症例 4
調査協力者　担当医師・両親

遺伝子変異　Arg248Cys
生年月日　H22.11. 2
調査時年齢　2 歳
性別　男
出生時父親年齢　37 歳/母親年齢 37 歳
出生時週数　37 週 5 日
出生前診断　有（CT）
分娩方法　帝王切開（胎児適応）
アプガースコア　6 点/1 分、8 点/5 分
体重 2800g
身長 39 ㎝
頭周囲長 37.5 ㎝
レントゲン所見　TD 所見
蘇生処置　有（出生直後から）
合併奇形　無

＜呼吸管理＞
HFO および SIMV を交互に
日齢 97　気管切開
日齢 131　在宅用呼吸器（トリロジー）
無気肺のエピソードあり
日齢 479　院内用呼吸器
自発呼吸と人工呼吸器の換気が合いにくいため、現在はトリロジーX にて管理中

＜栄養方法＞
離乳食摂取
呼吸機能の事を考慮して、水分制限を行っている

＜リハビリテーション＞
生後 1 か月より呼吸リハ開始
7 か月～1 歳 6 か月　ミルク経口量アップと離乳食の導入目的で ST 開始
ST→言語聴覚りは、嚥下状態の把握
現在作業療法開始
＜合併症＞
痙攣（4 か月より顔面半側痙攣）
脳波異常
てんかん
呼吸器感染症
洞性除脈
便秘
黒色表皮腫
甲状腺機能低下（治療はなし）
無気肺（H24,2 月）
大後頭孔狭窄
水頭症経度

＜精神・運動発達＞
介助をするとぼーろを手でつかみ、口元にもっていく
対立動作は不可
手足の動きあり
笑う
簡単な指示はわかる
自分の気もちをジェスチャーで伝える（いやいや、ちょうだい）
人見知りあり（人の区別ありそう）

＜歯芽＞
乳歯あり

＜その他＞
移動時ようのバギーあり（補助具装着）

症例 5
調査協力者　担当医師

遺伝子検査　Arg248Cys
生年月日　H23.8.9
調査時年齢　1 歳 5 か月
性別　男
出生時父親年齢　歳/母親年齢歳
出生時週数　38 週 2 日
出生前診断　無
分娩方法　経膣分娩
アプガースコア　2 点/1 分、6 点/5 分
体重 2528g
身長 37 ㎝
頭周囲長 37 ㎝
レントゲン所見　TD 様所見
蘇生処置　有（出生直後から酸素投与）
合併奇形　無

＜呼吸管理＞
HFO
生後 38 日　気管切開
生後 107 日　在宅呼吸器導入
生後 178 日　在宅へ
汎用人工呼吸器レジェンドエアー（エアロックス社）
努力呼吸があるのに 1 回換気量が増えるエ

ピソードがあるのでPIP設定が難しい
本人の呼吸flowを器械が感知しにくい

＜栄養管理＞
経鼻栄養から開始
現在経鼻（ミルク、白湯）+離乳食（1～2回/日）+経口（ミルク、イオン飲料、果汁）

＜リハビリテーション＞
呼吸リハ　1回/週
保育士訪問　1～2回/月
歯科衛生士訪
＜合併症＞
痙攣（間代性、眼球変位）
脳波異常なし
呼吸器感染症
便秘
黒色表皮腫（全体的に浅黒いが、足が特に著明）
褥瘡
遷延性肺高血圧（出生後7日間NO使用）
気管支内肉芽
右鼠径ヘルニア

<精神・運動発達＞
追視
音のする方を見る
なん語
笑う
泣く
顔をしかめる
人見知りあり
人に来てもらいたいときは鈴を鳴らす
手ではらいのける

＜その他＞
猿線あり

＜リハビリテーション＞
現在は施行せず
入院中は関節リハを行っていた。
リハビリで手足の動きはました

＜精神・運動発達＞
手足の動きあり
あやすと笑う
顔をしかめる
人の区別はできている印象（家族）

＜歯芽＞
乳歯と永久歯あり

＜在宅療法＞
訪問看護5回/週
往診1回/週

症例6
調査協力者　担当医師・母親

遺伝子検査　未
生年月日　H21. 10.30
調査時年齢　3歳2ヶ月
性別　男
出生時父親年齢　昭和54年生/母親年齢昭和55年生
出生時週数　38週0日
出生前診断　超音波およびCT
四肢短縮・胸郭低形成・羊水過多
分娩方法　経膣分娩（C/Sのリスクよりも母体の安全性のため）
アプガースコア　8点/1分、9点/5分
体重2362g
身長40㎝
頭周囲長36㎝
胸囲26㎝
レントゲン所見　情報なし
蘇生処置　有（酸素投与）
合併奇形　無

＜呼吸管理＞
看取り予定であり、最初は酸素投与のみであったが24時間経過後も自発呼吸および酸素投与にて生存していたため、積極的な医療介入となる。
nasalCPAPおよび酸素投与を行っていたが、急変時の気道維持が困難である事から気管切開を日齢187で施行（在宅療法に向けての意味合いもある）
生後320日に呼吸不全をおこしたため、呼吸器導入（フィリップスLTV1200）
現在在宅でクリーンエアVEILA使用

