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SREBP-2 negatively regulates FXR-dependent transcription of *FGF19* in human intestinal cells

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ABSTRACT

Sterol regulatory element-binding protein-2 (SREBP-2) is a basic helix-loop-helix-leucine zipper transcription factor that positively regulates transcription of target genes involved in cholesterol metabolism. In the present study, we have investigated a possible involvement of SREBP-2 in human intestinal expression of fibroblast growth factor (FGF)19, which is an endocrine hormone involved in the regulation of lipid and glucose metabolism. Overexpression of constitutively active SREBP-2 decreased *FGF19* mRNA levels in human colon-derived LS174T cells. In reporter assays, active SREBP-2 overexpression suppressed GW4064/FXR-mediated increase in reporter activities in regions containing the IR-1 motif (+848 to +5200) in the FGF19 gene. The suppressive effect disappeared in reporter activities in the region containing the IR-1 motif when the mutation was introduced into the IR-1 motif. In electrophoretic mobility shift assays, binding of the FXR/retinoid X receptor α heterodimer to the IR-1 motif was attenuated by adding active SREBP-2, but SREBP-2 binding to the IR-1 motif was not observed. In chromatin immunoprecipitation assays, specific binding of FXR to the IR-1-containing region of the FGF19 gene (+3214 to +3404) was increased in LS174T cells by treatment with cholesterol and 25-hydroxycholesterol. Specific binding of SREBP-2 to FXR was observed in glutathione-S-transferase (GST) pull-down assays. These results suggest that SREBP-2 negatively regulates the FXR-mediated transcriptional activation of the FGF19 gene in human intestinal cells.

1. Introduction

Several transcription factors are involved in the regulation of cholesterol/bile acid metabolism, including sterol regulatory element-binding protein-2 (SREBP-2), SREBP1-a, liver X receptor and farnesoid X receptor (FXR) [1–3].

SREBP-2 is a basic helix-loop-helix-leucine zipper transcription factor, which is synthesized as a precursor protein that is localized to the endoplasmic reticulum [4]. It is activated in a cholesterol-dependent manner: when cellular sterol levels fall, the precursor is transported to the Golgi apparatus by SREBP cleavage-activating protein and is cleaved by two proteases, designated site-1 and site-2 proteases, to release the mature, transcriptionally active form [4]. The active form enters the nucleus and binds to specific sterol regulatory elements (SREs) in the promoter regions of cholesterogenic genes to positively regulate the transcription of target genes resulting in the maintenance of cellular cholesterol levels [4,5]. SREBP-2 controls the expression of numerous genes involved in cholesterol homeostasis including 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), 3-hydroxy-3-methylglutaryl-CoA synthase (HMGCS), low density lipoprotein receptor (LDLR) and Niemann–Pick C1-Like 1 (NPCIL1) [6–9].

FXR is a nuclear receptor that binds to the IR-1 (inverted repeat separated by 1 nucleotide), ER-8 (everted repeat separated by 8 nucleotides), and DR-1 (direct repeat separated by 1 nucleotide) motifs in regulatory regions of genes [10]. FXR is activated by bile acids and positively regulates the transcription of target genes involved in bile acid, lipid and glucose metabolism [11,12,2,13]. FXR also plays an essential role in the negative regulation of cholesterol levels [14]. Increases in the hepatic and serum cholesterol levels are observed in *Fxr*-null mice [15].

Fibroblast growth factor (FGF)15/19 acts on several organs including the liver as an endocrine hormone, regulating various metabolic processes that involve bile acids, lipids, and

Abbreviations: ChIP, chromatin immunoprecipitation; DR-1, direct repeat separated by 1 nucleotide; EMSA, electrophoretic mobility shift assay; ER-8, everted repeat separated by 8 nucleotides; FXR, farnesoid X receptor; FGF19, fibroblast growth factor 19; GST, glutathione-S-transferase; IR-1, inverted repeat separated by 1 nucleotide; LDLR, low-density lipoprotein receptor; PXR, pregnane X receptor; SREBP-2, sterol regulatory element-binding protein-2; RXRα, retinoid X receptor α.

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carbohydrates [16–19]. Previous reports have suggested that FGF19 regulates bile acid homeostasis by downregulating the hepatic expression of CYP7A1, a rate-limiting enzyme of hepatic bile acid synthesis [16,20,21]. In addition to the regulation of various metabolic processes, FGF19 is involved in liver cell proliferation [22,23].

