*Original Article*

# Humoral and cellular factors inhibit phosphate-induced vascular calcification during the growth period

**Yuki Kamei,[1,2] Yosuke Okumura,[1] Yuichiro Adachi,[1] Yuki Mori,[1] Maiko Sakai,[1] Kohta Ohnishi,[1] Hirokazu Ohminami,[1] Masashi Masuda,[1] Hisami Yamanaka-Okumura,[1,3] and Yutaka Taketani[1,*]**

[1]Department of Clinical Nutrition and Food Management and [2]Department of Food and Nutrition, Tokushima University Graduate School of Medical Nutrition, 3-18-15 Kuramoto-cho, Tokushima 770-8503, Japan
[3]Department of Food Science and Nutrition, Doshisha Women's College of Liberal Arts, Teramachi Nishi-iru, Imadegawa-dori, Kamigyo-ku, Kyoto 602-0893, Japan

(Received 7 February, 2023; Accepted 25 February, 2023; Released online in J-STAGE as advance publication 28 June, 2023)

**Hyperphosphatemia is an independent and non-classical risk factor of cardiovascular disease and mortality in patients with chronic kidney disease (CKD). Increased levels of extracellular inorganic phosphate (Pi) are known to directly induce vascular calcification, but the detailed underlying mechanism has not been clarified. Although serum Pi levels during the growth period are as high as those observed in hyperphosphatemia in adult CKD, vascular calcification does not usually occur during growth. Here, we have examined whether the defence system against Pi-induced vascular calcification can exist during the growth period using mice model. We found that calcification propensity of young serum (aged 3 weeks) was significantly lower than that of adult serum (10 months), possibly due to high fetuin-A levels. In addition, when the aorta was cultured in high Pi medium *in vitro*, obvious calcification was observed in the adult aorta but not in the young aorta. Furthermore, culture in high Pi medium increased the mRNA level of tissue-nonspecific alkaline phosphatase (TNAP), which degrades pyrophosphate, only in the adult aorta. Collectively, our findings indicate that the aorta in growing mouse may be resistant to Pi-induced vascular calcification via a mechanism in which high serum fetuin-A levels and suppressed TNAP expression.**

***Key Words*: chronic kidney disease, hyperphosphatemia, fetuin-A, pyrophosphate, alkaline phosphatase**

Inorganic phosphate (Pi) is an essential nutrient as a component of bone, cell membranes, and adenosine triphosphate, and to maintain these functions, serum Pi levels are normally maintained within a certain range (2.5–4.5 mg/dl).[1,2] However, hyperphosphatemia is caused by chronic kidney disease (CKD) due to decreased Pi excretion. Hyperphosphatemia is a leading cause of vascular calcification, and closely related to cardiovascular mortality.[3] Vascular calcification, the pathological deposition of hydroxyapatite crystals in the blood vessel wall, is a risk factor for arterial stiffness and cardiovascular disease, especially in patients with CKD.[4,5] Vascular calcification is classified into intimal, valvular, and medial types, the last of which is commonly observed in patients with CKD.[6] Although the detailed mechanism of vascular calcification has not been clarified, both passive deposition of hydroxyapatite and active calcification similar to the process involved in bone formation are involved. The key step in vascular calcification is considered to be the osteochondrogenic differentiation of vascular smooth muscle cells in response to various external stresses,[7] such as hyperphosphatemia, diabetes, hypoxia, and ageing. Conversely, there are several mechanisms that inhibit vascular calcification, including humoral factors such as fetuin-A and inorganic pyrophosphate (PPi). A decrease in the circulation of these inhibitors can also promote vascular calcification.[8]

A recent intervention study reported that strict control of serum Pi levels improved the coronary artery calcification score in dialysis patients.[9] This suggests that lowering serum Pi levels by medication of Pi-binding agents or dietary Pi restriction by avoidance of foods related to increasing serum Pi levels may be effective in the prevention of vascular calcification,[10] however those treatments are not always successful easily. For example, adherence of Pi-binding agents for hyperphosphatemia is often not good due to polypharmacy problem.[11,12] On the other hand, dietary Pi restriction also has low adherence and may cause protein energy wasting or malnutrition with several nutrients in relation to low protein diet.[13] Therefore, a different approach against vascular calcification should be needed.

Notably, serum Pi levels change over lifetime. Levels are highest in early life-stages and gradually decrease with aging as long as renal function is normal.[14,15] Because low serum Pi during the growth period leads to bone dysplasia and short stature, maintaining high serum Pi levels during this life stage must be essential for normal bone modeling.[15] Interestingly, serum Pi during the growth period (4–7 mg/dl) is as high as that in cases of hyperphosphatemia among patients with end-stage CKD (5–10 mg/dl). Nevertheless, vascular calcification does not occur during the growth period in healthy individuals. This is probably related to the increased demand for Pi in bone, however there is no evidence showing that active bone modeling prevents vascular calcification. Moreover, even if Pi is used for bone formation, arteries continued to be exposed to high serum Pi level, so some mechanism should be needed to prevent vascular calcification. Therefore, we hypothesized that an underlying mechanism prevents vascular calcification during the growth periods, while allowing hard tissues to be calcified. To examine whether the defence system against Pi-induced vascular calcification can exist during the growth period, here we identified candidates for humoral and cellular factors among the known inhibitor or stimulator of vascular calcification by comparing mice at the growth stage with adult mice.

