Sirtuin 1 Retards Hyperphosphatemia-Induced Calcification of Vascular Smooth Muscle Cells

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Objective—Arterial calcification is associated with cardiovascular disease as a complication of advanced atherosclerosis. Aged vascular cells manifest some morphological features of a senescent phenotype. Recent studies have demonstrated that mammalian sirtuin 1 (SIRT1), a histone deacetylase (HDAC), is an exciting target for cardiovascular disease management. Here, we investigated the role of SIRT1 in a calcification model of vascular smooth muscle cells (SMCs).

Methods and Results—In adenine-induced renal failure rats with hyperphosphatemia, massive calcification was induced in the aortic media. Senescence-associated β-galactosidase (SAβ-gal) activity, a marker of cellular senescence, in medial SMCs was significantly increased, and its induction was positively associated with the degree of calcification. In cultured SMCs, inorganic phosphate (Pi) stimulation dose-dependently increased SAβ-gal-positive cells, and Pi-induced senescence was associated with downregulation of SIRT1 expression, leading to p21 activation. The activation via SIRT1 downregulation was blunted by inhibition of Pi cotransporter. Activation of SIRT1 by resveratrol significantly reduced the senescence-associated calcification. Conversely, SIRT1 knockdown by small interfering RNA accelerated the Pi-induced SMC senescence and subsequent calcification. In addition, SIRT1 knockdown induced phenotypic change from a differentiated state to osteoblast-like cells. The senescence-related SMC calcification was completely prevented by p21 knockdown. In addition to Pi-induced premature senescence, SMCs with replicative senescence were also more sensitive to Pi-induced calcification compared with young SMCs, and this finding was attributable to augmented p21 expression.

Conclusion—SIRT1 plays an essential role in preventing hyperphosphatemia-induced arterial calcification via inhibition of osteoblastic transdifferentiation. In addition, Pi-induced SMC calcification may be associated with both premature and replicative cellular senescence. (Arterioscler Thromb Vasc Biol. 2011;31:00-00.)

Key Words: cellular senescence • hyperphosphatemia • longevity gene SIRT1 • vascular calcification • vascular smooth muscle cell

Atherosclerotic vascular damage associated with aging manifests several features, namely atherosclerosis, sclerosis, and calcific change, finally leading to cardiovascular events. These pathological changes result in arterial wall thickening (localized morphological changes) and arterial stiffening (functional changes). Arterial calcification makes the management of hemodynamics more difficult in the elderly, because ectopic calcium deposition in the aorta and arteries contributes to vessel wall stiffening and loss of elastic recoil. These pathological conditions result in unstable hemodynamic consequences, finally leading to a decline in end-organ perfusion and subsequent ischemic events. Recently, several reports have demonstrated that aortic calcification detectable on chest X-ray examination is a strong predictor of future cardiovascular events beyond traditional risk factors. Arterial calcification is anatomically separated into two types, intimal and medial calcification. Intimal calcification, which is seen as patchy scattered deposits only occurring within atherosclerotic plaques, is shown to be associated with plaque vulnerability. On the other hand, medial calcification, which is frequently seen in the elderly and in diabetes and chronic renal failure, is observed as continuous linear deposits along the internal elastic lamina. Advanced atherosclerosis with both types of calcified lesions is the consequence of overlapping pathological mechanisms.

Ectopic calcification in the vasculature has been shown to result from passive precipitation of calcium with aging and osteoporosis, the so-called calcium shift theory, as a previous hypothesis. However, accumulating recent evidence has shown it to be attributable to an active “cell-mediated process” resembling osteogenesis in bone rather than passive mineral precipitation in vascular smooth muscle cells (SMCs).

Silent information regulator-2 (Sir2), an NAD+-dependent HDAC, is highly conserved in organisms ranging from Archaea...
to humans.10 In yeast, Sir2 has been shown to play critical roles in DNA repair, stress resistance, and longevity. Mammalian sirtuin 1 (SIRT1), the closest homolog of Sir2, regulates the cell cycle, apoptosis, and metabolism by interacting with a number of molecules, including p53, PML, Foxo, Ku70, and peroxisome proliferator-activated receptor-γ.11 A previous study has shown that SIRT1 antagonizes p53-mediated premature senescence in mouse embryo fibroblasts.12 In addition, we have recently demonstrated that SIRT1 inhibits oxidative stress-induced premature senescence in vascular endothelial cells.13 However, the detailed mechanism of how SIRT1 affects vascular SMC senescence and arterial calcification remains unclear.

In this study, we hypothesized that SIRT1 plays an important role in preventing arterial calcification due to renal failure, in association with modulation of cellular senescence. Here, we demonstrated the protective potential of SIRT1 against hyperphosphatemia-induced premature and replicative senescence and subsequent calcification in SMCs.

**Methods**

**Aortic Calcification in Renal Failure Rats**

Renal failure was induced in rats by a 0.75% adenine-containing diet as previously described.14 All procedures and animal care were in accordance with the Guide for the Care and Use of Laboratory Animals of the University of Tokyo. Detailed methods are described in the supplemental materials, available online at http://atvb.ahajournals.org.

**Induction of SMC Calcification**

Primary human aortic SMCs (HASMCs) were treated with a pathologically concentrated inorganic phosphate (Pi) up to 3.2 mmol/L in culture medium as previously described.15 To quantitatively measure Pi-induced calcification, two distinct experiments were performed as previously described:

1. Intracellular calcium deposition as determined by o-cresolphthalein complexone method, and
2. Visualization of mineralization as determined by von Kossa staining. Detailed methods are described in the supplemental materials.

**Senescence-Associated β-Galactosidase Staining**

To assess senescent changes in the phenotype of cultured HASMCs or aortic medial cells of rats, staining for senescence-associated β-galactosidase (SAβ-gal), a well-established biomarker of cellular senescence, was performed. Detailed methods are described in the supplemental materials.

**Knockdown of SIRT1 or p21 by Small Interfering RNA**

HASMCs were transfected with 200 pmol/L small interfering RNA (siRNA) for SIRT1, p21<sup>WAF1/CIP1</sup>, or both. Detailed methods are described in the supplemental materials.

**Real-Time Polymerase Chain Reaction Analysis: Osteoblastic Markers**

To examine whether Pi stimulation induces change to an osteoblastic phenotype, the expression of Runx2/Cbfa-1 and alkaline phosphatase, which are well known to be representative osteoblastic markers, was checked using real-time polymerase chain reaction analysis. In addition, the effect of knockdown of SIRT1, p21, or both by siRNA on the osteoblastic phenotypic change in HASMCs was examined. Primer sequences are shown in the Supplemental Figure.

**Results**

**Association of Senescent Vascular Cells With Aortic Medial Calcification in Renal Failure Rats**

The adenine-fed rats had severe renal failure, with a huge increase in serum creatinine (3.0±0.9 mg/dL in renal failure rats versus 0.3±0.0 mg/dL in control rats), similar to a previous report.16 The renal failure rats showed an approximately 2.0-fold increase in serum phosphorus (18.9±4.7 mg/dL) compared with control rats (9.8±0.9 mg/dL). Histological assessment using von Kossa staining showed that the aorta in renal failure rats had extensive linear calcification, which was localized in the aortic media, resembling the typical Mönckeberg’s pattern (Figure 1A). Numerous SAβ-gal positive cells were found in the aortic media of renal failure rats, whereas the aortic wall in control rats did not contain senescent cells (Figure 1B). The senescent cells were mainly localized to the calcified area and its surrounding area, which was defined as the area not stained black by von Kossa staining. Quantitative assessment showed that the number of senescent cells with high SAβ-gal activity was positively correlated with the calcified area in the aortic media (Figure 1C).

**Pi Induces Cellular Senescence in Cultured SMCs**

On the basis of our results obtained from animal experiments, we hypothesized that senescent SMCs in the aortic media are strongly associated with the development of arterial calcification. Therefore, the effect of excessive Pi stimulation (2.6 mmol/L) on cellular senescence in cultured SMCs was examined. SAβ-gal-positive senescent HASMCs were significantly induced by not only angiotensin II (Ang II) but also Pi stimulation (Figure 2A). Notably, Pi stimulation increased
calcium deposition; however, Ang II alone did not (Figure 2B). It suggests that high-dose Pi condition, but not stress by Ang II alone, is indispensable to induce SMC calcification. These findings also suggest that intracellular Pi influx at least is essential to induce this SMC calcification model.

In addition, to determine how many days after the initiation of Pi stimulation the cells showed a senescent phenotype and subsequent calcification, the time-dependent effects of Pi stimulation on both SAβ-gal activity and calcium deposition were examined. As shown in Figure 2C, SAβ-gal-positive cells were significantly increased by Pi stimulation even on day 1, although calcium deposition was not markedly increased at the same time point. A statistically significant increase in calcium deposition was found from day 3 and later. Cotreatment with PFA, an inhibitor of NPC, showed significant inhibition of Pi-induced senescence (Figure 2D). Our previous report showed that treatment with PFA completely inhibited Pi-induced SMC calcification,\textsuperscript{15} suggesting the importance of increased intracellular influx of phosphate in Pi-induced SMC senescence.

