Membrane and Integrative Nuclear Fibroblastic Growth Factor Receptor (FGFR) Regulation of FGF-23*

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Background: Mechanisms whereby local bone-derived factors regulate FGF-23 are unclear.

Results: Low and high molecular weight FGF-2 stimulated FGF-23 promoter activity in osteoblasts through membrane FGFR-mediated PLCy and MAPK activation of NFAT and Ets1 and integrative nuclear FGFR1 signaling involving cAMP/CBP/CREB signaling pathways, respectively.

Conclusion: Membrane FGFR and intranuclear FGFR/CBP pathways regulate FGF-23 transcription.

Significance: Paracrine/autocrine FGFR signaling pathways are implicated in the regulation of FGF-23 gene transcription, but the molecular pathways remain poorly defined. We used low molecular weight (LMW, 18 kDa) FGF-2 and high molecular weight (HMW) FGF-2 isoforms, which, respectively, activate cell surface FGFR receptors and intranuclear FGFR1, to determine the roles of membrane FGFRs and integrative nuclear FGFR1 signaling (INFS) in the regulation of FGF-23 gene transcription in osteoblasts. We found that LMW-FGF-2 induced NFAT and Ets1 binding to conserved cis-elements in the proximal FGF-23 promoter and stimulated FGF-23 promoter activity through PLCy/calcineurin/NFAT and MAPK pathways in SaOS-2 and MC3T3-E1 osteoblasts. In contrast, HMW-FGF-2 stimulated FGF-23 promoter activity in osteoblasts through a cAMP-dependent binding of FGFR1 and cAMP-response element-binding protein (CREB) to a conserved cAMP response element (CRE) contiguous with the NFAT binding site in the FGF-23 promoter. Mutagenesis of the NFAT and CRE binding sites, respectively, inhibited the effects of LMW-FGF-2 and HMW-FGF-2 to stimulate FGF-23 promoter activity. FGF-2 activation of both membrane FGFRs and INFS-dependent FGFR1 pathways may provide a means to integrate systemic and local regulation of FGF-23 transcription under diverse physiological and pathological conditions.

FGF-23 is a member of the hormone-like FGF gene family that also includes FGF-19 and FGF-21 (1). FGF-23 is predominately secreted by osteoblasts and osteocytes in bone (2). Hormone-like FGF-23 evolved from paracrine/autocrine FGFs through the emergence of a novel C terminus that permits diffusion and heparin-independent binding to a binary membrane receptor complex consisting of FGFR and α-Klotho, a type I membrane β-glycosidase (4–9). FGF-23 activation of FGFR/α-Klotho complexes in the kidney leads to reductions in Npt2a co-transporters and inhibition of renal tubular phosphate reabsorption and reductions in circulating 1,25(OH)2D levels through inhibition of Cyp27b1 and stimulation of Cyp24 enzymes regulating vitamin D metabolism (3–9).

Pathological increments in circulating FGF-23 concentrations underlie acquired and hereditary forms of hypophosphatemic rickets, whereas decrements in FGF-23 cause hereditary tumoral calcinosis (10). Physiologically, FGF-23 participates in systemic and local regulatory networks that control serum phosphate and 1,25(OH)2D levels. As a counter-regulatory hormone for 1,25(OH)2D, elevations of FGF-23, which is induced by 1,25-(OH)2D, parathyroid hormone (PTH), or calcium (11), results in reductions in serum phosphorus levels and suppression of 1,25(OH)2D production (14, 15). FGF-23 also coordinates bone mineralization with renal handling of phosphate through poorly defined local processes that involve classical paracrine FGFR1 activation (1, 11–22).

Emerging data suggests that the earlier evolved paracrine/autocrine FGFR signaling pathways remain linked to the more recent hormonal FGF-23 (1, 10, 23). First, FGF-23 is increased in osteoglyphic dysplasia, which is caused by activating mutations in FGFR1 (24, 25). Second, ligands for FGFR1, including FGF-1, LMW–FGF-2, and FGF-7, as well as HMW–FGF-2, are significantly increased in the Hyp and/or Dmp1 knock-out mouse models of FGF-23 excess (10, 26–28). Third, pharmacological inhibition of FGFR1 blocks FGF-23 transcription in bone both in vitro and ex vivo (10, 29, 30). Fourth, the administration of monoclonal FGFR1 activating antibodies stimulates FGF-23 production and induces hypophosphatemia (31). Finally, and most specifically, conditional deletion of FGFR1 in osteocytes of the Hyp mouse model reduces FGF-23 expression in bone (32).

