Genome-wide transcriptome analysis identifies novel gene signatures implicated in human chronic liver disease

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Smalling RL, Delker DA, Zhang Y, Nieto N, Mcguiness MS, Liu S, Friedman SL, Hagedorn CH, Wang L. Genome-wide transcriptome analysis identifies novel gene signatures implicated in human chronic liver disease. Am J Physiol Gastrointest Liver Physiol 305: G364–G374, 2013. First published June 27, 2013; doi:10.1152/ajpgi.00077.2013.—The molecular mechanisms behind human liver disease progression to cirrhosis remain elusive. Nuclear receptor small heterodimer partner (SHP/Nr0b2) is a hepatic tumor suppressor and a critical regulator of liver function. SHP expression is diminished in human cirrhotic livers, suggesting a regulatory role in human liver diseases. The goal of this study was to identify novel SHP-regulated genes that are involved in the development and progression of chronic liver disease. To achieve this, we conducted the first comprehensive RNA sequencing (RNA-seq) analysis of Shp−/− mice, compared the results with human hepatitis C cirrhosis RNA-seq and nonalcoholic steatohepatitis (NASH) microarray datasets, and verified novel results in human liver biospecimens. This approach revealed new gene signatures associated with chronic liver disease and regulated by SHP. Several genes were selected for validation of physiological relevance based on their marked upregulation, novelty with regard to liver function, and involvement in gene pathways related to liver disease. These genes include peptidoglycan recognition protein 2, dual specific phosphatase-4, tetrapsan 4, thrombospondin 1, and SPARC-related modular calcium binding protein-2, which were validated by qPCR analysis of 126 human liver specimens, including steatosis, fibrosis, and NASH, alcohol and hepatitis C cirrhosis, and in mouse models of liver inflammation and injury. This RNA-seq analysis identifies new genes that are regulated by the nuclear receptor SHP and implicated in the molecular pathogenesis of human chronic liver diseases. The results provide valuable transcriptome information for characterizing mechanisms of these diseases.

and relevant animal models should be invaluable in developing new preventative, diagnostic, and therapeutic strategies. This study uses mice deficient in orphan nuclear receptor small heterodimer partner (SHP), officially known as nuclear receptor subfamily 0, group B, member 2 (Nr0b2), as a model to investigate genomic changes involved in chronic liver disease development and progression. SHP represses multiple genes involved in hepatic lipid metabolism and inflammation (6, 26, 27, 47) and has been shown to be critical for liver functions, including bile acid synthesis (42). SHP is functionally implicated in cholestatic liver injury due to bile acid feeding (41) or bile duct ligation (34), fatty liver (15, 16), liver fibrosis (46), and liver cancer (50). In addition, SHP is a newly identified regulator of hepatocyte apoptosis (48) and DNA methylation (45, 49). More importantly, SHP levels are down-regulated in human cirrhosis (46) and HCC (12). This makes SHP-deficient (Nr0b2−/−; referred to as Shp−/−) mice, which spontaneously develop hepatoma (50), a suitable model to identify and study molecular mechanisms leading to chronic liver disease and HCC. Recent studies suggest that SHP modulates hepatitis B virus biosynthesis through different nuclear receptors in human Huh7 and HepG2 hepatoma cells (31), but its role in HCV replication has not been explored. However, bile acid receptor farnesoid X receptor, a transactivator of SHP, is involved in HCV replication (38), indicating potential regulation of HCV by SHP as well.

RNA sequencing (RNA-seq) is a comprehensive means of measuring gene expression that provides more detailed information than gene arrays. Analyzing 5′-capped RNA using RNA-seq methods has been shown to be more sensitive than analyzing poly(A)-selected RNA, since it detects mRNAs with short or absent 3′-ends, detects more differentially expressed genes (DEGs), and identifies unannotated Pol II RNAs (33). We used this approach to identify DEGs that may be regulated by SHP and play a role in the development of chronic liver disease. We identified gene expression changes in Shp−/− mouse liver using RNA-seq analysis and investigated the enrichment of this signature in chronic hepatitis C cirrhosis and NASH liver biospecimens (2), respectively.

