Increased Expression of Fibroblast Growth Factor 21 (FGF21) during Chronic Undernutrition Causes Growth Hormone Insensitivity in Chondrocytes by Inducing Leptin Receptor Overlapping Transcript (LEPROT) and Leptin Receptor Overlapping Transcript-like 1 (LEPROTL1) Expression

**Background:** FGF21 causes GH insensitivity.

**Results:** Increased FGF21 expression induces LEPROT and LEPROTL1 expression. Inhibition of LEPROT or LEPROTL1 in growth plate chondrocytes prevents the FGF21-mediated inhibition of the GH stimulatory effects on chondrocyte function and IGF-1 expression.

**Conclusion:** FGF21 prevents the GH effects on chondrocytes by activating LEPROT and LEPROTL1.

**Significance:** LEPROT and LEPROTL1 mediate the FGF21 inhibition of GH action.

During calorie restriction in mice, increased expression of FGF21 causes growth attenuation and growth hormone (GH) insensitivity. Previous evidence also indicates that fasting-associated increased expression of leptin receptor overlapping transcript (LEPROT) and LEPROTL1-like 1 (LEPROTL1) (two proteins that regulate intracellular protein trafficking) reduces GH receptor cell-surface expression in the liver. Thus, we hypothesized that FGF21 causes GH insensitivity through regulation of LEPROT and/or LEPROTL1 expression. After 4 weeks of food restriction, LEPROT and LEPROTL1 mRNA expression in the liver and in the tibial growth plate of wild-type (WT) mice was increased compared with WT mice fed ad libitum. In Fgf21 knock-out (KO) mice, LEPROT and LEPROTL1 mRNA expression in food-restricted and fed ad libitum was similar, with the exception of a subgroup of food-restricted Fgf21 KO mice treated with recombinant human (rh) FGF21 that experienced increased LEPROT and LEPROTL1 mRNA expression compared with untreated food-restricted Fgf21 KO mice.

In cultured growth plate chondrocytes, FGF21 stimulated LEPROT and LEPROTL1 mRNA expression, with such effect being prevented in chondrocytes transfected with FGFR1 siRNA or ERK1 siRNA. In cells transfected with control siRNA, GH increased [3H]thymidine incorporation, collagen X, and IGF-1 mRNA expression, with all effects being prevented by rhFGF21. In addition, rhFGF21 decreased 125I-GH binding. In LEPROT siRNA- and/or LEPROTL1 siRNA-transfected cells, rhFGF21 did not prevent the GH stimulatory effects on thymidine incorporation, collagen X, and IGF-1 expression; furthermore, rhFGF21 did not prevent 125I-GH binding. Consistent with the effects of rhFGF21, LEPROT overexpression in chondrocytes resulted in the inhibition of GH action. Our findings indicate that the increased expression of FGF21 during chronic undernutrition inhibits GH action on chondrocytes by activating LEPROT and LEPROTL1.

FGF21 is a member of a subfamily of FGFs (which includes FGF15/19 and FGF23) (1, 2) characterized by the lack of the FGF heparin-binding domain. Thus, these FGFs can act locally at/near the site of synthesis and additionally may diffuse away from their tissue of synthesis and function as endocrine factors. During fasting, increased expression of FGF21 induces gluconeogenesis, fatty acid oxidation, and ketogenesis; as a result, FGF21 is considered a key regulator of the metabolic adaptation to fasting (3–5). In addition, it has been shown that transgenic mice overexpressing FGF21 exhibit reduced bone growth and hepatic GH insensitivity (6).

In many experimental models food restriction is associated with reduced skeletal growth, with such association primarily resulting from impaired growth hormone action (7–16). In a previous study we demonstrated that mice undergoing 4 weeks of food restriction exhibited increased expression of Fgf21 (both in the liver and in the growth plate), growth hormone (GH) insensitivity, and a concomitant reduction of their body linear growth and growth plate chondrogenesis (17). Interest-
ingly, food-restricted Fgf21 knock-out mice experienced an attenuated reduction of their linear growth, tibial growth, and tibial growth plate thickness when compared with food-restricted wild-type mice. In addition, food-restricted Fgf21 knock-out mice were more GH-sensitive than food-restricted wild-type mice (i.e., they displayed greater GH receptor and IGF-1 expression both in the liver and in the tibial growth plate). In a subsequent study (18) we also showed that FGF21 can directly modulate growth plate chondrocyte function, as both β-Klotho and FGFR1 and FGFR3 (two of the FGF receptors known to bind FGF21) are expressed in chondrocytes. In the same study we demonstrated that FGF21 specifically antagonize the promoting effects of GH on cultured chondrocyte proliferation and differentiation. Thus, these findings indicate that FGF21 inhibits growth plate chondrogenesis and longitudinal bone growth during undernutrition both systemically and locally in the growth plate.

