification compared with VSMCs treated with high Pi alone or with high Pi and Ca without miRNA inhibitors, as assessed by the quantification of the calcium content (Figure 6a; Supplementary Figure 3A). However, no clear differences in the degree of calcification were detected between untreated VSMCs and those treated with a single miRNA inhibitor or an inhibitor of miR-680, which was the most highly upregulated miRNA in the miRNA array (Figures 2b and 6a). The calcium content in the cultured VSMCs treated with all of the miRNA inhibitors was reduced by 30% compared with cells that were not treated with inhibitors but were cultured with high Pi and Ca (Figure 6a), and the calcium content was reduced by 40% in cells cultured in high Pi alone (Supplementary Figure 3A). The VSMCs treated with only one miRNA inhibitor showed no significant reduction of calcium content (Figure 6a; Supplementary Figure 3A). The results obtained by von Kossa staining were consistent with those obtained by the quantification of calcium content (Figure 6b). These results suggest that the miRNAs are involved in Pi- and Ca-induced VSMC calcification and that inhibiting their expression can reduce the degree of calcification.

Finally, to further confirm that miR-135*, miR-762, miR-714, and miR-712* target the calcium transport proteins

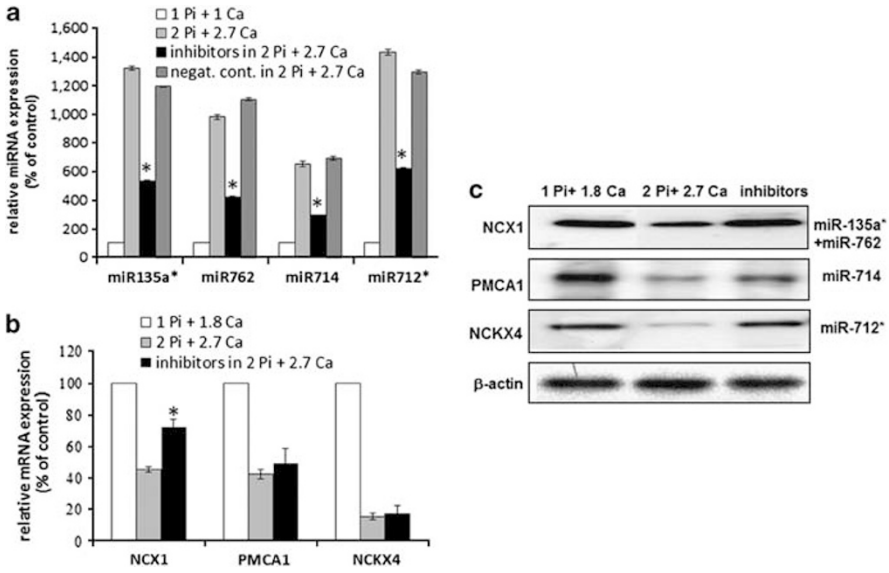

Figure 5 miRNA inhibitors restore the expression levels of the potential corresponding target genes. (**a**) The effects of miRNA inhibitors on the expression levels of miR-135a*, miR-762, miR-714, and miR-712*, as determined by qRT–PCR. Cultured VSMCs were treated with 1 mM Pi + 1.8 mM Ca or 2 mM Pi + 2.7 mM Ca without miRNA inhibitors or with 2 mM Pi + 2.7 mM Ca after treatment with a miRNA inhibitor. miScript inhibitor Negative Control (Qiagen) was used as a negative control. $^*P<0.05$ compared with cultured VSMCs treated with 2 mM Pi + 2.7 mM Ca without miRNA inhibitors by one-way ANOVA followed by Student–Newman–Keul's test. The experiments were repeated in triplicate, and the data are presented as the mean ± s.d. (**b**) The effects of miRNA inhibitors on the expression levels of NCX1, PMCA1, and NCKX4 in cultured VSMCs as determined by qRT–PCR. $^*P<0.05$ compared with cultured VSMCs treated with 2 mM Pi + 2.7 mM Ca for 7 days without miRNA inhibitors by one-way ANOVA followed by Student–Newman–Keul's test. The experiments were repeated in triplicate, and the data are presented as the mean ± s.d. (**c**) The effects of miRNA inhibitors on the expression levels of NCX1, PMCA1, and NCKX4 in cultured VSMCs as determined by western blot. β-Actin was used as a loading control. The experiments were repeated in triplicate.