### Downregulation of SIRT1 by Pi

Treatment of HASMCs with Pi caused downregulation of SIRT1 expression in a time-dependent manner (Figure 3A). The decline was dependent on Pi concentration (data not shown). An increase in acetylation of both substrates of SIRT1, histone-3 and p53 (a nonhistone substrate), was found according to the decline in SIRT1 deacetylase activity. In addition, expression of p21, a downstream molecule of p53, was significantly induced by Pi as well. Quantitative assessment showed that an increase in these expression levels of Ac-p53 and p21 on day 3 and day 6 was statistically significant compared with the pretreatment levels, suggesting that downregulation of SIRT1 activity may mediate the subsequent increase in Ac-p53 and p21 expression.

To address whether SIRT1 downregulation-related SMC senescence and calcification are reversible or not, the effects of continuation or termination of high-dose Pi were examined. As shown in Figure 3B, the continuation of Pi up to day 10 was associated with SIRT1 downregulation and subsequent upregulation of Ac-p53 and p21, leading to induction of senescence-related calcification. However, the slight increase in senescent cells was not statistically significant, although calcification was significantly induced. Of note, the Pi-induced downregulation of SIRT1 was almost completely reversed by withdrawal (termination) of Pi stimulation (exchange of Pi from 2.6 mmol/L to 1.4 mmol/L as a normal level on day 6) as shown in Figure 3B. According to the restoration of SIRT1, levels of both Ac-p53 and p21 were also decreased without more progression. In addition, termination of Pi showed no progression of senescence-related calcification; however, preexisting senescent cells and calcification on day 6 continued without regression.

Next, NPC inhibition by PFA completely blunted Pi-induced SIRT1 downregulation and subsequent activation of its downstream p53/p21 pathway (Figure 3C).

### Regulation of SIRT1 Modulates Pi-Induced SMC Senescence and Calcification

The effects of modulation of SIRT1 activity on Pi-induced cellular senescence were investigated. First, sirtinol, a chemical inhibitor of SIRT1, induced an increase in SAβ-gal-positive cells even under a normal Pi (1.4 mmol/L), and the
increased number of senescent cells induced by Pi was significantly augmented by sirtinol (Figure 4A). Sirtinol dose-dependently augmented Pi-induced calcification, although no augmentation was found under a normal Pi (Figure 4B and 4C). Conversely, treatment with resveratrol, an activator of SIRT1, significantly reduced both Pi-induced senescent transition and calcification in a dose-dependent manner (Figure 4D to 4F).

Second, complete knockdown of SIRT1 by siRNA caused a significant increase in acetylation of both substrates (histone-3 and p53) and p21 expression (Figure 5A). Similarly to sirtinol, SIRT1 inhibition by siRNA also augmented not only senescent transition (Figure 5A, bottom) but also calcium deposition (Figure 5C, top). In contrast, real-time polymerase chain reaction analysis showed that Pi induced the expression of two representative osteoblastic markers, Runx-2/Cbfa-1 and alkaline phosphatase (Figure 5C, bottom) with statistical significance. In addition, complete knockdown of SIRT1 using siRNA significantly accelerated the Pi-induced osteoblastic phenotypic change, suggesting that modulation of SIRT1 is associated with osteoblastic phenotypic change in SMCs.

Figure 3. Inorganic phosphate (Pi) stimulation leads to sirtuin 1 (SIRT1) downregulation and subsequent p21 activation. A, The effect of Pi on SIRT1 expression and its downstream pathway was examined. Treatment of human aortic SMCs (HASMCs) with Pi (2.6 mmol/L) showed downregulation of SIRT1 expression, leading to an increase in acetylation of its substrates (Ac-H3 and Ac-p53) and p21 expression. Bottom: Quantitative analysis showed that Pi gradually induced not only SIRT1 downregulation but also upregulation of Ac-p53 and p21. B, To address whether SIRT1 downregulation-related senescence and subsequent calcification are reversible, the effects of continuation or termination of high-dose Pi were examined. Termination (on day 6) of Pi showed no progression of senescence-related calcification in association with restoration of SIRT1, whereas continuation (up to day 10) of Pi stimulation showed further progression of calcification. C, Treatment with PFA, an NPC inhibitor, completely reversed Pi-induced SIRT1 downregulation. A decline in Ac-H3 and Ac-p53 reflected the restoration of SIRT1 deacetylase activity. Pi-induced p21 activation was significantly inhibited by inhibition of Pi transport.

increased number of senescent cells induced by Pi was significantly augmented by sirtinol (Figure 4A). Sirtinol dose-dependently augmented Pi-induced calcification, although no augmentation was found under a normal Pi (Figure 4B and 4C). Conversely, treatment with resveratrol, an activator of SIRT1, significantly reduced both Pi-induced senescent transition and calcification in a dose-dependent manner (Figure 4D to 4F).

Second, complete knockdown of SIRT1 by siRNA caused a significant increase in acetylation of both substrates (histone-3 and p53) and p21 expression (Figure 5A). Similarly to sirtinol, SIRT1 inhibition by siRNA also augmented not only senescent transition (Figure 5A, bottom) but also calcium deposition (Figure 5C, top).

Although stimulation with Ang II alone could increase the number of SAβ-gal-positive cells, it did not increase calcium deposition. To understand the mechanism of these discrepant phenomena, the effect of Ang II alone on osteoblastic phenotypic change was examined. Ang II alone did not increase the expression of Runx2 in the absence of Pi stimulation, unlike Pi stimulation (Figure 5B).

To understand the detailed mechanism by which SIRT1 modulates senescence-related calcification, the effect of SIRT1 on phenotypic change in HASMCs was examined. Pi inhibited the expression of caldesmon, a differentiated SMC lineage marker, and complete knockdown of SIRT1 augmented the Pi-induced partial downregulation of caldesmon (Figure 5C, middle). In contrast, real-time polymerase chain reaction analysis showed that Pi induced the expression of two representative osteoblastic markers, Runx-2/Cbfa-1 and alkaline phosphatase (Figure 5C, bottom) with statistical significance. In addition, complete knockdown of SIRT1 using siRNA significantly accelerated the Pi-induced osteoblastic phenotypic change, suggesting that modulation of SIRT1 is associated with osteoblastic phenotypic change in SMCs.
Inhibition of Senescence-Related Calcification in SMCs by p21 Knockdown

To address the association of p21 with senescence-related calcification, knockdown of p21 using siRNA was performed. Treatment of p21 siRNA (up to 200 pmol/L) completely inhibited p21 (Figure 5D). p21 knockdown completely inhibited Pi-induced senescence and subsequent calcification (Figure 5E).

Regulation of NPC-Mediated Runx2 Expression by SIRT1/p21 Pathway

As the next step, the role of SIRT1 in NPC-mediated Runx2/Cbfa1 expression was examined. First, complete knockdown of SIRT1 did not show any change in both osteoblastic markers, Runx2 and alkaline phosphatase, in a normal Pi (Supplemental Figure 1). As shown in Figure 5F, Pi-induced Runx2 was significantly blunted by PFA, an NPC inhibitor. SIRT1 activation by resveratrol inhibited Pi-induced Runx2 activation. The Runx2 induction was augmented by knockdown of SIRT1 by siRNA, and the activation was completely inhibited by PFA. Surprisingly, Runx2 activation was strongly inhibited by knockdown of p21 alone. In addition, the inhibition of Runx2 induction by double knockdown of SIRT1 and p21 was less than that by single knockdown of SIRT1.

To address a difference in senescent induction by Pi or Ang II, immunohistolohical assessment of SIRT1 in HASMCs was examined (Supplemental Figure II). Although SIRT1 was predominantly localized in nucleus without Pi, the translocation of SIRT1 to cytoplasm was observed after Pi stimulation for 24 hours, and its expression disappeared in both areas on day 6. In contrast, Ang II stimulation did not show the dynamic translocation.

High Sensitivity of SMCs With Replicative Senescence to Pi-Induced Calcification

Not only Pi-induced "premature senescence" in HASMCs but also the effects of Pi on "replicative senescence" were evaluated. Senescent cells (passage 18) were more sensitive to Pi-induced calcification compared with young cells (passage 7) (Figure 6A). SIRT1 expression was downregulated in senescent cells compared with young cells, and the down-regulation was significantly augmented by Pi stimulation (Figure 6B, top). In parallel with this finding, senescent cells showed an increase in Ac-p53 and p21 expression. Statistical analyses using densitometric measurement showed that (1) downregulation of SIRT1 and upregulation of Ac-p53 and p21 were augmented by replicative senescence, and (2) Pi inhibited the SIRT1-p21 pathway even in cells with replicative senescence (passage 18) (Figure 6B, bottom).

Discussion

Vascular aging, leading to cardiovascular disease, manifests complex and diverse vascular changes (eg, impairment of distensibility due to loss of arterial elasticity). Arterial wall stiffening resulting from ectopic calcification is a complication of advanced atherosclerosis and makes the management of hemodynamics more difficult in the elderly. Few reports have addressed whether cellular senescence is associated with SMC calcification. This study showed the importance of SIRT1, a longevity gene, in arterial calcification in association with cellular senescence.

First, our data obtained from animal experiments clearly showed the association of senescent SMCs with aortic medial calcification in the renal failure rats with hyperphosphatemia. Senescent cells showed significant colocalization with calcium deposition. In addition, many senescent cells could be detected before microscopic calcification occurred at 4 weeks after the start of renal failure induction (data not shown), suggesting that the transition to a senescent phenotype in medial SMCs may be associated with the initiation and progression of calcification. Therefore, hyperphosphatemia, a potent uremic factor, may be a stimulator to induce senescent phenotypic transition of medial SMCs.

