**FGF19 Signaling Cascade Suppresses APOA Gene Expression**

Indumathi Chennamsetty, Thierry Claudel, Karam M. Kostner, Michael Trauner, Gert M. Kostner

**Objective**—Lipoprotein(a) is a highly atherogenic lipoprotein, whose metabolism is poorly understood. Currently no safe drugs exist that lower elevated plasma lipoprotein(a) concentrations. We therefore focused on molecular mechanisms that influence apolipoprotein(a) (APOA) biosynthesis.

**Methods and Results**—Transgenic human APOA mice (tg-APOA mice) were injected with 1 mg/kg of recombinant human fibroblast growth factor 19 (FGF19). This led to a significant reduction of plasma APOA and hepatic expression of APOA. Incubation of primary hepatocytes of tg-APOA mice with FGF19 induced ERK1/2 phosphorylation and, in turn, downregulated APOA expression. Repression of APOA by FGF19 was abrogated by specific ERK1/2 phosphorylation inhibitors. The FGF19 effect on APOA was attenuated by transfection of primary hepatocytes with siRNA against the FGF19 receptor 4 (FGFR4). Using promoter reporter assays, mutation analysis, gel shift, and chromatin immuno-precipitation assays, an Ets-1 binding element was identified at −1630/−1615bp region in the human APOA promoter. This element functions as an Elk-1 binding site that mediates repression of APOA transcription by FGF19.

**Conclusion**—These findings provide mechanistic insights into the transcriptional regulation of human APOA by FGF19. Further studies in the human system are required to substantiate our findings and to design therapeutics for hyperlipoprotein(a).


**Key Words:** apolipoproteins ■ fibrinolysis ■ gene expression ■ lipoproteins ■ molecular biology

Increased plasma lipoprotein(a) [Lp(a)] concentrations correlate with atherothrombotic diseases and evidence is accumulating that this association might be causal.1-5 Plasma Lp(a) levels are highly variable among individuals ranging from <1 mg/dL to more than 200 mg/dL and the consensus report of the European Atherosclerosis Society recommends a cut-off for aggressive treatment at Lp(a) levels of >50 mg/dL.6 Unfortunately, only very few drugs affect plasma Lp(a) levels, except for nicotinic acid, that maximally reduces Lp(a) by 30% with an unknown mode of action.7

Recently we reported that patients suffering from obstructive cholestasis had strongly reduced plasma Lp(a) concentrations.12 This was also verified in transgenic APOA (tg-APOA) mice expressing the human APOA gene controlled by its native promoter, undergoing bile duct ligation as well as in tg-APOA mice fed a chow containing 0.2% cholic acid where the transcription of APOA in the liver and the concentration of plasma APOA were dramatically reduced. In a series of experiments we finally provided evidence that the DR-1 at −826/−814bp of the APOA promoter functions as a negative FXR response element. However, the downregulation of APOA transcription by this DR-1 element did not fully account for the almost complete disappearance of plasma APOA in tg-APOA mice fed a CA containing chow. Therefore we searched for additional negative regulators mediated by FXR signaling.

FGF15/19 plays an important regulatory role in hepatic bile acid metabolism. The key enzyme in bile acid biosynthesis CYP7A1 was shown to be downregulated indirectly by FXR after intestinal induction of FGF19 expression in humans13 and its ortholog FGF15 in mice.14 FGF19 binds to FGFR4...
on liver cells and suppresses the expression CYP7A1 in human hepatocytes in a signaling cascade involving the MAPK/ERK1/2 pathway. These findings prompted us to test whether this pathway might be also operative in APOA suppression.

In the present study we show that APOA transcription is indeed suppressed via the FXR-FGF15/19-FGFR4 axis. FGF15/19 binding to its receptor FGFR4 on liver cells activates MAPK/ERK1/2 that in turn displaces phosphorylated Elk-1 to the nucleus. Elk-1 binds to a negative control element located at −1630/−1615bp of the human APOA promoter thereby suppressing APOA transcription and plasma APOA concentrations.

Materials and Methods

Chemicals

Recombinant FGF19 was purchased from R&D systems (Vienna, Austria), and cholic acid was from Sigma (Vienna, Austria). The reagents PD98059, SB203580, and SP600125 were from CalBiochem (Germany); U0126 was from Upstate Biotech (Lake Placid, NY). Collagenase was from Worthington Corporation (NJ).

