FGF23 Suppresses Chondrocyte Proliferation in the Presence of Soluble α-Klotho both in Vitro and in Vivo*\[1\]  

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**Background:** The role of elevated FGF23 in the development of growth retardation associated with X-linked hypophosphatemic rickets (XLH) remains elusive. Here, we postulated that elevated FGF23 in these tissues may affect the development of growth retardation associated with dysregulated phosphate metabolism. However, the precise mechanisms of growth retardation in XLH remain elusive. We, therefore, postulated that FGF23 suppressed chondrocyte proliferation in the presence of soluble α-Klotho (sKL). In vitro and ex vivo studies revealed that FGF23 formed a protein complex with sKL through KL1 internal repeat and suppressed the linear growth of metatarsals in the presence of sKL, which was antagonized by co-incubation with neutralizing antibodies against FGF23 or by knocking-down FGF3 expression. Additionally, FGF23 binding to FGFR3 was enhanced in the presence of sKL. Histologically, the length of the proliferating zone was diminished and was associated with decreased chondrocyte proliferation. FGF23/sKL suppressed Indian hedgehog (Ihh) expression and administration of Ihh protein partially rescued the suppressive effect of FGF23/sKL on metatarsal growth. Intraperitoneal administration of sKL in Hypa mice, a murine model for XLH, caused a decrease in the length of the proliferating zone associated with decreased chondrocyte proliferation without altering circulating phosphate levels. These findings suggest that suppression of chondrocyte proliferation by FGF23 could have a causative role in the development of growth retardation in XLH.

Emerging evidence from clinical and animal studies demonstrates pivotal roles of fibroblast growth factor 23 (FGF23)2 signaling in the regulation of phosphate homeostasis (1–5). Osteoblast-lineage specific cells, especially osteocytes, produce large amounts of FGF23, but other tissues including small intestine, heart, and ventrolateral thalamic nucleus and thymus also produce FGF23 as well although the physiological significance of FGF23 produced from these tissues remains to be defined (6, 7). FGF23 transduces its signals through its specific FGF receptors, which requires α-Klotho, a 130-kDa single-pass transmembrane protein, as a co-receptor (8, 9). Contrary to a widely accepted tenet that membrane-bound α-Klotho is mandatory for FGF23 to activate its downstream signaling pathways in physiological conditions (7, 8), accumulating evidence highlights the possibility that FGF23 may stimulate its downstream signaling pathways in cells that lack or have little expression of membrane-bound α-Klotho. Although membrane-bound α-Klotho is not expressed in the skeleton, FGF23 may be operative in skeletal cells (10, 11). Sitarra et al. generated a mouse model where both Fgfg3 and Slc34a1, encoding for the type IIa sodium-phosphate (Na1/Pi) co-transporter, were deleted to reverse the hyperphosphatemia noted in Fgfg3-null mice (10). These double mutants exhibited similar skeletal phenotypes to Fgfg3-null mice, despite a correction in serum phosphate levels, suggesting the possibility of a phosphate-independent action of FGF23 in the skeleton. In the same vein, adenosinergic transduction of FGF23 in rat calvarial osteoblasts has been shown to stimulate osteoblastogenesis and mineralization (11). In addition, there is mounting evidence that soluble α-Klotho (sKL) has been implicated to have biological functions in experimental models. For example, sKL has been shown to inhibit insulin and IGF-I signaling (12). Another levels of examples include the antagonistic effects of sKL on Wnt signaling and TGF-β signaling pathways (13, 14). Of note is that sKL has also been implicated to mediate FGF23 signaling in cells which do not express membrane-bound α-Klotho (15). Combined together, these lines of evi-

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‡ The abbreviations used are: FGF, fibroblast growth factor; XLH, X-linked hypophosphatemic rickets; sKL, soluble α-Klotho; Ihh, Indian hedgehog.
dence indicate that FGF23 may have a non-canonical function such that FGF23 can exert its signals in cells that do not express functional membrane-bound α-Klotho such as chondrocytes, which may be more relevant in the presence of sKL.

