Conversion of a Paracrine Fibroblast Growth Factor into an Endocrine Fibroblast Growth Factor*

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Background: The role of heparan sulfate (HS) in endocrine FGF signaling has not been defined.

Results: Endocrine FGF mutants devoid of HS binding retain full metabolic activity.

Conclusion: HS is dispensable for the metabolic activity of endocrine FGFs.

Significance: The study provides new insights into the composition of the cell surface signaling complex of endocrine FGFs.

FGFs 19, 21, and 23 are hormones that regulate in a Klotho co-receptor-dependent fashion major metabolic processes such as glucose and lipid metabolism (FGF21) and phosphate and vitamin D homeostasis (FGF23). The role of heparan sulfate glycosaminoglycan in the formation of the cell surface signaling complex of endocrine FGFs has remained unclear. Here we show that heparan sulfate is not a component of the signal transduction unit of FGF19 and FGF23. In support of our model, we convert a paracrine FGF into an endocrine ligand by diminishing heparan sulfate-binding affinity of the paracrine FGF and substituting its C-terminal tail for that of an endocrine FGF containing the Klotho co-receptor-binding site to home the ligand into the target tissue. In addition to serving as a proof of concept, the ligand conversion provides a novel strategy for engineering endocrine FGF-like molecules for the treatment of metabolic disorders, including global epidemics such as type 2 diabetes and obesity.

FGF signaling is essential for mammalian development and metabolism (1, 2). The 18 mammalian FGFs are grouped into five paracrine-acting subfamilies and one endocrine-acting subfamily comprising FGF19, FGF21, and FGF23 (2, 3). FGFs mediate their actions by binding and activating FGF receptor (FGFR) tyrosine kinases. There are four FGFR genes in mammals (FGFR1–4) (4), and tissue-specific alternative splicing of FGFR1–3 generates “b” and “c” splice isoforms with distinct ligand-binding specificity (5–10). Paracrine FGFs direct multiple processes during embryogenesis, including gastrulation, somitogenesis, organogenesis, and tissue patterning (2, 11, 12) and also regulate tissue homeostasis in the adult (13, 14). Endocrine FGFs control major metabolic processes such as bile acid homeostasis (15), hepatic glucose and protein metabolism (16, 17) (FGF19), glucose and lipid metabolism (18–21) (FGF21), and phosphate and vitamin D homeostasis (22, 23) (FGF23). Thus, these ligands have attracted much attention as potential drugs for the treatment of various inherited or acquired metabolic disorders (1, 24).

FGFs share a core homology region of ~120 amino acids that fold into a β-trefoil (25–28) consisting of 12 β strands in paracrine FGFs (β1–β12) and 11 β strands in endocrine FGFs (β1–β10 and β12) (3, 29). The conserved core region is flanked by divergent N and C termini, which play a critical role in conferring distinct biological activity on FGFs (3, 8). All FGFs interact with pericellular heparan sulfate (HS) glycosaminoglycans, albeit with different affinities (30). The HS-binding site of FGFs is comprised of the β1–β2 loop and the region between β10 and β12 strands (3). HS interacts with both side chain and main chain atoms of the HS-binding site in paracrine FGFs (see Fig. 1A) (31). The HS-binding site of endocrine FGFs deviates from the common conformation adopted by paracrine FGFs such that interaction of HS with backbone atoms of the HS-binding site is precluded (29). As a result, compared with paracrine FGFs, endocrine FGFs exhibit poor affinity for HS (1, 30). The poor HS affinity enables these ligands to diffuse freely away from the site of their secretion and enter the blood circulation to reach their distant target organs (29, 30). By contrast, because of their high HS affinity (30), paracrine FGFs are mostly immobilized in the vicinity of the cells secreting these ligands and hence can only act within the same organ. There is emerging evidence that differences in HS-binding affinity among paracrine FGFs translate into the formation of ligand-specific gradients in the pericellular matrix (32, 33), which contribute to the distinct functions of these ligands (1, 2).
In addition to controlling ligand diffusion in the extracellular space, HS promotes the formation of the 2:2 paracrine FGF-FGFR signal transduction unit (see Fig. 1D) (31, 34). HS engages both ligand and receptor to enhance the binding affinity of FGF for receptor and promote dimerization of ligand-bound receptors. Because of their poor HS-binding affinity, endocrine FGFs rely on Klotho co-receptors to bind their cognate FGFFR (35–38). Klotho co-receptors are single-pass transmembrane proteins that non-covalently associate with FGFRs to enhance the binding affinity of endocrine FGFs for their cognate FGFRs in target tissues (35–38). αKlotho is the co-receptor for FGF23 (36, 38), and βKlotho is the co-receptor for both FGF19 and FGF21 (35, 37). The C-terminal region of endocrine FGFs mediates binding of these ligands to the FGFR-α/βKlotho co-receptor complex (29, 41–45).

Endocrine FGFs still possess residual HS-binding affinity, and moreover, there are differences in this residual binding affinity among the endocrine FGFs (see Fig. 3A) (29). These observations raise the possibility that HS may play a role in endocrine FGF signaling (see Fig. 1D). Indeed, there are several reports showing that HS can promote endocrine FGF signaling in the presence and even in the absence of Klotho co-receptor. It has been shown that HS augments the mitogenic signal elicited by endocrine FGFs in BaF3 cells overexpressing FGF-2 and Klotho co-receptor by at least 2-fold (46). In addition, HS alone enables endocrine FGFs to induce proliferation of BaF3 cells overexpressing FGF-2 (47, 48). Compared with paracrine FGFs, however, significantly higher concentrations of both ligand and HS are needed, and the proliferative response of cells to endocrine FGFs still lags behind that of paracrine FGFs by about an order of magnitude (48).