*To whom correspondence should be addressed.
E-mail: taketani@tokushima-u.ac.jp

doi: 10.3164/jcbn.23-11

**Table 1.** Oligonucleotide primers for real-time PCR analysis

| Gene name | Forward primer (5'-3') | Reverse primer (5'-3') |
|---|---|---|
| β-actin | CTGACCCTGAAGTACCCCATTGAACA | CTGGGGTGTTGAAGGTCTCAAACATG |
| Abcc6 | ACCATGAGCTTTGCCACCTTT | AGCCAGTACCCGAACAACAC |
| Enpp1 | CTGGTTTTGTCAGTATGTGTGCT | CTCACCGCACCTGAATTTGTT |
| Ank | ATGTGGATGAGTCTGTGGGGAG | TGGCTACGAAAACAACCTGAGC |
| TNAP | GTTGCCAAGCTGGGAAGAACAC | CCCACCCCGCTATTCCAAAC |
| Fetuin-A | GAAACAAGACGGCCAGTTCA | TGGACCACGTTGGTATCGTT |
| Rplp0 | GGGCATCACCACGAAAATCTC | CTGCCGTTGTCAAACACCT |
| MGP | GGCAACCCTGTGCTACGAAT | CCTGGACTCTCTTTTGGGCTTTA |
| αSMA | GTCCCAGACATCAGGGAGTAA | TCGGATACTTCAGCGTCAGGA |
| SM22α | TTCTTGAAGGCAGCTGAAGA | GCACTGCTGCCATATCCTTA |
| Pit-1 | TCTGACCTTCACTCCGAGTCTG | AAGGATCTGCAGGAACTGGAAG |
| Runx2 | TGCACCTACCAGCCTCACCATAC | GACAGCGACTTCATTCGACTTCC |
| IL-6 | CTGATGCTGGTGACAACCAC | TCCACGATTTCCCAGAGAAC |

Abcc6, ATP binding cassette sub-family C member 6; Enpp1, ectonucleotide pyrophosphatase/phosphodiesterase 1; Ank, progressive ankylosis protein; TNAP, tissue nonspecific alkaline phosphatase; Rplp0, ribosomal protein lateral stalk subunit P0; MGP, matrix gla protein; αSMA, α-smooth muscle actin; SM22α, smooth muscle 22α; Pit-1, sodium-dependent phosphate transporter 1; Runx2, Runt-related transcription factor 2; IL-6, interleukin 6.

## Materials and Methods

**Animals.** C57BL/6J male mice aged 3 weeks or 9–10 months were purchased from The Jackson Laboratory Japan, Inc. (Yokohama, Japan) and Japan SLC, Inc. (Shizuoka, Japan). All animals were kept at the Division for Animal Research Resources and Genetic Engineering, Support Center for Advanced Medical Sciences, Tokushima University Graduate School under pathogen-free conditions. They were maintained in a climate-controlled room (22 ± 2°C) under a 12/12 h light/dark cycle with free access to distilled water and standard chow (MF; Oriental Yeast Co., LTD., Tokyo, Japan). All mice were euthanized under anesthesia for sample collection. All animal studies were approved by the animal experimentation committee of Tokushima University and conducted in accordance with the guidelines for the management and handling of experimental animals. (Approval No. T2019-27 and T2022-28).

**Determination of serum calcification propensity.** Calcification propensity was evaluated *in vitro* by overloading Ca and Pi into serum samples as previously reported with a few modifications.[16] In brief, 80 µl of serum was mixed with 20 µl of NaCl (140 mM), 50 µl of Pi stock solution (24 mM), and 50 µl of Ca stock solution (40 mM) in a 96-well plate. The plate was then covered with a thin adhesive sealing film and incubated at 37°C for 600 min. Optical density at 650 nm ($OD_{650}$) was measured every 60 min with a microplate reader (SpectraMax ABS; Molecular Devices, San Jose, CA).

**Plasma biochemical parameters.** Blood samples were collected into tubes with heparin (Mochida Pharmaceutical Co., Ltd., Tokyo, Japan) by inferior vena cava puncture. After centrifugation for 15 min at 5,000 × *g*, the supernatant was collected as plasma. For the pyrophosphate assay, each plasma sample was immediately filtered by a membrane filter (Nanosep 30 K, Pall, Port Washington), snap-frozen in liquid nitrogen, and stored at −80°C until analysis. Plasma phosphate was estimated by the *p*-methyl aminophenol method using Phospha-C test (FUJIFILM Wako Pure Chemical Corp., Osaka, Japan). Plasma calcium (Ca) was estimated by the methylxylenol blue method using Calcium-E test (FUJIFILM Wako Pure Chemical Corp.). Plasma fetuin-A was estimated by a Quantikine® ELISA kit (R&D Systems, Minneapolis, MN). Plasma PPi was estimated by PPi Light™ (Lonza, Basel, Switzerland). Alkaline phosphatase activity was estimated by measuring the absorbance of *p*-nitrophenol converted from *p*-nitrophenyl phosphate (FUJIFILM Wako Pure Chemical Corp.) as a substrate.

**Real-time RT-PCR analysis.** Total RNA was isolated from homogenized liver, femur and aorta tissue by using RNAiso Plus (Takara Bio Inc., Shiga, Japan) and then digested by recombinant DNase I (Worthington Biochemical, Lakewood). Femur without bone marrow flushing was subjected to homogenize. First-strand cDNA was synthesized from 1.0 µg of total RNA by using M-MLV Reverse Transcriptase (Nippon Gene, Tokyo, Japan), Oligo (dT) Primer (Invitrogen, Waltham, MA), and dNTP Mixture (Promega, Madison, WI). After cDNA synthesis, real-time PCR was performed with appropriate forward and reverse primers and Fast SYBR™ Green master mix (Applied Biosystems, Waltham, MA) using a real-time PCR system (StepOne Plus; Applied Biosystems). The primer sequences and target genes, including abbreviations, are summarized in Table 1. Each gene was quantified as the mRNA level normalized to a housekeeping gene (β-actin for liver and femur, Rplp0 for aorta) using the comparative Ct method.

***Ex vivo* culture of aortic tissue.** Thoracic aortas were isolated, perfused with phosphate-buffered saline (PBS), and maintained in Dulbecco's modified Eagle's medium (high glucose; Sigma, St. Louis, MO) containing 10% fetal bovine serum (Cosmo Bio Co., LTD., Tokyo, Japan), 100 U/ml penicillin, and 100 µg/ml streptomycin (Nacalai Tesque, Inc., Kyoto, Japan) at 37°C in a humidified atmosphere of 5% $CO_2$ and 95% air. Calcification was induced by incubating each aorta in 5 mM high Pi medium. To examine the effect of tissue-nonspecific alkaline phosphatase (TNAP) inhibitor on aortic calcification, levamisole was added at a final concentration of 1 mM. The aorta was then incubated for 8 days with replacement of the medium every 2 days. The first region 2–3 mm of the descending aorta was used for histology and remaining sample was used for Ca deposition or real-time PCR analysis.

