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ORIGINAL RESEARCH

Nephronectin Expression is Inhibited by Inorganic Phosphate in Osteoblasts

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Abstract

Nephronectin (Npnt), an extracellular matrix protein, is known to be a ligand of integrin α8β1, and it has also been known to play critical roles as various organs. In the present study, elevated extracellular inorganic phosphate (Pi) strongly inhibited the expression of *Npnt* in MC3T3-E1 cells, while the existence of extracellular calcium (Ca) was indispensable for its effect. Furthermore, Pi-induced inhibition of *Npnt* gene expression was recovered by inhibitors of both sodium-dependent Pi transporter (Pit) and fibroblast growth factor receptors (Fgfrs). These results demonstrated that *Npnt* gene expression is regulated by extracellular Pi via Pit and Fgfrs.

Keywords Nephronectin · Phosphate · Calcium · MC3T3-E1 · FGFR

Introduction

Nephronectin (Npnt) was initially identified in MC3T3-E1 cells, a mouse osteoblastic cell line, as a ligand of integrin α8β1, while it was also shown to possess the MAM (meprin A5 protein and receptor protein-tyrosine phosphatase m) domain and RGD (Arg-Gly-Asp) motif, which have important roles in adhesion, spreading, and survival of MC3T3-E1 cells [1]. In other studies, Kahai et al. demonstrated that osteoblasts with overexpression of Npnt caused earlier formation of bone nodules as compared to control mice [2], while Npnt was also identified in kidney tissues [3] and mice lacking Npnt showed renal agenesis at birth [4]. Together, these results suggest that Npnt has significant roles in bone and kidney development. In our previous studies, we reported that *Npnt* expression was suppressed by transforming growth factor-β (Tgf-β), tumor necrosis factor-α (Tnf-α), oncostatin M (Osm), and interleukin 1β (IL-1β) [5–8].

Phosphate is associated with various biological processes, including membrane integrity, synthesis of nucleic acid, energy metabolism, and intracellular signaling [9]. Also, inorganic phosphate (Pi) itself triggers signaling to regulate gene expressions [10–12]. In clinical findings, the concentration of intracellular phosphate is influenced by pH, hormones, and subcellular compartmentalization, and circulating phosphate levels are maintained within a narrow normal range [13]. But, in chronic hemodialysis patients, because of a failure of the phosphate coordination mechanism, an elevated circulating phosphate level increases the risk of mortality [14, 15].

A high-phosphate diet is known to induce high-turnover bone disease in rodent CKD models [16]. It is shown that continuous intravenous phosphate loading and oral phosphate loading in uremic rats increased serum Fgf23 [17]. Fgf23 is produced mainly by osteocytes and prevents phosphate overload through increasing renal excretion and mediation of vitamin D metabolism, in which Fgf23 induces inhibition of 1α-hydroxylase and stimulation of 24-hydroxylase [18]. Our previous studies showed the direct effect of $1,25\text{-}(OH)_2D_3$ and FGF family proteins on Npnt expression [19, 20]; then, we studied the effect of Pi which also

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is associated with chronic kidney disease mineral and bone disorder (CKD-MBD).

In the present study, we examined the effects of Pi on *Npnt* expression. The results demonstrated that *Npnt* gene expression is regulated by extracellular Pi together with Ca via a Pi transporter (Pit) and fibroblast growth factor receptors (Fgfrs).

Materials and Methods

Cell Culture

The osteoblast-like cell line MC3T3-E1 was maintained in MEMα with L-glutamine and phenol red medium (WAKO Pure Chemical Industries, Ltd., Cat. No. 135-15175), supplemented with 10% fetal bovine serum (FBS) (Life Technologies, USA) and 1% penicillin–streptomycin at 37 °C in a CO_2 incubator (5% CO_2, 95% air). Mouse primary osteoblasts were isolated from calvaria of neonatal ddY mice and grown in MEMα supplemented with 10% FBS. For the experiments, cells were plated in 6-well plates (Thermo Scientific Inc., Cat. No. 140675).