FGF19 is predominantly expressed in the intestine [16]. The expression is positively regulated by bile acid-mediated signaling via the nuclear receptors FXR and pregnane X receptor (PXR) [20,24,25]. The FGF19 gene contains several FXR-responsive elements in the second intron and promoter region [20,26]. The IR-1 motif, which is a typical FXR-responsive element, is present in the second intron of the FGF19 gene.

Ileal mRNA levels of the FXR target genes, *Fgf15* and *Shp*, levels were increased in mice fed a high-cholesterol diet [27]. These observations suggest that ileal SREBP-2 negatively regulates ileal FXR signaling. In the present study using human intestinal LS174T cells, we demonstrate that SREBP-2 negatively regulates the FXR-mediated transcription of *FGF19* in human intestine. These results suggest a novel mechanism for SREPB-2-mediated regulation of cholesterol/bile acid metabolism.

2. Materials and methods

2.1. Materials

Bovine serum albumin (BSA), cholesterol, 25-hydroxycholesterol, protease inhibitor cocktail, and proteinase K were purchased from Sigma–Aldrich (St. Louis, MO, USA). Fetal calf serum and [γ-^{32}P]ATP were purchased from Nichirei (Tokyo, Japan) and Perkin-Elmer Life and Analytical Sciences (Waltham, MA, USA), respectively. GW4064 was provided by Dr. Timothy M. Wilson (GlaxoSmithKline, Research Triangle Park, NC, USA).

2.2. Cell culture

LS174T cells were obtained from the Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). These cells were cultured in minimum essential medium containing 10% fetal calf serum, nonessential amino acids, and penicillin–streptomycin (Invitrogen, Carlsbad, CA, USA).

2.3. Plasmid preparation

FGF19 luciferase constructs (pFGF19(−2 k-intron) and pFGF19(intron)) were previously prepared [26]. A mutation construct (pFGF19(intron-FXREmut)) was prepared using the QuikChange Lightning Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). Expression plasmids, pTarget/hSREBP2 [28], pTarget/hFXR [26] and pTNT/hRXRα [29] were prepared previously.

2.4. Analyses of mRNA levels

A transient transfection assay was performed using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. LS174T cells were seeded in a 12-well plate at 4×10^5 cells/well for 24 h before transfection. Either the pTargeT/hSREBP-2 or the empty pTargeT (1 μg) was transfected and cells were incubated for an additional 24 h. Thereafter, the cells were harvested and subjected to quantitative reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted using the acid guanidine thiocyanate–phenol–chloroform method and cDNA was synthesized using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Quantitative PCR was performed using Go-Taq qPCR Master Mix (Promega, Madison, WI, USA) with the TP800 Thermal Cycler Dice Real Time System (Takara Bio, Otsu, Japan). Relative mRNA levels were calculated by the comparative threshold cycle method. The following specific forward and reverse primers were used for real-time quantitative PCR: *HMGCR* sense, 5′-CTGCACCATGCCATCTATAG-3′ and antisense, 5′-GACAATTCCCCAGCCATTAC-3′; *HMGCS1* sense, 5′-GATGAAAAGC ACAGAAGAAC-3′ and antisense, 5′-CCTCACAGAGTATCTTAATG-3′; *FGF19* sense, 5′-CACGGGCTCTCCAGCTGCTTCCTGCG-3′ and antisense, 5′-TCCTCCTCGAAAGCACAGTCTTCCTCCG-3′; *IBABP* sense, 5′-CAGAC TTTGCACCTCTGGCT-3′ and antisense, 5′-TCAGAACCTGAGTCAGCG CC-3′. *GAPDH* sense, 5′-AACAGCCTCAAGATCATCAGC-3′ and antisense, 5′-GGATGATGTTCTGGAGAGCC-3′.

2.5. Reporter assay

LS174T cells were seeded in a 48-well plate at 3×10^4 cells/well 24 h before transfection. The reporter constructs, pTarget or pTarget/hSREBP2, pTarget/hFXR and pRL-SV40 (Promega) were cotransfected by the calcium phosphate method using the Cellphect Transfection Kit (GE Healthcare, Piscataway, NJ, USA). The cells were washed with phosphate-buffered saline 12 h after transfection and then treated with GW4064 (0.1 μM) or vehicle (0.1%, dimethyl sulfoxide (DMSO)) in fetal calf serum-free medium for 24 h. Thereafter, the cells were harvested and firefly and *Renilla* luciferase activities were determined using Dual-Luciferase Reporter Assay System (Promega).

