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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*

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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

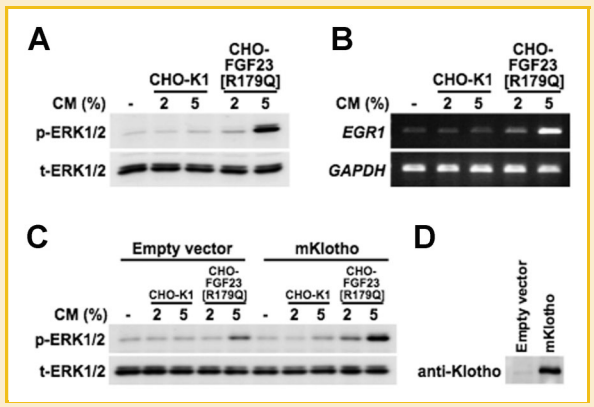

Fig. 1. Responsiveness of HEK293 cells to FGF23[R179Q]. A,B: Phosphorylation of ERK1/2 and expression of *EGR1* were induced by FGF23[R179Q]. HEK293 cells were starved in serum-free, Pi-free medium for 3 h, and treated with concentrated CM from CHO-FGF23[R179Q] or parental CHO-K1 cells for 30 min. The 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.

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

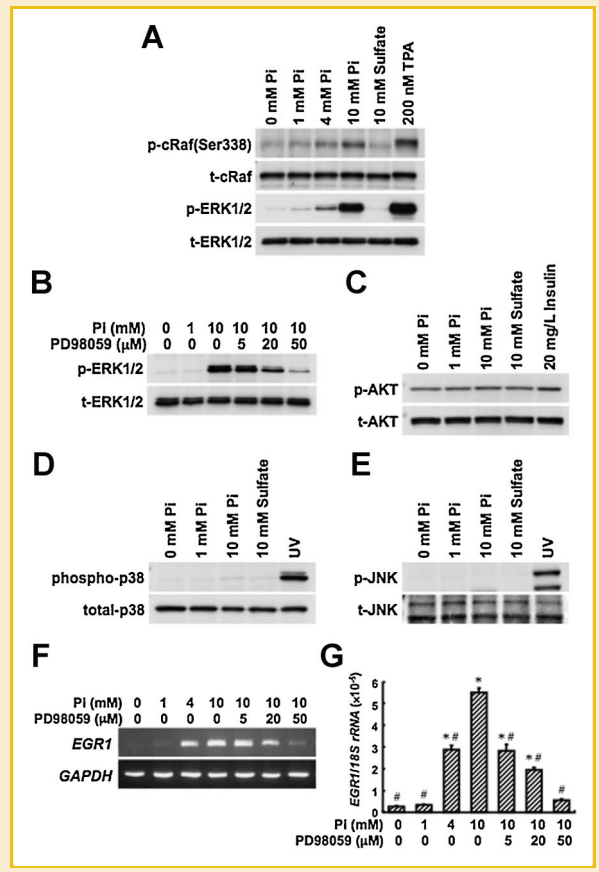

Fig. 2. Increased extracellular Pi induced activation of the Raf/MEK/ERK pathway. A: Phosphorylation of c-Raf and ERK1/2 was induced by an increase in extracellular Pi. HEK293 cells were starved in serum- and Pi-free medium for 3 h, and then treated with the indicated concentrations of Pi for 15 min. 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.