Our analysis identified novel and distinct patterns of gene expression changes that were common to Shp−/− mice, hepatitis C cirrhosis, and/or NASH livers relative to controls. These changes were verified by analyzing a large collection of human liver biospecimens, including steatosis, fibrosis, NASH cirrhosis, alcohol cirrhosis, and hepatitis C cirrhosis, as well as mouse models of liver injury and inflammation. Our study identifies novel genes whose expression levels are altered in several major chronic liver diseases and that show physiologically relevant responses to liver injury and inflammation. The
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involvement of these genes in chronic liver disease pathogenesis warrants further detailed investigation.

Glossary

BDL Bile duct ligation
CCl₄ Carbon tetrachloride
Cyp2a5 Cytochrome P-450, family 2, subfamily a, polypeptide 5
DAVID The Database for Annotation, Visualization and Integrated Discovery
DDC 3,5-Diethoxycarbonyl-1,4-dihydrocollidine
DEGs Differentially expressed genes
DNMT1 DNA methyltransferase 1
DUSP4 Dual specific phosphatase-4
Egr-1 Early growth response 1
FDR False discovery rate
FXR Farnesoid X receptor
GO Gene ontology
Cyp7a1 Cholesterol 7 α-hydroxylase
HCC Hepatocellular carcinoma
HCV Hepatitis C virus
KEGG Kyoto Encyclopedia of Genes and Genomes
MAPK Mitogen activated protein kinase
MIAME Minimum Information about a Microarray Experiment
Mmd2 Monocyte to macrophage differentiation-associated 2
NAFLD Nonalcoholic fatty liver disease
NASH Nonalcoholic steatohepatitis
PGLYRP2 Peptidoglycan-recognition protein 2
RNA-seq RNA sequencing
SHP Small heterodimer partner (nuclear receptor subfamily 0, group B, member 2, Nr0b2)
SMOC2 SPARC-related modular calcium binding protein-2
THBS1 Thrombospondin 1
TSPAN4 Tetraspanin 4
Vaspin Visceral adipose tissue derived serine protease inhibitor
WT Wild type

MATERIALS AND METHODS

Animals and human liver samples. Shp⁻/⁻ mice were generated as previously described (42). Briefly, the livers of 8-wk-old male Shp⁻/⁻ mice on a C57BL/6 background and wild-type (WT) C57BL/6 mice fed a normal chow diet were harvested at 12:00 noon, when SHP/Nr0b2 gene expression is lowest because of its circadian rhythm (32). Total RNA was used for RNA-seq and qPCR (n = 3 mice/genotype).

Experimental fibrosis was induced in 2-mo-old male B6129SF2/J mice. Briefly, mice were challenged with CCl₄ (10% vol/vol in corn oil, 5 μl/g body wt ip two times a week) for 6 wk, or mice were fed a 0.1% 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC)-supplemented diet for 6 wk. Whole liver lysates were analyzed. Experimental inflammation was induced by low and high doses of lipopolysaccharide (LPS) in primary hepatocytes according to an established protocol (18). Hepatocytes were isolated from WT and Shp⁻/⁻ mice following our published method (15).

Alcohol-induced liver injury and isolation of hepatic stellate cells. Hepatic stellate cells (HSC) from ethanol-treated rats were generated as described (5). Rats (300 g female Sprague-Dawley, n = 10/group) were fed the control or ethanol Lieber-DeCarli diets for 8 mo (23). Animals received humane care according to the criteria outlined in the Guide for Care and Use of Laboratory Animals. Details regarding pathology of the liver of the control and alcohol-fed rats are described (5). Animal care and use protocols were approved by the Institutional Animal Care and Use Committee at the University of Utah and Mount Sinai School of Medicine.

The human biospecimens were obtained from liver explants taken during the time of surgery from patients with hepatitis C cirrhosis, alcohol cirrhosis, NASH cirrhosis and fibrosis, and donor livers histologically diagnosed with fibrosis, steatosis, or as normal livers. The biometric and diagnostic details for the human liver specimens procured via the Liver Tissue Cell Distribution System (Minneapolis, MN) are described in supporting documents (data not shown). Procurement and use of additional hepatitis C cirrhosis and normal samples under Institutional Review Board approval has been previously described (33).