**Evaluation of calcification.** To evaluate Ca deposition, each incubated aorta was immersed in 0.6 N HCl for 24 h at room temperature. The amount of dissolved Ca was determined by the methylxylenol blue method described above and corrected by wet tissue weight. For histological analysis, each cultured aorta was fixed overnight by 4% paraformaldehyde/PBS and embedded in paraffin wax. The paraffin block was dissected in 5-µm sections, subjected to Von Kossa staining as previously described,[17,18] and counterstained with hematoxylin-eosin (HE).

For Alizarin red staining, tissue sections were treated with 1% alizarin red solution (pH 6.4) for 5 min, and then washed with distilled water.

**Statistical analysis.** Welch's *t* test was used for two-group comparisons and one-way analysis of variance (ANOVA) with Tukey-Kramer post hoc test was used to compare data among three groups or more. All statistical analysis was performed using GraphPad Prism 9 (GraphPad Software, San Diego, CA). A $p$ value of <0.05 was considered to be statistically significant.

## Results

**Young serum more strongly inhibits the ripening of calciprotein particles.** To test the hypothesis that a humoral factor may contribute to the inhibition of Pi-induced vascular calcification during the growth period, we compared the calcification propensity of serum between young mice (aged 3 weeks) and adult mice (10 months). Without serum, a supersaturated solution of Ca and Pi immediately formed a visible white precipitation with a high $OD_{650}$ (Fig. 1A, 0 min). Addition of serum inhibits this precipitation due to the formation of colloidal particles of Ca, Pi, and fetuin-A, known as calciprotein particles (CPPs). Here, a sharp increase in $OD_{650}$ was observed at 120–300 min after the start of incubation with adult serum (Fig. 1A), which may reflect the transition from amorphous primary CPP to more crystalline secondary CPP.[16] With young serum, an increase in $OD_{650}$ was observed at 240–420 min after the start of incubation (Fig. 1A). At 600 min, $OD_{650}$ was significantly lower in young serum than in adult serum (Fig. 1B). Correspondingly, the amount of precipitation at the endpoint was clearly smaller in young serum than in adult serum (Fig. 1C). These results suggest that, as compared with adult serum, young serum can inhibit more strongly the ripening of CPPs from amorphous primary particles to more crystalline secondary participles.

**Plasma fetuin-A levels is higher in young mice than adult mice.** Next, we compared endogenous calcification stimulators and inhibitors in plasma between young mice and adult mice to investigate the factors involved in the differences in calcification propensity. Plasma Pi and Pi × Ca product levels were significantly higher in young mice than in adult mice (Table 2). In addition, the level of inorganic pyrophosphate (PPi), one of the potent calcification inhibitors,[19] was significantly lower in young plasma than in adult plasma (Table 2). This may not be due to low hepatic PPi production but high femoral expression of tissue-nonspecific alkaline phosphatase (TNAP),[20] which degrades PPi, and high plasma alkaline phosphatase activity in young plasma (Table 2, Fig. 2A and B). On the other hand, plasma concentrations and liver mRNA levels of fetuin-A, which is a systemic inhibitor of calcification and major components of the CPP,[21] were significantly higher in young mice (Table 2 and Fig. 2B). These data suggest that high levels of fetuin-A may counteract the high vulnerability to calcification due to high Pi, high calcium, and low PPi in young plasma.

**Young aorta is more resistant to Pi-induced vascular calcification.** An alternative hypothesis is that the young aorta *per se* may have an inhibitory mechanism against Pi-induced calcification. To test this hypothesis, we compared the susceptibility to Pi-induced vascular calcification between young aorta and adult aorta. It is difficult to evaluate this susceptibility *in vivo* because multiple macro- and micro-environmental factors affect vascular calcification; therefore, we used an *ex vivo* culture model to compare the young and adult aorta under the same environment. Interestingly, young aorta did not exhibit calcification under the high Pi condition (Fig. 3A and C), whereas adult aorta showed obvious calcification (Fig. 3B and C). These results suggest that young aorta may be more resistant to Pi-induced vascular calcification as compared with adult aorta.