＜栄養管理＞
VGチューブにて経管栄養
シリンジ・カップフィーディングでミルクや水分摂取は可能

経口での離乳食摂取も可能だが、本人が離乳食を嫌がる。
赤ちゃんせんべいは自分でつかんで摂取可能

＜リハビリテーション＞
H25. 5.16 か訪問リハビリテーション開始

＜合併症＞
難聴（補聴器装着）
脳波異常
てんかん（痙攣は不明）
浮腫（時々認める）
呼吸器感染症
便秘
水頭症
大後頭孔狭窄（手術は脳外科に不可能と言われている）
鼠径ヘルニア（手術施行）

＜精神・運動発達＞
手足の動きあり
寝返りあり
あやすと笑う
顔をしかめる
人の区別はできている印象（家族）
自分の意思を伝える（母に来てほしいときの合図）
眠たくなると自分でガーゼを顔の上にのせる
わざとチューブを抜く
お姉ちゃんが踊っていると、体全体を動かして真似をしている
足を上げてしばらくの間挙上したままにできる

＜歯芽＞
乳歯あり

D．考察
本研究においては、胎児骨系統疾患を妊娠中から適正に診断して、その後の妊娠管理や分娩形式の決定に役立てるという目標が相当部分実現してきたと思われる。全国各地の医療機関から胎児骨系統疾患フォーラムに診断依頼が来る状況となっており、可能な限り正確な診断に努めている。そのための学術講演会も毎年定期的に開催しており、成果がでている。病名変更は平成２４年度に実現して、新聞報道もされており、実際に患児を育てておられるご家族からも歓迎されている。引き続き長期生存例の発育調査を継続していきたい。

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胎児骨系統疾患の診断に有用な胎児ＣＴの被ばく量軽減の試み　第49回日本周産期・新生児学会　平成25年7月14～16日　パシフィコ横浜

澤井英明、山田崇弘、佐藤秀平、室月淳、堤誠司、篠塚憲男、林聡、高橋雄一郎、佐世正勝、宮嵜治　胎児骨系統疾患の診断に有用な３次元ヘリカルＣＴによる胎児被ばく線量の軽減に向けた取り組み：全国調査による診断参考レベルの推定　第65回日本産科婦人科学会　平成25年5月10日～12日　ロイトン札幌

H. 知的所有権の取得状況

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

Ⅱ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

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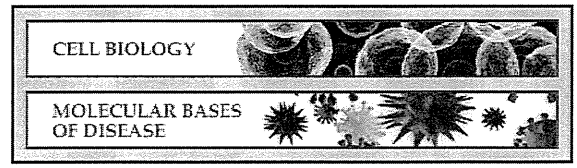
澤井英明	遺伝子診断による早期診断　骨系統疾患	周産期医学	44(2)	219-222	2014

Ⅲ. 業績別刷

Cell Biology:

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



Masanobu Kawai, Saori Kinoshita, Akihito Kimoto, Yasuhiro Hasegawa, Kazuaki Miyagawa, Miwa Yamazaki, Yasuhisa Ohata, Keiichi Ozono and Toshimi Michigami

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

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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.
Results: FGF23 suppresses chondrocyte proliferation in cooperation with soluble α-Klotho.
Conclusion: Elevated FGF23 could have a causative role in the development of growth retardation in XLH.
Significance: This may provide insights into the unrecognized function of FGF23 signaling in chondrocyte biology.

Fibroblast growth factor-23 (FGF23) is well established to play crucial roles in the regulation of phosphate homeostasis. X-linked hypophosphatemic rickets (XLH) is characterized by impaired mineralization and growth retardation associated with elevated circulating FGF23 levels. Administration of phosphate and calcitriol is effective in improving growth retardation, but is not sufficient to fully reverse impaired growth, suggesting the existence of a disease-specific mechanism in the development of growth retardation in addition to dysregulated phosphate metabolism. However, the precise mechanisms of growth retardation in XLH remain elusive. Here, we 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 FGFR3 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 *Hyp* 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, produces large amounts of FGF23, but other tissues including small intestine, heart, and ventrolateral thalamic nucleus and thymus produce FGF23 as well although the physiological significance of FGF23 produced from these tissues remain 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). Sitara *et al.* generated a mouse model where both *Fgf23* and *Slc34a1*, encoding for the type IIa sodium-phosphate (Na^+/Pi) co-transporter, were deleted to reverse the hyperphosphatemia noted in *Fgf23*-null mice (10). These double mutants exhibited similar skeletal phenotypes to *Fgf23*-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, adenoviral 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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[S] This article contains supplemental Figs. S1–S4.
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[2] 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 *Fgfr3* deficient mice without altering circulating phosphate and vitamin D levels, indicating that signals exerted from FGFR3 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 TransGenic Inc. Expression vector containing human FGFR3 (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 (αMEM) supplemented with 5% FBS and ITS.

Isolation of Primary Chondrocytes—Primary chondrocytes were isolated from rib cages obtained from 3-day old C57BL6/J 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 reaction 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 using ImmunoCruz Staining Systems (Santa Cruz Biotechnology). The TUNEL assay was performed using the *In situ* Apoptosis Detection kit (Takara Bio Inc.) according to the manufacturer's protocol.