The FGF-2 gene produces 18-kDa low molecular weight (LMW)2 FGF-2 and 22–34-kDa high molecular weight FGF-2

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2 The abbreviations used are: LMW, low molecular weight; HMW, high molecular weight; FSK, forskolin; Hyp, hypophosphatemic mouse XLH homologue; NLS, nuclear localization sequence; CREB, cAMP-response element-binding protein; INFS, integrative FGFR1 nuclear pathway; PIC, protease inhibitor mixture; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; PLCγ, phospholipase Cγ; SP, secretory peptide; CsA, cyclosporin A; NFAT, nuclear factor of activated T cells.
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isoforms created by alternative initiation codons (33). Membrane signaling involves extracellular LMW-FGF-2 formation of ternary complexes with cell surface FGFRs and heparin-sulfate proteoglycans (34). Cell surface FGFRs are principally coupled to PI3K/Akt, RAS/MAPK, and PLCγ intracellular signaling pathways (35). In contrast, HMW-FGF-2 isoforms have an N-terminal nuclear localization sequence (NLS) that leads to nuclear localization and activation of intracellular FGFR1/CBP/CREB signaling pathways (also called integrative FGFR1 nuclear pathway or INFS) (36). INFS appears to be the earliest evolved FGFR signaling mechanism (37). To understand the roles of membrane FGFR1 and INFS in regulating FGF-23 transcription, we examined the effects LMW-FGF-2 and HMW-FGF-2 on FGF-23 transcription in osteoblasts cell lines.

EXPERIMENTAL PROCEDURES

Cell Culture and Promoter Analysis—MC3T3-E1 osteoblast precursor cells and SaOS-2 osteoblast cells were cultured according to American Type Culture Collection guidelines. Briefly, 3–5 × 10^4 cells were seeded in 6-cm diameter tissue culture plates in α-MEM (Life Technologies, Grand Island, NY) with 10% fetal calf serum at 37 °C in the presence of 5% CO_2 in a humidified incubator. Cells were plated 18 h before transfection and fed fresh medium 4 h before transfection. FGF-23 promoters (mFGF-23 and hFGF-23) DNA were constructed into a pGL3 basic reporter gene (Promega, Madison, WI). To create mutations of NFAT, CREB, or both NFAT/CREB sites in the FGF-23 promoter, a GENEART Site-directed Mutagenesis System (Life Technologies) was used by following the manufacturer’s instructions. All FGF-23 reporter plasmid DNAs were introduced into MC3T3-E1 or SaOS-2 cells using cationic liposomes (LipofectAMINE2000, Life Technologies). Co-transfections (0.25 μg of FGF-23 promoter plasmid DNAs with FGFR1, FGFR2, FGFR3, FGFR4, HMW-FGF-2, or FGFR1(TK–), FGFR1(SP–/NLS), FGFR1(TK–/SP–/NLS) plasmid DNAs) was carried out for 16–18 h, and then cells were washed twice with phosphate-buffered saline and incubated in fresh medium containing 10% fetal calf serum for 38 h. LMW-FGF-2, Cyclosporine A (Sigma), or U0126 (Cell Signaling Technology) containing 10% fetal calf serum for 38 h. LMW-FGF-2, Cyclosporine A (Sigma), or U0126 (Cell Signaling Technology) was added to the cell cultures 24 h before the cells were harvested. To standardize the transfection efficiency, 0.1 μg of pRL-CMV vector (pRL Renilla reniformis luciferase control reporter vector; Promega) was cotransfected in all experiments. Cells were harvested 72 h after transfection and lysed in 50 μl of reporter lysis buffer (Promega). A luciferase assay (20 μl of cell lysed) was performed using a dual luciferase assay kit (Promega), and activity was measured with an Opticon 1 luminometer (MGM Instruments, Inc., Hamden, CT). Promoter activity (mean ± S.D. of triplicate samples in relative fold-changes) is represented by the reporter activity normalized to the pRL-CMV control. Graphs represent typical results of 3–4 separate experiments. To induce osteogenic differentiation for MC3T3 and SaOS-2 cells, cells were first cultured in the α-MEM containing 10% FBS for 3 days to grow cells to about 80% confluence. Then, these cells were cultured in serum-free medium supplemented with 100 μg/ml of ascorbic acid, 10 mM β-glycerophosphate for another 18 days. Supernatants from day 21 cells cultured in the conditional medium were collected and concentrated using Amicon Ultra-4 Centrifugal Filters (10k) (Millipore, Temecula, CA). Full-length FGF-23 levels were measured using the FGF-23 ELISA kit (Kainos Laboratories, Tokyo, Japan) following the manufacturer’s recommendations.

