The Orphan Nuclear Receptor ERRγ\textsuperscript{γ} Regulates Hepatic CB1 Receptor-Mediated Fibroblast Growth Factor 21 Gene Expression

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Abstract

Background

Fibroblast growth factor 21 (FGF21), a stress inducible hepatokine, is synthesized in the liver and plays important roles in glucose and lipid metabolism. However, the mechanism of hepatic cannabinoid type 1 (CB1) receptor-mediated induction of FGF21 gene expression is largely unknown.

Results

Activation of the hepatic CB1 receptor by arachidonyl-2'-chloroethylamide (ACEA), a CB1 receptor selective agonist, significantly increased FGF21 gene expression. Overexpression of estrogen-related receptor (ERR) \textsuperscript{γ} increased FGF21 gene expression and secretion both in hepatocytes and mice, whereas knockdown of ERR\textsuperscript{γ} decreased ACEA-mediated FGF21 gene expression and secretion. Moreover, ERR\textsuperscript{γ}, but not ERR\textsuperscript{α} and ERR\textsuperscript{β}, induced FGF21 gene promoter activity. In addition, deletion and mutation analysis of the FGF21 promoter identified a putative ERR\textsuperscript{γ}-binding motif (AGGTGC, a near-consensus response element). A chromatin immunoprecipitation assay revealed direct binding of ERR\textsuperscript{γ} to the FGF21 gene promoter. Finally, GSK5182, an ERR\textsuperscript{γ} inverse agonist, significantly inhibited hepatic CB1 receptor-mediated FGF21 gene expression and secretion.

Conclusion

Based on our data, we conclude that ERR\textsuperscript{γ} plays a key role in hepatic CB1 receptor-mediated induction of FGF21 gene expression and secretion.
Introduction

The three estrogen-related receptors (ERRs), termed α, β, and γ, belong to the NR3B subfamily of the nuclear receptor superfamily. ERRs bind to the estrogen response element as dimers or to the half-site core sequence (TNAAGGTCA) as monomers. ERR isoforms are expressed in the pancreas, heart, brain, and liver [1–3]. ERRγ plays important regulatory roles in various metabolic events. ERRs are regulated by the peripheral circadian clock in key metabolic tissues, such as muscle, white or brown adipocytes, and liver [4]. ERRγ plays an essential role in the maturation of glucose-response β-cells [5]. In brown adipose tissue, ERRγ induces Uncoupling Protein 1 (UCP1) expression and fatty acid oxidation [6]. It is also important in cancer therapy, where it is used as a marker of clinical course and in the selection of appropriate therapies [7]. ERRγ suppressed tumor growth and the proliferation of prostate cancer cells [8]. We also reported that ERRγ is involved in insulin-mediated inhibition of hepatic gluconeogenesis [9]. In addition, GSK5182 controls ERRγ-induced hepcidin gene expression and improves Salmonella typhimurium infection by modulating host iron homeostasis [10]. Previously, we demonstrated that hepatic ERRγ regulates the expression of gluconeogenic genes and blood glucose levels in a mouse model of type 2 diabetes and plays a key role in hepatic insulin signaling mediated by lipin1 [11, 12]. Moreover, ERRγ displays endogenous ligand-independent constitutive transcriptional activity that depends on its interaction with coactivators or corepressors. PKB/Akt suppresses the transcriptional activity of ERRγ by promoting the phosphorylation of ERRγ at S179 and by eliciting translocation of ERRγ from the nucleus to the cytoplasm [9]. GSK5182, a 4-hydroxytamoxifen analog, is a selective inverse agonist of ERRγ [13]. GSK5182 inhibits ERRγ transcriptional activity increasing the interaction between ERRγ and the corepressor of SMILE [14].

