Diurnal Regulation of the Early Growth Response 1 (Egr-1) Protein Expression by Hepatocyte Nuclear Factor 4α (HNF4α) and Small Heterodimer Partner (SHP) Cross-talk in Liver Fibrosis*

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Early growth response 1 (Egr-1) protein is a critical regulator of genes contributing to liver fibrosis; however, little is known about the upstream transcriptional factors that control its expression. Here we show that Egr-1 expression is tightly regulated by nuclear receptor signaling. Hepatocyte nuclear factor 4α (HNF4α) activated the Egr-1 promoter through three DR1 response elements as identified by trans-activation assays. Deletion of these response elements or knockdown of HNF4α using siRNA largely abrogated Egr-1 promoter activation. HNF4α activity, as well as its enrichment on the Egr-1 promoter, were markedly repressed by small heterodimer partner (SHP) co-expression. Egr-1 mRNA and protein were transiently induced by HNF4α. On the contrary, HNF4α siRNA reduced Egr-1 expression at both the mRNA and protein levels, and overexpression of SHP reversed these effects. Conversely, knockdown of SHP by siRNA elevated Egr-1 protein. Interestingly, Egr-1 mRNA exhibited diurnal fluctuation, which was synchronized to the cyclic expression of SHP and HNF4α after cells were released from serum shock. Unexpectedly, the levels of Egr-1 mRNA and protein were highly up-regulated in Hnf4a−/− mice. Both HNF4α and Egr-1 expression were dramatically increased in SHP−/− mice with bile duct ligation and in human cirrhotic livers, which was inversely correlated with diminished SHP expression. In conclusion, our study revealed control network for Egr-1 expression through a feedback loop between SHP and HNF4α.

Hepatic fibrosis or cirrhosis is a scarring process characterized by both increased deposition of extracellular matrix proteins and reduced breakdown of extracellular matrix after liver injury (1). The early growth response 1 (Egr-1) gene, a zinc finger transcription factor, was shown to play roles in multiple pathways and processes, including proliferation, differentiation, and inflammation during cholestatic liver injury (2–5). In addition to its critical role in mediating inflammatory responses, Egr-1 is also involved in regulating the expression of genes contributing to liver fibrosis, such as transforming growth factor β1 (TGF-β), platelet-derived growth factor (PDGF), and fibroblast growth factor (FGF) (6). Recent studies have shown that TGF-β induces a rapid but transient expression of Egr-1 that results in stimulation of collagen gene expression (7). Insulin-like growth factor (IGF)-binding protein 5 induces a fibrotic phenotype via the activation of MAPK signaling and the induction of Egr-1, resulting in activation of genes involved in fibrogenesis (8). These studies suggest that Egr-1 may play an important role in the pathogenesis of fibrosis.

Nuclear receptor small heterodimer partner (SHP, NROB2) is a unique member of the nuclear receptor superfamily that contains the dimerization and ligand binding domain, but lacks the conserved DNA binding domain. Numerous studies have shown that SHP is a transcriptional repressor of a number of genes critical to hepatic bile acid, cholesterol, triglyceride, glucose, and drug metabolism (9–15). Recent studies demonstrated that SHP has a preventative role in liver fibrosis (10). Activation of hepatic stellate cells, the major source of extracellular matrix in the liver, plays an important role in the development of cirrhosis through a progressive trans-differentiation from a resting phenotype toward a myofibroblast-like phenotype. Exposure of hepatic stellate cells to farnesoid X receptor (FXR) ligands causes a 3-fold increase of SHP and reduces α1 collagen and TGF-β, indicating a protective effect of SHP in liver fibrosis (16). In addition, SHP−/− mice showed increased sensitivity to liver damage induced by bile duct ligation (BDL) (17). These studies provide evidence that SHP may play a role in protecting against liver fibrosis. However, the target genes of SHP involved in regulating fibrosis remain to be determined.

In this study, we show that Egr-1 is a direct target of SHP. SHP represses Egr-1 gene transcription via cross-talk with hepatocyte nuclear factor 4α (HNF4α). HNF4α binds to and acti-
vates the Egr-1 promoter, inducing Egr-1 mRNA and protein. This transcriptional induction is inhibited by SHP. We further show that Egr-1 is strongly up-regulated in human cirrhotic livers, which is associated with the up-regulation of HNF4α and down-regulation of SHP. This study reveals a novel pathway by which SHP and HNF4α regulate liver fibrosis through targeting Egr-1.