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—Knock-down 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 1×10^7 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 Vivo sKL Administration—From postnatal day 7, 0.02 mg/kg of sKL was intraperitoneally administered in wild-type or *Hyp* mice for 3 days.

In Vivo BrdU Assay—BrdU (100 μg/gBW) and FdU (12 μg/gBW) were injected intraperitoneally as described previously (20). Two hours after injection, tibiae were collected and fixed in 4% PFA overnight at 4 °C. Tibiae were decalcified using 20% EDTA (pH 7.4) for 14 days and subjected to immunohistochemistry for BrdU. Following this, blocking sections were incubated with a mouse monoclonal anti-BrdU antibody (1:100, Calbiochem) overnight at 4 °C. Sections were then incubated with a biotinylated goat anti-mouse secondary antibody, followed by incubation with streptavidin-biotinylated HRP complex, and visualized with 3,3′-diaminobenzidine.

Generation of Conditioned Media—The expression vector containing Indian hedgehog (pcDNA3.1-Ihh) was kindly provided by Dr. Nishimura (Osaka University School of Dentistry). pcDNA3.1-Ihh or an empty vector was transfected into HEK293 cells using FuGene HD reagent (Promega) in DMEM containing 10% fetal calf serum. Two days post-transfection; conditioned media were collected and centrifuged to remove cell debris.

Measurement of Serum Parameters—Measurement of plasma phosphate and FGF23 concentrations was carried out using P-test Wako (Wako) and sandwich ELISA (Kainos Laboratory), respectively, following manufacturers' instructions.

Statistical Analysis—All data are expressed as the mean ± S.E. Results were analyzed for significant differences using the Student's *t* test or ANOVA followed by the Bonferroni multiple comparison *post hoc* test. Significance was set at $p < 0.05$.

RESULTS

FGF23 Elicits Its Signals in the Presence of Soluble α-Klotho in Chondrocytes in Vitro—To investigate whether FGF23 exerts direct effects on chondrocytes, we examined the expression profile of α-Klotho in chondrogenic ATDC5 cells and primary chondrocytes since α-Klotho has been shown to be required for FGF23 to mediate its signals. *α-Klotho* transcripts were detectable in these cells by real-time RT-PCR, but expression levels were more than hundred times lower than those in the anterior pituitary, known to abundantly express *α-Klotho* (supplemental Fig. S1*A*). Consistent with the extremely low levels of *α-Klotho* transcripts in chondrogenic cells, immunohistochemical analysis failed to detect α-Klotho protein expression in metatarsals (supplemental Fig. S1*B*). In addition, the expression profile of *α-Klotho* did not show any alteration during *in vitro* chondrogenesis (supplemental Fig. S1*A*). These lines of evidence led us to speculate that α-Klotho is unlikely to be functional in chondrogenic cells. Consistent with this speculation, recombinant FGF23 protein failed to induce ERK1/2 phosphorylation in chondrogenic cells, although FRS2α was weakly phosphorylated by FGF23 at a dose of 100 ng/ml (Fig. 1, *A* and *C*). These data indicate that FGF23 alone is not likely to exert its action on chondrogenic cells due to the lack of functional α-Klotho expression. Because there is evidence that sKL has an important role in mediating FGF23 signaling pathways in cells that do not express functional α-Klotho, we tested the concept that FGF23 may elicit its signals in the presence of sKL