Yet little is known on the underlying molecular mechanisms of the FGF21-mediated growth attenuation during undernutrition. Previous evidence indicates that fasting-associated increased expression of LEPROT and LEPROTL1 (two transmembrane proteins involved in the regulation of intracellular protein trafficking) reduces GH receptor abundance on the hepatocyte cell membrane (19). Both proteins are also described as endospanin 1 and endospanin 2, respectively (20). Based on this evidence, we hypothesized that the FGF21-dependent reduced GH action in the liver and in the growth plate during chronic undernutrition is mediated by the increased expression of LEPROT and/or LEPROTL1. To test our hypothesis we first studied the expression of LEPROT and LEPROTL1 in the liver and in the growth plate of FGF21 knock-out and wild-type mice at the end of 4 weeks of food restriction. Then we evaluated the effects of rhFGF21 on the expression of LEPROT and LEPROTL1 in cultured growth plate chondrocytes. Last, we evaluated the effects of rhFGF21 on GH binding and GH action in cultured chondrocytes transfected with LEPROT siRNA and/or LEPROTL1 siRNA.

**MATERIALS AND METHODS**

**Generation of Mice with Targeted Disruption of Fgf21**—To generate mice with targeted disruption of the Fgf21 locus, a 6.5-kb Nhel genomic fragment containing all three exons of the mouse Fgf21 gene was subcloned and used as a gene-targeting vector by replacing part of exon 1 (30 bp downstream of the ATG), all of exon 2, and the 5′ region of exon 3 with a neomycin resistance gene (pGTN29; New England Biolabs, Ipswich, MA) thus deleting ~1200 bp of the genomic Fgf21 sequence and deleting the 3′ part of exon 1, all of exon 2, and the 5′ region of exon 3. Founder mice were subsequently backcrossed onto the C57/BL6 line at least 10 times before investigation.

**Animal Care**—Animal care was in accordance with the Guide for the Care and Use of Laboratory Animals (DHEW Publication (NIH) 85–23, revised 1988). All procedures were approved by the Institutional Animal Care and Use Committee of Drexel University College of Medicine. Homozygous Fgf21 knock-out mice were cross-bred with wild-type C57/BL6 mice; heterozygous Fgf21 knock-out mice (F1) were then cross-bred to obtain homozygous WT and homozygous Fgf21 knock (KO) mice for subsequent investigation.

Mice were maintained in a temperature (22 °C)-, humidity-, and light (12 h of light, 12 h of darkness)-controlled environment. Three-week-old KO and WT mice were housed separately and allowed ad libitum access to chow and water for 1 week (“pre-study”). During this week the mean daily food consumption for the KO and WT mice was measured. At the beginning of the study period (4 weeks), mice were assigned to four groups: two groups (WT-ad libitum and KO-ad libitum) fed ad libitum with standard chow and two groups (WT-restricted and KO-restricted) fed with a special diet (5001, Animal Specialties And Provisions, LLC., Quakertown, PA, containing 2× the amount of vitamins and minerals compared with standard chow) provided at 50% of the amount of food consumed during the pre-study by WT and KO mice, respectively. At the end of each week, the mean daily amount of chow consumed by the WT-ad libitum and KO-ad libitum mice was calculated separately; at the beginning of the following week, WT-restricted and KO-restricted mice were given 50% of the mean daily amount of food consumed in the previous week by the WT-ad libitum and KO-ad libitum, respectively. A subgroup of KO-restricted mice received recombinant human FGF21 (5 μg/g wt) or vehicle administered intraperitoneally daily for the whole duration of the study. At the end of the study, all mice were euthanized, and their livers and tibial growth plates removed and stored at −80 °C for subsequent analysis.

