# Diet Polyphenol Curcumin Stimulates Hepatic Fgf21 Production and Restores Its Sensitivity in High-Fat-Diet–Fed Male Mice

Kejing Zeng,[1,2]* Lili Tian,[2,4] Rucha Patel,[5] Weijuan Shao,[2] Zhuolun Song,[2,4] Ling Liu,[2] Justin Manuel,[3] Xuezhong Ma,[3] Ian McGilvray,[3] Carolyn L. Cummins,[4,5] Jianping Weng,[1] and Tianru Jin[2,4,6,7]*

[1]Department of Endocrinology and Metabolism, the Third Affiliated Hospital of Sun Yat-Sen University, Guangdong Provincial Key Laboratory of Diabetology, Guangzhou, 510630, China; [2]Toronto General Research Institutes and [3]Transplant Core Laboratory, Multi-organ transplantation, University Health Network, Toronto, Ontario M5G 1L7, Canada; [4]Banting and Best Diabetes Centre, University of Toronto, Toronto, Ontario M5G 2C4, Canada; [5]Faculty of Pharmacy, University of Toronto, Toronto, Ontario M5S 3M2, Canada; and [6]Department of Physiology, Faculty of Medicine, and [7]Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario M5S 1A8, Canada

We found previously that short-term curcumin gavage stimulated mouse hepatic fibroblast growth factor 21 (Fgf21) expression. Here we conducted mechanistic exploration and investigated the potential pathophysiological relevance on this regulation. Fgf21 stimulation was observed at messenger RNA and protein levels in mice with daily curcumin gavage for 4 or 8 days and in primary hepatocytes with curcumin treatment. Using peroxisome proliferator-activated receptor $\alpha$ (PPAR$\alpha$) agonist and antagonist, along with luciferase reporter and chromatin immune-precipitation approaches, we determined that curcumin stimulates Fgf21 transcription in a mechanism involving PPAR$\alpha$ activation. High-fat diet (HFD) feeding also increased mouse hepatic and serum Fgf21 levels, whereas dietary curcumin intervention attenuated these increases. We found that HFD feeding reduced hepatic expression levels of genes that encode FGFR1 and $\beta$Klotho, PGC1$\alpha$, and the targets of the PPAR$\alpha$–PGC1$\alpha$ axis, whereas concomitant curcumin intervention restored or partially restored their expression levels. Importantly, hepatocytes from HFD-fed mice showed a loss of response to FGF21 treatment on Erk phosphorylation and the expression of *Egr1* and *cFos*; this response was restored in hepatocytes from HFD-fed mice with curcumin intervention. This investigation expanded our mechanistic understanding of the metabolic beneficial effects of dietary curcumin intervention involving the regulation of Fgf21 production and the attenuation of HFD-induced Fgf21 resistance. (***Endocrinology*** **158: 277–292, 2017)**

Fibroblast growth factor 21 (Fgf21) is mainly produced in and released by the liver in response to fasting (1–4). The lack of the conventional heparin binding domain of this unique FGF family member allows this hepatokine to diffuse away from its site of origin and function as a hormone (4–6). Intensive investigations have revealed that the elevation of Fgf21 production during fasting occurs via a mechanism involving activation of peroxisome proliferator–activated receptor $\alpha$ (PPAR$\alpha$) (1, 2, 7, 8). During the adaptive starvation response, Fgf21 may induce hepatic expression or activation of peroxisome proliferator-activated receptor $\gamma$ coactivator 1-$\alpha$ (PGC1$\alpha$), which facilitates fatty acid oxidation, tricarboxylic acid cycle flux, and gluconeogenesis (9).

Over the past a few years, we have seen growing evidence for the therapeutic potential of Fgf21 in metabolic disorders, including diabetes and its complications,

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*These authors contributed equally to this study.

Abbreviations: ChIP, chromatin immunoprecipitation; Fgf21, fibroblast growth factor 21; F/I, forskolin and IBMX; HFD, high-fat diet; IBMX, 3-isobutyl-1-methylxanthine; IPGTT, intraperitoneal glucose tolerance test; LFD, low-fat diet; LUC, luciferase; mRNA, messenger RNA; PCR, polymerase chain reaction; PPAR$\alpha$, peroxisome proliferator-activated receptor $\alpha$; PPRE, peroxisome proliferator-activated receptor response element; T2D, type 2 diabetes.

obesity, dyslipidemia, and liver steatosis, in humans and in animal models (4, 10–15); however, animals or human subjects with obesity or diabetes were paradoxically found to have elevated plasma Fgf21 levels, raising the hypothesis that obesity is an Fgf21-resistant state (16–21). Indeed, Fisher *et al.* (16) have shown that high-fat/high-sucrose diet–induced obese mice displayed an attenuated response to Fgf21 treatment, associated with reduced messenger RNA (mRNA) expression of FGF receptors in the liver and white adipose tissue. In a mouse model with mild nonalcoholic fatty liver disease, Rusli *et al.* (22) demonstrated that an elevated plasma Fgf21 level was associated with decreased expression of the gene that encodes the Fgf21 co-receptor βKlotho.

Curcumin, anthocyanin, and resveratrol are the most studied dietary plant polyphenols (23, 24). As a traditional medicine and the major component of turmeric, curcumin possesses antimicrobial, insecticidal, cardioprotective, radioprotective, and anticancer properties (24). Importantly, several preclinical investigations have shown the effect of curcumin in improving insulin signaling and reducing body weight gain in high-fat diet (HFD)-fed mice (25–27) and have attributed such effects to the anti-inflammation and antioxidative stress functions (25, 26, 28, 29). A recent small-scale clinical trial indicated that curcumin intervention in prediabetic subjects lowered the number of individuals who eventually developed type 2 diabetes (T2D) (30). Thus, the nutraceutical curcumin possesses great potential in the prevention and treatment of obesity, diabetes, and other metabolic disorders in which insulin resistance plays a fundamental role.

We demonstrated very recently that daily curcumin gavage for 6 days improved insulin signaling in mice that received dexamethasone injections (31). In that study, we also noticed the stimulation of hepatic Fgf21 protein expression by curcumin gavage (31). Here we conducted a mechanistic exploration examining Fgf21 induction by curcumin treatment *in vivo* and *in vitro* and investigated the potential pathophysiological relevance of the role of curcumin on Fgf21 production and function in the HFD-fed mouse model. Our findings suggest the existence of both a short-term stimulatory effect of curcumin on Fgf21 production and a long-term effect of dietary curcumin intervention on attenuating Fgf21 resistance in mice consuming a HFD.

## Materials and Methods

### Reagents

Curcumin was purchased either from Sigma-Aldrich (St. Louis, MO) for *in vitro* cell culture experiments or from Organika Health Products (Richmond, BC, Canada; a 95% standardized curcumin extract) for short-term gavage or long-term dietary intervention in mice. The PPARα antagonist GW6471 (G5045) and the agonist Wy14643 (C7081) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Actinomycin D was purchased from Calbiochem (La Jolla, CA). Human recombinant Fgf21 was from Novoprotein Scientific Inc. (Summit, NJ). The cyclic adenosine monophosphate–promoting agents forskolin and 3-isobutyl-1-methylxanthine (IBMX) were from Sigma-Aldrich.

### Animals and animal experimental design

Eight-week-old, male C57BL/6J mice purchased from Charles River Laboratories (St. Laurent, QC, Canada) were housed at constant temperature (22°C) under a 12-hour light/dark cycle with free access to food and water. The animal experiments and protocols were approved by the University Health Network Animal Care Committee and performed in accordance with the guidelines of the Canadian Council of Animal Care.

#### *Experiment 1*

The protocol for 6 days curcumin gavage was previously presented (31). Briefly, male mice received either sesame oil (solvent) or curcumin (500 mg/kg body weight/d) gavage every day for 5 consecutive days. After 1 day of rest, an intraperitoneal insulin tolerance test was performed at day 7. A booster curcumin (or solvent) gavage was given on day 8. The mice were killed for liver tissue and serum collection on day 10. This experiment is defined as 6-day curcumin gavage.

#### *Experiment 2*

The same experiment as experiment 1 was performed, except the dosage of curcumin was reduced to 100 mg/kg body weight/d for 4 or 8 days before animals were killed for liver tissue and serum collection.

#### *Experiment 3*

Eight-week-old male mice were fed a low-fat diet (LFD) or an HFD (60% Kcal from soybean fat) with or without curcumin (4 g/kg diet) for 12 weeks (26). An intraperitoneal glucose tolerance test (IPGTT) was performed as previously described (26).

### Cell culture

The human HepG2 and mouse Hepa1-6 cell lines were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum (32). C57BL/6J male mice (8–12 weeks old) were used for primary hepatocyte isolation as described previously (33, 34). Human hepatocytes were isolated from a 16-year-old male donor without a known hepatic disease, with the 2-step collagenase isolation method in the laboratory approved with Good Laboratory Practice. The study was approved by the Research Ethics Board of the University Health Network.