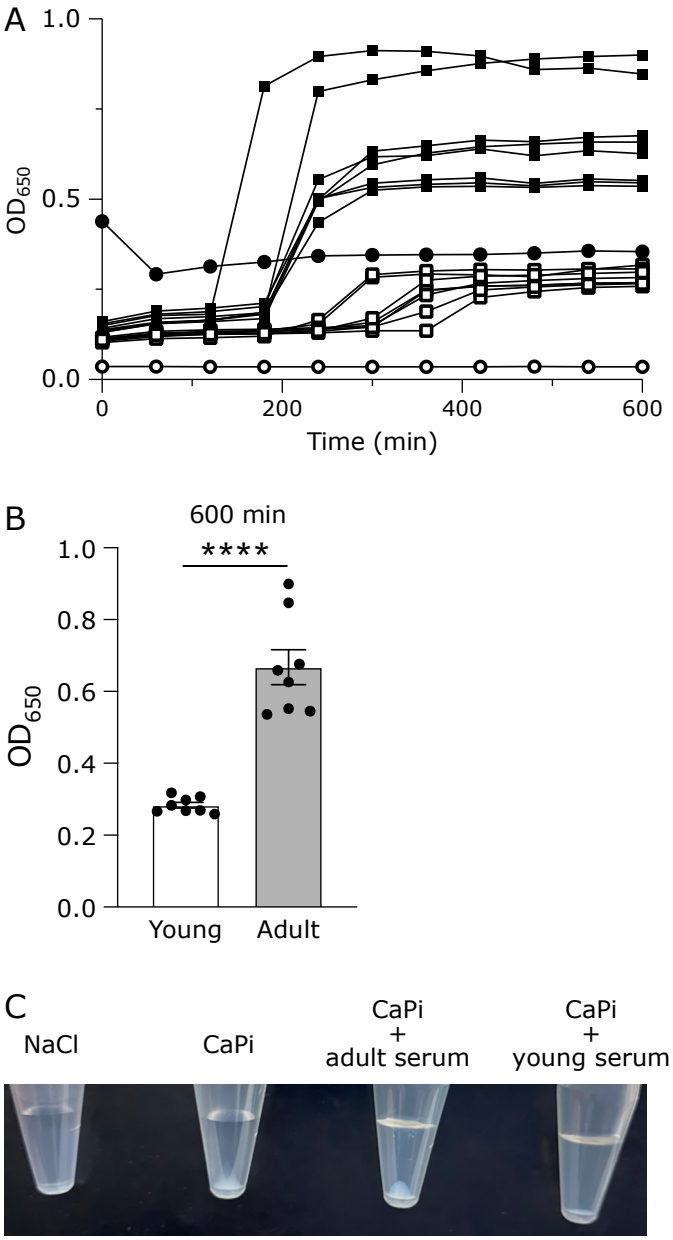


**Fig. 1.** Calcification propensity of young and adult serum. The calcification propensity of serum was compared between young (3 weeks) and adult (9 months) mice. Calcium and phosphate (CaPi) were added to serum for 600 min, and optical density at 650 nm ($OD_{650}$) was measured every 60 min. (A) Changes in $OD_{650}$. Young serum is shown as open squares, adult serum as closed squares, NaCl (negative control) as open circles, and CaPi (positive control) as closed circles. (B) $OD_{650}$ at endpoint (600 min). Data are expressed as mean ± SEM ($n$ = 8, ****$p$<0.0001). (C) Images of precipitation at the endpoint (600 min) after centrifugation.

**Pi-induced TNAP mRNA upregulation is suppressed in young aorta.** Then, we compared the mRNA expression of genes involved in vascular calcification between young and adult aorta cultured under both normal control (CP) and high Pi (HP) conditions. The mRNA levels of MGP, Enpp1, αSMA, Pit-1, and Runx2 were not significantly different among the four groups, while the mRNA expression of SM22α was significantly lower in the adult HP group than adult CP Group (Fig. 4A–F). Notably, the mRNA expression of TNAP and IL-6 was significantly


doi: 10.3164/jcbn.23-11

**Table 2.** Comparison of plasma biochemical data between young (3 weeks) and adult (10 months) mice

| | Young | Adult |
|---|---|---|
| Pi (mg/dl) | 8.88 ± 0.59 | 7.25 ± 0.24* |
| Ca (mg/dl) | 8.01 ± 0.14 | 7.63 ± 0.13 |
| Pi × Ca ($mg^2/dl^2$) | 71.44 ± 5.78 | 55.27 ± 1.92* |
| PPi (μmol/L) | 0.12 ± 0.03 | 1.04 ± 0.12*** |
| ALP activity (μmol/L/min) | 114.40 ± 3.43 | 15.11 ± 1.27**** |
| Fetuin-A (μg/ml) | 171.78 ± 7.92 | 76.72 ± 6.32**** |

Data are expressed as mean ± SEM ($n = 6$). *$p<0.05$, ***$p<0.001$, ****$p<0.0001$ vs young mice.

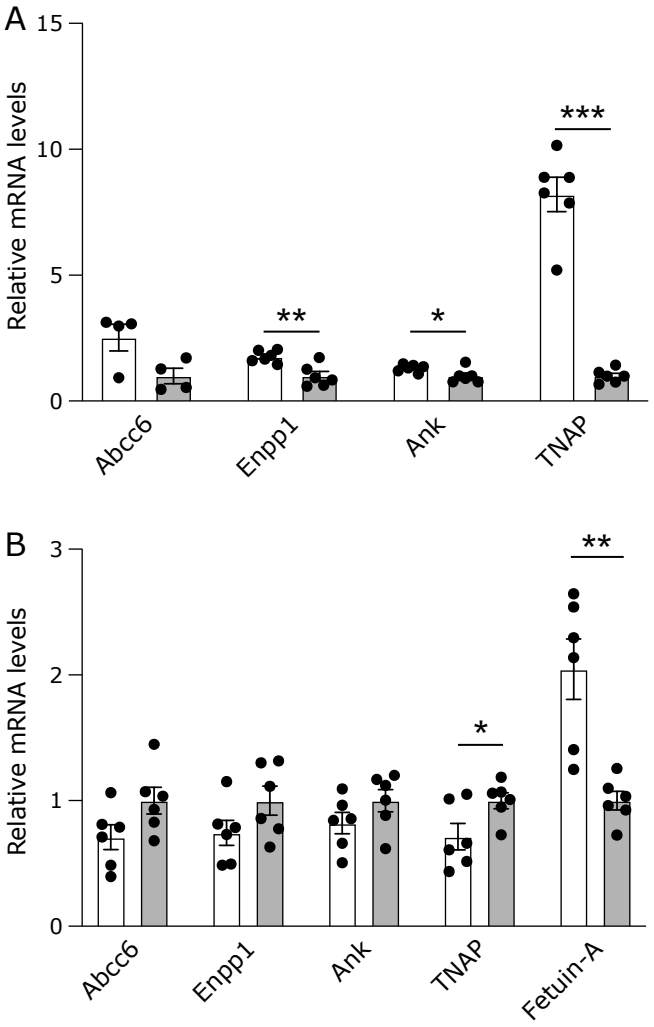


**Fig. 2.** Expression of genes associated with inhibition of Pi-induced vascular calcification in the liver and femur of young and adult mice. Relative mRNA levels of Abcc6, Enpp1, Ank, TNAP, and fetuin-A in the femur (A) and liver (B) of young (3 weeks, open column) and adult (10 months, grayish column) mice were measured by real-time RT-PCR. β-Actin was measured as an endogenous control. Quantitative data are expressed as mean ± SEM ($n = 4–6$, *$p<0.05$, **$p<0.01$, ***$p<0.001$).

