between collagen fibrils [9]. Disorders in Pi homeostasis such as hypophosphatemic rickets/osteomalacia manifest as defective skeletal mineralization, indicating the central role of extracellular Pi in the mineralization of bone and cartilage. It has been reported that Pi stimulates the maturation and apoptosis of differentiated chondrocytic cells [10]. In addition, it has recently been demonstrated that Pi stimulates the expression of the gene for matrix Gla protein (MGP), which is expressed in maturated chondrocytes and involved in mineralization [11]. In the osteoblastic cell line MC3T3-E1, extracellular Pi regulated the expression of several genes including that for osteopontin [12,13]. These results indicate the critical roles of extracellular Pi in mature chondrocytes and osteoblasts, which express the genes closely involved in mineralization, and most of the genes identified as being regulated by extracellular Pi in these cells are involved in the mineralization process, such as those for osteopontin and MGP.

In contrast to the critical roles of extracellular Pi in the mineralization process, its physiological roles in the early stages of chondrocytic differentiation including in proliferating chondrocytes have not been fully studied. Mwale et al. determined the tissue content of Pi in growth plates, and demonstrated that progressive accumulation of Pi ion started from the proliferative zone and peaked in the hypertrophic zone [14]. According to their results, the content of Pi in the proliferative zone reached several times higher levels compared with that in reserve zone, suggesting that the proliferating chondrocytes are exposed to rather high concentrations of Pi, especially in fetuses and neonates where Pi in extracellular fluid is often higher than 2 mM. In addition, the expression of type III sodium/phosphate (Na^+/Pi) cotransporters has been demonstrated in immature chondrocytes, suggesting Pi to be involved in the commitment of chondrogenic cells to differentiation [15]. Considering these findings together with the previous reports demonstrating that an increase in extracellular Pi caused to the altered gene expression in osteoblasts and mature chondrocytes [12,13], we have hypothesized that the modest increase in the extracellular Pi in the proliferative zone also might have some influence on proliferation and/or differentiation of the cells through the alteration of gene expression.

Based on these backgrounds, here we focused on the roles of extracellular Pi in the early stages of chondrocytic differentiation. The murine mesenchymal cell line ATDC5 is a commonly used cell model of endochondral bone formation that can reproduce the multistep chondrocytic differentiation process encompassing the stages from mesenchymal condensation to calcification *in vitro* [16–18]. We noticed the up-regulation of *cyclin D1* (*Ccnd1*) and the down-regulation of *alkaline phosphatase 2, liver* (*Alpl; Akp2*) encoding tissue non-specific liver/kidney/bone-type of ALP by increased extracellular Pi (10 mM) in the proliferating stage of ATDC5 cells expressing *SOX9* and *Col2a1* from our cDNA microarray analysis. *Cyclin D1* has been reported to be expressed in proliferating chondrocytes and regulated by PTHrP and TGF-β [19–21], and the up-regulation of its expression by extracellular Pi came to our attention, because increased extracellular Pi has been rather recognized as an inducer of apoptosis in mature chondrocytes [10,22,23]. As to the expression of *Alpl*, it is reasonable that its expression is reduced by a high level of extracellular Pi, since ALP is an enzyme that produces Pi. In the present study, we have investigated the molecular mechanisms by which the signaling triggered by increased extracellular Pi regulates the expression of *cyclin D1* and *Alpl* in proliferating chondrocytes.

Materials and methods

Cell culture

ATDC5 cells were maintained in a 1:1 mixture of Dulbecco's modified Eagle's and Ham's F12 (DMEM/F12) medium (Sigma Aldrich, St. Louis, MO) supplemented with 5% fetal bovine serum (FBS: Invitrogen, Carlsbad, CA) and 1% insulin–transferrin–selenium-G supplement (ITS: Invitrogen; insulin 10 μg/ml, sodium selenite 6.7 μg/ml and transferrin 5.5 mg/ml as a final concentration) at 37 °C in a 5% CO_2 atmosphere. Cells were subcultured every four days using 0.05% trypsin/0.53 mM EDTA (Invitrogen). Pi-free medium was obtained from Nikken (Tokyo, Japan). The composition of the Pi-free medium was the same as that of DMEM, except that it did not contain Pi. Media containing various concentrations of Pi were prepared by adding sodium phosphate buffer to the Pi-free medium.

For chondrogenic induction, ATDC5 cells were inoculated into 6-well culture plates (5×10^5 cells/well) and cultured in DMEM/F12 medium supplemented with 5% FBS and 1% ITS at 37 °C in a 5% CO_2 atmosphere. Three days later, the medium was changed to alpha minimal essential medium (αMEM) supplemented with 5% FBS and 1% ITS, and the culture plates were sealed with adhesive tape to facilitate mineralization, as previously described [24,25]. The medium was replaced every 3 days.

Isolation of primary chondrocytes

Animal protocols were approved by the Institutional Animal Care and Use Committee at Osaka Medical Center and Research Institute for Maternal and Child Health. Primary chondrocytes were isolated from the ventral part of the rib cages of 4-day old ICR mice following a previous report [26] with some modifications. In brief, the cartilage dissected from rib cages was rinsed in PBS, and incubated with actinase E (2 mg/ml in PBS, Kaken Pharmaceutical Co. Ltd., Tokyo, Japan) for 30 min at 37 °C to detach the soft tissue from the cartilage. Then, the cartilage was rinsed three times with PBS, treated with collagenase (3 mg/ml, Wako, Osaka, Japan) in 10 ml of DMEM for 90 min at 37 °C, and transferred to a 50-ml tube. After several rounds of pipetting, the tube was stood for 5 min and the supernatant containing soft tissue was discarded. The remaining cell clumps were washed with DMEM containing 10% FBS and then with PBS, and passed through a 100-μm cell strainer to be collected as chondrocytic cells. The collected cells were cultured in DMEM supplemented with 10% FBS and 50 μg/ml of ascorbic acid (Sigma Aldrich) until use for experiments.

Inhibitors

The MEK inhibitor PD98059 and the inhibitor of phosphate transporter phosphonoformic acid (PFA) were purchased from Sigma Aldrich. When used in the experiments, they were added to the medium 30 min before the addition of sodium phosphate buffer.

Assay for increases in cell number

Cells were plated in 96-well culture plates at a density of 1×10^3 cells/well (designated as day 0), and cultured in the media containing various concentrations of Pi or 4 mM sodium sulfate. The media containing various concentrations of Pi or 4 mM sulfate were prepared by adding sodium phosphate buffer or sodium sulfate buffer to Pi-free media supplemented with 5% FBS (Nikken). The actual concentrations of Pi added to the cells were determined using a Phosphor-C test kit (Wako, Osaka, Japan). Then, the cell number in each well was evaluated by an MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] assay using a CellTiter 96® aqueous one solution cell proliferation assay kit (Promega, Madison, WI) according to the manufacturer's instructions.

Reverse transcription-polymerase chain reaction (RT-PCR) and real-time PCR

Total RNA (2.5 μg) treated with DNase (Qiagen Inc.) was reverse transcribed using random hexamer (Promega) and SuperScript II reverse transcriptase (Invitrogen). PCR was performed using rTaq

polymerase (Takara Bio Inc.) and the specific primer sets summarized in Supplemental Table. Amplification of the expected fragments was confirmed by sequencing of the products. For real-time PCR, we utilized TaqMan® Gene Assays with the 7300 Real Time PCR System (Applied Biosystems, Tokyo, Japan). The ID numbers for the assays are Mm99999915_g1 for *Gapdh*, Mm00432359_m1 for *cyclin D1* (*Ccnd1*), Mm00475831_m1 for *alkaline phosphatase* (*Alpl*), Mm00489382_g1 for *Pit-1* (*Slc20a1*), and Mm0122230_m1 for *Pit-2* (*Slc20a2*). To generate a standard curve for real-time PCR, the amplicons of interest were first cloned into the pT7-Blue vector (Novagen, Madison, WI), and serial 10-fold dilutions of the constructed plasmid were included in the assay. Samples were analyzed in triplicate. The copy number of the target cDNA in each sample was estimated by referring to the standard curve, which was standardized by that of *Gapdh* in each sample.

Western blotting

Whole cell extracts were harvested in RIPA buffer [1% Triton, 1% Na deoxycholate, 0.1% SDS, 150 mM NaCl, 10 mM Tris–Cl (pH 7.4), 5 mM EDTA, 1 mM orthovanadate, 1 mM NaF, and protease inhibitor cocktail (Complete™; Roche Diagnostics)]. The cell lysates containing 10 μg of each protein were then subjected to SDS-PAGE, and transferred to PVDF membranes (Biorad, Hercules, CA). After blocking with BlockAce reagent (Dainippon Pharmaceuticals, Osaka, Japan) or Blocking-one reagent (Nacalai Tesque, Kyoto, Japan), the membranes were incubated with the following primary antibody; anti-cyclin D1 antibody (Cell Signaling Technology Inc., Beverly, MA), anti-GAPDH antibody (Santa Cruz, Santa Cruz, CA), anti-phosphotyrosine antibodies (PY20 and PY350; Santa Cruz), anti-phospho ERK1/2 antibody, anti-ERK1/2 antibody, anti-phospho c-Raf (Ser 338) antibody, anti-c-Raf antibody, anti-phospho Akt antibody, anti-Akt antibody, anti-phospho p38MAPK antibody, anti-p38MAPK antibody, anti-phospho FRS2α (Tyr196) antibody (Cell Signaling), or anti-total FRS2α antibody (Santa Cruz). After incubation with the corresponding HRP-conjugated secondary antibody, the proteins were visualized using the enhanced chemiluminescence detection system (GE Healthcare, Buckinghamshire, UK). In some experiments, densitometry was performed using NIH Image 1.63 software to evaluate the intensity of the signals.

Two-dimensional (2-D) electrophoresis

Two-dimensional (2-D) electrophoresis was performed using the IPGphor™ Isoelectric Focusing (IEF) System (Amarsham Biosciences) and Miniprotean II 1-D Cell (Biorad) according to the manufacturers' instructions. After starvation in serum-free medium for 24 h, ATDC5 cells were incubated in the presence of 1 mM Pi or 10 mM Pi for 30 min. Cells were then harvested in NEA buffer [10 mM HEPES–KOH (pH 7.8), 10 mM KCl, 0.1 mM EDTA, 0.1% Nonidet P-40, 1 mM DTT, 1 mM sodium orthovanadate, 10 mM NaF and protease inhibitor cocktail Complete™ (Roche)], and after centrifugation, the supernatant was collected as a cytosolic fraction. Five to 10 μg of protein from the fraction was precipitated with methanol and dissolved in the sample buffer [8 M urea, 4% CHAPS, 40 mM Tris base, 1 mM sodium orthovanadate, 10 mM NaF and protease inhibitor cocktail Complete™]. Next the samples were applied to Immobiline™ DryStrips (pH 4–7 and pH 6–11, 7 cm; Amersham Biosciences) and the DryStrips were incubated in IPG buffer. After incubation for 15 h, IEF was carried out using IPGphor. The DryStrips were then subjected to SDS-PAGE, followed by silver staining or Western blotting.

Assay for alkaline phosphatase activity

ATDC5 cells were plated in 6-well plates at a density of 2×10^5 cells/well. On the next day, after being washed in Pi-free, serum-free medium twice, the cells were incubated in the media containing 5% FBS and various concentrations of Pi. When simultaneous treatment with PD98059 or PFA was performed, the inhibitors or the corresponding vehicles were added to the cells 30 min before the addition of Pi, and were included in the treatment media until the harvesting of the cell lysates. Twenty-four hours after the addition of Pi, whole cell lysates were harvested in a buffer containing 10 mM Tris–HCl (pH 7.4). After freeze–thawing and sonication, the lysates were treated with 0.05% Triton X-100 at 4 °C for 30 min, followed by centrifugation at 15,000 rpm for 15 min. The supernatant of each sample was subjected to a measurement of ALP activity by the Lowry method using *p*-nitrophenylphosphate as a substrate in glycine alkaline buffer containing 10 mM $MgCl_2$ [27]. The assay reaction was performed at 37 °C for 30 min, and the ALP activity of each sample was determined by referring to a *p*-nitrophenol standard (Sigma). The ALP activity of each sample was normalized to the amount of protein.

Gene silencing

Gene silencing was performed using the siPORT Amine transfection agent (Applied Biosystems) and *Silencer*® Select siRNAs (Applied Biosystems) by the reversal transfection method, where the cell suspension was mixed with the transfection mixture containing siPORT Amine and siRNAs and then plated into culture plates. As the *Pit-1*-specific siRNA, *Silencer*® Select siRNAs s73888 and s73889 were utilized. For the silencing of *Pit-2*, siRNAs s73890 and s73891 were used. A negative control siRNA with a scrambled sequence was also included in the experiments.

Pi uptake assay

ATDC5 cells transfected with siRNAs were subjected to a Pi uptake assay. Cells were trypsinized, and 4.6×10^4 cells were applied to each well of 24-well culture plates with transfection mixture containing 3 μl/well of siPORT Amine and 60 nM of siRNAs. Three days later, Pi uptake was assayed following the method described by Jonsson et al. [28] with some modifications. In brief, cells were rinsed twice with uptake buffer (150 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 5 mM glucose, and 10 mM cyclohexylaminopropane sulfonic acid, pH 9.5), before the addition of 150 μl/well of the same buffer supplemented with 5×10^5 c.p.m. of ^{32}P-orthophosphate and 0.2 mM KH_2PO_4. After 10 min of incubation at 37 °C, the radioactive buffer was rapidly aspirated and replaced by 500 μl/well of ice-cold STOP solution (5 mM sodium arsenate and 150 mM choline chloride, pH 7.4). The cells were then lysed with 0.5 mM NaOH (250 μl/well) and total radioactivity was determined by a liquid scintillation counter. The data was standardized with the protein content of total cell lysates harvested from the identical wells.

Statistical analysis

Data were analyzed using the one-way analysis of variance (ANOVA). The methods of Tukey or Student–Newman–Keuls were used as post hoc tests.