RNA isolation and RNA-seq. Total and 5’-capped RNA was purified from mouse and human liver biospecimens as previously described (4). For RNA-Seq of Shp⁻/⁻ mice, the quality of RNA and the PCR libraries used for RNA-seq were as previously described (5), and single 36-bp reads were obtained using the Illumina RNA-seq protocol (30, 33).

qRT-PCR and immunohistochemistry validation. cDNA was prepared from total RNA and reverse transcribed as previously described (46). Primers were designed to qPCR amplify 70- to 200-bp regions of selected genes that were highly differentially expressed in Shp⁻/⁻ mice and hepatitis C cirrhosis and/or NASH. Sequences are available on request. Thermal cycling was carried out using a Roche Light Cycler 480 (Roche Applied Science). The Student’s unpaired t-test was used in data analysis; P < 0.05 was considered significant. Error bars represent the SE of the mean. Gene signatures were validated by qPCR in a subset of human liver specimens and mouse models of liver fibrosis and inflammation.

Sections for immunohistochemistry (IHC) were prepared from snap-frozen human liver tissue specimens fixed in optimum cutting temperature compound. Detection of dual specific phosphatase-4 (DUSP4) protein levels was carried out using a DUSP4 mouse monoclonal antibody (SAB1403748-100UG; Sigma-Aldrich). Immunofluorescent labeling was done using Alexa Fluor 594 Goat Anti-Mouse IgG (H+L) from Life Technologies (A-11005). Sections were counterstained with ProLong Gold Antifade Reagent with DAPI.

Bioinformatics. RNA-seq reads were aligned to the July 2007 mouse reference sequence genome (GRcM37/mm9) for the Shp⁻/⁻ mice (n = 3) (GEO accession no.: GSE43893) and the February 2009 human reference sequence genome (GRCh37/hg19) for hepatitis C cirrhosis (n = 6) using the Noalign short-read alignment software (36). Sample reads were visualized, and DEGs were identified as previously described (29, 33). The NASH dataset (n = 7) was prepared on the GE Healthcare Gene Expression Bioarrays (Codelink) platform (GEO accession no.: GSE17470). The data used were Minimum Information about a Microarray Experiment compliant (2). Genes with a log-transformed false discovery rate (FDR) of >10 (i.e., an untransformed FDR of <0.05 or 5 false positives/100 observations) and greater than or equal to ±1.5 normalized fold change in expression relative to controls were considered significantly differentially expressed (33). We normalized the log-transformed reads of the DEGs to the average of the controls within each dataset and then used these values to produce hierarchical clustering and generate heat maps of the genes. Enrichment of the Shp⁻/⁻ gene signature in HCV cirrhosis and NASH datasets was statistically determined using Fisher’s exact test to generate the two-sided P value and the hypergeometric distribution statistic.

Pathway analysis. The Database for Annotation, Visualization, and Integrated Discovery (DAVID) software (13, 14) was used to identify pathways that were differentially altered in Shp⁻/⁻ mice, hepatitis C cirrhosis, and NASH datasets. Briefly, we queried the DEG lists against the human and mouse genome for hepatitis C cirrhosis and NASH, and Shp⁻/⁻ mice, respectively, to generate functional annotations of the gene sets. Kyoto Encyclopedia of Genes and Genomes
(KEGG) and Gene Ontology (GO) pathways that were enriched at a significance level of $P \leq 0.05$ were selected. Individual GO pathways were combined into groups based on functional similarity and common genes.

RESULTS

RNA-seq analysis identifies and predicts SHP-regulated genes. RNA-seq analysis of Shp$^{-/-}$ mice serves as an efficient approach to identify and predict genes regulated by SHP. In the Shp$^{-/-}$ mice, SHP (Nr0b2) exon 1 was replaced as described (42); therefore, sequencing reads were not observed in exon 1 (Fig. 1A) but reads were observed flanking the exon. The analysis of 5'-capped RNA detects both spliced and unspliced isoforms. Therefore, some mRNA transcripts may have been generated from an alternative promoter, but no functional SHP mRNA has been detected in Shp$^{-/-}$ mice (42).