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

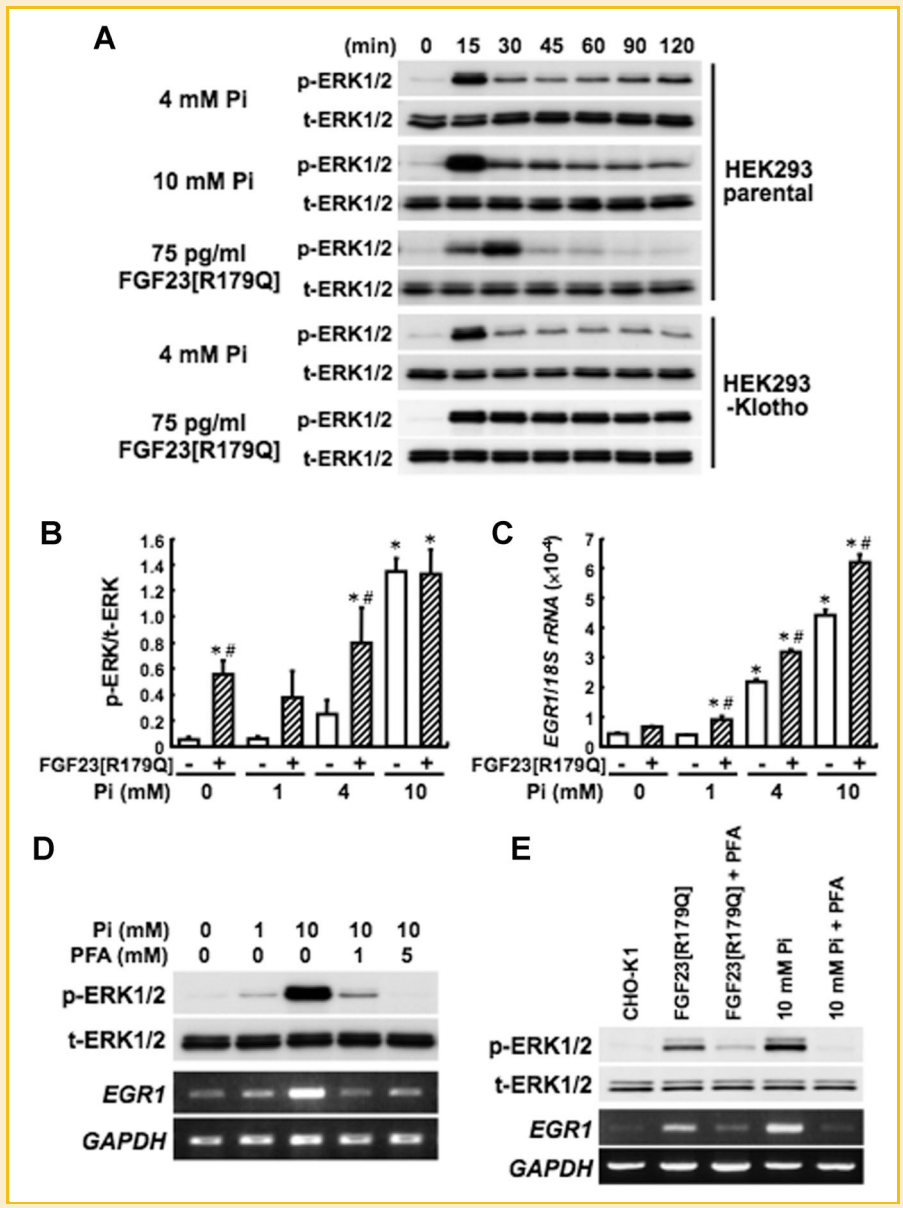

Fig. 3. Commonality and possible interaction between the signals induced by extracellular Pi and FGF23[R179Q]. A: Time-course study of the effects of increased extracellular Pi and FGF23[R179Q] on phosphorylation of ERK1/2. After 3 h of starvation in serum-free, Pi-free medium, HEK293 and HEK293-Klotho 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.