EXPERIMENTAL PROCEDURES

Cell Lines, Animals, and Human Liver Specimens—Human cervix adenocarcinoma cells (HeLa, ATCC CCL-2), human hepatoma cells (HuH7, Health Science Research Resources Bank JCRB0403; HepG2, ATCC HB-8065), and mouse hepatoma cell line Hepa1 (ATCC CRL-1830) were maintained in Dulbecco’s modified Eagle’s medium with 100 units of penicillin-G-streptomycin sulfate/ml and 10% heat-inactivated FBS. Human stellate LX2 cells (a gift from Dr. Scott Friedman) were maintained in 2% FBS. The SHP+/+ (WT) and SHP−/− mice have been described previously (15, 18). Livers were obtained from 6-week-old male liver-specific Alb-Hnf4α+/− (Alb-WT) or Alb-Hnf4α+/− (knock-out) mice. An acute, conditional liver-specific Hnf4a F/F knock-out was obtained by treating Hnf4a F/F Alb-Hnf4a F/F (knock-out) mice with a purified diet containing 0.1% tamoxifen citrate for 5 days, removing the diet, and killing the mice 2 weeks later. Protocols for animal use were approved by the Institutional Animal Care and use Committee at the University of Utah. Livers from five normal livers and eight cirrhotic specimens were obtained through the Liver Tissue Cell Distribution System (Minneapolis, MN).

Plasmids, siRNAs, and Antibodies—The mouse Egr-1 (Gene ID 13653) promoter luciferase reporter (Egr-1 Luc) and its deletion mutation constructs were engineered in our laboratory. Each DNA fragment of the mouse Egr-1 promoter was inserted into the KpnI and Xhol sites of pGL3-basic (Promega). The integrity of the recombinant plasmids was verified by sequence analysis. Expression plasmids for FXR, RXR, RAR, HA-HNF4α, HNF4α S78D, HNF4α S304D, and FLAG-SHP were described previously (19–21). The following antibodies were used for co-immunoprecipitation (Co-IP), chromatin IP (ChIP), and Western blotting (WB): M-200 sheep anti-rabbit or mouse IgG Dynabeads (Invitrogen Dynal As), rabbit normal IgG (Sigma, H-6908), Egr-1 (Cell Signaling, 4153), HNF4α (Cell Signaling, 3113), HNF4α (Santa Cruz Biotechnology, sc-6556), β-actin (Sigma, A-1978), tubulin (Sigma, T-6199), and SHP (PPMX, L:-76571). HNF4α (SASI_Hs01_00124507) and non-specific (SIC001) siRNAs were purchased from Sigma. SHP siRNA (ON-TARGETplus SMARTpool NR082, L-003410) was purchased from Thermo Scientific Dharmacon RNAi Technologies.

Transient Transfection and Promoter Activity Assays—For transient transfection assays, HeLa, HuH7, or LX2 cells were co-transfected with the mouse Egr-1 Luc reporter, pcDNA3-HA-HNF4α, or pcDNA3-FLAG-SHP as indicated in the figure legends. Empty vector DNA was added as needed so that the same amounts of expression vector DNA were present in each transfection. Transfection was carried out using Lipofectamine 2000 (Invitrogen) in 24-well plates. Thirty-six hours after transfection, cells were collected, and luciferase activities were measured and normalized against Renilla activities (Promega). Consistent results were observed in three independent triplicate transfection assays in each experiment.