Chromatin Immunoprecipitation (ChIP) Assays—ChIP assays were performed with a kit from Cell Signaling Technology (Danvers, MA) according to the manufacturer’s instructions with the following modifications. Approximately 4 × 10^7 MC3T3-E1 or SaOS-2 cells in 100-cm^2 culture dishes were cross-linked in 1% formaldehyde solution (Fisher Scientific, Pittsburgh, PA) for 10 min at room temperature, the cross-linking was stopped by adding 1 M glycine for 5 min at room temperature. Cells were washed twice with ice-cold 1X phosphate-buffered saline solution (PBS) (Life Technologies) and harvested in 1-ml of 1X PBS + protease inhibitor mixture (PIC) + phenylmethylsulfonyl fluoride (PMSF) (Sigma). Cells were then pelleted by centrifugation at 1500 × g for 5 min at 4 °C and the pellet was resuspended in 10 ml of ice-cold buffer A with DTT, PIC, and PMSF on ice for 10 min. Cell nuclei were pelleted by centrifugation at 3000 × g for 5 min at 4 °C. The nuclei pellet was then washed in 10 ml of ice-cold buffer B with DTT, and resuspended in 1.0 ml of buffer B with DTT. Micrococcal nuclease was added to the nuclei and incubated for 20 min at 37 °C. Digestion was stopped by adding 100 μl of 0.5 M EDTA, and the nuclei were pelleted by centrifugation at 13,000 × g for 1 min at 4 °C. The nuclear pellet was resuspended in 0.5 ml of 1X ChIP buffer with PIC and PMSF for sonication using the VirSonic ultrasonic cell disruptor 100 (VirTis, Gardiner, NY) to shear the DNA to an average length of 300 to 500 base pairs (six, 15-s bursts on ice). Samples were stored at –80 °C before use. For chromatin immunoprecipitation, 200 μl of the cross-linked chromatin preparation were added to 800 μl of ChIP buffer with PIC. Immunoprecipitations were carried out with 2 μg of antibodies (CREB, Est-1, or NFATc1 from Santa Cruz Biotechnology, Santa Cruz, CA, and FGFR1 from Cell Signaling) and 0.2 μg of p-CREB (Santa Cruz). Histone H3 rabbit mAb (2 μg) and normal rabbit IgG (1 μg) were used as positive or negative controls, respectively. After overnight immunoprecipitation on a rotator at 4 °C, ChIP samples were then washed and eluted and DNA was uncross-linked with NaCl and subsequently treated with proteinase K, Tris-HCl, and EDTA. DNA was purified and subjected to PCR amplification of the FGF-23 promoter DNA containing putative binding sites for CREB and NFAT transcription factors using specific primers (mouse: forward primer, 5'-tgaccagaggtcagata-3', reverse primer, 5'-gaagtgggaggtctctg-3'; human: forward primer, 5'-cgctctggggtctttgca-3', reverse primer, 5'-tgctctggggtctttgca-3').

RT-PCR—Total RNA was extracted from SaOS-2 and MC3T3-E1 osteoblasts using an RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. cDNAs were synthesized using by a iScript^TM cDNA Synthesis Kit (Bio-Rad). RT-PCR was performed to examine the FGF-23 mRNA, FGFR1, FGFR2, FGFR3, and FGFR4 in the SaOS-2 and MC3T3-E1 osteoblasts using a pair of primers as follows: human FGF-23, 5'-cagatgaggtctctcag-3' (forward) and 5'-ccagccatctctcttgctg-3' (reverse); mouse FGF-23, 5'-caacctgagagctgctgg-3' (forward) and 5'-ctctgtctctgctttc-3' (reverse); human FGFR1, 5'-aaggcaacaccaagcttgg-3' (forward) and 5'-ccaaatctgctatctcacc-3' (reverse); human FGFR2, 5'-ctgtgctaggaagcaag-3'.

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LMW-FGF-2 Stimulates FGF-23 Promoter Activity Principally through FGFR1—To study the role of specific FGFRs in FGF-23 gene regulation, human p700-FGF-23 proximal promoter and mouse p600-FGF-23 proximal promoter-luciferase constructs were, respectively, co-transfected with vector DNA, FGFR1, FGFR2, FGFR3, or FGFR4 in SaOS-2 and MC3T3-E1 cells and stimulated with LMW-FGF-2 (50 ng/ml). We found that LMW-FGF-2 resulted in a minimal 2-fold increase in promoter activity in control (vector-transfected) SaOS-2 cells, reflecting endogenous FGFR activation (Fig. 1D). Co-transfection of FGFR1, FGFR2, FGFR3, or FGFR4 in the absence of LMW-FGF-2, also resulted in a minimal 2–3-fold increase in FGF-23 promoter activity as compared with vector control in SaOS-2 cells. LMW-FGF-2 in FGFR1-cotransfected SaOS-2 cells resulted in a marked 9-fold increase of FGF-23 promoter activity (Fig. 1D). In contrast, LMW-FGF-2 resulted in only a 3–4-fold increase in FGFR2-, FGFR3-, or FGFR4-cotransfected SaOS-2 cells (Fig. 1D). Similar results were found in MC3T3-E1 cells (Fig. 1F).

We found that LMW-FGF-2 resulted in a dose-dependent increase in promoter activity, reaching an approximate 2–3-fold increase in both cell types at LMW-FGF-2 concentrations of 50 ng/ml (Fig. 1, G and J). LMW-FGF-2 also stimulated increments in endogenous FGF-23 transcripts in SaOS-2 and MC3T3-E1 osteoblasts as assessed by RT-PCR (Fig. 1, H and J).