The endocannabinoid system, which consists of two G protein-coupled receptors, CB1 and CB2 (cannabinoid receptor type 1 and 2), is an endogenous lipid signaling pathway. Anandamide and 2-arachidonyl glycerol (2-AG) are the two best characterized endocannabinoid activators of CB1 and CB2 [15]. The CB1 receptor is expressed in the brain, vascular tissues, heart, and liver, whereas the CB2 receptor is expressed in most immune cells. 2-AG synthesis is achieved through the hydrolysis of diacylglycerol (DAG) by DAG lipases (DAGLα and DAGLβ) [16, 17]. Activation of the hepatic CB1 receptor promotes fatty acid synthesis and diet-induced obesity [18, 19]. The synthesis of 2-AG is promoted by alcohol-mediated upregulation of DAGLβ in hepatic stellate cells. 2-AG activates the CB1 receptor on adjacent hepatocytes by a paracrine mechanism [20]. Arachidonyl-2′-chloroethylamide (ACEA) is a synthetic CB1 receptor selective agonist [21], while AM251 is a CB1 receptor selective antagonist [22]. Previously, we reported that activation of the CB1 receptor inhibits insulin receptor signaling through cAMP-responsive element-binding protein 3-like 3 (CREBH)-mediated lipin1 gene expression in the liver [23]. We also reported that ERRγ regulates cytochrome P450 2E1 (CYP2E1) expression and oxidative liver injury by alcohol via the hepatic CB1 receptor [24].

Fibroblast growth factor (FGF) 21 is a member of the FGF family [25] and is a metabolic hormone secreted predominantly by hepatocytes [26]. Unlike the classical members of the FGF family, FGF21 does not have heparin-binding properties, which enables its release into the circulation [27]. Therefore, FGF21 acts through cell surface receptors composed of classic FGF receptors complexed with β-Klotho [28]. FGF21 regulates carbohydrate and lipid metabolism. For example, FGF21 increases glucose uptake via the induction of glucose transporter 1 in adipose tissue [29]. FGF21 also increases fatty acid oxidation and gluconeogenesis in the liver [30–32]. FGF21 protects against acetaminophen (APAP)-induced hepatotoxicity by increasing the peroxisome proliferator-activated receptor coactivator (PGC)-1α-mediated antioxidant capacity [33]. FGF21 expression is increased in the liver in the fasting condition by activation
of the nuclear receptor peroxisome proliferator-activated receptor (PPAR) α. It is also increased in the fed condition to regulate PPARγ in adipose tissue [31, 34]. Recently, the nuclear receptor retinoic acid receptor-related orphan receptor α (RORA), retinoic acid receptor β (RARβ), and farnesoid X receptor (FXR) were shown to play a role in regulating FGF21 in the liver [35–37]. Moreover, growth hormone receptor (GHR) signaling directly stimulates FGF21 gene transcription in the liver by janus kinase 2 (JAK2)-signal transducer and activator of transcription 5 (STAT5) [38]. However, the mechanism of hepatic cannabinoid type 1 (CB1) receptor-mediated induction of FGF21 gene expression is largely unknown.

Previously, we reported that activation of the CB1 receptor induces CREBH gene expression and its transcriptional activity in the liver [23], and that CREBH regulates FGF21 promoter activity [39]. We also found that hepatic ERRγ expression is induced by ethanol via the activation of CB1 receptor signaling [24]. In this study, we demonstrated that the orphan nuclear receptor ERRγ is responsible for CB1 receptor-mediated FGF21 expression. Hepatic ERRγ gene expression is induced by activation of the CB1 receptor, and knockdown of ERRγ gene expression prevents CB1 receptor-mediated FGF21 expression. Moreover, FGF21 gene expression and secretion are inhibited by an inverse agonist of ERRγ. Collectively, this study demonstrates that ERRγ is a novel regulator of FGF21 gene expression and secretion.

Materials and Methods

Ethics Statement

Animal experiments were approved by the Chonnam National University Animal Care and Use Committee (No. CNU-IACUC-YB-2014-39).

Chemicals

ACEA was purchased from Tocris Bioscience. GSK5182 was synthesized as described previously [13]. GSK5182 was used at a concentration of 10 μM in vitro and 40 mg/kg in mouse experiments.