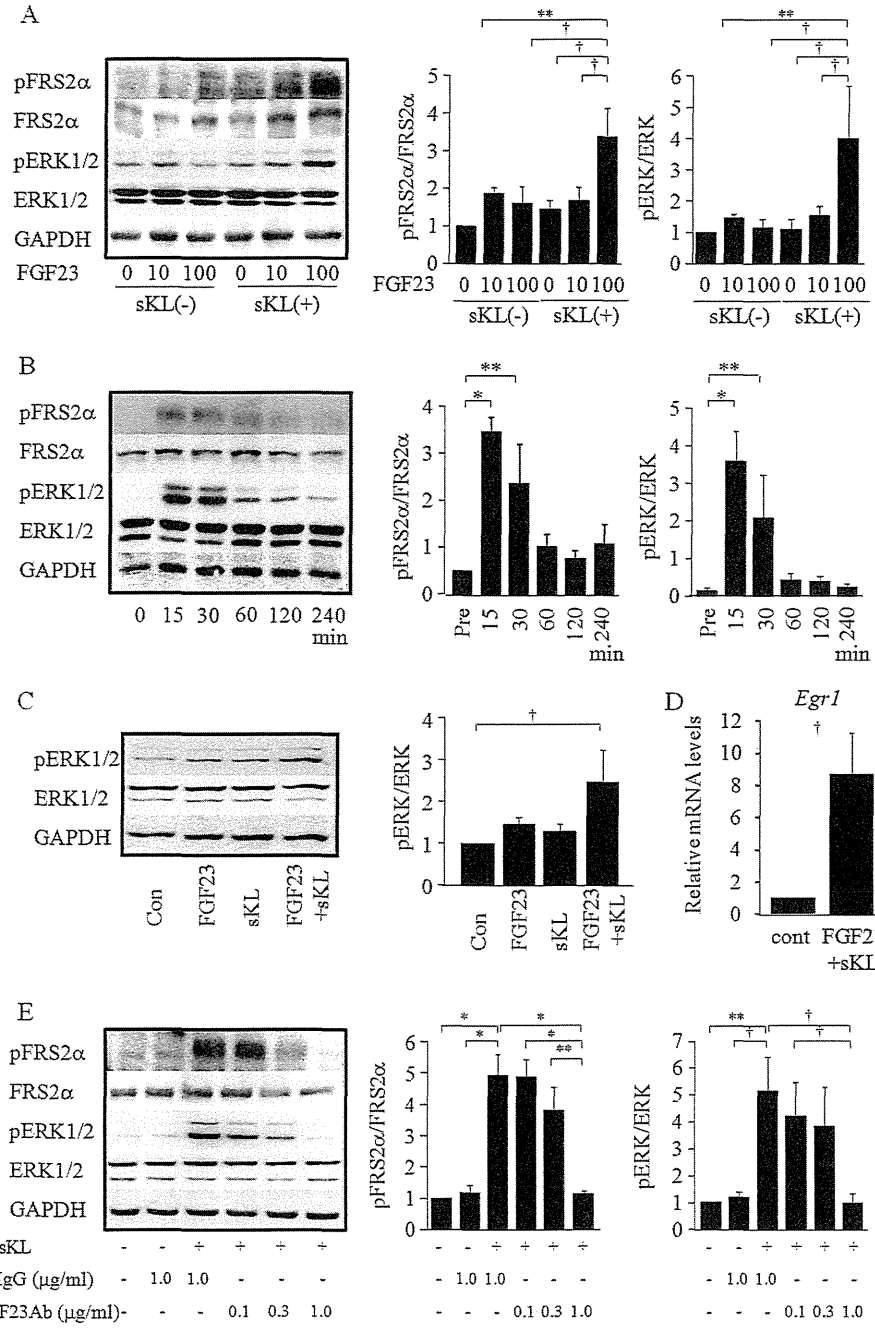

FIGURE 1. **FGF23 activates downstream signaling pathways in the presence of sKL in chondrogenic cells.** *A–C*, ATDC5 cells were treated with FGF23 in the presence or absence of sKL (100 ng/ml) for 15 min (*A*) or FGF23 (100 ng/ml) and sKL (100 ng/ml) for the indicated period (*B*). Primary chondrocytes were treated with FGF23 (100 ng/ml), sKL (100 ng/ml), or both for 15 min (*C*). Western blot analysis was performed using whole cell lysates and quantification was performed using densitometry ($n = 3$–4). *D*, ATDC5 cells were treated with FGF23 (100 ng/ml) and sKL (100 ng/ml) for one hour. Expression of *Egr1* was determined by real-time RT-PCR ($n = 3$). *E*, ATDC5 cells were treated with FGF23 (100 ng/ml) and sKL (100 ng/ml) in the presence or absence of neutralizing antibodies for FGF23 (anti-FGF23 Ab) at the indicated concentration for 15 min. Normal mouse IgG was used as a control for anti-FGF23 Ab. Western blot analysis was performed using whole cell lysates and quantification was performed using densitometry ($n = 3$). The figures shown are the representative from at least three independent experiments. The values were expressed as mean ± S.E. *, $p < 0.001$; **, $p < 0.01$; †, $p < 0.05$.

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. 2*A*). 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. 2*D*). Based on these observations, we next examined the effect of sKL with respect to FGF23 signaling in chondrogenic cells. FGF23 in the presence

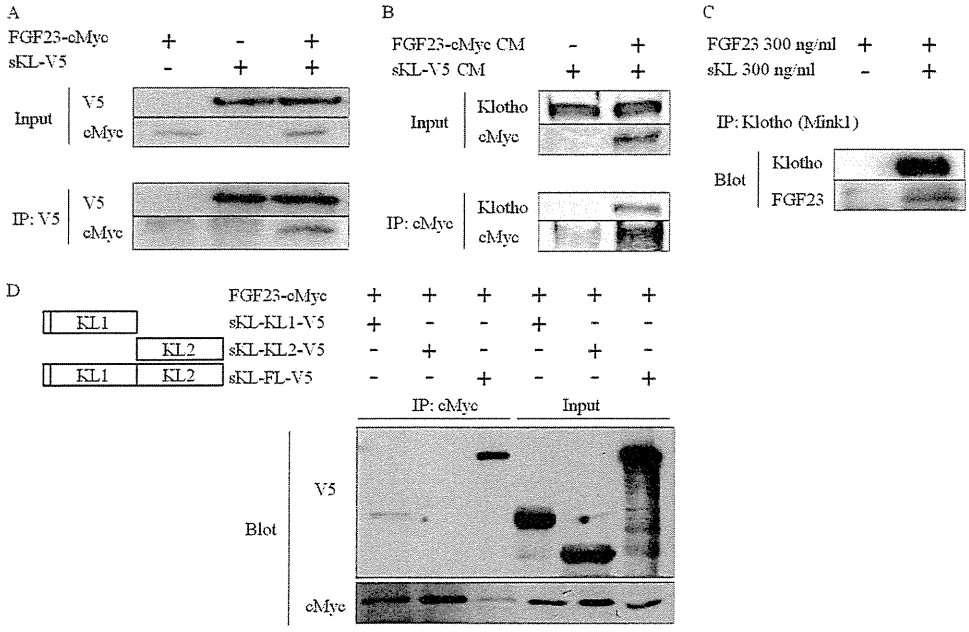

FIGURE 2. **FGF23 makes a protein complex with sKL.** *A*, HEK293 cells were transfected with FGF23-cMyc or sKL-V5 expression vectors. Immunoprecipitation with V5 was followed by Western blot analysis for cMyc. *B*, conditioned-media containing FGF23-cMyc were mixed with sKL-containing conditioned-media. 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. 1*D*). 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. 1*E*).