2.6. Electrophoretic mobility shift assay (EMSA)

Human SREBP-2, FXR and retinoid X receptor α (RXRα) proteins were synthesized *in vitro* with pTNT/hSREBP-2 [28], pTNT/hFXR [26] and pTNT/hRXRα [29], respectively, using the TNT Quick Coupled Transcription/Translation System (Promega). The EMSA was performed as previously described [26]. The binding reaction was performed in a reaction mixture (15 μl) containing 10 mM Tris–HCl (pH 8.0), 5% glycerol, 100 mM KCl, 1 mM DTT, 1 μg of poly (dI–dC) (GE Healthcare), and 1 μl each of synthesized SREBP-2-, FXR- and/or RXRα-containing lysate or the control lysate. Reaction mixtures were preincubated on ice for 30 min before adding the ^{32}P-labeled probe.

2.7. Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed as previously described [26] with minor modifications. After crosslinking the cells with 1% formaldehyde followed by sonication, the supernatant was obtained by centrifugation, and a portion of the supernatant was retained as the input sample. The supernatant was incubated with Protein G-coupled Dynabeads (Invitrogen) at 4 °C for 1 h, and the beads were then removed. The supernatant was then incubated with normal mouse IgG (Millipore, Billerica, MA, USA) or anti-human FXR mouse monoclonal antibody (A9033; Perseus Proteomics, Tokyo, Japan) at 4 °C overnight. Protein G-coupled Dynabeads was added to the reaction and the mixture was further incubated at 4 °C for 12 h to immunoprecipitate the chromatin–antibody complex. The bound chromatin was eluted and incubated at 65 °C overnight to decrosslink. After treatment with RNase A and proteinase K, genomic DNA was purified with a Wizard SV gel (Promega) and PCR was performed using KOD FX Neo (Toyobo, Osaka, Japan). The following specific forward and reverse primers were used for the PCR: FXRE sense, 5′-AGGAGCCTGGGTTGCTGCAATGAGC-3′ and antisense, 5′-GAAGGGCTGGGCAGTAAACACACTG-3′; NC (negative control) sense, 5′-CCATCTCCCAGCCCACCAG-3′ and antisense, 5′-CCCCCG CTGCTTCCACACAG-3′.

2.8. GST pull-down assay

GST-SREBP-2 proteins were prepared as previously described [28]. GST and glutathione-Sepharose 4B (GE Healthcare) were incubated with 5 μg of GST or GST-SREBP-2 in binding buffer (50 mM Tris–HCl, pH 8.0, 100 mM NaCl, and 1 mM EDTA) at 4 °C for 1 h and washed three times with the same buffer. The protein-bound beads were incubated with 3 μl of the *in vitro*-synthesized FXR containing lysate or control lysate at room temperature for 1 h in the binding buffer. The beads were washed five times with the binding buffer, and the bound proteins were eluted with SDS–PAGE sample buffer (31 mM Tris–HCl, pH 6.8, 1% SDS, 5% sucrose, 0.1 μg/μl bromophenol blue, and 5% 2-mercaptoethanol) and subjected to immunoblotting with anti-hFXR antibody (Perseus Proteomics).

2.9. Statistical analysis

Mean values ± S.D. are shown. Data were analyzed with the unpaired Student's *t*-test or by analysis of variance followed by the Turkey's test (GraphPad Prism ver. 4.0c software; GraphPad Software, San Diego, CA, USA). Differences at $p < 0.05$ were considered statistically significant.