To decipher the role of HS in endocrine FGF signaling, we generated FGF19 and FGF23 mutant ligands devoid of HS binding and compared their signaling capacity with that of wild-type ligands. Our data show that the mutated ligands retain full metabolic activity, demonstrating that HS does not participate in the formation of the endocrine FGF signaling complex. In support of this finding, we convert a paracrine FGF into an endocrine ligand by diminishing HS-binding affinity and replacing the C-terminal tail of the paracrine FGF with that of an endocrine FGF. Importantly, the ligand conversion provides a novel strategy for engineering endocrine FGF-like ligands for therapeutic purposes.

**EXPERIMENTAL PROCEDURES**

**Purification of FGF, FGFR, and Klotho Proteins**—The N-terminally hexahistidine-tagged, mature forms of human FGF19 (Arg²⁶⁻Lys²¹⁶), human FGF21 (His²⁹⁻Ser²⁰⁹; see Fig. 5A), and human FGF23 (Tyr²⁵⁻Ile²⁵¹; see Fig. 5A) were refolded in vitro from bacterial inclusion bodies and purified by published protocols (49, 50). HS-binding site mutants of FGF19 (K149A) and FGF23 (R140A/R143A) were purified from bacterial inclusion bodies by similar protocols as the wild-type proteins. To minimize proteolysis of FGF23 wild-type and mutant proteins, arginine residues 176 and 179 of the proteolytic cleavage site RXXR were replaced with glutamine as it occurs in the phosphate wasting disorder autosomal dominant hypophosphatemic rickets (22, 51). Human FGF1 (Met¹⁻Asp¹⁵⁵), human FGF2 (Met¹⁻Ser¹⁵⁵; see Fig. 5A), and human FGF homologous factor 1B (FH1B; Met¹⁻Thr¹⁸¹) were purified by published protocols (50, 52). Chimeras composed of the core domain of FGF2 (Met¹⁻Met¹⁵¹) and the C-terminal region of either FGF21 (Pro¹⁶⁸⁻Ser²⁰⁹) or FGF23 (Arg¹⁶¹⁻Ile²⁵¹) (termed FGF²WTcore-FGF2¹C-tail and FGF²WTcore-FGF2³C-tail, respectively; see Fig. 5A) were purified by the same protocol as that for native FGF2 (50). Analogous chimeras containing three mutations in the HS-binding site of the FGF2 core (K128D/R129Q/K134V) (termed FGF²ΔHBScore-FGF2¹C-tail and FGF²ΔHBScore-FGF2³C-tail, respectively; see Fig. 5A) were purified from the soluble bacterial cell lysate fraction by ion exchange and size exclusion chromatographies. To minimize proteolysis of the chimeras containing the C-terminal sequence Arg¹⁶¹⁻Ile²⁵¹ of FGF23, arginine residues 176 and 179 of the proteolytic cleavage site RXXR were located within this sequence were replaced with glutamine as it occurs in autosomal dominant hypophosphatemic rickets (22, 51). In addition, to prevent disulfide-mediated dimerization of FGF2 and chimeric FGF2 proteins, cysteine residues 78 and 96 were mutated to serine. The N-terminally hexahistidine-tagged C-terminal tail peptide of FGF23 (Ser¹⁸⁰⁻Ile²⁵¹, termed FGF²³C-tail) was purified by a published protocol (41). The ligand-binding domain of human FGFR1c (Asp¹⁴²⁻Arg³⁶⁵) was refolded in vitro from bacterial inclusion bodies, and purified by published protocols (49, 50). The ectodomain of murine αKlotho (Ala³⁵⁻Lys⁹²) and the ectodomain of murine βKlotho (Phe⁵³⁻Leu⁹⁵) were expressed in HEK293 cells as fusion proteins with a C-terminal FLAG tag (36, 53). The binary complex of FGFR1c ligand-binding domain with αKlotho ectodomain (referred to as αKlotho-FGFR1c complex) was prepared by a published protocol (41). The binary complex of FGFR1c ligand-binding domain with βKlotho ectodomain (referred to as βKlotho-FGFR1c complex) was prepared in the same fashion as the αKlotho-FGFR1c complex.

