THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 51, pp. 39105–39113, December 22, 2006

Disruption of an SP2/KLF6 Repression Complex by SHP Is Required for Farnesoid X Receptor-induced Endothelial Cell Migration*[S]

Received for publication, August 11, 2006, and in revised form, October 10, 2006 Published, JBC Papers in Press, October 27, 2006, DOI 10.1074/jbc.M607720200

Amitava Das[‡1], Martin E. Fernandez-Zapico[‡], Sheng Cao[‡], Janet Yao[‡], Stefano Fiorucci[§], Robert P. Hebbel[¶], Raul Urrutia[‡], and Vijay H. Shah[‡2]

From the [‡]Gastroenterology Research Unit, Department of Physiology and Cancer Cell Biology Program, Mayo Clinic, Rochester, Minnesota 55905, the [§]Dipartimento di Medicina Clinica e Sperimentale, Clinica di Gastroenterologia ed Endoscopia Digestiva, University of Perugia, Perugia 06122, Italy, and the [¶]Vascular Biology Center, Division of Hematology, Oncology, and Transplantation, University of Minnesota Medical School, Minneapolis, Minnesota 55454

The farnesoid X receptor (FXR) signaling pathway regulates bile acid and cholesterol homeostasis. Here, we demonstrate, using a variety of gain- and loss-of-function approaches, a role of FXR in the process of cell motility, which involves the small heterodimeric partner (SHP)-dependent up-regulation of matrix metalloproteinase-9. We use this observation to reveal a transcriptional regulatory mechanism involving the SP/KLF transcription factors, SP2 and KLF6. Small interference RNA-based silencing studies in combination with promoter, gel shift, and chromatin immunoprecipitation assays indicate that SP2 and KLF6 bind to the matrix metalloproteinase-9 promoter and together function to maintain this gene in a silenced state. However, upon activation of FXR, SHP interacts with SP2 and KLF6, disrupting the SP2/KLF6 repressor complex. Thus, together, these studies identify a mechanism for antagonizing Sp/KLF protein repression function via SHP, with this process regulating endothelial cell motility.

Farnesoid X Receptor (FXR)[3] is a member of the nuclear receptor superfamily of transcription factors. In response to ligand binding, FXR regulates expression of genes involved in bile acid, cholesterol, and triglyceride metabolism (1–9). FXR heterodimerizes with the 9-*cis*-retinoic acid receptor α, which allows binding to a specific DNA sequence composed of two inverted hexamer repeats separated by one nucleotide (IR-1), thereby regulating target gene transcription (10–12). An alternate mechanism of regulation occurs through FXR-dependent up-regulation of the atypical nuclear receptor, small heterodimeric partner (SHP). Although SHP lacks a DNA binding domain, it regulates transcription by several putative mechanisms that are not fully understood (13–15).

Recent work has delineated a requisite role for Sp/KLF transcription factors for diverse biological functions (16–19). The Sp/KLF family contains 24 identified members, including SP1–8 and KLF1–16, which bind with varying affinity to GC-rich DNA sequences of target gene promoters (20). Interestingly, these proteins may function as transcriptional activators or repressors, although the mechanisms by which specificity of effect is achieved are not well defined.

Because bile acids such as chenodeoxycholic acid (CDCA) are the natural ligands for FXR (21–23), prior investigations have largely been pursued in cells with active bile acid signaling, such as hepatocytes, cholangiocytes, and enterocytes (4, 24, 25). However, more recent work has expanded the scope of this nuclear receptor system into a diversity of cell types and functions, including vascular wall cells (26–28), thereby suggesting potentially important and heretofore unrecognized actions that may be achieved by the FXR pathway. In this study, we delineate a signaling pathway by which FXR promotes endothelial cell motility through transcriptional activation of matrix metalloproteinase-9 (MMP-9). This pathway requires SHP inhibition of an SP2/KLF6 repressor complex. Thus, whereas SP2 and KLF6 repress MMP-9 promoter activity under basal conditions, activation of FXR results in a process by which SHP displaces SP2/KLF6 from the MMP-9 promoter, thereby up-regulating MMP-9 expression and function. Together, these studies identify SHP as a key disruptor of SP2/KLF6-mediated gene silencing to allow transcriptional activation by FXR. The functional importance of this pathway is supported by its biological role in FXR-induced endothelial cell motility.

EXPERIMENTAL PROCEDURES

Cell Culture—Blood outgrowth endothelial cells (BOECs) are a blood-derived endothelial cell commonly utilized for investiga-

* This work was supported by National Institutes of Health Grants R01-59615 and R01-59388 (to V. H. S.) and DK 52913 (to R. U.), by Pancreatic SPORE Career Development Award P50 CA102701 and Mayo Clinic Cancer Center (to M. E. F.-Z.), by American Heart Association Scientist Development Grant AHA0435063N (to S. C.), and by National Institutes of Health Grant PO1-HL076540 (to R. P. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S4.

[1] To whom correspondence may be addressed: GI Research Unit, Alfred 2-435, Mayo Clinic Rochester, 200 First St. SW, Rochester, MN 55905. Tel.: 507-255-1607; Fax: 507-255-6318; E-mail: das.amitava@mayo.edu.

[2] To whom correspondence may be addressed: GI Research Unit, Alfred 2-435, Mayo Clinic Rochester, 200 First St. SW, Rochester, MN 55905. Tel.: 507-255-5040; Fax: 507-255-6318; E-mail: shah.vijay@mayo.edu.

[3] The abbreviations used are: FXR, farnesoid X receptor; BOEC, blood outgrowth endothelial cell; CDCA, chenodeoxycholic acid; 6-ECDCA, 6-ethyl-chenodeoxycholic acid; SHP, small heterodimeric partner; MMP-9, matrix metalloproteinase-9; Sp, stimulatory protein; KLF, Kruppel-like factor; siRNA, short interfering RNA; ChIP, chromatin immunoprecipitation; RLA, relative luciferase activity; RT, reverse transcription; qRT-PCR, quantitative real-time PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; VEGF, vascular epidermal growth factor; rFXR, retroviral FXR; rSHP, retroviral SHP; EMSA, electrophoretic mobility shift assay.

tions of vascular cell motility and remodeling (29). BOECs (P4–P6) were prepared from human blood using primary cell isolation conditions that have been previously described (29). Cells were grown in EBM-2 medium supplemented with EGM-2, 10% fetal bovine serum, and 1% streptomycin/penicillin. Cells were incubated with CDCA, 6-ECDCA, or equivalent volume of vehicle (Me_2SO) at concentrations and durations that were based on prior work (27) and that are indicated in individual experiments. HepG2 cells were also used in some experiments in which the molecular intervention was not feasible in BOECs.

Retroviral Overexpression—FXR and SHP plasmid constructs (from Benjamin Shneider and David Mangelsdorf) and lacZ were subcloned into the retroviral vector pMMP using standard molecular approaches to generate pMMP-rFXR, pMMP-rSHP, and pMMP-rLacZ (30). All constructs were sequenced for confirmation. In brief, to generate retrovirus, 5×10^6 293T/17 cells in 100-mm dishes were co-transfected with three plasmids, 1.5 μg of pMD.MLV gag.pol, 0.5 μg of pMD.G, and 2 μg of relevant retroviral vector using Effectene^{TM} transfection reagent (Qiagen). Cell culture medium containing retrovirus was collected 48 h after transfection. For cell transduction, 2 ml of viral stock and 8 ml of fresh Dulbecco's modified Eagle's medium were mixed and added in a 100-mm dish of 0.5×10^6 BOECs with 8 μg/ml Polybrene. Transduction efficiency using this approach was uniformly >90%. Cells were used for experiments after 18–24 h.

Microarray Analysis—Cells were incubated with vehicle or CDCA (100 μM) for 48 h. Total RNA was isolated using an RNeasy kit according to the manufacturer's instruction (Qiagen), and 3 μg was used for the probe preparation using the GEArray AmpoLabeling-LPR kit and biotin-16,2′-deoxyuridine-5′-triphosphate. GEArray Q Series Human Endothelial Cell Biology Gene Array (HS-036) membrane was used for hybridization with the synthesized probe and detected by using the Chemiluminescent detection kit (Super Array Bioscience Corp., Frederick, MD) according to the manufacturer's protocol. Changes in expression were assessed by the software provided by the manufacturer.

siRNA Gene Silencing—siRNA targeting human FXR, SHP, MMP-9, SP2, KLF6, and a scrambled control were obtained from Ambion (Austin, TX). Cells were transfected with siRNA using Oligofectamine (Invitrogen) as we described previously (16). Conditions and concentrations required for specificity of knockdown with high transfection efficiency were individually established for each of the siRNA (see supplemental materials).