Growth retardation is mainly caused by a defect in chondrogenesis and is one of the significant complications in children suffering from a disorder with dysregulated phosphate and vitamin D metabolism. X-linked hypophosphatemic rickets (XLH) is a disorder with a loss-of-functional mutation in the PHEX gene (16). These patients exhibit elevated serum FGF23 levels associated with decreased serum phosphate and 1,25-dihydroxyvitamin D levels. The skeletal phenotype of these patients includes growth retardation as well as rickets and impaired mineralization. Administration of phosphate and calcitriol is effective in improving rickets and growth retardation, but it is well recognized that impaired linear growth still remains despite the correction in biochemical markers and rachitic changes (17). Abnormal phosphate and vitamin D metabolism is likely to be the leading cause of growth retardation associated with XLH, but the fact that normalization of dysregulated phosphate and vitamin D levels did not fully reverse impaired growth led us to speculate that there could be a disease-specific mechanism that modulates the development of growth retardation in addition to dysregulated phosphate and vitamin D metabolism. Interestingly, Liu et al. showed that shortening of the tibia in Hyp mice, a murine model for XLH carrying a 3′-deletion in the Phex gene, was partially rescued by crossing these mice with Fgr3 deficient mice without altering circulating phosphate and vitamin D levels, indicating that signals exerted from FGF3 may be partly responsible for the development of growth retardation in Hyp mice (18). These lines of evidence prompted us to hypothesize that elevated FGF23 levels may be at least in part responsible for the development of growth retardation in XLH patients through activating FGF receptors in chondrogenic cells.

**EXPERIMENTAL PROCEDURES**

*Mice—* C57BL/6j mice were purchased from CLEA Japan, Inc. and Hyp mice on a C57BL/6j background were kindly provided by Dr. T. Tanaka (Okayama University Graduate School of Medicine). Mice were maintained with free access to water and standard chow (CE-2, CLEA Japan, Inc) on a 12:12 h LD cycle in a pathogen-free animal facility. All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee of Osaka Medical Center and Research Institute for Maternal and Child Health.

*Reagents and Cell Lines—* Recombinant human FGF23 and neutralizing antibody against FGF23 were kindly provided by Kyowa Hakko Kirin Co., Ltd. Mouse anti-Klotho antibody (Mink1) was a kind gift from Drs. A. Imura and Y. Nabeshima (Institute of Biomedical Research and Innovation). Recombinant mouse soluble α-Klotho and rat anti-Klotho antibody (AF1819) were purchased from R&D Systems. Rat anti-Klotho antibody (Clone KM2076, KO603) was obtained from Trans-Genic Inc. Expression vector containing human FGF3 (pcDNA3-hFGFR3) was kindly provided from Dr. K. Hasegawa (Okayama University Graduate School of Medicine). ATDC5 cells were obtained from the Human Science Research Resources Bank (Osaka, Japan) and maintained in DMEM/F12 medium supplemented with 5% fetal bovine serum and 1% insulin-transferrin-selenium-G supplement. For chondrogenic induction, cells were incubated with alpha minimal essential medium supplemented with 5% FBS and ITS.

*Isolation of Primary Chondrocytes—* Primary chondrocytes were isolated from rib cages obtained from 3-day-old C57BL/6j mice as previously described (19). Briefly, cartilage was incubated with actinase E (2 mg/ml in PBS, Kaken Pharmaceutical Co. Ltd., Tokyo, Japan) for 30 min at 37 °C, followed by digestion with collagenase (3 mg/ml, Wako) for 90 min at 37 °C. Pellets collected by the centrifuge were washed, passed through a 100-μm cell strainer, and used as primary chondrocytes. Primary chondrocytes were cultured in DMEM supplemented with 10% FBS and 50 μg/ml of ascorbic acid.

*Generation of Truncated skL Mutant—* Truncated mutants containing KL1 internal repeat (skL-KL1: aa1–536) or KL2 internal repeat (skL-KL2: aa537–958) were created by subcloning the corresponding PCR products into pENTR vector using pENTR Directional TOPO cloning kit (Invitrogen) and transferred to pcDNA3.2/V5 vectors using LR recombination system (Invitrogen).

*Metatarsal Organ Culture—* Middle metatarsals were isolated from E15.5 C57BL/6j mouse embryos and incubated in αMEM containing 0.5% BSA, 50 μg/ml ascorbic acid, and 1 mM β-glycerol phosphate as previously described (20). Stimulants were added to culture media on day 0, day 1, and every other day from day 1 for indicated periods. For BrdU labeling, metatarsals were incubated with BrdU for 3 h according to the manufacturer’s instructions (Calbiochem). To visualize calcium deposition, metatarsals were labeled with calcein (500 ng/ml) for 2 h. Bones were then fixed with 4% PFA, embedded in paraffin, and processed for hematoxylin and eosin staining and immunohistochemistry.