ChIP Assays—ChIP assays were carried out essentially as described previously (20). Briefly, HeLa cells or HuH7 cells were incubated with 1% formaldehyde at 25°C for 10 min. Nuclei were isolated and sonicated to shear the DNA into 0.3–1.0 kb. Chromatin was precleared in the presence of 20 μl of normal serum and 30 μl of M-200 sheep anti-rabbit or mouse IgG Dynabeads. Precleared chromatin samples were subjected to immunoprecipitation at 4°C overnight in the presence of 2 μg of antibodies against FLAG, HA, HNF4α, or rabbit normal IgG. After the complex was collected by incubation in 30 μl of sheep anti-rabbit or mouse IgG Dynabeads and centrifugation, the beads were washed five times, and the chromatin immune complex was eluted. Then the cross-links were reversed, and the DNA was purified and used as a template in PCR. Real-time PCR and PCR were performed using ChIP primer sets specific for the HNF4α binding sites in mouse Egr1 (mEgr-1) and human EGR1 (hEGR-1) promoters: mouse p1 forward, 5′-TGCCCACACTTGTGGATG-3′ and reverse, 5′-CAAGGGTC-TGGAAACAGCAGG-3′; mouse p2 forward, 5′-AAAGTGGGATCTCTCAAGGG-3′ and reverse, 5′-GCAGGGTCATTGCTTCTAGG-3′; mouse p3 forward, 5′-TAAAGGGTGCTCCTGACTG-3′ and reverse, 5′-AGGCTATTCTCCTCGTCCTG-3′; human p1 forward, 5′-GGTGCTCTGCAATAGGGC-3′ and reverse, 5′-CCCGGACTCCCTTATTTT-3′; human p2 forward, 5′-AGTGGCGGCTGATCTTTCC-3′ and reverse, 5′-TATCGCTGTCTTCAGGCC-3′; human p3 forward, 5′-CCACCTGGACTGTAAAGGG-3′ and reverse, 5′-CGAAGGTGTCTCTAGTTCC-3′; human p4 forward, 5′-ATGACACTGGAAGGG-3′ and reverse, 5′-AACATCATCCTGTTTC-3′ and reverse, 5′-AGAGGAAAGGAGGAGGTG-3′ serve as negative controls.

Real-time Quantitative PCR (qPCR) Analysis—Quantitative PCR was performed as described previously (18, 20, 21). In brief, total RNA was isolated using TRIzol, cDNA was synthesized, and real-time PCR was carried out using the SYBR Green PCR master mix (Applied Biosystems). The melting curve data were collected to check PCR specificity. Each cDNA sample was run in triplicate, and the corresponding no-reverse transcription (RT) mRNA sample was included as a negative control. The amount of PCR products was measured by threshold cycle (Ct) values, and the relative ratio of specific genes to HPRT1 for each sample was then calculated. The sequences for the primers are as follows: forward primer, 5′-CGTCTGTAGTTAGGTAGTG-3′ and reverse primer, 5′-CACACAGAGGGGCCAC-3′; mouse Hprt1; forward primer, 5′-TGACACTGGCAAAACATGCA-3′ and reverse primer, 5′-GGTCCTTTCACCACAGCAGCT-3′ for human HPRT1; forward primer, 5′-GACAGGTTATCCAGGCTACCA-3′ and reverse primer, 5′-GCGAGGAAAGGAGGTAGAG-3′ for mouse Egr-1; forward primer, 5′-AGACCTTGACCCGAGGTG-3′ and
reverse primer, 5′-AGATGGTGCTAGGACGAGG-3′ for human EGR-1; forward primer, 5′-CACCCTGCATCTGAGCAGCAGC-3′ and reverse primer, 5′-GCGAACCCCAAGGAGGAGAAG-3′ for mouse SHP; forward primer, 5′-GGTGCCACGCTATCTCAAGA-3′ and reverse primer, 5′-GGACTTCCAAGCAGCACCAGT-3′ for human SHP; forward primer 5′-TGGGCAAGATTGACACCTG-3′ and reverse primer, 5′-AGGTTGAGGGGATCGTGGT-3′ for mouse HNF4α; forward primer, 5′-CAGGAGTCTAGATCAGTGATGA-3′ and reverse primer, 5′-CAGCACCCAGT-3′ for human SHP; and forward primer, 5′-GCTGCAACTGCTTCGAGGAGGT-3′ and reverse primer, 5′-GATGTCTCTCCTGGCATGAGTC-3′ for mouse E2F1.