### Fgf21, triglyceride, and free fatty acid measurements

The quantitative measurement of Fgf21 in mouse serum was performed with the Fgf21 Immunoassay Kit (catalogue number 32180) provided by Antibody and Immunoassay Services (The University of Hong Kong). Liver triglyceride contents were determined using the Serum Triglyceride Determination Kit

(TR0100; Sigma-Aldrich). Details are provided in the Supplemental Methods.

### Preparation of cell lysates and Western blotting

Whole-cell lysates from mouse liver tissue, primary hepatocytes, or cultured cell lines were prepared and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by Western blotting as previously described (35). Antibodies for Western blotting are listed in Table 1.

### RNA extraction, real-time reverse transcription polymerase chain reaction, and the assessment of the effect of curcumin treatment on Fgf21mRNA stability

TRI reagent (Sigma-Aldrich) was used for RNA extraction. Real-time polymerase chain reaction (PCR) was performed as previously described (36). PCR primers are listed in Supplemental Table 1. The method for assessing the effect of curcumin on *Fgf21* mRNA stability is detailed in Supplemental Methods.

### Luciferase assay, chromatin immunoprecipitation, and GAL4 nuclear receptor assay

The construction of the fusion gene constructs in which the expression of the luciferase (LUC) reporter is driven by an ~1.5-kb or ~3.1-kb mouse *Fgf21 5′* flanking sequence is detailed in the Supplemental Methods. The LUC assay was conducted as previously described (33). The procedures for chromatin immunoprecipitation (ChIP) and quantitative ChIP (qChIP), as well as GAL4 nuclear receptor assay, are detailed in the Supplemental Methods. Supplemental Table 1 also contains the primers used in ChIP and qChIP.

### Statistics

Quantitative results are expressed as a mean ± standard deviation. For the comparison of 2 groups, the 2-tailed, unpaired Student $t$ test was used in the statistical analysis, with significance at $P < 0.05$. Comparisons between groups with 1 treatment were determined by one-way analysis of variance with a *post hoc* Bonferroni test.

## Results

### Curcumin treatment increases hepatic Fgf21 expression

We found previously that 6-day curcumin gavage in dexamethasone-injected mice improved insulin signaling and increased hepatic Fgf21 protein levels (31). Because dexamethasone can stimulate Fgf21 expression as well (37), we tested whether short-term curcumin gavage in mice on chow diet increases Fgf21 expression at both mRNA and protein levels in the absence of dexamethasone injection. As shown in Fig. 1(A), 6-day curcumin

**Table 1. Antibodies Used**

| Peptide/ Protein Target | Antigen Sequence (if known) | Name of Antibody | Manufacturer, Catalog No. and/or Name of Individual Providing the Antibody | Species Raised in; Monoclonal or Polyclonal | Dilution Used | RRID |
|---|---|---|---|---|---|---|
| Akt | C-terminal sequence (mouse) | Anti-Akt antibody | Cell Signaling Technology (9272) | Rabbit polyclonal | 1:1000 | AB_329827 |
| pAkt at S473 | 471–475 (Q-F-S-Y-S) (human) | Anti-pAkt (ab-473) | Signalway Antibody (21054) | Rabbit polyclonal | 1:1000 | AB_2629463 |
| $\beta$-actin | Not available | Anti-$\beta$-Actin (C4) | Santa Cruz Biotechnology (SC47778) | Mouse monoclonal | 1:2000 | AB_626632 |
| FGF21 | Not available | Anti-FGF21 | Abcam (AB171941) | Rabbit monoclonal | 1:1000 | AB_2629460 |
| GAPDH | 1–335 (human) | Anti-GAPDH (FL-335) | Santa Cruz Biotechnology (SC25778) | Rabbit polyclonal | 1:2000 | AB_10167668 |
| Heme oxygenase 1 | 1–100 (N terminal, human) | Anti-Heme oxygenase 1 | Abcam (Ab68477) | Rabbit polyclonal | 1:1000 | AB_11156457 |
| NF-$\kappa$B P-65 | 527–531 (human) | Anti-NF-$\kappa$B P-65 (Ab-529) | Signal Way Antibody (21210) | Rabbit polyclonal | 1:1000 | AB_2629462 |
| PPAR$\alpha$ | 1–98 (human) | Anti-PPAR$\alpha$ (H-98) | Santa Cruz Biotechnology (9000) | Rabbit polyclonal | 1:1000[a] | AB_2165737 |
| pPPAR$\alpha$ (S12) | ICPLSpPLEADDL (mouse) | Anti-pPPAR$\alpha$ (S12) | Abcam (AB3484) | Rabbit polyclonal | 1:1000 | AB_303844 |
| Rabbit IgG | Not applicable | Rabbit IgG | Santa Cruz Biotechnology (SC2027) | Rabbit polyclonal | 1:25[a] | AB_737197 |
| RNA polymerase II | 1–224 (human) | Anti-RNA polymerase II | Santa Cruz Biotechnology (SC9001) | Rabbit polyclonal | 1:25[a] | AB_2268548 |

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NF, nuclear factor; RRID, Research Resource Identifier.

[a]Diluted 1:25 for ChIP assay.

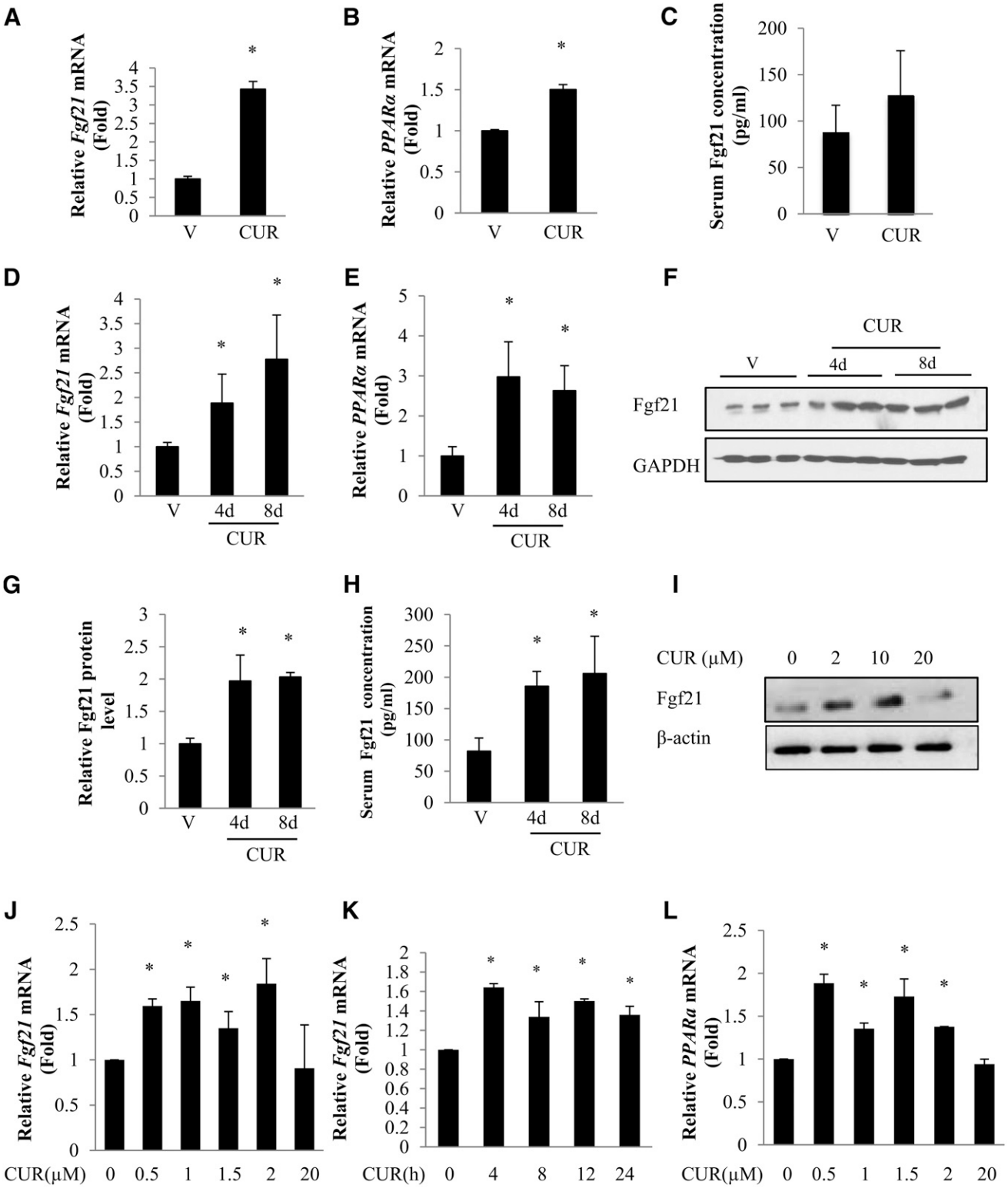


**Figure 1.** Curcumin increases hepatic Fgf21 expression in mice and mouse primary hepatocytes. Hepatic *Fgf21* (A) and *PPARα* (B) mRNA levels in mice receiving daily curcumin (500 mg/kg body weight) or control vehicle gavage for 6 days, assessed by quantitative reverse transcription PCR. (C) Random serum Fgf21 levels of the mice depicted in A. Hepatic *Fgf21* (D) and *PPARα* (E) mRNA levels in mice receiving curcumin (100 mg/kg body weight) or control vehicle gavage for 4 or 8 days. (F) Western blotting of hepatic Fgf21 protein expression in aforementioned mice depicted in panel D. (G) Quantification of panel F. (H) Random serum Fgf21 levels of the mice depicted in panel D. (I) Mouse primary hepatocytes were treated with the indicated doses of curcumin for 6 hours followed by cell harvesting and Western blotting with the indicated antibodies. (J–L) Mouse primary hepatocytes were treated with the indicated doses of curcumin for 6 hours (J and L) or with 2 μM curcumin for the indicated time intervals followed by cell harvesting and quantitative reverse transcription PCR against indicated genes. Values are means ± SD (n = 6 for A–C, n = 5 for D–H, and n ≥ 3 for J–L). Panel I is a represented blot of 3 independent experiments. *$P < 0.05$ vs the corresponding control. CUR, curcumin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; V, control vehicle.