To further investigate the specific role of FGFR1, we tested the effects of LMW-FGF-2 to activate the FGF-23 promoter in the presence of a co-transfected wild-type FGFR1 or FGFR1(TK−) mutant cDNA construct that lacks the tyrosine kinase domain and forms non-functional dimers with endogenous FGFR1 receptors. Co-transfection of FGFR1 cDNA resulted in a 6–10-fold stimulation in FGF-23 promoter activity for SaOS-2 and MC3T3-E1 osteoblasts (Fig. 1, K and L). This response was enhanced compared with the 2–3-fold induction of FGF-23 by endogenous FGFRs. In contrast, overexpression of the dominant-negative FGFR1(TK−) mutant resulted in inhibition of LMW-FGF-2-mediated FGFR1 stimulation of the FGF-23 promoter in MC3T3-E1 osteoblasts in a dose-dependent manner (Fig. 1M). LMW-FGF-2 also stimulated FGFR1-mediated FGF-23 gene expression in MC3T3-E1, which was inhibited by co-transfection of the domi-
nant-negative FGFR1(TK/H11002) construct (Fig. 1N), as determined by RT-PCR of FGF-23 mRNA (top) and FGF-23 protein (bottom).

To assess the effects of LMW-FGF-2 on FGF-23 protein expression in cultured osteoblasts, we compared FGF-23 secretion in the medium of MC3T3-E1 osteoblasts after transfection with wild-type FGFR1 or FGFR1(TK/H11002) mutant constructs. FGF-23 levels in conditioned medium were 10 pg/dl before LMW-FGF-2 stimulation and increased to 40 pg/dl after administration of LMW-FGF-2 to cells transfected with FGFR1. In contrast, FGFR1(TK/H11002)-transfected osteoblasts did not increase the FGF-23 levels in response to LMW-FGF-2 stimulation (Fig. 1J).

FGFR-dependent Activation of the FGF-23 Promoter through PLCγ and Calcineurin-NFAT Signaling Cascade—Cell surface FGFR1 is coupled to at least three major signaling pathways, including PI3K/AKT, Ras/MAPK, in which ERK plays a central role, and PLCγ pathways (40, 41). We have previously shown that ERK inhibitors (PD98059, which inhibits MAPK kinase and U0126, which inhibits MEK1 and MEK2) inhibit LMW-FGF-2-mediated FGFR1 activation of the FGF-23 promoter in MC3T3-E1 cells (32). In contrast, the phosphoinositide 3-kinase inhibitor wortmannin had no effect of LMW-FGF-2 stimulation of FGF-23 promoter activity in MC3T3-E1 osteoblasts (32).

Because the calcineurin-NFAT cascade is an important downstream target of PLCγ (42) and Ets-1 is a key cis-acting element in the MAPK/ERK signaling cascade (43), we examined whether the proximal FGF-23 promoter possessed NFAT and Ets-1 binding sites. We found a putative consensus Ets-1 site that overlaps an NFAT site adjacent to a CREB binding site at positions 182 and 165 bp in the mouse FGF-23 promoter (44). These sites were conserved in the human FGF-23 promoters.

To establish that LMW-FGF-2 stimulates NFAT or Ets-1 binding to the consensus NFAT or Ets-1 binding site in the FGF-23 promoter, we performed ChIP assays using an NFAT
or Ets-1 antibody and RT-PCR on the immunoprecipitates with primers to amplify the region spanning the putative NFAT or Ets-1 binding sites. We found that stimulation of either SaOS-2 or MC3T3-E1 osteoblasts with LMW-FGF-2 resulted in binding of NFAT and Ets-1 to the endogenous FGF-23 proximal promoter region (Fig. 2, A and B). This binding was inhibited by treatment of osteoblasts with the calcineurin inhibitor CsA (Fig. 2, A and B) or with the MEK1 and MEK2 inhibitor U0126 (Fig. 2, A and B). Interestingly, inhibition of calcineurin by CsA or ERK1/2 by U0126 also blocks Ets-1 and NFAT binding to the FGF-23 promoter in both osteoblast cell lines (Fig. 2, A and B).

To evaluate the role of phospholipase Cγ-induced NFAT signaling, we used a NFAT reporter gene (Qiagen, Valencia, CA) to test the ability of LMW-FGF-2 to stimulate NFAT promoter activity in SaOS-2 and MC3T3-E1 osteoblasts. Incubation with LMW-FGF-2 stimulated NFAT activity by ~3-fold in both SaOS-2 and MC3T3-E1 osteoblasts (Fig. 2, C and E). To test the ability of the calcineurin inhibitor CsA to block LMW-FGF-2 stimulation of NFAT and FGF-23 promoter activity, we treated the cells with CsA. Incubation with CsA resulted in inhibition of FGF-2 stimulation of NFAT and FGF-23 reporter activity in both SaOS-2 and MC3T3-E1 osteoblasts, achieving an almost complete inhibition at concentrations of 1.0 μM (Fig. 2, C–F).