Results

Expression of cyclin D1 and alkaline phosphatase are regulated by extracellular Pi

To confirm the regulation of *cyclin D1* and *Alpl* expression by extracellular Pi, we treated ATDC5 cells with various concentrations of extracellular Pi for 24 h (Figs. 1A–C). Since the accumulation of Pi starts in proliferating zone of growth plates [14], the Pi concentration in the extracellular matrix in the proliferating zone might be rather high, especially in fetuses and neonates. Therefore, we selected 10 mM for the higher concentration of Pi as the experimental

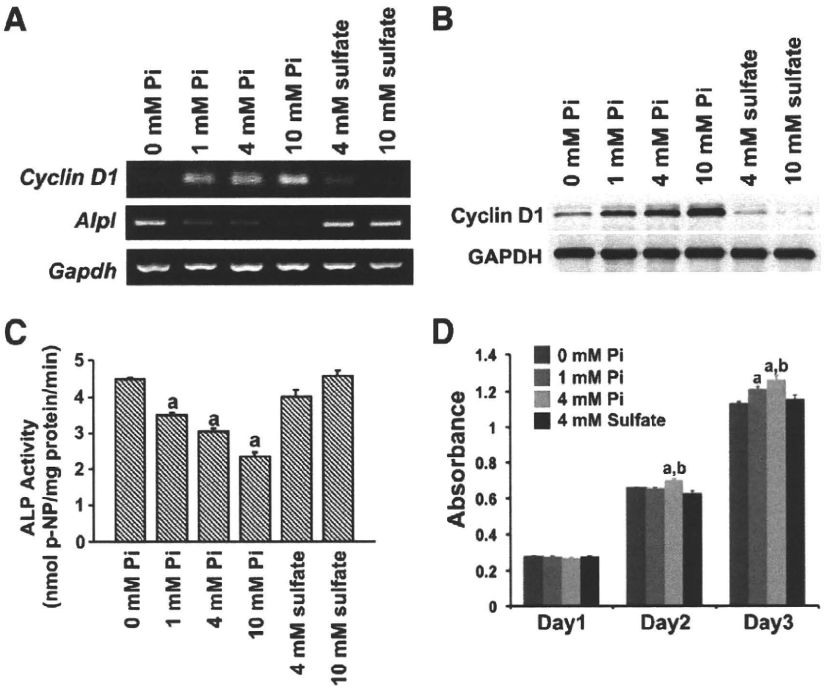

Fig. 1. Expression of cyclin D1 and alkaline phosphatase (ALP) is regulated by extracellular Pi. A) RT-PCR analyses for *cyclin D1* and *alkaline phosphatase* (*Alpl*). ATDC5 cells were incubated for 24 h in the presence of various concentrations of extracellular Pi or sulfate as a negative control before total RNA was extracted. Media containing indicated concentrations of Pi or sulfate were prepared by addition of sodium salt buffer solutions to Pi-free medium. The experiments were performed in the presence of 5% FBS, which provided additional 0.1 mM Pi. Therefore, the actual concentrations of Pi added to the cells, which were determined using a Phosphor-C test kit (Wako), were 0.1 mM higher than the indicated levels. The media added with sulfate also contained 0.1 mM Pi provided as FBS. The extracted total RNA was subjected to RT-PCR for *cyclin D1*, *Alpl* and *Gapdh*. The expression of *cyclin D1* was up-regulated, while that of *Alpl* was down-regulated at higher concentrations of extracellular Pi. B) Effects of increased extracellular Pi on cyclin D1 protein amount. Total cell lysates were harvested from ATDC5 cells treated as in (A), and subjected to Western blotting using antibodies against cyclin D1 and GAPDH. C) Effects of increased extracellular Pi on ALP activity. Cell lysates were harvested from ATDC5 cells treated as in (A, B), and subjected to measurements of ALP activity. [a]Significantly different from the value in the cells cultured in the presence of 0 mM Pi ($p<0.001$).D) Effects of extracellular Pi on the increase in cell number. ATDC5 cells were plated in 96-well culture plates at a density of 1×10^3 cells/well (designated as day 0), and cultured in the presence of various concentrations of Pi or 4 mM sulfate. The experiments were performed in the presence of 5% FBS as in (A–C). The cell number in each well was evaluated by an MTS assay on days 1, 2 and 3, represented by the corrected absorbance at O.D. 490 nm. The results were described as the mean ± S.E.M. ($n=4$). [a,b]Significantly different from the value in the cells cultured in the presence of 0 mM Pi (a; $p<0.01$) or in those cultured in the presence of 4 mM sulfate (b; $p<0.01$).

condition. Sodium sulfate was utilized as a negative control. The increase in Pi up-regulated cyclin D1 expression, while it down-regulated ALP expression, at both the mRNA and protein levels, in a dose-dependent manner (Figs. 1A–C).

Since the increase in extracellular Pi induced the up-regulation of *cyclin D1* expression, elevated levels of extracellular Pi may promote the proliferation of cells. However, in mature chondrocytes, it has been reported that increased levels of extracellular Pi induce apoptosis [10,22]. Therefore, we examined the effect of elevated Pi levels on the proliferation of ATDC5 cells using the MTS assay. In our preliminary experiments to see the effects of sulfate, 10 mM sodium sulfate significantly decreased the cell number compared with the untreated control after 2 days of culture (data not shown), and it was unlikely to be suitable as a negative control. Therefore, we selected 4 mM sodium sulfate as a negative control, and examined the effects of 0–4 mM Pi (Fig. 1D). The experiments shown in Fig. 2 were performed in the presence of 5% FBS, which provided additional 0.1 mM Pi. The actual concentrations of Pi added to the cells were determined using a Phosphor-C test kit (Wako). The media added with 4 mM sulfate also contained 0.1 mM Pi, which was provided as FBS. The cell number after 2 days of culture was significantly increased in the presence of 4 mM Pi (actual concentration 4.1 mM) compared with the presence of 0 mM Pi (actual concentration 0.1 mM) or 4 mM sodium sulfate in the media (Fig. 1D), suggesting that extracellular Pi accelerates the proliferation rather than induces apoptosis at the early stages of chondrocytic differentiation.

ERK1/2 phosphorylation is responsible for the up-regulation of cyclin D1 and down-regulation of alkaline phosphatase expression by increased extracellular Pi

By hypothesizing that the extracellular Pi concentration functions as an extracellular stimulus and exerts signals into cells, we examined the effects of increased extracellular Pi on the phosphorylation status of proteins. We performed Western blotting with anti-p-Tyr antibody after 2-D electrophoresis using cytoplasmic fractions harvested from the cells incubated in the presence of 1 mM or 10 mM Pi for 30 min, and found that only a limited number of signals were enhanced by treatment with increased Pi, and the intensity of the other signals was not obviously changed (data not shown). It has been reported that an increase in extracellular Pi induces the phosphorylation of ERK1/2 in osteoblastic MC3T3-E1 cells and maturated ATDC5 cells [11,29]. Therefore, we subjected the same samples to 2-D electrophoresis followed by immunoblotting using anti-ERK1/2 antibody, which suggested that ERK1/2 is included among the proteins whose phosphorylation at Tyr is enhanced by increased extracellular Pi (data not shown). A conventional Western blot analysis confirmed that increased extracellular Pi induced the phosphorylation of ERK1/2 in proliferating ATDC5 cells (Fig. 2A). The treatment with a MEK inhibitor, PD98059, abolished the phosphorylation of ERK1/2 induced by 10 mM Pi, confirming that MEK was involved in the phosphorylation induced by extracellular Pi (Fig. 2B). We also examined the effects of the increase in extracellular Pi on the phosphorylation of c-

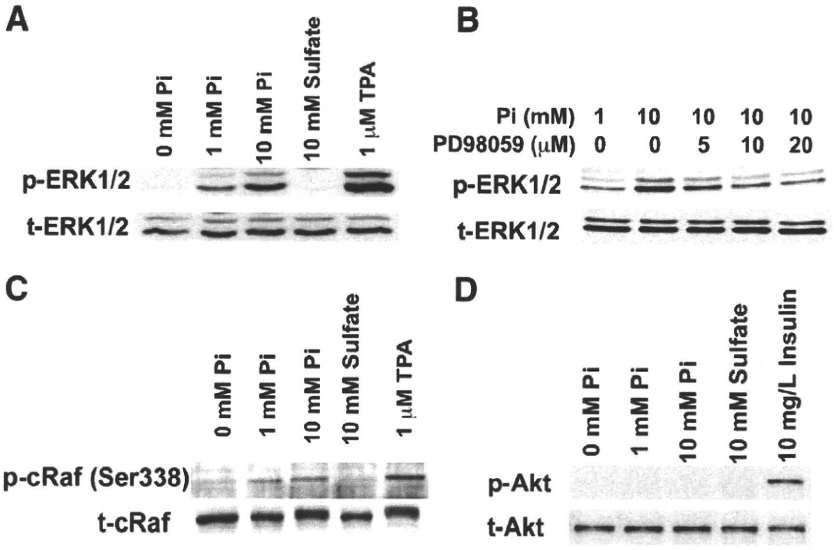

Fig. 2. Increased extracellular Pi activates the c-Raf/MEK/ERK pathway. A) Phosphorylation of ERK1/2 induced by increased extracellular Pi. ATDC5 cells were starved in serum-free, Pi-free media for 24 h, and then treated for 30 min with the indicated concentrations of Pi, 10 mM sulfate as a negative control, or 1 μM TPA as a positive control. The experiments were performed in the absence of FBS. After the treatment, whole cell lysates were harvested and subjected to Western blotting using antibodies against total and phosphorylated ERK1/2. B) A MEK inhibitor PD 98059 abolished the phosphorylation of ERK1/2 induced by increased extracellular Pi. ATDC5 cells were starved in serum-free, Pi-free media for 24 h, and then treated with the indicated concentrations of PD98059 or vehicle. Thirty minutes later, sodium phosphate buffer was added to provide the indicated concentrations of Pi, and the cells were incubated for a further 30 min. After the incubation, whole cell lysates were harvested and subjected to Western blotting. C) Phosphorylation of c-Raf induced by increased extracellular Pi. Cell lysates prepared in (A) were subjected to Western blotting with antibodies against c-Raf phosphorylated at Ser338 and total c-Raf. D) Increased extracellular Pi failed to induce the phosphorylation of Akt. Total cell lysates were harvested from ATDC5 cells treated with the indicated concentrations of Pi or sulfate for 30 min, and subjected to Western blotting using the antibodies against the indicated molecules. Total cell lysates from the cells treated with 10 mg/l insulin were included as the positive control.

Raf, which lies upstream of MEK. The increase in extracellular Pi resulted in the phosphorylation of c-Raf at Ser338 (Fig. 2C), suggesting that the phosphorylation of ERK1/2 was mediated by c-Raf. We examined the effects of extracellular Pi on the phosphorylation of Akt, which was not altered by the increase in extracellular Pi (Fig. 2D). The phosphorylation of p38MAPK was not induced by the increase in extracellular Pi, either (data not shown).

Treatment with PD98059 cancelled the up-regulation of cyclin D1 expression induced by the extracellular Pi in a dose-dependent manner (Figs. 3A and C). Similarly, the down-regulation of ALP expression was also abolished by PD98059 (Figs. 3B and D), indicating that extracellular Pi regulates the expression of these genes through the MEK/ERK pathway.

Phosphorylation of FRS2α is required for the activation of the Raf/MEK/ERK pathway in response to the increased extracellular Pi

Then, to explore the molecular mechanism underlying the connection between the increased extracellular Pi and the activation of Raf/MEK/ERK pathway, we examined the effects of increased extracellular Pi on the phosphorylation of FGF receptor substrate 2α (FRS2α). FRS2α functions upstream of Raf/MEK/ERK cascade in the signaling by FGFs, which plays a critical role in chondrocyte proliferation and differentiation. ATDC5 cells expressed FGFRs 1, 2 and 3 as confirmed by RT-PCR (data not shown). We treated the cells with various concentrations of extracellular Pi, and examined the effects on phosphorylation of FRS2α at Tyr196 by Western blot. As expected, treatment with 50 ng/ml of basic FGF (R&D Systems, Minneapolis, MN) induced the phosphorylation of FRS2α (Fig. 4). Interestingly, an increase in extracellular Pi also induced the phosphorylation of FRS2α in a dose-dependent manner, while the treatment with 10 mM sodium sulfate did not (Fig. 4). These results suggest that an increase in extracellular Pi leads to the activation of Raf/MEK/ERK pathway through the phosphorylation of FRS2α.

Pit-1 is involved in the activation of FRS2α, Raf/MEK/ERK pathway and gene regulation induced by increased extracellular Pi

As the next step, we investigated whether Na^+/Pi cotransporters are involved in the responsiveness of ATDC5 cells to extracellular Pi. We examined the temporal expression pattern of the two genes encoding type III Na^+/Pi cotransporters, *Pit-1* (*Glvr-1; Slc20a1*) and *Pit-2* (*Ram-1; Slc20a2*), during the chondrogenic differentiation of ATDC5 cells (Fig. 5A). When the cells were cultured in differentiation medium, the expression of *Col2a1* increased from 0 to 4 weeks of culture, and diminished after 6 weeks. The expression of *Pit-1* peaked at the time-point of 0 weeks and steadily declined. On the other hand, the expression level of *Pit-2* was rather constant (Fig. 5A). The expression of other Na^+/Pi cotransporters, types IIa, IIb and IIc, was not detected by RT-PCR (data not shown). As to the effects of increased extracellular Pi on the levels of *Pit-1* and *Pit-2*, 24-h treatment with Pi did not cause any obvious change in the expression of these genes (data not shown), although we cannot exclude the possibility that longer treatment might alter it. Treatment with PFA, an inhibitor of Na^+/Pi cotransporters, abolished the phosphorylation of c-Raf and ERK1/2 induced by increased extracellular Pi in a dose-dependent manner (Fig. 5B). Consistent with this observation, the up-regulation of cyclin D1 expression and down-regulation of ALP activity induced by the increase in extracellular Pi were abolished by treatment with PFA (Figs. 5C and D).

We further investigated the responsiveness to extracellular Pi, using primary chondrocytes isolated from cartilage of mouse rib cages. The production of cartilaginous matrices by the isolated cells was confirmed by alcian blue staining (Fig. 6A). *Sox9* and *Col2a1* were strongly expressed (Fig. 6B), suggesting that most of the cells are at the proliferating stage. The expression of *Col10a1* was also detected (Fig. 6B). *Pit-1* and *Pit-2* also were expressed in these cells, and 24-h treatment with increased extracellular Pi did not result in any obvious change in the expression of these genes (Fig. 6C). The increase

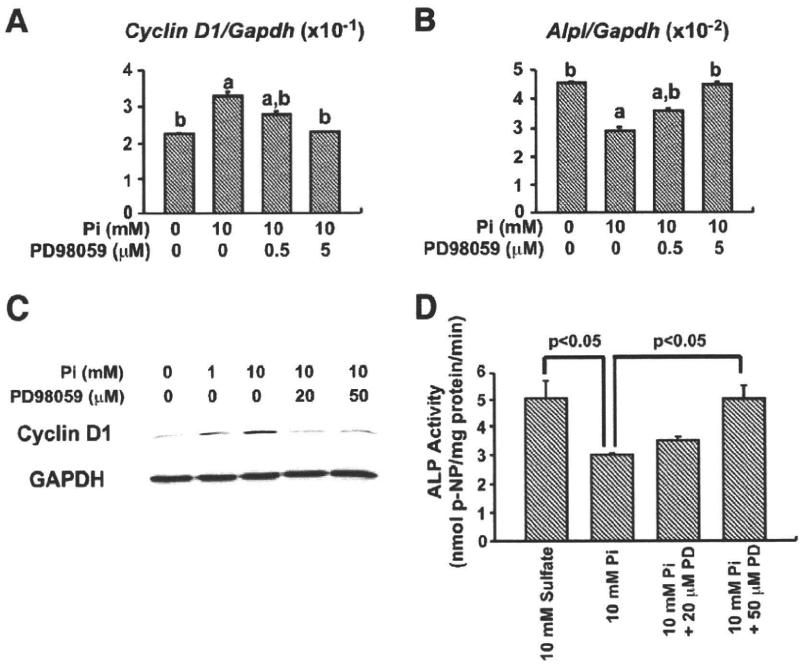

Fig. 3. A MEK inhibitor PD98059 abolished the effects of increased extracellular Pi on the expression of cyclin D1 and ALP. A, B) Real-time PCR for *cyclin D1* (A) and *Alpl* (B). After ATDC5 cells were treated with the indicated concentrations of PD98059 or vehicle in the absence of Pi for 30 min, sodium phosphate buffer was added to the media to make the indicated concentrations of Pi, and the cells were incubated for a further 24 h. The experiments were performed in the presence of 5% FBS, which provided additional 0.1 mM Pi. The media added with sulfate also contained 0.1 mM Pi provided as FBS. After the incubation, total RNA was extracted, and real-time PCR was performed with the TaqMan system. The calculated copy number of amplicons was standardized based on that of *Gapdh*. The data are expressed as the mean ± S.E.M. (n = 3). [a,b]Significantly different from the value in the cells incubated in the presence of 1 mM Pi (a; $p<0.01$) or in those cultured in the presence of 10 mM Pi without PD98059 (b; $p<0.01$), respectively. The experiments were performed three times, and similar results were obtained. C) Treatment with PD98059 abolished the Pi-induced increase in cyclin D1 protein. Whole cell lysates were prepared from ATDC5 cells treated with various concentrations of extracellular Pi for 8 h together with PD98059 or vehicle in the presence of 5% FBS as in (A, B), and subjected to Western blotting with anti-cyclin D1 antibody. D) Effects of extracellular Pi and PD98059 on ALP activity. ATDC5 cells were treated with 10 mM of extracellular Pi in the presence or absence of PD98059, or 10 mM sulfate, for 24 h. Cell lysates were then harvested and subjected to measurements of ALP activity. The experiments were performed in the presence of 5% FBS. The data are expressed as the mean ± S.E.M. (n = 3).