Animals

Male 8-week-old C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME, USA) were used for this study. Mice were maintained at 24°C on a 12:12 h light-dark cycle. CB1 receptor-knockout (CB1−/−) mice were kindly provided by Dr. George Kunos at the National Institute on Alcohol Abuse and Alcoholism/NID as described previously [40, 41]. Eight-week-old male CB1−/− mice were used to obtain primary hepatocytes. All animals were allowed ad libitum access to food and water. For the compound studies, ACEA administration (10 mg/kg, intraperitoneal injection) was performed in wild-type mice. Ad-GFP and Ad-FLAG-ERRγ were injected via the tail vein, and mice were sacrificed on day 3 after the injection. To identify the effect of ERRγ, control and recombinant shERRγ adenoviruses were injected into mice in the presence or absence of ACEA (10 mg/kg, intraperitoneal injection). GSK5182 was administered by intraperitoneal injection (40 mg/kg). Liver tissues from chronic alcohol diet mice were used to measured FGF21 gene expression as described previously [24]. Briefly, alcohol was administered for 4 weeks (chronic alcohol model) and GSK5182 was given by oral gavage administration once daily for the last 2 weeks of alcohol feeding. The mice were monitored once-daily after experimental injection. Mice were injected with Rompon (BAYEL) and Zoletil50 (Virbac) and sacrificed by exsanguination according to the protocol of the Chonnam National University Animal Care and Use Committee (No. CNU-IACUC-YB-2014-39).
Plasmids and DNA constructs

The FGF21-Luc (-2078bp/+129bp) construct was described previously [42]. FGF21 ERR response element mut-Luc (-1032bp CAAGGTGCTT-1022bp to -1032bp CAAAATGCTT-1022bp) and FGF21 ERR mut-Luc were generated using the QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). ERRα, ERRβ, and ERRγ constructs were described previously [43]. All plasmids used were confirmed by complete sequence analysis.

Recombinant adenoviruses

Ad-GFP, Ad-FLAG-ERRγ, Ad-US, and Ad-shERRγ were described previously [14]. All viruses were purified via CsCl2.

Cell culture and transient transfection assays

HepG2 (human hepatoma cells) and 293T (human embryonic kidney cells) cells were obtained as described previously [44]. AML12 cells (mouse immortalized hepatocytes) were cultured in DMEM/F-12 medium (Gibco-BRL, Grand Island, NY, USA) supplemented with insulin-transferrin-selenium (Gibco-BRL), dexamethasone (40 ng/ml; Sigma, St. Louis, MO, USA), and antibiotics in a humidified atmosphere containing 5% CO2 at 37°C. Transient transfections were conducted using LipofectAmine 2000 reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer’s instructions. The cells were treated with 10 μM GSK5182 unless noted otherwise. After 48 h of transfection, the cells were harvested, and luciferase activity was measured and normalized to β-galactosidase activity.

Culture of primary hepatocytes

Mouse primary hepatocytes were isolated from C57BL/6J or CB1−/− mice (male, 20–22 g) by collagenase perfusion [45]. Rat primary hepatocytes were prepared from Male 8-week-old Sprague-Dawley rats (Damul Science, Daejeon, Korea) by a collagenase perfusion method, as described previously [46]. After being allowed to adhere for 12 h, cells were infected with the indicated adenoviruses for overexpression or knockdown. Hepatocytes were treated with 10 μM ACEA and 10 μM GSK5182.