Reagents

To prepare Pi, 500 mM NaH_2PO_4 was combined with 500 mM Na_2HPO_4 and the pH was adjusted to 7.2; then, 500 mM Pi solutions were produced. The indicated concentrations of Pi represent the amount added to the basal levels, without considering the amount contained in 10% FBS (0.3 mM of Pi). As a negative control, 12 mM sodium sulfate was added to MEMα. BGJ398 (Cat. No. 872511-34-7-5mg) was purchased from Selleckchem. Phosphonoformic acid (PFA) (Cat. No. P6801-250MG) was purchased from SIGMA. For Ca treatment, we prepared 500 mM $CaCl_2$ solution. The indicated concentrations of Ca represent the amount added to the basal level, without considering the amount contained in 10% FBS (0.6 mM of Ca). Ca-free medium was obtained from SIGMA (Cat. No. M8165-500ML).

Cell Viability Assay

Cells were plated in 24-well plates (Thermo Scientific Inc., Cat. No. 142475) at 5×10^4 cells/well, then incubated in a CO_2 incubator (5% CO_2, 95% air) for 1 day. Next, growth medium was aspirated from each well and 200 µL/well was added along with 1 mmol/L of 0.25% trypsin (WAKO Pure Chemical Industries, Ltd., Cat. No. 209-16941). After peeling the cells from the plates, growth medium was added at 800 µL/well; then, 20 µL 0.4% trypan blue (WAKO Pure Chemical Industries, Ltd., Cat. No. 207-03252) was mixed with 20 µL of medium and the number of living cells was counted.

RT-PCR

Total RNA was extracted using TRIzol reagent (Life Technologies, Cat. No. 15596018); then, SuperScript III (Life Technologies, Cat. No. 18080-044) was used for reverse transcription. For *Npnt* mRNA expression analysis, real-time PCR was performed using a StepOneTM Real-time PCR System (Applied Biosystems, USA) with SYBR Green Fast PCR Master Mix (Applied Biosystems, Cat. No. 4385612) and the following specific PCR primers: *Gapdh*, 5′-AAA TGGTGAAGGTCGGTGTG-3′ and 5′-TGAAGGGGTCGT TGATGG-3′; and *Npnt*, 5′-CACGAGTAATTACGGTTG ACAACAG-3′ and 5′-CTGCCGTGGAATGAACACAT-3′.

Western Blotting

Cell lysates were collected using Sample Buffer Solution with Reducing Reagent (6×) for SDS-PAGE (Nacalai Tesque, Cat. No. 09499-14), then electrophoresed onto a 10% SDS polyacrylamide gel and blotted onto PVDF membranes. The membranes were incubated with anti-nephronectin (R&D Systems, Cat. No. AF4298) and anti β−actin (MERCK, Cat. No. A5060) as the first antibodies, then further probed with anti-mouse IgG horseradish peroxidase-linked (GE Healthcare UK, Cat. No. NA931V) and anti-goat IgG horseradish peroxidase-linked (NOVUS, Cat. No. NB7352) secondary antibodies. Proteins were visualized using ECL Prime Western Blotting Detection reagent (GE Healthcare, Cat. No. #RPN2232). Intensity of the chemiluminescent bands was quantitatively analyzed using Versa Doc 5000 MP (Bio-Rad Laboratories, Hercules, CA, USA). The ratio for intensity of a band for phosphorylated protein to that for total protein was calculated.

Statistical Analysis

All results are expressed as the mean ± standard deviation (SD). As for the results shown in Figs. 1a, 2, 3, 4 and Fig. S1, statistical analysis was performed using one-way ANOVA, while those shown in Fig. 1b and Fig. S2 were obtained using a two-tailed Student's *t* test. A p value of < 0.05 or < 0.01 was considered to indicate statistical significance.