Semiquantitative and Quantitative RT-PCR—Total RNA was extracted from cells as described above. 1.0 μg of total RNA was used for the cDNA synthesis using oligo(dT) primer of SuperScript III First-Strand Synthesis System (Invitrogen) and appropriate forward and reverse primers of FXR and GAPDH for the semiquantitative PCR. Thermal cycling conditions were: 2 min at 94 °C, followed by 40 cycles of 94 °C for 15 s, 55 °C for 30 s, and 72 °C for 60 s and a final extension by 1 cycle of 72 °C for 10 min in a Hybaid PCR express instrument. For TaqMan-based quantitative real-time PCR analysis (qRT-PCR) 25 ng of each cDNA was added to the TaqMan Universal PCR Master Mix along with 900 nM of each primer and 200 nM of probe according to the manufacturer's instruction (Applied Biosystems, Foster City, CA) (7, 12). Real-time fluorescence monitoring was performed with the Applied Biosystems 7500 Real Time PCR System instrument. Amplification of human GAPDH and eukaryotic 18 S rRNA (Applied Biosystems) was used in the same reaction of all samples as an internal control. FXR, SHP, MMP-9, SP2, and KLF6 mRNA was normalized to GAPDH mRNA and shown as the -fold change.

Gelatin Zymography—Conditioned media samples from cells incubated with vehicle, CDCA, or 6-ECDCA for 48 h were mixed with electrophoresis loading buffer and subjected to 8% SDS-PAGE co-polymerized with gelatin type B (2 mg/ml). After washing with 2.5% Triton X-100 and incubation in zymogram developing buffer, the gels were stained with 0.5% Coomassie, and then destained. Gelatinolytic activities were detected as transparent bands (31).

MMP-9 Enzyme-linked Immunosorbent Assay—Total human MMP-9 levels were measured from cultured BOEC supernatants by enzyme-linked immunosorbent assay (R&D Biosystems, Minneapolis, MN) based on the manufacturer's instructions.

Cell Motility—Chemotaxis was measured by using a modified Boyden chamber assay (8-μm pore size, Neuro Probes, Inc., Gaithersburg, MD) as we described previously (32). Briefly, the bottom wells of the chamber were filled with 26 μl of serum-free media containing 10 ng/ml VEGF, and covered with a polycarbonated filter. 2×10^4 serum-starved cells were added into the upper chamber of each well. Cells were incubated for 6 h at 37 °C with CDCA or vehicle to stimulate FXR-dependent gene transcription. Migrated cells at the lower surface of filters were fixed and stained using Hema3 stain (Biochemical Sciences, Inc., Swedesboro, NJ). Cellular migration was determined by counting the number of stained cells on membranes in five randomly selected, non-overlapping high power fields. Data are presented as percent change in VEGF-induced chemotaxis.

Luciferase Reporter Assay—HepG2 cells were transfected with wild-type or mutated Sp1 human MMP-9-promoter-luciferase reporter constructs (33) (Dr. H. Sato, Japan) and 0.01 μg of *Renilla* luciferase reporter vector to control for transfection efficiency (pRL-TK) using Lipofectamine 2000 (Invitrogen). 10 h later, culture medium was changed, and cells were cultured for an additional day then stimulated with vehicle or 50 μM CDCA for 6 h. Luciferase assays were conducted using a dual luciferase kit (Promega, Madison, WI) as described previously (16).

Electrophoretic Mobility Shift Assay—5.0 μg of nuclear extract from vehicle or CDCA (50 μM)-stimulated cells was incubated in a binding buffer (50 mM Hepes, pH 7.9, 1.0 mM EDTA, 100 mM KCl, 20% glycerol, 2.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1.5 μg of poly(dI-dC)) for 10 min at room temperature. The ^{32}P-labeled probe encoding the Sp1 binding site of MMP-9 promoter 5′-ATTCCTTCCGCCCCCAGATG-3′ was added for 20 min. In some experiments, an excess of cold probe, at the indicated dilutions, was added concomitant with the addition of radiolabeled probe. The mixture was electrophoresed at 12.5 V/cm on 4% nondenaturing polyacrylamide gel in 0.5 × TBE (Tris borate-EDTA). Gels were vacuum-dried and autoradiographed (16).

Chromatin Immunoprecipitation Assay—Cells were transfected with either control pcDNA or pSP2 or pKLF6 and then

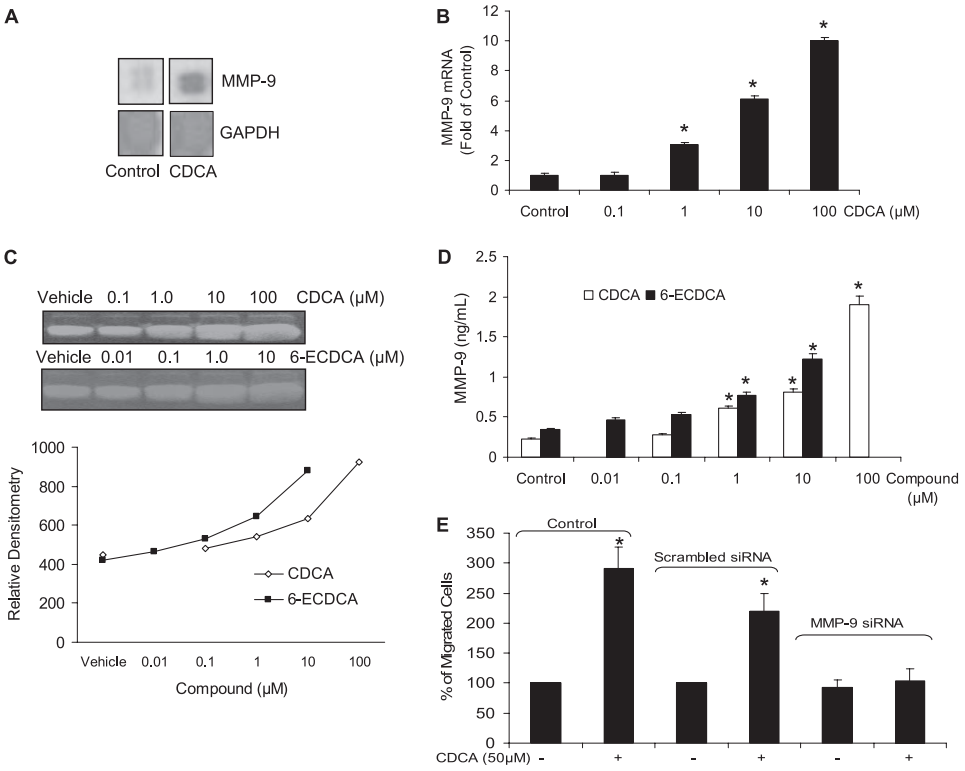

FIGURE 1. **FXR ligands increase MMP-9 mRNA/protein levels and MMP-9-dependent cell motility.** *A*, BOECs were incubated with vehicle or CDCA, and RNA was isolated for microarray analysis using a focused endothelial cell microarray. Prominent up-regulation of MMP-9 was detected in BOECs incubated with CDCA. *B*, BOECs were incubated for 6 h with vehicle or increasing concentrations of CDCA. MMP-9 transcript levels were measured by qRT-PCR. MMP-9 mRNA levels increased in association with increasing concentrations of CDCA ($n = 3$; *, $p < 0.05$). *C*, BOECs were incubated with various concentrations of CDCA or 6-ECDCA for 48 h, and conditioned media were collected for gelatin zymography. Increasing MMP-9 gelatinase activity was observed in association with increasing concentrations of CDCA or 6-ECDCA. The depicted blot is representation of an experiment that was replicated three times. *D*, MMP-9 concentration was measured from conditioned media using a human total MMP-9 enzyme-linked immunosorbent assay kit. Significant increases in MMP-9 concentration were observed in response to increasing concentrations of CDCA or 6-ECDCA ($n = 3$; *, $p < 0.05$). *E*, migration assay was performed using untransfected, control BOECs as well as BOECs transfected with 30 nM of either scrambled siRNA or MMP-9 siRNA. Cells were incubated with vehicle or 50 μM CDCA, and migration in response to a VEGF chemotactic gradient was measured using a Boyden chamber. BOECs transfected with MMP-9 siRNA evidenced no migration in response to CDCA, whereas control cells and cells transfected with scrambled siRNA showed significant migration ($n = 3$; *, $p < 0.05$).

