A Liver-Specific Long Noncoding RNA With a Role in Cell Viability Is Elevated in Human Nonalcoholic Steatohepatitis

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Hepatocyte apoptosis in nonalcoholic steatohepatitis (NASH) can lead to fibrosis and cirrhosis, which permanently damage the liver. Understanding the regulation of hepatocyte apoptosis is therefore important to identify therapeutic targets that may prevent the progression of NASH to fibrosis. Recently, increasing evidence has shown that long noncoding (lnc) RNAs are involved in various biological processes and that their dysregulation underlies a number of complex human diseases. By performing gene expression profiling of 4,383 lncRNAs in 82 liver samples from individuals with NASH (n = 48), simple steatosis but no NASH (n = 11), and healthy controls (n = 23), we discovered a liver-specific lncRNA (RP11-484N16.1) on chromosome 18 that showed significantly elevated expression in the liver tissue of NASH patients. This lncRNA, which we named lnc18q22.2 based on its chromosomal location, correlated with NASH grade (r = 0.51, P = 8.11 × 10−7), lobular inflammation (r = 0.49, P = 2.35 × 10−6), and nonalcoholic fatty liver disease activity score (r = 0.48, P = 4.69 × 10−6). The association of lnc18q22.2 to liver steatosis and steatohepatitis was replicated in 44 independent liver biopsies (r = 0.47, P = 0.0013). We provided a genetic structure of lnc18q22.2 showing an extended exon 2 in liver. Knockdown of lnc18q22.2 in four different hepatocyte cell lines resulted in severe phenotypes ranging from reduced cell growth to lethality. This observation was consistent with pathway analyses of genes coexpressed with lnc18q22.2 in human liver or affected by lnc18q22.2 knockdown.

Conclusion: We identified an lncRNA that can play an important regulatory role in liver function and provide new insights into the regulation of hepatocyte viability in NASH. (HEPATOLOGY 2017;66:794-808).

Nonalcoholic fatty liver disease (NAFLD) is a spectrum of conditions ranging from hepatocellular steatosis through steatohepatitis to fibrosis and irreversible cirrhosis. It is currently the most prevalent chronic liver disease and highly associated with metabolic syndrome and obesity.1 Nonalcoholic steatohepatitis (NASH) is the progressive form of NAFLD. The major features of NASH include not only a fatty liver and inflammation but also hepatocyte apoptosis.2 NASH can be severe and can
lead to fibrosis and cirrhosis, which permanently damage and scar the liver, disrupting hepatic function. Preventing NASH from progressing to fibrosis and cirrhosis is therefore crucial. However, treatment options remain limited and are restricted to lifestyle improvement and body weight control. Understanding the regulation of hepatocyte apoptosis will contribute to the identification of molecular targets that prevent NASH progression.

Transcriptome analysis has been used to identify molecular mechanisms involved in NAFLD and NASH. Previously, we profiled genome-wide transcripts in multiple tissue types from a Dutch obesity cohort using microarray to identify novel protein-coding genes in NASH patients, including tissue-specific adipokines and the cholesteryl ester transfer protein. In recent years, with the advance of RNA sequencing (RNA-seq) technology, a large proportion of the human genome has been found to produce functional RNA molecules rather than encoding proteins, and these functional RNAs are known as “noncoding RNAs.” Noncoding RNAs are classified into different groups that include microRNAs, small interfering RNAs, piwi-interacting RNAs, and the largest group, long noncoding RNAs (lncRNAs). An lncRNA is defined as a noncoding transcript longer than 200 nucleotides with an exon–intron structure. To date, more than 15,000 lncRNAs have been annotated in the human genome.