Animal Experiments

All animal experiments were performed following approval of the protocol by the Austrian Federal Ministry of Science and Research (Vienna, Austria). tg-APOA mice carrying human APOA gene controlled by its native flanking region in YAC18 were hosted under standard 12 hour light/12 hour dark cycle and fed standard rodent chow diet and water ad libitum. Ten- to 12-week-old female tg-APOA mice were injected intraperitoneally recombinant FGF19 (1 mg/kg body weight) (n=5 mice) or vehicle (PBS) (n=6 mice), followed by plasma and tissue harvesting after 16 hours. Plasma concentrations of APOA were measured enzymatically by an in-house DELFIA method.

Plasmids

Expression plasmids encoding human Elk-1 (pcDNA3-Elk-1) was generously provided by Dr Robert Hipskind (IGM Montpellier, France). The human APOA promoter constructs were obtained by PCR amplification as a template as described previously.

Transient Transfection and Reporter Gene Assays

Reporter gene assays were performed in HepG2 cells. Cells at 60% to 70% confluency were transiently transfected with the indicated reporter or expression plasmids using FuGENE 6 reagent as described previously.

Site-Directed Mutagenesis

Mutagenesis was performed using the Quik Change site-directed mutagenesis system (Stratagene, La Jolla, CA), according to the manufacturer’s protocol. The oligonucleotide Mut (5’- GCGGTAGG TTTTACCATTATCATTTAATGGTTGCGCTGTC-3’) was used to introduce mutations into the full length hAPOA −1952/+52. Mutated bases are in bold.

Electrophoretic Mobility Shift Assays

Human Elk-1 protein was synthesized in vitro using the TNT T7 Quick Coupled Transcription/Translation System (Promega, Madison, WI). The sense and antisense oligonucleotide probes of Elk-1 Wt (5’-ACCATTATCAGGAGTGGTTGCGCTGTC-3’) and an Elk-1 consensus element-containing oligonucleotide (5’-GGTCCCTAAGCCTGACCCG GAAAGTTGCGTCAAGTTC-3’) were annealed and radioactively labeled. In vitro translated human Elk-1 (2.0 μL) was incubated for 20 minutes at room temperature in a total volume of 10 μL with binding buffer (Gel shift assay system, Promega, Madison, WI) before the labeled probe was added. Binding reactions were further incubated for 30 minutes and resolved by 6% nondenaturing polyacrylamide gel electrophoresis in 0.25X Tris-Borate-EDTA buffer at room temperature and 120 V for 4.0 hours. The gel was dried and exposed to an X-ray film. In supershift assays, anti–Elk-1 antibody (sc-355x, Santa Cruz Biotechnology) was added for 1 hour on ice prior to the addition of probes. For competition experiments, unlabeled probes were included in the binding reaction at the indicated excess concentrations.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) assay was performed with primary mouse hepatocytes treated with vehicle or FGF19 for 6 hours using ChIP-IT Express kit (Active Motif, Rixensart, Belgium) according to the manufacturer’s instructions. Chromatin was immunoprecipitated using 2 μg anti–Elk-1 and 1 μg anti-IgG antibody. DNA extractions were PCR amplified using the following flanking primers covering Ets-1 element and the PCR products (166bp) were analyzed by agarose gel electrophoresis. (ChIP FWD 5’ CCTATGTCGGCCACTGGAT 3’; ChIP REV 5’ AGGGTGCACTACCTGGACTA 3’).

Statistics

Statistical analyses of the experiments were performed with GraphPad Prism 5.0. Two-tailed, unpaired Student t-test was applied to determine statistical significance (**P<0.01; ***P<0.001; P<0.05).

Cell isolation, culture, treatments, real-time PCR, immunoblotting, and siRNA transfections are described in detail in the online-only Data Supplement.

Results

FGF19 Inhibits APOA Expression In Vitro and In Vivo in Transgenic APOA Mice

We showed previously that cholic acid feeding dramatically suppressed APOA expression in liver and APOA abundance in plasma of tg-APOA mice. The in vitro effect observed when primary mouse hepatocytes were incubated with FXR ligands, however, was less pronounced. We therefore considered the possibility, that FGF19/15 might have an additive effect.