Second, we also confirmed the association of Pi-induced SMC senescence with calcification in vitro experiments. Senescent SMCs were significantly increased by Pi even on day 1, although calcium deposition was not markedly in-
Figure 5. Augmentation of senescence-related smooth muscle cell (SMC) calcification by sirtuin 1 (SIRT1) knockdown in association with osteoblastic phenotypic change and prevention of inorganic phosphate (Pi)-induced changes by p21 knockdown. A, To achieve SIRT1 knockdown in human aortic SMCs (HASMCs), small interfering RNA (siRNA) was simultaneously administered at the start of Pi stimulation (2.6 mmol/L). Complete inhibition of SIRT1 showed a significant increase in acetylation of both substrates (Ac-H3 and Ac-p53), p21 expression and senescence-associated \( \beta \)-galactosidase (SA\( \beta \)-gal)-positive cells. B, Angiotensin II (Ang II) alone (10 pmol/L) did not increase the expression of Runx2 in the absence of Pi stimulation, unlike Pi stimulation. C, top: SIRT1 knockdown by siRNA significantly accelerated Pi-induced calcification \((n=6)\), whereas control (Ctrl) siRNA did not. C, middle and bottom: Western blots showed that Pi partially inhibited the expression of a differentiated SMC marker, caldesmon, and complete knockdown of SIRT1 by siRNA augmented its downregulation. Real-time polymerase chain reaction analysis showed that Pi induced the expression of Runx-2 and alkaline phosphatase (ALP). Complete knockdown of SIRT1 significantly accelerated the Pi-induced osteoblastic markers. A.U. indicates arbitrary units. *\( P<0.05 \) vs control without Pi stimulation (left column), **\( P<0.05 \) vs Pi-stimulated cells with SIRT1 siRNA (sixth column from left). D and E, Knockdown of p21 by siRNA (200 pmol/L) significantly reduced the senescent phenotypic change and subsequent calcification \((n=6)\). F, The role of SIRT1/p21 axis in NPC-mediated Runx2 expression was evaluated. Augmentation of Pi-induced Runx2 expression by SIRT1 knockdown was significantly inhibited by double knockdown of SIRT1 and p21. *\( P<0.05 \) vs control without Pi stimulation (left column), **\( P<0.05 \) vs Pi-stimulated cells with SIRT1 siRNA (sixth column from left).
creased at the same time point. A statistically significant increase in calcium deposition was found from day 3 and later. Considering these data, we hypothesize that (1) calcium deposition may be more readily induced in senescent cells compared with nonsenescent cells, and (2) Pi-induced senescent change is observed earlier than calcium deposition. In other words, senescent transition associated with Runx2 induction may lead to progressive calcification.

Senescent SMCs were associated with the SIRT1-related p53/p21 pathway, based on the findings that SIRT1 knockdown augmented not only cellular senescence but also calcification. In addition, p21 knockdown completely inhibited senescence-related calcification induced by Pi. This raises the question of how cellular senescence in SMCs is associated with calcification. Our experiments to understand the detailed mechanisms by which SIRT1 modulates senescence-related calcification showed that Pi-induced SIRT1 downregulation led to the phenotypic change from a differentiated state to osteoblast-like cells in SMCs. It has been reported that Pi induces osteoblastic change, in which NPC plays a role in inducing Runx2/Cbfa-1 expression, in SMCs. As the next step, to determine how SIRT1 regulates NPC-mediated Runx2 expression, we examined the effects of knockdown of SIRT1, p21, or both by siRNA on Pi-induced Runx2 expression. Our data shown in Figure 5F suggested that (1) NPC plays an essential role in Pi-induced Runx2 expression, (2) SIRT1 has an inhibitory effect on NPC-mediated Runx2 expression, (3) knockdown of p21 alone ameliorates Runx2 induction, and (4) p21-related osteoblastic change is at least in part dependent on SIRT1.

There is now the new question of how SIRT1 regulates Runx2 regulation. A report by Jeon has shown that acetylation of Runx2 itself is important in osteoblast differentiation, and it is downregulated by HDAC activities. Based on this evidence, SIRT1, 1 of the HDACs, may be able to deacetylate Runx2, leading to inhibition of Runx2-related osteoblastic transition in SMCs. Therefore, the inhibition of SIRT1 by hyperphosphatemia may lead to Runx2 activation via its hyperacetylation. Further investigation of the detailed mechanism of the SIRT1/p21/osteoblastic gene axis is needed. These data clearly suggest that SIRT1 activation may inhibit the hyperphosphatemia-induced osteoblastic phenotypic change of SMCs, and the degree of change may be dependent on SIRT1 expression level. It is possible that the inhibition of SIRT1 expression by Pi alone is "partial," because complete downregulation of SIRT1 by siRNA worsened the dynamic phenotypic change compared with Pi only.

We have already shown that tumor necrosis factor-α, a potent atherogenic cytokine, augmented Pi-induced SMC calcification, as previously described. In addition, tumor necrosis factor-α significantly decreased Pi-induced SIRT1 downregulation further (data not shown). According to these results, we currently hypothesize that hyperphosphatemia induces SIRT1 downregulation and subsequent osteoblastic phenotypic change in SMCs, leading to calcification, and these changes are worsened by some harmful atherogenic factors, which decrease SIRT1 expression/activity further. These results provide a new insight, showing that SIRT1 plays an essential role in the prevention of arterial calcification and that the beneficial effect may be associated with an inhibition in Pi-induced SMC senescent transition.

In addition, Ang II did not increase calcium deposition, although the stimulation increased the number of senescent cells. Of note, Ang II alone did not increase Runx2 expression in the absence of Pi (Figure 5B). This result suggests that SMC senescence shows two different features: one is SAB-
gal-positive cells with an increase in Runx2 and the other is SAβ-gal-positive cells without. First, it has recently been reported that SMCs with replicative senescence, rather than the cells without senescence, show hypersensitivity in response to induction of calcification with the more induction of osteoblastic markers, suggesting that the induction of osteoblastic transdifferentiation is strongly associated with the senescent change in SMCs. In addition, the translocation of SIRT1 to cytoplasm was observed after Pi stimulation for 24 hours, although SIRT1 predominantly localized in nucleus without Pi. In contrast, Ang II did not show the dynamic translocation. Thinking about the mechanism for regulating the activity of HDACs, including SIRT1, recent several reports show the importance of their coordinated shuttling between nucleus and cytoplasm. A report demonstrates that HDAC7, an HDAC, represses the transcriptional activity of Runx2 and that osteogenic stimuli induce export of HDAC7 from nucleus, leading to a decline in the repressive potentials of HDAC7 for Runx2. On the basis of our findings and a previous report, the reason that stimulation with Ang II alone did not induce Runx2 expression and subsequently SMC calcification may in part depend on the difference of SIRT1 translocation after stimulation. Therefore, we strongly hypothesize that in the senescent SMCs with upregulation of p21, Pi stimulation, but not Ang II stimulation, may activate Runx2 via at least two phenomena, the hyperacetylation of Runx2 by SIRT1 downregulation and the dynamic SIRT1 translocation, leading to marked osteoblastic transdifferentiation and subsequent calcification. In addition, we have another hypothesis. In general, it has been shown that high-dose Pi navigates release of matrix vesicles from SMCs in parallel with osteoblastic transdifferentiation. The vesicles play an essential role in the initiation of hydroxyapatite aggregation, so-called nucleation. Accumulating recent reports show that the nanocrystal formation as an initial step under hyperphosphatemia accelerates the harmful cascade of osteoblastic transdifferentiation in SMCs via endocytosis. Maybe Ang II alone does not induce the nanocrystal formation and the cascade of osteoblastic change. Therefore, we explain that the difference of senescent phenotypic changes in SMCs between both stimulations, Pi and Ang II alone, may depend on (1) SIRT1 translocation and (2) nanocrystal formation to accelerate calcification. Further investigation to address the detailed mechanisms by which SIRT1 regulates osteoblastic transdifferentiation in SMCs under the cellular senescence is needed.

Are SIRT1 downregulation-related SMC senescence and subsequent calcification reversible or not? To answer this question, the effects of continuation or termination of high-dose Pi were examined. As shown in Figure 3B, termination (on day 6) of Pi showed no progression of senescence-related calcification in association with the restoration of SIRT1, whereas continuation (up to day 10) of Pi stimulation showed further progression of calcification. It is suggested that a therapeutic strategy to manage hyperphosphatemia to the normal range of serum phosphate concentration may lead to at least termination of progressive calcification via reversal of SIRT1 activity.

Cellular senescence has been shown to have two features: not only stress-induced premature senescence but also replicative senescence, indicating a limited number of divisions in culture. In fact, both endothelial cells and SMCs derived from human atherosclerotic plaques show a senescent phenotype earlier than do cells from normal vessels. Notably, we found that senescent HASMCs were significantly more sensitive to Pi-induced calcification compared with young cells. These results suggest that calcium deposition may be more readily induced in arterial medial SMCs with replicative senescence. This insight may explain the mechanisms by which arterial calcification occurs in the elderly more frequently than in the young population. Therefore, these observations support our hypothesis that arterial calcification is accelerated by both senescent types (premature and replicative senescence) in SMCs. To explore new therapeutic strategies against arterial calcification, it is essential to investigate how to maintain a higher SIRT1 level in the vasculature, leading to prevention of medial SMC senescence and which drug is capable of achieving it.