Increasing evidence has shown that lncRNAs play important roles in numerous physiological processes by regulating gene expression and modulating protein function through a variety of mechanisms. Dysregulation of lncRNAs has also been shown to contribute to the progression of many diseases, including liver disease. Individual lncRNAs associated with metabolic disorders and liver diseases have been identified in mice and humans. For instance, lncLSTR, a liver-enriched lncRNA, was identified as a putative regulator of plasma triglyceride levels in mice, but no human orthologue was found. An antisense lncRNA to apolipoprotein A1 (APOA1-AS) has been shown to negatively regulate the expression of APOA1, a major component of high-density lipoprotein. The lncRNAs Meg3 and MALAT-1 may be involved in hepatocellular carcinoma (HCC) by regulating gene expression and alternative splicing, respectively. Although over 1,000 lncRNAs have been associated with NAFLD, their role in this disease remains largely unknown. Moreover, their potential as noninvasive biomarkers is largely unexplored as noncoding RNAs can form stable secondary structures that can be detected in circulating exosomes.

In this study, we report the discovery of lnc18q22.2, a liver-specific lncRNA (RP11-484N16.1) involved in cell viability with elevated expression in the liver of NASH patients. An overview of this study is presented in Fig. 1. The involvement of lncRNAs in NASH was first identified by association analyses between the expression levels of 4,383 lncRNAs and detailed histological analysis of NASH phenotypes in 82 liver samples. Expression of lnc18q22.2 was significantly elevated in NASH patients, a finding that we replicated in an independent data set. We then assessed lnc18q22.2’s structure, abundance, and cellular location. Further, we investigated its downstream effect by silencing its expression in four hepatocyte cell lines (HCC–derived HepG2, Huh7, and Hep3B cell lines and the nontumorous cell line immortalized human hepatocytes [IHH]) and two nonhepatocyte cell lines as controls (HEK293T and HeLa). This lnc18q22.2
knockdown resulted in negative regulation of cell viability in hepatocytes. Finally, the underlying processes were assessed by pathway analysis of coexpressed genes and of genes affected by lnc18q22.2 knockdown using RNA-seq.

Materials and Methods

LIVER BIOPSIES AND NASH PHENOTYPES

Our study cohort consisted of liver biopsies from 82 severely obese Dutch individuals with a body mass index between 30 and 73 who underwent elective bariatric surgery in the Department of General Surgery, Maastricht University Medical Center. The collection and processing of liver biopsies are described in detail in the Supporting Information. Exclusion criteria for this cohort were individuals with acute or chronic inflammatory disease (e.g., autoimmune disease) or degenerative disease, who reported alcohol consumption of >10 g/day, or who used anti-inflammatory drugs. NASH phenotypes were analyzed by an experienced pathologist who was blinded to the clinical and biochemical parameters. NASH staging and grading was performed according to the Brunt scoring system. Moreover, each individual was scored for different histological parameters of liver pathology: steatosis, fibrosis, inflammation (lobular inflammation, large lipogranulomas, portal inflammation), liver cell injury (ballooning), and glycogenated nuclei according to the scoring system described by Kleiner et al. (Supporting Table S1). The NAFLD activity score (NAS) was calculated according to the Kleiner scoring system. Circulating levels of the liver enzymes aspartate aminotransferase and alanine aminotransferase were also measured and used for the analysis. The study was approved by the Medical Ethics Board of the Maastricht University Medical Center, in line with the ethical guidelines of the 1975 Declaration of Helsinki. Informed consent was obtained in writing from each participant.

MICROARRAY DATA GENERATION AND lncRNA PROBE MAPPING

RNA was isolated and profiled as described. The average RNA integrity number for RNA quality was 7.6, with a range of 5.7-9.3. Whole-transcriptome expression profiling was performed on 82 liver samples using Illumina Human HT12 Bead Chips (Illumina, San Diego, CA). Although not designed for lncRNA quantification, this platform contains probes for transcripts with unknown function and without significant coding potential. In order to identify which probes cover lncRNA genes, two human lncRNA annotation databases were used: GENCODE version 19 (July 2013, 13,870 annotated lncRNAs; http://www.gencodegenes.org/stats/archive.html#a19) and the Human Body Map catalog (>8,000 annotated

intergenic lncRNAs, a subclass of lncRNAs; http://www.broadinstitute.org/genome_bio/human_lincrnas/?q=home). lncRNAs were quantified using probes that mapped to one or both database annotations and did not overlap with protein-coding genes or other lncRNAs. The final list of probes used to determine lncRNA expression contained 4,468 probes covering 4,383 lncRNAs. Data were log2-transformed, quantile-normalized, and corrected for batch effects.(20)