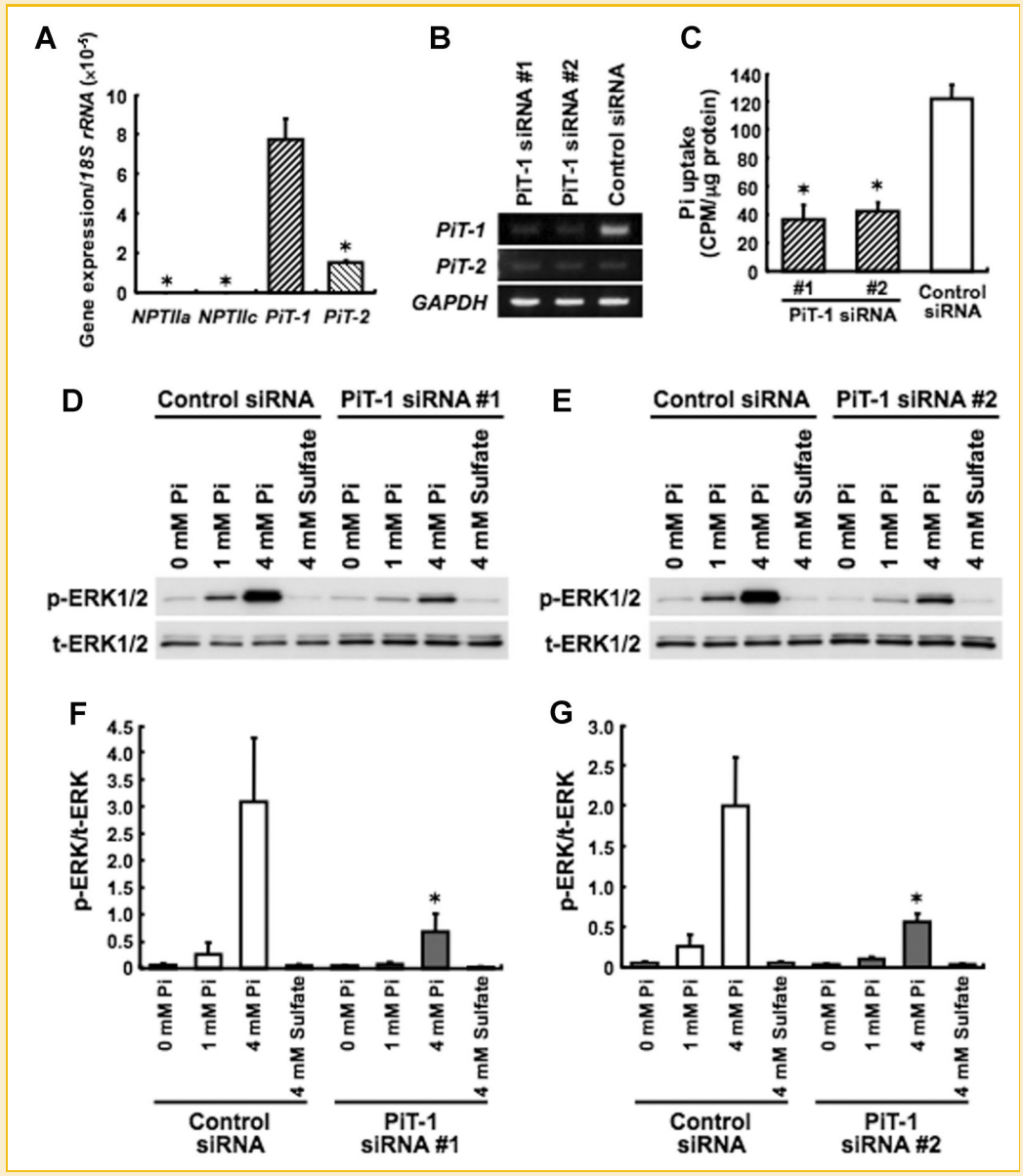

Fig. 4. Type III Na^+/Pi cotransporter PiT-1 was involved in the responsiveness to extracellular Pi in HEK293 cells. A: Expression of Na^+/Pi cotransporters in HEK293 cells. Total RNA extracted from HEK293 cells was subjected to real-time PCR for type II Na^+/Pi cotransporters, *NPTIIa* and *NPTIIc*, 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