Results

We examined the *Npnt* expression mechanism by determining the level of extracellular Pi in the osteoblastic cell line MC3T3-E1. Initially, we treated MC3T3-E1 cells with 1, 2, 4, 8, or 12 mM Pi or 12 mM sulfate for 24 h, then analyzed

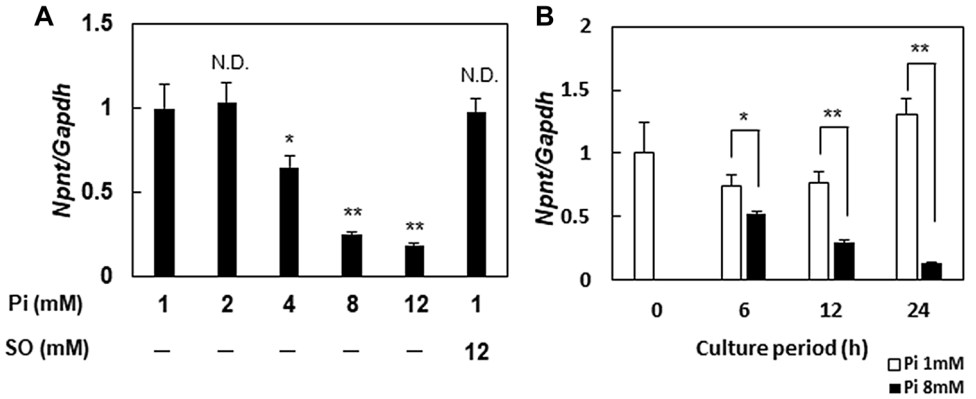

Fig. 1 Pi suppresses Npnt expression in dose- and time-dependent manners. **a** MC3T3-E1 cells were treated with 1, 2, 5, 8, or 12 mM Pi, or 12 mM sulfate (SO_4) as a negative control for 24 h. Values are shown as the mean ± SD of three samples as compared to the level in cells treated with 1 mM Pi. $*p < 0.05$, $**p < 0.01$; relative to level in cells treated with 1 mM Pi (ANOVA). **b** Time-dependent effects of Pi on *Npnt* mRNA expression in MC3T3-E1 cells. Cells were treated with 8 mM Pi for 6, 12, or 24 h. mRNA levels for *Npnt* and *Gapdh* were examined using real-time PCR. Values are shown as the mean ± SD of three samples as compared to the value at 0 h. $*p < 0.05$, $**p < 0.01$; relative to level in cells treated with 1 mM Pi at each time point (Student's *t* test)