**Chondrocyte Culture**—The cartilaginous portions of metatarsal bones isolated from C57BL/6N mouse embryos (19 days post coitus) were dissected, rinsed in PBS, then incubated in 0.2% trypsin for 1 h and 0.2% collagenase for 3 h. Cell suspension was aspirated repeatedly and filtered through a 70-μm cell strainer, rinsed first in PBS then in serum-free DMEM, and counted. Chondrocytes were seeded in 100-mm dishes at a density of 5×10⁴/cm² in DMEM with 100 units/ml penicillin and 100 μg/ml streptomycin, 50 μg/ml ascorbic acid, and 10% FBS. The culture medium was changed at 72-h intervals. Upon confluence, cells were subcultured, and their chondrogenic phenotype was confirmed by studying the expression of type I collagen, type II collagen, and type X collagen by immunocytochemistry. In our culture conditions the percentage of cells expressing type I, type II, and type X collagen was 3.9, 97.1, and 22.0%, respectively. Subsets of cells with confirmed chondrogenic phenotype (with at least 95% of the cells positive for type II collagen-positive and negative for type I collagen-negative) were treated without or with graded concentrations of rhFGF21 (0.01–0.1–1–5–10 μg/ml, recombinant human, Lilly Research Laboratories) for up to 14 days. In another series of experiments cells were cultured for 24 h without or with graded concentrations of rhFGF21 (0.01–0.1–1–5–10 μg/ml). After 24 h, culture medium containing FGF21 was removed and replaced with fresh serum-free DMEM without or with recombinant mouse GH (10 ng/ml, National Hormone and Peptide Program, Harbor-UCLA Medical Center, Torrance, CA).

**siRNA and Plasmid Transfection**—Chondrocytes were transfected with pools of siRNAs targeted for LEPROT (sc-105612, Santa Cruz), LEPROTL1 (sc-146710, Santa Cruz), FGF21 (sc-39485, Santa Cruz), FGF1 (sc-29317, Santa Cruz), and ERK1 (sc-29308, Santa Cruz). A pool of siRNAs consisting of scrambled sequences was similarly transfected as control siRNA.
FGF21 Inhibits GH Action through LEPROT and LEPROTL1

mRNA Expression of LEPROT and LEPROTL1 in Wild-type and FgF21 Knock-out Mice Fed Ad Libitum or Food-restricted—In food-restricted wild-type mice the mRNA expression of LEPROT and LEPROTL1 in the liver (Fig. 1) and in the tibial growth plate (Fig. 2) was significantly greater than that measured in wild-type mice fed ad libitum. In contrast, we found no statistical difference in the mRNA expression of either LEPROT or LEPROTL1 when comparing FgF21 knock-out mice fed ad libitum or food-restricted (AL and FR) (Fig. 1 and 2). In the subgroup of food-restricted mice treated daily with rhFGF21 throughout the study period (5 μg/g wt), the mRNA expression of both LEPROT and LEPROTL1 was significantly increased compared with that measured in the untreated KO-FR subgroup both in the liver and in the tibial growth plate (Figs. 1 and 2).

Effects of rhFGF21 on the mRNA Expression of LEPROT and LEPROTL1 in Chondrocytes—To evaluate the direct effects of FGF21 on LEPROT and LEPROTL1 expression in growth plate chondrocytes, we added graded concentrations of rhFGF21 (0.01–0.1–1–5–10 μg/ml) to the culture medium. After 24 h, rhFGF21 significantly increased the mRNA expression of LEPROT and LEPROTL1 (assessed by real-time PCR, Fig. 3A) in a concentration-dependent fashion.

(Santa Cruz) and introduced into cells using Lipofectamine 2000 (Invitrogen) according to the procedure recommended by the manufacturer. In another series of experiments, chondrocytes were transfected with the expression plasmid for the LEPROT (Endospanin 1)-pcDNA3 (kindly provided by Dr. Ralf Jockers, Institut Cochin, Paris, France). The empty pcDNA3 vector DNA was similarly transfected as control. The expression vector was introduced to cells using Lipofectamine PLUS (Invitrogen) according to the procedure recommended by the manufacturer. One day before transfection cells were plated in 500 μl of growth medium without antibiotics such that they were 30–50% confluent at the time of transfection. The transfected cells were cultured in DMEM containing 10% FCS for 72 h after transfection.

[^H]Thymidine Incorporation—To assess cell proliferation, we measured[^H]thymidine incorporation into newly synthesized DNA. At the indicated time points during the culture period, 2.5 μCi/well of[^H]thymidine (25 Ci/mmol; Amersham Biosciences) was added to the culture medium for an additional 3 h. Cells were released by trypsin and collected onto glass fiber filters. Incorporation of[^H]thymidine was measured by liquid scintillation counting. The data represent the percentage of control from three independent experiments.