in extracellular Pi induced the phosphorylation of ERK1/2, which was cancelled by treatment with PFA (Fig. 6D). In addition, increased extracellular Pi up-regulated the expression of *cyclin D1* and down-regulated that of *Alpl*, both of which were also abolished by treatment with PFA (Figs. 6E and F), indicating that primary chondrocytes respond to increased extracellular Pi in a Na^+/Pi cotransporter-dependent manner. Treatment with 1 mM Pi did not result in the reduction of *Alpl* expression compared with 0 mM Pi in primary chondrocytes although 10 mM Pi markedly suppressed it (Fig. 6F), which was different from the case in ATDC5 cells where 1 mM Pi clearly suppressed both *Alpl* mRNA level and ALP activity (Fig. 1). There might be some difference between primary chondrocytes and ATDC5 cells in terms of sensitivity to lower levels of extracellular Pi.

Finally, we examined the effects of knockdown of *Pit-1* or *Pit-2*, on the responsiveness of ATDC5 cells to the increase in the extracellular Pi concentration. RT-PCR analyses together with the Pi-uptake assay confirmed the successful knockdown of the expression of *Pit-1* or *Pit-2* by transfection of corresponding siRNAs (Figs. 7A and B). The decrease in the uptake of Pi by knockdown of *Pit-1* or *Pit-2* was rather modest, probably due to the functional redundancy of these two molecules in the uptake of Pi. Then, we examined the effects of knockdown of these genes on the phosphorylation of ERK1/2 induced by increased extracellular Pi. We treated the cells with 0–4 mM Pi in these experiments, because induction of ERK1/2 phosphorylation by these concentrations of extracellular Pi was clearly dose-dependent in the cells transfected with control siRNA. Interestingly, knockdown of *Pit-1*, but not *Pit-2*, diminished the phosphorylation of ERK1/2 induced by increased extracellular Pi (Figs. 7C–F), indicating the critical role of Pit-1 in the responsiveness of ATDC5 cells to extracellular Pi. Phosphorylation of FRS2α induced by an increase in extracellular Pi was cancelled by knockdown of *Pit-1* expression, either (Fig. 8).

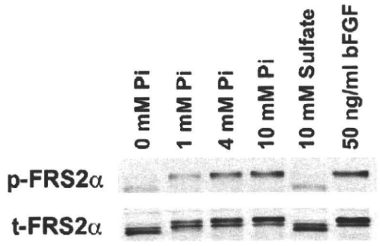

Fig. 4. Increased extracellular Pi resulted in the phosphorylation of FRS2α at Tyr196. ATDC5 cells were starved in serum-free, Pi-free media for 24 h, and then treated for 30 min with the indicated concentrations of Pi, 10 mM sulfate as a negative control, or 50 ng/ml basic FGF (bFGF) as a positive control, in the absence of FBS. After the treatment, whole cell lysates were harvested and subjected to Western blotting using antibodies against phosphorylated or total FRS2α.

Discussion

Here we have provided evidence that extracellular Pi triggers signal transduction and leads to altered gene expression in the early stages of chondrocytic differentiation. It is interesting that the increase in the concentration of extracellular Pi induced an increase in the expression of *cyclin D1* in both ATDC5 cells and isolated primary chondrocytes (Figs. 1 and 5). To date, a line of evidence has demonstrated the roles of cyclin D1 in skeletogenesis. Mice lacking a *cyclin D1* gene exhibit severe postnatal growth retardation and skeletal abnormalities [30]. Cyclin D1 is a positive regulator of progression through the G1 phase of the cell cycle, and is induced to express by many mitogenic stimuli. It regulates cell cycle progression through its association with and activation of cyclin-dependent

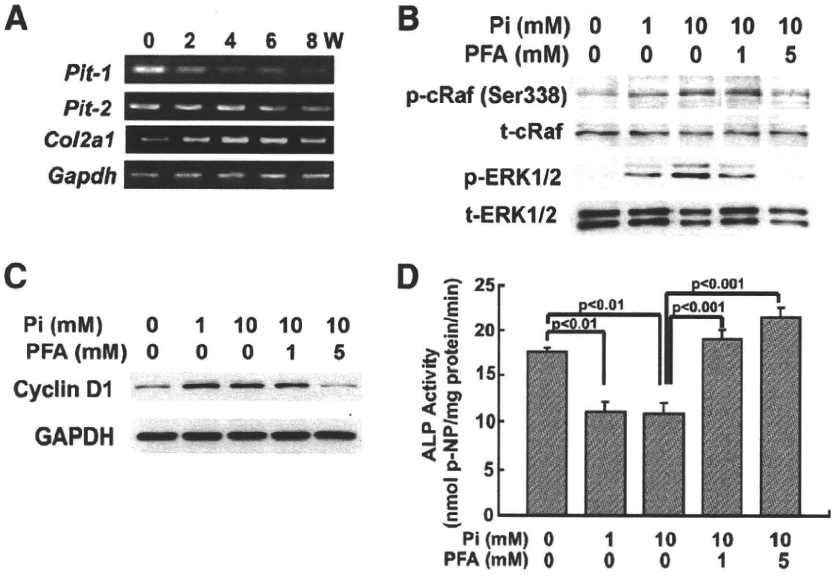

Fig. 5. Expression of type III Na^+/Pi cotransporters in ATDC5 cells and the effects of phosphonoformic acid (PFA) on the responsiveness to increased extracellular Pi. A) Temporal expression pattern of type III Na^+/Pi cotransporters *Pit-1* and *Pit-2* during the chondrogenic differentiation of ATDC5 cells. ATDC5 cells were cultured in differentiation medium containing 5% FBS and 1% ITS for the indicated period, and subjected to RT-PCR analyses for *Pit-1*, *Pit-2*, *Col2a1* and *Gapdh*. The expression of *Col2a1*, which is a marker of proliferating chondrocytes, increased from 0 to 4 weeks of culture, and diminished after 6 weeks. The expression of *Pit-1* was intense early on in the culture but declined steadily. The expression of *Pit-2* was quite consistent. B) Treatment with PFA abolished the phosphorylation of c-Raf and ERK1/2 induced by the increased concentration of extracellular Pi. ATDC5 cells were starved in serum-free, Pi-free media for 24 h, and then treated with the indicated concentrations of PFA or vehicle. Thirty minutes later, sodium phosphate buffer was added to provide the indicated concentrations of Pi, and the cells were incubated for a further 30 min in the absence of FBS, before cell lysates were harvested for Western blotting. C) Treatment with PFA abolished the Pi-induced increase in cyclin D1 protein. ATDC5 cells were treated with the various concentrations of extracellular Pi for 24 h in the presence or absence of PFA, and the cell lysates were subjected to Western blotting using antibodies against cyclin D1 and GAPDH. The experiments were performed in the presence of 5% FBS which provided 0.1 mM Pi, and the actual concentrations of Pi in the media were 0.1 mM higher than the indicated levels. D) Treatment with PFA abolished the Pi-induced increase in ALP activity. ATDC5 cells were treated with the various concentrations of extracellular Pi for 24 h in the presence or absence of PFA as in (C), and the cell lysates were subjected to measurements of ALP activity. The experiments were performed in the presence of 5% FBS. The data are expressed as the mean ± S.E.M. (n = 3).

kinases 4 and 6 [31–33]. In chondrocytes, Beier et al. have previously identified cyclin D1 as a target of PTHrP and TGF-β [19,20], both of which play critical roles in the proliferation of chondrocytes. They demonstrated that the TGF-β pathway activates the transcription factor ATF-2, whereas PTHrP uses the related transcription factor CREB, to stimulate the cyclin D1 promoter, and that the inhibition of cyclin D1 expression caused a delay in progression of chondrocytes through the G1 phase and decreased proliferation [19]. In addition, it is also reported that TGF-β stimulates cyclin D1 expression through activation of β-catenin in chondrocytes [21]. These findings indicate that cyclin D1 plays a central role in the proliferation of chondrocytes. Recently, it has been reported that cyclin D1 is a target for the proliferative effects of PTH and PTHrP in immature osteoblastic cells as well [34].

The up-regulation of the cyclin D1 expression by the increased extracellular Pi suggests that signaling induced by the extracellular Pi facilitates the proliferation of early chondrocytes. Consistent with this notion, the results of MTS assays suggested that an increase in the concentration of extracellular Pi favored the proliferation of ATDC5 cells (Fig. 1D), which presents a striking contrast to reports that apoptosis was induced by increased extracellular Pi in mature chondrocytes [10,22]. The effects of extracellular Pi on chondrocytes might change during the process of differentiation. In fact, when cultured in the differentiation medium for 8 weeks, ATDC5 cells lost the expression of *cyclin D1*, and an increase in extracellular Pi failed to induce its expression (data not shown). This observation was consistent with the previous report demonstrating that *cyclin D1* expression in growth plate was specific for proliferating chondrocytes by *in situ* hybridization [35]. The target genes of the signaling triggered by extracellular Pi might differ among cell types, probably due to the difference in the expressed transcription factors and/or co-factors. Indeed, it has been reported that the transcription factor ATF-3 is up-regulated during chondrocyte differentiation and represses the transcription of *cyclin D1* gene in maturing chondrocytes [36]. We speculate that the ERK1/2 activation induced by an increase in extracellular Pi failed to induce the transcription of *cyclin D1* gene in maturing chondrocytes due to the expression of ATF-3.

Recently, a series of *in vitro* and *in vivo* studies have suggested critical roles of the MEK/ERK pathway in chondrogenesis. It has been reported that the up-regulation of *Sox9* expression by FGFs was mediated by the MEK/ERK pathway in primary chondrocytes and a mesenchymal cell line, C3H10T1/2 [37]. In addition, it was also reported that transgenic mice expressing a constitutively active form of MEK1 in chondrocytes showed achondroplasia-like dwarfism with impaired hypertrophic differentiation [38]. In the current study, we revealed that the increased extracellular Pi resulted in the phosphorylation of FRS2α and the activation of Raf/MEK/ERK pathway. The commonality between the signaling triggered by the increased extracellular Pi and that induced by FGFs suggests that the extracellular Pi might modulate the actions of FGFs in chondrogenesis.

The involvement of Na^+/Pi cotransporters in the signaling of extracellular Pi is an important issue in our study. The Na^+/Pi cotransporters in mammals are classified into 3 groups: types I, II and III [39]. Type I cotransporters transport organic anions. Type IIa and IIc cotransporters are predominantly expressed in renal proximal tubules, while type IIb is detected in the intestine. Type III Na^+/Pi cotransporters Pit-1 and Pit-2 are expressed ubiquitously. Taking the findings in the knockdown experiments together with the temporal expression pattern of *Pit-1* into consideration, the signaling induced by extracellular Pi is likely to play an important role in the early stages of chondrocytic differentiation as well as in mature chondrocytes and osteoblasts.

Our results suggest that chondrocytes sense and respond to the increased extracellular Pi via Pit-1. FRS2α, which functions upstream of Raf/MEK/ERK pathway in FGF signaling, is also suggested to play a role. At the moment, we do not know the precise molecular