incubated with vehicle or CDCA (50 μM). Cells were cross-linked with formaldehyde for 10 min at 37 °C, then harvested in SDS lysis buffer (Upstate Biotechnology) and sheared to fragment DNA. Samples were then immunoprecipitated using agarose-conjugated antibodies to SP2 or KLF6 (Santa Cruz Biotechnology, Santa, Cruz, CA), control (IgG) antibody, or agarose beads alone. After removal of the cross-links, immunoprecipitated DNA was purified using phenol/chloroform extraction (500 μl) and ethanol precipitation. DNA was used for PCR with sense primer 5′-ATT CAG CCT GCG GAA GAC AGG G-3′ and antisense primer 5′-TGA TGG AAG ACT CCC TGA GAC TTC-3′ encoding the Sp1 binding site of MMP-9 promoter and detected by visualizing PCR products on an agarose gel as we previously described (16).

Co-immunoprecipitation and Western Blot Analysis—Cells overexpressing rSHP-His were lysed in a buffer (50 mM Tris, pH 7.5/150 mM NaCl/3 mM $MgCl_2$/1 mM EDTA/0.5% Nonidet P-40/10% glycerol). Lysates were precleared with control IgG and Protein A-Sepharose beads, and then incubated overnight with Anti-His antibody (Santa Cruz Biotechnology). After incubation with Protein A-Sepharose beads and washing, samples were separated by SDS-PAGE, transferred to nylon membranes, and immunoblotted with SP2 or KLF6 antibody. To study the endogenous interaction of SHP with SP2/KLF6, nuclear extract of cells treated with either vehicle or CDCA (50 μM) were immunoprecipitated with SP2 or KLF6 antibody (Santa Cruz Biotechnology) and immunoblotted with SHP antibody (Santa Cruz Biotechnology) as described above. Proteins were also probed separately with human FXR, SP2, KLF6, and Histone H1 (Santa Cruz Biotechnology), MMP-9 (BD Biosciences), and β-actin (Sigma).

Statistical Analysis—The data in the *bar graphs* represent the mean ± S.D. of at least three independent experiments, each performed with duplicate samples. Blots, autoradiographs, and micrographs represent typical experiments reproduced at least three times with similar results. Statistical analyses were performed using a Student's *t* test, with a two-tailed value of $p < 0.05$ considered significant.

RESULTS

FXR Regulates Cell Motility via the Up-regulation of MMP-9—Our work initiated from the observation that FXR is expressed in BOECs and regulates cell motility via the up-regulation of MMP-9, a key cellular protease important in cell motility (Fig. 1, *A–E*, and supplemental Fig. S1*A*). Initially, the FXR-mediated up-regulation of MMP-9 was identified using a pathway-specific array approach (Fig. 1*A*). These results were confirmed by qRT-PCR, which showed that the FXR ligand, CDCA, induced a concentration-dependent up-regulation of MMP-9 mRNA levels, with a 6-fold increase observed in response to 10 μM CDCA (Fig. 1*B*). In addition to these observations at the mRNA level, we also found that FXR activation resulted in a 2-fold increase in MMP-9 enzymatic activity and 3-fold increase in MMP-9 protein levels as measured by gelatin zymography (Fig. 1*C*) and enzyme-linked immunosorbent assay, respectively (Fig. 1*D*). Moreover, similar results were obtained with the synthetic FXR agonist, 6-ECDCA (Fig. 1, *B–D*). Lastly, cells were incubated with the natural FXR ligand, CDCA, under the same conditions used above to stimulate FXR-dependent gene transcription, and then chemotaxis was examined in response to the chemotactic agent, VEGF. Interestingly, cells incubated with CDCA evidenced enhanced migration compared with those incubated with vehicle (Fig. 1*E*).

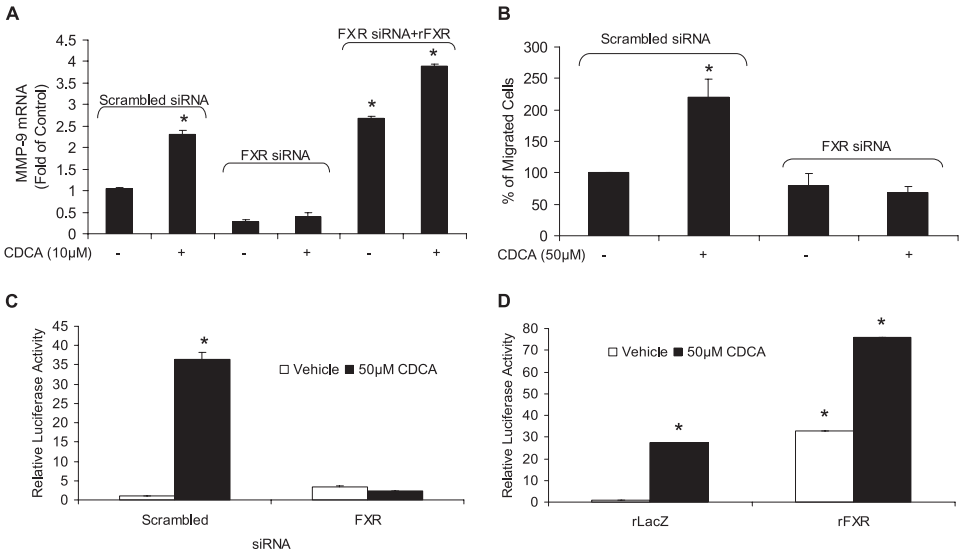

FIGURE 2. **FXR is required for bile acid activation of MMP-9 gene transcription and MMP-9-dependent cell motility.** *A*, BOECs were transfected with 30 nM of either FXR siRNA or scrambled siRNA sequence and then incubated with vehicle or 10 μM CDCA for 6 h. Total RNA was isolated and used for qRT-PCR of MMP-9 and GAPDH. CDCA-induced MMP-9 up-regulation was detected in BOECs transfected with scrambled siRNA but not in cells transfected with FXR siRNA. However, overexpression of FXR with pMMP-rFXR transduction rescued back the CDCA-induced MMP-9 mRNA up-regulation in BOECs previously transfected with 30 nM FXR siRNA ($n = 3$; *, $p < 0.05$). *B*, BOECs were transfected with 30 nM of scrambled siRNA or FXR siRNA and incubated with vehicle or 50 μM CDCA. Migration toward a VEGF gradient was measured using a Boyden chamber. CDCA-induced migration in cells transfected with scrambled siRNA but was absent in BOECs transfected with 30 nM FXR siRNA ($n = 3$; *, $p < 0.05$). *C*, HepG2 cells were co-transfected with 30 nM of either scrambled siRNA or FXR siRNA and wild-type MMP-9 promoter luciferase constructs and incubated with vehicle or 50 μM CDCA for 6 h. Relative luciferase activity (RLA) was significantly increased in scrambled siRNA-transfected cells in the presence of CDCA (50 μM), whereas FXR silencing abolished this effect ($n = 3$; *, $p < 0.05$). *D*, HepG2 cells were transduced with pMMP-rlacZ or pMMP-rFXR. 24 h after transduction, cells were transfected with wild-type MMP-9 promoter luciferase reporter construct and incubated with vehicle or CDCA. RLA was significantly increased in cells overexpressing FXR both basally and in response to CDCA ($n = 3$; *, $p < 0.05$).

To more firmly establish the MMP-9 protease in cell migration, we silenced MMP-9 using a specific siRNA. MMP-9 protein levels were markedly reduced in the presence of MMP-9 siRNA as observed by Western blot (supplemental Fig. S1*B*). Also, MMP-9 mRNA levels were significantly reduced by 80% in the presence of MMP-9 siRNA, whereas GAPDH expression remained unchanged as observed by qRT-PCR (supplemental Fig. S1, *C* and *D*). Interestingly, under these conditions migration was not observed in cells transfected with MMP-9 siRNA indicating that this molecule is required to trigger FXR-dependent cell motility.