gavage increased hepatic *Fgf21* mRNA level, associated with *PPARα* elevation [Fig. 1(B)]. Plasma Fgf21 levels in mice in this set of experiments showed a trend toward an increased response to curcumin gavage, but the difference did not reach statistical significance [Fig. 1(C)]. To confirm the *in vivo* stimulatory effect, we repeated the curcumin gavage experiment in C57BL/6 mice and reduced the dosage from 500 to 100 mg/kg body weight/d. Figure 1(D) and 1(E) shows that curcumin gavage for either 4 or 8 days increased hepatic *Fgf21* and *PPARα* mRNA levels. We also observed an increase of Fgf21 expression at the protein level [Fig. 1(F) and 1(G)]. In this experiment, the effect on random plasma Fgf21 level elevation was observed for mice receiving either 4- or 8-day curcumin gavage (Fig. 1H).

We then conducted *in vitro* experiments. As shown in Fig. 1(I) and 1(J) and in Supplemental Fig. 1A, Fgf21 mRNA and protein expression in mouse primary hepatocytes can be stimulated by 12 hours of curcumin treatment at dosages varying from 0.5 to 10 μM but not at 20 μM, although the activation on Fgf21 protein expression at 0.5 μM was not always observable. Figure 1(K) shows that *Fgf21* mRNA expression can be stimulated by 2 μM curcumin at time courses from 4 to 24 hours. Stimulated *Fgf21* mRNA expression is associated with elevated expression of *PPARα* [Fig. 1(L)]. The stimulatory effect of curcumin on FGF21 was also observed in human hepatocytes [Supplemental Fig. 1(B) and 1(C)].

The Fgf21 protein level in the human HepG2 cell line was very low in our study, and in the mouse Hepa1-6 cell line it was below the detection limit by Western blotting [Supplemental Fig. 2(A) and 2(B)]. Indeed, we could not detect *Fgf21* mRNA expression in the Hepa1-6 cell line by reverse transcription PCR [Supplemental Fig. 2(C)].

Because curcumin was shown to exert anti-inflammation and antioxidative stress effects in hepatocytes and elsewhere (25, 26), we also tested its effect on reducing nuclear factor κB and increasing heme oxygenase-1 expression in mouse hepatocytes (Supplemental Fig. 3). Because these effects were achieved only at concentrations higher than 2 μM, we suggest that curcumin-stimulated Fgf21 expression is likely independent of its anti-inflammation and antioxidative stress effects.

## PPARα is among mediators that convey the stimulatory effect of curcumin on Fgf21 transcription

PPARα positively regulates *Fgf21* transcription, and 2 evolutionarily conserved PPAR response elements (PPREs) were located on the *Fgf21* gene promoter regions (1, 8). We have hence constructed the *Fgf21*-LUC reporter gene constructs in which the expression of the LUC reporter is driven by either ~1.5 kb or ~3.1 kb mouse *Fgf21* gene 5′ flanking region [Fig. 2(A)]. Figure 2(B) and 2(C) shows that when either the 1.5 kb or the 3.1 kb *Fgf21*-LUC reporter plasmid was transiently transfected into HepG2 cells, 4 hours of curcumin treatment increased the LUC activity. The stimulatory effect of the cyclic adenosine monophosphate/protein kinase A activation agents [forskolin and IBMX (F/I)] on *Fgf21*-LUC reporter expression was also shown in Fig. 3(B) and 3(C), and Supplemental Fig. 4 shows that in the baby hamster kidney fibroblast naive cell system, curcumin, or F/I also stimulated the activity of the 1.5 kb *Fgf21*-LUC.

To determine the involvement of PPARα on curcumin-stimulated Fgf21 expression, PPARα antagonist GW6471 and agonist Wy14643 were used. Figure 2(D) and 2(E) shows that although GW6471 did not inhibit Fgf21 expression on its own, it blocked the stimulatory effect of curcumin treatment. Figure 2(F) and 2(G) shows that both curcumin and Wy14643 increased Fgf21 expression, and there was no appreciable synergistic effect when both curcumin and Wy14643 were used. We were unable to detect appreciable stimulation on PPARα expression at the protein level by curcumin treatment [Fig. 2(E) and 2(G)]. However, curcumin treatment (at both 1.5 and 2 μM) reduced PPARα (S12) phosphorylation, which has been suggested to be inversely associated with its activity (38) [Fig. 2(H)]. Figure 2(I) shows that curcumin treatment generated no appreciable effect on attenuating the degradation of *Fgf21* mRNA in the presence of actinomycin D. These observations collectively suggest that curcumin stimulates *Fgf21* transcription via a mechanism involving PPARα activation.

## Four-hour curcumin treatment increases the binding of PPARα to the Fgf21 gene promoter

ChIP was then used to test whether curcumin treatment increases binding of PPARα to *Fgf21* promoter. Figure 3(A) shows the overall organization of mouse *Fgf21* intron–exon organization and the 5′ flanking region, including the locations of two PPREs. Figure 3(B) and 3(C) shows that the anti-PPARα antibody, but not the control rabbit IgG, pulled down DNAs that contain PPRE1 and PPRE2. We were also able to pull down PPRE1 and PPRE2 with the RNA polymerase II antibody [Fig. 3(B) and 3(C)]. None of the 3 antibodies pulled down the intron II fragment that contains no PPRE [Fig. 3 (D)]. Figure 3(E) and 3(F) shows the qChIP results, which indicate that treatment of mouse hepatocytes with the PPARα agonist Wy14643 for 0.5 hours enriched the binding of PPARα to both PPRE1 and PPRE2. The enriched binding of PPARα to PPRE1 and PPRE2 was