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 FGF23/sKL in the presence or absence of 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 *Fgfr3* 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. 4*G*, 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 *Fgfr3*. 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. 5*A*). In addition, binding of FGF23 to FGFR3 was enhanced as well when sKL was present (Fig. 5*B*). These data indicate that one of the mechanisms by which sKL enhanced FGF23 signaling pathway would be mediated through enhancing the accessibility of FGF23 to its receptors.

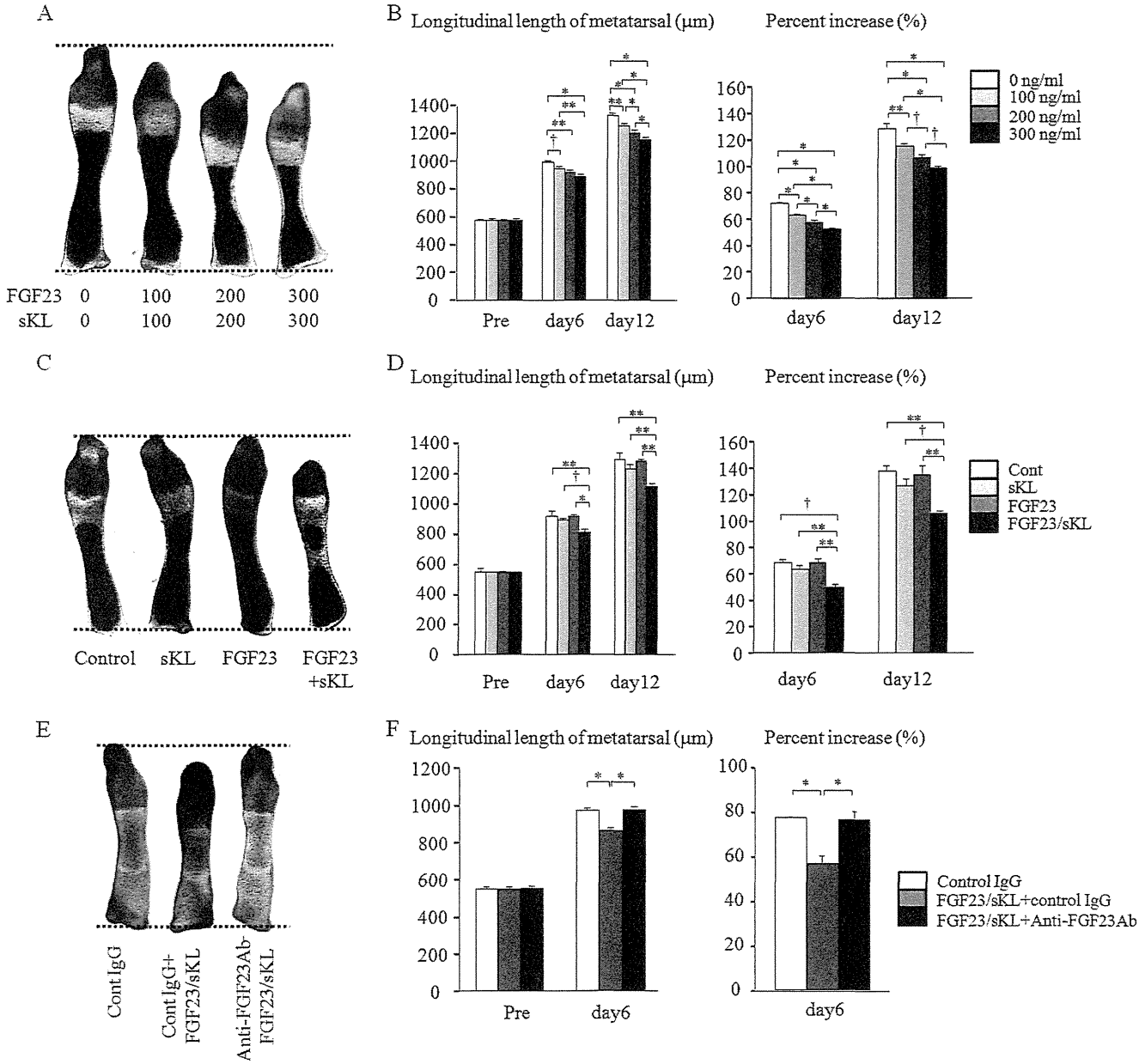

FIGURE 3. **FGF23 impairs longitudinal growth of metatarsals in cooperation with sKL.** *A–D*, metatarsals were cultured for 12 days in various concentrations of FGF23 and sKL as indicated (*A* and *B*) or treated with FGF23 (300 ng/ml), sKL (300 ng/ml), or both (*C* and *D*). Longitudinal lengths and percent increases in metatarsals were measured ($n = 3–4$). *E* and *F*, metatarsals were cultured with FGF23 (300 ng/ml) and sKL (300 ng/ml) in the presence or absence of neutralizing antibodies for FGF23 (anti-FGF23 Ab) (3 μg/ml) for 6 days, and longitudinal lengths and percent increases in metatarsals were measured ($n = 3–4$). Normal mouse IgG was used as a control for anti-FGF23 Ab. The values were expressed as mean ± S.E. *, $p < 0.001$; **, $p < 0.01$; †, $p < 0.05$.