How does SIRT1 exert protective effects against SMC calcification? This study clearly showed that inhibition of SIRT1 was associated with increases in both Ac-p53 and p21 expression. These findings were significantly induced by not only replicative senescence but also Pi-induced premature senescence. SIRT1-mediated deacetylation of p53 inhibits p53-dependent transactivation of target genes, including p21. A report showed that a decline in cellular deacetylase activity increases the half-life of endogenous p53, suggesting that p53 acetylation is also associated with p53 stabilization. Therefore, the increased Ac-p53 by Pi-induced SIRT1 downregulation may induce SMC senescence because of a decline in degradation of p53, leading to calcification. In addition, p53 itself can inhibit SIRT1 transcription because the SIRT1 promoter has two response elements to p53. Further investigation to address how the SIRT1-p53 negative regulatory pathway is associated with SMC calcification is needed.

On the other hand, regarding p21 activation, it is reported that inhibition of p21 expression in the vasculature significantly attenuates cellular senescence, leading to prevention of atherosclerosis. This evidence suggests a pivotal role of p21 in the development of atherosclerosis. p21 activation has been shown to be regulated by a pathway that is p53 dependent, p53 independent, or both. Okamoto et al have demonstrated that inhibition of HDAC by trichostatin A showed activation of p21 promoter activity by the Sp1 site even in vascular SMCs, and the induction of p21 was independent of the p53 pathway. The p21 transcriptional activation in response to HDAC inhibitors was mediated by histone hyperacetylation in its promoter region. Based on these findings, Pi-induced p21 activation via SIRT1 downregulation may be in part involved in a p53-independent pathway, leading to a senescent phenotype of SMCs. Further investigation exploring which molecule activates the p21 promoter under hyperphosphatemia is needed.

Conclusion

We showed that SIRT1 exerts a protective role in hyperphosphatemia-based arterial calcification via inhibition...
of osteoblastic transdifferentiation, in association with cross-talk between calcification and cellular senescence. This ability of SIRT1 may orchestrate an analogous protective/longevity paradigm even in vascular SMCs, leading to maintenance of healthy elasticity of the arterial wall. Strategies to maintain a higher level of SIRT1 activity may provide novel therapeutic opportunities for the prevention of arterial calcification.

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Disclosures
None.
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Sirtuin SIRT1 retards hyperphosphatemia-induced calcification of vascular smooth muscle cells

Methods

Aortic calcification in renal failure rats

Renal failure was induced in rats by a 0.75% adenine-containing diet as previously described.28 Twelve-week-old male Wistar rats (Nippon Clea Inc., Japan) were pair-fed standard CE-2 chow (containing 1.2% calcium and 0.6% phosphorus; Nippon Clea Inc.) in the control group or CE-2 chow containing 0.75% adenine (Sigma) in the renal failure group for 4 weeks. Then, the diet was returned to normal chow for an additional 4 weeks. After induction of renal failure for 8 weeks in total, the rats were sacrificed to collect samples. After perfusion with saline at a constant, nonpulsatile pressure of 100 mmHg, the aorta was immediately embedded in OCT compound frozen section and sequentially cut into cross-sections with 5-μm thickness from each part of the aorta. To detect calcification in the aortic wall, each cross-section was subjected to von-Kossa staining to demonstrate mineralization. The calcified area and number of
SAβ-gal-positive cells in the cross-section were measured by image analysis software (ImageJ, Scion Image, Maryland, USA). All procedures and animal care were in accordance with the Guide for the Care and Use of Laboratory Animals of the University of Tokyo.

**Induction of SMC calcification**

Primary human aortic SMC (HASMC), derived from the internal thoracic artery (Clonetics), were treated with a pathological concentration of inorganic phosphate (Pi) in culture medium. To set up the calcification medium, a mixed solution of Na$_2$HPO$_4$ and NaH$_2$PO$_4$ whose pH was adjusted to 7.4 was added to serum-supplemented DMEM to final doses of up to 3.2 mmol/L as previously described.$^{29}$ To quantitatively measure Pi-induced calcification, two distinct experiments, (1) intracellular calcium (Ca) deposition as determined by o-cresolphthalein complexone method and (2) visualization of mineralization as determined by von-Kossa staining, were performed as previously described.$^{28}$ We have previously confirmed that excessive Pi stimulation dose- and time-dependently induced calcium deposition in HASMC, whereas a normal Pi dose (1.4 mmol/L), equivalent to the human physiological level of serum phosphate, did not.$^{29}$
**Senescence-associated β-galactosidase (SAβ-gal) staining**

To assess senescent changes in the phenotype of cultured HASMC or aortic medial cells of rats with/without renal failure, staining for senescence-associated β-galactosidase (SAβ-gal), a well-established biomarker of cellular senescence, was performed at pH 6.0, as opposed to endogenous lysosomal enzyme detected at pH 4.0 in normal cells, as previously described. Numbers of SAβ-gal-positive cells were quantitatively counted in the aortic wall or cultured HASMC. As a positive control in *in vitro* experiments, angiotensin II (AngII) was used to induce transition to a senescent phenotype in HASMC.

**Knockdown of SIRT1 or p21 by small interfering RNA**

HASMC were transfected with 200 pmol/L small interfering RNA (siRNA) for SIRT1 (GAT GAA GTT GAC CTC CTC A and TGA AGT GCC TCA GAT ATT A, Santa Cruz Biotechnology) or control (Cntl) using silMPORTER (Upstate). In addition, knockdown of p21[^WAF1/CIP1] was performed using 100 to 200 pmol/L siRNA for p21 (CGA CUG UGA UGC GCU AAU G, CCU AAU CCG CCC ACA GGA A, CGU CAG AAC CCA UGC GGC A, and AGA CCA GCA UGA CAG AUU U) by the same
method. To inhibit p21 expression effectively and completely, four kinds of sequences of p21 siRNA were used. HASMC were treated simultaneously with these siRNAs at the start of Pi stimulation.

**Western blot and SDS-PAGE**

Protein expression was assessed by Western blot analysis with chemiluminescence detection. SIRT1 was detected using a rabbit polyclonal anti-SIRT1 antibody (Abcam), and p53, acetylated p53 (Ac-p53; Lys-382), acetylated histone-3 (Ac-H3), p21, caldesmon and β-tubulin were detected with monoclonal antibodies (Santa Cruz Biotechnology). The expression levels of Ac-p53 and Ac-H3 were used to reflect SIRT1 activity as a deacetylase. Caldesmon was used to reflect a lineage marker of SMC differentiation.

**Real-time PCR analysis: Osteoblastic markers**

Primer sequences were as follows:

ALP; (forward) ACCATTCCCACGTCTTCACATTTG,

(reverse) AGACATTCTCTCGTTCACCGCC,

Runx-2/Cbfa-1; (forward) TCTGGCCTTCCACTCTCAGT,
GACTGGCGGGGTGAAGTAA,
SIRT1; (forward) CCTGACTTCAGGTCAAGGGATGGTA,
(reverse) CTGATTAAAAATATCTCCTCGTACAG,
β-actin; (forward) CTGGAACCGGTGAAGGTGACA,
(reverse) AAGGGACTTCCTGTAACAATGC.

Materials

Angiotensin II (AngII) was used to induce senescent phenotypic change in cultured HASMC as a positive control. To inhibit activity of Na-dependent phosphate cotransporter (NPC) stimulated by treatment with exogenous Pi, phosphonoformic acid (PFA; SIGMA), a chemical inhibitor, was used. Sirtinol (a chemical inhibitor; Calbiochem) or resveratrol (an activator of SIRT1; WAKO) was used for modulation of SIRT1 activity. Localization of SIRT1 in HASMC was detected using its antibody (Santa Cruz: sc-15404).

Immunohistological staining

To address a difference in senescent induction by Pi or AngII, the localization of SIRT1 in HASMC was compared using immunohistological assessment. SIRT1 specific
antibody showed localization and its translocation in HSMC before or after stimulation of Pi alone (2.6 mM) or AngII alone (10 pmol/L). Nucleus was detected by DAPI stain.

**Statistical analysis**

All results are presented as mean ± standard error (SE). Differences between the groups were analyzed using ANOVA, followed by Fisher’s PLSD test. A value of $P < 0.05$ was considered to be significant. All *in vitro* experiments were performed at least three times.

**Figure legends**

**Supplemental figure 1. Deterioration of osteoblastic transition by SIRT1 knockdown under high-dose Pi stimulation**

The effect of SIRT1 knockdown on osteoblastic markers, Runx2 and ALP, in the condition of normal Pi or high-dose Pi was examined. Complete knockdown of SIRT1 showed significantly augmented expression of both osteoblastic markers, Runx2 and ALP, in a high-dose Pi condition; however, augmentation was not found in a normal Pi condition. These data suggest that intracellular Pi influx by Pi stimulation is essential to
induce SMC calcification in association with osteoblastic phenotypic change, and the osteoblastic transition may be correlated with NPC, a cotransporter of Pi.

**Supplemental figure 2. Translocation of SIRT1 in HASMC is induced by Pi, but not AngII.**

To address a difference in senescent induction by Pi or AngII, immunohistological assessment of SIRT1 in HASMC was examined. SIRT1 was predominantly localized in nucleus without Pi. Dynamic translocation of SIRT1 to cytoplasm was observed after Pi stimulation (2.6 mM) for 24 hr and its expression disappeared in both areas on day 6. In contrast, AngII alone (10 pmol/L) did not show the dynamic translocation. SIRT1 siRNA shows complete knockdown of SIRT1 expression. DAPI shows nuclear stain.
Supplement Material  Figure I

Runx2 (A.U.)