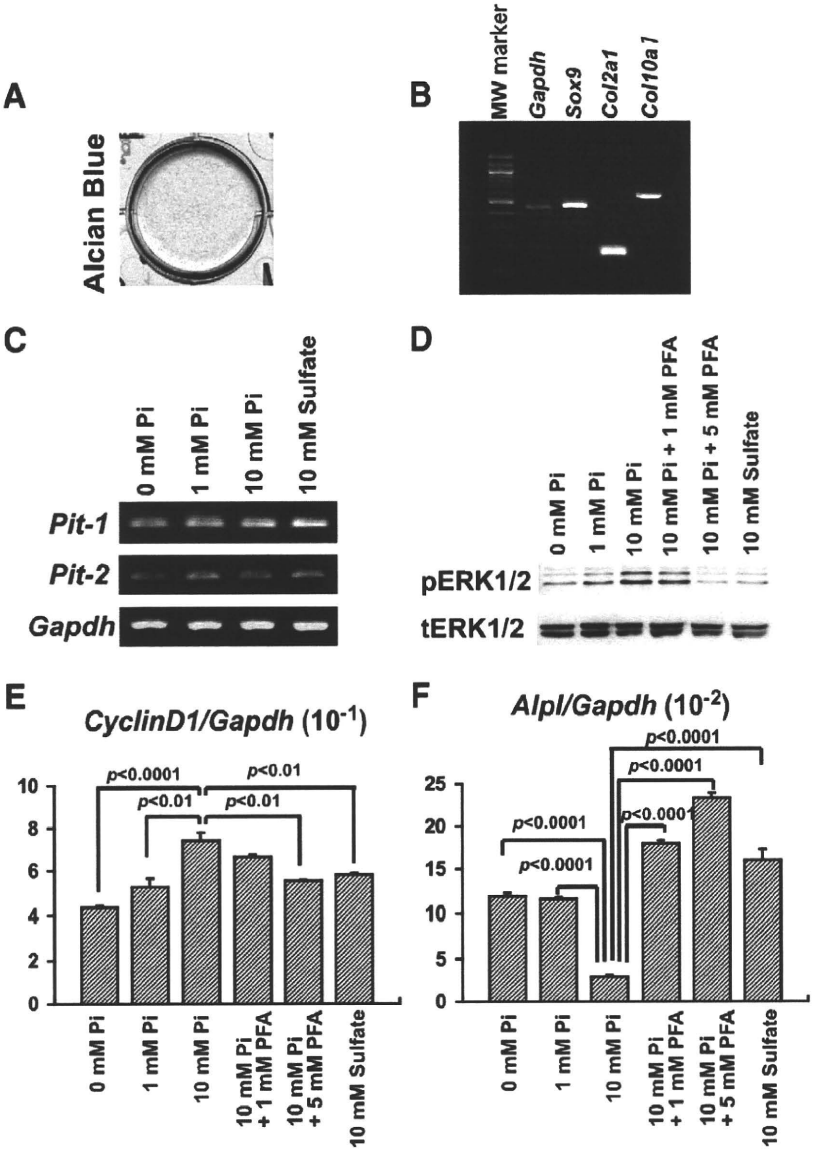

Fig. 6. Responsiveness of isolated primary chondrocytes to increased extracellular Pi and involvement of Na^+/Pi cotransporters. A, B) Characterization of the isolated primary chondrocytes. Primary chondrocytes isolated from mouse rib cartilage were inoculated in 6-well plates (2×10^5 cells/well), and cultured in DMEM with 10% FBS and 50 μg/ml of ascorbic acid for 2 days. The cells were then subjected to alcian blue staining (A) or RT-PCR analyses for the expression of chondrocytic marker genes (B). C) Expression of *Pit-1* and *Pit-2* in primary chondrocytes. Isolated primary chondrocytes were inoculated in 6-well plates (2×10^5 cells/well), and cultured in DMEM with 10% FBS and 50 mg/ml of ascorbic acid for 2 days. Then, the cells were washed in Pi-free media twice, and incubated in media containing the indicated concentrations of Pi or sulfate for 24 h before total RNA was extracted for RT-PCR. The 24-h treatment with increased extracellular Pi did not alter the expression of *Pit-1* and *Pit-2*. The experiments were performed in the presence of 5% FBS, and the actual concentrations of Pi added to the cells were 0.1 mM higher than the indicated levels. The media added with sulfate also contained 0.1 mM Pi provided as FBS. D) Increased extracellular Pi induced the phosphorylation of ERK1/2 in primary chondrocytes in a Na^+/Pi cotransporter-dependent manner. Primary chondrocytes (2×10^5 cells/well in 6-well plates) were cultured in DMEM with 10% FBS and 50 μg/ml of ascorbic acid for 2 days. The cells were starved in serum-free, Pi-free media for 24 h, and then treated with the indicated concentrations of PFA or vehicle. Thirty minutes later, sodium phosphate buffer was added to provide the indicated concentrations of Pi, and the cells were incubated for a further 30 min before cell lysates were harvested for Western blotting. Sodium sulfate buffer was used as a negative control. The experiments were performed in the absence of FBS. E, F) Increased extracellular Pi up-regulated *cyclin D1* (E) and down-regulated *Alpl* (F) expression in primary chondrocytes in a Na^+/Pi cotransporter-dependent manner. Primary chondrocytes cultured for 2 days were washed with serum-free, Pi-free media twice. The cells were then treated with the indicated concentrations of PFA or vehicle, and 30 min later, sodium phosphate buffer was added to prepare the indicated concentrations of extracellular Pi. As in (C), the experiments were performed in the presence of 5% FBS. After a 24-h incubation, total RNA was extracted, and real-time RT-PCR was performed.

mechanisms by which cells transduce the signals triggered by extracellular Pi from Pit-1 to FGFRs and Raf/MEK/ERK pathway. However, considering that knockdown of *Pit-2* expression had no apparent effects on the phosphorylation of ERK1/2 induced by increased extracellular Pi although Pit-2 contributed to the uptake of Pi as did Pit-1 (Fig. 7), the transduction of the signals triggered by extracellular Pi might involve unclarified Pit-1-specific function other than the transport of Pi. Based on the findings in the current study, we are hypothesizing that an increase of extracellular Pi might cause some modification including phosphorylation or conformational change of Pit-1 itself, which might lead to the direct or indirect interaction between Pit-1 and FGFRs and alter the autophosphorylation activity of FGFRs. Most tissues express FGFRs in a redundant manner, and we propose that a complex containing Pit-1 and FGFR might play a critical role in sensing of extracellular Pi. It was recently reported that stable PiT-1 depletion reduced tumor growth when engineered HeLa cells were injected into nude mice, and the proliferation of PiT-1-depleted cells were rescued by the PiT-1 mutant

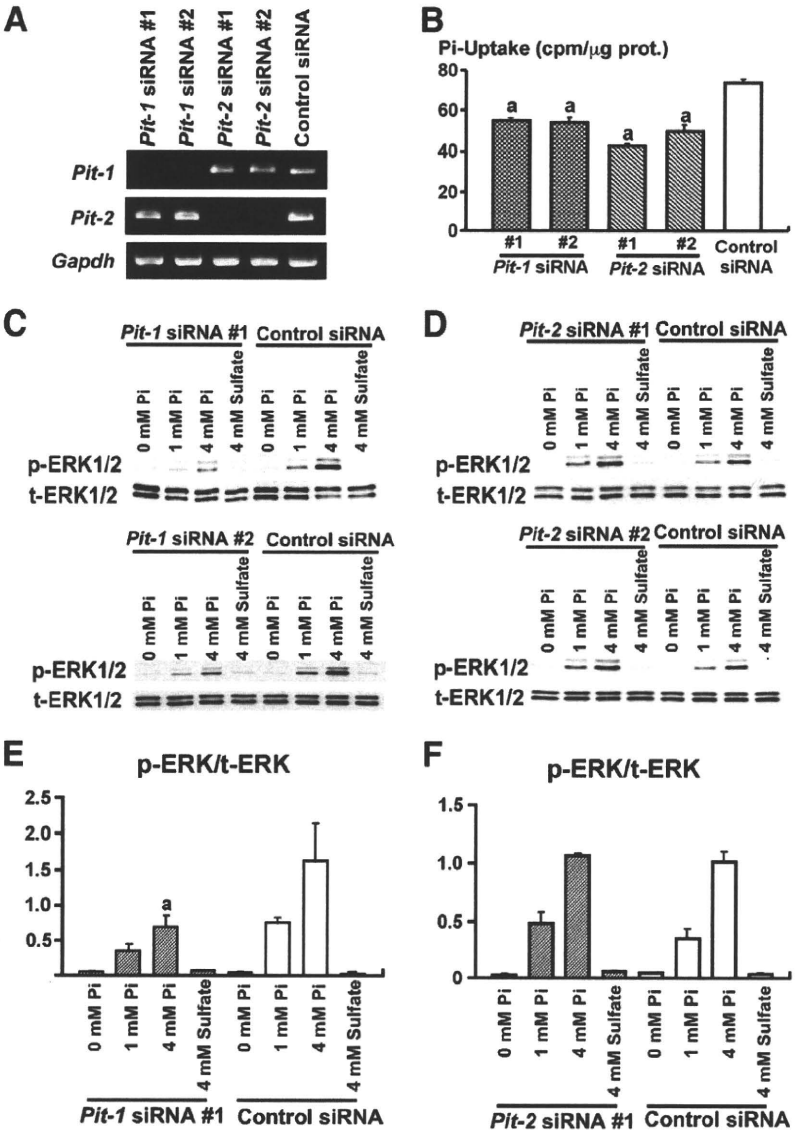

Fig. 7. Effects of knockdown of type III Na^+/Pi cotransporters on the ERK1/2 phosphorylation induced by the increased extracellular Pi. A) Effects of *Pit-1* or *Pit-2* knockdown on the expression of these genes. ATDC5 cells were transfected with *Pit-1*-specific siRNA, *Pit-2*-specific siRNA, or a negative control siRNA (60 nM each). To knockdown the expression of *Pit-1* or *Pit-2*, two siRNAs were used for each target, designated as #1 and #2. Three days later, total RNA was extracted, and subjected to RT-PCR. B) Effects of *Pit-1* or *Pit-2* knockdown on Pi uptake in ATDC5 cells. Cells were transfected with *Pit-1*-specific siRNA, *Pit-2*-specific siRNA, or a negative control siRNA (60 nM each) as in (A). Three days later, a Pi uptake assay was performed using ^{32}P-labeled orthophosphate. The radioactivity was standardized based on the protein content of cells with the same transfection. The data are expressed as the mean ± S.E.M. (n = 4). [a]Significantly different from the value in the cells transfected with control siRNA ($p<0.0001$). C–F) Western blot analyses to examine the effects of knockdown of *Pit-1* (C, E) or *Pit-2* (D, F) expression on ERK1/2 phosphorylation induced by increased extracellular Pi. ATDC5 cells were transfected with *Pit-1*-specific siRNA, *Pit-2*-specific siRNA, or a negative control siRNA (60 nM each). Two days later, the medium was changed to serum-free, Pi-free medium to starve the cells. After 24 h of starvation, the cells were treated with the indicated concentrations of Pi or sulfate for 30 min. Then, cell lysates were harvested for Western blotting using antibodies against phosphorylated or total ERK1/2. To knockdown the expression of *Pit-1* or *Pit-2*, two siRNAs were used for each target, to confirm that similar results were obtained (C, D). Each experiment was performed three times. The densitometric ratio of phosphorylated ERK1/2 to total ERK1/2 in the experiments using the *Pit-1* siRNA #1 or *Pit-2* siRNA #1 is depicted in (E) and (F), respectively. In these graphs, the data are shown as the mean ± S.E.M. (n = 3). [a]Significantly different from the value in the cells transfected with control siRNA and treated with the same concentration of Pi ($p<0.05$).

lacking Pi transport activity [40]. The report also supports the idea of the novel function of Pit-1 in addition to its Pi transport activity. Experiments using various Pit-1 and FGFR mutants might be useful to test our hypothesis.

In conclusion, proliferating chondrocytes are capable of changing the expression of selective genes such as those for cyclin D1 and ALP in response to extracellular Pi, a process which is mediated by the type III Na^+/Pi cotransporter Pit-1 and c-Raf/MEK/ERK pathway. The signaling of extracellular Pi appears to facilitate the proliferation of immature chondrocytes, which is different from the case in mature chondrocytes.

Supplementary materials related to this article can be found online at doi:10.1016/j.bone.2010.08.006.

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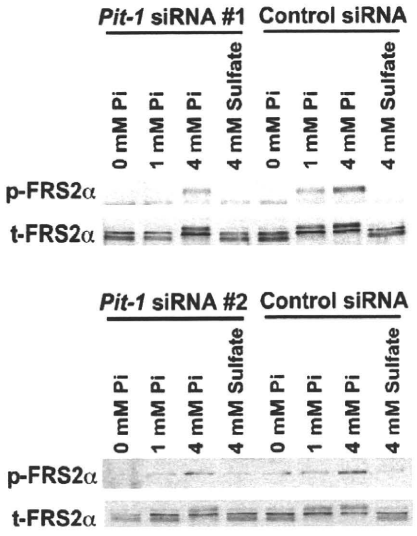

Fig. 8. The knockdown of *Pit-1* expression cancelled the phosphorylation of FRS2α induced by increased extracellular Pi. ATDC5 cells were transfected with two *Pit-1*-specific siRNAs (designated as #1 and #2) or a negative control siRNA (60 nM each). Two days later, the medium was changed to serum-free, Pi-free medium to starve the cells. After 24 h of starvation, the cells were treated with the indicated concentrations of Pi or sulfate for 30 min, before cell lysates were harvested for Western blotting.

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ARTICLE

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Both FGF23 and Extracellular Phosphate Activate Raf/MEK/ERK Pathway via FGF Receptors in HEK293 Cells

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ABSTRACT

Fibroblast growth factor 23 (FGF23) is a phosphaturic hormone produced by bone and exerts its function in the target organs by binding the FGF receptor (FGFR) and Klotho. Since recent studies suggested that extracellular inorganic phosphate (Pi) itself triggers signal transduction and regulates gene expression in some cell types, we tested the notion that extracellular Pi induces signal transduction in the target cells of FGF23 also and influences its signaling, utilizing a human embryonic kidney cell line HEK293. HEK293 cells expressed low levels of *klotho*, and treatment with a recombinant FGF23[R179Q], a proteolysis-resistant mutant of FGF23, resulted in phosphorylation of ERK1/2 and induction of *early growth response-1* (*EGR1*) expression. Interestingly, increased extracellular Pi resulted in activation of the Raf/MEK/ERK pathway and expression of *EGR1*, which involved type III sodium/phosphate (Na^+/Pi) cotransporter PiT-1. Since the effects of an inhibitor of Na^+/Pi cotransporter on FGF23 signaling suggested that the signaling triggered by increased extracellular Pi shares the same downstream cascade as FGF23 signaling, we further investigated their convergence point. Increasing the extracellular Pi concentration resulted in the phosphorylation of FGF receptor substrate 2α (FRS2α), as did treatment with FGF23. Knockdown of *FGFR1* expression diminished the phosphorylation of both FRS2α and ERK1/2 induced by the Pi. Moreover, overexpression of FGFR1 rescued the decrease in Pi-induced phosphorylation of ERK1/2 in the cells where the expression of *PiT-1* was knocked down. These results suggest that increased extracellular Pi triggers signal transduction via PiT-1 and FGFR and influences FGF23 signaling in HEK293 cells. J. Cell. Biochem. 111: 1210–1221, 2010. © 2010 Wiley-Liss, Inc.

KEY WORDS: PHOSPHATE; FGF23; FGF RECEPTOR; SIGNALING; Na^+/Pi COTRANSPORTER

Phosphorus plays critical roles in various biological processes. In extracellular fluid, most of the phosphorus is present as inorganic phosphate (Pi). Fibroblast growth factor 23 (FGF23) is a circulating phosphaturic factor that plays a central role in the renal reabsorption of Pi and metabolism of vitamin D [Quarles, 2008]. Two genetic disorders, autosomal-dominant hypophosphatemic rickets (ADHR; OMIM # 193100) and hyperphosphatemic familial tumoral calcinosis (OMIM # 211900), are caused by activating and inactivating mutations in the *FGF23* gene, respectively [ADHR Consortium, 2000; Araya et al., 2005; Larsson et al., 2005]. FGF23 is also responsible for tumor-induced hypophosphatemic osteomalacia (TIO) [Shimada et al., 2001]. FGF23 increases renal Pi excretion by reducing the expression of type IIa and IIc sodium/phosphate (Na^+/Pi) cotransporters (NPTIIa and NPTIIc) in the brush-border membrane of proximal tubules and decreases the production of 1,25-dihydroxyvitamin D [1,25$(OH)_2$D] by suppressing the expression of 25-hydroxyvitamin D-1α-hydroxylase (1α-hydroxylase) and inducing that of 25-hydroxyvitamin D-24-hydroxylase (24-hydroxylase) [Segawa et al., 2003; Shimada et al., 2004a,b].

FGF23 is produced by bone and functions mainly in kidney in an endocrine fashion, like FGF19 and FGF21. It has been demonstrated that low heparin-binding affinity confers its endocrine function [Goetz et al., 2007]. To exert its effects, FGF23 requires α-Klotho (Klotho). FGF23 binds to FGF receptor (FGFR)–Klotho complex and induces the phosphorylation of FGFR substrate 2α (FRS2α) and ERK1/2 downstream, resulting in the expression of *early growth*

Additional Supporting Information may be found in the online version of this article.

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response-1 (*Egr1*), gene which encodes a transcription factor [Kurosu et al., 2006; Urakawa et al., 2006]. Among FGFRs, FGFR1 is suggested to be the biologically relevant receptor for FGF23 [Liu et al., 2008; Gattineni et al., 2009]. Since Klotho is expressed in distal but not proximal tubules, and the expression of FGFR1 is strongest in distal tubules in kidney, it is suggested that the distal tubule is the target of FGF23 signaling in kidney [Kuro-o et al., 1997; Liu et al., 2008]. In fact, the administration of FGF23 to mice results in the phosphorylation of ERK1/2 in distal tubules [Farrow et al., 2009]. It remains unclear how the initial signaling triggered by FGF23 in distal tubules results in action in proximal tubules.