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. 6*E*). 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

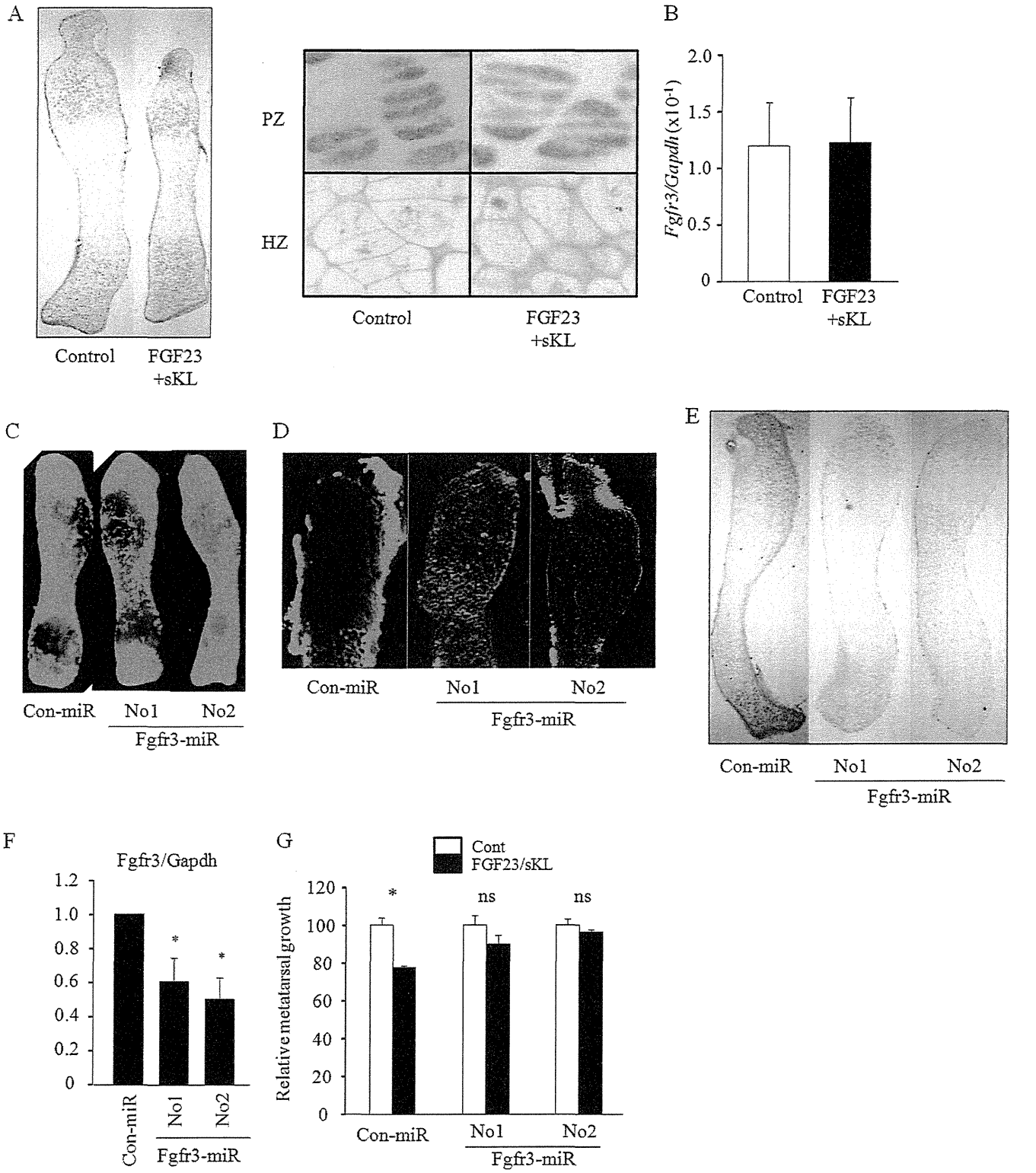

FIGURE 4. **FGFR3 partly mediates the suppressive effect of FGF23/sKL on metatarsal growth.** *A* and *B*, metatarsals were cultured in the presence or absence of FGF23 (300 ng/ml) and sKL (300 ng/ml) for 6 days. FGFR3 expression was analyzed by immunohistochemistry (*A*) and real-time RT-PCR (*B*) ($n = 3$). *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. 7*A*), which was associated with comparable expression levels of *Sox9* and *Col2a1* between the two groups (Fig. 7*B*). 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. 8*C*), 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. 9*A*). In line with