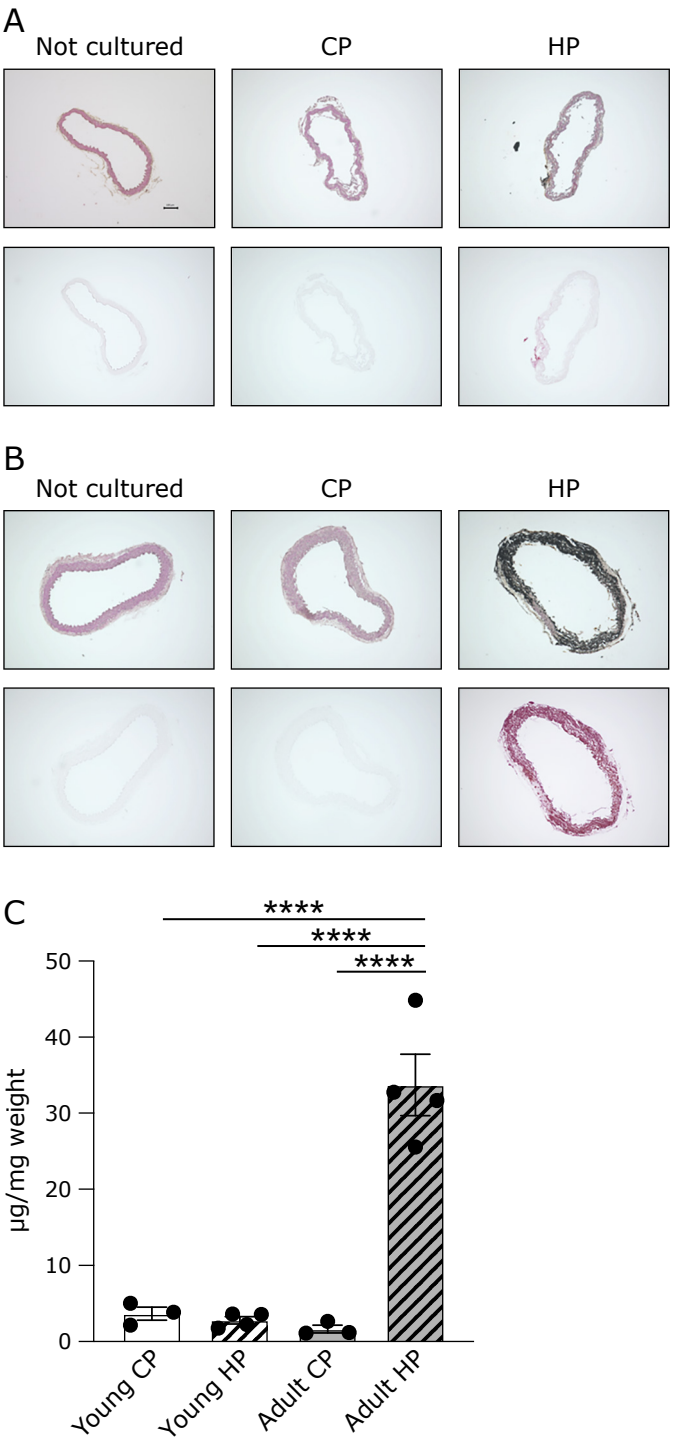


**Fig. 3.** Effect of incubation in high Pi on vascular calcification in the aorta of young and adult mice. The descending aorta was harvested from young (3 weeks) and adult (10 months) and immediately treated for histology (Not cultured), or incubated for 8 days in either control Pi (CP, 1 mM) or high Pi (HP, 5 mM) medium. (A, B) Paraffin sections of young (A) and adult (B) aorta were stained by Von Kossa staining and HE staining (upper row), or Alizarin Red staining (lower row). Scale bar = 100 μm. (C) Cultured aortas were decalcified by HCl, and dissolved Ca was determined. Total Ca was corrected by wet tissue weight. Data are expressed as mean ± SEM ($n = 3–4$, ****$p<0.0001$).

higher in the adult HP group than in any other group (Fig. 4G and H). Because TNAP is known to have an important role in the development of vascular calcification,[22] we further explored the role of TNAP on vascular calcification in our aortic culture model by evaluating the effect of the TNAP inhibitor levamisole.