Finally, to confirm the role of NFAT signaling through the identified cis-acting element, we mutated the NFAT site in both the human and mouse FGF-23 promoter activities. Cyclosporine A (1 μM) blocks the effect of LMW-FGF-2 on NFAT reporter gene expression. D and F, LMW-FGF-2 stimulates FGF-23 promoter activity in both SaOS-2 and MC3T3-E1 osteoblasts. Cyclosporine A (1 μM) blocks the effect of LMW-FGF-2 on FGF-23 reporter gene expression. G and H, LMW-FGF-2 stimulates both human and mouse FGF-23 promoter activities. NFAT site mutation blocks LMW-FGF-2 effect. Data are expressed as the mean ± S.D. from three independent experiments. *, p < 0.05 versus control vector group.

FIGURE 2. LMW-FGF-2 up-regulates FGF-23 gene transcription via NFAT and MAPK pathways. A and B, LMW-FGF-2 enhanced NFAT and Ets-1 binding to the endogenous FGF-23 promoter as determined by CHIP assay using NFAT or Ets-1 antibody. First lane, 100-bp DNA standard; second lane, input ChIP DNA; third lane, nonspecific IgG; fourth lane, untreated control cells; fifth lane, FGFR1 co-transfected cells; sixth lane, FGFR1 co-transfected cells treated with LMW-FGF-2 (50 ng/ml) for 24 h; seventh lane, FGFR1 co-transfected cells treated with LMW-FGF-2 (50 ng/ml) in the presence of CsA (1 μM) for 24 h; eighth lane, FGFR1 co-transfected cells treated with LMW-FGF-2 (50 ng/ml) in the presence of U0126 (10 μM) for 24 h; and ninth lane, negative control. C and E, LMW-FGF-2 stimulates NFAT activity in both SaOS-2 and MC3T3-E1 osteoblasts. Cyclosporine A (1 μM) blocks the effect of LMW-FGF-2 on NFAT reporter gene expression. D and F, LMW-FGF-2 stimulates FGF-23 promoter activity in both SaOS-2 and MC3T3-E1 osteoblasts. Cyclosporine A (1 μM) blocks the effect of LMW-FGF-2 on FGF-23 promoter gene expression. G and H, LMW-FGF-2 stimulates both human and mouse FGF-23 promoter activities. NFAT site mutation blocks LMW-FGF-2 effect. Data are expressed as the mean ± S.D. from three independent experiments. *, p < 0.05 versus control vector group.
Integrative Nuclear FGFR1 Signaling

To explore the possibility for a role of INSF signaling, we initially tested “membrane arrested” FGFR1–4, a chimeric receptor in which the transmembrane domain of FGFR1 is replaced with the transmembrane domain of FGFR4 to limit receptor internalization, and the FGFR1(SP−/H11002/NLS)-mutated receptor, in which the secretory peptide (SP) required for plasma membrane insertion has been replaced with a NLS to promote nuclear uptake. Co-transfection of wild-type FGFR1 had no effect on FGF-23 promoter activity in the absence of ligand. However, addition of LMW-FGF-2 resulted in a 4-fold enhancement of FGF-23 promoter activity in FGFR1-transfected cells compared with control cells. Cells stimulated with LMW-FGF-2 alone only resulted in a 2-fold enhancement of FGF-23 promoter activity, reflecting the function of endogenous FGFRs. Co-transfection of the chimeric FGFR1/FGFR4 construct did not result in LMW-FGF-2 stimulation of FGF-23 promoter activity in both human and mouse osteoblast cells, whereas FSK (10 µM) alone showed little effect. Combination of LMW-FGF-2 and FSK enhances FGF-23 promoter activity by more than 5-fold as compared with controls. E and F, dose-dependent up-regulation of FGF-23 promoter activity by HMW-FGF-2 with fixed FSK (10 µM). G and H, dose-dependent up-regulation of FGF-23 promoter activity by FSK with fixed HMW-FGF-2 (0.25 µg). Data are expressed as the mean ± S.D. from three independent experiments. Values sharing the same superscript in different groups are not significantly different. *, p < 0.05; **, p < 0.01 versus control vector group, respectively.

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These findings are consistent with the presence of INSF signaling in osteoblasts. To directly test the role of INSF signaling in the regulation of FGF-23 promoter activity, we transfected a HMW-FGF-2 cDNA that encodes the intracellular ligand for intranuclear FGFR1 (36) into osteoblasts. We also tested the co-dependence of cAMP on HMW-FGF-2 regulation of FGF-23 promoter activity, because FGFR1 and HMW-FGF-2 co-localize in the nuclear matrix to activate gene transcription through interactions with the CBP (36, 45, 46).