In addition to kidney, parathyroid has been recently identified as a target organ for FGF23 both in vivo and in vitro [Ben-Dov et al., 2007; Krajisnik et al., 2007]. In bovine parathyroid cells, it was reported that FGF23 induced the expression of *Egr1* and decreased the PTH mRNA expression and secretion [Krajisnik et al., 2007]. In rats, it was demonstrated that the parathyroid gland expressed Klotho and two kinds of FGFRs, and that FGF23 activated MAPK pathway through ERK1/2 phosphorylation and increased *Egr1* gene in the parathyroid, to decrease serum levels of PTH [Ben-Dov et al., 2007]. These results indicate that MAPK pathway is likely to be the main signaling pathway of the FGF23–Klotho axis both in kidney and parathyroid gland.

Reflecting endocrine function of FGF23, its circulating levels are controlled by various systemic factors, of which $1,25(OH)_2D$ has the best defined regulatory role [Liu et al., 2006]. Its expression might be regulated by the level of Pi in serum as well. In patients with chronic kidney disease (CKD) with hyperphosphatemia, plasma FGF23 concentrations are increased [Larsson et al., 2003; Weber et al., 2003; Pande et al., 2006; Fliser et al., 2007; Westerberg et al., 2007]. However, an increase in extracellular Pi did not directly stimulate FGF23 expression in osteoblasts [Liu et al., 2006]. It has been reported that acute changes of serum Pi did not modify FGF23 levels in the healthy human [Ito et al., 2007]. Although Pi loading increases FGF23 levels in mice [Perwad et al., 2005], it is unclear whether dietary Pi regulates FGF23 levels in humans [Ferrari et al., 2005; Nishida et al., 2006]. The precise mechanism by which Pi controls the production of FGF23 by bone remains unknown at the moment.

Several studies have suggested that increased extracellular Pi triggers signal transduction and results in altered gene expression in some cell types. In the osteoblastic cell line MC3T3-E1, extracellular Pi regulated the expression of several genes including that for osteopontin [Beck et al., 2000, 2003]. In addition, elevated Pi levels induced the pathological calcification of vascular tissue by inducing the expression of osteoblast-specific genes via a type III Na^+/Pi cotransporter, Pit-1, in vitro [Jono et al., 2000; Li et al., 2006; Mizobuchi et al., 2006]. Although it has been reported that an increase in extracellular Pi did not directly stimulate FGF23 expression in osteoblasts as described above [Liu et al., 2006], the responsiveness to extracellular Pi might be retained in other tissues including the targets of FGF23 signaling such as kidney and parathyroid. If this is the case, the signaling triggered by extracellular Pi in these tissues might influence the signaling evoked by FGF23 and modulate the feedback mechanism for the control of FGF23 production.

In the present study, to test the notion that extracellular Pi itself might exert signals in the target cells of FGF23 as well and influence its signaling, we investigated the responsiveness to extracellular Pi and the effects of Pi levels on FGF23 signaling using a human embryonic kidney cell line, HEK293.

MATERIALS AND METHODS

CELL CULTURE AND GENERATION OF STABLE HEK293–KLOTHO TRANSFECTANTS

HEK293 human embryonic kidney cells and CHO-K1 Chinese hamster ovarian cells were cultured in DMEM and F-12 medium (Sigma–Aldrich, St. Louis, MO), respectively, supplemented with 10% fetal bovine serum (FBS; JRH Biosciences, Inc., Lenexa, KS) at 37°C in a 5% CO_2 atmosphere. Pi-free DMEM was obtained from Nikken (Tokyo, Japan). Media containing various concentrations of Pi were prepared by adding sodium phosphate buffer to Pi-free DMEM (pH 7.3).

To generate HEK293 stably overexpressing Klotho (designated HEK293–Klotho), an expression plasmid encoding the full-length mouse Klotho (pcDNA3-klotho; a gift from Prof. Y.-I. Nabeshima) was introduced into HEK293 cells using FuGENE®6 (Roche Diagnostics, Mannheim, Germany), and stable transfectants were selected for resistance to G418 (Promega Corporation, Madison, WI). The expression of Klotho was confirmed by Western blotting using anti-Klotho antibody (1:1,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

CONSTRUCTION OF THE EXPRESSION PLASMIDS ENCODING FGF23 AND FGFR1

To construct the expression plasmids encoding human FGF23, we first performed PCR-based cloning of cDNA for human FGF23 using RNA derived from human heart (Takara Bio, Inc., Shiga, Japan). Then, the cDNA was cloned in frame into a pcDNA4/Myc-His vector (Invitrogen, Carlsbad, CA), after the stop codon was mutated to a *Bam*HI recognition site, resulting in a plasmid encoding FGF23 fused to Myc-tag at the C-terminus (designated as pcDNA-hFGF23-Myc-His). We also introduced a mutation from G to A at nucleotide 576 of the FGF23 coding sequence by PCR-based site-directed mutagenesis, leading to an amino acid substitution of glutamine for arginine at codon 179 (R179Q). The resultant plasmid was designated as pcDNA-hFGF23[R179Q]-Myc-His. The FGF23[R179Q] mutant is resistant to proteolysis. Human FGFR1 cDNA was purchased from Invitrogen as an Ultimate™ Human ORF Clone and cloned into pcDNA3.1/nV5-DEST™ using the Gateway® Technology (Invitrogen) to generate the expression iplasmid pcDNA-FGFR1.

PREPARATION OF FGF23[R179Q]-CONTAINING CONDITIONED MEDIUM

The expression plasmid pcDNA-hFGF23[R179Q]-Myc-His was introduced into CHO-K1 cells, and stable transfectants (CHO-FGF23[R179Q]) were selected for resistance to Zeocin (Invitrogen). The expression of FGF23[R179Q] was confirmed by Western blotting using anti-c-Myc antibody (1:500; Santa Cruz Biotechnology, Inc.). Serum-free 48-h conditioned medium (CM) was harvested from CHO-FGF23[R179Q] as well as parental CHO-K1 cells. After

removal of the cell debris by centrifugation, the supernatant was subjected to ultrafiltration using Centricut (Kurabo, Osaka, Japan) until a 10-fold concentration was achieved. The concentration of FGF23 in the CM was determined with a FGF23 ELISA kit (Kainos Laboratories, Inc., Tokyo, Japan).

INHIBITORS

The MEK inhibitor, PD98059, and the inhibitor of Na^+/Pi cotransporters, PFA, were purchased from Sigma–Aldrich. When these inhibitors were used in the experiments, they were added to the medium 30 min before the addition of sodium phosphate buffer.

WESTERN BLOTTING

Total cell lysate was harvested in RIPA buffer [1% Triton X-100, 1% Na deoxycholate, 0.1% SDS, 150 mM NaCl, 10 mM Tris–HCl (pH 7.4), and 5 mM EDTA] containing 1 mM orthovanadate, 1 mM NaF, and a protease inhibitor cocktail (Complete™; Roche Diagnostics). Ten micrograms of protein was subjected to SDS–PAGE, and transferred to a PVDF membrane (Bio-Rad Laboratories, Hercules, CA). Blocking One-P reagent (Nacalai Tesque, Kyoto, Japan) was used for blocking. The membranes were incubated with the following primary antibody; anti-phospho-p44/42 MAP kinase (Thr202/Tyr204) antibody (1:1,000; Cell Signaling Technology, Inc., Beverly, MA), anti-p44/42 MAP kinase antibody (1:1,000; Cell Signaling Technology, Inc.), anti-phospho-c-Raf (Ser338) antibody (1:1,000; Cell Signaling Technology, Inc.), anti-c-Raf antibody (1:1,000; Cell Signaling Technology, Inc.), anti-phospho-Akt (Ser473) antibody (1:1,000; Cell Signaling Technology, Inc.), anti-Akt antibody (1:1,000; Cell Signaling Technology, Inc.), anti-phospho-p38 MAP kinase (Thr180/Tyr182) antibody (1:1,000; Cell Signaling Technology, Inc.), anti-p38 MAP kinase antibody (1:1,000; Cell Signaling Technology, Inc.), anti-phospho-SAPK/JNK (Thr183/Tyr185) antibody (1:1,000; Cell Signaling Technology, Inc.), anti-SAPK/JNK antibody (1:1,000; Cell Signaling Technology, Inc.), anti-phospho-FRS2α (Tyr196) antibody (1:1,000; Cell Signaling Technology, Inc.), anti-phospho-FGFR (Tyr653/654) antibody (1:1,000; Cell Signaling Technology, Inc.), anti-FRS2α antibody (1:1,000; Santa Cruz Biotechnology, Inc.), anti-FGFR1 (Flg; C-15) antibody (1:2,000; Santa Cruz Biotechnology, Inc.), or anti-Klotho antibody (1:1,000; Santa Cruz Biotechnology, Inc.). After incubation with the corresponding secondary antibody, signals were visualized with the enhanced chemiluminescence system (GE Healthcare, Buckinghamshire, UK). In some experiments, densitometry was performed using NIH Image 1.63 software to evaluate the intensity of the signals.

REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR) AND REAL-TIME PCR

Total RNA was isolated using TRIzol® Reagent (Invitrogen). Total RNA (2.5 μg) treated with DNase (Qiagen, Inc., Valencia, CA) was reverse transcribed using random hexamer (Promega Corporation) and Superscript II reverse transcriptase (Invitrogen). PCR was performed using rTaq polymerase (Takara Bio, Inc.) and the specific primer sets summarized in the Supplemental Table. Amplification of the expected fragments was confirmed by sequencing of the products. For real-time PCR, we utilized TaqMan® Gene Expression Assays with a 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA). The ID numbers for the assays are; Hs99999901_s1 for *18S ribosomal RNA*, Hs00152928_m1 for *EGR1*, Hs00193673_m1 for *PiT-1/SLC20A1*, Hs00198840_m1 for *PiT-2/SLC20A2*, Hs00161828_m1 for *NPTIIa/SLC34A1*, Hs02341449_m1 for *NPTIIc/SLC34A3*, and Hs00183100_m1 for *klotho*. To generate a standard curve for real-time PCR, the amplicons of interest were first cloned into the pT7-Blue vector (Merck Chemicals Ltd, Darmstadt, Germany) and serial 10-fold dilutions of the constructed plasmid were included in the assay. Samples were analyzed in triplicate. The copy number of the target cDNA in each sample was estimated by referring to the standard curve, which was standardized to that of *18S rRNA* in each sample.

GENE SILENCING

A reverse transfection method was used for gene silencing, in which the cells were transfected as they adhered to culture plates after trypsinization. The siPORT™ Amine transfection agent (Applied Biosystems) and 50 nM Silencer® Select Pre-designed siRNA (Applied Biosystems) were diluted in Opti-MEM I Reduced-Serum Medium (Invitrogen) and mixed with HEK293 cells. The siRNA ID numbers are; s13087 and s13088 for *PiT-1*, s5165 and 1216 for *FGFR1*, and s5173 and s5174 for *FGFR2*. A negative control siRNA with a scrambled sequence was also included in the experiments. For the cotransfection of the expression plasmid and siRNAs, the plasmid was diluted in Opti-MEM I together with siRNA.

PI UPTAKE ASSAY

Pi uptake was assayed following the method described by Jonsson et al. [2001] with some modifications. The cells were washed with uptake buffer (150 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 5 mM glucose, and 10 mM cyclohexylaminopropane sulfonic acid; pH 9.5) and incubated with uptake buffer containing 5×10^5 cpm of ^{32}P-orthophosphate and 0.2 mM KH_2PO_4 for 10 min at 37°C. After the radioactive buffer was removed, the Pi uptake was stopped by adding ice-cold stop solution (5 mM sodium arsenate and 150 mM choline chloride; pH 7.4). The cells were lysed in 0.5 M NaOH and the radioactivity was measured with a liquid scintillation counter in the Cherenkov P mode. Radioactivity (cpm) levels were corrected based on the amount of protein in the total cell lysate harvested from the cells with the same transfection.

STATISTICAL ANALYSIS

Data were analyzed using the one-way analysis of variance (ANOVA). The methods of Tukey or Student–Newman–Keuls were used as post hoc tests.

RESULTS

RESPONSIVENESS OF HEK293 CELLS TO A PROTEOLYSIS-RESISTANT FGF23[R179Q] MUTANT

First, we examined the responsiveness of parental HEK293 cells to FGF23, utilizing the CM obtained from CHO cells stably expressing the gain-of-function mutant FGF23[R179Q]. FGF23[R179Q] is known to be resistant to proteolysis and is widely used as a tool

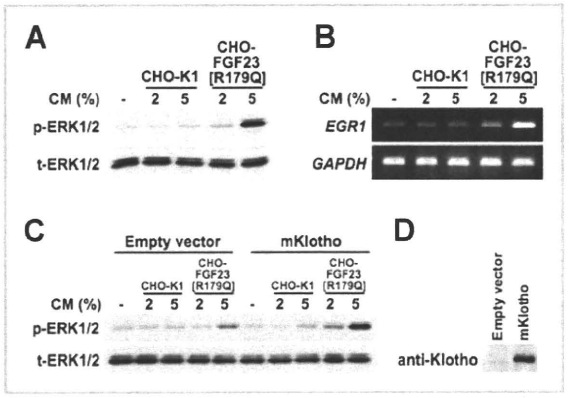

Fig. 1. Responsiveness of HEK293 cells to FGF23[R179Q]. A,B: Phosphorylation of ERK1/2 and expression of *EGR1* were induced by FGF23[R179Q]. HEK293 cells were starved in serum-free, Pi-free medium for 3 h, and treated with concentrated CM from CHO-FGF23[R179Q] or parental CHO-K1 cells for 30 min. The addition of the CM from CHO-FGF23[R179Q] cells at final concentrations of 2% or 5% provided 30 and 75 pg/ml of FGF23, respectively. After the 30-min treatment, cells were harvested for Western blotting using antibodies against phosphorylated or total ERK1/2 (A), or RT-PCR analyses for *EGR1* and *GAPDH* (B). C: Effects of exogenous expression of Klotho on the phosphorylation of ERK1/2 induced by FGF23[R179Q]. The expression plasmid mKlotho-EGFP encoding mKlotho tagged with EGFP at the carboxy-terminus or the corresponding empty vector was introduced into HEK293 cells. Forty-eight hours after the transfection, the cells were starved in serum-free, Pi-free medium for 3 h, and then treated with CM from CHO-FGF23[R179Q] or CHO-K1 cells for 30 min, before whole cell lysate was harvested for Western blotting. D: Confirmation of the exogenous expression of Klotho. The cell lysates used in (C) were subjected to Western blotting with antibody against Klotho.