To verify the effect of FGF19 on human APOA expression, primary hepatocytes isolated from tg-APOA mice were incubated with different concentrations of recombinant FGF19. Analysis of mRNA levels by real-time quantitative PCR revealed a significant dose- and time-dependent decrease in APOA mRNA levels suggesting a transcriptional effect (Figure 1A and 1C). Western blot analysis confirmed that this FGF19-mediated repression also occurs at the protein level in cell lysates (Figure 1B). In addition, APOA secretion into the medium was reduced by 48% (Figure 1I in the online-only Data Supplement). Cell viability assessed by trypan blue exclusion test revealed that all concentrations of FGF19 were well tolerated (data not shown). As a positive control, we measured the expression levels of the known FGF19 target gene Cyp7a1. As expected FGF19 treatment dose-dependently downregulated Cyp7a1 mRNA levels (Figure II in the online-only Data Supplement). Furthermore, FGF19 treatment also suppressed the APOA expression in primary hepatocytes isolated from tg-APOAfxr−/− mice confirming a direct role of FGF19 on APOA repression (Figure III in the online-only Data Supplement).
As expected, the repression of APOA transcription was further amplified by the addition of the FXR ligand cholic acid in the presence of FGF19 (Figure 1D and 1E). In primary hepatocytes from tg-APOA mice, cholic acid treatment alone led to repression of APOA by 47%. A comparable additional repression by 40% was seen by the addition of 40 ng/mL FGF19 (Figure 1D).

To verify the physiological relevance of the FGF19 effect, tg-APOA mice were injected intraperitoneally FGF19 (1 mg/kg) and analyzed for APOA expression. Treatment with FGF19 resulted in significantly decreased plasma APOA levels by 32% (Figure 2A), and hepatic APOA mRNA by 47% (Figure 2B) when compared to vehicle-treated controls. FGF19 treatment led to a strong inhibition of Cyp7a1 but Shp mRNA levels were unaltered (Figure 2C and 2D).

Because Shp was described as another transcriptional repressor activated by bile acids, we addressed in parallel the effect of FGF19 on Shp expression. As shown in Figure IV in the online-only Data Supplement, FGF19 did not affect Shp mRNA and protein expression in primary mouse hepatocytes.

To rule out any additional effects mediated by Shp on APOA we next investigated the effect of specifically knocking down Shp in primary hepatocytes. Shp specific siRNA led to a significant knockdown of Shp at the protein level (Figure VA in the online-only Data Supplement). siRNA-mediated knockdown of Shp did not affect FGF19-mediated repression of APOA transcription (Figure VB in the online-only Data Supplement), suggesting that FGF19-mediated inhibition of APOA expression is independent of Shp. Altogether, FGF19 represses APOA expression in a time- and dose-dependent manner without involvement of Shp.

To study the role of FGFR4 in FGF19-mediated repression of APOA, primary hepatocytes from tg-APOA mice were transfected with siRNA to FGFR4 to block FGF19/FGFR4 signaling and then treated with FGF19. Knockdown of FGFR4 attenuated FGF19 repression of APOA compared with control siRNA further underlining the role of FGF19/FGFR4 pathway in the repression of APOA (Figure VI in the online-only Data Supplement).

Identification of the FGF19 Activated Protein Kinase Involved in APOA Repression

To identify kinases activated by FGF19, primary hepatocytes were treated with 40 ng/mL FGF19 and the phosphorylation of individual MAP kinases was followed. Cell lysates were analyzed by immunoblotting using phospho-specific MAP-kinase antibodies. FGF19 (40 ng/mL) time-dependently stimulated phosphorylation of ERK1/2 (Figure 3A); phosphorylation remained consistent for 24 hours. FGF19 did not activate JNK and p38 in our experiments, which is consistent with previous reports. Taken together these results demonstrated that FGF19
specifically activated the MEK/ERK1/2 pathway in primary hepatocytes from tg-APOA mice.