RNA Extraction and Real Time PCR—Total RNA was extracted from liver and the tibial growth plate and from cultured chondrocytes isolated from fetal (day post coitus 20) mouse metatarsal growth plates using the Qiagen RNeasy Mini kit (Qiagen Inc., Valencia CA). The recovered RNA was further processed using a First Strand cDNA synthesis kit for RT-PCR (avian myeloblastosis virus) (Roche Diagnostics) to produce cDNA. 1 μg of total RNA and 1.6 μg of oligo-p(dT)15 primer were incubated for 10 min at 25 °C followed by incubation for 60 min at 42 °C in the presence of 20 units of avian myeloblastosis virus reverse transcriptase and 50 units of RNase inhibitor in a total 20-μl reaction. The cDNA products were directly used for PCR or stored at −80 °C for later analysis. Real-time quantitative PCR was carried out using a StepOne Real time PCR System (Applied Biosystems, Foster City, CA) in a final volume of 25 μl containing 1 μl of cDNA, 12.5 μl of 2X SYBR Green master mix (Applied Biosystems), 0.1 μM primers (Applied Biosystems) in DNase-free water. Primers used were: mouse β-actin (5’-TGT GAT GGT GGG AAT GGG TCA GAA-3’ and 5’-TGG GTG GCC AGA TCT TCT CCA TGT-3’); IGF-1(5’-GGG CAT TGT GGA TGA TTG CTA-3’ and 5’-TGG AAC GAG CTG ACT TTG TAG CTT-3’; mouse metatarsal growth plates using the Qiagen RNeasy Mini kit (Qiagen Inc., Valencia CA). The recovered RNA was further processed using a First Strand cDNA synthesis kit for RT-PCR (avian myeloblastosis virus) (Roche Diagnostics) to produce cDNA. 1 μg of total RNA and 1.6 μg of oligo-p(dT)15 primer were incubated for 10 min at 25 °C followed by incubation for 60 min at 42 °C in the presence of 20 units of avian myeloblastosis virus reverse transcriptase and 50 units of RNase inhibitor in a total 20-μl reaction. The cDNA products were directly used for PCR or stored at −80 °C for later analysis. Real-time quantitative PCR was carried out using a StepOne Real time PCR System (Applied Biosystems, Foster City, CA) in a final volume of 25 μl containing 1 μl of cDNA, 12.5 μl of 2X SYBR Green master mix (Applied Biosystems), 0.1 μM primers (Applied Biosystems) in DNase-free water. Primers used were: mouse β-actin (5’-TGT GAT GGT GGG AAT GGG TCA GAA-3’ and 5’-TGG GTG GCC AGA TCT TCT CCA TGT-3’); IGF-1(5’-GGG CAT TGT GGA TGA TTG CTA-3’ and 5’-TGG AAC GAG CTG ACT TTG TAG CTT-3’; mouse metatarsal growth plates using the Qiagen RNeasy Mini kit (Qiagen Inc., Valencia CA). The recovered RNA was further processed using a First Strand cDNA synthesis kit for RT-PCR (avian myeloblastosis virus) (Roche Diagnostics) to produce cDNA. 1 μg of total RNA and 1.6 μg of oligo-p(dT)15 primer were incubated for 10 min at 25 °C followed by incubation for 60 min at 42 °C in the presence of 20 units of avian myeloblastosis virus reverse transcriptase and 50 units of RNase inhibitor in a total 20-μl reaction. The cDNA products were directly used for PCR or stored at −80 °C for later analysis. Real-time quantitative PCR was carried out using a StepOne Real time PCR System (Applied Biosystems, Foster City, CA) in a final volume of 25 μl containing 1 μl of cDNA, 12.5 μl of 2X SYBR Green master mix (Applied Biosystems), 0.1 μM primers (Applied Biosystems) in DNase-free water. Primers used were: mouse β-actin (5’-TGT GAT GGT GGG AAT GGG TCA GAA-3’ and 5’-TGG GTG GCC AGA TCT TCT CCA TGT-3’); IGF-1(5’-GGG CAT TGT GGA TGA TTG CTA-3’ and 5’-TGG AAC GAG CTG ACT TTG TAG CTT-3’); mouse collagen X (5’-ATA AGA ACG GCA CGC CTA CGA TGT-3’ and 5’-CTG CAT TGG GCA ATT GGA ACC ATG CTT-3’); mouse LEPROT (19, 20, 22). Cultured chondrocytes were incubated with 105 cpm human 125I-EFG in the presence or absence of 10 μg of unlabeled EGF. After incubation, cultured cells were processed according the protocol described for GH binding.