**Analysis of FGF-Heparin and FGF-FGFR-α/βKlotho Interactions by Surface Plasmon Resonance Spectroscopy**—Surface plasmon resonance (SPR) experiments were performed on a Biacore 2000 instrument (Biacore AB), and the interactions were studied at 25 °C in HBS-EP buffer (10 mM HEPES-NaOH, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% (v/v) polysorbate 20). To study endocrine FGF-heparin interactions, a heparin chip was prepared by immobilizing biotinylated heparin (Sigma-Aldrich) on flow channels of a research grade streptavidin chip (Biacore AB). The coupling density was ~5 fmol mm⁻² of flow channel. To measure binding of chimeric FGF2 proteins to heparin, biotinylated heparin was coupled to a streptavidin chip at a ~4-fold lower density as judged based on the binding responses obtained for FGF1. To study FGF-FGFR-α/βKlotho interactions, FGF chips were prepared by covalent coupling of FGF proteins through their free amino groups on flow channels of research grade CM5 chips (Biacore AB). The proteins were injected over a chip at a flow rate of 50 μl min⁻¹, and at the end of each protein injection (180 and 300 s, respectively), HBS-EP buffer (50 μl min⁻¹) was flowed over the chip to monitor dissociation for 180 or 240 s. The heparin chip surface was regenerated by injecting 50 μl of 2.0 M NaCl in 10 mM sodium acetate,
pH 4.5. For FGF chips, regeneration was achieved by injecting 2.0 M NaCl in 10 mM sodium/potassium phosphate, pH 6.5. To control for nonspecific binding in experiments where an FGF ligand was immobilized on the chip, FHF1B, which shares structural similarity with FGFs but does not exhibit any FGFR binding (52), was coupled to the control flow channel of the chip (52). In experiments where heparin was immobilized on the chip, the control flow channel was left blank. The data were processed with BiaEvaluation software (Biacore AB). For each protein injection over the heparin chip, the nonspecific responses from the control flow channel were subtracted from the responses recorded for the heparin flow channel. Similarly, for each protein injection over a FGF chip, the nonspecific responses from the FHF1B control flow channel were subtracted from the responses recorded for the FGF flow channel. Where possible, equilibrium dissociation constants (KD values) were calculated from fitted saturation binding curves. Fitted binding curves were judged to be accurate based on the distribution of the residuals (even and near 0) and (52). To examine whether the K149A mutation abrogates residual heparin binding of FGF19, increasing concentrations of wild-

**FIGURE 1.** Side by side comparison of the HS-binding site of FGF2, FGF19, and FGF23 and working model of the endocrine FGF signaling complex. A, interactions of FGF2 (shown as cartoon representation) with a heparin hexasaccharide (shown as sticks) as observed in the crystal structure of the 2:2 FGF2-FGFR1c dimer (Protein Data Bank code 1FQ9) (31). The heparin hexasaccharide consists of three disaccharide units of 1→4-linked N-sulfated 6-O-sulfated α-glucosamine and 2→O-sulfated L-iduronic acid. Note that the heparin hexasaccharide interacts with both side chain and backbone atoms of residues in the HS-binding site of FGF2. Dashed lines denote hydrogen bonds. Lys128, Arg129, and Lys134, which make the majority of hydrogen bonds with the heparin hexasaccharide, are boxed. The β-strand nomenclature follows the original FGF1 and FGF2 crystal structures (25–28). Please note that compared with the prototypical β-trefoil fold seen in soybean trypsin inhibitor (Protein Data Bank code 1TIE) (64) and interleukin 1β (Protein Data Bank code 1I1B) (65), the β10-β11 strand pairing in FGF2 and other paracrine FGFs is less well defined. B and C, cartoon representation of the crystal structures of FGF19 (Protein Data Bank code 2P23) (29) (B) and FGF23 (Protein Data Bank code 2P39) (29) (C) shown in the same orientation as the FGF2 structure in A. The side chains of residues that map to the corresponding HS-binding sites of these ligands are shown as sticks. Residues selected for mutagenesis to knock out residual HS binding in FGF19 and FGF23 are boxed. NT and CT indicate N and C termini of the FGFs. D, schematic of the endocrine FGF-FGFR-Klotho signal transduction unit. For comparison, a schematic of the paracrine FGF-FGFR-HS signaling unit is shown that was made based on the crystal structure of the 2:2:2 FGF2-FGFR1c-HS complex (Protein Data Bank code 1FQ9) (31). HS engages both paracrine FGF and receptor to enhance binding of FGF to its primary and secondary receptors thus promoting receptor dimerization. A question mark denotes whether or not HS is also a component of the endocrine FGF signaling complex.
type FGF19 were passed over a heparin chip. Thereafter, the FGF19K149A mutant was injected over the heparin chip at the highest concentration tested for the wild-type ligand. The effect of the R140A/R143A double mutation in the HS-binding site of FGF23 on residual heparin binding of FGF23 was examined in the same fashion as was the effect of the HS-binding site mutation in FGF19.

To verify that the K128D/R129Q/K134V triple mutation in the HS-binding site of the FGF2 core domain diminishes heparin-binding affinity of the FGF2 core, increasing concentrations of FGF2\textsubscript{HBScore-FGF21C-tail} and FGF2\textsubscript{HBScore-FGF23C-tail} were passed over a heparin chip. As a control, binding of FGF2\textsubscript{WTcore-FGF21C-tail} and FGF2\textsubscript{WTcore-FGF23C-tail} to heparin was studied.

To examine whether the FGF2\textsubscript{HBScore-FGF21C-tail} chimera can compete with FGF21 for binding to the βKlotho–FGFR1c complex, increasing concentrations of FGF2\textsubscript{HBScore-FGF21C-tail} were mixed with a fixed concentration of βKlotho–FGFR1c complex in HBS-EP buffer, and the mixtures were passed over a chip containing immobilized FGF21 (~19 fmol mm\textsuperscript{-2} of flow channel). As controls, the binding competition was carried out with FGF21 or FGF2 as the competitor in solution. As an additional specificity control, binding of the FGF2\textsubscript{HBScore-FGF21C-tail} chimera with FGF21 for binding to the βKlotho–FGFR1c complex was studied. βKlotho–FGFR1c complex was mixed with FGF2\textsubscript{HBScore-FGF21C-tail} or FGF21 at a molar ratio of 1:10, and the mixture was injected over a chip containing immobilized FGF23 (~12 fmol mm\textsuperscript{-2} of flow channel).