*Western Blot Analysis—* To prepare whole cell lysates, cells were solubilized 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, and protease inhibitor mixture (Complete TM; Roche). Equal amounts of protein were separated by SDS-PAGE and transferred electrophoretically to PVDF membranes. Membranes were blocked in BlockAce reagent (Dainippon Pharmaceuticals, Osaka, Japan) or Blocking ONE P reagent (Nacalai Tesque), immunoblotted with anti-ERK (1:1000, 9102, Cell Signaling), anti-pERK (1:1000, 9101, Cell Signaling), anti-FRS2α (1:1000, sc-8318, Santa Cruz Biotechnology), anti-pFRS2α (1:1000, 3864, Cell Signaling), or anti-GAPDH (1:2000, sc-20357, Santa Cruz Biotechnology) and developed with horseradish peroxidase-coupled secondary antibodies, followed by enhancement with a chemiluminescence (ECL) detection system (GE Healthcare).

*Co-immunoprecipitation—* Cells were solubilized in IP buffer (5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40, 10% glycerol, and 10 mM Tris-HCl, pH 8.0) containing a protease inhibitor mixture (Complete TM, EDTA-free; Roche Diagnostics) and centrifuged. Supernatants were incubated with antibody, followed by immunoprecipitation with protein A/G-Sepharose (Santa Cruz Biotechnology) at 4 °C overnight. Where conditioned media were used for co-immunoprecipitation, condi-
tioned media obtained from either CHO cells stably expressing FGF23R179Q which is a proteolysis-resistant mutant or HEK293 cells stably expressing soluble α-Klotho were mixed and incubated with antibody, followed by immunoprecipitation with protein A/G-Sepharose (Santa Cruz Biotechnology) at 4 °C overnight. Samples were washed five times with IP buffer and then subjected to Western blot analysis.

**Immunohistochemistry**—Samples were fixed in 10% buffered formalin and immunohistochemical analysis was performed using paraffin-embedded samples. Femurs were decalcified using 20% EDTA solution for 7 days before paraffin-embedding. After deparaffinization and rehydration, antigen retrieval was performed using citrate buffer at 95 °C for 60 min (for Sox9 and Ihh), pepsin solution (Cat AP-9007-005, Thermo Scientific) at 37 °C for 10 min (for Col2a1) or proteinase K solution (0.4 mg/ml, Cat S3020, DAKO) at room temperature for 5 min (for Fgfr3). Endogenous peroxidase activity was quenched using ImmunoCruz staining systems (Santa Cruz Biotechnology). Following blocking, sections were incubated with anti-Sox9 antibody (1:100; sc-20095, Santa Cruz Biotechnology), anti-Ihh antibody (1:50; sc-1196 Santa Cruz Biotechnology), anti-Col II mouse monoclonal antibody (Clone 2B1.5, MS-235-R7, Thermo Fisher Scientific, Waltham, MA), anti-Fgfr3 antibody (1:100, SAB4500888, Sigma) overnight at 4 °C. Normal IgG was used as a negative control. Sections were then incubated with a biotinylated secondary antibody, followed by incubation with streptavidin-biotinylated HRP complex, and visualized with 3, 3’-diaminobenzidine.

**In Situ Hybridization**—Digoxigenin-labeled riboprobes were synthesized by an in vitro transcription reaction using a DIG RNA labeling kit according to manufacturer’s protocol (Roche Applied Science). Paraffin-embedded sections were deparaffinized, rehydrated, and incubated with proteinase K solution. Sections were then fixed with PFA, treated with 0.25% acetic acid, and incubated with a prehybridization buffer, followed by hybridization with digoxigenin-labeled riboprobes. After hybridization, sections were incubated with anti-DIG-alkaline phosphatase-conjugated antibody and visualized by 4-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate.

**Adenovirus-mediated Knock-down Experiments**—Knockdown of Fgfr3 expression in metatarsals was performed based on adenovirus-mediated expression of the microRNA (miRNA) system using the BLOCK-iT™ Pol II miR RNAi Expression Vector Kit with EmGFP (Invitrogen). Recombinant adenoviruses were prepared using the ViraPower™ Adenovirus Expression System (Invitrogen) according to the manufacturer’s protocol. Each metatarsal was infected with 1 × 10⁷ particles of adenovirus in the presence of poly-L-lysine for 4 h on day 0, 1, and every other day from day 1.