3. Results

3.1. Association of SREBP-2 activity with the expression of FXR target genes in LS174T cells

LS174T cells were treated with cholesterol (10 μg/ml) and 25-hydroxycholesterol (1 μg/ml) for 24 h to inactivate endogenous SREBP-2 and verify the intestinal crosstalk between SREBP-2 and FXR signaling: nuclear SREBP-2 protein levels and mRNA levels of SREBP-2 or the FXR target genes were determined. Nuclear SREBP-2 protein levels were clearly decreased in LS174T cells after cholesterol and 25-hydroxycholesterol treatment (Fig. 1A). Consistent with the decrease in nuclear SREBP-2 protein levels, mRNA levels of the SREBP-2 target genes, *HMGCS1* and *HMGCR* significantly decreased (Fig. 1B). In contrast, mRNA levels of the FXR target genes, *FGF19* and *IBABP* significantly increased. These results suggest that SREBP-2 negatively regulates the expression of FXR target genes by suppressing FXR signaling. To test this possibility, dominant active SREBP-2 was overexpressed in LS174T cells, and mRNA levels of SREBP-2 and FXR target genes were measured. *HMGCS1* mRNA levels significantly increased in LS174T cells overexpressing active SREBP-2 whereas *FGF19* mRNA levels significantly decreased (Fig. 1C).

3.2. Role of SREBP-2 in the transcription of FGF19 gene in LS174T cells

Reporter assays were performed using the pFGF19(intron) construct containing the second intron of the FGF19 gene (+848 to +5200) and the pFGF19(intron-FXREmut) construct in which the FXR responsive element (IR-1) in the second intron was mutated to further investigate whether the suppressive effect of SREBP-2 depended on FXR-responsive element. Increased reporter activity of pFGF19(intron) in LS174T cells treated with GW4064 was attenuated by overexpressing SREBP-2 (Fig. 2). In contrast, reporter activity of pFGF19(intron-FXREmut) was unaltered by treatment with GW4064 and/or SREBP-2 overexpression.