To test whether the FGF2\textsubscript{HBScore-FGF23C-tail} chimera can compete with FGF21 for binding to the βKlotho–FGFR1c complex, increasing concentrations of FGF2\textsubscript{HBScore-FGF23C-tail} were mixed with a fixed concentration of βKlotho–FGFR1c complex in HBS-EP buffer, and the mixtures were passed over a chip containing immobilized FGF21 (~19 fmol mm\textsuperscript{-2} of flow channel). As controls, the binding competition was carried out with FGF21 or FGF2 as the competitor in solution. As an additional specificity control, binding of the FGF2\textsubscript{HBScore-FGF23C-tail} chimera with FGF23 for binding to the βKlotho–FGFR1c complex was studied. βKlotho–FGFR1c complex was mixed with FGF2\textsubscript{HBScore-FGF23C-tail} or FGF23 at a molar ratio of 1:10, and the mixture was injected over a chip containing immobilized FGF23 (~12 fmol mm\textsuperscript{-2} of flow channel).

To measure binding of FGFR1c to each of the three endocrine FGFs, increasing concentrations of FGFR1c ligand-binding domain were injected over a chip containing immobilized FGF19, FGF21, and FGF23 (~30 fmol mm\textsuperscript{-2} of flow channel). As a control, binding of FGFR1c to FGF2 immobilized on a chip was studied. As additional controls, binding of the βKlotho–FGFR1c complex to FGF23 and binding of FGFR1c to the C-terminal tail peptide of FGF23 were measured.

Analysis of Phosphorylation of FRS2α and 44/42 MAP Kinase in Hepatoma and Epithelial Cell Lines—To examine whether the FGF19\textsuperscript{K149A} and FGF23\textsuperscript{R140A/R143A} mutants can activate FGFR in an α/βKlotho-dependent fashion, induction of tyrosine phosphorylation of FGFR substrate 2α (FRS2α) and downstream activation of MAP kinase cascade was used as readout

**FIGURE 2. Sequence alignment of the endocrine FGFs.** The amino acid sequences of the mature human FGF19, FGF21, and FGF23 ligands are aligned. Also included in the alignment is the human sequence of FGF2, a prototypical paracrine FGF, which was used in this study to convert into endocrine FGF ligands. Residue numbers are in parentheses to the left of the alignment. Secondary structure elements are labeled, and residues containing these elements for known secondary structures are boxed. Gaps (dashes) were introduced to optimize the sequence alignment. The β-trefoil core domain for known FGF crystal structures is shaded gray. Dark gray bars on top of the alignment indicate the location of the HS-binding regions. HS-binding residues selected for mutagenesis are shaded dark gray.
for FGFR activation. Subconfluent cultures of the H4IIE rat hepatoma cell line, which endogenously expresses βKlotho (35), were serum-starved for 16 h and then stimulated for 10 min with the FGF19K149A mutant or wild-type FGF19 (0.2 ng ml⁻¹ to 2.0 μg ml⁻¹). Similarly, subconfluent cultures of a HEK293 cell line ectopically expressing the transmembrane isoform of murine αKlotho (36) were treated with the FGF23R140A/R143A mutant or wild-type FGF23 (0.1–100 ng ml⁻¹). After stimulation, the cells were lysed (53), and cellular proteins were resolved on SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The protein blots were probed with antibodies to phosphorylated FRS2α, phosphorylated 44/42 MAP kinase, total (phosphorylated and nonphosphorylated) 44/42 MAP kinase, and αKlotho. Except for the anti-αKlotho antibody (KM2119) (54), all antibodies were from Cell Signaling Technology.

Analysis of Egr1 Protein Expression in an Epithelial Cell Line—To examine whether the FGF2HBScore-FGF21C-tail and FGF2HBScore-FGF23C-tail chimeras can activate FGFR in an HS-dependent fashion, induction of protein expression of the transcription factor Egr1 (early growth response 1), a known downstream mediator of FGF signaling, was used as readout for FGFR activation. HEK293 cells were serum-starved overnight and then stimulated for 90 min with FGF2HBScore-FGF21C-tail or FGF2HBScore-FGF23C-tail (0.1 and 0.3 nM). Cell stimulation with FGF2WTcore-FGF21C-tail, FGF2WTcore-FGF23C-tail, FGF21, and FGF23 served as controls. To test whether the FGF2HBScore-FGF21C-tail chimera can activate FGFR in a βKlotho-dependent fashion, HEK293 cells transfected with murine βKlotho were serum-starved overnight and then stimulated for 90 min with FGF2HBScore-FGF21C-tail or FGF21 (3–300 ng ml⁻¹). After stimulation, the cells were lysed (53), and cellular proteins were resolved on SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The protein blots were probed with antibodies to Egr1 and GAPDH. The anti-Egr1 antibody was from Cell Signaling Technology, and the anti-GAPDH antibody was from Abcam.