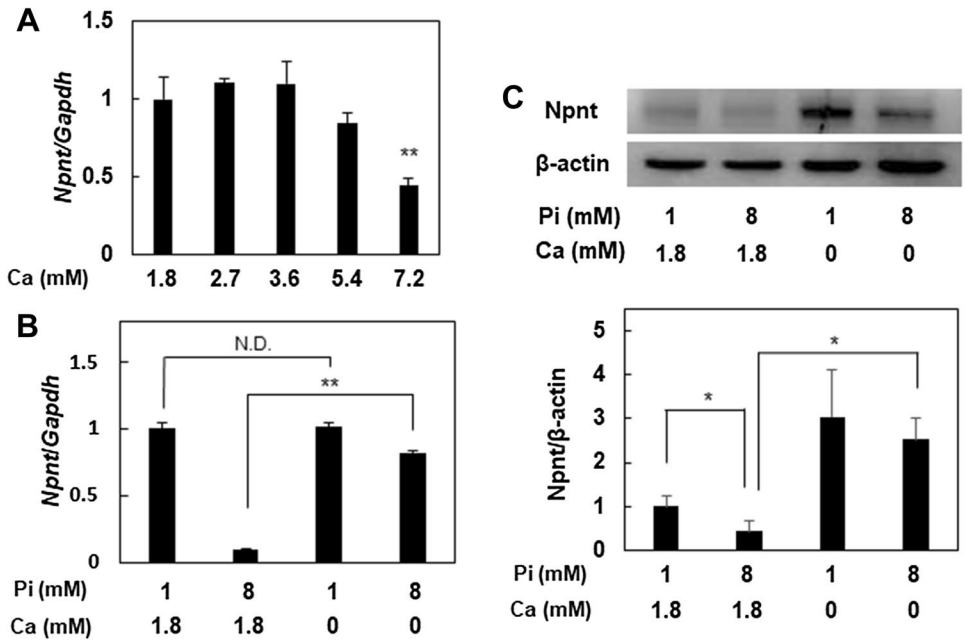

Fig. 2 Suppression of *Npnt* by extracellular Pi requires extracellular calcium. **a** MC3T3-E1 cells were treated with 1.8, 2.7, 3.6, 5.4, or 7.2 mM Pi for 24 h. Values are shown as the mean ± SD of 3 samples as compared to the level in cells treated with 1.8 mM Ca. $**p < 0.01$; relative to level in cells treated with 1.8 mM Ca (ANOVA). **b** Effect of extracellular Ca on suppression of *Npnt* by extracellular Pi. MC3T3-E1 cells are treated with 1.8 mM or without extracellular Ca for 24 h. Real-time PCR was performed using cDNA derived from total cellular RNA from each sample to determine the expression levels of *Npnt* and *Gapdh* mRNAs. Results are shown as the mean ± SD of three samples as compared to the group of Pi (1 mM) and Ca (1.8 mM) $**p < 0.01$ (ANOVA). **c** Western blotting analysis of Npnt protein levels in cells treated with or without Pi and Ca. Cell lysates were collected after 24 h of incubation. The densitometric ratio of Npnt to β-actin is depicted. Results are shown as the mean ± SD of three samples. $*p < 0.05$ (ANOVA)

Npnt mRNA expression. More than 4 mM of extracellular Pi suppressed the expression of *Npnt* mRNA (Fig. 1a). Because an extracellular Pi concentration of 8 mM showed marked reduction in *Npnt* mRNA expression, we examined the effect of Pi on the nephronectin gene expression at this Pi concentration, though Pi concentration of 8 mM in human blood exceeds the levels seen in the clinical presentation of hyperphosphatemia. To examine the influence of the cell

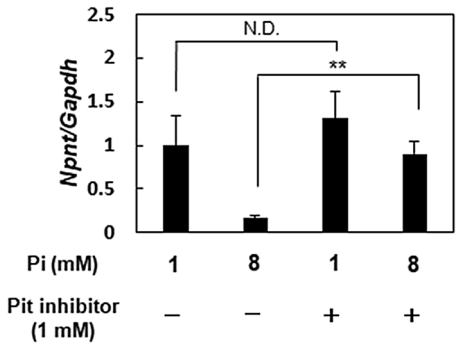

Fig. 3 Pit involvement in expression of *Npnt*. MC3T3-E1 cells were pre-treated with 1 mM of a Pit inhibitor (PFA) for 1 h, and then with 8 mM of Pi and the Pit inhibitor for 24 h. Real-time PCR was performed using cDNA derived from total cellular RNA from each sample to determine the expression levels of *Npnt* and *Gapdh* mRNAs. Results are shown as the mean ± SD of three samples as compared to the group without treatment. $^{**}p < 0.01$ (ANOVA)

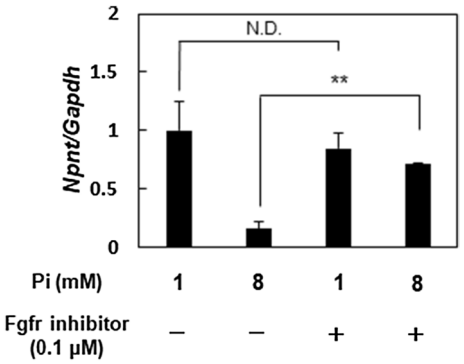

Fig. 4 Fgfrs involvement in expression of *Npnt*. MC3T3-E1 cells were pre-treated with 0.1 μM of an Fgfr inhibitor (BGJ398) for 1 h, and then with 8 mM of Pi and the Fgfr inhibitor for 24 h. Real-time PCR was performed using cDNA derived from total cellular RNA from each sample to determine the expression levels of *Npnt* and *Gapdh* mRNAs. Results are shown as the mean ± SD of three samples as compared to the group without treatment. $^{**}p < 0.01$ (ANOVA)