In addition to the pharmacological experiments shown above, a series of molecular gain- and loss-of-function approaches were also used to confirm that CDCA activation of MMP-9 gene transcription was indeed mediated directly by FXR. First, cells were transfected with FXR siRNA or scrambled siRNA. A concentration-dependent decrease in FXR mRNA levels was observed in response to FXR siRNA in cells with 30 nM FXR siRNA effectively knocking down 85% of FXR mRNA levels (supplemental Fig. S2, *A–C*). CDCA increased MMP-9 mRNA levels in cells transfected with scrambled siRNA but not in cells transfected with FXR siRNA as assessed by qRT-PCR (Fig. 2*A*). Furthermore, retroviral FXR (rFXR) overexpression rescued the MMP-9-silencing effect of FXR siRNA both basally as well as in response to CDCA (Fig. 2*A*). In control experiments, rFXR robustly increased both FXR mRNA and protein levels by severalfold (supplemental Fig. S2*D*). Lastly, siRNA transfection of BOECs also suppressed BOEC migration indicating that the effects of CDCA on cell motility were also occurring through an FXR-dependent mechanism (Fig. 2*B*). Therefore, these studies demonstrate that FXR regulates cell motility through an MMP-9-dependent mechanism.

FXR Activation of MMP-9 Gene Transcription Requires SHP—To search for biochemical mechanisms used by FXR to regulate gene transcription, we used HepG2 cells, a commonly used cell for transcription mechanism studies relating to FXR (4). First, we co-transfected HepG2 cells with a human wild-type MMP-9 promoter and either FXR siRNA or scrambled siRNA and then incubated cells with vehicle or 50 μM CDCA. CDCA significantly increased relative luciferase activity in HepG2 cells co-transfected with scrambled siRNA, as compared with FXR siRNA (Fig. 2*C*). Next, we overexpressed FXR or retrovirus encoding lacZ in HepG2 cells transfected with wild-type MMP-9 promoter and then incubated cells with vehicle or CDCA. Interestingly, the relative luciferase activity was significantly increased by >2.5-fold in HepG2 cells overexpressing FXR as compared with lacZ control, both in the presence and absence of CDCA (Fig. 2*D*).

One prototypical pathway by which FXR regulates target genes is through heterodimerization with nuclear receptors such as retinoic acid receptor and binding to consensus IR-1 repeats located within target promoters (11, 12). Another indirect pathway occurs through transcriptional activation of the atypical non-DNA binding nuclear receptor SHP (13). To differentiate between the roles of these two pathways, we performed computer-assisted analysis of the published nucleotide sequence upstream of the transcriptional start site of the human MMP-9 gene, which failed to identify an IR-1 repeat (data not shown). Consequently, we focused our attention on SHP as a mediator of FXR activation of MMP-9 gene transcription. For this purpose, first, we measured mRNA and protein levels of SHP in response to increasing concentrations of CDCA. A concentration-dependent increase in SHP mRNA levels was observed in cells incubated with CDCA as analyzed by qRT-PCR (Fig. 3*A*). A concurrent concentration-dependent increase in SHP protein levels were also observed as analyzed by Western blot (Fig. 3*B*). In addition, CDCA induced up-regulation of SHP mRNA levels was not observed in cells transfected with FXR siRNA, confirming SHP as an FXR target gene (Fig. 3*C*). Interestingly, an increase in SHP mRNA levels (2.26-fold) was also observed in response to MMP-9 siRNA (supplemental

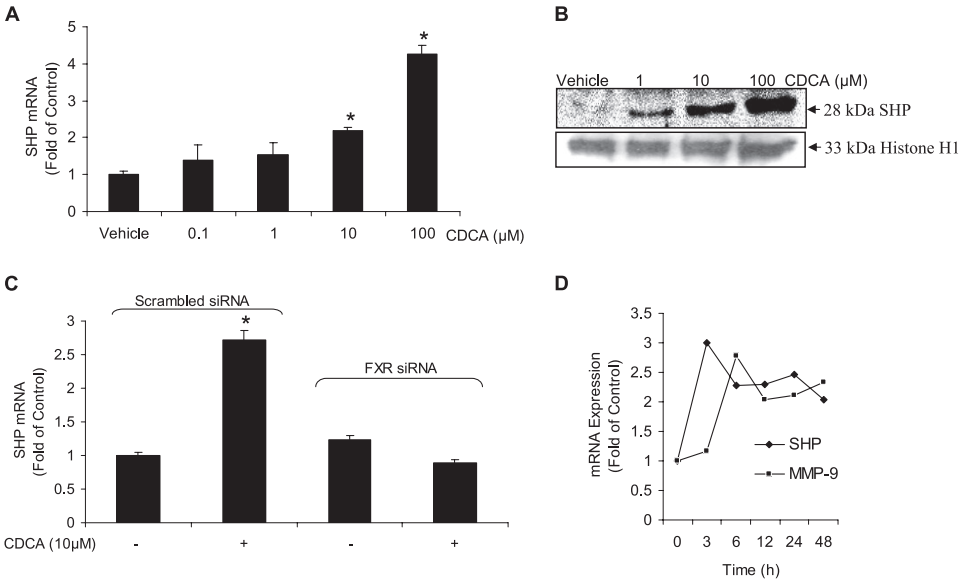

FIGURE 3. **CDCA up-regulates SHP through FXR in BOECs.** *A*, BOECs were incubated with various concentration of CDCA for 6 h, and total RNA was isolated and used for qRT-PCR analysis. SHP mRNA levels were significantly increased with increasing concentrations of CDCA ($n = 3$; *, $p < 0.05$). *B*, BOECs were incubated with increasing concentration of CDCA for 48 h and nuclear fraction was extracted and used for Western blot analysis. SHP protein levels were observed to increase with increasing concentrations of CDCA. Histone H1 antibody used as loading control. The depicted blot was one of the representative experiments that were reproduced three times. *C*, SHP transcript levels were measured by qRT-PCR in BOECs transfected with 30 nM of FXR siRNA or scrambled siRNA. CDCA significantly increased SHP mRNA expression in BOECs transfected with scrambled siRNA but not in cells transfected with FXR siRNA ($n = 3$; *, $p < 0.05$). *D*, BOECs were treated with 50 μM CDCA or vehicle for various time periods. Total RNA was isolated and used for qRT-PCR analysis of SHP mRNA and MMP-9 mRNA expression. SHP mRNA expression was significantly increased by 3 h, whereas MMP-9 mRNA expression was increased after 6 h.