In both SaOS-2 and MC3T3-E1 osteoblasts, transfection of HMW-FGF-2 (0.25 μg) alone resulted in an approximate 2-fold stimulation of FGF-23 promoter activity (Fig. 3, C and D). Surprisingly, addition of only forskolin (FSK, 10 μM) to the medium had no significant effects to stimulate the FGF-23 promoter activity in either SaOS-2 or MC3T3-E1 osteoblasts. However, in the presence of fixed amounts of FSK (10 μM), we observed a marked accentuation of the ability of HMW-FGF-2 to stimulate FGF-23 promoter activity. The combined effect of HMW-FGF-2 and FSK resulted in a 5–6-fold increase in FGF-23 promoter activity (Fig. 3, C and D). In the presence of FSK, a dose-dependent effect of HMW-FGF-2 to increase the FGF-23 promoter activity in both SaOS-2 and MC3T3-E1 osteoblasts was observed (Fig. 3, E and F). Conversely, in cells transfected with fixed amounts of transfected HMW-FGF-2 (0.25 μg), FSK also show a dose-dependent stimulation of FGF-23 promoter activity (Fig. 3, G and H). Thus, consistent with current models of INSF signaling, HMW-FGF-2 and cAMP-dependent pathways act in a cooperative fashion.

Next, we examined if CREB binds to the CRE site of the FGF-23 promoter located adjacent to the NFAT cis-element. We performed ChIP assays using CREB and pCREB antibodies. We found low levels of CREB binding to the CRE site in unstimulated conditions in both SaOS-2 and MC3T3-E1 osteoblasts (Fig. 4, A and B). FSK treatment increased pCREB binding to the CRE site in the FGF-23 promoter. To investigate if HMW-FGF-2 and FGFR1 form a nuclear signaling complex that binds to the CRE site in the FGF-23 promoter, we next performed ChIP assays using CBP or FGFR1 antibodies. In SaOS-2 osteoblasts, we detected
FGFR1 but not CBP binding to the CREB site in the FGF-23 promoter under unstimulated conditions (Fig. 4C). Transfection of FGFR1 or treatment with FSK resulted in an increase in both FGFR1 and CBP binding. Transfection of HMW-FGF-2 alone and in combination with FSK treatment and FSK treatment plus FGFR1 co-transfection, maximally increased binding of FGFR1 to the endogenous FGF-23 promoter (Fig. 4C). Similar increments in FGFR1 and CBP binding to CREB were observed in MC3T3-E1 transfected with HMW-FGF-2 and treated with FSK, and co-transfected with FGFR1 (Fig. 4D). MC3T3-E1 osteoblasts differed from SaOS-2 in the lack of CBP binding after FGFR1 transfection or FSK treatment alone (Fig. 4D).

To investigate the functional relevance of the ChIP assay results, we performed mutagenesis of the CREB binding site. Mutation of CREB completely blocked the ability of combined HMW-FGF-2 and FSK to stimulate FGF-23 promoter activity in SaOS-2 (Fig. 4E). In MC3T3-E1 osteoblasts the response to HMW-FGF-2 and FSK stimulation by FGF-23 promoter activity was also inhibited (Fig. 4F).

To further explore the role of FGFR1, we examined the effect of co-transfection of FGFR1 on combined HMW-FGF-2 and FSK-stimulated FGF-23 promoter activity. Transfection of FGFR1 resulted in a 10–15-fold increase in FGF-23 promoter activity in SaOS-2 and MC3T3 osteoblast cells cultured in a conditioned medium containing 50 μg/ml of ascorbic acid and 5 mM β-glycerophosphate for 3 weeks. Treatment of these cells with Su5402 (10 μM) during the last 24 h of culture blocks FGF-23 expression. Western blot analysis of FGFR1 cytoplasmic and nuclear localization stimulated by forskolin with or without HMW-FGF-2 co-transfections. β-Actin or Lamin B blot was used for internal controls. H, the dominant-negative FGFR1 (TK−/SP−/NLS) mutant blocked the effect of HMW-FGF-2/cAMP on FGF-23 promoter activity in a dose-dependent manner. Data are expressed as the mean ± S.D. from three independent experiments. Values sharing the same superscript in different groups are not significantly different. *, p < 0.05; **, p < 0.01 versus control vector group, respectively.
tein levels in conditioned media (Fig. 5, E and F) in both cell lines. We further demonstrated that HMW-FGF-2/cAMP-induced INSF signaling involves the translocation of FGFR1 from cytoplasmic into nuclear (Fig. 5G). We showed that osteoblasts expressed a low level of endogenous FGFR1 as determined by Western blot. Then, we overexpressed a human FGFR1 cDNA by transient transfection into SaOS-2 cells and studied the integrative nuclear FGFR1 translocation stimulated by HMW-FGF-2 with or without forskolin treatment. We found that cAMP increased nuclear FGFR1 accumulation in osteoblasts. Co-transfection of HMW-FGF-2 also increased the nuclear FGFR1 level. Moreover, a combination of forskolin and HMW-FGF-2 further increased FGFR1 translocation from cytosolic to nuclear (Fig. 5G). Dominant-negative FGFR1 (TK−/SP−/NLS) mutant blocks HMW-FGF-2/cAMP-induced INSF signaling (Fig. 5I).