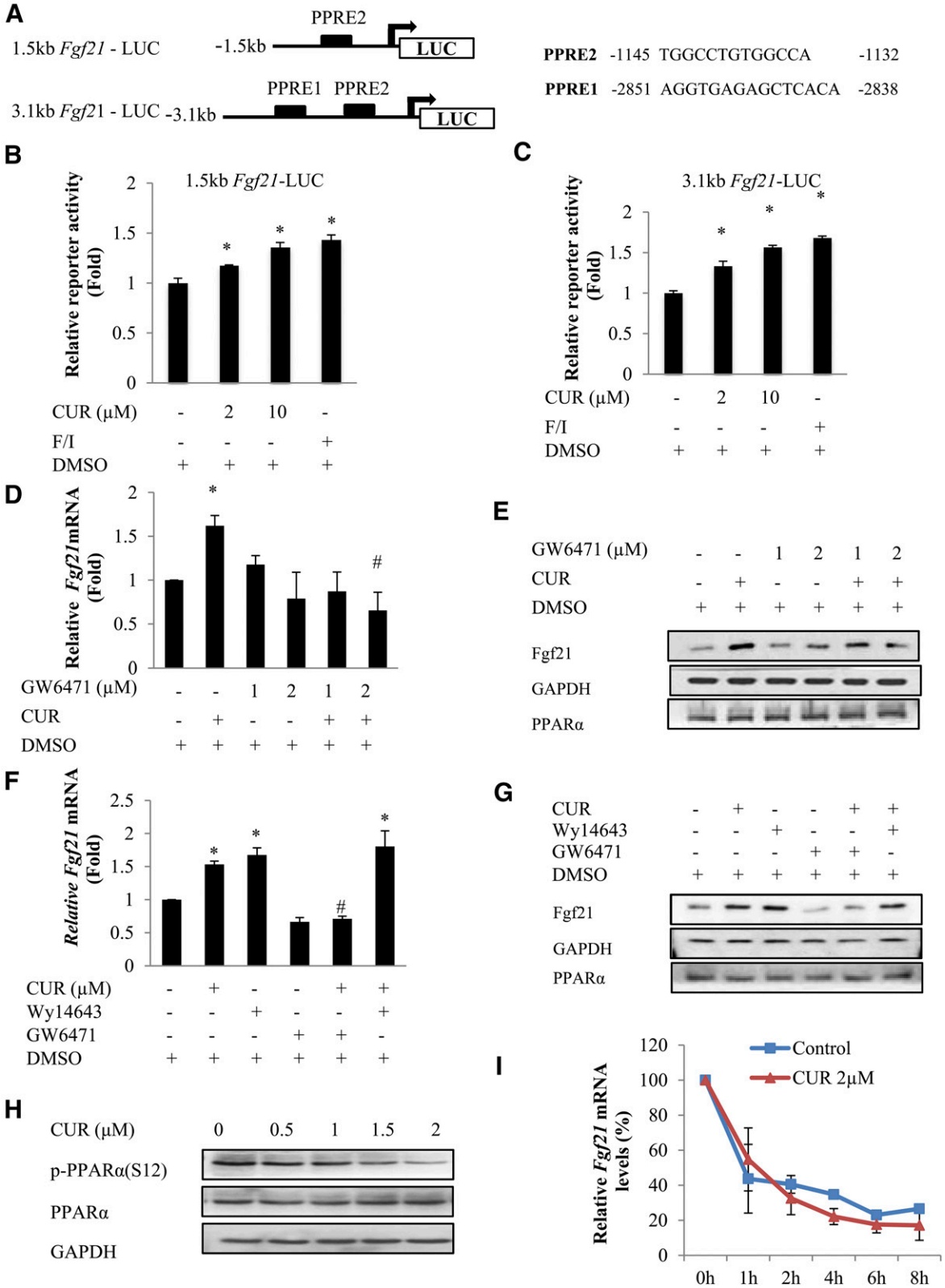


**Figure 2.** PPARα is among the mediators that convey the stimulatory effect of curcumin on Fgf21 expression. (A) Illustration of *Fgf21*-LUC reporter gene constructs including the positions of PPRE1 and PPRE2. (B, C) Effect of 4-hour treatment with curcumin (at the indicated dosages) or F/I (10 μM each) on the activity of indicated *Fgf21*-LUC reporter in HepG2 cells. Values were presented as fold change with untreated sample defined as 1-fold. Effect of curcumin (2 μM) on *Fgf21* mRNA (D) or Fgf21 and PPARα protein (E) expression in mouse primary hepatocytes in the absence and presence of the indicated dosages of the PPARα antagonist GW6471. Effect of curcumin (2 μM) on *Fgf21* mRNA (F) or Fgf21 and PPARα protein (G) expression in mouse primary hepatocytes in the absence and presence of GW6471 (2 μM) or the PPARα agonist Wy14643 (10 μM). For panels D–G, cells were pretreated with GW6471 or Wy14643 for 1 hour, followed by 4 hours of curcumin treatment. (H) Effect of 4 hours of curcumin treatment (at the indicated dosage) on PPARα S12 phosphorylation. (I) Effect of curcumin (2 μM) on *Fgf21* mRNA expression

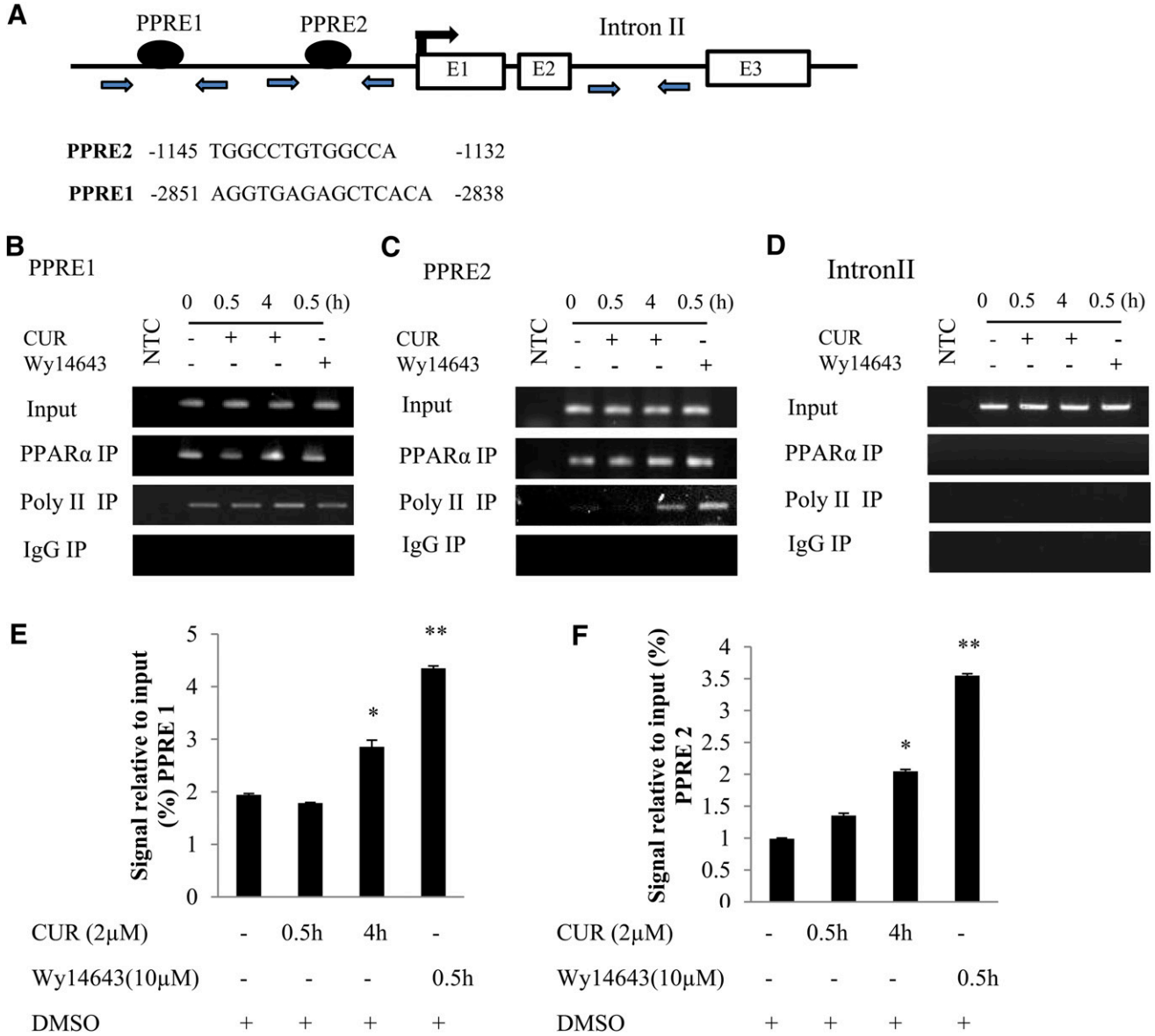


**Figure 3.** Curcumin treatment increases the binding of PPAR$\alpha$ to the mouse *Fgf21* gene promoter. (A) Illustration of the organization of the mouse *Fgf21* gene and its proximal 5′ flanking region (not to scale), including the positions of primers used for ChIP. The detailed positions and nucleotide sequences of PPRE1 and PPRE2 are shown. (B–D) Agarose gel electrophoreses (2%) show the detection of PPRE1, PPRE2, or Intron II containing DNA fragments by PCR after ChIP (with the indicated antibody) in mouse primary hepatocytes with the indicated treatment of the indicated time. qChIP shows the effect of Wy14643 (10 μM) or curcumin (2 μM) treatment on the occupancy of PPAR$\alpha$ on PPRE1 (E) and PPRE2 (F).. Values are means ± SD (n ≥ 3). * $P < 0.05$ vs the corresponding control treatment. CUR, curcumin; DMSO, dimethyl sulfoxide; IgG, immunoglobulin G; IP, immunoprecipitation; NTC, no template control; Poly II, RNA polymerase II.

also observed in mouse hepatocytes treated with curcumin for 4 hours but not for 0.5 hours [Fig. 3(E) and 3(F)], suggesting that curcumin may not serve as a direct ligand of PPAR$\alpha$. Indeed, we conducted a GAL4 nuclear receptor/upstream activation sequence–luciferase reporter assay in HEK293 cells. Curcumin did not activate PPAR$\alpha$, although the vitamin D receptor could be activated with 10 μM curcumin treatment. Furthermore, conditioned medium of mouse primary hepatocytes treated with curcumin cannot stimulate the GAL4 nuclear receptor/upstream activation sequence–luciferase reporter (detailed in Supplemental Fig. 5)

**Figure 2. (Continued).** in the absence or presence of actinomycin D (10 μg/ml) for the indicated time. The expression levels are presented as percentage change, with *Fgf21* mRNA levels in cells receiving no treatment defined as 100%. For panels B, C, D, F, and I, values are presented as means ± SD (n = 3). *$P < 0.05$ vs the corresponding control treatment. Panels E, G, and H are representative Western blots of 3 independent experiments. CUR, curcumin; DMSO, dimethyl sulfoxide; GAPDH, glyceraldehyde 3-phosphate dehydrogenase;

### HFD feeding increases hepatic Fgf21, whereas curcumin intervention attenuates the increase

Beneficial metabolic effects of curcumin intervention in mice were reported previously (25, 26). We repeated the mouse study with 12 weeks of dietary curcumin intervention. Figure 4(A–C) shows the attenuation of curcumin intervention on HFD-induced body weight increase. Figure 4(D) and 4(F) shows that there was no appreciable difference in food intake among the three groups of mice during the experimental period. Figure 4(G) and 4(H) shows our IPGTT results, indicating that curcumin intervention attenuated the impairment on glucose disposal induced by HFD consumption.