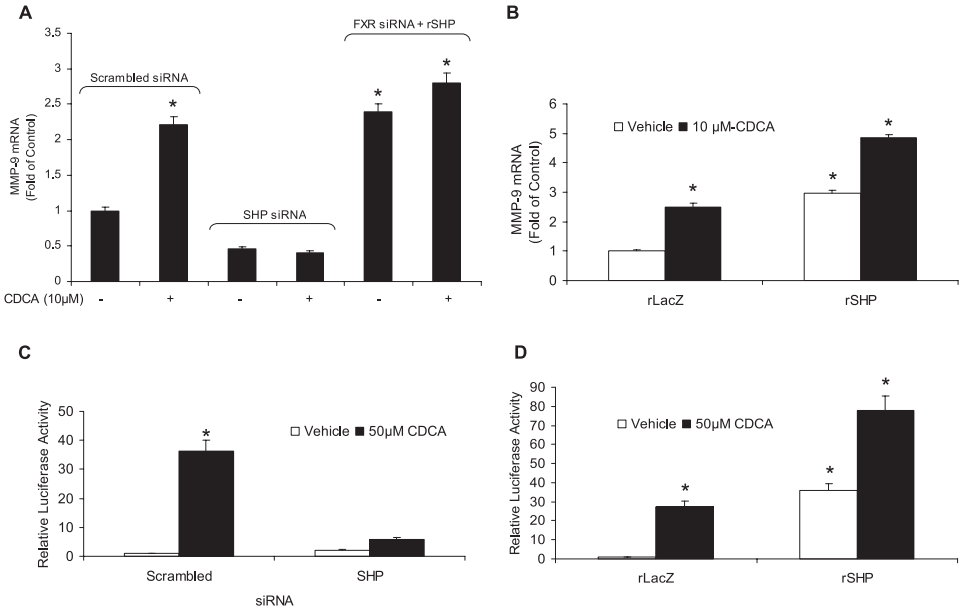

FIGURE 4. **FXR activation of MMP-9 gene transcription requires SHP.** *A*, BOECs were transfected with 30 nM of SHP siRNA or scrambled siRNA, and MMP-9 transcript levels were analyzed using qRT-PCR. CDCA significantly up-regulated MMP-9 mRNA levels in scrambled siRNA-transfected BOECs but not in BOECs transfected with SHP siRNA ($n = 3$; *, $p < 0.05$). Retroviral overexpression of SHP (*rSHP*) rescued MMP-9 mRNA expression in BOECs previously transfected with FXR siRNA ($n = 3$; *, $p < 0.05$). *B*, BOECs were transduced with pMMP-rSHP or pMMP-rLacZ. Overexpression of SHP in BOECs increases basal and CDCA (10 μM)-induced MMP-9 mRNA levels as compared with control ($n = 3$; *, $p < 0.05$). *C*, HepG2 cells were co-transfected with 30 nM of both scrambled siRNA or SHP siRNA and a wild-type MMP-9 promoter luciferase reporter construct. Cells were incubated with vehicle or 50 μM CDCA for 6 h. Increase in RLA in response to CDCA was observed in cells transfected with scrambled siRNA but not with SHP siRNA ($n = 3$; *, $p < 0.05$). *D*, HepG2 cells were transduced with pMMP-rlacZ or pMMP-rSHP. 24 h after transduction, cells were transfected with wild-type MMP-9 promoter luciferase reporter construct. Both basal and CDCA-induced increases in RLA were potentiated by overexpression of SHP ($n = 3$; *, $p < 0.05$).

Fig. S3*A*). However, a time kinetic analysis using qRT-PCR revealed that the CDCA-induced increase in SHP mRNA levels preceded the increase in MMP-9 mRNA levels (Fig. 3*D*).

To determine if SHP is required for CDCA activation of MMP-9 gene transcription, we next examined if gene silencing of SHP could block CDCA-induced MMP-9 up-regulation. Indeed, MMP-9 mRNA levels did not increase in response to CDCA in BOECs transfected with SHP siRNA (Fig. 4*A*). Furthermore, retroviral SHP (rSHP) overexpression rescued the MMP-9-silencing effect of FXR siRNA both basally as well as in the presence of CDCA (Fig. 4*A*). In control experiments, a concentration-dependent decrease in SHP mRNA level was observed in cells transfected with SHP siRNA with 30 nM SHP siRNA reducing SHP mRNA levels by 70% and blocking CDCA induced up-regulation of SHP mRNA (supplemental Fig. S3, *B* and *C*). Next, overexpression of SHP mRNA levels was pursued using a retroviral system (rSHP), which increased SHP mRNA levels in transduced cells by >4-fold (supplemental Fig. S3*D*). Overexpression potentiated both basal and CDCA-induced up-regulation of MMP-9 mRNA levels by 2-fold (Fig. 4*B*). Subsequently, these results were corroborated using the MMP-9 promoter in HepG2 cells. siRNA-based silencing of SHP abolished the CDCA-induced increase in MMP-9 promoter activity (Fig. 4*C*). Additionally, transduction experiments using rSHP significantly increased the relative MMP-9 luciferase activity by >2-fold both basally and in response to CDCA (Fig. 4*D*). These studies establish that FXR activates MMP-9 gene transcription through SHP and led us to further identify the components of this pathway.

SHP-mediated Antagonism of SP2 and KLF6 Repression Is Required for the Up-regulation of MMP-9 by FXR—The MMP-9 promoter contains an Sp1 binding site and indeed prior studies have demonstrated

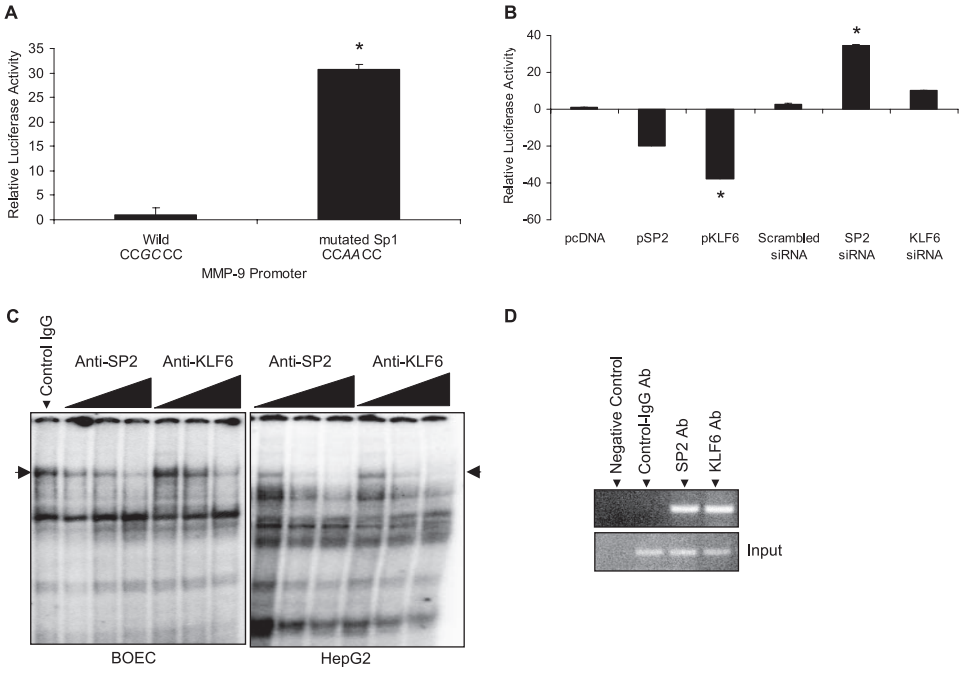

FIGURE 5. **SP2 and KLF6 repress MMP-9 promoter activity.** *A*, HepG2 cells were transfected with either wild-type or mutated Sp1 human MMP-9 promoter luciferase reporter constructs. Cells were harvested, and RLA was determined as described under "Experimental Procedures." RLA was significantly increased in cells transfected with mutated Sp1 promoter luciferase reporter as compared with a wild-type promoter ($n = 3$; *, $p < 0.05$). *B*, HepG2 cells were co-transfected with wild-type MMP-9 promoter luciferase reporter and with control pcDNA, pSP2, pKLF6, or with SP2 or KLF6 siRNA. Cells were harvested, and RLA was determined. SP2 and KLF6 overexpression decreased the basal RLA of wild-type MMP-9 promoter. Conversely, siRNA silencing of SP2 and KLF6 blocked SP2 and KLF6 repression of MMP-9 promoter ($n = 3$; *, $p < 0.05$). *C*, EMSA was performed using nuclear extracts of BOECs or HepG2 cells, increasing concentrations (2, 5, and 10 μg) of SP2 or KLF6 antibody, and probes encoding Sp1 binding site of MMP-9 promoter. Decrease in protein binding to Sp1 motif was observed with increasing concentrations of antibody (*arrow*). The depicted blots are representative of an experiment that was replicated more than three times. *D*, ChIP assay was performed using HepG2 cells overexpressing pcDNA or pSP2 or pKLF6 and immunoprecipitated with SP2 and KLF6 antibody. Both SP2 and KLF6 remains bound to Sp1 motif of MMP-9 promoter. Binding was not detected when PCR was run in absence of DNA (*Negative Control*) nor when control IgG was used in place of immunoprecipitating antibody (*Control IgG Ab*). The depicted blot is representative of an experiment that was replicated three times.