viability by Pi treatment, we counted the number of viable cells after treatment with each concentration of Pi for 24 h, then elevated Pi has no significant influence on cell viability (Fig. S1). Next, we examined the time-dependent effects of different levels of extracellular Pi on *Npnt* mRNA expression. Significant suppression of that expression was found at 6 h after exposure to 8 mM of extracellular Pi (Fig. 1b). These results revealed that *Npnt* gene expression is affected by Pi in both time- and dose-dependent manners. The suppression of *Npnt* gene expression by elevated Pi was also apparent in primary osteoblasts (Fig. S2).

A previous study showed that not only extracellular Pi, but also extracellular calcium (Ca) and the Ca-Pi complex play important roles in bone remodeling [21]. We also examined the effect of extracellular Ca on the mechanism of *Npnt* expression. MC3T3-E1 cells were treated with 1.8, 2.7, 3.6, 5.4, or 7.2 mM Ca for 24 h. *Npnt* expression in cells treated with 7.2 mM Ca was suppressed, whereas the lower levels did not cause a significant change (Fig. 2a). Next, the effect of Pi without Ca was examined using Ca-free medium. Suppression of *Npnt* expression induced by extracellular Pi disappeared in cells treated with Ca-free medium despite exposure to a high level of extracellular Pi (Fig. 2b), with the same trend seen for the expression level of Npnt protein (Fig. 2c).

Pi is a negatively charged anion that must be actively transported to gain entry into cells. The highly expressed transmembrane type III sodium-dependent Pit, also called Slc20a, has been proposed to transport Pi into cells [22]. To investigate whether Pit regulates inhibition of *Npnt* expression caused by an elevated level of extracellular Pi, we used phosphonoformic acid (PFA), which was used as a Pit inhibitor in several studies [23–25]. Inhibition of *Npnt* expression induced by extracellular Pi was abrogated by PFA (Fig. 3), suggesting that Pi transported into cells via Pit regulates *Npnt* expression.

Several studies have shown that extracellular Pi activates Fgfrs, which in turn regulate the expressions of *cyclin D1* and *dentin matrix protein 1* (*Dmp1*) [11, 12]. To examine whether *Npnt* expression is regulated by Pi via Fgfrs, we treated MC3T3-E1 cells with the higher level of Pi and BGJ398, an Fgfr inhibitor. After 24 h of treatment with BGJ398, the Pi-induced inhibition of *Npnt* expression was abolished (Fig. 4). These results suggest that *Npnt* expression is regulated by Pi via Fgfrs.

Discussion

The present findings demonstrate that elevated extracellular Pi suppresses *Npnt* expression in both time- and dose-dependent manners and also show involvement of Pi-mediated suppression of *Npnt* expression via Fgfrs (Fig. 5). Notably, the existence of extracellular Ca is indispensable for suppression of *Npnt* by extracellular Pi, while several other studies have found that basic calcium phosphate crystals can induce expression of a variety of inflammatory substances, e.g., interleukin (IL)-1 and IL-6 [26, 27].

Previously, it was reported that Pi-triggered signal transduction is involved in mitogen-activated protein kinase (MAPK) and Akt/mechanistic targets of the rapamycin complex 1 (mTORC1) pathways [12, 28]. More recently, our previous study demonstrated that Fgf2 suppresses *Npnt* expression via JNK (c-Jun N-terminal kinase) and PI3K (phosphoinositide-3 kinase) pathways [20]. Thus, it is considered possible that Pi-mediated suppression of *Npnt* expression is involved in MAPK- and PI3K-mediated mTORC1 pathways.