ALP (A.U.)

Pi  -  -  -  +  +  +
Ctrl siRNA  -  +  -  -  +  -
SIRT1 siRNA  -  -  +  -  -  +
**Summary**

**목적**
동맥의 석회화는 진행된 죽상동맥경화의 합병증으로 심혈관질환과 관련되어 있다. 노인의 혈관세포는 노화에 의한 특징적인 구조적인 특성을 보인다. 최근 연구는 포유류의 histone deacetylase인 SIRT1 (sirtuin 1)이 심혈관질환의 새로운 치료제를 개발하는데 이용될 수 있는 표적임을 보여주고 있다. 우리는 혈관 평활근세포를 이용한 혈관석회화 모델에서 SIRT1의 역할에 대해 알아보고자 하였다.

**방법 및 결과**
아데닌에 의한 신부전, 고인산혈증 백서 모델에서 대동맥의 중막(media)에서 광범위한 혈관석회화가 관찰되었다. 세포노화의 지표인 노화관련 β-galactosidase(SA β-gal)의 활성도는 중막의 혈관 평활근세포에서 유의하게 증가하였으며 이는 혈관석회화 정도와 양의 상관관계를 보였다. 배양된 혈관 평활근세포에서 무기인산은 SA β-gal 양성인 세포를 용량의존적으로 증가시켰다. 인산에 의해 유도된 세포노화는 SIRT1 발현 감소에 의한 p21 활성화와 관련되었다. 인산에 의해 유도된 세포노화는 SIRT1 발현 감소에 의한 p21 활성화와 관련되었다. 인산의 공동전달체를 차단함으로써 SIRT1 저하에 의한 활성화는 소실되었다. 레스베라트롤(resveratrol)에 의한 SIRT1의 활성화는 세포노화에 의한 혈관석회화를 유의하게 감소시켰다. 반면 small interfering RNA로 SIRT1의 발현을 억제하면 인산에 의한 혈관 평활근세포의 노화 및 혈관석회화가 급속하게 진행하였다. 또한 SIRT1 발현 억제는 혈관 평활근세포가 조골세포 유사세포(osteoblast-like cells)의 형태로 변화되는 것을 유도하였다. 세포노화와 관련된 혈관 평활근세포의 석회화는 p21의 발현억제에 의해 완전하게 예방되었다. 인산에 의해 유도된 조기 세포노화와 같이 세포복제에 의해 노화(replicative senescence)된 혈관 평활근세포가 젊은 혈관 평활근세포에 비해 인산에 의한 혈관석회화에 보다 취약하였고 이러한 결과는 항진된 p21 발현과 관련되었다.

**결론**
SIRT1은 혈관 평활근세포가 조골세포 (osteoblast)로의 transdifferentiation을 억제함으로써 고인산혈증에 의한 혈관석회화를 예방하는데 중요한 역할을 한다. 또한 인산에 의해 유도된 혈관 평활근세포의 석회화는 조기 세포노화 및 세포복제에 의한 노화 모두와 관련이 있는 것으로 생각된다.
혈관석회화는 혈관에 무기질이 침착됨으로써 혈관의 경직도가 증가하고 축상동맥경화반의 파열을 초래하여 심혈관질환의 발생 및 합병증의 위험성을 증가시킨다. 특히 고령 환자에게는 동반 질환 이 없이도 흔히 관찰되는 대표적인 혈관 노화 현상이나 아직까지 원인 및 기전에 관한 연구는 초보적인 단계에 머물러 있는 상태이다.
혈관석회화는 노화, 당뇨병, 만성 신부전, 죽상동맥경화 등에 따른 이차적인 현상이라고 생각되어 왔으나 최근 혈관석회화의 병태 생리에 대한 연구가 진행되면서 혈관석회화는 독립적인 기전에 의해 이루어지는 질병으로서 혈관의 파열, 축상동맥경화반의 파열을 초래하여 심혈관질환의 발생 및 합병증의 위험성을 증가시킨다.

특히, 미국 UCLA의 Demer 그룹에 의해 혈관내피세포로 조골세포와 유사한 역할을 하는 CVC (Calcifying vascular cell)가 존재한다는 사실이 밝혀졌고, 그 기전에 관한 연구가 활발히 진행되고 있다. 특히, 미국 UCLA의 Demer 그룹에 의해 혈관내피세포로 조골세포와 유사한 역할을 하는 CVC (Calcifying vascular cell)가 존재한다는 사실이 밝혀졌고, 그 기전에 관한 연구가 활발히 진행되고 있다. 하지만 아직까지 혈관석화화는 아직까지 혈관석회화를 일으키는 근원세포의 기원과 그 특성을 명확하게 규정한 연구 결과는 많지 않으며 혈관석화화 과정에 영향을 미치는 여러 인자의 상호 관계 및 기전에 관한 연구는 초기 단계에 머물러 있는 실정이다. Sirtuin (SIRTs)은 항상성 유지 및 외부 스트레스에 대한 반응을 조절하는 물질로 대부분의 sirtuin (SIRT1, SIRT2, SIRT3, SIRT5)은 NAD⁺-dependent deacetylation 과정을 촉매하며 SIRT4, SIRT6은 ADP-ribosylation을 매개한다. 효모균에서 Sir2 (silent information regulator 2)는 budding 및 수명을 조절하는 역할을 담당하며 외부 스트레스나 식이 제한으로부터 개체의 생존을 유지하는데 중요한 역할을 한다. 포유류에서는 Sir2의 homolog인 SIRT1이 세포노화, 세포고사, 인슐린 감수성, 염증반응, 유전자 안정성, 산화 스트레스 등 여러 기전에 관여하는 것으로 알려져 있다.

비록 효모균에서 Sir2가 외부 스트레스에 대한 개체 보호효과를 가짐으로써 수명을 연장시키는 사실이 알려져 있지만 포유류에서도 SIRT1의 노화를 억제하고 수명을 연장시키는 기전에 대한 연구가 흥미롭게도 같은 방향으로 진행되고 있다. 특히, 포유류에서도 흥미로운 점은 식이 제한 모델에서 식이 제한에 의해 SIRT1의 발현이 증가된다는 사실이 알려져 있으며, 포유류에서도 수명을 연장시키고 노화 관련 여러 장기의 변화를 조절하고 노화 관련 질병을 예방하는 데 관여할 것으로 기대되고 있다. 따라서 최근에는 SIRT1 activator의 임상적 효능에 대해 많은 관심을 가지고 연구가 진행 중에 있다.

혈관 노화에 있어서 SIRT1의 역할에 대해서도 많은 관심을 받고 있다. SIRT1은 p53에 의해 유도된 세포노화를 억제하고 혈관내피세포에서 산화 스트레스에 의한 세포조기노화를 차단한다는 사실이 보고된 바 있다. 하지만 아직까지 SIRT1이 혈관의 노화를 억제하는 기전에 대해서는 잘 밝혀져 있지 못한 상태이다. 본 논문에서 동경대학교 노년내과학 교실의 연구자들은 신부전에 의한 고인산혈증 모델에서
SIRT1이 혈관석화화 과정을 조절하는 기전에 대해 밝히고자 하였다. 신부전 환자에서는 혈관석화화가 흔히 관찰되며 만성 신부전 환자의 주된 사망원인이다. 심혈관질환이라는 점을 고려하면 연구자들의 실험 모델은 임상적으로도 혈관석화화 연구를 수행하는데 적절한 모델이라고 생각된다. 연구자들이 새롭게 밝혀낸 사실은 SIRT1이 고인산 혈증에 의해 유도된 혈관석화화 과정을 차단하는 보호작용을 하며 특히 혈관석화화 과정은 노화된 혈관 병합근세포에서 보다 쉽게 유도되기 때문에 SIRT1 활성도를 혈관석화화에 보다 취약한 것으로 생각되는 노인에서 어떻게 하면 높게 유지할 수 있는가를 찾아내는 것이 향후 혈관석화화를 억제할 수 있는 새로운 치료법 개발을 위해 필수적일 것으로 제시하였다. 또한 SIRT1의 혈관석화화 억제 기전이 p53/p21 경로를 통한다는 사실을 밝히므로써 혈관 노화/석화화의 상호 관계성을 이해하는데 도움이 되게 하였다. 즉, 혈관세포의 노화는 석화화에 보다 취약해지는데 이러한 기전이 같은 경로에 의해 발생한다는 사실을 밝혔다. 이러한 일련의 연구를 통해 이전까지 수동적인 과정이라고 생각되었던 혈관석화화가 동동적인 자체 기전에 의해 발생한다는 사실을 알게 되었고 또한 혈관석화화를 촉진 또는 억제하는 다양한 기전을 밝혀냄으로써 향후 연구의 고려로 인해 임상에서 더욱 문제가 될 것으로 생각되는 혈관석화화의 근본적인 치료방법을 개발하는데 한걸음 나아갈 수 있을 것으로 기대된다.

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Sirtuin 1 Retards Hyperphosphatemia-Induced Calcification of Vascular Smooth Muscle Cells

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Objective—Arterial calcification is associated with cardiovascular disease as a complication of advanced atherosclerosis. Aged vascular cells manifest some morphological features of a senescent phenotype. Recent studies have demonstrated that mammalian sirtuin 1 (SIRT1), a histone deacetylase, is an exciting target for cardiovascular disease management. Here, we investigated the role of SIRT1 in a calcification model of vascular smooth muscle cells (SMCs).