Analysis of CYP7A1 and CYP8B1 mRNA Expression in Murine Liver Tissue—To examine the metabolic activity of the FGF19K149A mutant in vivo, 6–8-week old C57BL/6 mice were fasted overnight and then given intraperitoneally a single dose (1 mg kg body weight⁻¹) of FGF19K149A or FGF19 as a control. 6 h after the injection, the mice were sacrificed, and liver tissue was excised and frozen. Total RNA was isolated from liver tissue, and mRNA levels of cholesterol 7α-hydroxylase (CYP7A1) and sterol 12α-hydroxylase (CYP8B1) were measured using quantitative real time RT-PCR as described previously (15, 55). The Institutional Animal Care and Use Committee at the University of Texas Southwestern Medical Center at Dallas had approved the experiments.

Measurement of Serum Phosphate in Mice—To examine the metabolic activity of the FGF23R140A/R143A mutant in vivo, 4–5-week old C57BL/6 mice were given intraperitoneally a single dose (0.29 mg kg body weight⁻¹) of FGF23R140A/R143A or FGF23 as a control.

To test whether the FGF2HBScore-FGF23C-tail chimera exhibits FGF23-like metabolic activity, 5–6-week old C57BL/6 mice were given a single injection of FGF2HBScore-FGF23C-tail (0.21 mg kg body weight⁻¹). As controls, the mice were injected with FGF2WTcore-FGF23C-tail or FGF23. To confirm that αKlotho is required for the metabolic activity of the FGF2HBScore-FGF23C-tail chimera, 7–8-week old αKlotho knock-out mice (Lexicon Genetics) were injected once with FGF2HBScore-FGF23C-tail or FGF23 as a control (0.51 mg kg body weight⁻¹). Before and 8 h after each protein injection, blood was drawn from the cheek pouch and spun at 3,000 × g for 10 min to obtain serum. Phosphate concentration in serum was measured using the Phosphorus Liqui-UV Test (Stanbio Laboratory).

Analysis of CYP27B1 mRNA Expression in Murine Renal Tissue—The ability of the FGF2HBScore-FGF23C-tail chimera to reduce renal expression of 25-hydroxyvitamin D₃ 1α-hydroxylase (CYP27B1) was used as another readout for FGF23-like...
metabolic activity. C57BL/6 mice injected with FGF2/H9004HBScore-FGF23C-tail, FGF2WTcore-FGF23C-tail, or FGF23 were sacrificed 8 h after the protein injection, and renal tissue was excised and frozen. CYP27B1 mRNA levels in total renal tissue RNA were measured using real time quantitative PCR as described previously (56, 57).

**Insulin Tolerance Test in Mice**—The ability of the FGF2/H9004HBScore-FGF21C-tail chimera to potentiate the hypoglycemic effect of insulin was used as readout for FGF21-like metabolic activity (58). 8–12-week old C57BL/6 mice were kept on normal chow. On the day of the insulin tolerance test, the mice were fasted for 4 h and then bled from the cheek pouch for measuring fasting blood glucose levels. Thereafter, the mice were administered intraperitoneally insulin (0.5 unit kg body weight⁻¹) alone or insulin (0.5 units kg body weight⁻¹) plus FGF2/H9004HBScore-FGF21C-tail chimera (0.3 mg kg body weight⁻¹). As a control, the mice were co-injected with insulin plus FGF21. At the indicated time points after the injection (see Fig. 7G), blood was drawn from the tail vein. Glucose concentrations in the blood samples were determined using Bayer Contour® blood glucose test strips (Bayer). These experiments and all the in vivo experiments with FGF23 ligands were approved by the Harvard University Animal Care and Research committee board.

**Statistical Analysis**—The data are expressed as the means ± S.E. A Student’s t test or analysis of variance was used as appropriate to make statistical comparisons. A value of p < 0.05 was considered significant.

**RESULTS AND DISCUSSION**

**HS Is Dispensable for the Metabolic Activity of FGF19 and FGF23**—To engineer endocrine FGFs devoid of HS binding, we compared the FGF19 crystal structure (Protein Data Bank code 2P23) (29) with that of FGF2 bound to a heparin hexasaccharide (Protein Data Bank code 1FQ9) (31). This analysis shows that solvent-exposed residues Lys149, Gln150, Gln152, and Arg157 of FGF19 lie at the corresponding HS-binding site of this ligand and hence could account for the residual HS binding of FGF19 (Figs. 1, A and B, and 2). Likewise, comparative analysis of the FGF23 crystal structure (Protein Data Bank code 2P39) (29) with that of heparin-bound FGF2 (Protein Data Bank code 1FQ9) (31) points to Arg48, Asn49, Arg140, and Arg143 as candidates mediating the residual HS binding of this ligand (Figs. 1, A and C, and 2). As a control, the mice were co-injected with insulin plus FGF21. At the indicated time points after the injection (see Fig. 7G), blood was drawn from the tail vein. Glucose concentrations in the blood samples were determined using Bayer Contour® blood glucose test strips (Bayer). These experiments and all the in vivo experiments with FGF23 ligands were approved by the Harvard University Animal Care and Research committee board.

**Statistical Analysis**—The data are expressed as the means ± S.E. A Student’s t test or analysis of variance was used as appropriate to make statistical comparisons. A value of p < 0.05 was considered significant.
FGF19<sup>K149A</sup> mutant was as effective as wild-type FGF19 in inducing tyrosine phosphorylation of FRS2α and downstream activation of MAP kinase cascade (Fig. 4A). These data show that elimination of residual HS binding has no impact on the ability of FGF19 to signal in cultured cells. To test whether the same holds true for FGF23 signaling, we transfected HEK293 cells, which naturally express two of the three cognate receptors of FGF23, namely FGFR1c and FGFR3c (36), with the transmembrane isoform of Klotho, the co-receptor of FGF23, and treated these cells with the FGF23<sup>R140A/R143A</sup> double mutant or wild-type FGF23. The FGF23<sup>R140A/R143A</sup> mutant had the same capacity as wild-type FGF23 in inducing phosphorylation of FRS2α and downstream activation of MAP kinase cascade (Fig. 4B). These data show that similar to FGF19, FGF23 does not need to bind HS to activate FGFR in cultured cells.