Early growth response 1 (Egr-1), a SHP-repressed target we recently identified (46), was highly upregulated in Shp$^{-/-}$ mice (Fig. 1B) as was mitogen-activated protein kinase (MAPK) pathway activator monocyte-to-macrophage differentiation-associated 2 (19), which suggests it is repressed by SHP (Fig. 1C). Cholesterol 7 $\alpha$-hydroxylase (Cyp7a1), an enzyme of bile acid biosynthesis known to be repressed by SHP (42, 47), was highly overexpressed in Shp$^{-/-}$ compared with the WT mice (Fig. 1D). The adipocytokine Serpina12 (or vaspin, visceral adipose tissue-derived serine protease inhibitor) is increased in NAFLD (1). This gene was decreased in Shp$^{-/-}$ mice (Fig. 1E), which are resistant to high cholesterol diet-induced fatty liver (15). Similarly, cytochrome P-450, family 2, subfamily a, polypeptide 5 (Cyp2a5), which is induced in xenobiotic-induced hepatotoxicity

Fig. 1. Identification of novel candidate small heterodimer partner (SHP)-regulated genes by RNA sequencing (RNA-seq) analysis of wild-type (WT) and Shp$^{-/-}$ liver. A: Integrated Genome Browser visualization tracks from RNA-seq reads depict complete loss of SHP (nuclear receptor subfamily 0, group B, member 2 (Nr0b2)) exon 1 expression in Shp$^{-/-}$ mice. B: known SHP target early growth response 1 (Egr-1) is increased 9.1-fold in Shp$^{-/-}$ mice compared with WT. C: potential SHP target monocyte-to-macrophage differentiation-associated 2 (Mmd2) shows a dramatic 8.0-fold increase above WT Mmd2 levels. D: cholesterol 7 $\alpha$-hydroxylase (Cyp7a1) shows 4.2-fold increased expression in Shp$^{-/-}$ mice compared with WT mice. E: expression of Serpina12 is decreased 3.8-fold in Shp$^{-/-}$ mice compared with WT across exons 3, 4, and 5. F: cytochrome P-450, family 2, subfamily a, polypeptide 5 (Cyp2a5) is decreased 2.6-fold in Shp$^{-/-}$ compared with WT mice.
and microbial hepatitis (21), was significantly downregulated (Fig. 1F). Cyp2a5 and Serpina12 represent genes that are likely activated by SHP through transcription-independent mechanisms. Our approach consistently identifies known SHP-regulated genes, and we subsequently demonstrate its utility in predicting new SHP targets.

Bioinformatics analysis identifies DEGs common to Shp<sup>−/−</sup> mice, hepatitis C cirrhosis, and NASH. The bioinformatics analysis methods and statistical criteria applied to the RNA-seq and microarray data allowed rigorous identification of DEGs in Shp<sup>−/−</sup> mice, NASH, and hepatitis C cirrhosis. We identified 1,161 DEGs in Shp<sup>−/−</sup> mice, 93 of which were enriched in the set of 3,326 genes differentially expressed in hepatitis C cirrhosis ($P = 0.0011$) (Fig. 2A, left), and 68 were enriched in the 2,050 DEGs in NASH ($P = 1.919E-006$) (Fig. 2B, left). Sixty-seven genes were significantly upregulated in hepatitis C cirrhosis and Shp<sup>−/−</sup> mice, whereas 26 were commonly downregulated below 1.5-fold; 25 were upregulated above 5-fold in hepatitis C cirrhosis (Fig. 2A, middle and right). Shp<sup>−/−</sup> mice and NASH shared 52 upregulated genes; 22 were upregulated above 3-fold in NASH, and 16 were downregulated below 1.5-fold (Fig. 2B, middle and right). Notably, 13 genes had a similar differential expression pattern in all three conditions (Fig. 2C), suggesting that these genes may play important roles in the development of NASH and cirrhosis and may serve as biomarkers for the presence of these diseases. The fold changes and number of DEGs in hepatitis C cirrhosis and NASH were consistently higher than those for the same genes in Shp<sup>−/−</sup> mice. This likely reflects the greater accumulation of genomic changes over time in the disease states compared with early changes in 2-mo-old Shp<sup>−/−</sup> mice.