RNA isolation and analysis

Total RNA was isolated using TRIzol reagent (Invitrogen), in accordance with the manufacturer’s instructions, and real time quantitative PCR (qPCR) analysis was conducted using the following primers: ERRγ (mouse/human), 5’-AAGATCGACACATTGATTCCAGC-3’ (Forward) and 5’-CATGGTTGAACCTGAATTCACCAC-3’ (Reverse); FGF21 (mouse), 5’-CTGCTG GGGTGCTTCAACG-3’ (Forward) and 5’-CTGCGCTCACCTGCTTAC-3’ (Reverse); and FGF21 (human), 5’-GGGATGTGGAGCTGGAAGTG-3’ (Forward) and 5’-TGGACCAGGA AGGACTCAC-3’ (Reverse). All data were normalized to β-actin (mouse/human) expression, which was determined using 5’-TCTGGCACCACCCCTTCCAG-3’ (Forward) and 5’-TCGTA GATGGGCACAGTGTGG-3’ (Reverse) primers.

Western blot analysis

Mouse liver tissue or cultured cells were lysed with RIPA buffer and subjected to immunoblot analysis as described previously [47]. The membranes were probed with anti-ERRγ (Cell Signaling Technology, Danvers, MA, USA; diluted 1:1000), anti-β-actin (AbFrontier, Seoul, Korea; diluted 1:5000) [24] and anti-FGF21 (Abcam, Cambridge, UK; diluted 1:1000) [48] antibodies.
Measurement of FGF21 levels

Total FGF21 was extracted from cell culture medium or mouse serum. FGF21 was analyzed with a Quantikine FGF21 ELISA kit (R&D Systems, Minneapolis, MN, USA).

Chromatin immunoprecipitation (ChIP) assay

Chromatin immunoprecipitation (ChIP) assays were performed according to the manufacturer’s protocol (Upstate Biotechnology, Lake Placid, NY, USA). Immunoprecipitation was performed using an anti-ERRγ antibody or IgG (as a negative control). After recovery of DNA, qPCR was performed using primers encompassing the FGF21 promoter region. The primers used for PCR were as follows: -1.95 kb/-1.75 kb, 5’-TGGGTTCTCTGACTTGACCG-3’ (Forward) and 5’-CTACTCCCCAGAGCATCTAGC-3’ (Reverse); and -0.95 kb/-0.75 kb, 5’-ACTCCTCTTACACACTGCTG-3’ (Forward) and 5’-TGGGATCTAGCTCTTGGGTC-3’ (Reverse).

Statistical analyses

The results are presented as mean ± SD. Statistical differences in one factor between two groups were determined using the unpaired Student’s t test. Multiple-group comparisons were made using ANOVA. All analyses were performed using Prism 5 (GraphPad Software, La Jolla, CA).

Results

The hepatic FGF21 mRNA level is increased by ACEA, a CB1 receptor agonist

Previous reports demonstrated that activation of the CB1 receptor induces CREBH and ERRγ gene expression and CREBH regulates FGF21 promoter activity [23, 24, 39]. Therefore, we hypothesized that ERRγ may regulate CB1 receptor-mediated FGF21 gene expression. We used ACEA, a CB1 receptor selective agonist, for CB1 receptor activation [21]. ACEA treatment led to significant increases in both ERRγ and FGF21 mRNA levels within 1 h and reached a maximum level at 6 h in HepG2 cells and at 3 h in rat primary hepatocytes and AML12 cells (Fig 1A–1C). In mouse primary hepatocytes, ACEA also significantly increased ERRγ and FGF21 mRNA levels. However, ACEA had no effect on ERRγ and FGF21 mRNA levels in CB1−/− mouse primary hepatocytes (Fig 1D). Together with this direct effect of ACEA on hepatocytes, we measured hepatic ERRγ and FGF21 mRNA levels in ACEA-injected C57BL/6J mice. ACEA significantly increased ERRγ and FGF21 mRNA levels in mouse liver in a time-dependent manner (Fig 1E). These results demonstrate that ACEA increases ERRγ and FGF21 mRNA levels.