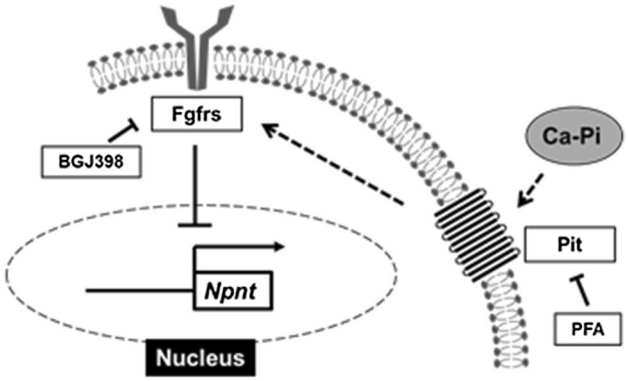

Fig. 5 Down-regulation of *Npnt* gene expression by Pi via Pit and activated Fgfrs. Extracellular Pi has effects on the cell molecular mechanism through Pit and is inhibited by PFA, and suppresses *Npnt* gene expression via Fgfrs

Others have shown that elevated extracellular Pi induces the expression of Fgf2 and Fgfr1. However, Pi-induced phosphorylation of Fgf receptor substrate 2α (FRS2α) likely occurs prior to increased production of Fgf2 and Fgfr1 [12]. We speculated that *Npnt* expression is suppressed in an autocrine manner by Fgf2 and determined Fgf2 concentrations in medium used for culturing cells with elevated Pi, though the level of Fgf2 was not increased, thus supporting the possibility that elevated Pi activates Fgfrs without a requirement for Fgf. An elevated level of Pi might regulate phosphorylateion or conformational change of Pit1, possibly leading to an interaction between Pit1 and Fgfrs, thus changing the autophosphorylation activety of Fgfrs [11].

The effects of phosphate on osteoblastic differentiation have been reported by several investigators. Okamoto et al. showed that Pi increased reactive oxygen species (ROS) generation in MC3T3-E1 cells [29], while another study reported that Npnt is also associated with osteoblastic differentiation [2]. In the present experiments, we examined alkaline phosphatase (ALP) activity induced by both BMP-2 and β−glycerophosphate/ascorbic acid in MC3T3-E1 cells with higher levels of Pi and an Fgfr inhibitor. Although increase in ALP activity induced by BMP-2 was decreased by elevated Pi, its reduction by elevated Pi was not restored by Fgfr inhibitor. On the other hand, when the induction of osteoblast differentiation by treatment of β−glycerophosphate and ascorbic acid was performed, ALP activation was decreased by elevated Pi, and its decrease in ALP activity by elevated Pi was restored by Fgfr inhibitor (data not shown). The above results indicate that the effects of Pi and the Fgfr inhibition on BMP-2-induced ALP activity were different from those on β−glycerophosphate and ascorbic acid-induced ALP activities. Nevertheless, Npnt has essential roles in several different organs and the influence of Pi toward Npnt requires further investigation.

Conclusion

Our results revealed that elevated extracellular Pi with Ca induces suppression of *Npnt* expression via Fgfrs.

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Author Contributions TK, AY, KS, KY, NM, HO, AS, and RK involved in study concept. AY, KS, and RK: involved in formal analysis; AY, HO, AS, and RK acquired funding; TK investigated the study; TK, AY, and RK applied methodology for the study; AY and RK administrated the project; HO, AS, and RK collected the resources; AY and RK supervised and validated the study; TK and AY wrote the original manuscript; TK, AY, and RK reviewed and edited the original manuscript.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflicts of interest in regard to this study.

Human and Animal Rights and Informed Consent All animal experiments were performed in accordance with the guide for care and use of laboratory animals and approved by the Showa University Animal Care Use and Committee.

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