**In Vitro and ex Vivo Treatment with FGF23 and/or sKL**—Where FGF23 and sKL combinational treatment was used, FGF23 and sKL were mixed and pre-incubated for 5 min at room temperature before adding to culture media.
in chondrogenic cells. First, we analyzed whether FGF23 can form a protein complex with sKL. Co-immunoprecipitation analysis using HEK293 cells over-expressing FGF23 and/or sKL revealed that FGF23 can form a protein complex with sKL in these cells (Fig. 2A). Because both sKL and FGF23 are secretory molecules, we next performed co-immunoprecipitation analysis using the conditioned media containing FGF23 or sKL, or solutions of recombinant FGF23 and/or sKL proteins and found that FGF23 and sKL can make a protein complex in a solution as well (Fig. 2, B and C). To determine the responsible domain of this interaction, we generated truncated mutants containing either KL1 internal repeat or KL2 internal repeat and found that KL1 internal repeat was involved in the interaction between sKL and FGF23 (Fig. 2D). Based on these observations, we next examined the effect of sKL with respect to FGF23 signaling in chondrogenic cells. FGF23 in the presence of sKL in chondrogenic cells.
FGF23 makes a protein complex with sKL

A, HEK293 cells were transfected with FGF23-cMyc or sKL-V5 expression vectors. Immunoprecipitation with V5 was followed by Western blot analysis for cMyc. B, conditioned media containing FGF23-cMyc were mixed with sKL-containing conditioned media. Immunoprecipitation with cMyc was followed by Western blot analysis for Klotho (AF1819). C, 300 ng of recombinant FGF23 and recombinant sKL were mixed in one milliliter solution and immunoprecipitation with Klotho (Mink1) was followed by Western blot analysis for Klotho (KM2076). D, HEK293 cells were transfected with FGF23-cMyc, and full-length (sKL-FL-V5) or truncated sKL (sKL-KL1-V5 or sKL-KL2-V5) expression vectors. Immunoprecipitation with cMyc was followed by Western blot analysis for V5. The figures shown are the representative from at least three independent experiments.

FGF23 Signaling in Chondrocytes

FIGURE 2. FGF23 makes a protein complex with sKL. A, HEK293 cells were transfected with FGF23-cMyc or sKL-V5 expression vectors. Immunoprecipitation with V5 was followed by Western blot analysis for cMyc. B, conditioned media containing FGF23-cMyc were mixed with sKL-containing conditioned media. Immunoprecipitation with cMyc was followed by Western blot analysis for Klotho (AF1819). C, 300 ng of recombinant FGF23 and recombinant sKL were mixed in one milliliter solution and immunoprecipitation with Klotho (Mink1) was followed by Western blot analysis for Klotho (KM2076). D, HEK293 cells were transfected with FGF23-cMyc, and full-length (sKL-FL-V5) or truncated sKL (sKL-KL1-V5 or sKL-KL2-V5) expression vectors. Immunoprecipitation with cMyc was followed by Western blot analysis for V5. The figures shown are the representative from at least three independent experiments.

of sKL (FGF23/sKL) induced FRS2α and ERK1/2 phosphorylation both in ATDC5 and primary chondrocytes in a dose- and time-dependent manner, although sKL alone did not show any effect on phosphorylation of these proteins (Fig. 1, A--C). In addition, FGF23/sKL induced the expression of Early growth response 1 (Egr1), a target gene of FGF23 signaling, in ATDC5 cells (Fig. 1D). Importantly, phosphorylation of FRS2α and ERK1/2 by FGF23/sKL was completely blocked by co-incubation with neutralizing antibodies raised against FGF23 (anti-FGF23 Ab), confirming the specificity of FGF23 in activating FRS2α and ERK1/2 signaling in chondrogenic cells (Fig. 1E).

FGF23 Impairs Linear Growth of Metatarsals in the Presence of sKL ex Vivo—To better understand the role of FGF23/sKL signaling in chondrocyte biology, we introduced an ex vivo metatarsal organ culture system to analyze the effect of FGF23/sKL on metatarsal growth. Neither FGF23 alone nor sKL alone exhibited any effects on the linear growth of metatarsals, but when metatarsals were treated with FGF23 in the presence of sKL, their linear growth was significantly impaired in a dose-dependent manner (Fig. 3, A--D). To confirm the specificity of FGF23 signaling in the suppression of metatarsal growth, we treated metatarsals with anti-FGF23 Ab. Although treatment with anti-FGF23 Ab alone did not affect the linear growth of metatarsals compared with control-IgG treatment (supplemental Fig. S2), the inhibitory effect of FGF23/sKL on metatarsal linear growth was completely abolished by co-incubation with anti-FGF23 Ab (Fig. 3, E and F).