**ERK1/2 Inhibitors Attenuate FGF19-Mediated Inhibition of APOA Expression in Mouse Primary Hepatocytes**

To further investigate the signaling pathways involved in FGF19-mediated inhibition of APOA gene transcription, mouse primary hepatocytes were treated with several specific inhibitors of MAP kinases. U0126 and PD98059 alone, inhibitors of the MEK/ERK1/2 pathway, strongly stimulated APOA mRNA expression. Pretreatment with U0126 and PD98059 attenuated the inhibitory effect of FGF19 on APOA expression in mouse primary hepatocytes. SP600125, a JNK inhibitor, and SB203580, a p38 kinase inhibitor, had no effect on FGF19-mediated repression of APOA (Figure 3B and 3C). Taken together these results demonstrate that MEK/ERK1/2 inhibitors, but not JNK and p38MAPK inhibitors, blocked FGF19-mediated inhibition of APOA expression.

**Mapping of the Promoter Region Responsive to FGF19 Signaling**

To identify promoter elements responsible for the observed FGF19 effects, a 2-kb fragment of human APOA promoter (hAPOA –1952/+52) was cloned into a pGL3-luciferase reporter plasmid. In addition a series of 5’ deletion constructs were generated as shown in Figure 4A. HepG2 cells were then transiently transfected with these constructs of the hAPOA promoter in the absence or presence of FGF19 (40 ng/ml)
ng/mL). Incubation with FGF19 strongly lowered the activity of the hAPOA\(-1952/+52\) promoter by 48% (Figure 4B). However, the repression was relieved for \(-1446, -757, -657, -477,\) and \(-148\) promoter constructs indicating that the region between \(-1952\) to \(-1446\)bp of the human APOA promoter contains a response element mediating the suppression of APOA transcriptional activity by FGF19.

Next, to study the effect of kinase inhibitors on human APOA promoter activity, HepG2 cells were transfected with full length hAPOA\(-1952/+52\) promoter reporter plasmidpretreated for 1 hour with specific MAP kinase inhibitors, followed by treatment with vehicle or FGF19. Pretreatment with MEK/ERK1/2 inhibitors U0126 and PD98059, but not the other inhibitors, reversed the inhibitory effects of FGF19 on human APOA promoter activity (Figure 4C).

Notably, in silico Matinspector promoter analysis suggested the presence of an Ets-1 binding motif located between nucleotides \(-1630\) and \(-1615\) of the APOA gene promoter. To test whether this Ets-1 site could mediate FGF19 dependent repression of human APOA promoter, we introduced mutations in the full-length human APOA\(-1952/+52\) promoter \((-1952/+52\text{ Wt})\) and generated the mutant construct \((-1952/+52\text{ Mut})\) as shown in Figure 5A. Mutation of this site completely abolished the FGF19-mediated repression of APOA promoter activity (Figure 5B). Taken together, these results demonstrate that the Ets-1 response element located between nucleotides \(-1630\) and \(-1615\) of human APOA promoter is a negative response element responsible for FGF19-mediated repression of human APOA promoter activity involving MAPK/ERK1/2.

\textbf{Elk-1 Binds to an Ets-1 Motif in the Human APOA Promoter} \newline

Elk-1 has been shown to be a well-characterized common nuclear substrate for activated ERK1/2\textsuperscript{21-22} that belongs to the family of Ets domain containing transcription factors. By Western blot analysis we ascertained that Elk-1 indeed is phosphorylated by FGF19 in primary mouse hepatocytes (Figure 6A). To provide additional evidence that Elk-1 indeed binds to the Ets-1 binding motif at the \(-1630\) and \(-1615\)bp region of the human APOA promoter, gel shift assays with in
vitro translated human recombinant Elk-1 were performed using probes that cover the Ets-1 element. Consensus Elk-1 probe was used as a positive control. Elk-1 bound the labeled consensus probe (Figure 6B, lane 2) and to the Elk-1 wild type (Wt) probe (Figure 6B, lane 5), but not to the probe carrying the mutated Elk-1 element (Mut) (Figure 6B, lane 6). The formation of Elk-1–DNA complex was specifically competed by unlabeled cold Wt– probe (Figure 6C, lanes 3 and 4), whereas the Mut– probe did not compete for binding (Figure 6C, lanes 5 and 6). The intensity of Elk-1–DNA complex formation was decreased by the addition of a specific anti–Elk-1 antibody.