that Sp1 is important for regulating MMP-9 gene transcription (33). Interestingly, the expanding family of Sp/KLF proteins regulates target genes through DNA binding to Sp1 motifs. Furthermore, intersection of FXR signaling with Sp/KLF protein gene regulation is unexplored. To examine this further, we transfected HepG2 cells with wild-type reporter luciferase construct or one with a point mutation in the Sp1 site. The Sp1 mutant construct evidenced a severalfold increase in MMP-9 promoter activity, suggesting that repressor protein binding to the Sp1 motif of the MMP-9 promoter may regulate MMP-9 gene transcription (Fig. 5*A*). Because Sp1-like proteins may function as repressors, we comprehensively examined the expression of most Sp/KLF transcription factors, including SP1-SP8 and KLF1-KLF16, by RT-PCR in BOECs and HepG2 cells. SP2, SP3, and KLF6 were constitutively expressed in BOECs, whereas SP1–SP8 and KLF2, KLF6, and KLF11 were constitutively expressed in HepG2 cells (data not shown). To identify which of these proteins may bind to the Sp1 motif and repress MMP-9 gene transcription, we overexpressed HepG2 cells with each of the Sp/KLF proteins that were constitutively expressed (SP1–SP8, KLF2, KLF6, and KLF11) and then measured wild-type MMP-9 promoter activity (supplemental Fig. S4*A*). Of these proteins, SP2 and KLF6 resulted in repression of MMP-9 promoter activity (Fig. 5*B*, Western blot analysis confirmed the overexpression of SP2 or KLF6, supplemental Fig. S4*B*). Conversely, siRNA-based silencing of SP2 and KLF6 increased MMP-9 promoter activity as compared with scrambled siRNA (Fig. 5*B*). In control experiments, SP2 siRNA and KLF6 siRNA effectively silenced the respective genes (supplemental Fig. S4, *C* and *D*). To confirm that SP2 and KLF6 could bind to the Sp1 site contained within the MMP-9 promoter, we performed an electrophoretic mobility shift assay (EMSA) using SP2 or KLF6 antibodies. Cell nuclear extracts were incubated with increasing concentrations of SP2, KLF6, or control IgG antibody and a radiolabeled Sp1 oligonucleotide motif from the MMP-9 promoter. As seen in Fig. 5*C*, with BOECs and HepG2 lysates, increasing concentration of either SP2 or KLF6 antibody led to a decrease in protein binding to the Sp1 motif of MMP-9 promoter (Fig. 5*C*, *left panel*: BOECs and *right panel*: HepG2). Finally we performed chromatin immunoprecipitation assays (ChIP) using SP2, KLF6, or control IgG antibody as outlined under "Experimental Procedures." These studies indicated that both SP2 and KLF6 are indeed bound to the Sp1 motif of the MMP-9 promoter (Fig. 5*D*). Thus, both SP2 and KLF6 bind to the Sp1 motif of the MMP-9 promoter to repress transcription.

Next, we investigated how SP2 and KLF6 function downstream of FXR and SHP to coordinate MMP-9 gene transcription. First, HepG2 cells overexpressing SP2 or KLF6 were transfected with wild-type MMP-9 promoter luciferase reporter construct and incubated with CDCA or vehicle. Incubation of cells with CDCA reversed the repression of MMP-9 promoter conferred by SP2 or KLF6 (Fig. 6*A*). Interestingly, a role for SHP as an antagonist of KLF proteins is unknown. To examine if SHP could bind SP2 or KLF6 and thereby integrate signals from FXR to the MMP-9 promoter, we performed EMSA using nuclear extracts prepared from cells that had been incubated with increasing concentrations of CDCA to up-regulate SHP. A concentration-dependent decrease in protein binding to a radiolabeled Sp1 motif from the MMP-9 promoter was observed in response to increasing concentrations of CDCA (Fig. 6*B*, similar data were obtained with HepG2 lysates, not shown). Confirmation of the role of SHP in the competition of Sp1-like protein binding to the Sp1 site of the MMP-9 promoter was then pursued in complementary ways. We performed ChIP using SP2, KLF6, or control IgG antibody with lysates from HepG2 cells incubated with either vehicle or CDCA. FXR acti-

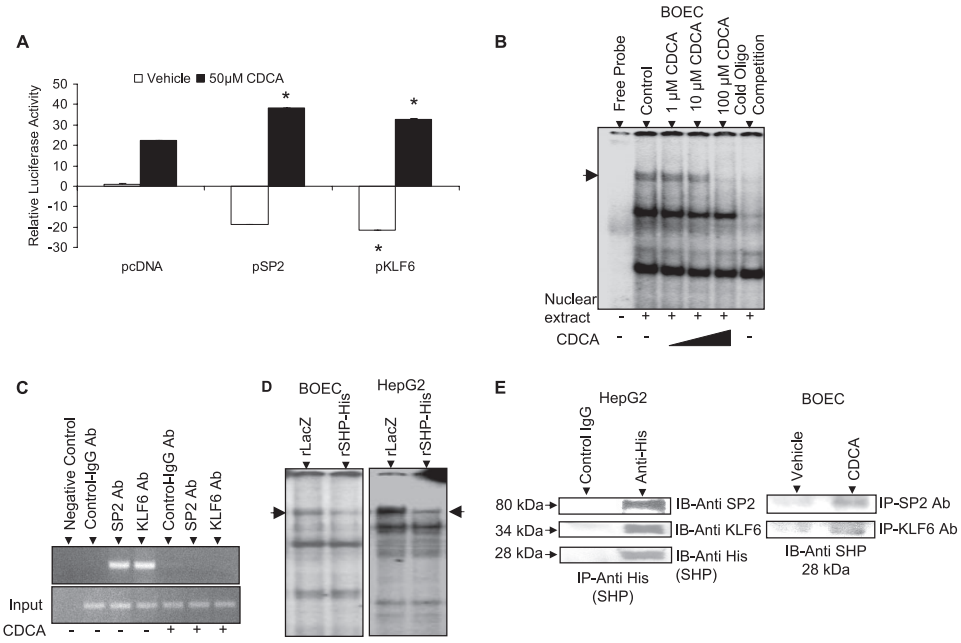

FIGURE 6. **FXR activation of SHP antagonizes SP2/KLF6 repression of MMP-9.** *A*, HepG2 cells were co-transfected with wild-type MMP-9 promoter luciferase reporter and either control pcDNA, pSP2, or pKLF6 and incubated with vehicle or 10 μM CDCA for 6 h. Promoter activity assay demonstrates that CDCA reverses the repressor effect of SP2 and KLF6 overexpression ($n = 3$; *, $p < 0.05$). *B*, EMSA was performed using nuclear extracts of BOECs incubated with various concentrations of CDCA and probes encoding Sp1 binding sites of MMP-9 promoter. A specific decrease in protein binding on the Sp1 motif of MMP-9 promoter was observed in response to increasing CDCA concentration (*arrow*). The depicted blot is representative of an experiment that was replicated more than three times. *C*, ChIP assay was performed in HepG2 cells overexpressing either pcDNA control or pSP2 or pKLF6 and incubated with vehicle or CDCA (50 μM). Samples were immunoprecipitated using agarose-conjugated antibodies to SP2 or KLF6 (Santa Cruz Biotechnology), or control (IgG) antibody. CDCA dissociated SP2 and KLF6 binding to Sp1 motif. Binding was not detected when PCR was run in absence of DNA (*Negative Control*) nor when control IgG was used in place of immunoprecipitating antibody (*Control IgG Ab*). The depicted blot is representative of an experiment that was replicated three times. *D*, EMSA was performed using BOECs or HepG2 cells transduced with rSHP-His or rlacZ. Overexpression of rSHP-His in BOECs or HepG2 competes off protein bound to Sp1 motif of MMP-9 promoter (*arrow*). The depicted blots are representative of an experiment that was replicated more than three times. *E*, protein co-immunoprecipitation was performed using nuclear extracts of HepG2 cells transduced with rSHP-His, using control IgG or Anti-His antibody. Immunoprecipitates were analyzed by SDS-PAGE and immunoblotted with SP2 or KLF6 antibody. Immunoprecipitation of His-SHP coprecipitated SP2 and KLF6 (*left panel*). In a separate set of experiments, endogenous interaction between SHP and SP2/KLF6 was studied using nuclear extracts of BOECs treated with vehicle or 50 μM CDCA. These nuclear extracts were immunoprecipitated with either SP2 or KLF6 antibody and immunoblotted with SHP antibody. SHP was co-immunoprecipitated with SP2 or KLF6 in the CDCA-treated group (*right panel*). The depicted blots are representative of an experiment that was replicated three times.