RESULTS

mRNA Expression of LEPROT and LEPROTL1 in Wild-type and FgF21 Knock-out Mice Fed Ad Libitum or Food-restricted—In food-restricted wild-type mice the mRNA expression of LEPROT and LEPROTL1 in the liver (Fig. 1) and in the tibial growth plate (Fig. 2) was significantly greater than that measured in wild-type mice fed ad libitum. In contrast, we found no statistical difference in the mRNA expression of either LEPROT or LEPROTL1 when comparing FgF21 knock-out mice fed ad libitum or food-restricted (AL and FR) (Fig. 1 and 2). In the subgroup of food-restricted mice treated daily with rhFGF21 throughout the study period (5 μg/g wt), the mRNA expression of both LEPROT and LEPROTL1 was significantly increased compared with that measured in the untreated KO-FR subgroup both in the liver and in the tibial growth plate (Figs. 1 and 2).

Effects of rhFGF21 on the mRNA Expression of LEPROT and LEPROTL1 in Chondrocytes—To evaluate the direct effects of FGF21 on LEPROT and LEPROTL1 expression in growth plate chondrocytes, we added graded concentrations of rhFGF21 (0.01–0.1–1–5–10 μg/ml) to the culture medium. After 24 h, rhFGF21 significantly increased the mRNA expression of LEPROT and LEPROTL1 (assessed by real-time PCR, Fig. 3A) in a concentration-dependent fashion.
Consistent with the effects observed in knock-out mice lacking the expression of FGF21, silencing FGF21 expression in cultured chondrocytes with siRNA did not cause any change in mRNA expression of LEPROT and LEPROTL1 and did not prevent the stimulatory effects of GH on IGF-1 mRNA expression, total thymidine incorporation, and collagen X mRNA expression when compared with control siRNA-transfected chondrocytes (supplemental figure).

To determine whether the effects of rhFGF21 were specific, we measured LEPROT and LEPROTL1 mRNA expression in chondrocytes transfected with FGFR1 (one of the receptors known to bind FGF21) siRNA or ERK-1 (a member of the FGF21-related intracellular signaling cascade) siRNA. In transfected cells, 0.1 ng/ml FGF21 (the lowest effective FGF21 concentration) did not induce any change in LEPROT or LEPROTL1 mRNA expression (Fig. 3B).

**Functional Interaction between FGF21, LEPROT/LEPROTL1, and GH in Chondrocytes**—To determine whether FGF21 antagonizes GH binding in chondrocytes via LEPROT or LEPROTL1, we cultured mouse growth plate chondrocytes without or with 0.1

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**FIGURE 1.** Effects of food restriction on LEPROT and LEPROTL1 mRNA and protein expression in the liver of wild-type and Fgf21 knock-out mice. At the end of the 4-week study, mice were euthanized, and their livers were removed. Total RNA and cell lysates were obtained from the liver and processed as described under “Materials and Methods.” mRNA expression was detected by real-time PCR, and protein expression was detected by Western blot. The LEPROT and LEPROTL1 mRNA expression was normalized by β-actin in the same samples. A, mRNA expression in WT mice fed ad libitum (AL) (n = 6) or food-restricted (FR) (n = 10), B, mRNA expression in Fgf21 knock-out (KO) mice fed ad libitum (AL) (n = 5), food-restricted (FR) (n = 11), or food-restricted and injected daily with recombinant human FGF21 (FR +) (n = 5). C, LEPROT and LEPROTL1 protein expression.

**FIGURE 2.** Effects of food restriction on LEPROT and LEPROTL1 mRNA and protein expression in the tibial growth plate of wild-type and Fgf21 knock-out mice. At the end of the 4-week study, mice were euthanized, and their tibial growth plates were removed. Total RNA and cell lysates were obtained from the growth plates and processed as described under “Materials and Methods.” mRNA expression was detected by real-time PCR, and protein expression was detected by Western blot. The LEPROT and LEPROTL1 mRNA expression was normalized by β-actin in the same samples. A, mRNA expression in WT mice fed ad libitum (AL) (n = 6) or food-restricted (FR) (n = 10), B, mRNA expression in Fgf21 knock-out (KO) mice fed ad libitum (AL) (n = 5), food-restricted (FR) (n = 11), or food-restricted and injected daily with recombinant human FGF21 (FR +) (n = 5). C, LEPROT and LEPROTL1 protein expression.
FGF21 Inhibits GH Action through LEPROT and LEPROTL1