Methods and Results—In adenine-induced renal failure rats with hyperphosphatemia, massive calcification was induced in the aortic media. Senescence-associated β-galactosidase (SAβ-gal) activity, a marker of cellular senescence, in medial SMCs was significantly increased, and its induction was positively associated with the degree of calcification. In cultured SMCs, inorganic phosphate (Pi) stimulation dose-dependently increased SAβ-gal-positive cells, and Pi-induced senescence was associated with downregulation of SIRT1 expression, leading to p21 activation. The activation via SIRT1 downregulation was blunted by inhibition of Pi cotransporter. Activation of SIRT1 by resveratrol significantly reduced the senescence-associated calcification. Conversely, SIRT1 knockdown by small interfering RNA accelerated the Pi-induced SMC senescence and subsequent calcification. In addition, SIRT1 knockdown induced phenotypic change from a differentiated state to osteoblast-like cells. The senescence-related SMC calcification was completely prevented by p21 knockdown. In addition to Pi-induced premature senescence, SMCs with replicative senescence were also more sensitive to Pi-induced calcification compared with young SMCs, and this finding was attributable to augmented p21 expression.

Conclusion—SIRT1 plays an essential role in preventing hyperphosphatemia-induced arterial calcification via inhibition of osteoblastic transdifferentiation. In addition, Pi-induced SMC calcification may be associated with both premature and replicative cellular senescence. (Arterioscler Thromb Vasc Biol. 2011;31:2054-2062.)

Key Words: cellular senescence • hyperphosphatemia • longevity gene SIRT1 • vascular calcification • vascular smooth muscle cell

Atherosclerotic vascular damage associated with aging manifests several features, namely atherosclerosis, sclerosis, and calcific change, finally leading to cardiovascular events. These pathological changes result in arterial wall thickening (localized morphological changes) and arterial stiffening (functional changes).1 Arterial calcification makes the management of hemodynamics more difficult in the elderly, because ectopic calcium deposition in the aorta and arteries contributes to vessel wall stiffening and loss of elastic recoil.2 These pathological conditions result in unstable hemodynamic consequences, finally leading to a decline in end-organ perfusion and subsequent ischemic events. Recently, several reports have demonstrated that aortic calcification detectable on chest X-ray examination is a strong predictor of future cardiovascular events beyond traditional risk factors.3

Arterial calcification is anatomically separated into two types, intimal and medial calcification.4 Intimal calcification, which is seen as patchy scattered deposits only occurring within atherosclerotic plaques, is shown to be associated with plaque vulnerability.5 On the other hand, medial calcification, which is frequently seen in the elderly and in diabetes and chronic renal failure, is observed as continuous linear deposits along the internal elastic lamina.6 Aadvanced atherosclerosis with both types of calcified lesions is the consequence of overlapping pathological mechanisms.

Ectopic calcification in the vasculature has been shown to result from passive precipitation of calcium with aging and osteoporosis, the so-called calcium shift theory, as a previous hypothesis.7 However, accumulating recent evidence has shown it to be attributable to an active “cell-mediated process” resembling osteogenesis in bone rather than passive mineral precipitation in vascular smooth muscle cells (SMCs).8,9

Silent information regulator-2 (Sir2), an NAD+-dependent HDAC, is highly conserved in organisms ranging from Archaea...
to humans. In yeast, Sir2 has been shown to play critical roles in DNA repair, stress resistance, and longevity. Mammalian sirtuin 1 (SIRT1), the closest homolog of Sir2, regulates the cell cycle, apoptosis, and metabolism by interacting with a number of molecules, including p53, promyelocytic leukemia protein, Foxo, Ku70, and peroxisome proliferator-activated receptor-γ.11 A previous study has shown that SIRT1 antagonizes p53-mediated premature senescence in mouse embryo fibroblasts.12 In addition, we have recently demonstrated that SIRT1 inhibits oxidative stress-induced premature senescence in vascular endothelial cells.13 However, the detailed mechanism of how SIRT1 affects vascular SMC senescence and arterial calcification remains unclear.

In this study, we hypothesized that SIRT1 plays an important role in preventing arterial calcification due to renal failure, in association with modulation of cellular senescence. Here, we demonstrated the protective potential of SIRT1 against hyperphosphatemia-induced premature and replicative senescence and subsequent calcification in SMCs.

Methods

Aortic Calcification in Renal Failure Rats
Renal failure was induced in rats by a 0.75% adenine-containing diet as previously described.14 All procedures and animal care were in accordance with the Guide for the Care and Use of Laboratory Animals of the University of Tokyo. Detailed methods are described in the supplemental materials, available online at http://atvb.ahajournals.org.

Induction of SM C Calcification
Primary human aortic SMCs (HASMCs) were treated with a pathologic concentration of inorganic phosphate (Pi) up to 3.2 mmol/L in culture medium as previously described.29 To quantitatively measure Pi-induced calcification, two distinct experiments were performed as previously described: (1) intracellular calcium deposition as determined by o-cresolphthalein complexone method, and (2) visualization of mineralization as determined by von Kossa staining. Detailed methods are described in the supplemental materials.

Senescence-Associated β-Galactosidase Staining
To assess senescent changes in the phenotype of cultured HASMCs or aortic medial cells of rats, staining for senescence-associated β-galactosidase (SA β-gal), a well-established biomarker of cellular senescence, was performed. Detailed methods are described in the supplemental materials.

Knockdown of SIRT1 or p21 by Small Interfering RNA
HASMCs were transfected with 200 pmol/L small interfering RNA (siRNA) for SIRT1, p21^WAF1/CIP1, or both. Detailed methods are described in the supplemental materials.

Real-Time Polymerase Chain Reaction Analysis: Osteoblastic Markers
To examine whether Pi stimulation induces change to an osteoblastic phenotype, the expression of Runx-2/Cbfa-1 and alkaline phosphatase, which are well known to be representative osteoblastic markers, was checked using real-time polymerase chain reaction analysis. In addition, the effect of knockdown of SIRT1, p21, or both by siRNA on the osteoblastic phenotypic change in HASMCs was examined. Primer sequences are shown in Supplemental Figure I.

Results

Association of Senescent Vascular Cells With Aortic Medial Calcification in Renal Failure Rats
The adenine-fed rats had severe renal failure, with a huge increase in serum creatinine (3.0±0.9 mg/dL in renal failure rats versus 0.3±0.0 mg/dL in control rats), similar to a previous report.14 The renal failure rats showed an approximately 2.0-fold increase in serum phosphorus (18.9±4.7 mg/dL) compared with control rats (9.8±0.9 mg/dL). Histological assessment using von Kossa staining showed that the aorta in renal failure rats had extensive linear calcification, which was localized in the aortic media, resembling the typical Mönckeberg’s pattern (Figure 1A). Numerous SA β-gal-positive cells were found in the aortic media of renal failure rats, whereas the aortic wall in control rats did not contain senescent cells (Figure 1B). The senescent cells were mainly localized to the calcified area and its surrounding area, which was defined as the area not stained black by von Kossa staining. Quantitative assessment showed that the number of senescent cells with high SA β-gal activity was positively correlated with the calcified area in the aortic media (Figure 1C).

Pi Induces Cellular Senescence in Cultured SMCs
On the basis of our results obtained from animal experiments, we hypothesized that senescent SMCs in the aortic media are strongly associated with the development of arterial calcification. Therefore, the effect of excessive Pi stimulation (2.6 mmol/L) on cellular senescence in cultured SMCs was examined. SA β-gal-positive senescent HASMCs were significantly induced by not only angiotensin II (Ang II) but also Pi.

Figure 1. Presence of senescent vascular cells colocalized with calcification in aortic media of renal failure rats. A, Rats with severe renal failure had massive calcification throughout the aorta (right) compared with control rats (left) (n=5). Yellow arrows indicate calcified area. Morphological assessment by von Kossa staining showed extensive calcification in the aortic media of renal failure rats. Scale bar=500 μm. B, Senescent vascular cells (senescence-associated β-galactosidase [SA β-gal]-positive: blue) were significantly detected throughout the calcified area (Calc) in renal failure rats, whereas these senescent cells were not present in control rats. Scale bar=100 μm. C, Localized association between calcification and senescent cells is shown in renal failure rats. SA β-gal-positive cells were frequently found in areas with marked calcification. D, The association of the number of SA β-gal-positive cells with the calcified area in each photograph was evaluated. The senescent cell number was linearly correlated with the area of calcification in the aortic media of renal failure rats (calcified area in media: percentage).
stimulation (Figure 2A). Notably, Pi stimulation increased calcium deposition; however, Ang II alone did not (Figure 2B). It suggests that high-dose Pi condition, but not stress by Ang II alone, is indispensable to induce SM C calcification. These findings also suggest that intracellular Pi influx at least is essential to induce this SM C calcification model.

In addition, to determine how many days after the initiation of Pi stimulation the cells showed a senescent phenotype and subsequent calcification, the time-dependent effects of Pi stimulation on both SA β-gal activity and calcium deposition were examined. As shown in Figure 2C, SA β-gal-positive cells were significantly increased by Pi stimulation even on day 1, although calcium deposition was not markedly increased at the same time point. A statistically significant increase in calcium deposition was found from day 3 and later. Cotreatment with phosphonoformic acid, an inhibitor of Na-dependent phosphate cotransporter (NPC), showed significant inhibition of Pi-induced senescence (Figure 2D). Our previous report showed that treatment with PFA completely inhibited Pi-induced SM C calcification, suggesting the importance of increased intracellular influx of phosphate in Pi-induced SM C senescence.