**Co-IP Assays**—Co-IP was performed as described previously with minor modifications (18). Extracts from Huh7 or HepG2 cells were incubated in lysis buffer (20 mM KOH-HEPES, pH 8.0, 0.2 mM EDTA, 5% glycerol, 250 mM NaCl, 0.5% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM dithiothreitol, and protease inhibitors) with antibody against HNF4α or rabbit immunoglobulin G (IgG) at 4 °C for 4 h to overnight, and the immune complex was collected by incubation with 30 μl of M-280 sheep anti-rabbit IgG Dynabeads for 4 h. Immunoprecipitates were washed four times with lysis buffer supplemented with NaCl to 400 mM and subjected to Western blotting.

**Western Blotting in Cells and Liver Tissues**—Hepa1 or Huh7 cells were pelleted by centrifugation and resuspended in lysis buffer (50 mM Tris, pH 8.0, 1% Nonidet P-40, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS) with protease inhibitors (Thermo Fisher Scientific, 78410). After incubation on ice for 10 min followed by sonication and centrifugation, the protein concentration of cell lysates was determined using the DC protein assay (Bio-Rad, 500-0112). Cell lysates (30 μg) were resolved by SDS-PAGE and transferred to nitrocellulose membranes according to standard procedures. Membranes were washed in Tris-buffered saline containing 0.05% Tween 20 (TBST), blocked for 1 h with TBST containing 5% nonfat milk, and then incubated with primary antibodies at a 1:1,000 dilution in TBST containing 5% nonfat milk overnight at 4 °C. Membranes were then washed with TBST before incubation with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Membranes were washed four times with TBST before antibody binding was visualized with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific 34080) according to the manufacturer’s protocol. For Western blotting using mouse liver, 100 μg of liver fragments from three to four individual 6-week-old Alb-HNF4α−/−, ErT2-HNF4α−/− mice or their respective controls were homogenized in lysis buffer and subjected to Western blotting as described above. For Western blotting using human liver specimens, five normal donor livers and eight cirrhotic livers were homogenized and subjected to Western blotting. Equal loading of protein was verified with β-actin or tubulin, respectively.

**BDL**—The experiments were performed at the University of Kansas Medical Center, and the studies were approved by the Institutional Animal Care and Use Committee of KUMC. Mice were maintained on standard laboratory chow on a 12-h light/
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FIGURE 2. Attenuation of the Egr-1 promoter activity by deletion of HNF4α response elements. A, diagram showing the location of three putative HNF4α binding sites in the Egr-1 promoter and its deletion mutation constructs, B and C, mutagenesis analysis. Transient transfection assays of mEgr-1 promoter luciferase reporter activity by HNF4α (200 ng) in HeLa (B) and Huh7 (C) cells are shown. The experiments were done as in Fig. 1. *, p < 0.01 versus control pcDNA group; †, p < 0.01 versus HNF4α activation of Egr-1 Luc-1731. D, mutagenesis analysis of mEgr-1 Luc-787 mutation constructs. Top, transient transfection assays of mEgr-1 Luc-787 activity by HNF4α (200 ng) and SHP (200 ng) in HeLa cells. *, p < 0.01 versus control pcDNA group; †, p < 0.01 versus HNF4α activation of Egr-1 Luc-787. Bottom, HNF4α site 1 was deleted (del-12), partially deleted (del-5), mutated (mut-1), or mutated to a conserved DR1 site (DR1), the latter was used as a positive control. Underlining, mutated nucleotides.

Statistical Analysis—Data are expressed as mean ± S.E. Statistical analyses were carried out using Student’s unpaired t test; p < 0.01 was considered statistically significant.