Finally, we examined the effects of inactivation of the CREB and NFAT binding sites for the FGF-23 promoter by LMW-FGF-2 and HMW-FGF-2 stimulation of FGF-23 transcription (Fig. 6, A and B). LMW-FGF-2 stimulated FGF-23 promoter activity 2–3-fold, whereas HMW-FGF-2 plus FSK stimulated FGF-23 promoter activity ~5-fold in the wild-type promoter. Addition of LMW-FGF-2 to the combined HMW-FGF-2 plus FSK did not stimulate the FGF-23 promoter activity further (Fig. 6, C and E, 4th column). Mutation of the CREB binding site completely eliminated effects of both LMW-FGF-2, and combined HMW-FGF-2 and FSK to stimulate FGF-23 promoter activity in SaOS-2 osteoblasts (Fig. 6D). Mutation of the CREB binding site also inhibited LMW-FGF-2 and HMW-FGF-2 plus FSK stimulation of the FGF-23 promoter in MC3T3-E1 osteoblasts (albeit less completely than in SaOS-2 cells) (Fig. 6F).

Thus, CREB binding to CRE is important for both LMW- and HMW-FGF-2 signaling in osteoblasts. Mutation of the NFAT binding site prevented the effects of LMW-FGF-2 and HMW-FGF-2 plus FSK significantly to the stimulated FGF-23 promoter activity in both SaOS-2 and MC3T3-E1 osteoblasts (Fig.

FIGURE 6. Membrane and INSF FGFR1 signaling regulates FGF-23 in a cooperative fashion. A and B, human and mouse FGF-23 promoter DNA sequence containing wild-type NFAT and CREB sites, and NFAT site mutation, CREB mutation, or both NFAT and CREB site mutations. Effect of LMW-FGF-2 (50 ng/ml), HMW-FGF-2 and FSK, or HMW-FGF-2 and FSK plus LMW-FGF-2 on the promoter activity of the wild-type FGF-23 reporter (C and E), CREB mutant (D and F), NFAT mutant (G and I), or CREB/NFAT mutant (H and J), respectively. Data are expressed as the mean ± S.D. from three independent experiments. Values sharing the same superscript in different groups are not significantly different. *, p < 0.05; **, p < 0.01 versus control vector group, respectively.
6, G and I), although the magnitude of the response was less (compare Fig. 6, G and I with C and E). Mutation of both CREB and NFAT binding sites resulted in loss of LMW-FGF-2 and HMW-FGF-2 plus FSK effects in both SaSO-2 and MC3T3-E1 osteoblasts (Fig. 6, H and J).

DISCUSSION

Our studies show that LMW-FGF-2 and HMW-FGF-2 activate the FGF-23 promoter in osteoblasts through the respective FGFR membrane and INFS pathways (Fig. 7). LMW-FGF-2 activation of membrane FGFRs is coupled to FGF-23 gene transcription through activation of PLCγ-dependent NFAT and MAPK-dependent signaling leading to binding of phosphorylated NFAT and ETS-1 to binding sites in the proximal FGF-23 promoter. In contrast, HMW-FGF-2 acts in concert with cAMP-dependent pathways to stimulate FGF-23 promoter activity through a FGFR/HMW-FGF-2/CBP/CREB complex that binds to the CRE in the proximal FGF-23 promoter. To our knowledge this is the first report demonstrating a role for the intracrine FGFR1 and CREB nuclear regulator complex regulating FGF-23 hormone transcription in osteoblasts (47).

The close proximity between NFAT and CRE binding sites and the effects of mutations of either NFAT or CRE to attenuate HMW-FGF-2/FSK stimulation of the FGF-23 promoter suggest that the NFAT and CRE sites may be functionally coupled. Cross-talk between these signaling pathways could occur at multiple levels, including MAPK activation of both ETS-1 and NFAT, and LMW-FGF-2 and HMW-FGF-2 activating cAMP-dependent CREB/INFS pathways. However, HMW-FGF-2 in the presence of FSK is more potent than LMW-FGF-2 in activating the FGF-23 promoter in both human-derived SaOS-2 and mouse-derived MC3T3-E1 osteoblasts.

These findings are important for several reasons. First, we establish the importance of HMW-FGF-2 regulation of the FGF-23 promoter activity in vitro and establish the involvement of the INFS pathway in regulating FGF-23 gene transcription. Our observations are consistent with the findings that transgenic overexpression of HMW-FGF-2, the ligand for nuclear FGFR1, stimulates FGF-23 expression in bone and that HMW-FGF-2 is increased in bone of adult Hyp mice (26), that the conditional deletion of FGFR1 in osteocytes reduces FGF-23 in the Hyp mouse models of increased FGF-23 (32), and the deletion of HMW-FGF-2 in mice reduces FGF-23 expression in vivo (48).

Second, by showing an additional role of paracrine/autocrine FGFR1 membrane signaling, our data confirm that local matrix-derived factors play an essential role in the regulation of FGF-23 gene transcription. Indeed, local FGFs and FGFR signaling are important in skeletal development and physiology (49–51). These local factors likely coordinate FGF-23 transcription to match bone influx and efflux of phosphorus with the overall mineral metabolism. Thus, membrane and integrative nuclear FGFR signaling may provide a physiological “axis mundi” for integration of multiple factors regulating FGF-23 in bone.