The increases in serum and hepatic triglyceride and serum free fatty acid content with HFD feeding were attenuated with curcumin intervention [Fig. 5(A–C)]. In contrast to what we observed with short-term curcumin gavage, long-term curcumin intervention attenuated the increase of serum Fgf21 level induced by HFD feeding [Fig. 5(D)]. Therefore, we examined hepatic Fgf21 levels in these mice. As shown in Fig. 5(E) and 5(F), HFD feeding increased hepatic Fgf21 protein levels, and this increase was completely blocked with curcumin intervention. Figure 5(G) shows the stimulation of HFD feeding on hepatic *Fgf21* mRNA expression, and this stimulation was also blocked with dietary curcumin intervention. Figure 5(H) shows that, when compared with LFD, 12 weeks of HFD feeding reduced the *PPARα* mRNA level, whereas curcumin intervention partially restored the *PPARα* mRNA level.

### Curcumin intervention restores or partially restores the expression of genes that are altered by HFD feeding

Elevated plasma Fgf21 levels in obese mice were shown to be associated with reduced *Fgfr1* or *βKlotho* expression (16, 22). In our HFD-fed mouse model, hepatic *Fgfr1* and *βKlotho* levels were also reduced, whereas curcumin intervention partially restored their expression levels [Fig. 6(A) and 6(B)]. Figure 6(C) shows that HFD feeding also reduced hepatic levels of *PGC1α*, a key mediator of Fgf21 hepatic function (9), and this reduction was completely blocked by curcumin intervention.

*Acox1*, *Pdk4*, and *Ehhadh* are suggested downstream targets of the PPARα/Fgf21/PGC1α axis, importantly involved in lipid β-oxidation (7). The hepatic expression levels of these three genes were reduced in HFD-fed mice, whereas 12-week dietary curcumin intervention restored the levels of *Acox1* and *Ehhadh* but not *Pdk4* [Fig. 6(D)].

*Pnpla2*, *Lipe*, and *Mgll* encode adipose triglyceride lipase, hormone-sensitive lipase, and monoacylglycerol lipase. Figure 6(E–G) shows the repressive effect of HFD on their expression. Curcumin intervention at least partially restored their expression levels. The stimulation on hepatic PGC1α/*Ppargc1a*, *Acox1*, *Pdk4*, and *Ehhadh* expression was also observed in LFD-fed mice with 6-day curcumin gavage [Fig. 6(H) and 6(I)].

### Curcumin intervention likely attenuates Fgf21 resistance

The fact that long-term curcumin intervention attenuates hepatic Fgf21 production in HFD-fed mice prompted us to wonder whether curcumin attenuates Fgf21 resistance. To prove that Fgf21 resistance can be assessed *in vitro*, we treated mouse hepatocytes with 50 nM FGF21 overnight. Figure 7(A) shows that the treatment reduced *Fgfr1* and *βKlotho* levels. Overnight high-dose Fgf21 treatment also attenuated the response of cells to further insulin treatment on Akt phosphorylation (Supplemental Fig. 6).

We then tested the *in vitro* effect of FGF21 in mouse hepatocytes on Erk phosphorylation. Supplemental Fig. 7 and Fig. 7(B) show that cells treated with 1, 2, or 10 nM FGF21 for 60 minutes (but not for 5 or 30 minutes) exhibited elevated Erk phosphorylation. Figure 7(C) and 7(D) show that the activation was absent in hepatocytes isolated from HFD-fed mice and was restored in hepatocytes isolated from HFD-fed mice with curcumin intervention. Figure 7(E–J) shows that the stimulatory effect of 1 and 10 nM FGF21 on the expression of *Egr1* and *cFos* was absent in hepatocytes isolated from HFD-fed mice and restored in hepatocytes isolated from HFD-fed mice with curcumin intervention.

## Discussion

After the recognition of Fgf21 as a novel hepatic hormone with potential in the treatment of T2D, obesity, liver steatosis, and other metabolic disorders (4, 10, 13, 14, 39, 40), great efforts have been made to explore the mechanisms underlying its production under physiological and pathophysiological conditions (16, 41–44). Despite its therapeutic potential, elevated plasma Fgf21 levels were reproducibly demonstrated in subjects with T2D, coronary heart disease, and nonalcoholic fatty liver disease (20, 45–49) and in various animal models of diabetes or obesity (16, 20, 50, 51). In the current study, we have demonstrated the acute stimulatory effect of the dietary polyphenol compound curcumin on hepatic Fgf21 production in mice on chow diet or in hepatocytes *in vitro*, and our mechanistic exploration suggests that the stimulation of Fgf21 production involves the activation of the nuclear receptor PPARα, a key stimulator for hepatic *Fgf21* gene transcription (1, 2, 8). More importantly, we

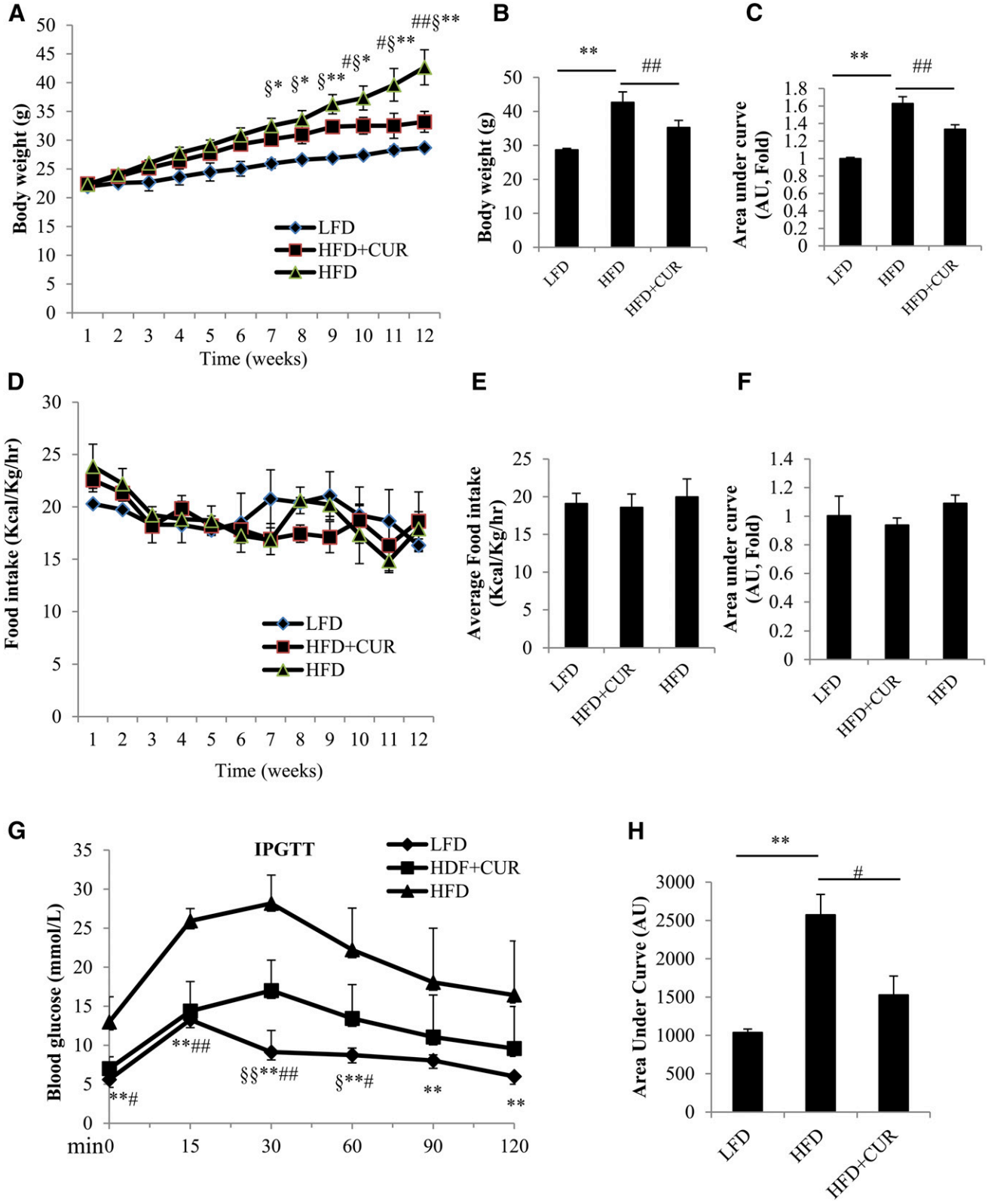


**Figure 4.** Twelve-week dietary curcumin intervention improves metabolic profiles of mice consuming HFD. (A) Body weight measurement during the 12-week experimental period. (B) Body weight at the end of the 12-week experimental period. (C) Area under the curve (AU) of panel A. (D) Food intake during the 12-week experimental period. (E) Average of food intake in 3 groups of mice. (F) AU of panel D. (G) IPGTT performed at week 8. (H) AU for panel G. n = 5 per group. CUR, curcumin. * or **, HFD vs LFD; § or §§, LFD vs HFD+CUR; # or ##, HFD vs HFD+CUR. CUR, curcumin.