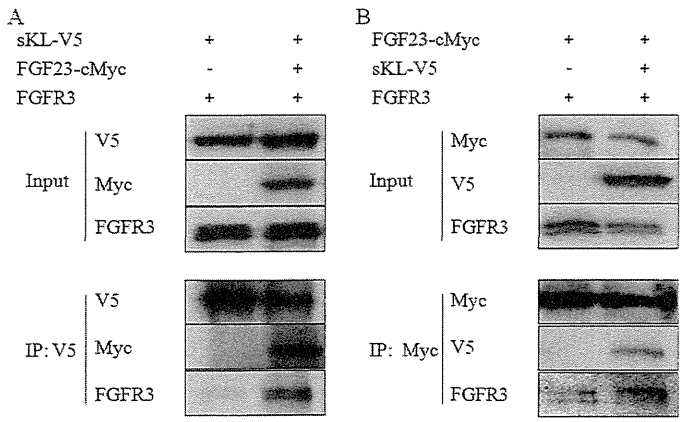

FIGURE 5. **Interaction between FGF23 and FGFR3 is enhanced in the presence of sKL.** *A*, HEK293 cells were transfected with FGFR3 and sKL-V5 expression vectors in the presence or absence of FGF23-cMyc expression vector. Immunoprecipitation with V5 was followed by Western blot analysis for cMyc and FGFR3. *B*, HEK293 cells were transfected with FGFR3 and FGF23-cMyc expression vectors in the presence or absence of sKL-V5 expression vector. Immunoprecipitation with cMyc was followed by Western blot analysis for V5 and FGFR3. The figures shown are the representative from at least three independent experiments.