Third, defining cis-elements in the proximal FGF-23 promoter that are necessary for FGF-23 gene transcription provides a molecular framework for understanding how diverse local and systemic pathways act coordinately to regulate FGF-23 promoter activity. For example, calcium has recently been shown to regulate FGF-23 expression in bone (11). Our findings suggest that this may be mediated through calcineurin/NFAT pathways. In addition, circulating FGF-23 concentrations decrease rapidly and disproportionately to reductions in parathyroid hormone levels after successful renal transplantation (52). Involvement of NFAT in FGF-23 gene transcription suggests that treatment with calcineurin inhibitors to prevent transplant rejection may have a direct role in suppressing FGF-23 gene transcription, a possibility requiring further study. Our findings may also explain the variable and inconsistent actions of phenylthiohydantoin to stimulate FGF-23 gene tran-
scription (1). Indeed, phenylthiohydantoin, which is coupled to both intracellular calcium and cAMP signaling, induces FGF-23 transcription in UMR106 osteoblast-like cells via activation of nuclear receptor-related protein 1 (Nurr1) (53). Our results showing that forskolin alone is insufficient to activate the proximal FGF-23 promoter and that cAMP and HMW-FGF-2 have synergistic effects, and data showing the cooperation of nuclear FGFRI and Nurr1 in the activation of neuronal genes, such as tyrosine hydroxylase (54), predict that phenylthiohydantoin actions might be influenced by the activity of FGFRI in bone.

There are gaps in our knowledge of how LMW-FGF-2 and HMW-FGF-2 are regulated in response to systemic and local factors that impact FGF-23 production and bone remodeling. One possibility is that alteration in the bioactivity of LMW-FGF-2 stored in the extracellular matrix is a mechanism whereby the extracellular protein, dentin matrix protein-1 (DMP1), and the transmembrane enzymes, phosphate regulating gene with homologies to endopeptidases on the X chromosome (PHEX) and ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1), regulate FGF-23 gene transcription (10). Consistent with this possibility, LMW-FGF-2 administration in vivo induces hypophosphatemia and impairs matrix mineralization (55, 56). The Hyp mouse model of X-linked hypophosphatemic rickets (XLRH) also has elevated expression of HMW-FGF-2 in osteoblasts, but the mechanisms linking PHEX and HMW-FGF-2 expression remain unknown.

Interestingly, HMW-FGF-2 is regulated by inflammation and oxidative stress, which are additional factors implicated in the regulation of FGF-23 (57). In this regard, HMW-FGF-2 is regulated by H-ras, cytokines, such as interleukin 1β and tumor necrosis factor-α, heat shock, and oxidative stress (33). Activating mutations of HRAS also lead to hypophosphatemia in association with skeletal dysplasia in subjects with epidermal nevus syndrome (58). It would be of interest to know if HMW-FGF-2 is involved in this response. Finally, iron deficiency and inflammation also play an important role in non-replicating osteocytes.

In conclusion, FGFR1, which is involved in the regulation of FGF-23 in osteoblasts, because these two receptors are expressed in low levels in the osteoblast cell lines that we used to test FGF-23 promoter activity, and more importantly, global ablation of FGFR3 and FGFR4 in Hyp mice results in increased, not decreased, FGF-23 expression (61). Our studies do not preclude, however, a role of membrane FGFR2 signaling in the regulation of FGF-23 gene transcription, because FGFR2 is expressed in osteoblasts and is known to regulate bone formation (62). However, like FGFR3 and FGFR4, the transmembrane domain of FGFR2 lacks the characteristics of the FGFR1 transmembrane domain that facilitates the nuclear translocation of FGFR1 (47). Indeed, comparison of the effects of FGFR1 and FGFR2 on FGF-23 gene transcription showed that FGFR1 had a significantly greater effect, possibly due to its dual role to stimulate both membrane and integrative nuclear signaling. This interpretation is supported by the attenuation of FGFR1 function by substituting the transmembrane domain of FGFR4 in the FGFR1–4 chimeric construct.

Our investigations are potentially limited by the use of only the proximal promoter of FGF-23 to define the molecular pathways linking FGFR1 and LMW- and HMW-FGF-2. This approach would miss additional potential enhancers and transcriptional binding sites located at considerable distances from the transcription start site. Nevertheless, 1,25(OH)2D treatment of URM-106 osteoblasts resulted in enhancement in histone H4 acetylation in the proximal FGF-23 promoter between −57 and 259 bp (44), suggesting that the proximal promoter is important in FGF-23 gene transcription. Chip-seq investigations in human and mouse osteoblasts will be required to completely define the NFAT, ETS-1, CREB, and other potential binding sites that mediate FGFR1 regulation of FGF-23 gene transcription.

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