vation by CDCA dissociated bound SP2 and KLF6 from the Sp1 motif of the MMP-9 promoter (Fig. 6*C*). Additionally, cells were transduced with the retroviral constructs, rlacZ control or rSHP-His, and then prepared for EMSA using radiolabeled Sp1 oligonucleotide. Overexpression of SHP led to decreased protein binding to the Sp1 motif. Together, these results indicate that SHP competes off repressor protein binding to the Sp1 site in the MMP-9 promoter (Fig. 6*D*, *left panel*: BOECs and *right panel*: HepG2). Finally we directly examined if SHP binds to SP2 and KLF6 using protein co-immunoprecipitation assay. We used a His-tagged SHP retroviral overexpression system to allow epitope-based immunoprecipitation and detection of SHP. HepG2 cells were transduced with rSHP-His, and samples were immunoprecipitated with anti-His antibody or control IgG and immunoblotted with SP2 or KLF6 antibody. Indeed, both SP2 and KLF6 could be co-immunoprecipitated with SHP-His (anti-His antibody) (Fig. 6*E*, *left panel*), indicating that SHP can bind with these repressor proteins. Similar results were also obtained with BOECs (data not shown). To study the interaction between endogenous SHP and SP2/KLF6, in a separate set of experiments nuclear extract of BOECs treated with vehicle or CDCA were immunoprecipitated with SP2 or KLF6 antibody and immunoblotted with SHP antibody. Both SP2 and KLF6 co-immunoprecipitated with SHP in the CDCA-treated cells, thus indicating an interaction between endogenous SHP and SP2 or KLF6 (Fig. 6*E*, *right panel*). These studies support the role of sequestration of SP2 and/or KLF6 by SHP in the process of FXR activation of MMP-9 gene transcription.

DISCUSSION

Prototypical FXR target genes encode proteins relevant to bile acid biosynthesis and transport (1, 4). The present studies utilize complementary molecular approaches to provide several novel and fundamental mechanistic insights into how the FXR pathway achieves heretofore unrecognized effects on endothelial cell motility. These observations include, that 1) bile acids promote motility through FXR-mediated activation of MMP-9 gene transcription, 2) SHP mediates this process by displacing the DNA binding of Sp/KLF proteins, and 3) SP2 and KLF6 repressor function is susceptible to antagonism by FXR and SHP. This mechanism represents the intersection through which FXR signals to the MMP-9 promoter. Thus, these studies establish SP2 and KLF6 as integral co-regulatory proteins through which FXR regulates endothelial cell motility.

SHP is an atypical nuclear receptor that does not bind DNA directly. However, recent studies have shown that SHP regulates a number of target genes through heterodimerization with other nuclear receptors, through effects on heterochromatin (14, 15, 34) and competition mechanisms (35). Although most FXR target genes evidence SHP-mediated repression (13–15), an interesting feature of the present study is the stimulatory effect of SHP on MMP-9 gene transcription. Indeed, a recent microarray-based analysis of a transgenic mouse constitutively expressing SHP revealed a surprisingly large number of genes that were up-regulated by SHP as well (13). We considered two alternative mechanisms by which SHP might stimulate gene transcription in the absence of direct DNA binding. One, that SHP binds and potentiates a co-activator of MMP-9 or, alternatively, that SHP competes off a repressor of MMP-9. To dissect these possibilities we focused on the Sp1 motif of the

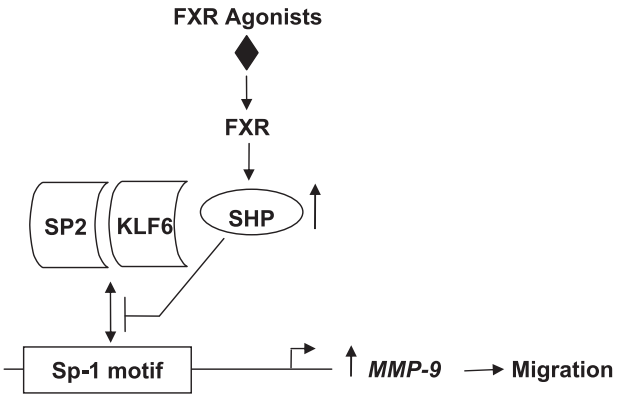

FIGURE 7. **Schematic representation of the model.** FXR activation up-regulates SHP, which, in turn dissociates an SP2/KLF6 repressor complex from the Sp1 motif of MMP-9. This de-repression increases MMP-9 expression and function resulting in increased cell motility.

MMP-9 promoter, because this site is important for gene transcription (33). Mutation of the Sp1 site of the MMP-9 promoter substantively increased promoter activity, supporting the concept that the Sp1 motif of MMP-9 might bind a repressor protein that is dissociated by SHP. Recent work from our group and others has begun to delineate an expanding role for Sp/KLF proteins (*i.e.* SP1–SP8 and KLF1–16), which bind to Sp1 motifs or GC-rich DNA sequences of target gene promoters (16–18). Although many of these proteins function as repressors, activation of gene transcription also can occur, depending upon molecular context. Therefore, we pursued a non-biased approach of overexpressing each of the family members that was constitutively expressed in our cells of interest. From these, only SP2 and KLF6 repressed MMP-9 promoter activity supporting a role for these two proteins in a repressor complex. However, upon FXR activation, this repressor complex is dissociated from the Sp1 site through binding with SHP, thereby allowing activation of MMP-9 gene transcription (Fig. 7). Thus, these studies identify integral signaling between FXR-SHP and Sp/KLF proteins.

FXR signaling is increasingly recognized to influence important biological pathways beyond the realm of bile acid homeostasis. Recent studies have begun to delineate important effects of bile acids on vascular cells through activation of FXR (26, 28). Our studies identify a role of FXR in the process of endothelial cell motility that is achieved through MMP-9. This observation may have significant biological implications, because endothelial cell motility is a fundamental step in the process of vascular remodeling and tissue repair. Indeed, recent studies have highlighted the key role of MMP-9 in the mobilization of endothelial/hematopoietic progenitor cells from the bone marrow (36) and integration of these cells into sites of vascular injury and remodeling (37). BOECs, similar to endothelial/hematopoietic progenitor cells, have been demonstrated to promote vascular remodeling in response to tissue injury, presumably owing to their highly replicative capacity (38). Because liver injury is associated with enhanced bile acid gradients, it is tempting to speculate that bile acids may provide signals for vascular remodeling and hepatic repair. Our results predict that bile concentrations may signal through FXR to regulate recruitment of blood/bone marrow-derived reparative cells to sites of liver injury. Indeed, bile acids promote liver regeneration, a process that is highly dependent on vascular remodeling (39). Furthermore, our recent studies demonstrate prominent recruitment of transplanted BOECs into liver of SCID mice that have undergone bile duct ligation, an experimental model of elevated bile acid gradients.[4] Since pharmacologic FXR agonists have been synthesized that have potency for FXR far exceeding that of natural bile acids, this direction may well be amenable to experimental therapeutics.

In summary, the present studies demonstrate a novel pathway whereby FXR-dependent up-regulation of MMP-9 occurs through SHP-dependent antagonism of SP2/KLF6 repression. The biological importance of this pathway is exemplified by its participation in the regulation of endothelial cell motility, thereby expanding both our biochemical understanding of FXR signaling and its cell biological function.

Acknowledgments—We gratefully acknowledge Benjamin Shneider (Mount Sinai Medical Center, New York, NY) and David Mangelsdorf (University of Texas Southwestern Medical Center, Dallas, TX) for providing FXR and SHP plasmid constructs, and Hiroshi Sato (Kanazawa University, Ishikawa, Japan) for providing the MMP-9 promoter luciferase reporter constructs. We also acknowledge Debabrata Mukhopadhyay (Mayo College of Medicine, Rochester, MN) and Saul Karpen (Baylor College of Medicine, Houston, TX) for helpful discussions and advice.