FGF Receptor 3 Mediates the Suppressive Effect of FGF23/sKL on Metatarsal Linear Growth—Next, we examined whether FGF receptor 3 (FGFR3) is involved in the suppressive effect of FGF23/sKL on metatarsal growth. We first investigated expression levels of FGFR3 in metatarsals treated with FGF23/sKL and found that expression and localization of FGFR3 was comparable between metatarsals treated with or without FGF23/sKL (Fig. 4, A and B). Second, we suppressed FGFR3 expression in metatarsals using adenovirus-mediated transduction of microRNA specific for Fgrf3 and examined the effect of FGF23/sKL on metatarsal growth. Transduction of adenovirus in metatarsals was determined by visualizing the fluorescence of EmGFP (Fig. 4, C and D), and immunohistochemistry and real-time PCR analyses for FGFR3 confirmed the efficient knock-down of FGFR3 in metatarsals (Fig. 4, E and F). As shown in Fig. 4G, FGF23/sKL showed an inhibitory effect on linear growth in metatarsals infected with control-miRNA, whereas the suppressive effect of FGF23/sKL on metatarsal growth was partly abolished in metatarsals infected with miRNA specific for Fgrf3. These findings imply that the effect of FGF23/sKL is at least in part mediated through FGFR3 in metatarsals.

Physical Interaction between FGF23 and FGFR3 Is Enhanced in the Presence of sKL—We next investigated the mechanism whereby sKL enhanced FGF23 signaling. Because FGFR3 is involved in the suppressive effect of FGF23/sKL on metatarsal growth, we assessed whether the binding of sKL or FGF23 to FGFR3 was affected by FGF23 or sKL, respectively. Co-immunoprecipitation analysis revealed that the interaction of sKL with FGFR3 was augmented in the presence of FGF23 (Fig. 5A). In addition, binding of FGF23 to FGFR3 was enhanced as well when sKL was present (Fig. 5B). These data indicate that one of the mechanisms by which sKL enhanced FGF23 signaling pathway was mediated through enhancing the accessibility of FGF23 to its receptors.
FGF23/sKL Impairs Chondrocyte Proliferation and Maturation—To elucidate the mechanisms by which FGF23/sKL impairs metatarsal growth, we performed histological analyses and found that the length of the proliferating zone was decreased in metatarsals treated with FGF23/sKL, whereas the length of the resting zone was longer in FGF23/sKL-treated metatarsals than that in controls, but the difference did not reach significance (Fig. 6, A and B). These findings raised the possibility that FGF23/sKL possessed a context-specific effect on chondrocyte proliferation where FGF23/sKL inhibited chondrocyte proliferation in the proliferating zone. To prove this possibility, we performed BrdU labeling of these metatarsals to determine chondrocyte proliferation. As shown in Fig. 6, C and D, the BrdU index was significantly lower in the proliferative zone of FGF23/sKL-treated metatarsals than that in controls, whereas the BrdU index was enhanced in the resting zone of FGF23/sKL-treated metatarsals. Because there is a possibility of the involvement of cell apoptosis for the decreased length of proliferating zone, we also analyzed whether cell apoptosis was enhanced by the treatment with FGF23/sKL. TUNEL assay revealed that cell apoptosis of proliferating chondrocyte was not different between metatarsals treated with or without FGF23/sKL (Fig. 6E). To further understand the mechanisms of decreased chondrocyte proliferation in the proliferating zone, we analyzed expression levels of Sox9, a master transcription factor for chondrocyte differentiation, and one of its target genes, Sox9.
FIGURE 4. FGFR3 partly mediates the suppressive effect of FGF23/sKL on metatarsal growth. A and B, metatarsals were cultured in the presence or absence of FGF23 (300 ng/ml) and sKL (300 ng/ml) for 6 days. FGFR3 expression was analyzed by immunohistochemistry (A) and real-time RT-PCR (B) (n = 3). C–F, metatarsal rudiments were infected with adenovirus harboring control miRNA (con-miR) or Fgfr3-specific miRNA (Fgfr3-miR) for 6 days. Fluorescence of EmGFP was detected in metatarsals (C). Frozen metatarsals were sectioned and transduction of adenovirus was visualized by detecting the fluorescence of EmGFP (D). FGFR3 expression was analyzed by immunohistochemistry (E) and real-time RT-PCR (F) (n = 3). G, metatarsals infected with adenovirus containing either con-miR or Fgfr3-miR were cultured in the presence or absence of FGF23 (300 ng/ml) and sKL (300 ng/ml) for 6 days. Relative percent increases in longitudinal metatarsal growth were calculated (n = 3–6). The figures shown are the representative from at least three independent experiments. The values were expressed as mean ± S.E. *, p < 0.05. ns, not significant.
genes, Col2a1, to determine whether transcriptional machinery of chondrogenesis is affected by FGF23/sKL treatment. Chondrogenesis of primary chondrocytes analyzed by Alcian blue staining was not affected by FGF23/sKL treatment (Fig. 7A), which was associated with comparable expression levels of Sox9 and Col2a1 between the two groups (Fig. 7B). Consistent with this, immunohistochemical analysis of metatarsals revealed that expression levels of Sox9 and Col2a1 were not altered by treatment with FGF23/sKL (Fig. 7, C and D), indicating that impaired proliferation of chondrocytes in the proliferating zone is unlikely to be caused by impaired transition of resting chondrocytes into proliferating chondrocytes. Next, we investigated whether maturation of hypertrophic chondrocytes was affected by FGF23/sKL treatment. In situ hybridization and real-time RT-PCR analyses demonstrated the decreased expression of Col10a1 in metatarsals treated with FGF23/sKL (Fig. 8, A and B). FGF23/sKL did not show any effect on Col10a1 expression in primary chondrocytes (Fig. 8C), suggesting that the effect of FGF23/sKL on decreased Col10a1 expression is unlikely due to the direct action of FGF23/sKL on hypertrophic chondrocytes. Interestingly, calcium deposition in the hypertrophic zone was impaired in metatarsals with FGF23/sKL (Fig. 8, D and E), indicating that FGF23/sKL delays maturation of hypertrophic chondrocytes.