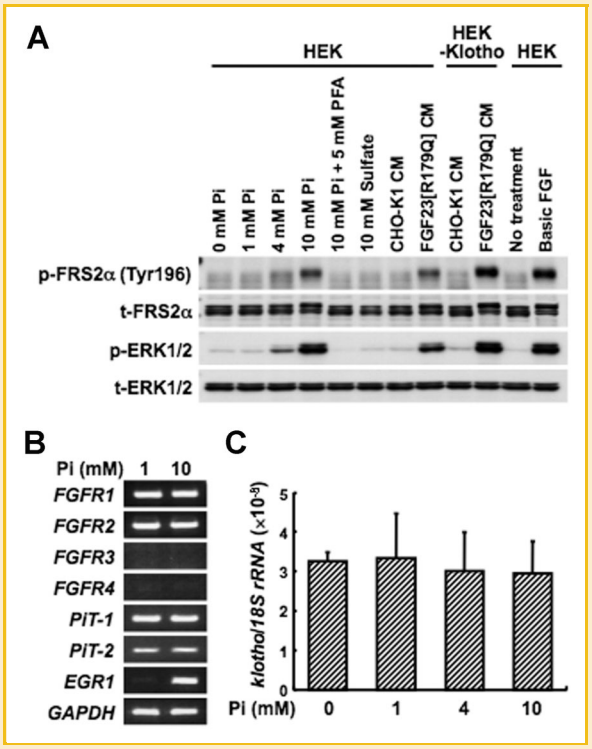

Fig. 5. Effects of the increase in extracellular Pi and FGF23[R179Q] on phosphorylation of FRS2α. A: The increase in extracellular Pi induced the phosphorylation of FRS2α. HEK293 cells (HEK) and HEK293–Klotho cells (HEK–Klotho) were starved in serum-free, Pi-free medium for 3 h, and then treated with 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