RESULTS

SHP Inhibits HNF4α Trans-activation of the Egr-1 Promoter—We searched nuclear receptor binding motifs and identified potential binding sites for FXR, RAR, and HNF4α within the mouse Egr-1 gene 5′-flanking region. To determine the role of these nuclear receptors in regulating Egr-1 promoter activity, HeLa cells were co-transfected with the Egr-1 promoter luciferase reporter (Egr-1 Luc) and the expression plasmids for FXR/RXR, RAR/RXR, and HNF4α at the indicated concentrations (Fig. 1A). Compared with pcDNA controls (−), overexpression of FXR/RXR and RAR/RXR did not affect Egr-1 promoter activity. In contrast, HNF4α significantly increased luciferase activity of the Egr-1 Luc reporter in a dose-dependent manner. SHP was previously shown to interact with and inhibit HNF4α trans-activation (21). As expected, co-expression of SHP abrogated the activity of HNF4α. Similar results were observed in Huh7 cells (Fig. 1B). On the other hand, S78D and S304D, the two HNF4α DNA binding domain mutants, failed to activate Egr-1 Luc reporter. The cross-linked chromatin was immunoprecipitated using anti-HA or anti-FLAG antibodies. The enriched DNA was quantified by real-time qPCR using EGR1 promoter primers hP1, hP2, and hP3. The amount of immunoprecipitated DNA in each sample is represented as signal relative to the total amount of input chromatin, expressed as Relative (rel) enrichment. Error bars represent S.E. from three independent measurements (*, p < 0.01 versus negative control siRNA).
Because Egr-1 is predominantly localized in hepatocytes, subsequent studies focused on using hepatocyte derived Hepa1 and Huh7 cells.

**Deletion of HNF4α Response Elements Attenuated Egr-1 Promoter Activity**—Three putative HNF4α DR1 binding sites were located in the Egr-1 promoter (Fig. 2A). We generated three mutant Egr-1 LuC constructs (Luc-934, Luc-787, and Luc-721). Deletion of HNF4α site 3 did not decrease the Luc-934 activity in HeLa cells, but markedly decreased it in Huh7 cells (Fig. 2, B versus C). Double deletion of site 3 and site 2 or triple deletion of all three sites attenuated Luc-787 or Luc-721 promoter activation by HNF4α in both HeLa and Huh7 cells. Although the mutant promoters showed somewhat different activities in HeLa and Huh7 cells, the data strongly suggest that all three sites bind HNF4α.

To confirm further that site 1 is important for HNF4α binding, we generated several site1 mutation constructs based on Egr-1 Luc-787. The Egr-1 Luc-787 promoter activity was abolished by deletion of site1 (del-12) (Fig. 2D). Deletion half of site 1 (del-5) or mutation of several nucleotides within the site 1 (mut-1) significantly decreased Egr-1 Luc-787 activity. We also mutated site 1 to a conserved DRI, which resulted in an increased Egr-1 Luc-787 activity by HNF4α compared with the native Egr-1 Luc-787.

**SHP Decreases HNF4α Recruitment to the Egr-1 Promoter**—The proximal Egr-1 promoter contains three putative HNF4α-binding elements (Fig. 3A). ChIP assays were used to determine binding of HNF4α and SHP to the Egr-1 promoter using primer sets mp1, mp2, and mp3 covering each HNF4α binding site, respectively. The mp4 primers were located upstream of the mouse Egr-1 promoter region which contains no HNF4α binding site and served as a negative control. Recruitment of HNF4α to all three sites was observed. Co-expression of SHP markedly attenuated HNF4α recruitment to all sites (Fig. 3B). In contrast, mp4 yielded no PCR products confirming specificity of the assay. SHP was shown to recruit mSin3A-Swi/Snf co-repressor complex to its target promoter (24). It is postulated that a similar co-repressor complex may be recruited to the Egr-1 promoter by SHP to compete for HNF4α binding.

Three potential HNF4α response elements were also predicted in the human EGR-1 promoter (Fig. 3C, left). HNF4α siRNA was used to knockdown endogenous HNF4α in Huh7 cells, and anti-HNF4α antibodies were used to immunoprecipitate HNF4α for ChIP analysis. Consistent with the results...
observed with the mouse Egr-1 promoter, HNF4α was recruited to all sites in the EGR-1 promoter, and its association with site 3, particularly site 2, but not site 1, was dramatically reduced by HNF4α siRNA (Fig. 3C, right). Taken together, the results suggest that both the mouse Egr-1 and human EGR-1 genes may be regulated by HNF4α in a similar fashion, i.e. by direct binding of HNF4α to its response elements in the promoters.