Hierarchical clustering reveals shared gene signatures in Shp<sup>−/−</sup> mice, chronic hepatitis C cirrhosis, and NASH. Unsupervised hierarchical clustering of all the genes common to NASH, hepatitis C cirrhosis, and Shp<sup>−/−</sup> mice datasets (Fig. 3A) demonstrated clear up- and downregulated gene signatures shared between the diseases and Shp<sup>−/−</sup> mice. Notably, several of the genes that showed significant differential expression in Shp<sup>−/−</sup> mice and hepatitis C cirrhosis and/or NASH (Fig. 2, A–C) clustered together independently of any selection criteria. Interestingly, more genes appeared to be upregulated in cirrhosis vs. NASH livers, which partly reflects a greater change in liver
cell types in cirrhosis compared with NASH, which is an earlier stage of liver disease.

Heat maps of significantly differentially expressed genes in Shp\(^{−/−}\) mice compared with WT were produced from supervised hierarchical clustering (Fig. 3B). This was also done for Shp\(^{−/−}\) mice and hepatitis C cirrhosis (Fig. 3C) and NASH (Fig. 3D) to highlight the shared DEGs with the most significant changes. The downregulated genes (group 1) (Fig. 3B) may represent genes...
silenced by hypermethylation when SHP repression of DNA methyltransferase 1 is released (45, 49). These genes could also be SHP transcriptional coregulators, or SHP-activated genes, such as peroxisome proliferator-activated receptor-γ (47). Group 2 genes (Fig. 3B), those significantly upregulated in Shp−/− mice, represented known and potential SHP direct or indirect targets for inhibition. Groups 3 (Fig. 3C) and 5 (Fig. 3D) represented genes that are repressed in hepatitis C cirrhosis and NASH, respectively, and are a part of the SHP regulatory network. Group 4 (Fig. 3C) and 6 (Fig. 3D) genes were also possible SHP targets such as peptidoglycan recognition protein 2 (PGLYRP2). These cluster analyses provide a better visualization of changes in the gene expression pattern shared between the Shp−/− mice and hepatitis C cirrhosis and/or NASH. Notably, there is more heterogeneity among the WT and control samples compared with the homogeneous gene expression pattern seen in Shp−/− mice, hepatitis C cirrhosis, and NASH.

qPCR and IHC validation of selected genes in a large set of human liver specimens. PGLYRP2, DUSP4, tetraspanin 4 (TSPAN4), thrombospondin 1 (THBS1), and SPARC-related modular calcium binding protein-2 (SMOC2), and SHP. Expression levels of each gene for each sample were normalized to the hypoxanthine phosphoribosyl transferase 1 expression level of that sample as an internal control. ¥P < 0.001, ¥¥P < 0.01, and *P < 0.05.

The no. of specimens in each group is as follows: HCV cirrhosis (n = 39), alcohol cirrhosis (n = 28), NASH cirrhosis (n = 13), fibrosis (n = 7), steatosis (n = 15), and control (n = 24).

Fig. 4. qPCR validation of new genes that are differentially expressed in Shp−/− mice, human liver steatosis, fibrosis, NASH, and alcohol and hepatitis C cirrhosis. A: from left to right: peptidoglycan recognition protein 2 (PGLYRP2), dual specific phosphatase-4 (DUSP4), and tetraspanin 4 (TSPAN4). B: from left to right: thrombospondin 1 (THBS1), SPARC-related modular calcium binding protein-2 (SMOC2), and SHP. Expression levels of each gene for each sample were normalized to the hypoxanthine phosphoribosyl transferase 1 expression level of that sample as an internal control. ¥P < 0.001, ¥¥P < 0.01, and *P < 0.05.

The no. of specimens in each group is as follows: HCV cirrhosis (n = 39), alcohol cirrhosis (n = 28), NASH cirrhosis (n = 13), fibrosis (n = 7), steatosis (n = 15), and control (n = 24). C: immunohistochemistry analysis of DUSP4 protein in human alcohol and hepatitis C cirrhosis compared with the normal liver. Two representative results from each group are shown. N, normal; AC, alcohol cirrhosis; HC, HCV cirrhosis. Top: DUSP4 protein expression. Bottom: DUSP4 overlay with DAPI staining. Magnification ×20.