Hepatic FGF21 is induced by ACEA

To determine whether the increases in ERRγ and FGF21 mRNA levels are correlated with protein levels, western blot analysis was performed to measure FGF21 protein after ACEA treatment in HepG2 and AML12 cells. ACEA significantly increased FGF21 protein levels (Fig 2A and 2B). ACEA also induced significant increases in the FGF21 protein level in mouse liver (Fig 2C). We also examined ACEA-induced FGF21 secretion levels in AML12 cells, rat primary hepatocytes, and serum of ACEA-injected mice. ACEA treatment led to significant increases in FGF21 secretion (Fig 2D–2F). These results indicate that the CB1 receptor-specific agonist ACEA increases the protein level and secretion of FGF21.
Overexpression of ERRγ increases hepatic FGF21 expression

Our previous study demonstrated that ethanol or ACEA induces ERRγ gene expression through CB1 receptor signaling in the liver [24]. In this study, we found that ACEA-mediated ERRγ and FGF21 expression (Figs 1 and 2). Therefore, we examined whether ERRγ regulates FGF21 expression by overexpression of ERRγ using Ad-ERRγ in HepG2 cells, AML12 cells, and mouse primary hepatocytes. Overexpression of ERRγ markedly increased FGF21 mRNA levels in HepG2 cells, AML12 cells, and mouse primary hepatocytes (Fig 3A–3C). Ad-ERRγ-infected mouse liver also showed significant increases in hepatic FGF21 mRNA (Fig 3D). To verify the effect of ERRγ on FGF21 secretion, we measured FGF21 levels in both AML12 cell

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Fig 1. ACEA induces FGF21 gene expression. (A–C) HepG2 cells, rat primary hepatocytes (RPH), and AML12 cells were treated with ACEA (10 μM) for the indicated time periods. (D) Wild-type or CB1−/− mouse primary hepatocytes (MPH) were treated with ACEA (10 μM) for 3 h. (E) Mice were treated with ACEA (10 mg/kg) for the indicated number of days. Livers were harvested for mRNA analysis. (A–E) FGF21 and ERRγ mRNA levels were measured by quantitative qPCR analysis and normalized to actin mRNA levels. All data are the means ± standard errors of at least three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001 by one-way ANOVA.

doi:10.1371/journal.pone.0159425.g001
culture medium and mouse serum after ERRγ overexpression. Ad-ERRγ increased FGF21 levels in the culture medium and mouse serum (Fig 3E and 3F). These results suggest that ERRγ is a regulator of FGF21 expression and secretion.

Knockdown of ERRγ decreases ACEA-mediated FGF21 expression

To evaluate the direct effect of ERRγ on hepatic FGF21 gene expression through the CB1 receptor, we examined ACEA-induced FGF21 expression after knockdown of ERRγ using an adenovirus harboring ERRγ-targeting shRNA (Ad-shERRγ). The ACEA-induced FGF21 mRNA level was dramatically reduced by knockdown of ERRγ in HepG2 cells, AML12 cells, and mouse primary hepatocytes (Fig 4A–4C). Similar to the results in hepatocytes, ACEA-induced FGF21 expression was significantly reduced in Ad-shERRγ-infected mouse liver (Fig 4D). Furthermore, ACEA-induced FGF21 secretion in culture media of AML12 cells was significantly decreased by shERRγ. Consistent with this finding, the ACEA-induced FGF21 level in mouse
serum was also significantly reduced by knockdown of ERRγ (Fig 4E and 4F). These results demonstrate that ERRγ is a key regulator of ACEA-mediated FGF21 gene expression and secretion.

**ERRγ activates the FGF21 gene promoter activity**

To explain the role of ERRγ in ACEA-mediated induction of FGF21 gene expression, we investigated whether ERRγ directly induces FGF21 gene transcription. First, we examined ACEA-

![Graphs showing the effect of ERRγ on FGF21 gene expression and secretion](Fig 3)