FGF23 Suppresses Indian Hedgehog Expression in the Presence of sKL—To further analyze the mechanisms whereby FGF23/sKL signaling impairs chondrocyte proliferation, we examined the expression of Indian hedgehog (Ihh) because Ihh is well known to be involved in chondrocyte proliferation and activation of FGFR3 signaling has been shown to result in decreased expression of Ihh (21–25). Ihh expression was significantly lower in primary chondrocytes and metatarsals treated with FGF23/sKL than that in controls (Fig. 9A). In line with
these observations, immunohistochemical analysis showed that Ihh expression was lower in metatarsals treated with FGF23/sKL than that in controls (Fig. 9B). These data led us to speculate that decreases in Ihh expression may at least in part mediate the inhibitory effect of FGF23/sKL on metatarsal growth. To test this hypothesis, we treated metatarsals with FGF23/sKL in the presence or absence of conditioned-media obtained from Ihh-overexpressing HEK293 cells (Ihh-CM). Addition of Ihh-CM to the culture media did not affect the longitudinal growth of metatarsals, but partially rescued the impaired longitudinal growth of metatarsals by FGF23/sKL (Fig. 9C). In line with this, the inhibitory effect of FGF23/sKL on metatarsal growth was weaker in metatarsals treated with cyclopamine, an antagonist for Ihh signaling pathway (supplemental Fig. S3). These data indicate that decreased expression of Ihh is at least in part responsible for FGF23/sKL-induced impairments in metatarsal longitudinal growth.

Administration of sKL Impairs Chondrocyte Proliferation in Hyp Mice—To understand the in vivo role of FGF23/sKL signaling in chondrocyte biology, we utilized Hyp mice, a murine model for XLH, and intraperitoneally administered sKL into these mice. Hyp mice at postnatal day 10 already exhibited higher circulating FGF23 levels than those of wild-type (WT) littermate controls (Fig. 10A). Administration of sKL did not show any influence on body weight gain in wild-type or Hyp mice (Fig. 10B, supplemental Fig. S4A), but caused a shortening of the longitudinal length of the tibia in Hyp mice (Fig. 10C), whereas sKL did not affect the length of the tibia in WT mice (supplemental Fig. S4B). Histological analysis of the tibial growth plate revealed that the length of the proliferating zone was lower in sKL-treated Hyp mice than that in saline-treated Hyp mice (Fig. 10D), which was accompanied by a decline in the BrdU index in sKL-treated Hyp mice (Fig. 10E). In contrast to Hyp mice, sKL did not alter these parameters in WT mice (supplemental Fig. S4, C and D). To exclude the possibility that shortening of the proliferating zone is a consequence of altered phosphate metabolism, we analyzed expression levels of genes involved in phosphate metabolism in the femur and the kidney. As shown in Fig. 10F and G and supplemental Fig. S4E, expression of Fgf23 in the femur and expressions of Cyp27b1, Cyp24a1, Slc34a1, and Slc34a3 in the kidney were comparable between Hyp mice treated with sKL or saline. Consistent with this, circulating levels of phosphate were not different between Hyp mice treated with sKL or saline (Fig. 10H and supplemental Fig. S4F).