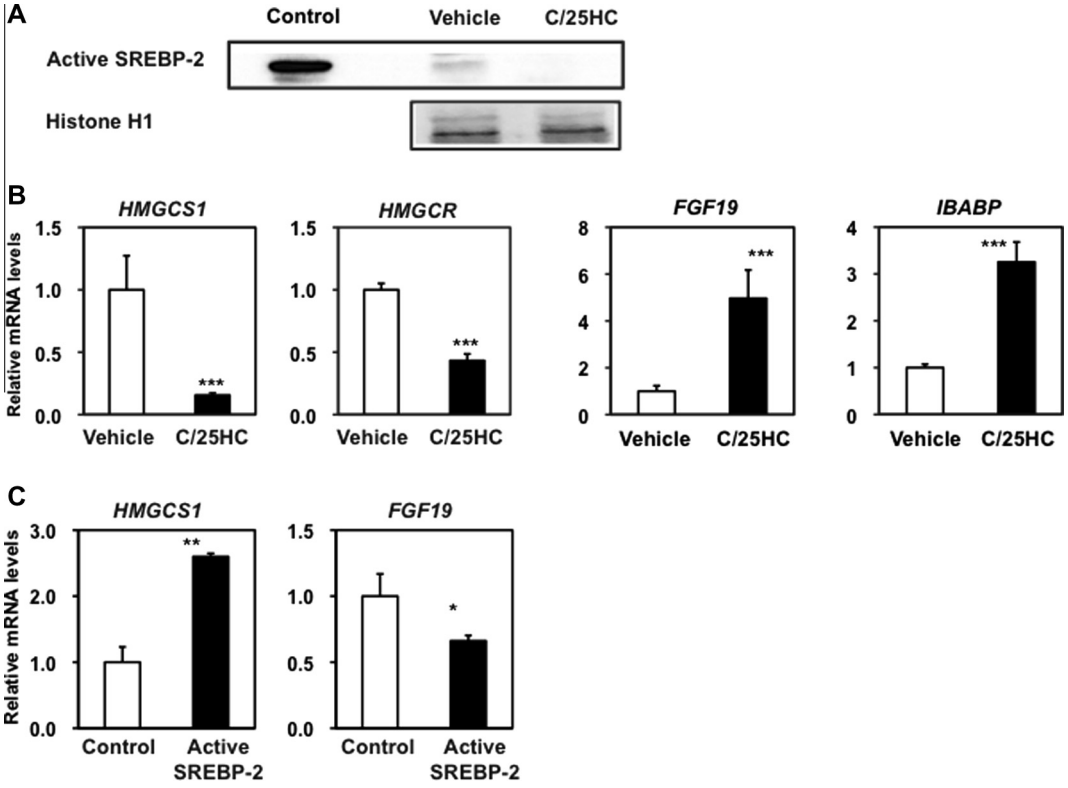

Fig. 1. mRNA levels of FXR target genes are altered in LS174T cells in a SREBP-2 dependent manner. (A and B) LS174T cells were treated with vehicle (0.2% ethanol) or 10 μg/ml cholesterol and 1 μg/ml 25-hydroxycholesterol (C/25HC) for 24 h, and then harvested. (A) Nuclear extracts of LS174T cells were subjected to immunoblotting to determine SREBP-2 and histone H1 protein levels. Control: *in vitro* translated SREBP-2. (B) mRNA levels of SREBP-2 target genes and FXR target genes were determined by quantitative RT-PCR. (C) LS174T cells were transfected with an active SREBP-2 expression vector or control vector as described under Section 2 for 24 h, and then the cells were harvested for quantitative RT-PCR. These mRNA levels were normalized with those of *GAPDH*. mRNA levels in vehicle treated cells or control cells are set at 1. Data represent the mean ± S.D. ($n = 3$). Significant differences were determined by Student's *t*-test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. The data are representative of two independent experiments.

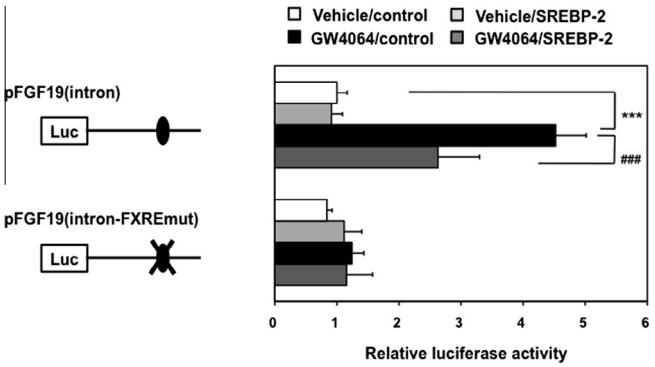

Fig. 2. SREBP-2 down-regulates FXRE-dependent transcriptional activation of *FGF19*. LS174T cells were transfected with the reporter plasmid indicated and either the SREBP-2 expression vector or the control vector as described in Section 2 for 12 h. Then, the cells were treated with vehicle (0.1% DMSO) or 0.1 μM GW4064 for 24 h and harvested for the determination of reporter activities. The reporter activities in the cells (vehicle/control) transfected with pFGF19(intron) plasmid are set at 1. Data represent the mean ± S.D. ($n = 4$). Significant differences were determined by one-way ANOVA with Tukey's post hoc test. $^{***}p < 0.001$ versus control vector and vehicle treated group. $^{\#\#\#}p < 0.001$ versus control vector and GW4064 treated group. The data are representative of two independent experiments.

3.3. SREBP-2-mediated inhibition of FXR binging to the FXR-responsive element

An EMSA was performed using an oligonucleotide probe containing the IR-1 motif in the second intron of the FGF19 gene to examine the influence of SREBP-2 on binding of FXR to the FXR-responsive element (Fig. 3A). A retarded band was observed using the IR-1 probe with both the FXR and RXRα proteins (Fig. 3B). The intensity of the retarded band was attenuated by adding SREBP-2 (lane 2 vs 3 and 4, Fig. 3B). This SREBP-2-mediated reduction disappeared when the amount of FXR, but not RXRα, was increased (lane 4, 5 and 6, Fig. 3B). In the presence of the SREBP-2 protein, a retarded band was observed using an oligonucleotide probe containing the sterol regulatory element (SRE) in the human low-density lipoprotein receptor (LDLR) gene whereas no retarded band was observed using the IR-1 probe (Fig. 3C).

ChIP assays were performed using LS174T cells treated with cholesterol and 25-hydroxycholesterol to investigate whether SREBP-2 suppresses binding of the FXR to the *FGF19* IR-1 motif in the human genome (Fig. 3D). As shown in Fig. 3E, the amount of FXR binding to the IR-1-containing region (+3214 to +3404) of the FGF19 gene increased in LS174T cells by treatment with cholesterol and 25-hydroxycholesterol.

These results suggest a protein–protein interaction between FXR and SREBP-2. Thus, we performed GST pull-down assays using the GST-SREBP-2 fusion protein. As expected, FXR directly bound to GST-SREBP-2, but not GST (Fig. 4).