**Fig 3. ERRγ overexpression induces FGF21 gene expression.** (A–C) HepG2 cells, AML12 cells, and mouse primary hepatocytes (MPH) were infected with Ad-GFP and Ad-ERRγ. (D) Ad-GFP or Ad-ERRγ was injected into male C57BL/6J mice via the tail vein. Mice were sacrificed at 5 days after injection. (A–D) FGF21 and ERRγ mRNA levels were measured by quantitative qPCR analysis and normalized to actin mRNA levels. (E) Culture media of adenovirus-infected AML12 cells was obtained for FGF21 secretion analysis. (F) Ad-GFP or Ad-ERRγ was injected via the tail vein into male C57BL/6J mice. Serum from these mice was analyzed for FGF21 secretion. All data are the means ± standard errors of at least three independent experiments. ***p < 0.001 by Student’s t-test.

doi:10.1371/journal.pone.0159425.g003
induced FGF21 promoter activity by knockdown of ERRγ using Ad-shERRγ. ACEA-induced FGF21 promoter activity was reduced by knockdown of ERRγ in AML12 cells (Fig 5A). Next, a reporter assay with co-transfection revealed that ERRγ specifically augmented the mouse FGF21 promoter activity, whereas ERRα and ERRβ had no effect (Fig 5B). On the other hand,
ERRγ activates mouse FGF21 gene promoter activity. (A) AML12 cells were transfected with mFGF21-Luc and then treated with ACEA for 3 h. (B) 293T cells were co-transfected with mFGF21-Luc along with expression vectors for ERRα, ERRβ, and ERRγ. (C) 293T cells were co-transfected with deletion mutants of mFGF21-Luc and ERRγ. (D) 293T cells were co-transfected with mFGF21-Luc, mFGF21 ERRE mut-Luc, and ERRγ. The alignment of potential ERRE sequences in the human and mouse FGF21 promoters is shown (top). (E) AML12 cells were transfected with mFGF21-Luc or mFGF21 ERRE mut-Luc and treated with ACEA for 3 h. (F-G) ChIP assay analysis was performed with Ad-GFP- or Ad-ERRγ-infected AML12 cells and ACEA treated AML12 cells. Cell extracts were immunoprecipitated with an anti-ERRγ antibody. Purified DNA samples were employed for PCR with primers that bind to the ERRE (-1.1 kb to -0.9 kb) and a distal site (-1.95 kb to -1.75 kb) in the FGF21 gene promoter. All data are the means ± standard errors of at least three independent experiments. **p < 0.01; ***p < 0.001 by one-way ANOVA.

doi:10.1371/journal.pone.0159425.g005
serial deletion of the FGF21 promoter showed that the region harboring -1.25 kb to -0.85 kb of the FGF21 promoter was activated by ERRγ, and a putative ERRγ-binding motif (AGGTGC, a near-consensus response element) was identified in the FGF21 promoter (Fig 5C). To further confirm the exact ERRγ-binding site in the FGF21 promoter, a reporter assay was performed using wild-type and point-mutated reporter constructs of the FGF21 promoter. ERRγ-dependent FGF21 promoter activity was significantly decreased in the ERRE-mutated reporter construct (Fig 5D).

Next, we examined the effect of ACEA on the ERRE in the mouse FGF21 promoter. FGF21 promoter activity was significantly increased by ACEA. However, this ACEA-mediated FGF21 promoter activity was decreased in ERRE-mutated reporter constructs (Fig 5E), suggesting that ACEA-dependent FGF21 promoter activation is mediated by ERRγ. To confirm the direct binding of ERRγ to the endogenous FGF21 promoter, ChIP assays were performed in AML12 cells. Overexpressed ERRγ and ACEA-induced endogenous ERRγ were recruited to the ERRγ consensus binding site in the FGF21 promoter, but not to the upstream region lacking an ERRE (Fig 5F and 5G). Overall, these data suggest that activation of the hepatic CB1 receptor-mediated ERRγ occupancy of the FGF21 promoter.