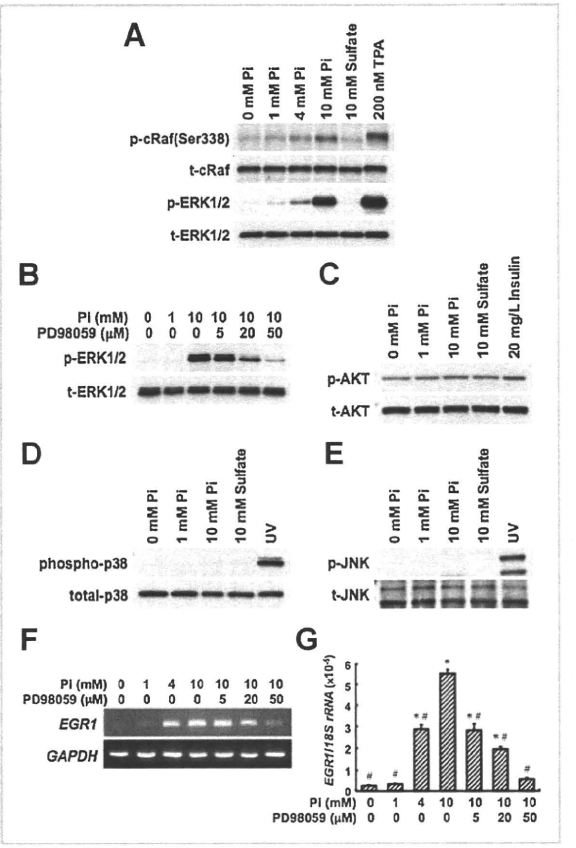

Fig. 2. Increased extracellular Pi induced activation of the Raf/MEK/ERK pathway. A: Phosphorylation of c-Raf and ERK1/2 was induced by an increase in extracellular Pi. HEK293 cells were starved in serum- and Pi-free medium for 3 h, and then treated with the indicated concentrations of Pi for 15 min. Sulfate was used as a negative control, while 12-*o*-tetradecanoylphorbol-13-acetate (TPA) was utilized as a positive control. Pi and sulfate were added to the medium as sodium salt buffer solutions. After the treatment, whole cell lysate was harvested and subjected to Western blotting with the antibodies against the indicated molecules. B: The MEK inhibitor PD98059 abolished the phosphorylation of ERK1/2 induced by the increase in extracellular Pi. HEK293 cells were starved in serum-free, Pi-free medium for 3 h, and then pre-treated with the indicated concentrations of PD98059 or vehicle for 30 min, after which the denoted concentrations of extracellular Pi were added. After incubation for another 15 min, whole cell lysate was harvested for Western blotting. Increased extracellular Pi did not induce the phosphorylation of AKT (C), p38MAPK (D), or JNK (E). HEK293 cells were starved in serum-free, Pi-free medium for 3 h, and treated with the indicated concentrations of Pi for 15 min before total cell lysate was harvested for Western blotting using the antibodies indicated. As a negative control, 10 mM sulfate was used. As a positive control, lysate from the cells treated with 20 mg/L insulin (C) or exposed to ultraviolet light (D,E) was utilized. F,G: Treatment with PD98059 abolished the induction of *EGR1* expression by the extracellular Pi. HEK293 cells were starved in serum-free, Pi-free medium for 3 h, and then treated with PD98059 or vehicle for 30 min, followed by the indicated concentrations of Pi. After incubation for another 30 min, total RNA was extracted for RT-PCR (F) or real-time PCR (G) analyses. In the real-time PCR analyses (G), the expression levels of *EGR1* were standardized based on those of *18S rRNA*. The data are expressed as the mean ± SD (n = 3). $^{*}P < 0.0001$ versus 0 mM Pi; $^{\#}P < 0.0001$ versus 10 mM Pi without PD98059. The experiments were performed three times, and similar results were obtained.

to examine FGF23 signaling [Saito et al., 2003]. The concentration of FGF23 in the CM was determined by ELISA. As a negative control, we used CM from CHO-K1 parental cells. Addition of FGF23[R179Q] to the HEK293 cells increased the phosphorylation of ERK1/2 and induced the expression of *EGR1*, a target of FGF23, in a dose-dependent manner (Fig. 1A,B). These results confirmed the responsiveness of these cells to FGF23. Real-time PCR detected weak expression of *klotho* in HEK293 cells (Fig. 5C). The overexpression of Klotho enhanced the phosphorylation of ERK1/2 induced by FGF23[R179Q] (Fig. 1C,D), which was consistent with previous reports [Kurosu et al., 2006; Urakawa et al., 2006].

EXTRACELLULAR Pi ACTIVATED THE Raf/MEK/ERK PATHWAY AND INDUCED *EGR1* EXPRESSION IN HEK293 CELLS

As an increase in extracellular Pi was reported to lead to the phosphorylation of ERK1/2 in certain cell types including osteoblasts [Beck and Knecht, 2003], we examined its effects in HEK293 cells. After 3 h of starvation in serum-free, Pi-free medium, cells were incubated with various concentrations of Pi. The increase in extracellular Pi caused an increase in the phosphorylation of ERK1/2 in a dose-dependent manner (Fig. 2A). In addition, c-Raf, located upstream of ERK1/2, was phosphorylated at Ser338 in response to Pi (Fig. 2A). Treatment with a MEK inhibitor, PD98059, abolished the phosphorylation of ERK1/2 induced by 10 mM Pi, confirming the involvement of MEK (Fig. 2B). These results indicate

that extracellular Pi activates the Raf/MEK/ERK pathway in HEK293 cells. Next, the effects of Pi on the phosphorylation of AKT, p38MAPK, and JNK were examined. The level of phosphorylated AKT was not altered by the increase in Pi (Fig. 2C), and p38MAPK and JNK were not phosphorylated, either (Fig. 2D,E). These results suggest that the extracellular Pi relatively specifically activates the Raf/MEK/ERK pathway.

Then the effect of extracellular Pi on the expression of *EGR1* was examined by RT-PCR and real-time PCR. The expression was up-regulated by the increase in Pi, an effect abolished by treatment with the MEK inhibitor PD98059 in a dose-dependent manner, indicating that extracellular Pi regulates the expression of *EGR1* through the MEK/ERK pathway (Fig. 2F,G).

COMMONALITY AND POSSIBLE INTERACTION BETWEEN THE SIGNALS INDUCED BY EXTRACELLULAR Pi AND FGF23[R179Q]

As described above, treatment with either FGF23[R179Q] or extracellular Pi induced the phosphorylation of ERK1/2 and expression of *EGR1* in HEK293 cells. Therefore, we examined the time course of ERK1/2 phosphorylation, using the parental HEK293 cells and HEK293-Klotho cells stably overexpressing Klotho. In the parental HEK293 cells, 4–10 mM Pi induced the phosphorylation of ERK1/2 within 15 min (Fig. 3A). Treatment with FGF23[R179Q] (75 pg/ml at final concentration) also induced phosphorylation within 15 min, with a maximum at 30 min. Thus, the time course of the phosphorylation of ERK1/2 induced by extracellular Pi was similar to that induced by FGF23[R179Q] in the HEK293 cells (Fig. 3A). Overexpression of Klotho in HEK293-Klotho cells did not alter the responsiveness to extracellular Pi, suggesting that the level of Klotho does not influence the responsiveness of the cells to extracellular Pi. As to the responsiveness to FGF23, addition of FGF23[R179Q] at the same concentration induced a prolonged phosphorylation of ERK1/2 in HEK293-Klotho cells compared with that in the parental cells (Fig. 3A). The stable overexpression of Klotho in HEK293-Klotho was confirmed by Western blotting (data not shown).

Since our data suggested that the signaling triggered by the increase in Pi and the FGF23 signaling share the same downstream cascade, we studied whether the extracellular Pi itself influences the responsiveness of cells to FGF23 by examining the effects of simultaneous treatment with Pi and FGF23[R179Q] on the phosphorylation of ERK1/2 and expression of *EGR1*. In HEK293 cells, the combined effects on the phosphorylation of ERK1/2 and the expression of *EGR1* seemed to be additive (Fig. 3B,C). Similar results were obtained in HEK293-Klotho cells which were more sensitive to FGF23 (data not shown).

Next, to investigate whether a Na^+/Pi cotransporter is involved in the responsiveness of HEK293 cells to extracellular Pi, we examined the effect of phosphonoformic acid (PFA), an inhibitor of Na^+/Pi cotransporters. Addition of PFA abolished the phosphorylation of ERK1/2 induced by the extracellular Pi (Fig. 3D). The induction of *EGR1* expression by the increase in Pi was also abrogated by treatment with PFA (Fig. 3D), indicating the involvement of Na^+/Pi cotransporters in the responsiveness of HEK293 cells to extracellular Pi. Interestingly, treatment with PFA canceled the phosphorylation of ERK1/2 and the up-regulation of *EGR1* induced by FGF23[R179Q] as well (Fig. 3E). These results suggest that the signaling triggered by extracellular Pi might interact with and modulate the FGF23 signaling.

INVOLVEMENT OF THE TYPE III Na^+/Pi COTRANSPORTER PiT-1 IN THE RESPONSIVENESS OF HEK293 CELLS TO EXTRACELLULAR Pi

Then, we performed real-time PCR analyses to determine the expression levels of various Na^+/Pi cotransporters. It was found that *PiT-1*, a type III Na^+/Pi cotransporter, was predominantly expressed in HEK293 cells. *PiT-2* was also expressed, but to a lesser extent. The expression of *NPTIIa* and *NPTIIc* was marginal (Fig. 4A). As to the expression of *NPTIIb*, it was not detected by RT-PCR (data not shown). The increase in extracellular Pi did not alter the expression of *PiT-1* after either 30 min or 24 h (Supplemental Fig. 1), although we cannot exclude the possibility that longer treatment with extracellular Pi might have an effect.

We further investigated the involvement of PiT-1 in the responsiveness of HEK293 cells to extracellular Pi by knocking down its gene. RT-PCR analyses confirmed the specific knockdown of *PiT-1* by the transfection of corresponding gene-specific siRNAs (Fig. 4B). In addition, an assay using ^{32}P-labeled orthophosphate demonstrated a decrease in the uptake of Pi in the cells transfected with *PiT-1*-specific siRNAs (Fig. 4C). Interestingly, the phosphorylation of ERK1/2 induced by increased extracellular Pi was diminished by knockdown of the expression of *PiT-1* (Fig. 4D–G), indicating that PiT-1 plays a critical role in the responsiveness of HEK293 to extracellular Pi.

EXTRACELLULAR Pi CAUSED THE PHOSPHORYLATION OF FGF RECEPTOR SUBSTRATE 2α (FRS2α) IN A Na^+/Pi COTRANSPORTER-DEPENDENT MANNER

To elucidate the point of convergence between the signaling induced by the extracellular Pi and the signaling by FGF23, we next examined the effects of Pi on the phosphorylation of FRS2α, which functions upstream of ERK1/2 in the signaling by FGFs, including FGF23. As expected, the phosphorylation of FRS2α was induced by the addition of FGF23[R179Q]. More interestingly, the increase in Pi also led to the phosphorylation of FRS2α, an effect abolished by treatment with PFA (Fig. 5A). Then, we analyzed the levels of *FGFRs* in HEK293 cells by RT-PCR and detected the endogenous expression of *FGFR1* or *FGFR2* (Fig. 5B). A 30-min treatment with Pi did not alter the expression of *FGFR1* or *FGFR2*, while it up-regulated that of *EGR1* (Fig. 5B). We also determined the expression of 22 kinds of *FGFs* by RT-PCR (Supplemental Fig. 2). Although some of them were endogenously expressed, the extracellular Pi did not cause any obvious change in their expression. The expression of *klotho*, which was detected by real-time PCR, was not altered by the 30-min treatment with Pi, either (Fig. 5C). These results indicate that the extracellular Pi induced the phosphorylation of FRS2α without altering the expression of *FGFs*, *FGFR*, and *klotho*.

FGFR1 MEDIATES THE SIGNAL TRANSDUCTION TRIGGERED BY AN INCREASE IN EXTRACELLULAR Pi DOWNSTREAM THE Na^+/Pi COTRANSPORTER PiT-1

Finally, we investigated whether FGFR1 or FGFR2 actually mediate the phosphorylation of FRS2α induced by extracellular Pi by