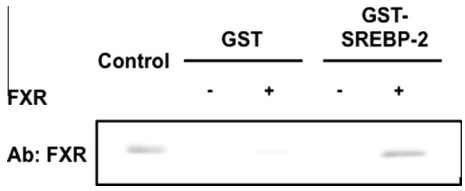

Fig. 4. SREBP-2 directly interacts with FXR. GST pull-down assays were performed as described in Section 2. GST or the GST-SREBP-2 protein bound to GSH-resin was incubated with *in vitro* synthesized FXR protein. Proteins bound to the resin were subjected to immunoblotting with anti-FXR antibody. Control: *in vitro* synthesized FXR protein. The data are representative of two independent experiments.

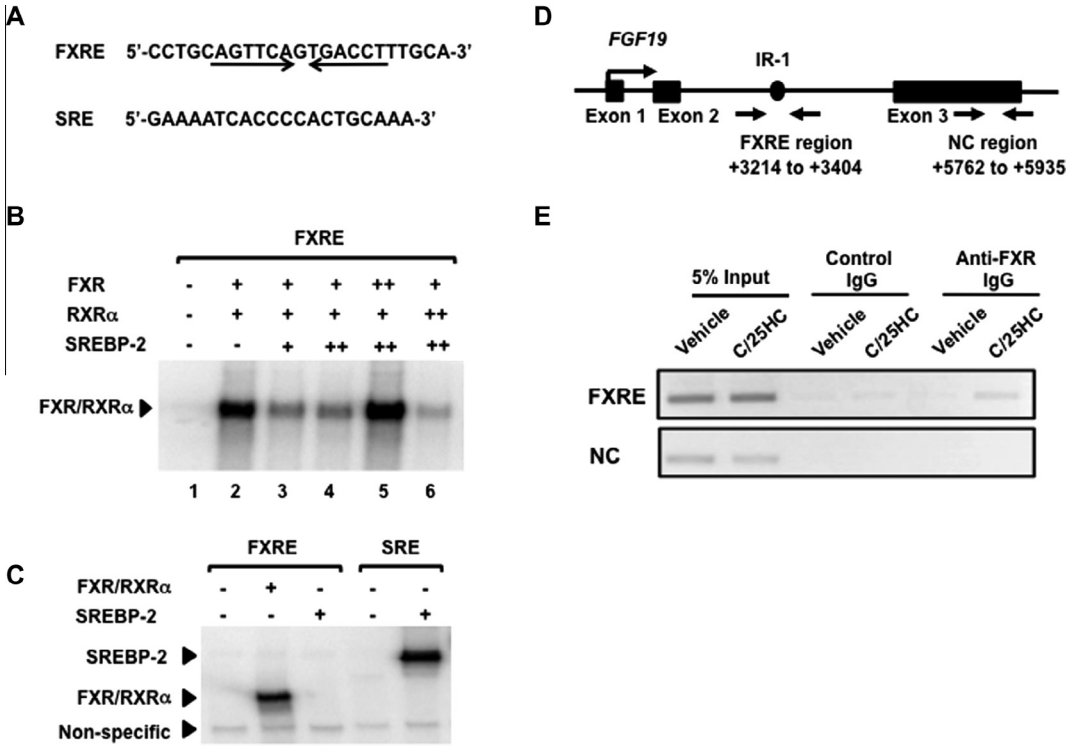

Fig. 3. SREBP-2 attenuates the binding of FXR/RXRα heterodimer to the IR-1 motif. (A) The oligonucleotide probe sequences used for the EMSA are shown. The FXRE probe contained the sequences of the IR-1 motif in the second intron of the FGF19 gene, and SRE probe contained the sequences of the SRE motif in promoter region of human LDLR gene. (B and C) The EMSAs were performed as described in Section 2 with ^{32}P-labeled probes and *in vitro* synthesized FXR, RXRα and SREBP-2 proteins. The data are representative of two independent experiments. (D) Regions analyzed by ChIP assays are shown. (E) LS174T cells were treated with vehicle (0.2% ethanol) or cholesterol (10 μg/ml) and 25-hydroxycholesterol (1 μg/ml) (C/25HC) for 8 h, and then ChIP assays were performed as described in Section 2. The immunoprecipitated DNA and DNA isolated before immunoprecipitation (Input) were analyzed by PCR with specific primers for the indicated regions. NC: negative control. The data are representative of two independent experiments.