The Induction of EGR-1 Expression by HNF4α Is Repressed by SHP—To determine further the regulation of Egr-1 by HNF4α, we overexpressed HNF4α in mouse hepatoma Hepa1 cells which had no detectable levels of HNF4α (data not shown) and performed qPCR analysis of Egr-1 mRNA. Interestingly, the endogenous Egr-1 mRNA was transiently induced by HNF4α at 8 h after transfection, which reached maximal induction by 24 h and declined to basal levels by 32 h (Fig. 4A, left). The Egr-1 protein showed parallel induction by HNF4α with time, although its increase prolonged to 32 h (Fig. 4A, right). Similarly, a time-dependent reduction of HNF4α protein was observed by 32 h. The stimulation of endogenous human EGR-1 protein by HNF4α was observed in Huh7 cells as well (Fig. 4B). At 24 h after knockdown of HNF4α by siRNA (Fig. 4C, left), EGR-1 mRNA (Fig. 4C, right) and protein (Fig. 4D, left) decreased in Huh7 and HepG2 cells (data not shown). SHP protein was also subsequently decreased by HNF4α siRNA, supporting SHP as a target of HNF4α (25, 26). In addition, knockdown of SHP using siRNA elevated EGR-1 protein but did not alter HNF4α protein levels (Fig. 4D, right), suggesting that SHP inhibition of EGR-1 is most likely through repressing HNF4α transactivation of the Egr-1 promoter (Figs. 1–3), but not by regulating HNF4α expression.

In agreement with the above observations, the induction of EGR-1 protein by HNF4α was antagonized by SHP overexpression (Fig. 4E, fourth versus second lane). The relative moderate increase in EGR-1 protein by HNF4α may be attributed to lower amounts of exogenously expressed HNF4α in the cells (Fig. 4, E versus B). Because HNF4α was able to up-regulate SHP protein (data not shown), the increased SHP may also counteract the effect of HNF4α by masking the activation of EGR-1 by HNF4α.

During this study, we noticed that there appeared to be a discoordination with regard to the time HNF4α and SHP exhibited their maximal efficacy. For instance, at 12 h after transfection, HNF4α induced EGR-1 mRNA, whereas SHP did not repress basal EGR-1 expression (Fig. 4F). By 24 h, the ability of HNF4α to elevate EGR-1 declined, whereas SHP exhibited a stronger ability to reduce basal EGR-1 levels (Fig. 4G). Thus, the highest activation of EGR-1 by HNF4α seemed to occur earlier, whereas the strongest ability of SHP inhibition of EGR-1 occurred later. In addition, the basal endogenous EGR-1 mRNA dropped about 4-fold at 24 h compared with 12 h (Fig. 4, F versus G, first lane). Because the expression of SHP shows a diurnal cycle in the liver (12), we examined EGR-1 expression in Huh7 cells that were briefly exposed to 50% horse serum, a condition that was used to induce cyclic SHP expression (12). SHP mRNA showed a small but nonsignificant increase 4 h after the cells were released from serum shock (Fig. 4H).

Intriguingly, SHP, HNF4α, and EGR-1 mRNAs showed similar diurnal cycles and reached their highest levels at the same time point (32 h). A noticeable difference was that the EGR-1 level dropped sharply at 36 h after its peak expression and reached minimal by 44 h, whereas SHP and HNF4α levels remained high after 32 h but decreased gradually with time. Based on this result, we propose that the up-regulation of EGR-1 at 32 h is mainly contributed by HNF4α activation, whereas its down-regulation after 32 h is more controlled by SHP inhibition.
Therefore, the EGR-1 gene displays a diurnal response to serum shock involving an early induction by HNF4α followed by a subsequent repression of SHP.

The Expression of Egr-1 Is Paradoxically Increased in HNF4α−/− Mice—Thus far, our in vitro studies identified HNF4α and SHP cross-talk in regulating Egr-1 expression. To test whether this regulatory mechanism exists in vivo, we performed qPCR and immunoblotting of Egr-1 mRNA and protein in liver-specific Alb-Hnf4α−/− mice. Unexpectedly, both Egr-1 mRNA and protein were markedly increased in livers of Alb-Hnf4α−/− mice (Fig. 5A). Similar results were obtained using the acute conditionally deleted ErT2-Alb-Hnf4α−/− mice (Fig. 5B). Another interesting observation was that SHP mRNA was markedly diminished in Alb-Hnf4α−/− (Fig. 5C, left), but not in ErT2-Alb-Hnf4α−/− mice (Fig. 5C, right). The decreased SHP may derepress Egr-1 and mask the lost activation of Egr-1 by HNF4α in Alb-Hnf4α−/− mice. The transcription factor E2F-1 is able to activate Egr-1 expression.4 E2F-1 mRNA was dramatically increased in ErT2-Alb-Hnf4α−/− mice (Fig. 5D, right), which may in part contribute to the high level of Egr-1. The E2F-1 target genes, including Cyclin E and Cdk1 (27, 28), were up-regulated in ErT2-Alb-Hnf4α−/− mice compared with the ErT2-WT mice, which was in agreement with the increased E2F-1 expression (Fig. 5E).