Fig. 3. Hierarchical clustering of DEGs in Shp−/− mice, chronic hepatitis C cirrhosis, and NASH. A: unsupervised hierarchical clustering of genes common to the NASH microarray and chronic hepatitis C cirrhosis and Shp−/− RNA-seq gene expression arrays. B: heat map showing supervised hierarchical clustering of genes with fold changes in expression greater than or equal to ±5-fold in Shp−/− compared with WT mice depicting potential SHP-activated genes in group 1 and SHP-repressed genes in group 2. C: heat map showing supervised hierarchical clustering of selected genes with fold changes greater than or equal to ±1.5 in both cirrhotic compared with normal livers and in Shp−/− compared with WT livers, demonstrating correlation between the disease state and Shp−/− gene expression, those repressed in chronic hepatitis C cirrhosis in group 3 and activated in group 4. D: similar to chronic hepatitis C cirrhosis, this heat map shows selected genes with fold changes greater than or equal to ±1.5-fold in NASH compared with normal livers common in Shp−/− compared with WT livers and has a similar pattern.

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modular calcium binding protein-2 (SMOC2) were highly differentially expressed and have barely characterized functions in liver disease. Intriguingly, our pathway analysis and literature search pointed to their possible involvement in liver disease. Therefore, we chose to further analyze these genes in a large set of human liver specimens, including normal liver, steatosis, fibrosis, NASH cirrhosis, alcohol cirrhosis, and hepatitis C cirrhosis. qPCR analysis showed that PGLYRP2 expression was sharply increased in alcohol and hepatitis C cirrhosis and in Shp−/− mice (Fig. 4A, left). TSPAN4 (Fig. 4A, right), THBS1 (Fig. 4B, left), and SMOC-2 (Fig. 4B, middle) were all upregulated in fibrosis and cirrhosis. These genes also showed increased expression in Shp−/− vs. WT mice. On the other hand, SHP levels were decreased in steatosis, fibrosis, and NASH cirrhosis, but not in hepatitis C cirrhosis (Fig. 4, right).

Next, we chose to examine DUSP4 protein expression by IHC in human liver specimens. DUSP4 protein expression was much higher in alcohol and hepatitis C cirrhotic livers compared with normal livers (Fig. 4C), which is consistent with increased mRNA levels.

Functional significance of selected genes in mouse models of liver inflammation, fibrosis, and alcohol injury. To further confirm SHP regulation of the selected genes, we analyzed their expression levels in Shp−/− and WT mice at different time points. Basal SHP mRNA levels exhibited diurnal fluctuation that was markedly higher at 24 h than at 6 h (Fig. 5A), consistent with SHP as a circadian clock-regulated gene (32, 46). The expression of Pglyrp2, Dusp4, Thbs1, and Tspan4 correlated negatively with SHP expression and so was higher at 6 h but lower at 24 h.

To provide initial evidence about the functional significance of the selected genes in liver inflammation, primary hepatocytes isolated from WT and Shp−/− mice were subjected to LPS treatment. Interestingly, short-time LPS treatment (6 h) decreased Pglyrp2, Dusp4, Thbs1, and Tspan4 mRNA in a dose-dependent fashion in the WT hepatocytes, but this effect of LPS was largely blocked in Shp−/− cells after 24 h (Fig. 5B). The levels of these genes were not further downregulated by LPS in WT cells at 24 h but were significantly induced in Shp−/− cells (Fig. 5B). This indicates a derepression effect by the loss of SHP. Notably, the increased expression of Pglyrp2 and Dusp4 in Shp−/− cells was enhanced by a high dose of LPS at 24 h.

Smoc2 exhibited a distinct expression profile. Its levels were not markedly altered by LPS in WT cells at 6 h but were highly induced in Shp−/− cells regardless of the presence of LPS (Fig. 5B). Although Smoc2 expression was ~50% reduced in Shp−/− cells at 24 vs. 6 h, it was consistently high compared with WT cells. LPS treatment induced an approximately twofold increase in Smoc2 expression in WT cells at 24 h.

We also investigated the relevance of these genes to bile acid injury using CCl4- and DDC-induced mouse fibrosis models. Pglyrp2 was induced by DDC but not by CCl4 (Fig. 5C). Dusp4 and Tspan4 were induced by DDC and CCl4, respectively, whereas Thbs1 and Smoc2 were markedly induced in both models.