GSK5182 inhibits CB1 receptor-induced FGF21 expression and FGF21 secretion

As an ERRγ inverse agonist, GSK5182 is used to inhibit ERRγ transactivation and its target gene expression [12, 13]. First, we examined the effect of GSK5182 on ACEA-mediated FGF21 promoter activation. Increased FGF21 promoter activity by ACEA was significantly decreased by GSK5182 (Fig 6A). To further clarify the direct effect of ERRγ on CB1 receptor-mediated FGF21 expression, AML12 cells were treated with ACEA in the presence or absence of GSK5182. GSK5182 dramatically decreased FGF21 gene expression in ACEA-treated HepG2 cells, AML12 cells, and mouse primary hepatocytes (Fig 6B–6D). In mice, the ACEA-treated group showed significant increases in ERRγ and FGF21 gene expression, while GSK5182 significantly suppressed the ACEA-mediated FGF21 mRNA level (Fig 6E). We also examined FGF21 secretion by ACEA in the presence or absence of GSK5182. GSK5182 significantly decreased ACEA-mediated FGF21 secretion in the cell culture medium (Fig 6F). In addition, the increased serum level of FGF21 induced by ACEA was also significantly reduced by GSK5182 treatment in mice (Fig 6G). Finally, we examined the effect of GSK5182 on FGF21 gene expression in a chronic alcohol-exposed C57BL6 mice model. Chronic alcohol exposure significantly increased hepatic ERRγ and FGF21 gene expression. The increase in FGF21 gene expression was decreased by GSK5182 treatment (Fig 6G). Taken together, these results indicate that the ERRγ inverse agonist GSK5182 inhibits CB1R-mediated FGF21 expression and secretion.

Discussion

FGF21 and ERRγ play important roles in hepatic metabolism. In this study, we revealed that ERRγ contributes to CB1 receptor-induced FGF21 gene expression. Activation of the hepatic CB1 receptor induced ERRγ and FGF21 gene expression, and knockdown of ERRγ gene expression prevented CB1 receptor-induced FGF21 gene expression. In addition, ERRγ induced FGF21 gene promoter activity by directly binding to the FGF21 promoter. Moreover, CB1 receptor-induced FGF21 gene expression was inhibited by the ERRγ inverse agonist GSK5182. FGF21 is regulated by the membrane receptor GHR via the JAK2-STAT5 pathway [38]. Until now, the other membrane receptor signaling pathways regulating FGF21 expression were largely unknown. In the present study, we demonstrated that activation of the hepatic CB1
receptor induced FGF21 gene expression via the induction of ERRγ gene expression. ACEA was used to activate the CB1 receptor because ACEA is the most selective synthetic agonist of the CB1 receptor. Activation of the hepatic CB1 receptor induced FGF21 expression and secretion (Figs 1 and 2). Several nuclear receptors are involved in regulation of FGF21 gene expression. To date, most studies have focused on transcription factors that control transcription in the -1200bp and -1000bp region of the FGF21 gene. FGF21 is induced by PPARα in the liver during fasting by direct binding to the PPAR response element (-1093bp and -1057bp) in the FGF21 promoter [31, 49]. FGF21 gene expression is induced by Nur77 during fasting via direct binding of Nur77 to the NGFI-B response element (-1282bp and -1248bp) in the promoter [50]. In addition, FXR, RORα, and LXR regulate FGF21 gene expression through direct binding to the FGF21 promoter [35, 37, 51]. Here, we found that overexpressed ERRγ induced FGF21 gene expression (Fig 3), and knockdown of ERRγ reduced CB1 receptor-induced FGF21 levels (Fig 4). Moreover, the present study provides an alternative mechanism by which FGF21 promoter activity is mediated by hepatic ERRγ via the putative ERRE sequence (-1032bp and -1022bp) (Fig 5). Previously, it was reported that rosiglitazone, a PPARγ agonist, induces FGF21 in white adipose tissue, but not in the liver. All trans-retinoic acid, an endogenous ligand of RARβ, induces FGF21 gene expression in liver tissue [36].