NCX1, PMCA1, and NCKX4, which pump Ca^{2+} out of the cytoplasm, we investigated whether the miRNA inhibitors could reduce the incremental rate at which intracellular Ca^{2+} concentrations increase in response to high Pi and Ca exposure. Interestingly, a mixture of the miRNA inhibitors reduced the maximum intracellular Ca^{2+} concentration of cultured VSMCs exposed to high Pi and Ca by 20% (Figure 6d; Supplementary Figure 3B). Taken together, these results suggest that the miRNAs targeting Ca^{2+} transporters are involved in Pi- and Ca-induced VSMC calcification and that inhibiting their expression can reduce the degree of calcification by decreasing intracellular Ca^{2+} concentrations.

DISCUSSION

In this study, we suggested that the expression of multiple miRNAs that target Ca efflux proteins is one of the etiologies of Pi- and Ca-induced VSMC calcification. We used *kl/kl* mice as a vascular calcification model. Hyperphosphatemia and hypercalcemia are potent inducers of VSMC calcification and are independently associated with increased cardiovascular mortality.[20] To address the mechanisms of vascular calcification caused by aberrant mineral homeostasis, we extracted RNA from aortic media isolated from 3-week-old WT and *kl/kl* mice for an miRNA array because aortic medial calcification begins 3 weeks after birth. In the miRNA array profile, we first found that many miRNAs were aberrantly expressed in the aortic media of *kl/kl* mice, and we investigated which miRNAs were important for vascular calcification. Determining the mRNA targets of miRNAs is complex because one miRNA could have multiple mRNA targets and vice versa.[15] During a database search for target genes of the most highly expressed miRNAs in *kl/kl* aortic media, we found that calcium ion transporters, such as NCX1, PMCA1, and NCKX4, frequently appeared as targets. We therefore assumed that their mRNAs were the targets of miR-135a*, miR-762, miR-714, and miR-712*.

We indirectly showed that NCX1, PMCA1, and NCKX4 could be the targets of miR-135a* and miR-762, miR-714, and miR-712* using gain-of-function and loss-of-function approaches. Western blot analysis clearly showed that the protein levels of NCX1, PMCA1, and NCKX4 were significantly decreased by treatment with miRNA mimics for miR-135a* and miR-762, miR-714, and miR-712*.

The protein levels of NCX1, PMCA1, and NCKX4 were restored by treatment with miRNA inhibitors. However, knocking down the miRNAs increased NCX1 mRNA levels but did not increase the other mRNA levels in parallel with their respective protein levels. Repression of target genes leads to a decrease in translational efficiency and/or a decrease in corresponding mRNA levels, but the relative contributions of miRNA to these two mechanisms are largely unknown.[21]

Although NCX1, the cardiac Na^+/Ca^{2+} exchanger, pumps Ca^{2+} out of cardiac myocytes, the role of NCX1 in VSMCs