To further confirm the interaction of the transcription factors Elk-1 with the Ets-1 element in the APOA promoter, we performed a ChIP experiment with primary mouse hepatocytes treated with FGF19. FGF19 treatment led to occupancy of the response element by Elk-1 (Figure 6D). As a negative control, equivalent amount of chromatin precipitated with IgG antibody resulted in no signal. These results further confirmed that Elk-1 binds to the response element at the −1630/−1615bp region of human APOA promoter.

Collectively, these data provide evidence that APOA is strongly repressed by the FGF19/MAPK/ERK1/2-Elk-1 signaling cascade.

Discussion

Lp(a) has been recognized as an important risk factor for cardiovascular diseases by interfering with several steps of hemostasis and fibrinolysis, by accumulating in the arterial intima because of its high affinity to proteoglycans and in addition by structural alterations under high oxidative stress thereby stimulating numerous inflammatory and immunologic pathways. Unfortunately, a final proof of concept by prospective intervention studies with Lp(a) lowering drugs are missing because there is currently no safe and effective medication available. Detailed knowledge of the Lp(a) and APOA metabolism might circumvent this problem and help to design more efficacious drugs for patients at increased risk for athero-thrombotic diseases.

As plasma Lp(a) levels are mainly controlled by its rate of biosynthesis, we focused our research to the transcriptional regulation of APOA. From previous work we knew that FXR ligands have a profound influence on APOA transcription and we identified a negative FXR response element at −830 to −815bp region of the APOA promoter that reduced APOA expression up to 60%. Promoter studies in combination
with in vitro findings however suggested that this negative FXR response element accounted only for part of the bile acid mediated transcriptional repression. FXR-signaling has been intensively studied with regard to bile acids metabolism. Among other pathways that are still under debate, ligand-activated FXR binds to promoter elements of small heterodimer partner and drives its transcription; small heterodimer partner in turn represses the activity of key genes such as CYP7A1. Independently of this pathway, FXR was found to transactivate mouse Fgf15, a gene that is highly expressed in the terminal ileum, and its human ortholog, FGF19, expressed in small intestine and liver. In our previous report we demonstrated that the overexpression of Shp did not affect APOA promoter activity. In the current study, silencing of Shp in primary mouse hepatocytes did not influence APOA expression (Figure V in the online-only Data Supplement). We therefore focused in the present work on a possible regulation by FGF15/19.

FGF15/19 belongs to the hormone-like endocrine subfamily of fibroblast growth factors.22 FGF19 and the mouse ortholog FGF15 are highly expressed in the ileum and circulate to the liver. Activated liver cells of human but not of mouse origin have also been found to express FGF19.13,14 FGF15/19 binds to its cognate receptor FGFR4 on liver cells repressing the transcription of several proteins involved in bile acid metabolism.15,16 In addition FGF15/19 plays important roles in hepatic lipid, protein and glycogen metabolism and is most relevant for the pathophysiology of type-2 diabetes mellitus and metabolic syndrome.26–28 FGF19 on the other hand has been related to tumorigenesis in humans and thus any medication influencing FGF19 expression needs to be carefully monitored for possible adverse effects.28

FGF15/19 expression in the intestine is under the control of FXR.14 Binding of bile acids, the natural ligands of FXR strongly induces FGF15/19 biosynthesis that bind to the tyrosine kinase receptor FGFR4 on liver cells. FGFR4 phosphorylation activates 2 distinct intracellular substrates: phospholipase C γ1 also named FGF receptor substrate FRS1 and FGFR substrate 2, named FSR2. In the FSR2 signaling cascade Ras-mitogen-activated protein kinase 1 and 2 (MEK) is phosphorylated which in turn activates further downstream substrates.29 Of particular interest for our work is the signaling cascade FGF19/FGFR4/ERK1/2. ERK1/2 phosphorylated by MEK binds and phosphorylates the ETS domain containing transcription factor Elk-1. Phosphorylated Elk-1 in turn binds to the ETS site in the promoter thereby regulating the transcriptional activity of target genes. Activation of Elk-1 was found to suppress promoter activity by recruiting corepressors and histone deacetylases, yet other pathways are not excluded.29–31 Conceivably, a similar mechanism could be responsible for the inhibition of APOA expression.