Downregulation of SIRT1 by Pi
Treatment of HASMCs with Pi caused downregulation of SIRT1 expression in a time-dependent manner (Figure 3A). The decline was dependent on Pi concentration (data not shown). An increase in acetylation of both substrates of SIRT1, histone-3 and p53 (a nonhistone substrate), was found according to the decline in SIRT1 deacetylase activity. In addition, expression of p21, a downstream molecule of p53, was significantly induced by Pi as well. Quantitative assessment showed that an increase in these expression levels of acetylated (Ac)-p53 and p21 on day 3 and day 6 was statistically significant compared with the pretreatment levels, suggesting that downregulation of SIRT1 activity may mediate the subsequent increase in Ac-p53 and p21 expression.

To address whether SIRT1 downregulation-related SM C senescence and calcification are reversible or not, the effects of continuation or termination of high-dose Pi were examined. As shown in Figure 3B, the continuation of Pi up to day 10 was associated with SIRT1 downregulation and subsequent upregulation of Ac-p53 and p21, leading to induction of senescent-related calcification. However, the slight increase in senescent cells was not statistically significant, although calcification was significantly induced. Of note, the Pi-induced downregulation of SIRT1 was almost completely reversed by withdrawal (termination) of Pi stimulation (exchange of Pi from 2.6 mmol/L to 1.4 mmol/L as a normal level on day 6) as shown in Figure 3B. According to the restoration of SIRT1, levels of both Ac-p53 and p21 were also decreased without more progression. In addition, termination of Pi showed no progression of senescent-related calcification; however, preexisting senescent cells and calcification on day 6 continued without regression.

Next, NPC inhibition by PFA completely blunted Pi-induced SIRT1 downregulation and subsequent activation of its downstream p53/p21 pathway (Figure 3C).

Regulation of SIRT1 Modulates Pi-Induced SM C Senescence and Calcification
The effects of modulation of SIRT1 activity on Pi-induced cellular senescence were investigated. First, sirtinol, a chem-
Inorganic phosphate (Pi) stimulation leads to sirtuin 1 (SIRT1) downregulation and subsequent p21 activation. A, The effect of Pi on SIRT1 expression and its downstream pathway was examined. Treatment of human aortic SMCs (HASMCs) with Pi (2.6 mmol/L) showed downregulation of SIRT1 expression, leading to an increase in acetylation of its substrates (acetylated [Ac]-H3 and Ac-p53) and p21 expression. Bottom: Quantitative analysis showed that Pi gradually induced not only SIRT1 downregulation but also upregulation of Ac-p53 and p21. B, To address whether SIRT1 downregulation-related senescence and subsequent calcification are reversible, the effects of continuation or termination of high-dose Pi were examined. As shown in 4th lane from left, termination (on day 6) of Pi showed no progression of senescence-related calcification in association with restoration of SIRT1, whereas continuation (up to day 10) of Pi stimulation showed further progression of calcification. C, Treatment with phosphonoformic acid (PFA), a Na-dependent phosphate cotransporter inhibitor, completely reversed Pi-induced SIRT1 downregulation. A decline in Ac-H3 and Ac-p53 reflected the restoration of SIRT1 deacetylase activity. Pi-induced p21 activation was significantly inhibited by inhibition of Pi transport.

Figure 3. Inorganic phosphate (Pi) stimulation leads to sirtuin 1 (SIRT1) downregulation and subsequent p21 activation. A, The effect of Pi on SIRT1 expression and its downstream pathway was examined. Treatment of human aortic SMCs (HASMCs) with Pi (2.6 mmol/L) showed downregulation of SIRT1 expression, leading to an increase in acetylation of its substrates (acetylated [Ac]-H3 and Ac-p53) and p21 expression. Bottom: Quantitative analysis showed that Pi gradually induced not only SIRT1 downregulation but also upregulation of Ac-p53 and p21. B, To address whether SIRT1 downregulation-related senescence and subsequent calcification are reversible, the effects of continuation or termination of high-dose Pi were examined. As shown in 4th lane from left, termination (on day 6) of Pi showed no progression of senescence-related calcification in association with restoration of SIRT1, whereas continuation (up to day 10) of Pi stimulation showed further progression of calcification. C, Treatment with phosphonoformic acid (PFA), a Na-dependent phosphate cotransporter inhibitor, completely reversed Pi-induced SIRT1 downregulation. A decline in Ac-H3 and Ac-p53 reflected the restoration of SIRT1 deacetylase activity. Pi-induced p21 activation was significantly inhibited by inhibition of Pi transport.
phenotype (D) and calcification (E). The inhibitory effect of resveratrol (100 μmol/L) showed an increase in the number of senescent cells compared with young cells, and the down-regulation was significantly augmented by Pi stimulation (Figure 6B, top). In parallel with this finding, senescent cells showed an increase in Ac-p53 and p21 expression. Statistical analyses using densitometric measurement showed that (1) downregulation of SIRT1 and upregulation of A-c-p53 and p21 were augmented by replicative senescence, and (2) Pi inhibited the SIRT1-p21 pathway even in cells with replicative senescence (passage 18) (Figure 6B, bottom).

**Discussion**

Vascular aging, leading to cardiovascular disease, manifests complex and diverse vascular changes (eg, impairment of distensibility due to loss of arterial elasticity). After arterial wall stiffness resulting from ectopic calcification is a complication of advanced atherosclerosis and makes the management of hemodynamics more difficult in the elderly. Few reports have addressed whether cellular senescence is associated with SMC calcification. This study showed the importance of SIRT1, a longevity gene, in arterial calcification in association with cellular senescence.

First, our data obtained from animal experiments clearly showed the association of senescent SMCs with aortic medial calcification in the renal failure rats with hyperphosphatemia. Senescent cells showed significant colocalization with calcium deposition. Intriguingly, numerous senescent cells could be detected before microscopic calcification occurred at 4 weeks after the start of renal failure induction (data not shown), suggesting that the transition to a senescent phenotype in medial SMCs may be associated with the initiation and progression of calcification. Therefore, hyperphosphatemia, a potent uremic factor, may be a stimulator to induce senescent phenotypic transition of medial SMCs.
Discussion

Augmentation of senescence-related smooth muscle cell (SMC) calcification by sirtuin 1 (SIRT1) knockdown in association with osteoblastic phenotypic change and prevention of inorganic phosphate (Pi)–induced changes by p21 knockdown. A, To achieve SIRT1 knockdown in human aortic SMCs (HASMCs), small interfering RNA (siRNA) was simultaneously administered at the start of Pi stimulation (2.6 mmol/L). Complete inhibition of SIRT1 showed a significant increase in acetylation of both substrates (acetylated [Ac]-H3 and Ac-p53), p21 expression and senescence-associated β-galactosidase (SA-β-gal)-positive cells. B, Angiotensin II (Ang II) alone (10 pmol/L) did not increase the expression of Runx2 in the absence of Pi stimulation, unlike Pi stimulation. C, top: SIRT1 knockdown by siRNA significantly accelerated Pi-induced calcification (n=6), whereas control (Ctrl) siRNA did not. C, middle and bottom: Western blots showed that Pi partially inhibited the expression of a differentiated SMC marker, caldesmon, and complete knockdown of SIRT1 by siRNA augmented its downregulation. Real-time polymerase chain reaction analysis showed that Pi induced the expression of Runx-2 and alkaline phosphatase (ALP). Complete knockdown of SIRT1 significantly accelerated the Pi-induced osteoblastic markers. A.U. indicates arbitrary units. *P<0.05 vs control without Pi stimulation (left column), **P<0.05 vs Pi-stimulated cells with SIRT1 siRNA (sixth column from left).

Figure 5. Augmentation of senescence-related smooth muscle cell (SMC) calcification by sirtuin 1 (SIRT1) knockdown in association with osteoblastic phenotypic change and prevention of inorganic phosphate (Pi)–induced changes by p21 knockdown. A, To achieve SIRT1 knockdown in human aortic SMCs (HASMCs), small interfering RNA (siRNA) was simultaneously administered at the start of Pi stimulation (2.6 mmol/L). Complete inhibition of SIRT1 showed a significant increase in acetylation of both substrates (acetylated [Ac]-H3 and Ac-p53), p21 expression and senescence-associated β-galactosidase (SA-β-gal)-positive cells. B, Angiotensin II (Ang II) alone (10 pmol/L) did not increase the expression of Runx2 in the absence of Pi stimulation, unlike Pi stimulation. C, top: SIRT1 knockdown by siRNA significantly accelerated Pi-induced calcification (n=6), whereas control (Ctrl) siRNA did not. C, middle and bottom: Western blots showed that Pi partially inhibited the expression of a differentiated SMC marker, caldesmon, and complete knockdown of SIRT1 by siRNA augmented its downregulation. Real-time polymerase chain reaction analysis showed that Pi induced the expression of Runx-2 and alkaline phosphatase (ALP). Complete knockdown of SIRT1 significantly accelerated the Pi-induced osteoblastic markers. A.U. indicates arbitrary units. *P<0.05 vs control without Pi stimulation (left column), **P<0.05 vs Pi-stimulated cells with SIRT1 siRNA (sixth column from left).
Second, we also confirmed the association of Pi-induced SMC senescence with calcification in in vitro experiments. Senescent SMCs were significantly increased by Pi even on day 1, although calcium deposition was not markedly increased at the same time point. A statistically significant increase in calcium deposition was found from day 3 and later. Considering these data, we hypothesize that (1) calcium deposition may be more readily induced in senescent cells compared with nonsenescent cells, and (2) Pi-induced senescent change is observed earlier than calcium deposition. In other words, senescent transition associated with Runx2 induction may lead to progressive calcification.