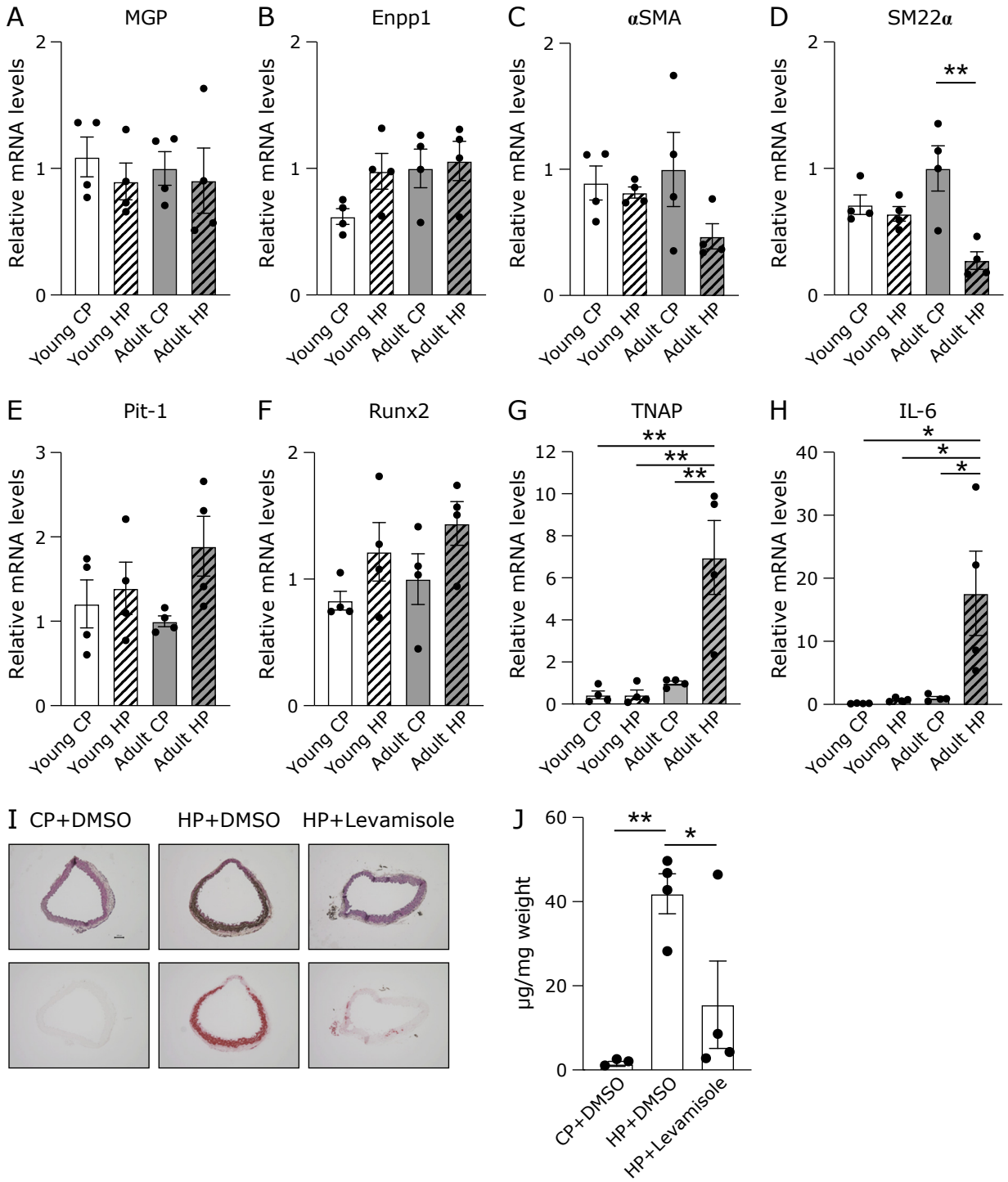


**Fig. 4.** Effect of incubation in high Pi on mRNA expression in the aorta of young and adult mice. (A–H) Expression of genes associated with vascular calcification. The descending aorta was harvested from young (3 weeks) and adult (10 months) mice, and then incubated for 8 days in control Pi (CP, 1 mM) or high Pi (HP, 5 mM) medium. Relative mRNA levels of MGP (A), Enpp1 (B), αSMA (C), SM22α (D), Pit-1 (E), Runx2 (F), TNAP (G), and IL-6 (H) in aorta of young and adult mice were measured by real-time RT-PCR. Rplp0 was used as an endogenous control. Quantitative data are expressed as mean ± SEM ($n = 4$, *$p<0.05$, **$p<0.01$) (I, J) Effect of levamisole on Pi-induced vascular calcification in adult mice. The descending aorta was harvested from adult mice (9 months), and then incubated for 8 days in control Pi medium (CP, 1 mM) plus dimethyl sulfoxide (DMSO), or high Pi medium (HP, 5 mM) plus DMSO, or HP plus 1 mM levamisole, and then prepared for histology and Ca determination. (I) Paraffin sections were stained by von Kossa staining and HE staining (upper row), or Alizarin Red staining (lower row). Scale bar = 100 μm. (J) Cultured aortas were decalcified by HCl, and dissolved Ca was determined. Total Ca was corrected by wet tissue weight. Data are expressed as mean ± SEM ($n = 3$–4, *$p<0.05$, **$p<0.01$).



doi: 10.3164/jcbn.23-11

As shown in Fig. 4I and J, levamisole significantly suppressed Pi-induced vascular calcification in adult aorta. These results suggest that the suppression of Pi-induced TNAP expression may contribute to resistance to vascular calcification during the growth period.

## Discussion

To our best knowledge, this is the first study to investigate why vascular calcification is not observed during the normal growth period even though serum Pi levels are higher than those observed in adult. Our results indicate that there are at least two contributing factors, fetuin-A, and TNAP, involved in the mechanism to protect the aorta from Pi-induced vascular calcification during the growth period. Vascular calcification can be closely related to bone calcification. Several reports have shown a negative correlation between vascular calcification and bone mineral density.[23,24] This is called the calcification paradox and have been major issues in the research field of calcification for many years.[25] Clarifying the mechanism that prevents vascular calcification during the growth period may be an important to understand the calcification paradox.

Higher serum calcification propensity is associated with more severe vascular calcification and mortality among patients with CKD.[26,27] In addition, high calcification propensity can predict cardiovascular mortality independent of renal function.[28] Despite the high Pi × Ca product and low PPi levels, our results revealed that the calcification propensity of young serum was lower than that of adult serum, suggesting that young serum is likely to contain either large amounts of calcification inhibitors or small amounts of calcification promoters. Given the role of fetuin-A in suppressing CPP ripening,[16] the low serum calcification propensity observed during the growth period may be explained, in part, by high fetuin-A levels. To date, several other inhibitors of CPP ripening such as albumin,[16] magnesium,[16,29] and zinc have been identified;[30,31] however, there are no studies demonstrating a relationship between their abundance in serum and CPP ripening during the growth period. Further research is needed to determine in detail the cause of the low calcification propensity of young serum.

Fetuin-A is secreted from the liver into the circulation and inhibits calcification by forming CPPs.[32] Many studies have demonstrated the relationship between plasma fetuin-A and vascular calcification or cardiovascular disease, including a study in paediatric CKD.[33–35] Fetuin-A is highly enriched in the mineralized bone matrix,[36] and mice lacking fetuin-A exhibit dysplasia of long bone.[37] Furthermore, CPPs extravasated into the bone marrow are deposited on the inner surface of the bone.[38] Therefore, the high levels of plasma fetuin-A observed during the growth period seem to be important not only for maintaining bone formation, but also for inhibiting vascular calcification by transporting Ca and Pi to the bone. However, further studies are needed to clarify why high levels of fetuin-A expression are maintained in the liver during the growth period.

In contrast to fetuin-A, plasma PPi levels were significantly lower in young mice than in adult mice. PPi inhibits calcification by binding to hydroxyapatite crystals and inhibiting their growth.[39] Lower plasma PPi levels are reported in patients with end-stage CKD and may be involved in vascular calcification.[40,41] The absence of vascular calcification during the growth period despite low plasma PPi concentrations suggests that PPi production in the vascular microenvironment is more important than circulating PPi in inhibiting calcification. In support of this, it has been reported that lethal vascular calcification occurs in mice overexpressing TNAP specifically within vascular smooth muscle cells even though circulating PPi levels do not decrease.[42]

The TNAP enzyme is expressed primarily in bone, kidney, and liver, and can strongly promote calcification by degrading PPi and producing Pi.[22] It is also expressed in vascular smooth muscle cells and upregulated under pro-calcific conditions. We demonstrated that the calcification propensity of young aorta was lower than that of adult aorta, and was possibly related to the suppression of Pi-induced TNAP expression. TNAP expression is regulated by Runx2, a master regulator of osteoblasts that activates TNAP expression via its binding enhancer region in mouse mesenchymal-like C3H10T1/2 cells.[43,44] However, our data demonstrated that neither young nor adult aorta showed a significant increase in Runx2 expression after incubation in high Pi medium.