In this study, we demonstrated that GSK5182 inhibited hepatic CB1 receptor-induced FGF21 gene expression via specific inhibition of the transcriptional activity of ERRγ. We also revealed that the hepatic CB1 receptor is a new membrane receptor regulating FGF21 gene expression, and that CB1 receptor-induced ERRγ is a new upstream regulator of FGF21 gene expression. Finally, ERRγ inverse agonist GSK5182 inhibited FGF21 gene expression in response to CB1 receptor activation. Previously, we reported that ERRγ regulates gluconeogenesis in the liver [11, 32]. ERRγ directly binds to the phosphoenolpyruvate carboxykinase (PEPCK) promoter and induces PEPCK gene expression. FGF21 increases hepatic gluconeogenesis through PGC-1α-mediated gluconeogenic gene expression [32]. Despite direct regulation of hepatic gluconeogenesis by ERRγ, ERRγ-induced FGF21 could be another pathway that regulates hepatic gluconeogenesis. In addition, activated CB1 receptor-induced ERRγ mediates gene expression of CYP2E1, which is a key enzyme generating alcohol-induced reactive oxygen species (ROS) in the liver [24]. In this study, hepatic ERRγ and FGF21 mRNA levels were significantly increased in the chronic alcohol-exposed mice liver (Fig 6H). Other studies suggest that APAP induces hepatic ROS generation and FGF21 production. APAP-induced FGF21 protects against hepatotoxicity via inducing antioxidant gene expression [33]. Furthermore, previous study suggests that FGF21 markedly reduces alcohol preference in mice [52]. This effect requires the FGF21 co-receptor beta-klotho in the central nervous system. Previous studies suggest that the hepatic CB1 receptor contributes to obesity in a diet induced obesity (DIO) mouse model by increasing free fatty acid synthesis [18]. Paradoxically, recombinant murine FGF21 treatment reverses hepatic steatosis by increasing energy expenditure in DIO mice [53].
Our previous study revealed that ERRγ gene expression is also higher in DIO mice [54]. Therefore, we speculate that intrinsic FGF21 might be involved in CB1 receptor signaling-mediated free fatty acid synthesis. However, excessive increases in FGF21 induced by ERRγ may have a beneficial effect on hepatic steatosis. The FGF21 level is higher under stressful conditions, including starvation and high fat diet, and FGF21 improves metabolic disorder under these conditions. Therefore, our study suggests that CB1 receptor-induced ERRγ contributes to the FGF21-mediated compensatory mechanism to oppose CB1 receptor-mediated diet-induced obesity.

Conclusions
Overall, our results reveal that ERRγ, induced via activation of the hepatic CB1 receptor, is a regulator of hepatic FGF21 gene expression and secretion. GSK5182 inhibited CB1 receptor-mediated FGF21 expression and secretion (Fig 6I), confirming that FGF21 is a direct target of ERRγ. In addition, chronic alcohol exposed mice showed increased FGF21 gene expression as a result of the induction of ERRγ gene expression. The identification of ERRγ as a mediator of FGF21 expression will increase current understanding of the mechanisms involved in controlling hepatic metabolism by FGF21.

Supporting Information
S1 Fig. Western blot (uncropped) for Fig 2A. (DOCX)
S2 Fig. Western blot (uncropped) for Fig 2B. (DOCX)
S3 Fig. Western blot (uncropped) for Fig 2C. (DOCX)
S4 Fig. Electrophoresis gel of the ChIP assay (uncropped) for Fig 5F. (DOCX)
S5 Fig. Electrophoresis gel of the ChIP assay (uncropped) for Fig 5G. (DOCX)

Acknowledgments
We would like to thank Soon-Young Na and Kun-Hee Kim for technical assistance.

Author Contributions
Conceived and designed the experiments: YSJ HSC. Performed the experiments: YSJ YSL KSK. Analyzed the data: YSJ. Contributed reagents/materials/analysis tools: RAH MSL IKL WIJ CHL JML DKK YHK SHK SJC JK. Wrote the paper: YSJ HSC.

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