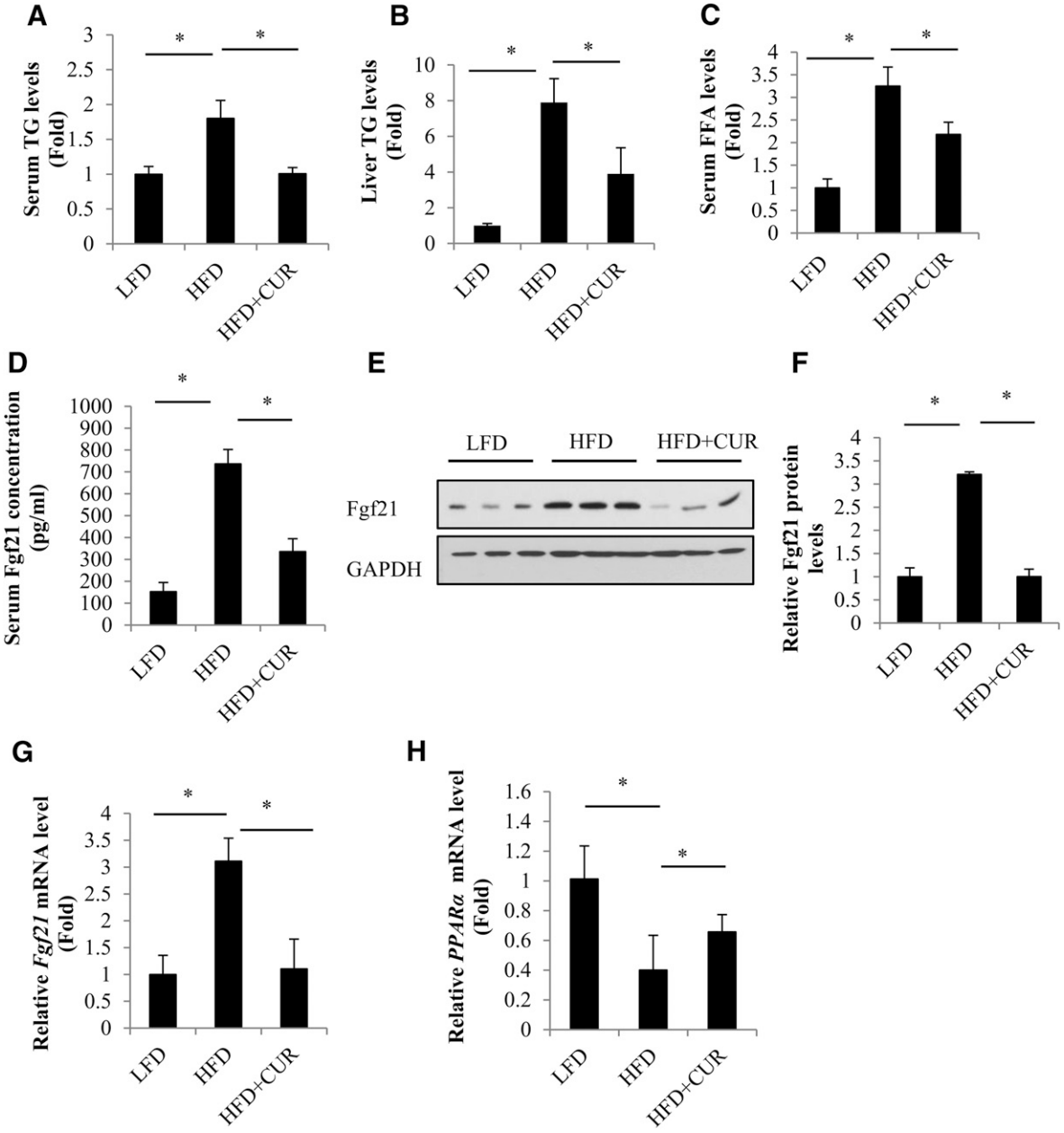


**Figure 5.** Twelve-week HFD feeding increases hepatic Fgf21 expression and plasma Fgf21 levels, whereas concomitant dietary curcumin intervention attenuated the increase. (A, B) Serum and liver triglyceride (TG) levels in the indicated groups of mice. (C) Serum free fatty acid (FFA) levels in the indicated groups of mice. (D, E) Serum Fgf21 hormone (D) and hepatic Fgf21 protein (E) levels in the indicated groups of mice. (F) Quantification of panel E. Hepatic *Fgf21* (G) and *PPARα* (H) mRNA levels. Values are means ± SD (n ≥ 5 per group of mice). $*P < 0.05$ vs the corresponding control treatment. CUR, curcumin.

have reproduced the observation that in the HFD-induced obese mouse model, hepatic Fgf21 mRNA and protein expression, as well as circulating Fgf21 level, were elevated. Our findings also revealed that long-term curcumin intervention attenuated the effect of HFD feeding, likely through the restoration of Fgf21 sensitivity.

The findings of our current study were initiated from a serendipitous observation. To determine whether curcumin administration could sensitize insulin signaling via a mechanism that is independent of its anti-inflammation and antioxidative stress properties, we tested the effect of short-term curcumin gavage in an intraperitoneal dexamethasone-injection–induced insulin-resistant mouse model (52). We found that in this model, short-term (*i.e.*, 6-day) curcumin gavage improved insulin signaling, and this improvement was associated with increased hepatic Fgf21 protein expression (31). Because dexamethasone was shown to induce hepatic Fgf21 expression as well by the activation of the glucocorticoid receptor (37), we conducted 2 sets of additional *in vivo* experiments with short-term curcumin gavage and observed that the stimulatory effect of curcumin on hepatic