To substantiate the findings in cells, we compared the metabolic activity of wild-type and mutated ligands in vivo. Mice were injected with the FGF19<sup>K149A</sup> mutant or wild-type FGF19, and liver gene expression of CYP7A1 and CYP8B1, which are key enzymes in the major bile acid biosynthetic pathway (59), was analyzed. Like wild-type FGF19, the FGF19<sup>K149A</sup> mutant markedly decreased CYP7A1 and CYP8B1 mRNA levels (Fig. 4C), demonstrating that knock-out of residual HS binding does not affect the metabolic activity of FGF19. To examine whether residual HS binding is also dispensable for the metabolic activity of FGF23, mice were injected with the FGF23<sup>R140A/R143A</sup> mutant or wild-type FGF23, and serum phosphate concentrations were measured. The FGF23<sup>R140A/R143A</sup> mutant reduced serum phosphate as effectively as wild-type FGF23 (Fig. 4D). These data show that, as in the case of FGF19, abolishment of residual HS binding does not impact the metabolic activity of FGF23, leading us to conclude that HS is not a component of the endocrine FGF signal transduction unit (Fig. 1D).

Conversion of a Paracrine FGF into an Endocrine Ligand Confirms That HS is Dispensable for the Metabolic Activity of Endocrine FGFs—If HS is dispensable for the metabolic activity of endocrine FGFs, then it should be feasible to convert a paracrine FGF into an endocrine FGF by eliminating HS-binding
affinity of the paracrine FGF and substituting its C-terminal tail for that of an endocrine FGF. Reducing HS-binding affinity will allow the ligand to freely diffuse and enter the blood circulation, while attaching the C-terminal tail of an endocrine FGF will home the ligand into its target tissues. We chose to convert FGF2, a prototypical paracrine FGF, into FGF23-like and FGF21-like ligands. FGF2 was selected as paracrine ligand for this protein engineering exercise because it preferentially binds to the “c” isoform of FGFR1, the principal receptor mediating the metabolic activity of FGF23 (60, 61) and FGF21 (35), respectively. In the crystal structure of heparin-bound FGF2 (Protein Data Bank code 1FQ9) (31), Lys128, Arg129, and Lys134 mediate the majority of hydrogen bonds with heparin, and hence mutation of these residues should cause a major reduction in HS-binding affinity of FGF2 (Figs. 1A, 2, and 5A). Accordingly, Lys128, Arg129, and Lys134 were mutated to aspartate, glutamine, and valine, respectively, which occupy the corresponding positions in FGF7 and FGF22. Based on the crystal structures of paracrine FGFs, these amino acid substitutions should disrupt the hydrogen bonding with HS without impacting the secondary structure of the HS-binding site. Next, the short C-terminal tail of the mutated FGF2 was replaced with the C-terminal tail of FGF23 (Arg161–Ile251) or the C-terminal tail of FGF21 (Pro168–Ser209) (Fig. 5A). The resulting chimeras were termed FGF2/H9004HBScore-FGF23C-tail and FGF2/H9004HBScore-FGF21C-tail (Fig. 5A). To demonstrate that reduction in HS-binding affinity is required for converting FGF2 into an endocrine ligand, we made two control chimeras in which the HS-binding site of the FGF2 core was left intact (FGF2WTcore-FGF23C-tail and FGF2WTcore-FGF21C-tail; Fig. 5A).