4. Discussion

In the present study, we have found that SREBP-2 is involved in the negative regulation of FGF19 expression in LS174T cells. We performed reporter, EMSA, ChIP, and GST-pull down assays to understand the molecular mechanism of the negative regulation. We demonstrated that SREBP-2 attenuated the binding of the FXR to the FXR-responsive element resulting in the suppression of FXR-mediated transactivation of the FGF19 gene in LS174T cells. We also demonstrated the direct binding of SREBP-2 to FXR using GST-pull down assays. Adding SREBP-2 decreased binding of the FXR/RXRα heterodimer to the FXR-responsive element. To more understand the detailed mechanisms, it seems to be important to identify the cellular site of the binding of SREBP-2 to the FXR. Further studies are needed to identify whether SREBP-2 interacts with the FXR in the nuclear or cytoplasm. These results suggest that activated SREBP-2 inhibits the binding of the FXR to the FXR-responsive element through a protein–protein interaction with FXR.

SREBP-2 can directly bind the sterol regulatory element (SRE) and also the E-box in the promoter region of cholesterogenic genes. SREBP-2 negatively regulates human *ABCA1* transcription through the E-box motif [30]. This negative regulation is due to direct binding of SREBP-2 to the E-box motif. In contrast, we have found that SREBP-2-mediated suppression of FGF19 transcription depends on FXR-responsive element and that SREBP-2 does not bind the FXR-responsive element (IR-1). Thus, SREBP-2 is likely to suppress the FGF19 transcription through an indirect mechanism.

There are a few findings in the SREBP-mediated negative regulation through indirect mechanism. SREPB-1c and SREPB-1a competes with peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) for direct interaction with hepatocyte nuclear factor-4α (HNF-4α) resulting in the suppression of HNF-4α-mediated transactivation of the target genes [31,32]. Recently, our group has reported that SREBP-2 directly interacts with PGC-1α, a major coactivator for HNF-4α, resulting in the suppression of HNF-4 signaling [28]. In this case, activated SREBP-2 has no influence on the HNF-4α binding to the HNF-4α-responsive element, but inhibited the binding of PGC-1α to HNF-4α on the motif. For the FGF19 gene regulation, SREBP-2 directly interacts with nuclear receptor, FXR, not co-activator. Activated SREBP-2 may suppress the binding of FXR to the FXR-responsive element, the IR-1 motif, through direct binding to FXR. The SREBP-2-mediated regulation of FGF19 gene is a novel suppressive mechanism for nuclear receptor signaling.

Co-activator PGC-1α is involved in the FXR-mediated trans-activation of the target genes [33,34]. Further studies are necessary to verify SREBP-2 binding to PGC-1α to suppress transcriptional activation of the FGF19 gene through FXR signaling.

Intestinal FGF15/19 is primarily regulated by bile acids through FXR signaling. FGF15/19 is also regulated by vitamin D/vitamin D receptor and vitamin A/RXRα signaling [35]. Our results demonstrate that regulation of FGF19 was also modulated by intestinal cholesterol/SREBP-2 signaling in conjunction with FXR signaling. It seems that ileal FGF15/19 is intricately regulated not only by bile acids but also by several food-derived components such as cholesterol and vitamins [35].

We have demonstrated here that FXR activation through the IR-1 motif in the second intron of the FGF19 gene is suppressed by activated SREBP-2. The IR-1 motif is a typical FXR responsive element that is present in the regulatory region of many FXR target genes [2,36]. It is likely that the transcription of many FXR target genes is suppressed by SREBP-2 activation.

There are unresolved issues with respect to the crosstalk between SREBP-2 and FXR signaling. It remains unclear whether the crosstalk between SREBP-2 and FXR is physiologically relevant to bile acid/cholesterol homeostasis. Under conditions of low cellular cholesterol, it may be reasonable to suppress FXR signaling to elevate cellular cholesterol levels. Further *in vivo* study may help to understand the physiological significance of the intestinal cross-talk between SREBP-2 and FXR.

In conclusion, our results demonstrate a direct interaction between SREBP-2 and FXR for the first time. Through this interaction, activated SREBP-2 may suppress FXR-mediated transcription of the FGF19 gene in human intestinal cells.

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