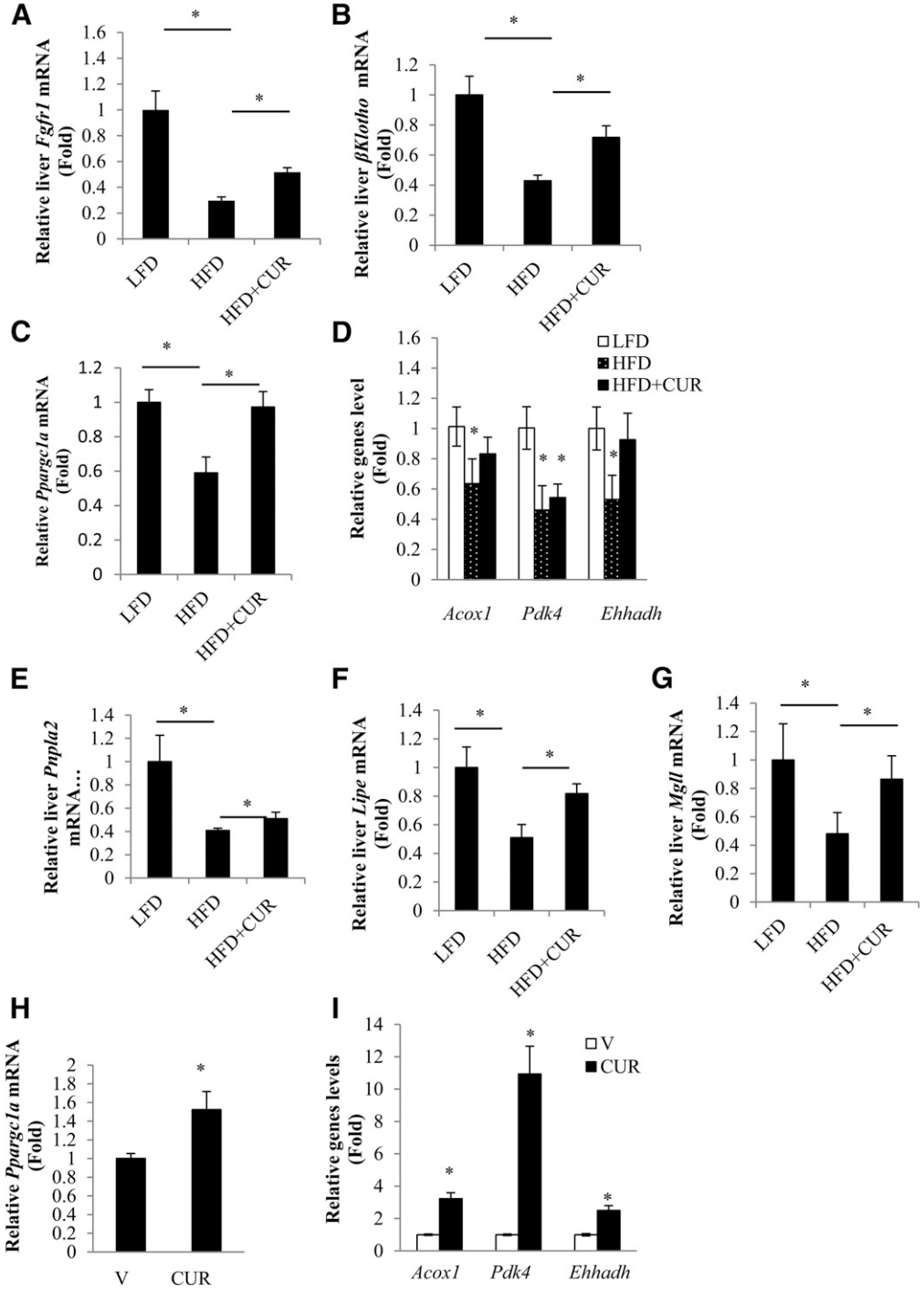


**Figure 6.** Twelve-week curcumin intervention attenuates the effect of HFD feeding on hepatic gene expression. Liver tissues from the indicated groups of mice were collected for detecting the expression of *Fgfr1* (A), *βKlotho* (B), *Ppargc1α* (C), as well as *Acox*, *Pdk4*, and *Ehhadh* (D). Expression levels of *Pnpla2* (E), *Lipe* (F), and *Mgll* (G) in the indicated groups of mice. Expression levels of *Ppargc1α* (H) as well as *Acox*, *Pdk4*, and *Ehhadh* (I) in mice received with or without 6-day curcumin gavage. Values are means ± SD (n = 5 for panels A–G; n = 6 for panels H and I). $^*P < 0.05$ vs the corresponding control treatment. CUR, curcumin; V, control vehicle.

Fgf21 and plasma Fgf21 levels occurred in the absence of dexamethasone administration. When the dosage of curcumin was 500 mg/kg body weight/d for 6 days, we observed elevated hepatic *Fgf21* mRNA levels associated with elevated *PPARα* mRNA expression but without the increase of ambient circulating Fgf21 levels. When we reduced the dosage of curcumin to 100 mg/kg body weight/d for either 4 or 8 days, elevated circulating Fgf21 became appreciable, associated with increased hepatic *Fgf21* mRNA and Fgf21 protein expression. Further investigations are needed to clarify whether multiple-day curcumin administration at a relatively higher dosage

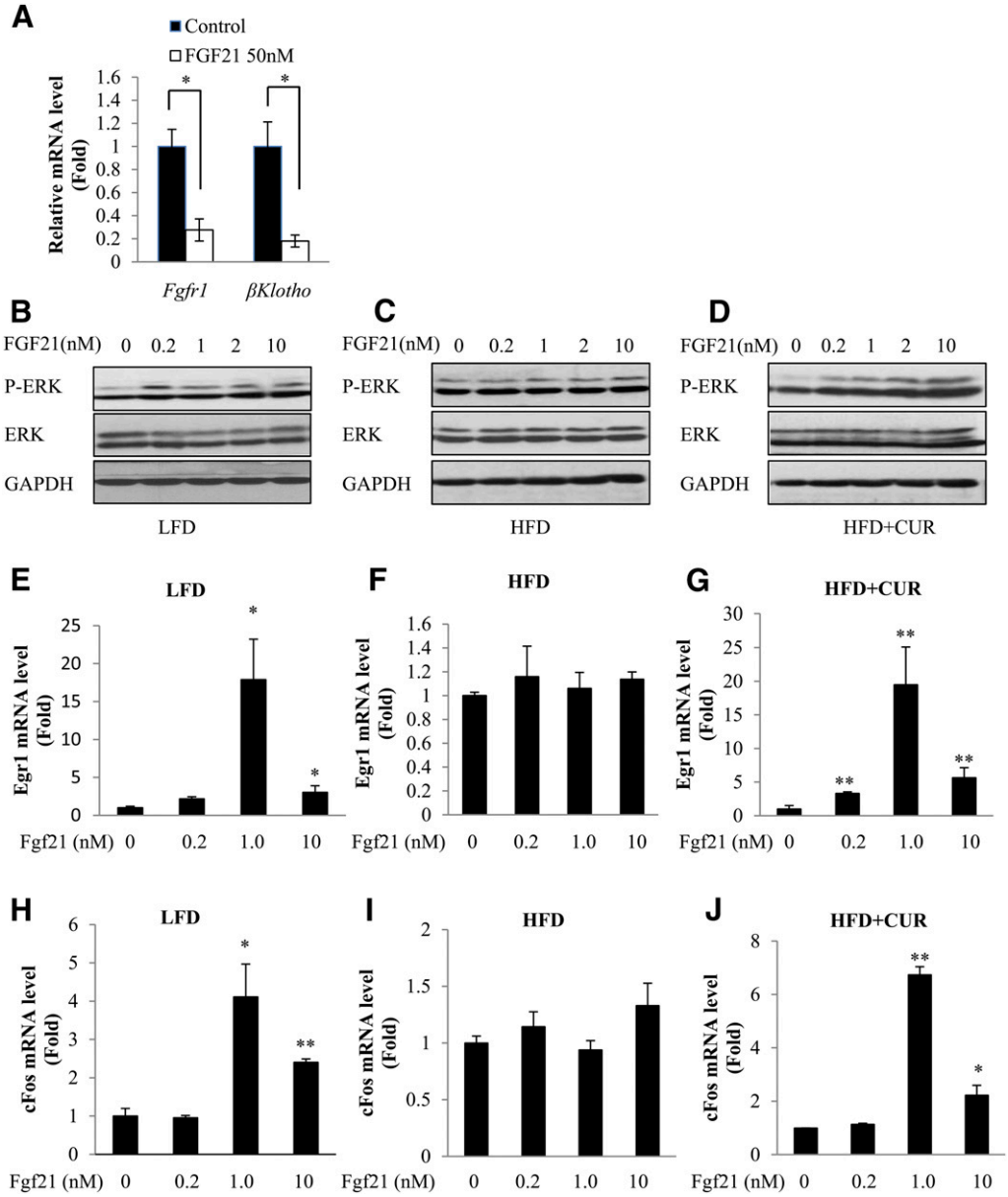


**Figure 7.** Curcumin intervention attenuates Fgf21 resistance in hepatocytes of HFD-fed mice. (A) Mouse primary hepatocytes were incubated with or without 50 nM human recombinant FGF21 for 16 hours. Cells were harvested for assessing *Fgfr1* and *βKlotho* mRNA expression by quantitative reverse transcription PCR. (B–D) Mouse hepatocytes were isolated from mouse fed with the indicated diet and treated with the indicated dose of human recombinant FGF21 for 60 minutes followed by Western blotting against indicated antibodies. Expression of *Egr1* (E–G) and *cFos* (H–J) in mouse hepatocytes isolated from mice fed the indicated diet and treated with the indicated dose of human recombinant FGF21 for 4 hours. $*P < 0.05$; $**P < 0.01$ vs the control treatment ($n \geq 4$ for panel A and E–J). Panels B–D are represented blots for 3 separated experiments. CUR, curcumin.

affects Fgf21 hormone production or release or whether this is simply due to the variations among the animals. In our long-term intervention study, we prepared the diet containing 4 g curcumin per kg weight, as we had previously (26); this concentration of curcumin is much lower than the one used in a previous study by another team (25).

The fundamental role of PPAR family members, especially PPARα, in regulating hepatic Fgf21 expression has been demonstrated previously (1, 2, 8, 51, 53, 54). It is well known that during starvation PPARα controls the utilization of fat as the alternative energy source, and it serves as the molecular target for the fibrate dyslipidemia drugs. Inagaki *et al.* (2) found that mouse hepatic Fgf21 expression is directly induced by PPARα in response to fasting, whereas Fgf21 mediates the pleiotropic effects of PPARα, including the stimulation of lipolysis in the white adipose tissue and ketogenesis in the liver. Badman *et al.* reported that hepatic Fgf21 expression and plasma Fgf21 levels were increased by fasting or ketogenic diet consumption but rapidly suppressed by refeeding (1). Indeed, circulation Fgf21 levels can be elevated 5- to 10-fold after prolonged fasting (55). Elevated hepatic Fgf21 expression

in response to ketogenic diet feeding is associated with upregulated expression of PPARα downstream targets. Badman *et al.* (1) have also demonstrated the *in vitro* stimulatory effect of the fibrate dyslipidemia drug on *Fgf21* mRNA expression in mouse hepatocytes. Furthermore, *PPARα*$^{-/-}$ mice were shown to be Fgf21 deficient and compromised during ketosis. In human subjects, the induction of FGF21 expression by prolonged fasting and PPARα activation was demonstrated by Galman *et al.* (56). Reduced *Fgf21* mRNA expression in PPARα-deficient mice was also observed by Lundasen *et al.* (8). These investigators have also demonstrated the *in vivo* stimulatory effect of Wy-14643, a potent PPARα agonist, on mouse hepatic *Fgf21* mRNA expression. This stimulation was repeated *in vitro* in human hepatocytes (8). Oishi *et al.* (53) found that injection of the PPARα ligand bezafibrate during the nighttime led to elevated Fgf21 expression, whereas bezafibrate-induced circadian Fgf21 expression was abolished in PPARα-deficient mice.