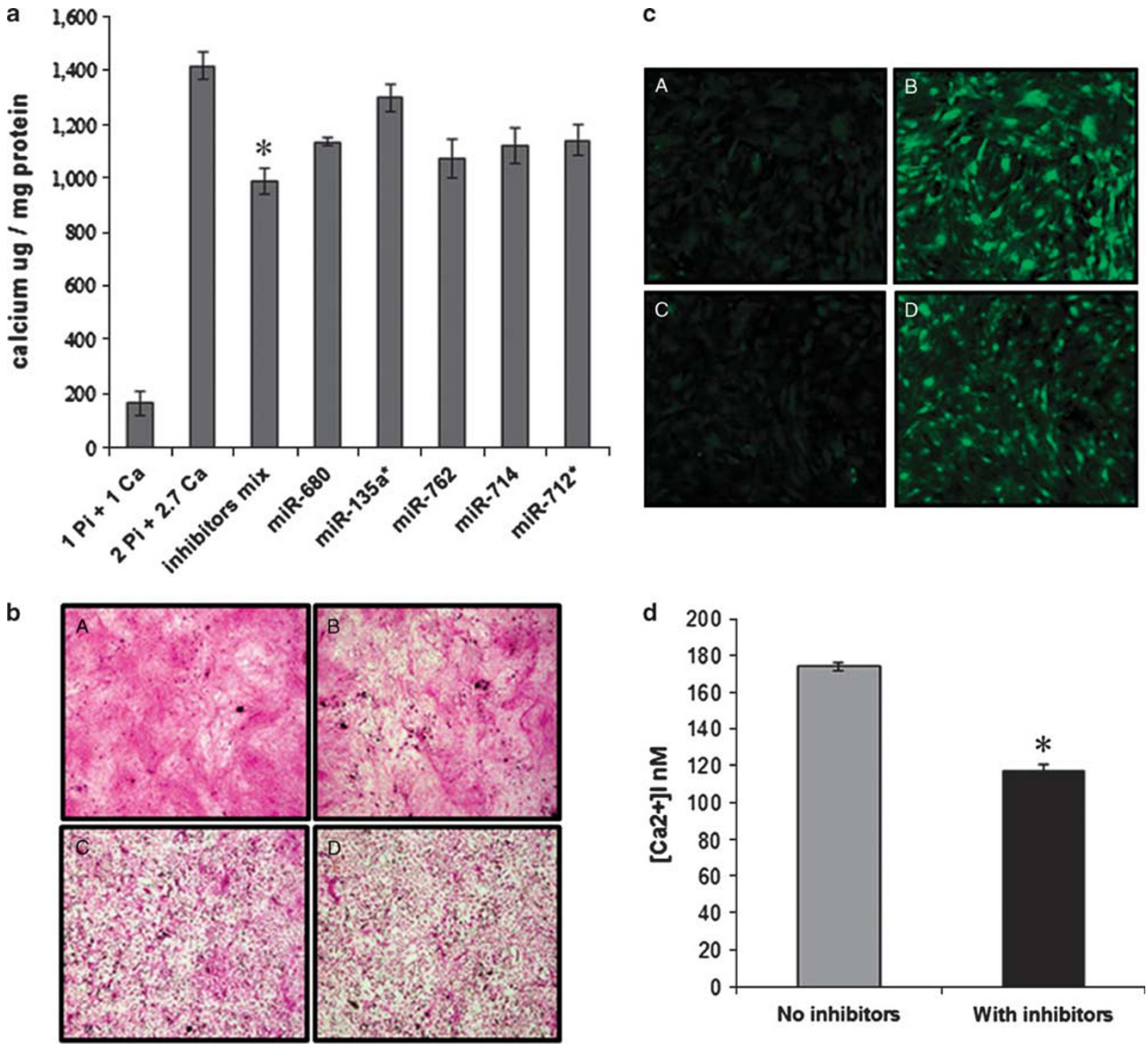

Figure 6 miRNA inhibitors decrease the degree of VSMC calcification. (**a**) Comparison of Ca content in cultured VSMCs transfected with or without miRNA inhibitors. Cultured VSMCs were exposed to 2 mM Pi + 2.7 mM Ca for 7 days after pretreatment with a mixture of inhibitors or an individual miRNA inhibitor. *$P<0.01$, compared with cultured VSMCs treated with 2 mM Pi + 2.7 mM Ca without pretreatment with miRNA inhibitors by one-way ANOVA followed by Student–Newman–Keul's test. The experiments were repeated in triplicate, and the data are presented as the mean ± s.d. (**b**) Representative images of von Kossa staining to show the effects of miRNA inhibitors on VSMC calcification. (A, B) Cultured VSMCs treated with 2 mM Pi + 1.8 mM Ca (A) and 2 mM Pi + 2.7 mM Ca (B) for 7 days after pre-transfection with the mixture of miRNA inhibitors. (C, D) Cultured VSMCs treated with 2 mM Pi + 2.7 mM Ca after pre-transfection with a miR-680 inhibitor (C) and an individual inhibitor for miR-135a*, miR-762, miR-714, or miR-712* (D). Scale bar: 100 μm. (**c**) Representative images of calcium imaging by Fluo4 staining. (A, C) The resting phase of VSMCs. (B, D) VSMCs after exposure to 2 mM Pi + 2.7 mM Ca medium. VSMCs were either not treated (A, B) or pretreated with the mixture of miRNA inhibitors (C, D). (**d**) The effect of miRNA inhibitors on the intracellular Ca^{2+} concentration in VSMCs. Data are presented as the mean ± s.d. ($n=12$). *$P<0.05$ *vs* VSMCs without miRNA inhibitor treatment.