Effects of FGF21 on LEPROT and LEPROTL1 mRNA expression in growth plate chondrocytes. Total RNA was extracted from chondrocytes and then processed as described under “Materials and Methods.” LEPROT and LEPROTL1 mRNA expression was evaluated by real-time PCR. The relative expression levels of mRNA were normalized by β-actin in the same samples. Results were expressed as fold change compared with control (untreated) chondrocytes (mean ± S.E.). A, chondrocytes were washed with fresh serum-free DMEM, seeded in 24-well plate, and cultured for 24 h in the absence or presence of graded concentrations of rhFGF21 (0.01–10 μg/ml). B, chondrocytes were washed with fresh serum-free DMEM, seeded in 24-well plate, transfected with control siRNA, FGFR1 siRNA, or ERK1 siRNA, and cultured for 24 h in the absence or presence of 0.1 μg/ml rhFGF21.

μg/ml rhFGF21 for 24 h. After removal of FGF21 from the culture medium, chondrocytes were incubated with 10 ng/ml radiolabeled recombinant mouse GH. In chondrocytes previously cultured in the presence of FGF21, 125I-GH cell surface binding was significantly decreased when compared with that in untreated cells. Such stimulatory effects were not prevented by the previous incubation with rhFGF21. Chondrocytes transfected with LEPROT siRNA or LEPORL1 siRNA exhibited ~80% reduction of LEPROT and LEPROTL1 mRNA expression, respectively (data not shown). In the LEPROT siRNA and LEPROTL1 siRNA-transfected cells, the FGF21-mediated inhibition of GH binding was fully neutralized (Fig. 4A). In similarly transfected chondrocytes, 125I-EGF binding was not affected by rhFGF21 (Fig. 4B).

We then studied the effects of FGF21 and GH in chondrocytes transfected with siRNAs directed to FGFR1 siRNA or ERK 1 siRNA. Mouse growth plate chondrocytes were first transfected with control siRNA, FGFR1 siRNA, or ERK 1 siRNA. All cells were cultured without or with 0.1 μg/ml rhFGF21 for 24 h. After removal of FGF21 from the culture medium, chondrocytes were incubated with 10 ng/ml recombinant mouse GH. In chondrocytes transfected with control siRNA and cultured in the absence of rhFGF21, GH significantly increased IGF-1 mRNA expression (assessed by real-time PCR) (Fig. 5A), cell proliferation (assessed by total thymidine incorporation (Fig. 5B), and collagen X mRNA expression (Fig. 5C) compared with that measured in untreated cells. Such stimulatory effects were absent in cells previously cultured in the presence of 0.1 μg/ml rhFGF21. In contrast, in FGFR1 siRNA-transfected or ERK 1 siRNA-transfected cells rhFGF21 did not prevent the GH stimulatory effects in chondrocytes.

Last, we evaluated the effects of FGF21 and GH on IGF-1 mRNA expression and chondrocyte function in control siRNA-transfected, LEPROT siRNA-transfected, and/or LEPROTL1 siRNA-transfected chondrocytes. As shown previously, in control siRNA-transfected chondrocytes 0.1 μg/ml rhFGF21 prevented the GH effects on IGF-1 mRNA expression (Fig. 6A), total thymidine incorporation (Fig. 6B), and collagen X mRNA expression (Fig. 6C). In contrast, in chondrocytes transfected with LEPROT siRNA and/or LEPROTL1 siRNA, all the GH stimulatory effects were not prevented by the previous incubation with rhFGF21. Chondrocytes transfected with LEPROT plasmid exhibited increased mRNA expression of LEPROT and a significantly reduced stimulation of GH on IGF-1 mRNA expression, total thymidine incorporation, and collagen X mRNA expression (Fig. 7).

These findings indicate that FGF21 specifically prevents the GH stimulatory effects on IGF-1 expression and chondrocyte function. Such antagonistic effect of FGF21 on GH action in chondrocytes is mediated by LEPROT and LEPORL1.