Because recombinant mouse FGF15 is unstable, we used recombinant human FGF19 for our studies. As shown in Figure 1, recombinant human FGF19 at a concentration of 40 ng/mL reduced APOA mRNA abundance by 40% in primary hepatocytes isolated from tg-APOA mice. This downregulation was time-dependent and reached maximal levels at 48 hours. The addition of the FXR ligand CA at a concentration of 200 μmol/L downregulated APOA mRNA abundance by additional 47% indicating that 2 FXR-mediated mechanisms act in parallel. FGF19 on the other hand did not affect the Shp mRNA expression in our experiments indicating that the FGF19 effect on APOA was small heterodimer partner independent. This is further corroborated by our observation that downregulation of baseline levels of Shp by specific siRNA did not influence APOA mRNA abundance (Figure V in the online-only Data Supplement).

FGF15/19-FGFR4 signaling is suggested to involve either one of the ERK1/2-JNK- or P38 MAPK pathways.23 To find out which of these MAP-kinases might be involved in FGF19-mediated APOA repression, primary hepatocytes from tg-APOA mice were preincubated for 1 hour with specific MAPK inhibitors followed by FGF19 incubation for 24 hours. The results displayed in Figure 3 proved that the effects of FGF19 on APOA repression was ERK1/2-mediated. FGF19-activated MEK/ERK1/2 in turn phosphorylated the downstream transcription factor Elk-1 (Figure 6A). Phosphorylated Elk-1 then suppressed APOA expression by binding to an Ets-1 motif in the promoter. Luciferase reporter assays using full-length (−1952/+52) and several 5’ truncations of the APOA promoter revealed that the regulatory sequence responding to FGF19-mediated signaling resides in the region −1630/−1615bp with the Ets-1 motif GAGT. Electrophoretic mobility shift assays and ChIP assays provide a final proof that the transcription factor Elk-1 binds the Ets-1 element at −1630/−1615bp region of human APOA promoter and is responsible for the FGF19-mediated repression of APOA transcription. There is however 1 caveat that needs to be considered: The results described here were obtained in a heterologous system, transgenic mice expressing human gene, and confirmation by studies in the human system will be necessary. Although we are working on this we were not able so far to get satisfactory results in primary human liver cell cultures because they quickly lose the expressionual activity of APOA.

Taken together we propose a dual regulatory mechanism of transcriptional APOA suppression by FXR signaling. One pathway operates via competition of activated FXR binding with HNF4α binding to the DR-1 element at −826/−814bp.12 The second pathway involves FGF15/19 binding to the FGFR4 on liver cells, the ERK1/2 phosphorylation cascade, and Elk-1 binding to its motif at −1630/−1615bp. We believe that these findings may serve as a basis for strategies in developing Lp(a) lowering drugs.

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Disclosures
None.
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References


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Supplemental Material:

Cell cultures.

Mouse primary hepatocytes were prepared from 7-9-week old tg-APOA mice. The mouse liver was perfused with collagenase solution and liver cells were collected. After filtration and centrifugation, the isolated hepatocytes were resuspended in DMEM (Gibco, Invitrogen, Lofer, Austria) supplemented with 20% (v/v) FCS (Sigma-Aldrich Chemie GmbH, Vienna, Austria), 100 units/ml penicillin, and 100 units/ml streptomycin, and plated in 6-well collagen-coated dishes (BD Biosciences, Erembodegem, Belgium) at a density of $1 \times 10^5$ cells/well at 37°C in an atmosphere of 5% CO₂ for 4 h.

Thereafter, cells were cultured in DMEM supplemented with 10% FCS, 100 units/ml penicillin/streptomycin for 16 h. Hepatocyte viability was monitored before plating by trypan blue exclusion, more than 85% of cells were consistently viable. Experiments were performed in serum-free DMEM supplemented with various concentrations of FGF19 (R&D Systems) for 24 h and harvested for RNA analysis. In some experiments cells were pre-treated with various kinase inhibitors for 1 h before the incubation with FGF19. Primary hepatocytes isolated from tg-APOA/Fxr⁻/⁻ mice were treated with FGF19 for 24 h and harvested for RNA analysis.

HepG2 cells were obtained from the American Type Culture Collection (Rockville, Maryland). The cells were maintained in DMEM containing 10% FCS, 100 units/ml penicillin/streptomycin.

RNA extraction, reverse transcription and real-time PCR.