Here we determined the stimulatory effect of curcumin treatment on *Fgf21* transcription and suggest that it is unlikely that curcumin treatment increases *Fgf21* mRNA stability because the decay of *Fgf21* mRNA in the presence of actinomycin D was not affected by curcumin treatment. In addition to reproducing the observation that the PPARα agonist Wy-14643 stimulates Fgf21 expression in mouse hepatocytes, we observed in this study that curcumin-stimulated *Fgf21* mRNA and Fgf21 protein expression can be blocked by the PPARα antagonist GW6471.

PPARα activation can be achieved by increasing its production, by reducing its phosphorylation at the S12 and S21 residues (38, 57, 58), or, more powerfully, by ligand-induced activation. Although we observed the stimulation of curcumin on *PPARα* mRNA expression *in vitro* and *in vivo* with short-term curcumin gavage or long-term dietary intervention, we did not observe an appreciable stimulation on PPARα protein levels with curcumin treatment in mouse hepatocytes. Nevertheless, we observed that curcumin treatment reduced PPARα S12 levels in mouse hepatocytes.

The most profound effect on PPARα activation is mediated by the interaction with its ligands (54). Our observations collectively suggest that curcumin cannot serve as a direct ligand for PPARα. In the HEK293 cell system, neither conditioned media from primary hepatocytes treated with 2 μM curcumin nor 10 μM curcumin on its own was able to stimulate GAL4-mPPARα, although we did observe a trend of activation with 10 μM curcumin on GAL4-RXR or GAL4-mVDR (Supplemental Fig. 5). In addition, we observed the stimulatory effect of curcumin in mouse primary hepatocytes on *Fgf21* promoter PPRE1 and PPRE2 occupancy by PPARα with curcumin treatment for 4 hours but not for 0.5 hours, although profound stimulation was clearly demonstrated with the PPARα agonist Wy14643 for 0.5 hours. Therefore, we suggest that either curcumin treatment increases intracellular endogenous PPARα ligand levels or that curcumin itself can be metabolized within hepatocytes, generating a yet to be defined PPARα ligand, or that curcumin treatment affects the activity of PPARα by modifying its phosphorylation status. Intensive further investigations are required to test these potential underlying mechanisms.

Animals or human subjects with obesity or diabetes were paradoxically found to have elevated plasma Fgf21 levels, raising the suggestion that obesity is an Fgf21-resistant state (16–21). We reproduced the observation that HFD feeding reduced hepatic expression of *Fgfr1* by Fisher *et al.* (16) and *βKlotho* by Rusie *et al.* (22). We have also demonstrated that mouse primary hepatocytes receiving overnight Fgf21 treatment showed reduced

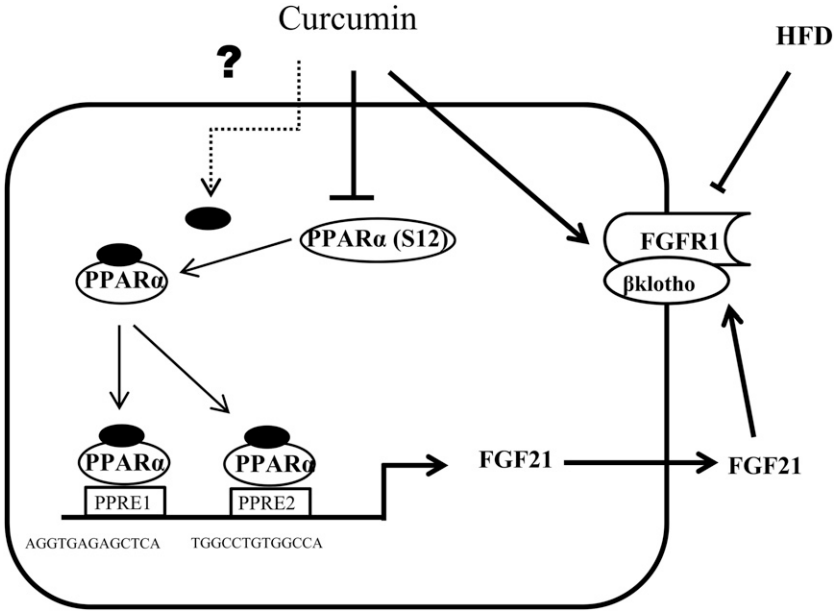


**Figure 8.** Illustration of the major findings of the study. Curcumin treatment activates PPARα. This can be achieved by attenuating its S12 phosphorylation, the generation of a yet to be identified ligand of PPARα, or the increase of the intracellular ligand levels. Activated PPARα upregulates *Fgf21* mRNA transcription and Fgf21 protein production, involving the elevation of binding of PPARα to PPREs. It is likely that long-term curcumin intervention blocks HFD-induced Fgf21 resistance, involving the attenuation of the repression on FGFR1 and βKlotho expression by HFD feeding and the activation of the PPARα/Fgf21//PGC1α transcriptional axis (not detailed on the figure).

expression of these two genes and that curcumin intervention restored their expression in HFD-fed mice, along with the restoration of expression of genes that are downstream targets of the PPARα/PGC1α axis and genes that are involved in fatty acid β-oxidations. Finally, we demonstrated that the direct effects of Fgf21 treatment in mouse hepatocytes on Erk phosphorylation and the expression of *Egr1* and *cFos* were blocked with HFD feeding and restored by curcumin intervention. These observations collectively suggest that curcumin intervention prevented the development of Fgf21 resistance in response to HFD consumption.

Profound metabolic beneficial effects of dietary curcumin intervention have been demonstrated in animal models and in clinical trials (25–27, 30). Many previous investigations have attributed the beneficial effects of dietary curcumin to its antioxidative stress and anti-inflammation properties, secondary to the body weight–lowering effect. We present here the *in vitro* and *in vivo* evidence for the role of curcumin in regulating Fgf21 production and function. Our *in vitro* study indicated that the dosage required for stimulating Fgf21 expression is much lower than that required for increasing HO1 and reducing nuclear factor κB (Supplemental Fig. 3). The bioavailability of curcumin and other dietary polyphenols is relatively low (59, 60), raising the possibility that the observed *in vivo* anti-inflammation and antioxidative stress effects of curcumin intervention may be secondary to the regulation of Fgf21 expression and function.

A very recent study revealed that, in human subjects, insulin resistance is negatively associated with plasma levels of betaine, a modified amino acid found in many foods, including whole grains and sugar beet (61). Dietary betaine supplementation was then demonstrated to increase Fgf21 levels, to improve glucose homeostasis, and to reduce hepatic steatosis in HFD-fed mice (61). In contrast to this recent betaine study, we observed the attenuation effect of long-term curcumin intervention on hepatic Fgf21 production and its plasma level elevation.

Figure 8 summarizes the major findings of this study. Our observations collectively suggest that curcumin activates PPARα indirectly. This can be achieved by blocking its S12 phosphorylation or by facilitating the generation of intracellular PPARα ligands. These mechanisms, however, are not mutually exclusive. Activated PPARα upregulates *Fgf21* transcription and protein production via binding to the 2 evolutionarily conserved PPREs. Importantly, long-term curcumin intervention blocks HFD-induced Fgf21 resistance, involving the attenuation of the reduction of FGFR1 and βKlotho expression by HFD feeding and the activation of the PPARα/Fgf21//PGC1α axis. This study has expanded the mechanistic exploration of the beneficial metabolic effects of curcumin from its anti-inflammation and antioxidative stress roles to the regulation of Fgf21 production and function. It remains to be assessed whether these effects apply to other dietary polyphenols.

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Address all correspondence and requests for reprints to: Tianru Jin, PhD, 10-354, Toronto Medical Discovery Tower, 101 College Street., Toronto, Ontario, Canada, M5G 1L7. E-mail: tianru.jin@utoronto.ca.

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Author contributions: K.Z., L.T., R.P., W.S., Z.S., and L.L. conducted the study and contributed the data for the manuscript. J.M., X.M., and I.M. provided human hepatocytes. C.L.C., J.W., and T.J. designed the experiments. J.W. provided funding support for the trainee. K.Z. and T.J. drafted the manuscript. K.Z., C.L.C., L.T., J.W., and T.J. edited the manuscript. All authors approved the version for the submission.

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