DISCUSSION

FGF21 is best known as an important metabolic regulator during adaptation to fasting, residing at the center of a complex network of transcriptional regulation underpinning energy homeostasis (23). Yet, recent evidence indicates that it may play a functional role in longitudinal bone growth. Transgenic mice overexpressing FGF21 exhibit shorter body length and tibiae when compared with wild-type mice (6). We have previously demonstrated that FGF21 expression is increased in the liver and in the growth plate of food-restricted wild-type mice (17). In food-restricted Fgf21 knock-out mice, the attenuated reduction of tibial growth and tibial growth plate thickness (compared with food-restricted wild-type mice) indicates that the increased expression of systemic FGF21 in food-restricted wild-type mice is causally related to the suppressed growth plate chondrogenesis and, in turn, suppressed longitudinal bone growth. In wild-type food-restricted mice, the increased expression of FGF21 also caused decreased GH binding and
FIGURE 4. Effects of FGF21 on GH and EGF binding at the cell surface of chondrocytes transfected with control siRNA, LEPROT siRNA, and/or LEPROT1 siRNA. 125I-GH and 125I-EGF binding activities were measured as described under "Materials and Methods" in chondrocytes transfected with control siRNA, LEPROT siRNA and/or LEPROT1 siRNA and cultured without or with 0.1 μg/ml rhFGF21. Results are expressed as % of control and represent mean values obtained from three independent experiments. A, GH binding activity; B, EGF binding activity.

FIGURE 5. Effects of FGF21 and GH on IGF-1 mRNA expression, total [3H]thymidine incorporation, and collagen X mRNA expression in chondrocytes transfected with control siRNA, FGFR1 siRNA, or ERK1 siRNA. Transfected chondrocytes were first cultured without or with 0.1 μg/ml rhFGF21 and then in the absence or presence of 10 ng/ml recombinant mouse GH. A, at the end of the culture period, total RNA was extracted from chondrocytes and then processed as described under "Materials and Methods." IGF-1 mRNA expression was evaluated by real time PCR. The relative expression levels of mRNA were normalized by β-actin in the same samples. Results were expressed as -fold change compared with control (untreated) chondrocytes (mean ± S.E.). B, at the end of culture period, 2.5 μCi/well of [3H]thymidine (Amersham Biosciences) was added to the culture medium for an additional 3 h. Incorporation of [3H]thymidine was measured by liquid scintillation counting. Results are expressed as % of untreated and represent mean values obtained from three independent experiments. C, collagen X mRNA expression was determined by real time PCR. Total RNA was extracted from transfected chondrocytes and then processed as described under "Materials and Methods." The relative expression levels of mRNA were normalized by β-actin in the same samples. Results were expressed as -fold change compared with control chondrocytes (mean ± S.E.).
IGF-1 expression both in the liver and in the tibial growth plate. The reduced IGF-1 expression in the liver suggests that the FGF21-dependent systemic GH insensitivity is mechanistically related to rodent undernutrition-associated growth failure. On the other hand, the reduced GH binding and IGF-1 expression in the tibial growth plate also implicates an antagonistic effect of FGF21 on GH action directly at the long bone growth plate.

The leptin receptor overlapping transcript (LEPROT, also known as endospanin-1 or obesity receptor gene-related protein, OB-RGRP) belongs to a family of genes that includes two other members: one in mammalian cells (LEPROT-like 1, LEPROTL1, or endospanin-2) (20, 24) and another in *Saccharomyces cerevisiae* (vacuolar protein sorting 55, VPS55) (25). These genes encode small proteins with four potential transmembrane domains. Experimental studies indicate that LEPROT may be implicated in the down-regulation of transmembrane protein levels and their targeting from late endosomes to lysosomes. Silencing of LEPROT increases leptin receptor localization on the plasma membrane (20, 22), whereas overexpression of LEPROT decreases cell-surface exposure of leptin receptor (20) and GH receptor (19). The effect of LEPROT on the intracellular trafficking of membrane receptors appears to be specific, as it has been shown that it does not affect EGF receptor trafficking (19, 20, 22). LEPROT and LEPROTL1 mRNAs are widely expressed in mammalian tissues and organs, including the liver. It has been demonstrated that transgenic mice overexpressing LEPROT and LEPROTL1 exhibit growth retardation and reduced GH sensitivity in the liver. In the same study the authors described the increased hepatic expression of LEPROT and LEPROTL1 during 24 h of fasting.