A limitation of this study is that the *ex vivo* experiment may not fully reflect *in vivo* conditions. In addition, this study focused only on known inhibitors or stimulators of calcification. Global multi-omics analysis will be helpful to identify other important factors, leading to understand the defense system against Pi-induced vascular calcification during the growth period.

In conclusion, our findings indicate that in growing mice the aorta may be resistant to Pi-induced vascular calcification, via a mechanism based on high serum fetuin-A levels and suppression of TNAP expression in response to high serum Pi. Further investigations based on these findings will contribute to the design of new therapeutic targets for vascular calcification in CKD patients.

## Author Contributions

YK: conceptualization, validation, investigation, visualization, methodology, and writing original draft. YO, YA, YM, and MS: validation and investigation. KO, HO, MM, and HYO: data curation, formal analysis, validation, methodology, and resources. YT: conceptualization, funding acquisition, validation, visualization, methodology, resources, supervision, project administration, writing original draft, review, and editing.

## Acknowledgments

We thank Ms. Maki Kishimoto, Ms. Miho Samori, Ms. Ayano Komatsubara, and Ms. Miyu Ehara (Department of Clinical Nutrition and Food Management, Tokushima University Graduate School of Medical Nutrition, Tokushima, Japan) for technical assistance. Funding was provided by Grant-in-Aid for Scientific Research (Grant No. 19H04053) from Japan Society for the Promotion of Science. This study was supported by Support Center for Advanced Medical Sciences, Tokushima University Graduate School of Biomedical Sciences, and JST SPRING (Grant No. JPMJSP2113), Japan.

## Conflict of Interest

YK, YO, YA, YM, MS, KO, HO, MM, HYO & YT declare no conflicts of interest.

## References

1 Takeda E, Yamamoto H, Nashiki K, Sato T, Arai H, Taketani Y. Inorganic phosphate homeostasis and the role of dietary phosphorus. *J Cell Mol Med* 2004; **8**: 191–200.

2 Michigami T, Kawai M, Yamazaki M, Ozono K. Phosphate as a signaling molecule and its sensing mechanism. *Physiol Rev* 2018; **98**: 2317–2348.