![Figure 5](https://via.placeholder.com/150)

Fig. 5. Functional analysis of selected genes in mouse liver inflammatory and injury models. A: qPCR of gene expression in hepatocytes isolated from 2-mo-old male mice that were cultured for 6 and 24 h. *P < 0.01, 24 h vs. 6 h. B: qPCR of gene expression from WT and Shp−/− hepatocytes treated with lipopolysaccharide (LPS) for 6 and 24 h. *P < 0.01, LPS 10 µg vs. LPS 1 µg or control in WT cells at 6 h; §P < 0.01, LPS 10 µg vs. control in Shp−/− cells at 6 h; ¶P < 0.01, Shp−/− vs. WT cells at 6 h; ‡P < 0.01, Shp−/− vs. WT cells at 24 h; §§P < 0.01, LPS vs. control at 24 h. C: qPCR of gene expression in mice fed with CCl4 or 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) to induce liver fibrosis. *P < 0.01, CCl4 or DDC vs. respective control.
SHP overexpression in Huh7 cells led to downregulation of Pglyrp2 and Thbs1 (Fig. 6A), whereas a moderate knockdown of SHP using RNAi led to upregulation of Thbs1 but not Pglyrp2 (Fig. 6B).

We next determined the expression of these genes in stellate (HSC) cells and their response to alcohol-induced liver injury. SHP was activated about 40-fold by alcohol, and Pglyrp2 was increased over 30-fold (Fig. 6D). Thbs1 and Tspan4 were induced three- and twofold, respectively (Fig. 6D).

Multiple pathways are commonly altered in Shp−/− mice, NASH, and hepatitis C cirrhosis. Having identified shared gene signatures within the datasets, we then used the DAVID functional annotation tool to uncover multiple pathways shared between Shp−/− mice and the liver diseases studied. We identified 171 GO pathways significantly enriched in Shp−/− mice. Fifty four percent of these were also found in hepatitis C cirrhosis and 51% in NASH (Fig. 7, A and B). These were among the most highly enriched pathways in the hepatitis C cirrhosis DEG set, supporting the potential role of the identified genes in development of chronic liver disease. We also identified 18 KEGG pathways that were significantly altered in Shp−/− mice, 44% of which were also enriched in hepatitis C cirrhosis and 50% were enriched in NASH (Fig. 7, A and B). Pglyrp2, Dusp4, Tspan4, Thbs1, and Smoc2 were novel genes implicated in cellular oxidation, migration, adhesion, intracellular signaling, and inflammation pathways (Fig. 7C). This is the first report to show such a wide-ranging effect of SHP deficiency on multiple pathways, and we highlight specific genes involved in physiological processes that are deregulated in major chronic liver diseases.

**DISCUSSION**

A revolution in the analysis of RNA has come through the development of deep sequencing technologies to map the entire transcriptome in cells and biospecimens (24, 25). Recently, this powerful approach revealed a comprehensive transcriptomic landscape in human HCC (17). However, such comprehensive RNA-seq analysis has not been performed in human liver NASH, fibrosis, or cirrhosis. In addition, establishing animal models that share common gene signatures with human liver diseases is essential to allow studies of genes important to human disease progression.

The present study is the first to conduct comparative RNA-seq analysis of human HCV cirrhosis specimens and Shp−/− (Nr0b2−/−) mice along with NASH gene array analysis. Thus, the results encompass several novel findings. The study revealed that SHP deficiency induced a significant shift in liver cellular metabolic and inflammatory states, and fibrogenic and oncogenic potential, which was also found in hepatitis C cirrhosis and NASH. Moreover, this study also identified new genes that are regulated by SHP and are differentially expressed during hepatic fibrosis and cirrhosis in humans. The findings are consistent with the role of SHP in regulating inflammatory response, lipid and xenobiotic metabolism, liver fibrogenesis, and oncogenesis (47) and suggests the dysregu-

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**Fig. 6.** Alteration in expression of selected genes in stellate cells from alcohol-induced liver injury. A and B: qPCR of gene expression in Huh7 cells with SHP overexpression (A) or knockdown (B). C: qPCR of gene expression in stellate cells [hepatic stellate cells (HSC)] isolated from alcohol-treated rats. *Significance was determined at P < 0.01.
lation of SHP participates in the biology of human chronic liver diseases. The overlap of genes we have found between murine and human conditions is suggested to represent critical gene expression changes in the progression or development of liver disease. Each disease condition, both mouse and human, arose from varying insults and early genomic changes, but they likely converge to disease development and/or progression through similar critical pathways. Our RNA-seq analysis has recapitulated known gene expression changes involved in chronic liver disease, such as increase in CYP7A1 and EGR1 expression, and we now present novel genes that are similarly implicated. The specific causal and/or functional roles of these genes will be thoroughly investigated in future studies.