Hnf4α−/− Mice Exhibit Hepatocyte Hypertrophy—Histology of liver sections was performed in Alb-Hnf4α−/− and ErT2-Alb-Hnf4α−/− mice. H&E staining revealed hepatocyte hypertrophy in both knockouts (Fig. 6A), which was more severe in the Alb-Hnf4α−/− mice. Neutral lipid accumulation appeared to be lower in Alb-Hnf4α−/− mice relative to ErT2-Alb-Hnf4α−/− mice, as examined by Oil red O staining (Fig. 6B). SHP deficiency resulted in diminished fatty liver (9, 14). The decreased SHP mRNA in Alb-Hnf4α−/− liver (Fig. 5C) may in part protect the mice from developing steatosis. Sirius Red staining did not identify liver fibrosis in either Hnf4α knock-out mouse model (Fig. 6C). A stress condition is likely required to assess the development of liver fibrosis in both mouse models. Increased hepatocyte proliferation was observed in ErT2-Alb-Hnf4α−/− mice,5 which may be associated with the up-regulation of E2F-1 (Fig. 5D, left).

The Expression of HNF4α and EGR-1 Is Inversely Correlated with SHP Expression in Human Cirrhotic Livers—We next determined whether HNF4α was elevated under a cholestatic condition that ultimately leads to fibrosis and cirrhosis. BDL was performed in SHP−/− mice to induce cholestasis. HNF4α mRNA was induced in WT mice with BDL, but to a much greater extent in SHP−/− mice without or with BDL (Fig. 7A). Consistent with the mouse models, up-regulation of HNF4α was also observed in human cirrhotic livers (Fig. 7B). Egr-1 protein expression was markedly high in SHP−/− livers (Fig. 7C) and in human cirrhotic livers (Fig. 7D), with a concomitant reduction of SHP expression (Fig. 7D). Taken together, these results establish an inverse correlation between HNF4α/EGR-1 and SHP expression under cholestatic/cirrhotic conditions.

4 Y. Zhang and L. Wang, unpublished data.
5 J. A. Bonzo and F. J. Gonzalez, unpublished data.
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DISCUSSION

Accumulating evidence suggests a critical role for Egr-1 in fibrosis. Elevated Egr-1 protein and/or mRNA has been widely detected in fibrotic tissues, including ureteral obstruction fibrotic kidneys in rats, lung tissues from patients with emphysema, and skin fibroblasts from patients with diffuse scleroderma (29–31). In addition to directly stimulating α-smooth muscle actin expression and myofibroblast trans-differentiation, Egr-1 also controls the expression of extracellular matrix genes contributing to fibrogenesis (29). Targeting the Egr-1 gene with specific DNA enzyme in a ureteral obstruction rat model blocked Egr-1 expression with a concomitant reduction in TGF-β, α-smooth muscle actin, and type I collagen mRNA expression, consequently inhibited interstitial fibrosis (29). Further studies showed that fibrosis of the lungs induced by TGF-β or interleukin-13 was markedly attenuated in Egr-1−/− mice (32, 33). Despite the importance of Egr-1 in fibrosis, the mechanisms responsible for elevated Egr-1 expression in fibrotic tissues remain largely unknown.