REFERENCES

1. Davis, R. A., Miyake, J. H., Hui, T. Y., and Spann, N. J. (2002) *J. Lipid Res.* **43,** 533–543
2. Hwang, S. T., Urizar, N. L., Moore, D. D., and Henning, S. J. (2002) *Gastroenterology* **122,** 1483–1492
3. Plass, J. R., Mol, O., Heegsma, J., Geuken, M., Faber, K. N., Jansen, P. L., and Muller, M. (2002) *Hepatology* **35,** 589–596
4. Denson, L. A., Sturm, E., Echevarria, W., Zimmerman, T. L., Makishima, M., Mangelsdorf, D. J., and Karpen, S. J. (2001) *Gastroenterology* **121,** 140–147
5. Urizar, N. L., Dowhan, D. H., and Moore, D. D. (2000) *J. Biol. Chem.* **275,** 39313–39317
6. Pineda Torra, I., Claudel, T., Duval, C., Kosykh, V., Fruchart, J. C., and Staels, B. (2003) *Mol Endocrinol* **17,** 259–272
7. Sirvent, A., Claudel, T., Martin, G., Brozek, J., Kosykh, V., Darteil, R., Hum, D. W., Fruchart, J. C., and Staels, B. (2004) *FEBS Lett.* **566,** 173–177
8. Claudel, T., Sturm, E., Duez, H., Torra, I. P., Sirvent, A., Kosykh, V., Fruchart, J. C., Dallongeville, J., Hum, D. W., Kuipers, F., and Staels, B. (2002) *J. Clin. Invest.* **109,** 961–971
9. Kast, H. R., Nguyen, C. M., Sinal, C. J., Jones, S. A., Laffitte, B. A., Reue, K., Gonzalez, F. J., Willson, T. M., and Edwards, P. A. (2001) *Mol Endocrinol* **15,** 1720–1728
10. Forman, B. M., Goode, E., Chen, J., Oro, A. E., Bradley, D. J., Perlmann, T., Noonan, D. J., Burka, L. T., McMorris, T., Lamph, W. W., Evans, R. M., and Weinberger, C. (1995) *Cell* **81,** 687–693
11. Zavacki, A. M., Lehmann, J. M., Seol, W., Willson, T. M., Kliewer, S. A., and Moore, D. D. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 7909–7914
12. Zhao, A., Lew, J. L., Huang, L., Yu, J., Zhang, T., Hrywna, Y., Thompson, J. R., de Pedro, N., Blevins, R. A., Pelaez, F., Wright, S. D., and Cui, J. (2003) *J. Biol. Chem.* **278,** 28765–28770
13. Boulias, K., Katrakili, N., Bamberg, K., Underhill, P., Greenfield, A., and Talianidis, I. (2005) *EMBO J.* **24,** 2624–2633
14. Kemper, J. K., Kim, H., Miao, J., Bhalla, S., and Bae, Y. (2004) *Mol. Cell. Biol.*

[4] D. A. Langer and V. H. Shah, unpublished studies.

24, 7707–7719
15. Gobinet, J., Carascossa, S., Cavailles, V., Vignon, F., Nicolas, J. C., and Jalaguier, S. (2005) *Biochemistry* **44,** 6312–6320
16. Cao, S., Fernandez-Zapico, M. E., Jin, D., Puri, V., Cook, T. A., Lerman, L. O., Zhu, X. Y., Urrutia, R., and Shah, V. (2005) *J. Biol. Chem.* **280,** 1901–1910
17. Nandan, M. O., Yoon, H. S., Zhao, W., Ouko, L. A., Chanchevalap, S., and Yang, V. W. (2004) *Oncogene* **23,** 3404–3413
18. Black, A. R., Black, J. D., and Azizkhan-Clifford, J. (2001) *J. Cell. Physiol.* **188,** 143–160
19. Slavin, D. A., Koritschoner, N. P., Prieto, C. C., Lopez-Diaz, F. J., Chatton, B., and Bocco, J. L. (2004) *Oncogene* **23,** 8196–8205
20. Fernandez-Zapico, M. E., Mladek, A., Ellenrieder, V., Folch-Puy, E., Miller, L., and Urrutia, R. (2003) *EMBO J.* **22,** 4748–4758
21. Makishima, M., Okamoto, A. Y., Repa, J. J., Tu, H., Learned, R. M., Luk, A., Hull, M. V., Lustig, K. D., Mangelsdorf, D. J., and Shan, B. (1999) *Science* **284,** 1362–1365
22. Parks, D. J., Blanchard, S. G., Bledsoe, R. K., Chandra, G., Consler, T. G., Kliewer, S. A., Stimmel, J. B., Willson, T. M., Zavacki, A. M., Moore, D. D., and Lehmann, J. M. (1999) *Science* **284,** 1365–1368
23. Pellicciari, R., Fiorucci, S., Camaioni, E., Clerici, C., Costantino, G., Maloney, P. R., Morelli, A., Parks, D. J., and Willson, T. M. (2002) *J. Med. Chem.* **45,** 3569–3572
24. Lu, T. T., Repa, J. J., and Mangelsdorf, D. J. (2001) *J. Biol. Chem.* **276,** 37735–37738
25. Inagaki, T., Moschetta, A., Lee, Y. K., Peng, L., Zhao, G., Downes, M., Yu, R. T., Shelton, J. M., Richardson, J. A., Repa, J. J., Mangelsdorf, D. J., and Kliewer, S. A. (2006) *Proc. Natl. Acad. Sci. U. S. A.* **103,** 3920–3925
26. Bishop-Bailey, D., Walsh, D. T., and Warner, T. D. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101,** 3668–3673
27. Fiorucci, S., Antonelli, E., Rizzo, G., Renga, B., Mencarelli, A., Riccardi, L., Orlandi, S., Pellicciari, R., and Morelli, A. (2004) *Gastroenterology* **127,** 1497–1512
28. He, F., Li, J., Mu, Y., Kuruba, R., Ma, Z., Wilson, A., Alber, S., Jiang, Y., Stevens, T., Watkins, S., Pitt, B., Xie, W., and Li, S. (2006) *Circ. Res.* **98,** 192–199
29. Lin, Y., Weisdorf, D. J., Solovey, A., and Hebbel, R. P. (2000) *J. Clin. Invest.* **105,** 71–77
30. Zeng, H., Sanyal, S., and Mukhopadhyay, D. (2001) *J. Biol. Chem.* **276,** 32714–32719
31. Huhtala, P., Tuuttila, A., Chow, L. T., Lohi, J., Keski-Oja, J., and Tryggvason, K. (1991) *J. Biol. Chem.* **266,** 16485–16490
32. Lee, J. S., Kang Decker, N., Chatterjee, S., Yao, J., Friedman, S., and Shah, V. (2005) *Am. J. Pathol.* **166,** 1861–1870
33. Sato, H., and Seiki, M. (1993) *Oncogene* **8,** 395–405
34. Boulias, K., and Talianidis, I. (2004) *Nucleic Acids Res.* **32,** 6096–6103
35. Fiorucci, S., Rizzo, G., Antonelli, E., Renga, B., Mencarelli, A., Riccardi, L., Orlandi, S., Pruzanski, M., Morelli, A., and Pellicciari, R. (2005) *J. Pharmacol. Exp. Ther.* **314,** 584–595
36. Heissig, B., Hattori, K., Dias, S., Friedrich, M., Ferris, B., Hackett, N. R., Crystal, R. G., Besmer, P., Lyden, D., Moore, M. A., Werb, Z., and Rafii, S. (2002) *Cell* **109,** 625–637
37. Kollet, O., Shivtiel, S., Chen, Y. Q., Suriawinata, J., Thung, S. N., Dabeva, M. D., Kahn, J., Spiegel, A., Dar, A., Samira, S., Goichberg, P., Kalinkovich, A., Arenzana-Seisdedos, F., Nagler, A., Hardan, I., Revel, M., Shafritz, D. A., and Lapidot, T. (2003) *J. Clin. Invest.* **112,** 160–169
38. Gulati, R., Jevremovic, D., Witt, T. A., Kleppe, L. S., Vile, R. G., Lerman, A., and Simari, R. D. (2004) *Am. J. Physiol. Heart Circ. Physiol.* **287,** H512–H517
39. Huang, W., Ma, K., Zhang, J., Qatanani, M., Cuvillier, J., Liu, J., Dong, B., Huang, X., and Moore, D. D. (2006) *Science* **312,** 233–236

Disruption of an SP2/KLF6 Repression Complex by SHP Is Required for Farnesoid X Receptor-induced Endothelial Cell Migration

Amitava Das, Martin E. Fernandez-Zapico, Sheng Cao, Janet Yao, Stefano Fiorucci, Robert P. Hebbel, Raul Urrutia and Vijay H. Shah

J. Biol. Chem. 2006, 281:39105-39113.
doi: 10.1074/jbc.M607720200 originally published online October 27, 2006