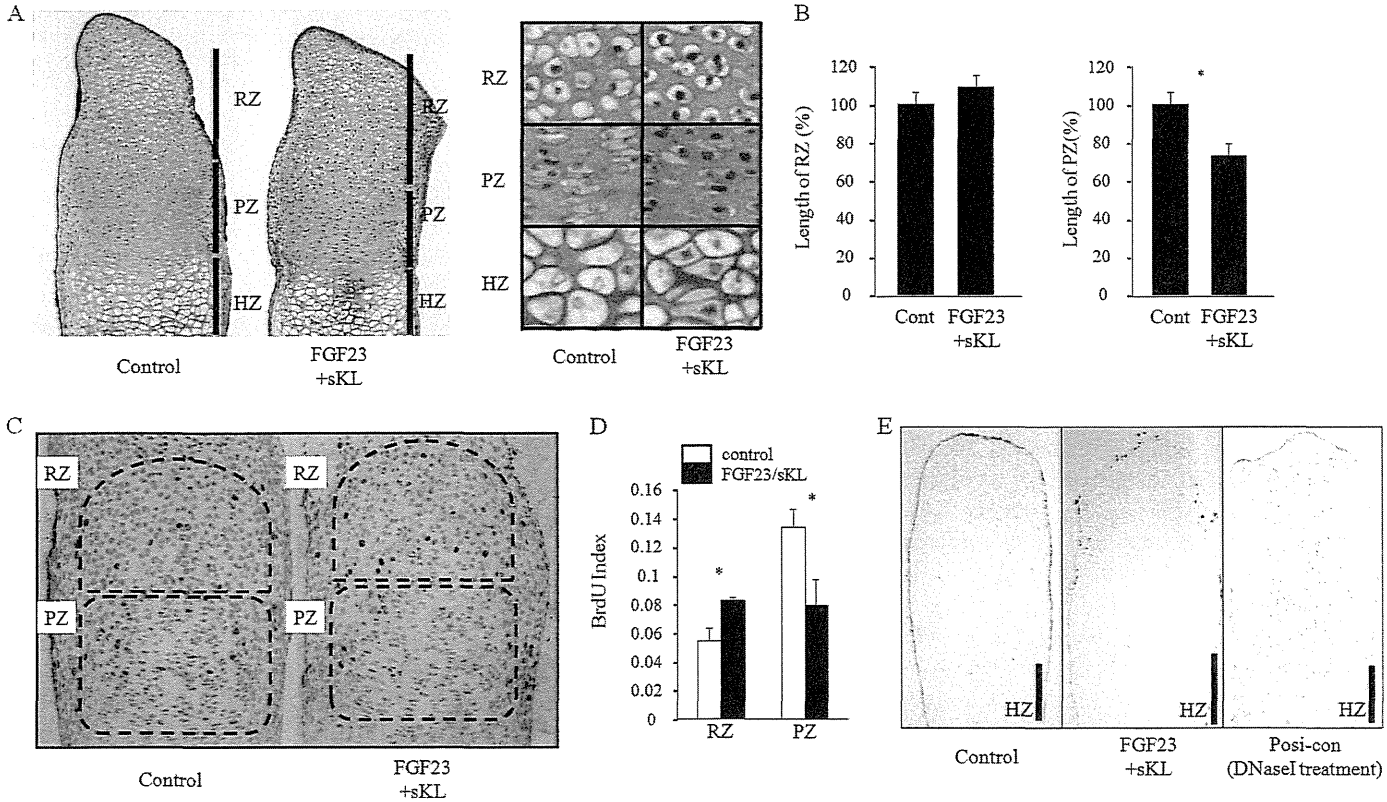

FIGURE 6. **FGF23 suppresses chondrocyte proliferation in the proliferating zone in the presence of sKL.** *A* and *B*, metatarsals were cultured for 12 days with or without FGF23 (300 ng/ml) and sKL (300 ng/ml). Paraffin-embedded sections were subjected to Hematoxylin and Eosin staining (*A*) and lengths of resting and proliferating zones were determined ($n = 5$) (*B*). *C–E*, metatarsals were cultured for 6 days with or without FGF23 (300 ng/ml) and sKL (300 ng/ml). BrdU staining (*C*) and TUNEL staining (*E*) were performed. The ratio of BrdU-positive cells over total cells was calculated (referred as BrdU index) ($n = 3–5$) (*D*). The figures shown are the representative from at least three independent experiments. The values were expressed as mean ± S.E. *, $p < 0.05$.

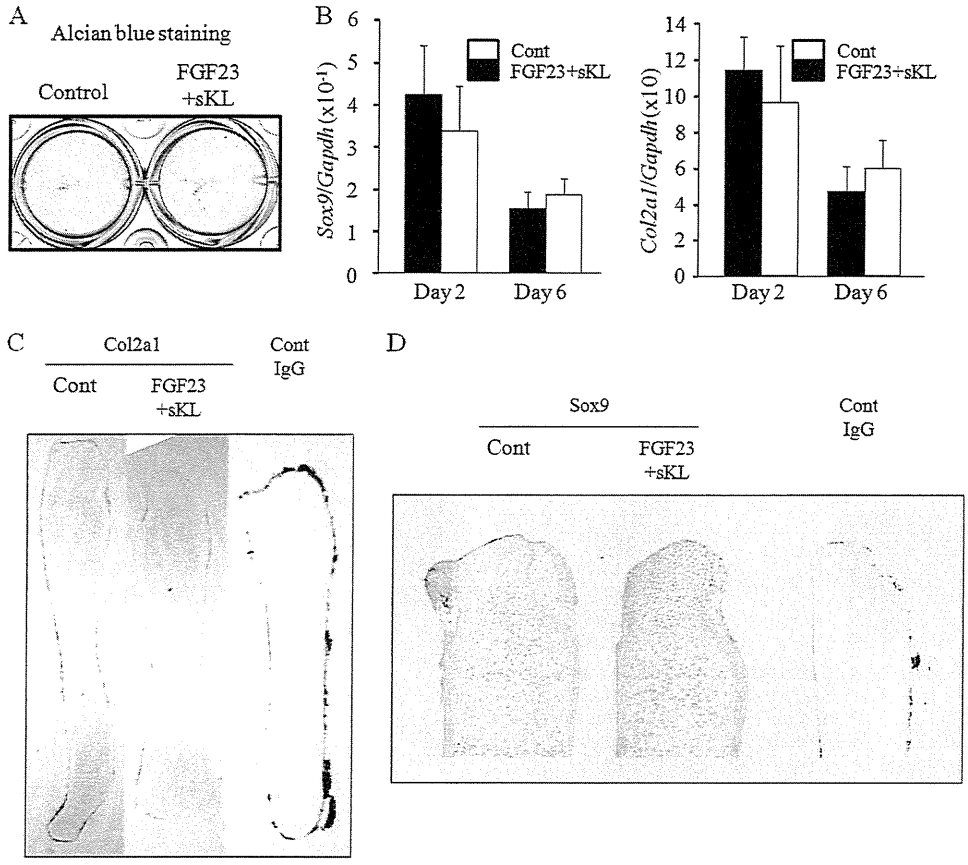

FIGURE 7. **FGF23/sKL does not affect the expression of Sox9 and Col2a1.** *A* and *B*, primary chondrocytes were grown to confluence and treated with chondrogenic media in the presence or absence of FGF23 (100 ng/ml) and sKL (100 ng/ml) for 6 days. Alcian blue staining was performed on day 6 (*A*), and expression levels of *Sox9* and *Col2a1* were determined by real-time RT-PCR on day 2 and 6 (*B*) ($n = 4$). *C* and *D*, 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 Col2a1 (*C*) and Sox9 (*D*) were analyzed by immunohistochemistry ($n = 3–4$). The figures shown are the representative from at least three independent experiments. The values were expressed as mean ± S.E.

these observations, immunohistochemical analysis showed that Ihh expression was lower in metatarsals treated with FGF23/sKL than that in controls (Fig. 9*B*). 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. 9*C*). 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. 10*A*). Administration of sKL did not show any influence on body weight gain in wild-type or *Hyp* mice (Fig. 10*B*, supplemental Fig. S4*A*), but caused a shortening of the longitudinal length of the tibia in *Hyp* mice (Fig. 10*C*), whereas sKL did not affect the length of the tibia in WT mice (supplemental Fig. S4*B*). 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. 10*D*), which was accompanied by a decline in the BrdU index in sKL-treated *Hyp* mice (Fig. 10*E*). 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. 10*F* and *G* and supplemental Fig. S4*E*, 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. 10*H* and supplemental Fig. S4*F*).

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-