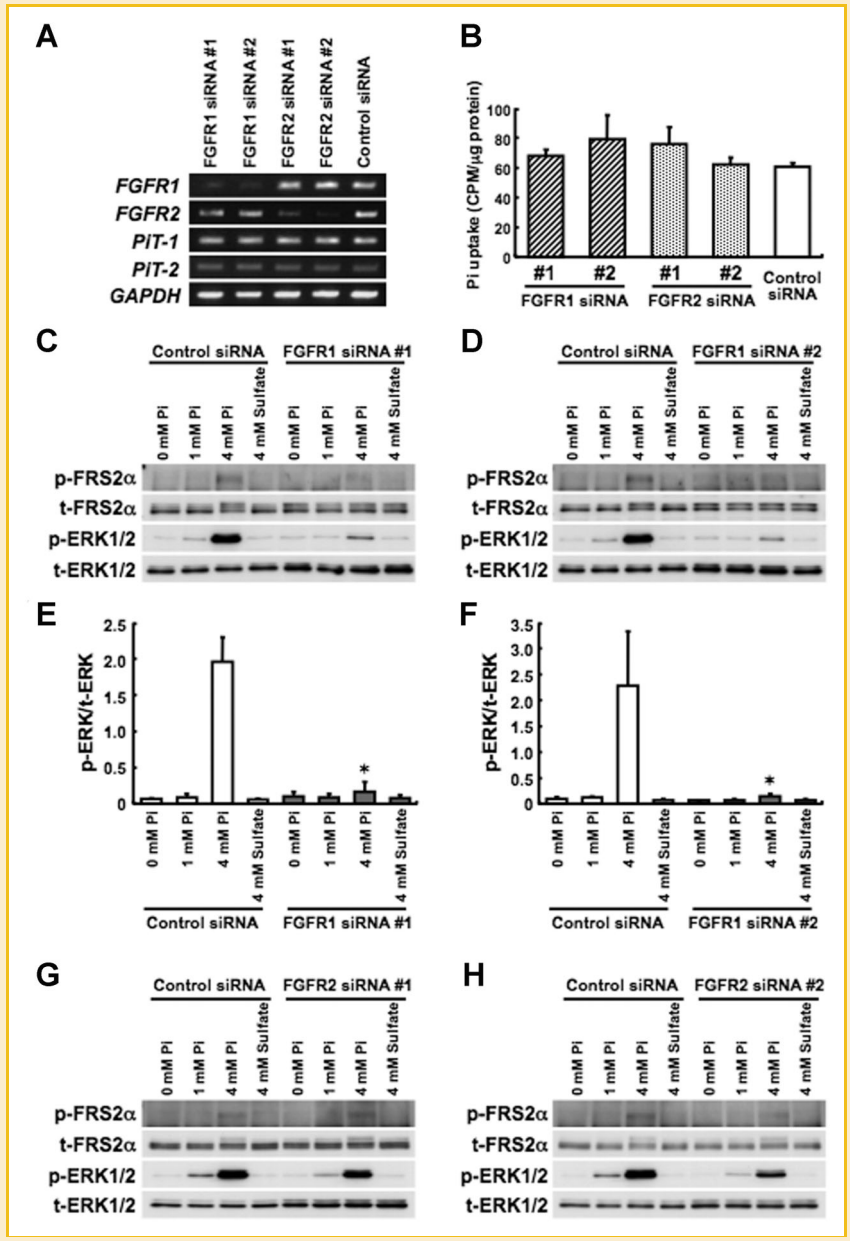

Fig. 6. Knockdown of the expression of *FGFR1* resulted in the decreased responsiveness to extracellular Pi. A: Confirmation of the specific knockdown of the expression of *FGFR1* and *FGFR2* by transfection of gene-specific siRNAs. HEK293 cells were transfected with *FGFR1*-specific siRNAs #1 and #2, *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.

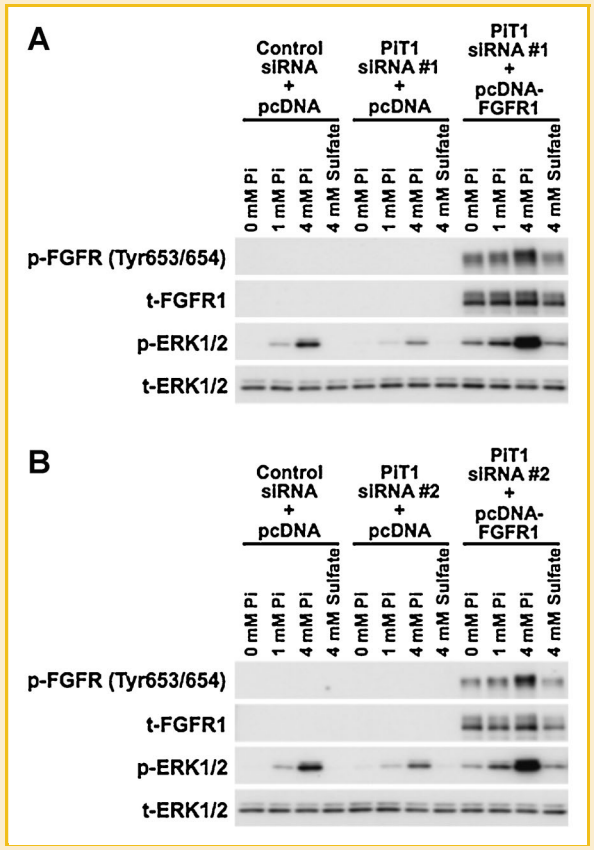

Fig. 7. Overexpression of FGFR1 rescued the decrease in the responsiveness to extracellular Pi in HEK293 cells where the expression of *PiT-1* was knocked down. A,B: HEK293 cells were transfected with *PiT-1*-specific siRNAs #1 (in A) and #2 (in B), or a negative control siRNA (50 nM each), together 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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