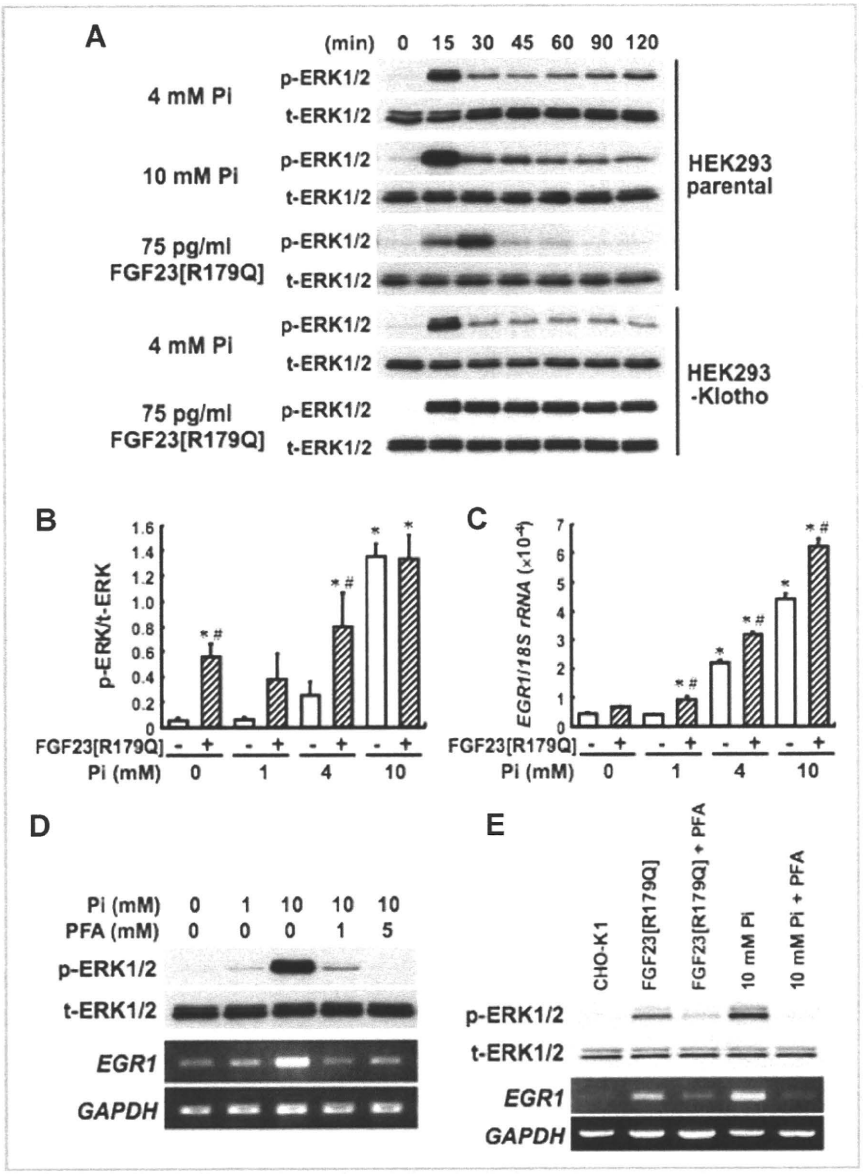

Fig. 3. Commonality and possible interaction between the signals induced by extracellular Pi and FGF23[R179Q]. A: Time-course study of the effects of increased extracellular Pi and FGF23[R179Q] on phosphorylation of ERK1/2. After 3 h of starvation in serum-free, Pi-free medium, HEK293 and HEK293-Klotho cells were treated with 4 or 10 mM of Pi or 75 pg/ml of FGF23[R179Q] for the period indicated before whole cell lysate was harvested for Western blotting. B: Effects of simultaneous treatment with FGF23[R179Q] and extracellular Pi on ERK1/2 phosphorylation in HEK293 cells. After 3 h of starvation, HEK293 cells were treated with the indicated concentrations of extracellular Pi with (hatched columns) or without (open columns) 30 pg/ml of FGF23[R179Q] for 15 min. Cell lysate was subjected to Western blotting using antibodies against phosphorylated or total ERK1/2. The densitometric ratio of phosphorylated ERK1/2 to total ERK1/2 in three experiments is depicted. The data are shown as the mean ± SD (n = 3). $^{*}P < 0.05$ versus the cells incubated with 0 mM Pi in the absence of FGF23[R179Q]. $^{\#}P < 0.05$ versus the cells incubated in the same concentration of Pi in the absence of FGF23[R179Q]. C: Effects of simultaneous treatment with FGF23[R179Q] and extracellular Pi on *EGR1* expression. Parental HEK293 cells were starved in serum-free, Pi-free medium for 3 h, and treated with the indicated concentrations of extracellular Pi with (hatched columns) or without (open columns) 50 pg/ml FGF23[R179Q] for 30 min. Real-time PCR was performed, and the expression levels of *EGR1* were standardized based on those of *18S rRNA*. The data are expressed as the mean ± SD (n = 3). $^{*}P < 0.01$ versus the cells incubated with 0 mM Pi in the absence of FGF23[R179Q]. $^{\#}P < 0.01$ versus the cells incubated in the same concentration of Pi in the absence of FGF23[R179Q]. D: Treatment with PFA, an inhibitor of Na^{+}/Pi cotransporters, abolished the phosphorylation of ERK1/2 and the expression of *EGR1* induced by the increase in extracellular Pi. After 3 h of starvation in serum-free, Pi-free medium, HEK293 cells were pre-treated with the indicated concentrations of PFA or vehicle for 30 min, and then treated with the indicated concentrations of Pi. The cells were incubated for another 15 min before total cell lysate was harvested for Western blotting using antibodies against phosphorylated or total ERK1/2. Total RNA was extracted after another 30-min incubation followed by RT-PCR analyses for *EGR1* and *GAPDH*. E: PFA abolished the phosphorylation of ERK1/2 and expression of *EGR1* induced by FGF23[R179Q]. After 3 h of starvation in serum-free medium, HEK293 cells were pre-treated with 5 mM PFA or vehicle for 30 min, and then treated with 5% CHO-K1 or FGF23[R179Q] CM (final concentration 75 pg/ml of FGF23) or 10 mM Pi. The cells were incubated for another 15 min before total cell lysate was harvested for Western blotting. Total RNA was extracted after another 30-min incubation.

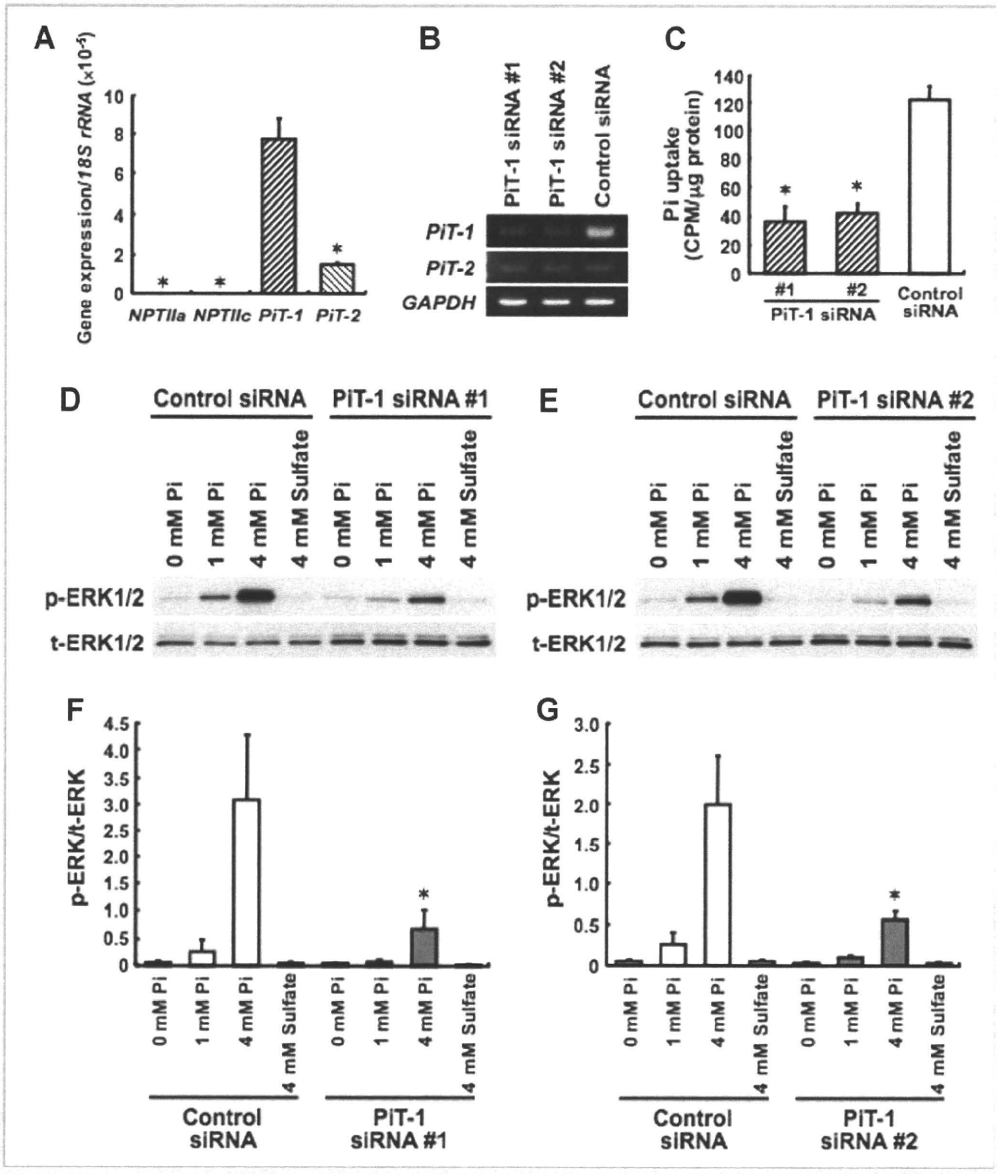

Fig. 4. Type III Na^+/Pi cotransporter PiT-1 was involved in the responsiveness to extracellular Pi in HEK293 cells. A: Expression of Na^+/Pi cotransporters in HEK293 cells. Total RNA extracted from HEK293 cells was subjected to real-time PCR for type II Na^+/Pi cotransporters, *NPTIIa* and *NPTIIc*, and type III Na^+/Pi cotransporters, *PiT-1* and *PiT-2*. The calculated copy number of amplicons was standardized based on that of *18S rRNA*. The data are expressed as the mean ± SD (n = 3). $^*P < 0.0001$ versus *PiT-1*. Experiments were performed three times, and similar results were obtained. B: Confirmation of the specific knockdown of the expression of *PiT-1* by transfection of gene-specific siRNAs. HEK293 cells were transfected with *PiT-1*-specific siRNA or a negative control siRNA (50 nM each). To knockdown the expression of *PiT-1*, two siRNAs were used, designated as #1 and #2. Seventy-two hours after the transfection, total RNA was extracted and subjected to RT-PCR for *PiT-1*, *PiT-2*, and *GAPDH*. C: Effects of knockdown of *PiT-1* on Pi uptake. HEK293 cells were transfected with *PiT-1*-specific siRNAs #1 and #2, or negative control siRNA, and 72 h later, a Pi uptake assay was performed using ^{32}P-labeled orthophosphate. The radioactivity was standardized based on the protein content of the cells with the same transfection. The data are expressed as the mean ± SD (n = 4). $^*P < 0.01$ versus control siRNA. D–G: Western blot analyses to examine the effects of knockdown of *PiT-1* expression on the phosphorylation of ERK1/2 induced by the increase in extracellular Pi. HEK293 cells were transfected with *PiT-1*-specific siRNAs #1 (in D and F) and #2 (in E and G), or a negative control siRNA (50 nM each). Seventy-two hours later, the medium was changed to serum-free, Pi-free medium to starve the cells. Following 3 h of starvation, the cells were treated with the indicated concentrations of extracellular Pi for 15 min before cell lysate was harvested for Western blotting. Each experiment was performed three times, and densitometry was carried out to evaluate the ratio of the intensity of the signals corresponding to phosphorylated ERK1/2 to that of total ERK1/2 (F,G). The data are shown as the mean ± SD (n = 3). $^*P < 0.01$ versus the cells transfected with control siRNA and treated with 4 mM Pi.

knocking down the expression of their genes. Transfection of *FGFR1*-specific or *FGFR2*-specific siRNA specifically silenced the expression of the corresponding gene (Fig. 6A). Neither transfection influenced the expression of *PiT-1* or *PiT-2*, or Pi uptake (Fig. 6A,B). However, silencing of the expression of *FGFR1* diminished the phosphorylation of both FRS2α and ERK1/2 induced by Pi (Fig. 6C–F). Silencing of the expression of *FGFR2* had similar but less extensive effects (Fig. 6G,H). In densitometry, the knockdown of

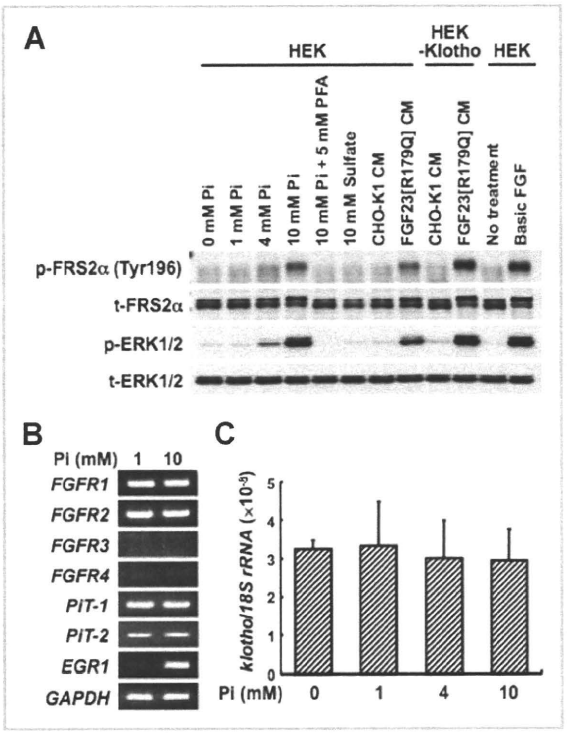

Fig. 5. Effects of the increase in extracellular Pi and FGF23[R179Q] on phosphorylation of FRS2α. A: The increase in extracellular Pi induced the phosphorylation of FRS2α. HEK293 cells (HEK) and HEK293-Klotho cells (HEK-Klotho) were starved in serum-free, Pi-free medium for 3 h, and then treated with the indicated stimulants for 15 min. FGF23[R179Q] was added at a final concentration of 75 pg/ml. PFA, an inhibitor of Na^+/Pi cotransporters, was added to the cells 30 min before the treatment with extracellular Pi. As a positive control, 100 ng/ml of basic FGF was used. Cell lysate was harvested and subjected to Western blotting with the antibodies against the indicated molecules. B: Effects of increased extracellular Pi on the expression of FGFRs and type III Na^+/Pi cotransporters in HEK293 cells. HEK293 cells were starved in serum-free, Pi-free medium for 3 h, and treated with 1 or 10 mM extracellular Pi for 30 min. Total RNA was extracted and subjected to RT-PCR for *FGFR1*, *FGFR2*, *FGFR3*, *FGFR4*, *PiT-1*, *PiT-2*, and *GAPDH*. The expression of *EGR1* was also examined to confirm its stimulation by the 30-min treatment with extracellular Pi. C: The expression of *klotho* was not affected by the extracellular Pi. HEK293 cells were starved in serum-free, Pi-free medium for 3 h, and treated with the indicated concentrations of Pi for 30 min before total RNA was extracted. The expression levels of *klotho* were standardized based on those of *18S rRNA*. The data are expressed as the mean ± SD (n = 3). There were no significant differences in the levels of *klotho*.

FGFR2 expression with #1 and #2 siRNAs, respectively, resulted in 53% and 46% decrease in the p-ERK/t-ERK in the cells treated with 4 mM Pi, although the effects were not statistically significant (data not shown).

Moreover, we examined whether overexpression of FGFR1 rescued the decrease in the responsiveness to extracellular Pi in the cells where the expression of *PiT-1* was knocked down. The expression plasmid encoding FGFR1 or the corresponding empty vector was introduced to HEK293 cells together with *PiT-1*-specific siRNAs or control siRNA, and their effects on the phosphorylation of ERK1/2 induced by an increase in extracellular Pi were determined (Fig. 7). The antibodies against FGFR phosphorylated at Tyr653/654 and total FGFR1 failed to detect the endogenous levels of these molecules in HEK293 cells, whereas the overexpressed levels were detectable. Transfection of *PiT-1*-specific siRNAs reduced the phosphorylation of ERK1/2 induced by an increase in extracellular Pi, which were completely rescued by the overexpression of FGFR1 (Fig. 7). Interestingly, even when the expression of *PiT-1* was reduced by siRNAs, an increase in extracellular Pi facilitated the phosphorylation of the overexpressed FGFR1.

DISCUSSION

Here we have provided evidence that the signaling triggered by extracellular Pi shares the same downstream cascade as FGF23 signaling, utilizing the HEK293 human embryonic kidney cell line. HEK293 is useful for investigating the signaling by FGF23, and previous studies demonstrated that treatment with FGF23 resulted in the phosphorylation of ERK1/2 and expression of *EGR1* when the Klotho protein was introduced into the cells [Kurosu et al., 2006; Urakawa et al., 2006]. In the current study, we used a proteolysis-resistant mutant, FGF23[R179Q], and found that it induced phosphorylation of ERK1/2 and expression of *EGR1* in HEK293 cells, both of which were augmented by the overexpression of Klotho, consistent with previous reports (Fig. 1) [Kurosu et al., 2006; Urakawa et al., 2006]. Since treatment with FGF23[R179Q]-CM resulted in a slight increase in the phosphorylation of ERK1/2 in CHO cells as well (data not shown), we cannot exclude completely the possibility that the effects of medium from the FGF23[R179Q]-expressing CHO cells could be due to a CHO-derived factor that is induced by FGF23. However, since the response of CHO cells was subtle and the overexpression of Klotho markedly increased the responsiveness of HEK293 cells to FGF23[R179Q]-CM, we assumed that the effects observed in HEK293 were exerted by FGF23[R179Q] itself. The responsiveness of parental HEK293 cells to FGF23[R179Q] despite their weak expression of *klotho* suggests cells with low levels of Klotho protein to be a target of FGF23 signaling in pathological conditions where FGF23 levels are extremely high, including CKD.