The genes we selected for validation were all significantly upregulated in Shp−/− mice, hepatitis C cirrhosis, and/or NASH and formed a part of the shared signatures created by hierarchical clustering analysis. Additionally, they were identified in inflammatory response, cell migration, or adhesion pathways, or their involvement in liver disease was suggested in the literature. PGLYRP2, which was upregulated in hepatitis C and alcohol cirrhosis biospecimens, belongs to an innate immunity protein family expressed in the liver (8) and was identified in the early inflammatory response of the gut in mice with colitis (37). Other genes were upregulated in Shp−/− mice and all fibrotic liver disease biospecimens, i.e., fibrosis and NASH, alcohol and hepatitis C cirrhosis. DUSP4 is a MAPK phosphatase (20) whose methylation was significantly correlated with recurrence-free survival in hepatitis C-induced HCC (7). TSPAN4 and Smoc2 were also upregulated in Shp−/− mice and fibrotic liver disease. A recent study showed that TSPAN4 was significantly overexpressed in HCC (22), and Smoc2 potentiates the angiogenic effects of growth factors (35). However, the functions of these genes in liver remain largely unstudied.

The marked increase of these genes in the fibrotic liver disease biospecimens, as well as in CCL4- and DDC-induced liver fibrosis in mice, suggests they are involved in the pathogenesis of fibrotic liver diseases in humans. Interestingly, P glyrp2, Dusp4, Tspan4, and Thbs1 expression is decreased by SHP, and LPS does not increase their expression in WT
hepatocytes. However, with SHP deficiency, as is found with fibrotic liver diseases in our study, expression of Pylrp2, Dusp4, and Tspan4 is induced and further increased on LPS treatment. THBS1 was previously implicated in congenital hepatic fibrosis (9), and its expression was gradually increased with the severity of fibrosis induced by diethyl nitrosamine in rats (10). Our results are consistent with this hypothesis. THBS1 mRNA was increased with CCl₄- and DDC-induced fibrosis in our mouse models and in the majority of fibrotic liver biospecimens, providing evidence that it is likely to be involved in human fibrotic liver disease.

SHP is most abundantly expressed in hepatocytes; therefore, the current study focused mostly on investigating that cell type. Intriguingly, our study for the first time revealed that SHP exhibited distinct and striking responses to alcohol-induced liver injury in nonparenchymal cells; it was highly activated in stellate cells by alcohol. The alteration in its gene expression in this cell population may explain why SHP levels were not downregulated in alcohol cirrhosis as seen in steatosis, when total liver mRNA was examined. Interestingly, induction of SHP in a rat biliary duct ligature model of liver fibrosis inhibited stellate cell function and protected against liver fibrosis (11). It remains to be determined whether posttranslational modifications, such as ubiquitination (51), or pathways through other nuclear receptors contribute to SHP expression in alcohol and HCV cirrhosis. In addition, how alcohol and HCV affect SHP levels in Kupffer cells needs to be investigated. On the other hand, the cell type-specific response to alcohol of Pglyrp2, and to a lesser extent Thbs1 and Tspan4 in stellate cells, suggests vital roles for these genes in alcohol-induced liver injury through to cirrhosis. In HSC cells, Pglyrp2 may be regulated in a SHP-independent manner.

In summary, our study provides new hypotheses regarding the molecular basis of chronic liver diseases in humans. The newly described genes that are similarly altered in Shp⁻/⁻ mice and chronic liver disease highlight the need for further studies of the possible mechanistic role of these genes in hepatic fibrogenesis and chronic liver disease in both patients and model systems such as Shp⁻/⁻ mice.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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