Senescent SMCs were associated with the SIRT1-related p53/p21 pathway, based on the findings that SIRT1 knockdown augmented not only cellular senescence but also calcification. In addition, p21 knockdown completely inhibited senescence-related calcification induced by Pi. This raises the question of how cellular senescence in SMCs is associated with calcification. Our experiments to understand the detailed mechanisms by which SIRT1 modulates senescence-related calcification showed that Pi-induced SIRT1 downregulation led to the phenotypic change from a differentiated state to osteoblast-like cells in SMCs. It has been reported that Pi induces osteoblastic change, in which NPC plays a role in inducing Runx2/Cbfα1 expression, in SMCs. As the next step, to determine how SIRT1 regulates NPC-mediated Runx2 expression, we examined the effects of knockdown of SIRT1, p21, or both by siRNA on Pi-induced Runx2 expression. Our data shown in Figure 5F suggested that (1) NPC plays an essential role in Pi-induced Runx2 expression, (2) SIRT1 has an inhibitory effect on NPC-mediated Runx2 expression, (3) knockdown of p21 alone ameliorates Runx2 induction, and (4) p21-related osteoblastic change is at least in part dependent on SIRT1.

There is the new question of how SIRT1 regulates Runx2 regulation. A report by Jeon has shown that acetylation of Runx2 itself is important in osteoblast differentiation, and it is downregulated by HDACs. Based on this evidence, SIRT1, 1 of the HDACs, may be able to deacetylate Runx2, leading to inhibition of Runx2-related osteoblastic change in SMCs. Therefore, the inhibition of SIRT1 by hyperphosphatemia may lead to Runx2 activation via its hyperacetylation. Further investigation of the detailed mechanism of the SIRT1/p21/osteoblastic gene axis is needed. These data clearly suggest that SIRT1 activation may inhibit the hyperphosphatemia-induced osteoblastic phenotypic change of SMCs, and the degree of change may be dependent on SIRT1 expression level. It is possible that the inhibition of SIRT1 expression by Pi alone is "partial," because complete downregulation of SIRT1 by siRNA worsened the dynamic phenotypic change compared with Pi only. We have already shown that tumor necrosis factor-α, a potent atherogenic cytokine, augmented Pi-induced SMC calcification, as previously described. In addition, tumor necrosis factor-α significantly decreased Pi-induced SIRT1 downregulation further (data not shown). According to these results, we currently hypothesize that hyperphosphatemia induces SIRT1 downregulation and subsequent osteoblastic phenotypic change in SMCs, leading to calcification, and these changes are worsened by some harmful atherogenic factors, which decrease SIRT1 expression/activity further. These results provide a new insight, showing that SIRT1 plays an essential role in the prevention of arterial calcification and that the beneficial effect may be associated with an inhibition in Pi-induced SMC senescent transition.
In addition, Ang II did not increase calcium deposition, although the stimulation increased the number of senescent cells. Of note, Ang II alone did not increase Runx2 expression in the absence of Pi (Figure 5B). This result suggests that SMC senescence shows two different features: one is SA β-gal-positive cells with an increase in Runx2 and the other is SA β-gal-positive cells without. First, it has recently been reported that SMCs with replicative senescence, rather than the cells without senescence, show hypersensitivity in response to induction of calcification with the more induction of osteoblastic markers; suggesting that the induction of osteoblastic transdifferentiation is strongly associated with the senescent change in SMCs. In addition, the translocation of SIRT1 to cytoplasm was observed after Pi stimulation for 24 hours, although SIRT1 predominantly localized in nucleus without Pi. In contrast, Ang II did not show the dynamic translocation. Thinking about the mechanism for regulating the activity of HDACs, including SIRT1, recent several reports show the importance of their coordinated shutting between nucleus and cytoplasm. A report demonstrates that HDAC7, an HDAC, represses the transcriptional activity of Runx2 and that osteogenic stimuli induce export of HDAC7 from nucleus, leading to a decline in the repressive potentials of HDAC7 for Runx2. On the basis of our findings and a previous report, the reason that stimulation with Ang II alone did not induce Runx2 expression and subsequently SM C calcification may in part depend on the difference of SIRT1 translocation after stimulation. Therefore, we strongly hypothesize that in the senescent SMCs with upregulation of p21, Pi stimulation, but not Ang II stimulation, may activate Runx2 via at least two phenomena, the hyperacetylation of Runx2 by SIRT1 downregulation and the dynamic SIRT1 translocation, leading to marked osteoblastic transdifferentiation and subsequent calcification. In addition, we have another hypothesis. In general, it has been shown that high-dose Pi navigates release of matrix vesicles from SM Cs in parallel with osteoblastic transdifferentiation. The vesicles play an essential role in the initiation of hydroxyapatite aggregation, so-called nucleation. A accumulating recent reports show that the nanocrystal formation as an initial step under hyperphosphatemia accelerates the harmful cascade of osteoblastic transdifferentiation in SM Cs via endocytosis. M aybe Ang II alone does not induce the nanocrystal formation and the cascade of osteoblastic change. Therefore, we explain that the difference of senescent phenotypic changes in SM Cs between both stimulations, Pi and Ang II alone, may depend on (1) SIRT1 translocation and (2) nanocrystal formation to accelerate calcification. Further investigation to address the detailed mechanisms by which SIRT1 regulates osteoblastic transdifferentiation in SM Cs under the cellular senescence is needed.

Are SIRT1 downregulation-related SMC senescence and subsequent calcification reversible or not? To answer this question, the effects of continuation or termination of high-dose Pi were examined. As shown in Figure 3B, termination (on day 6) of Pi showed no progression of senescence-related calcification in association with the restoration of SIRT1, whereas continuation (up to day 10) of Pi stimulation showed further progression of calcification. It is suggested that a therapeutic strategy to manage hyperphosphatemia to the normal range of serum phosphate concentration may lead to at least termination of progressive calcification via reversal of SIRT1 activity.

Cellular senescence has been shown to have two features: not only stress-induced premature senescence but also replicative senescence, indicating a limited number of divisions in culture. In fact, both endothelial cells and SMCs derived from human atherosclerotic plaques show a senescent phenotype earlier than do cells from normal vessels. Notably, we found that senescent HAMCs were significantly more sensitive to Pi-induced calcification compared with young cells. These results suggest that calcium deposition may be more readily induced in arterial medial SM Cs with replicative senescence. This insight may explain the mechanisms by which arterial calcification occurs in the elderly more frequently than in the young population. Therefore, these observations support our hypothesis that arterial calcification is accelerated by both senescent types (premature and replicative senescence) in SM Cs. To explore new therapeutic strategies against arterial calcification, it is essential to investigate how to maintain a higher SIRT1 level in the vasculature, leading to prevention of medial SMC senescence and which drug is capable of achieving it.

How does SIRT1 exert protective effects against SMC calcification? This study clearly showed that inhibition of SIRT1 was associated with increases in both A-c-p53 and p21 expression. These findings were significantly induced by not only replicative senescence but also Pi-induced premature senescence. SIRT1-mediated deacetylation of p53 inhibits p53-dependent transactivation of target genes, including p21. A report showed that a decline in cellular deacetylase activity increases the half-life of endogenous p53, suggesting that p53 acetylation is also associated with p53 stabilization. Therefore, the increased A-c-p53 by Pi-induced SIRT1 downregulation may induce SM C senescence because of a decline in degradation of p53, leading to calcification. In addition, p53 itself can inhibit SIRT1 transcription because the SIRT1 promoter has two response elements to p53. Further investigation to address how the SIRT1-p53 negative regulatory pathway is associated with SMC calcification is needed.

On the other hand, regarding p21 activation, it is reported that inhibition of p21 expression in the vasculature significantly attenuates cellular senescence, leading to prevention of atherosclerosis. This evidence suggests a pivotal role of p21 in the development of atherosclerosis. p21 activation has been shown to be regulated by a pathway that is p53 dependent, p53 independent, or both. Okamoto et al have demonstrated that inhibition of HDAC by trichostatin A showed activation of p21 promoter activity by the Sp1 site even in vascular SM Cs, and the induction of p21 was independent of the p53 pathway. The p21 transcriptional activation in response to HDAC inhibitors was mediated by histone hyperacetylation in its promoter region. Based on these findings, Pi-induced p21 activation via SIRT1 downregulation may be in part involved in a p53-independent pathway, leading to a senescent phenotype of SM Cs. Further investigation exploring which molecule activates the p21 promoter under hyperphosphatemia is needed.
Conclusion

We showed that SIRT1 exerts a protective role in hyperphosphatemia-based arterial calcification via inhibition of osteoblastic transdifferentiation, in association with crosstalk between calcification and cellular senescence. This ability of SIRT1 may orchestrate an analogous protective/longevity paradigm even in vascular SMCs, leading to maintenance of healthy elasticity of the arterial wall. Strategies to maintain a higher level of SIRT1 activity may provide novel therapeutic opportunities for the prevention of arterial calcification.

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Disclosures

None.

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