Interestingly, the increase in extracellular Pi resulted in activation of the Raf/MEK/ERK pathway and induction of the expression of *EGR1* in HEK293 cells (Fig. 2). Several studies have demonstrated that Pi itself functions as a signaling molecule and regulates gene expression in certain cell types including osteoblasts [Beck et al., 2000, 2003]. Moreover, an increase in extracellular Pi induced the calcification of vascular tissue by triggering the expression of osteoblast-specific genes [Jono et al., 2000; Li et al., 2006; Mizobuchi et al., 2006]. Our findings suggest that the responsiveness to Pi is retained in various tissues including the target cells of FGF23 signaling. In HEK293 cells, the increase in Pi induced activation of the Raf/MEK/ERK pathway but had no influence on the phosphorylation of AKT, p38MAPK, or JNK (Fig. 2A–E). In osteoblastic MC3T3-E1 cells and chondrocytic ATDC5 cells, an increase in extracellular Pi induced the phosphorylation of ERK1/2 to up-regulate mineralization-related genes [Beck and Knecht, 2003; Julien et al., 2007]. These results together with ours indicate the Raf/MEK/ERK pathway to play a central role in the transduction of the signaling triggered by the extracellular Pi. The

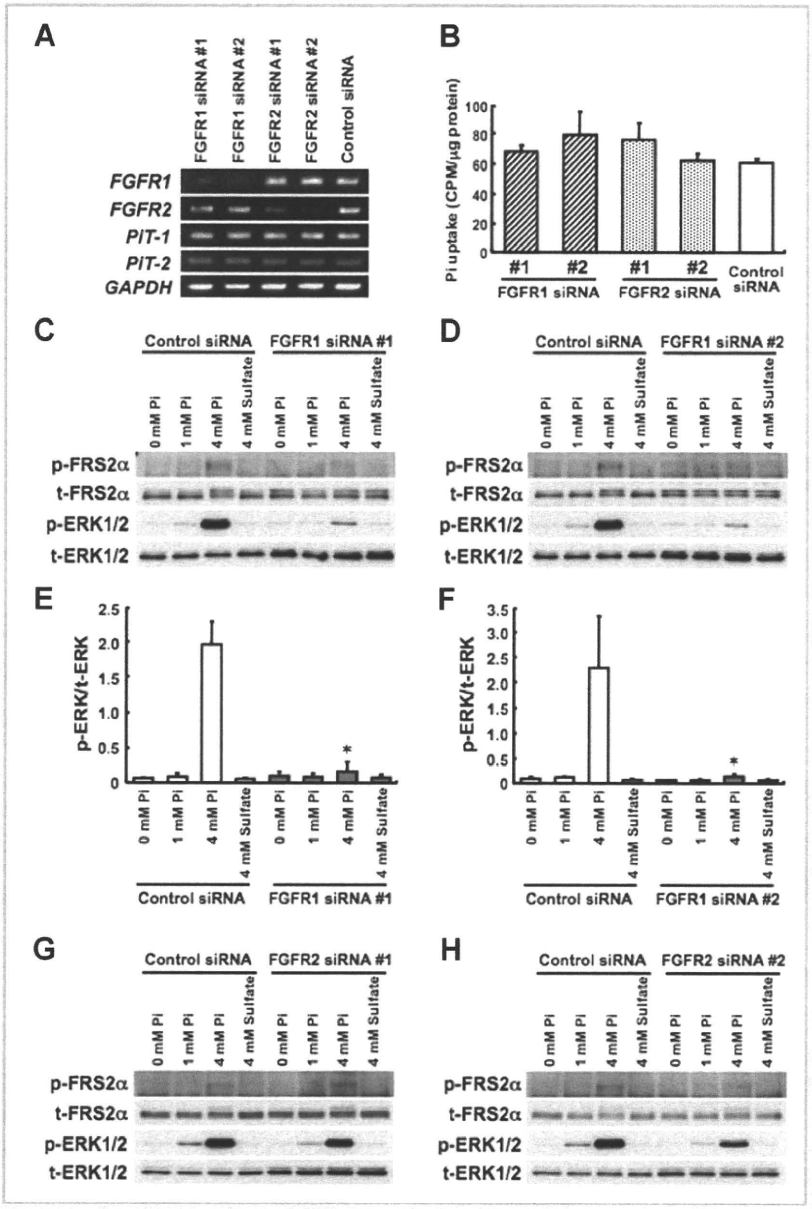

Fig. 6. Knockdown of the expression of *FGFR1* resulted in the decreased responsiveness to extracellular Pi. A: Confirmation of the specific knockdown of the expression of *FGFR1* and *FGFR2* by transfection of gene-specific siRNAs. HEK293 cells were transfected with *FGFR1*-specific siRNAs #1 and #2, *FGFR2*-specific siRNAs #1 and #2, or a negative control siRNA (50 nM each). Seventy-two hours after the transfection, total RNA was extracted and subjected to RT-PCR for *FGFR1*, *FGFR2*, *PiT-1*, *PiT-2*, and *GAPDH*. B: Knockdown of *FGFR1* or *FGFR2* had no influence on Pi uptake. HEK293 cells were transfected with *FGFR1*-specific siRNAs #1 and #2, *FGFR2*-specific siRNAs #1 and #2, or negative control siRNA. Seventy-two hours later, a Pi uptake assay was performed using ^{32}P-labeled orthophosphate. The radioactivity was standardized based on the protein content of cells with the same transfection. The data are expressed as the mean ± SD (n = 4). There were no significant differences in the five groups. C–F: FGFR1 was involved in the responsiveness of HEK293 to extracellular Pi. Western blot analyses were performed to examine the effects of *FGFR1*-specific siRNAs on the phosphorylation of FRS2α and ERK1/2 induced by extracellular Pi. HEK293 cells were transfected with *FGFR1*-specific siRNAs #1 (in C and E) and #2 (in D and F), or a negative control siRNA (50 nM each). Seventy-two hours after the transfection, the cells were starved in serum-free, Pi-free medium for 3 h, which was followed by the addition of the indicated concentration of extracellular Pi and incubation for another 15 min. Cell lysate was harvested for Western blotting with the antibodies against the indicated molecules (C,D). Each experiment was performed three times, and densitometry was carried out to evaluate the ratio of the intensity of the signals corresponding to phosphorylated ERK1/2 to that of total ERK1/2 (E,F). The data are shown as the mean ± SD (n = 3). $^{*}P < 0.0001$ versus the cells transfected with control siRNA and treated with 4 mM Pi. G,H: Effects of knockdown of *FGFR2* on phosphorylation of FRS2α and ERK1/2 induced by extracellular Pi. Experiments similar to those in (C,D) were performed by utilizing *FGFR2*-specific siRNAs #1 (in G) and #2 (in H). Cell lysate was harvested for Western blotting with the antibodies against the indicated molecules. Silencing of the expression of *FGFR2* had similar effects to the silencing of *FGFR1*, but to a lesser extent. Experiments were done three times, and similar results were obtained.

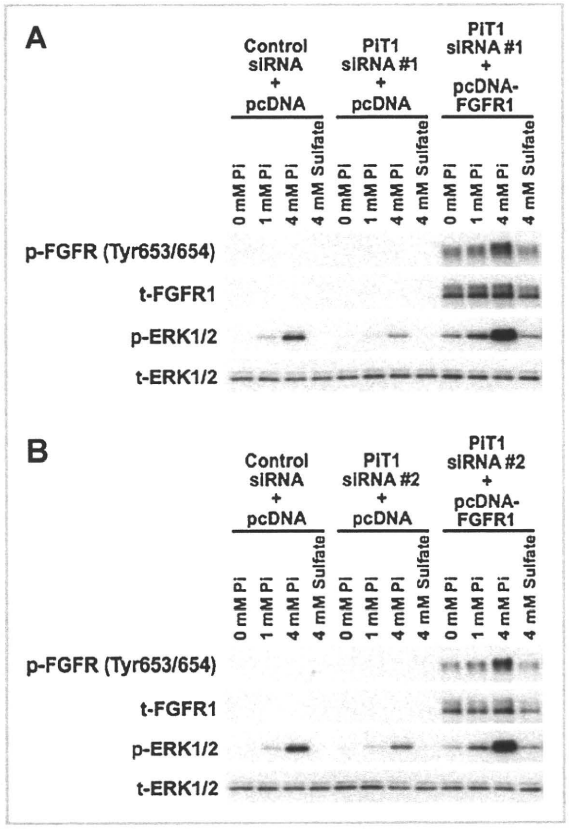

Fig. 7. Overexpression of FGFR1 rescued the decrease in the responsiveness to extracellular Pi in HEK293 cells where the expression of *PiT-1* was knocked down. A,B: HEK293 cells were transfected with *PiT-1*-specific siRNAs #1 (in A) and #2 (in B), or a negative control siRNA (50 nM each), together with pcDNA-FGFR1 (135 ng/well in 12-well culture plates) or pcDNA empty vector. Seventy-two hours later, the medium was changed to serum-free, Pi-free medium to starve the cells. Following 3 h of starvation, the cells were treated with the indicated concentrations of extracellular Pi for 15 min before cell lysate was harvested for Western blotting with the antibodies against the indicated molecules. The anti-phosphorylated FGFR (Tyr653/654) antibody detects the transfected levels of FGFRs only when phosphorylated at Tyr653/654, and the anti-total FGFR1 antibody also detects the overexpressed levels of the molecule. Although neither of these antibodies detected the endogenous levels of FGFR1 in HEK293 cells, the overexpressed levels were detectable.

target genes of the signaling triggered by extracellular Pi might differ among cell types. Since the concentrations of Pi used in the current study were above the physiological range, the relevance remains to be established.

Interestingly, the increase in Pi induced the expression of *EGR1*, a target of FGF23 signaling, in HEK293 cells (Fig. 2F,G). Moreover, the time course of the phosphorylation of ERK1/2 induced by the extracellular Pi was similar to that induced by FGF23[R179Q] (Fig. 3A). These results suggest the signaling induced by the Pi to share the same downstream cascade as the signaling evoked by FGF23. Their additive effects when combined and the abolishment of both signaling by PFA supported the idea of the interaction between these two pathways (Fig. 3B–E). Although FGF23 is a phosphaturic hormone, the feedback mechanism by which Pi controls the production of FGF23 by bone remains unknown. Our results suggest the possibility that extracellular Pi might regulate the production of FGF23 in bone through the modification of the expression of some targets of FGF23 in kidney and/or parathyroid.

The involvement of Na^+/Pi cotransporters in the signaling is an important area of study. Three classes of Na^+/Pi cotransporters have been identified in mammals [Virkki et al., 2007]. Type I is involved in the transport of organic ions [Busch et al., 1996]. Type IIa (NPTIIa) and type IIc (NPTIIc) are predominantly expressed in renal proximal tubules, while type IIb is detected in the intestine. Type III Na^+/Pi cotransporters, PiT-1 and PiT-2, are widely distributed, suggesting that they play a housekeeping role in Pi homeostasis [Kavanaugh et al., 1994; Kavanaugh and Kabat, 1996]. *PiT-1* was a major Na^+/Pi cotransporter in HEK293 cells, while the expression of *NPTIIa* and *NPTIIc* was marginal (Fig. 4A). *PiT-1*-specific siRNA reduced the phosphorylation of ERK1/2 induced by the increase in extracellular Pi, indicating essential roles for PiT-1 in the responsiveness to Pi in HEK293 cells (Fig. 4B–G). Although PiT-1 and PiT-2 have been thought to serve as housekeeping Na^+/Pi cotransporters, PiT-2 was recently demonstrated to participate in the reabsorption of Pi in the apical membrane of rat renal proximal tubules [Villa-Bellosta et al., 2009]. In addition, Nowik et al. [2008] demonstrated Pit-1 and Pit-2 to play a compensatory role in Pi reabsorption, using *NptIIa*-knockout mice. These results suggest that type III cotransporters are more than housekeeping transporters. HEK293 cells responded to the increase in Pi despite the marginal expression of *NPTIIa* and *NPTIIc*, indicating their dispensability in the signal transduction triggered by the extracellular Pi. Since *PiT-2* was modestly expressed in HEK293 cells (Fig. 4A), it too might play a part in the responsiveness to extracellular Pi, which would explain why there was still some phosphorylation of ERK1/2 in the cells transfected with *PiT-1*-specific siRNA.

We further investigated the convergence point of the signaling triggered by extracellular Pi and by FGF23 and found that the Pi induced phosphorylation of FRS2α (Fig. 5A). Results of RT-PCR and real-time PCR analyses suggested that FRS2α phosphorylation in response to the increase in extracellular Pi was unlikely to be caused by the altered expression of *FGFs*, *FGFRs*, or *klotho* (Fig. 5B,C and Supplemental Fig. 2). Therefore, we examined the effects of knocking down the expression of FGFRs on the responsiveness of HEK293 cells to extracellular Pi. Among the *FGFRs*, *FGFR1*, and *FGFR2* were expressed in HEK293 cells (Fig. 5B). Silencing of the *FGFR1* expression markedly diminished the phosphorylation of both FRS2α and ERK1/2 induced by increased Pi, while knockdown of *FGFR2* expression exerted minor effects (Fig. 6). In addition, the overexpression of FGFR1 completely rescued the decrease in the responsiveness to extracellular Pi in the cells where the *PiT-1* expression was knocked down (Fig. 7). These results indicate that FGFR1 plays a critical role downstream of PiT-1 in the signal transduction triggered by the increase in extracellular Pi.

Although most tissues express FGFRs in a redundant manner, FGFR1 in distal tubules is suggested to be the biologically relevant receptor for FGF23 in kidney [Liu et al., 2008; Gattineni et al., 2009]. Since our results suggest that FGFR1 is involved in transduction of the signal triggered by the increase in extracellular Pi, distal tubules are also likely to be the important targets of the signaling. Hence, the signaling induced by FGF23 and the extracellular Pi might converge

in distal tubule cells, probably at FGFR1. The knocking down of *FGFR1* or *FGFR2* influenced neither the expression of type III Na^+/Pi cotransporters nor uptake of Pi (Fig. 6A,B), indicating that increased Pi uptake via PiT-1 is not responsible for the phosphorylation of FRS2α and ERK1/2 induced by increased extracellular Pi. The precise mechanism that mediates the signaling from PiT-1 to FGFR1 remains to be elucidated. Since increased extracellular Pi did not cause the alteration in the mRNA levels of FGFs, FGFRs, and Klotho and the effects of Pi were rapid and appeared to be mediated by FGFR, we are currently hypothesizing an increase of extracellular Pi might cause some modification including phosphorylation or conformational change of PiT-1 itself, which might lead to the direct or indirect interaction between PiT-1 and FGFRs. Alternatively, Pi might stimulate the release of latent endogenous FGFs from the extracellular matrix. It has been reported that rapid effects of extracellular Pi in parathyroid cells is mediated by the activation of phospholipase A_2 and the production of arachidonic acid [Almaden et al., 2000], which also might be involved in the pathway identified in the current study.

In conclusion, we have evidenced that an increase in extracellular Pi triggers signal transduction via PiT-1 and FGFR1, leading to activation of the Raf/MEK/ERK pathway. The signaling induced by extracellular Pi and by FGF23 share the same downstream cascade and interact, suggesting Pi itself to have an influence on FGF23 signaling.

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