3 Palmer SC, Hayen A, Macaskill P, *et al*. Serum levels of phosphorus,

parathyroid hormone, and calcium and risks of death and cardiovascular disease in individuals with chronic kidney disease: a systematic review and meta-analysis. *JAMA* 2011; **305**: 1119–1127.
4 Rennenberg RJ, Kessels AG, Schurgers LJ, van Engelshoven JM, de Leeuw PW, Kroon AA. Vascular calcifications as a marker of increased cardiovascular risk: a meta-analysis. *Vasc Health Risk Manag* 2009; **5**: 185–197.
5 Blacher J, Guerin AP, Pannier B, Marchais SJ, London GM. Arterial calcifications, arterial stiffness, and cardiovascular risk in end-stage renal disease. *Hypertension* 2001; **38**: 938–942.
6 Sage AP, Tintut Y, Demer LL. Regulatory mechanisms in vascular calcification. *Nat Rev Cardiol* 2010; **7**: 528–536.
7 Durham AL, Speer MY, Scatena M, Giachelli CM, Shanahan CM. Role of smooth muscle cells in vascular calcification: implications in atherosclerosis and arterial stiffness. *Cardiovasc Res* 2018; **114**: 590–600.
8 Giachelli CM. Vascular calcification mechanisms. *J Am Soc Nephrol* 2004; **15**: 2959–2964.
9 Isaka Y, Hamano T, Fujii H, *et al*. Optimal phosphate control related to coronary artery calcification in dialysis patients. *J Am Soc Nephrol* 2021; **32**: 723–735.
10 Saito Y, Sakuma M, Narishima Y, Yoshida T, Kumagai H, Arai H. Greater consumption of noodle is associated with higher serum phosphorus levels: a cross-sectional study on healthy participants. *J Clin Biochem Nutr* 2021; **68**: 78–85.
11 Chiu YW, Teitelbaum I, Misra M, de Leon EM, Adzize T, Mehrotra R. Pill burden, adherence, hyperphosphatemia, and quality of life in maintenance dialysis patients. *Clin J Am Soc Nephrol* 2009; **4**: 1089–1096.
12 Covic A, Rastogi A. Hyperphosphatemia in patients with ESRD: assessing the current evidence linking outcomes with treatment adherence. *BMC Nephrol* 2013; **14**: 153.
13 Carrero JJ, Stenvinkel P, Cuppari L, *et al*. Etiology of the protein-energy wasting syndrome in chronic kidney disease: a consensus statement from the International Society of Renal Nutrition and Metabolism (ISRNM). *J Ren Nutr* 2013; **23**: 77–90.
14 Yamazaki Y, Imura A, Urakawa I, *et al*. Establishment of sandwich ELISA for soluble alpha-Klotho measurement: age-dependent change of soluble alpha-Klotho levels in healthy subjects. *Biochem Biophys Res Commun* 2010; **398**: 513–518.
15 Imel EA. Congenital conditions of hypophosphatemia in children. *Calcif Tissue Int* 2021; **108**: 74–90.
16 Pasch A, Farese S, Gräber S, *et al*. Nanoparticle-based test measures overall propensity for calcification in serum. *J Am Soc Nephrol* 2012; **23**: 1744–1752.
17 Fukuda-Tatano S, Yamamoto H, Nakahashi O, *et al*. Regulation of α-klotho expression by dietary phosphate during growth periods. *Calcif Tissue Int* 2019; **104**: 667–678.
18 Yimamu Y, Ohtani A, Takei Y, *et al*. 25-hydroxyvitamin D-1α-hydroxylase (CYP27B1) induces ectopic calcification. *J Clin Biochem Nutr* 2022; **71**: 103–111.
19 O'Neill WC, Sigrist MK, McIntyre CW. Plasma pyrophosphate and vascular calcification in chronic kidney disease. *Nephrol Dial Transplant* 2010; **25**: 187–191.
20 Jansen RS, Duijst S, Mahakena S, *et al*. ABCC6-mediated ATP secretion by the liver is the main source of the mineralization inhibitor inorganic pyrophosphate in the systemic circulation—brief report. *Arterioscler Thromb Vasc Biol* 2014; **34**: 1985–1989.
21 Schäfer C, Heiss A, Schwarz A, *et al*. The serum protein α2-Heremans-Schmid glycoprotein/fetuin-A is a systemically acting inhibitor of ectopic calcification. *J Clin Invest* 2003; **112**: 357–366.
22 Haarhaus M, Brandenburg V, Kalantar-Zadeh K, Stenvinkel P, Magnusson P. Alkaline phosphatase: a novel treatment target for cardiovascular disease in CKD. *Nat Rev Nephrol* 2017; **13**: 429–442.
23 Hyder JA, Allison MA, Barrett-Connor E, *et al*. Bone mineral density and atherosclerosis: the Multi-Ethnic Study of Atherosclerosis, Abdominal Aortic Calcium Study. *Atherosclerosis* 2010; **209**: 283–289.
24 Zhang Y, Feng B. Systematic review and meta-analysis for the association of bone mineral density and osteoporosis/osteopenia with vascular calcification in women. *Int J Rheum Dis* 2017; **20**: 154–160.
25 Persy V, D'Haese P. Vascular calcification and bone disease: the calcification paradox. *Trends Mol Med* 2009; **15**: 405–416.
26 Bundy JD, Cai X, Scialla JJ, *et al*. Serum calcification propensity and coronary artery calcification among patients with CKD: the CRIC (Chronic Renal Insufficiency Cohort) Study. *Am J Kidney Dis* 2019; **73**: 806–814.
27 Smith ER, Ford ML, Tomlinson LA, *et al*. Serum calcification propensity predicts all-cause mortality in predialysis CKD. *J Am Soc Nephrol* 2014; **25**: 339–348.
28 Eelderink C, Te Velde-Keyzer CA, Frenay AS, *et al*. Serum calcification propensity and the risk of cardiovascular and all-cause mortality in the general population: the PREVEND study. *Arterioscler Thromb Vasc Biol* 2020; **40**: 1942–1951.
29 Ter Braake AD, Eelderink C, Zeper LW, *et al*. Calciprotein particle inhibition explains magnesium-mediated protection against vascular calcification. *Nephrol Dial Transplant* 2020; **35**: 765–773.
30 Voelkl J, Tuffaha R, Luong TTD, *et al*. Zinc inhibits phosphate-induced vascular calcification through TNFAIP3-mediated suppression of NF-κB. *J Am Soc Nephrol* 2018; **29**: 1636–1648.
31 Nakatani S, Mori K, Sonoda M, *et al*. Association between serum zinc and calcification propensity ($T_{50}$) in patients with type 2 diabetes mellitus and *in vitro* effect of exogenous zinc on $T_{50}$. *Biomedicines* 2020; **8**: 337.
32 Cai MM, Smith ER, Holt SG. The role of fetuin-A in mineral trafficking and deposition. *Bonekey Rep* 2015; **4**: 672.
33 Ketteler M, Bongartz P, Westenfeld R, *et al*. Association of low fetuin-A (AHSG) concentrations in serum with cardiovascular mortality in patients on dialysis: a cross-sectional study. *Lancet* 2003; **361**: 827–833.
34 Wang AY, Woo J, Lam CW, *et al*. Associations of serum fetuin-A with malnutrition, inflammation, atherosclerosis and valvular calcification syndrome and outcome in peritoneal dialysis patients. *Nephrol Dial Transplant* 2005; **20**: 1676–1685.
35 Shroff RC, Shah V, Hiorns MP, *et al*. The circulating calcification inhibitors, fetuin-A and osteoprotegerin, but not Matrix Gla protein, are associated with vascular stiffness and calcification in children on dialysis. *Nephrol Dial Transplant* 2008; **23**: 3263–3271.
36 Ashton BA, Höhling HJ, Triffitt JT. Plasma proteins present in human cortical bone: enrichment of the αHS-glycoprotein. *Calcif Tissue Res* 1977; **22**: 27–33.
37 Seto J, Busse B, Gupta HS, *et al*. Accelerated growth plate mineralization and foreshortened proximal limb bones in fetuin-A knockout mice. *PLoS One* 2012; **7**: e47338.
38 Akiyama KI, Miura Y, Hayashi H, *et al*. Calciprotein particles regulate fibroblast growth factor-23 expression in osteoblasts. *Kidney Int* 2020; **97**: 702–712.
39 Meyer JL. Can biological calcification occur in the presence of pyrophosphate? *Arch Biochem Biophys* 1984; **231**: 1–8.
40 Lomashvili KA, Khawandi W, O'Neill WC. Reduced plasma pyrophosphate levels in hemodialysis patients. *J Am Soc Nephrol* 2005; **16**: 2495–2500.
41 Lomashvili KA, Narisawa S, Millán JL, O'Neill WC. Vascular calcification is dependent on plasma levels of pyrophosphate. *Kidney Int* 2014; **85**: 1351–1356.
42 Sheen CR, Kuss P, Narisawa S, *et al*. Pathophysiological role of vascular smooth muscle alkaline phosphatase in medial artery calcification. *J Bone Miner Res* 2015; **30**: 824–836.
43 Sun Y, Byon CH, Yuan K, *et al*. Smooth muscle cell-specific runx2 deficiency inhibits vascular calcification. *Circ Res* 2012; **111**: 543–552.
44 Weng JJ, Su Y. Nuclear matrix-targeting of the osteogenic factor Runx2 is essential for its recognition and activation of the alkaline phosphatase gene. *Biochim Biophys Acta* 2013; **1830**: 2839–2852.




doi: 10.3164/jcbn.23-11