FIGURE 6. FGF2/H9004HBScore-FGF23C-tail chimera exhibits FGF23-like activity. A and B, overlays of SPR sensorgrams illustrating inhibition by FGF2/H9004HBScore-FGF23C-tail (A) or FGF23 (B) of αKlotho-FGFR1c binding to FGF23 immobilized on a biosensor chip. Increasing concentrations of FGF2/H9004HBScore-FGF23C-tail or FGF23 were mixed with a fixed concentration of αKlotho-FGFR1c complex, and the mixtures were passed over a FGF23 chip. C, overlay of SPR sensorgrams illustrating failure of FGF2 to inhibit αKlotho-FGFR1c binding to FGF23. FGF2 and αKlotho-FGFR1c complex were mixed at a molar ratio of 15:1, and the mixture was passed over a biosensor chip containing immobilized FGF23. D and E, overlays of SPR sensorgrams illustrating no inhibition by FGF2/H9004HBScore-FGF23C-tail (D) or FGF23 (E) of βKlotho-FGFR1c binding to FGF21. FGF2/H9004HBScore-FGF23C-tail or FGF23 were mixed with βKlotho-FGFR1c complex at a molar ratio of 10:1, and the mixtures were passed over a biosensor chip containing immobilized FGF21. F, analysis of serum phosphate concentrations (serum Pi) in mice before and 8 h after intraperitoneal injection of FGF2/H9004HBScore-FGF23C-tail, FGF23, or vehicle. Wild-type mice and αKlotho knock-out mice were given 0.21 and 0.51 mg of protein, respectively, per kg of body weight. The data are presented as the means ± S.E. *** p < 0.001 by analysis of variance. G, quantitative analysis of CYP27B1 mRNA expression in renal tissue from mice injected with FGF2/H9004HBScore-FGF23C-tail, FGF23, or vehicle. 0.21 mg of protein/kg of body weight was injected. The data are presented as the means ± S.E. *** p < 0.001 by analysis of variance.
FGF2 is an obligatory co-factor in paracrine FGF signaling, the readout for FGFR activation. As shown in Fig. 5, known downstream mediator of FGF signaling, was used as a readout of protein expression of the transcription factor Egr1, a known downstream mediator of FGF signaling, was used as a readout of Egr1 expression in HEK293 cells stimulated with FGF21C-tail or FGF21. Numbers above the lanes give the amounts of protein added in ng ml⁻¹. GAPDH protein expression was used as a loading control. Note that the FGF21HBScore-FGF21C-tail chimera is more potent than native FGF21 at inducing Egr1 expression, suggesting that the chimera has agonistic properties. This is expected because the core domain of FGF2 has inherently greater binding affinity for FGFR than the core domain of FGF21 (see Fig. 8). A and C, analysis of blood glucose concentrations in mice before and at the indicated time points after intraperitoneal injection of insulin alone, insulin plus FGF21, or vehicle alone. 0.5 units of insulin/kg of body weight and 0.3 mg of FGF21 ligand/kg of body weight were injected. Blood glucose concentrations are expressed as percentages of preinjection values. The data are presented as the means ± S.E.

To determine whether the FGF21HBScore-FGF23C-tail and FGF21HBScore-FGF21C-tail chimeras have gained the ability to signal in a Klotho co-receptor-dependent, endocrine fashion, we first analyzed whether these chimeras can form ternary complexes with FGFR1c and Klotho co-receptor. To this end, an SPR-based binding competition assay was employed. FGF23 was immobilized onto an SPR biosensor chip, and mixtures of a fixed concentration of binary Klotho-FGFR1c complex were mixed with a fixed concentration of Klotho-FGFR1c complex, and the mixtures were passed over a FGF21 chip. C, overlay of SPR sensograms illustrating failure of FGF2 to inhibit Klotho-FGFR1c binding to FGF21. FGF2 and Klotho-FGFR1c complex were mixed at a molar ratio of 15:1, and the mixture was passed over a biosensor chip containing immobilized FGF21. D and E, overlays of SPR sensograms illustrating no inhibition by FGF21HBScore-FGF21C-tail (D) or FGF2 (E) of Klotho-FGFR1c binding to FGF23. FGF21HBScore-FGF21C-tail or FGF21 were mixed with Klotho-FGFR1c complex at a molar ratio of 10:1, and the mixtures were passed over a biosensor chip containing immobilized FGF23. F, immunoblot analysis for Egr1 expression in HEK293 cells stimulated with FGF21HBScore-FGF23C-tail or FGF21. Numbers above the lanes give the amounts of protein added in ng ml⁻¹. GAPDH protein expression was used as a loading control. Note that the FGF21HBScore-FGF21C-tail chimera is more potent than native FGF21 at inducing Egr1 expression, suggesting that the chimera has agonistic properties. This is expected because the core domain of FGF2 has inherently greater binding affinity for FGFR than the core domain of FGF21 (see Fig. 8). A and C, analysis of blood glucose concentrations in mice before and at the indicated time points after intraperitoneal injection of insulin alone, insulin plus FGF21, or vehicle alone. 0.5 units of insulin/kg of body weight and 0.3 mg of FGF21 ligand/kg of body weight were injected. Blood glucose concentrations are expressed as percentages of preinjection values. The data are presented as the means ± S.E.
effectively competed with immobilized FGF21 for binding to the βKlotho-FGFR1c complex (Fig. 7A), demonstrating that the chimera, like native FGF21 (Fig. 7B), is capable of binding to the binary complex of FGFR1c and βKlotho. Notably, native FGF2 failed to compete with FGF23 for binding to the αKlotho-FGFR1c complex (Fig. 6C) and with FGF21 for binding to the βKlotho-FGFR1c complex (Fig. 7C), because it lacks the Klotho co-receptor-binding domain. To further confirm the binding specificity of the FGF2ΔHBScore-FGF23C-tail chimera for the αKlotho-FGFR1c complex, FGF2ΔHBScore-FGF23C-tail chimera and βKlotho-FGFR1c complex were mixed at a molar ratio of 10:1, and the mixture was injected over a chip containing immobilized FGF21. FGF2ΔHBScore-FGF23C-tail, like native FGF23, failed to compete with FGF21 for binding to the βKlotho-FGFR1c complex (Fig. 6, D and E). Similarly, the FGF2ΔHBScore-FGF21C-tail chimera, like native FGF21, failed to compete with FGF23 for binding to the αKlotho-FGFR1c complex (Fig. 7, D and E). For the FGF2ΔHBScore-FGF21C-tail chimera, we investigated whether it is able to activate FGFR1c in a βKlotho-dependent fashion in cells. HEK293 cells were transfected with βKlotho and then stimulated with FGF2ΔHBScore-FGF21C-tail or FGF21C-tail. Similar to native FGF21, the FGF2ΔHBScore-FGF21C-tail chimera induced Egr1 protein expression in HEK293-βKlotho cells (Fig. 7F), indicating that the chimera is capable of activating FGFR1c in the presence of βKlotho.