has remained unclear.[22] PMCA1 and PMCA4, which are ubiquitously expressed calcium ATPases of the plasma membrane,[23] are Ca^{2+} pumps. NCKX4, a K^+-dependent Na^+/Ca^{2+} exchanger, catalyzes cytosolic Ca^{2+} efflux and is particularly important for neuronal Ca signaling. However, recent studies suggest that a newly discovered NCKX is present in VSMCs.[24] Because the Na^+/Ca^{2+} exchangers NCX1 and NCKX4 extrude Ca^{2+} from the cytosol in parallel with PMCA1,[25] we hypothesized that Pi- and Ca-induced calcification may be associated with an increase of intracellular Ca^{2+} because miRNAs upregulated by high Pi and Ca affect these Ca^{2+} exchanger proteins.

We propose that NCX1, PMCA1, and NCKX4, which are Ca^{2+} efflux channels, play critical roles in Pi- and Ca-induced VSMC calcification. All of their mRNA and protein levels decreased in the aortic media of *kl/kl* mice and in cultured VSMCs treated with high Pi alone or with high Pi and Ca, and this finding was paralleled by induction of miR-135a*, miR-762, miR-714, and miR-712*. Inhibitors directed against these miRNAs significantly attenuated the degree of VSMC calcification induced by high Pi alone or high Pi and Ca culture conditions for 7 days. In contrast, a control inhibitor of miR-680, which is the most significantly overexpressed miRNA in the aortic media of *kl/kl* mice, did not result in any significant change. We therefore believe that of the 17 miRNAs listed in Figure 2b, miR-135a*, miR-762, miR-714, and miR-712* are the specific miRNAs that are involved in calcification.

We hypothesize that the intracellular Ca^{2+} concentration is initially increased by Pi and Ca and that Ca^{2+} accumulates in the cytoplasm because of Ca^{2+} efflux failure. Indeed, we demonstrated using a Fluo4 experiment that miRNA inhibitors reduced the intracellular Ca^{2+} concentration ordinarily induced in cultured VSMCs by exposure to high Pi. We believe that the reduction of the peak Ca^{2+} concentration caused the attenuation of VSMC calcification, although we do not know whether this reduction lasts for 7 days. While the mechanisms by which an increase of intracellular Ca^{2+} concentration causes calcification are still unclear, it has been reported that Ca^{2+} influx channel blockers limit calcification progression.[26,27] In addition, intracellular mitochondrial calcification is observed in human vessel rings, though extracellular vesicle deposition does not occur in normal vessel rings when exposed to high Pi and Ca.[2]

Furthermore, other miRNAs in addition to miR-135a*, miR-762, miR-714, and miR-712* could be involved in devastating the vascular structure in *kl/kl* mice through different mechanisms. Carotid arteries treated with miR-126 display a marked reduction in plaque area, and miR-126 limits atherosclerosis, indicating that miR-126 has a vascular-protective function.[28] Moreover, we found miR-126-3p and miR-126-5p to be downregulated in the aortic media of *kl/kl* mice compared with WT mice, suggesting that the reduction of these miRNAs may contribute to the destruction of the aorta of *kl/kl* mice.

In summary, the present study reveals that miR-135a*, miR-762, miR-714, and miR-712* are overexpressed in the aortic media of *kl/kl* mice and cultured VSMCs exposed to high concentrations of Pi and Ca, resulting in the disruption of NCX1, PMCA1, and NCKX4 expression. Specific miRNA inhibitors partially restored NCX1, PMCA1, and NCKX4 expression levels, ameliorated the degree of calcification of cultured VSMCs, and reduced intracellular Ca^{2+} concentrations. These findings indicate that miR-135a*, miR-762, miR-714, and miR-712* could disrupt calcium transports, thereby increasing intracellular Ca^{2+} concentrations and, resulting in VSMC calcification. We therefore conclude that these miRNAs are involved in the etiology of Pi- and Ca-induced VSMC calcification and represent potential therapeutic targets.

Supplementary Information accompanies the paper on the Laboratory Investigation website (http://www.laboratoryinvestigation.org)

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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