Here we elucidate a transcriptional mechanism that controls Egr-1 expression through nuclear receptor-mediated signaling. Using a series of in vitro and in vivo approaches, we demonstrate that SHP directly represses Egr-1 promoter activity and gene transcription through HNF4α. Although liver fibrosis-associated Egr-1 induction results from elevated bile acids (34), the underlying mechanism is unclear. SHP plays a critical role in the negative feedback regulation of bile acid synthesis (11), and bile acid levels are elevated in SHP−/− mice due to loss of SHP inhibition of Cyp7a1 and Cyp8b expression (15, 35). SHP expression is decreased, whereas HNF4α is increased in cholestatic and cirrhotic livers. Therefore, the mechanism contributing to bile acid-induced Egr-1 in fibrotic and cirrhotic livers is at least in part attributable to the diminished SHP inhibition, with a concomitant activation by HNF4α.

Egr-1 stimulation is normally acute and transient (7), and sustained Egr-1 expression can be induced by tissue hypoxia or TGF-β (36). We observed a prolonged induction of EGR-1 by exogenously expressed HNF4α. Initially, we were surprised by the results that in the absence of Hnf4α, Egr-1 mRNA and protein are markedly elevated in both Alb-Hnf4α−/− (long term inactivation) and ErT2-Alb-Hnf4α−/− (short term inactivation) mice. This suggests that the influence of other factors may play a predominant role in governing Egr-1 expression under Hnf4α-deficient conditions. Mice lacking hepatic Hnf4α have increased levels of serum bile acids due to decreased Cyp8b activity (37). The SHP gene is activated by Hnf4α (25), and its levels are diminished in Alb-Hnf4α−/− mice. On the other hand, E2F-1 expression is induced in ErT2-Alb-Hnf4α−/− mice, which activates Egr-1 (4). The decreased SHP or the increased E2F-1, combined with the elevated bile acids and other unknown factors, may contribute to the elevation of Egr-1 in Alb-Hnf4α−/− mice. Thus, alteration of gene expression in vivo often reflects the consequences derived from both direct and indirect effects, and the final net outcome is strongly influenced by the physiological and pathological conditions.

Of particular interest, EGR-1 mRNA exhibits cyclic rhythm in Huh7 cells subjected to serum shock, which has not been reported before. Its diurnal fluctuation is synchronized with the cyclic expression of SHP and HNF4α. However, this is somewhat expected considering the fact that SHP is a clock-controlled gene and its interaction with HNF4α/LRH-1 shows rhythmic binding to the microsomal triglyceride transfer protein (MTP) promoter causing diurnal regulation of plasma triglycerides (12). Diurnal variations of bile acid concentration and composition are also observed in mice (38). Egr-1 expression displays circadian dependence in rat retina (39). It is highly possible that circadian regulation of hepatic Egr-1 expression exists in vivo in mice as well. It would be of great interest to...
explore in future studies. It should be restated that HNF4α exerts a dual role in activating both Egr-1 and SHP, and the activated SHP in turn inhibits the activity of HNF4α to induce Egr-1. Such regulation represents a fine tuning control of Egr-1 expression through a feedback loop between SHP and HNF4α that is likely mediated by the circadian clock.

A recent study showed that overexpression of HNF4α alleviated hepatic fibrosis in rat BDL or dimethylsulfoxide models (40). The authors propose that suppression of the epithelial-mesenchymal transition occurs through induction of E-cadherin. Egr-1 levels, as well as other fibrogenic genes, were not analyzed in the rat study, leaving unknown the association of the observed effect of HNF4α with Egr-1. It is presumed that HNF4α is a pleiotropic factor that regulates multiple cellular processes, and the consequences of its activation can be dramatically different depending on cellular milieu and contexts.

In conclusion, our studies present convincing evidence that the expression of Egr-1 is regulated by nuclear receptor signaling through SHP and HNF4α cross-talk. It helps better our understanding of the molecular basis that controls Egr-1 expression and function in liver fibrosis, which may pave the way of developing future therapeutic agents for the treatment of liver fibrosis.

Acknowledgments—We thank Dr. Scott Friedman for the LX2 cells and Drs. Frances Sladec and Akioyoshi Fukamizu for the HNF4α constructs. Normal human liver and cirrhotic liver specimens were obtained through the Liver Tissue Cell Distribution System (Minneapolis, MN), which is funded by National Institutes of Health Contract N01-DK-7-0004/HSN27200700004C.

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