DISCUSSION
Recent advances in our understandings highlight the multifaceted nature of FGF23 function beyond its pivotal roles in the regulation of phosphate and vitamin D metabolism. For exam-
ple, FGF23 has been recently shown to be responsible for the development of left ventricular hypertrophy through activating calcineurin-NFAT signaling pathway in mice (26). Non-canonical activity of FGF23 could be operative as well in chondrocytes as evidenced by the previous study in which Fgf23 and Slc34a1 genes were deleted in mice (10). The lack of Slc34a1 in Fgf23-deficient mice did not correct the decreased number of hypertrophic chondrocytes in Fgf23-deficient mice despite of the correction of serum phosphate levels, suggesting the presence of phosphate-independent action of FGF23 in chondrocytes; however, the precise mechanisms of phosphate-independent function of FGF23 in chondrocyte biology remain to be elucidated.

Initially, we demonstrated in vitro that FGF23 can mediate its signals in the presence of sKL. As previously reported, α-Klotho expression was extremely low in chondrogenic cells. In line with this, FGF23 alone could not activate ERK or induce Egr1 expression in chondrogenic cells. Since there is an increasing amount of evidence that demonstrates the biological function of sKL in mice (12–15), we assessed the functional interaction between FGF23 and sKL in chondrogenic cells. In the current study, we utilized ~130 kDa of sKL produced by ectodomain

FIGURE 8. Maturation of hypertrophic chondrocyte is impaired in metatarsals treated with FGF23/sKL. A and B, metatarsal rudiments were isolated from E 15.5 embryos and cultured in the presence or absence of FGF23 (300 ng/ml) and sKL (300 ng/ml) for 6 days. Expression levels of Col10a1 were analyzed by in situ hybridization (A) and real-time-RT-PCR (B) (n = 3). C, primary chondrocytes were treated with chondrogenic media in the presence or absence of FGF23 (100 ng/ml) and sKL (100 ng/ml) for 6 days. Expression levels of Col10a1 were determined by real-time RT-PCR (n = 3). D and E, metatarsals were cultured in the presence of various concentrations of FGF23 and sKL (D), or FGF23 (300 ng/ml), sKL (300 ng/ml), or both (E) for 12 days. On day 12, the calcein incorporated area was visualized and quantified (n = 3–4). RZ: resting zone, PZ: proliferating zone, HZ: hypertrophic zone. The figures shown are the representative from at least three independent experiments. The values were expressed as mean ± S.E. *, p < 0.01; **, p < 0.05. ns, not significant.
sheding based on a previous report showing that this type of sKL is the predominant sKL in human circulation (27). Interestingly, in vitro studies revealed that FGF23 exerted its signals in the presence of sKL in chondrogenic cells. The precise mechanisms whereby sKL mediates FGF23 signaling still need to be elucidated, but the finding that sKL forms a protein complex with FGF23 may raise the possibility that sKL may allow FGF23 to reach and bind to its specific receptors by making a complex with FGF23 in the circulation. Indeed, co-immunoprecipitation analysis revealed that the binding of FGF23 to FGFR3 was enhanced when sKL was present. However, it is still unclear as to whether FGF23/sKL complex is present in the circulation and further analyses are required to determine the significance of this complex in the in vivo physiological conditions.

Next, to elucidate the significance of FGF23/sKL signaling in chondrocyte biology, we introduced an ex vivo metatarsal organ culture system, which is a widely used procedure to recapitulate in vivo bone growth. Using this method, we found a unique phenotype with respect to chondrocyte proliferation such that FGF23/sKL suppressed proliferation in the proliferating zone. The finding that FGF23/sKL impaired chondrocyte proliferation in the proliferating zone led us to speculate that FGF23/sKL may recognize FGFR3 as its receptor because an activating mutation in the Fgfr3 gene has been shown to result in impaired chondrocyte proliferation (28–30). Based on this, we analyzed whether FGFR3 is involved in FGF23/sKL-mediated suppression of metatarsal growth and provided evidence that FGFR3 is at least in part involved in the FGF23/sKL-mediated action on metatarsal growth. However, given the fact that FGF23/sKL showed a non-significant suppression on the linear growth of metatarsals infected with adenovirus containing microRNA specific for Fgfr3, we could not exclude the possibility of the involvement of other types of FGF receptors, although it is still possible that residual expression of FGFR3 may contribute to the suppressive action in FGFR3-knocked down metatarsals. Similar to FGFR3, FGFR1 is abundantly expressed in the growth plate predominantly in perichondrium and hypertrophic chondrocytes, whereas FGFR3 exhibits abundant expression in resting and proliferating chondrocytes, with less expression in hypertrophic chondrocytes (31). The expression profile of FGFR2 is also different from FGFR3, and FGFR2 is mainly expressed in mesenchymal condensation (31). Although previous in vitro studies demonstrated the binding of FGF23 to multiple FGF receptors (8, 9), the compartment-specific expression profile of FGFRs in the growth plate would confer a specific binding partner for FGF23 in the presence of sKL such that FGF23 suppresses chondrocyte proliferation in the proliferating zone through the activation of FGFR3.