In a previous study (18) we have shown that FGF21 can directly inhibit GH binding in growth plate chondrocytes. Such inhibition does not result from a suppressed GH receptor (GHR) expression or from the proteolysis of the cell membrane-bound GHR; thus, it is plausible that the FGF21-dependent decreased GH binding likely results from a reduced GHR
intracellular recycling. Our present study demonstrated a significant increase of LEPROT and LEPROTL1 expression during prolonged food restriction in mice both in the liver and in the tibial growth plate. The concomitant increased expression in the same tissues of FGF21 during food restriction raises the question as to whether FGF21 functionally interacts with LEPROT and/or LEPROTL1. Several of our findings support this functional interaction. First, food-restricted wild-type mice exhibited a greater LEPROT and LEPROTL1 expression than wild-type mice fed ad libitum, whereas such increased expression was absent in food-restricted Fgf21 knockout mice. Second, the injection of rhFGF21 in food-restricted Fgf21 knockout mice increased LEPROT and LEPROTL1 expression in the liver and in the tibial growth plate. Third, rhFGF21 stimulated LEPROT and LEPROTL1 expression in control siRNA-transfected cultured chondrocytes but it did not in cells transfected with FGFR1 or ERK1 siRNAs. Fourth, rhFGF21 inhibited GH binding in cultured chondrocytes, but it did not inhibit EGF binding, suggesting that FGF21 effect is specific to the GH receptor and could be mediated by LEPROT as it has been previously shown that LEPROT regulates GH receptor, but not EGF receptor intracellular processing (22). All these findings indicate that FGF21 specifically and directly modulate LEPROT/LEPROTL1 expression and function in chondrocytes.

Based on the observations described above and on the evidence indicating that LEPROT and LEPROTL1 cause growth inhibition, we hypothesized that the FGF21 effects on growth plate chondrogenesis are mediated by LEPROT and LEPROTL1 in chondrocytes. In our study we first demonstrated that FGF21 directly antagonizes GH stimulatory effects on proliferation and differentiation and IGF-1 expression in growth plate chondrocytes. Such effects are FGF21-specific, as they are prevented in chondrocytes transfected with siRNAs directed to FGFR1 or ERK1, two components of the FGF21 signaling pathway. Furthermore, we have shown that the antagonistic properties of FGF21 on all of the GH stimulatory effects are prevented in LEPROT and/or LEPROTL1 siRNA-transfected chondrocytes. Because the concomitant silenced expression of LEPROT and LEPROTL1 induces the same quantitative effects of the silencing of either one of these two genes, it is plausible their activity in chondrocytes may overlap. Last, the overexpression of LEPROT in chondrocytes significantly reduces the GH stimulatory effects on chondrocyte function, thus suggesting that LEPROT does in fact mediate the antagonistic effects of FGF21 on GH action. The fact that LEPROT overexpression does not completely prevent GH effects raises the question as to whether other intracellular molecules may contribute to the FGF21 action in chondrocytes.

In conclusion, our findings indicate that the effects of FGF21 on growth plate chondrogenesis and bone growth during chronic undernutrition are direct and specific on chondrocytes. It is unclear whether FGF21 acts on the growth plate as a systemic (endocrine) and/or as a local (paracrine) growth factor, as its expression is increased during food restriction both in the liver and within the growth plate. Our findings also indicate that the FGF21 effects depend on the intracellular activity of

**FIGURE 7. Effects of LEPROT overexpression on GH activity in chondrocytes.** Chondrocytes transfected with LEPROT (endospanin 1)-pcDNA3 or the empty vector were cultured in the absence or presence of 10 ng/ml recombinant mouse GH. A, B, and D, at the end of the culture period, total RNA was extracted from chondrocytes and then processed as described under "Materials and Methods." LEPROT/LEPROTL1 (A), IGF-1 (B), and collagen X (D) mRNA expression was evaluated by real time PCR. The relative expression levels of mRNA were normalized by β-actin in the same samples. Results were expressed as -fold change compared with empty vector-transfected (untreated) chondrocytes (mean ± S.E.). C, at the end of culture period, 2.5 μCi/well of [3H]thymidine (Amersham Biosciences) was added to the culture medium for an additional 3 h. Incorporation of [3H]thymidine was measured by liquid scintillation counting. Results are expressed as % of empty vector-transfected and untreated cells and represent mean values obtained from three independent experiments.
LEPROT and/or LEPROT1, which in turn prevent GH binding and action on chondrocytes. Further studies are warranted to determine whether additional intracellular mediators play a mechanistic role in the FGF21 effects on systemic and local GH insensitivity and growth plate chondrogenesis.

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