Total RNA from cells and mouse tissues were isolated using Trizol (Invitrogen, Lofer, Austria) according to the manufacturer’s protocol. Quantitative real-time PCR was
performed on a Light Cycler 480 instrument (Roche Diagnostics, Mannheim, Germany) using the QuantiFast™ SYBR® Green PCR Kit (Qiagen, Hilden, Germany). Primer sequences were identical to those published previously. The gene expression values were normalized to cyclophilin-A as a housekeeping gene. The data were analyzed by the public domain program Relative Expression Software Tool – REST. Values are presented as mean ± SEM.

**Immunoblotting.**

Equivalent amounts of protein homogenates were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with rabbit polyclonal antibodies to human APOA (1:1250). Antibodies against SHP, Elk-1 and β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against ERK1/2, phospho-ERK1/2, JNK, phospho-JNK, p38, phospho-p38, phospho-Elk-1 were from Cell Signaling Technology (Beverly, MA). The immunoblots were visualized by Pierce® ECL chemiluminescence detection system (Thermo Scientific, Rockford, IL, USA). Densitometric analysis of the gels was carried out using ImageJ software.

**siRNA transfections.**

In siRNA experiments, mouse primary hepatocytes were transfected with 100 nM human synthetic predesigned short interfering RNA (siRNA) targeting FGFR4, SHP or non silencing siRNA (control) (Qiagen, Maryland, USA) using Hi-PerFect transfection reagent (Qiagen, Maryland, USA) following the manufacturer’s recommended protocol. Cells were then treated with FGF19 for 24 h and total RNA was prepared for real-time quantitative PCR analysis. SHP protein levels were analyzed by western blotting.
References:


Supplemental Figure I

APOA secretion from mouse primary hepatocytes:
Primary hepatocytes from tg-APOA mice were incubated for 24 h with 40 ng/ml of FGF19 or with vehicle. APOA levels in the medium were analyzed by DELFIA and expressed as mean ± SD from three independent experiments. ***p ≤ 0.001 when compared to vehicle treated control group.
Supplemental Figure II

Effect of FGF19 on the expression of \textit{Cyp7a1}.

Primary hepatocytes from \textit{tg-APOA} mice were incubated for 24 h with increasing concentrations of FGF19 (40 and 80 ng/ml) or vehicle. mRNA levels of known FGF19 target gene \textit{Cyp7a1} was analyzed as a positive control by real-time quantitative PCR. Results represent means ± SEM (** p \leq 0.001).
**Supplemental Figure III**

Effect of FGF19 on the expression of APOA in primary hepatocytes from tg-APOA/fxr<sup>-/-</sup> mice.

Primary hepatocytes from tg-APOA/fxr<sup>-/-</sup> mice were incubated with FGF19 (40 ng/ml) or vehicle for 24 h. APOA mRNA levels were analyzed by real-time quantitative PCR.

Results represent means ± SEM (** p ≤0.01).
Supplemental Figure IV

Effect of FGF19 on Shp expression.

Primary hepatocytes from tg-APOA mice were incubated for 24 h with increasing concentrations of FGF19 (40 and 80 ng/ml) or vehicle. (A) mRNA levels of Shp were analyzed by real-time quantitative PCR. Results represent means ± SEM of three independent experiments (** * p ≤0.001). (B) Western blot analysis of Shp protein expression in whole cell lysates from hepatocytes treated for 24 h with increasing concentrations of FGF19. β-actin expression was used as loading control.
Supplemental Figure V

Silencing of SHP does not affect APOA expression.

Primary mouse hepatocytes were transfected with 100 nM siRNA targeting SHP and control siRNA using hi-perfect transfection reagent and subsequently treated with or without FGF19 (40 ng/ml) for 24 h. (A) SHP protein expression in whole cell lysates was analyzed by Western blotting. (B) mRNA levels of APOA in primary hepatocytes treated with siRNA against SHP relative to control siRNA treatment. Data are expressed as means ± SEM (** p ≤0.01).
Knockdown of FGFR4 abolished FGF19 effect on APOA expression.

Primary mouse hepatocytes were transfected with 100 nM siRNA targeting FGFR4 and control siRNA using hi-perfect transfection reagent and subsequently treated with or without FGF19 (40 ng/ml) for 24 h. mRNA levels of APOA were analyzed relative to vehicle treated control siRNA treatment. Data are expressed as mean ± SEM (** p ≤0.01).