Multiple pathways have been shown to mediate the effect of FGFR3 activation with respect to the suppression of chondrocyte proliferation. Ihh has been implicated to be the downstream target of FGFR3 activation (21–23) and is well known to play a critical role in chondrocyte proliferation (24, 25). Based on these findings, we tested our speculation that Ihh may be the

![FIGURE 9. Indian Hedgehog partly mediates the suppressive effect of FGF23/sKL on the longitudinal growth of metatarsals. A and B, primary chondrocytes were treated with chondrogenic media in the presence or absence of FGF23 (100 ng/ml) and sKL (100 ng/ml) for 2 days. Metatarsal rudiments were cultured in the presence or absence of FGF23 (300 ng/ml) and sKL (300 ng/ml) for 6 days. Expression levels of Ihh were determined by real-time RT-PCR (n = 4) (A) and immunohistochemistry (n = 3) (B). C, metatarsals were treated with FGF23 (300 ng/ml) and sKL (300 ng/ml) for 6 days in the presence of 10% of Ihh conditioned media (Ihh-CM) or control conditioned media (Cont-CM) and longitudinal lengths and percent increases in metatarsals were measured (n = 3–4). Conditioned media obtained from HEK293 cells overexpressing empty vector were used as Cont-CM. PZ: proliferating zone. The figures shown are the representative from at least three independent experiments. The values were expressed as mean ± S.E. *, p < 0.001; **, p < 0.01; †, p < 0.05.](image-url)
downstream target of FGF23/sKL signaling and found that Ihh expression was reduced in the presence of FGF23/sKL and addition of Ihh protein partially reversed the impaired growth of metatarsals treated with FGF23/sKL. Since Ihh protein cannot fully rescue the growth impairment induced by FGF23/sKL signaling, other signals are likely involved in the action of FGF23/sKL on the suppression of chondrocyte proliferation. Because STAT1 has been shown to be activated in response to FGFR3 activation, which in turn results in the inhibition of chondrocyte proliferation (32), a signaling pathway through STAT1 activation may be involved in the FGF23/sKL-induced impairment in chondrocyte proliferation.

In XLH patients, it is well recognized that administration of phosphate and calcitriol is effective to improve linear growth, but is not sufficient to fully reverse impaired growth despite the correction in biochemical markers and rachitic changes (17, 33). This evidence may suggest the existence of factor(s) modulating the linear growth of XLH patients in addition to abnormal phosphate metabolism, and the current findings that FGF23 suppresses chondrocyte proliferation in the presence of sKL may at least in part explain the reason why the correction in serum phosphate levels by administration of phosphate and calcitriol cannot fully regain impaired growth in XLH patients. If this mechanism is operative, the blockade of FGF23 signaling as a strategy for the treatment of XLH patients would be very promising because suppressing FGF23 signaling may have an additional benefit such as enhancing chondrocyte proliferation beyond its capacity to correct phosphate and vitamin D metabolism. Indeed, recent in vivo animal studies have provided evidence for the striking effectiveness of the blockade of FGF23 signaling pathways by the anti-FGF23 neutralizing antibody in the improvement of rickets and growth retardation in Hyp mice (34).

The significance of sKL in chondrocyte biology in humans remains largely unknown. Recent development in the ELISA system to detect human sKL has revealed that significant amounts of sKL are present in the human circulation (35); however, it is still controversial as to whether sKL in the circulation has any biological functions despite evidence demonstrating the biological function of sKL in animal models (12–14). Nev-
et al. (2012) Regulation of phosphate metabolism, and this effect was partly mediated through FGFR3 and involved the suppression of Ihh expression. These lines of evidence add to our growing knowledge through FGFR3 and involved the suppression of Ihh expression. These lines of evidence add to our growing knowledge regarding signaling networks exerted by FGFR3 and provide insights into the unrecognized function of FGFR3 signaling that could be important for chondrocyte biology.

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