To provide definite proof for the ligand conversion, we tested the metabolic activity of the chimeras in vivo. Specifically, the ability of the FGF2ΔHBScore-FGF23C-tail chimera to lower serum phosphate and to reduce renal gene expression of CYP27B1, which catalyzes the conversion of vitamin D into its bioactive form, was examined. Mice were injected with FGF2ΔHBScore-FGF23C-tail or as controls, FGF23 or FGF2WTcore-FGF23C-tail, and serum phosphate concentrations and renal CYP27B1 mRNA levels were measured. Similar to native FGF23, the FGF2ΔHBScore-FGF23C-tail chimera caused a decrease in serum phosphate in wild-type mice (Fig. 6F). The chimera also induced a marked decrease in CYP27B1 mRNA levels, just like the native FGF23 ligand (Fig. 6G). These data show that the FGF2ΔHBScore-FGF23C-tail chimera acts as an FGF23-like hormone. Importantly, the FGF2WTcore-FGF23C-tail chimera failed to decrease serum phosphate or CYP27B1 mRNA levels (Fig. 6, F and G). This is expected because this chimera should be trapped in the vicinity of the injection site since it has high affinity for HS. Moreover, these data show that adding the Klotho co-receptor-binding site is not sufficient to convert a paracrine FGF into an endocrine ligand. To confirm that the metabolic activity of the FGF2ΔHBScore-FGF23C-tail chimera is dependent on αKlotho, αKlotho knock-out mice were injected with FGF2ΔHBScore-FGF23C-tail or FGF23 as a control, and serum concentrations of phosphate were measured. As shown in Fig. 6F, FGF2ΔHBScore-FGF23C-tail failed to lower serum phosphate, demonstrating that the chimera, like native FGF23 (Fig. 6F), requires αKlotho for metabolic activity.

To determine whether the FGF2ΔHBScore-FGF21C-tail chimera exhibits FGF21-like metabolic activity, its ability to potentiate the hypoglycemic effect of insulin was examined.
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(58). Mice were injected with insulin plus FGF2ΔHScore-FGF21C-tail, insulin plus FGF21, or insulin alone, and blood glucose concentrations were monitored for up to 1 h after the injection. Similar to FGF21, the FGF2ΔHScore-FGF21C-tail chimera enhanced the hypoglycemic effect of insulin (Fig. 7G), demonstrating that the chimera acts as an FGF21-like hormone.

Our ability to convert a paracrine FGF into an endocrine ligand by means of reducing HS-binding affinity of the paracrine FGF and adding the Klotho co-receptor-binding site substantiates that HS does not participate in the formation of the endocrine FGF signal transduction unit. The dispensability of HS for the metabolic activity of endocrine FGFs has an intriguing implication as to how these FGFs have evolved to become hormones. It appears that these ligands have lost the requirement to bind HS to signal while acquiring the ability to bind Klotho co-receptors.

In the target tissue, Klotho co-receptors constitutively associate with cognate receptors of endocrine FGFs to offset the inherently low receptor-binding affinity of endocrine FGFs (Fig. 8, B–D) (35–38). This low binding affinity is due to the fact that key receptor-binding residues in the β-trefoil core of endocrine FGFs are replaced by residues that are suboptimal for receptor binding (29). To measure the degree to which Klotho co-receptors enhance the receptor-binding affinity of endocrine FGFs, we conducted SPR experiments using FGF23 and FGR1c and αKlotho co-receptor as an example. The SPR data show that αKlotho enhances the affinity of FGF23 for FGR1c by over 20-fold (Fig. 8, D and E). The affinity of FGF23 for FGR1c in the presence of αKlotho is comparable with that of FGF2 for FGR1c in the absence of its HS co-factor (Fig. 8, A and E). It should be noted, however, that HS further increases the binding affinity of FGF2 for FGR1c by at least an order of magnitude (62, 63). Hence, the receptor-binding affinity of FGF23 in the presence of αKlotho co-receptor is still lower than that of FGF2 in the presence of HS co-factor. These observations imply that the signaling capacity of the endocrine FGF signal transduction unit should be weaker than that of the paracrine FGF signaling unit. Indeed, cell-based studies show that even in the presence of their Klotho co-receptor, endocrine FGFs are inferior to paracrine FGFs at activating FGR1-induced intracellular signaling pathways (35, 38).

Our finding that endocrine FGFs do not need to rely on HS for signaling has another important implication in regard to the role of Klotho co-receptors. Because FGR dimerization is a prerequisite for FGF signaling in general, we propose that Klotho co-receptors not only enhance the binding affinity of endocrine ligand for receptor but also promote receptor dimerization upon ligand binding. In other words, Klotho co-receptors must fulfill the same dual role that HS plays in signaling by paracrine FGFs (Fig. 1D). Definite proof for the proposed dual role of Klotho co-receptors awaits the determination of the crystal structure of an endocrine FGF-FGR-Klotho ternary complex, which is beyond the scope of this study.

The ligand conversion also provides an exciting framework for the rational design of endocrine FGF-like molecules for the treatment of metabolic disorders. An FGF23-like molecule, for example, could be used for the treatment of inherited or acquired hyper-phosphatemia, and an FGF21-like molecule could be used for the treatment of type 2 diabetes, obesity, and related metabolic disorders.

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