CORRELATION OF lncRNA EXPRESSION PROFILES WITH NASH PHENOTYPES

To determine the correlations between lncRNA expression and NASH phenotypes, Spearman’s rank correlation coefficients were determined between lncRNA expression values and the values of the measured traits, including NASH phenotypes. For these probe-level correlations, permutation testing was performed to estimate the false discovery rate (FDR)—corrected \( P \) values. To correct for age, gender, and body mass index, a linear model was run for all lncRNAs and their top significant phenotype associations. For these genome-wide correlations, we made use of the FDR-corrected \( P \) value.

REPLICATION OF THE ASSOCIATIONS

In order to replicate our findings, we downloaded a data set from the Gene Expression Omnibus (accession GSE33814).(21) The data set consisted of 44 human liver tissue samples obtained from patients with alcoholic steatohepatitis (ASH) and NASH (normal \( n = 13 \), steatosis \( n = 19 \), and steatohepatitis \( n = 12 \)) obtained from patients undergoing liver surgery for HCC, malignancies/metastatic diseases, or benign tumors of the liver and from organs dedicated to transplantation. However, the cohort contained both ASH and NASH. There was no individual phenotype information available, and we could not compute analysis for ASH and NASH patients separately. Healthy, nontumorous liver tissue with no detectable pathological changes was removed from patients undergoing surgical resection of liver metastases as control tissue. Whole-genome expression microarray SentrixH Human-6 v3 expression bead chips (Illumina) were used, which encompass probes for 48,804 genes including lncRNAs. These data were log2-transformed and quantile-normalized.

To test whether lnc18q22.2 was associated with liver cancer, we compared liver expression of lncRNA between 73 HCC patients and 85 healthy control samples, downloaded from the Sequence Read Archive (SRA; http://www.ncbi.nlm.nih.gov/sra/). As a positive control, we also checked two established cancer lncRNAs (\( MALAT1^{(15)} \) and \( HULC^{(22)} \)), both showing significant associations with HCC (\( MALAT, \ P = 3.00 \times 10^{-10}; HULC, \ P = 3.64 \times 10^{-5} \) (Supporting Fig. S5)). Moreover, no etiology information of HCC patients was available.

LIVER-SPECIFIC EXPRESSION AND STRUCTURE OF lnc18q22.2

To assess the expression level and transcript structure of lnc18q22.2 in the liver, we first compared its predicted structure as annotated in the GENCODE and Human Body Map databases. In addition, we extracted RNA-seq data on 30 tissue and 67 cell types through the SRA, including 63 liver samples and 34 HepG2 cell line samples.(23) Expression values in these samples were normalized with the trimmed mean of M values method using the R package “edgeR.”(24) The average of the normalized expression values per tissue or cell line was used. The RNA-seq read distribution showed a liver-specific structure for lnc18q22.2 and confirmed the liver specificity of its expression. Further, several RT-PCR and quantitative RT-PCR experiments were performed to validate the expression and structure of lnc18q22.2 and its cellular location, as described in detail in the Supporting Information. In brief, to evaluate the potential of lnc18q22.2 as a non-invasive biomarker, we measured the abundance of lnc18q22.2 in eight plasma samples and 1,141 whole-blood samples. We validated the transcript structure of lnc18q22.2 using quantitative PCR, followed by Sanger sequencing. We performed quantitative RT-PCR to validate the association of lnc18q22.2 in 33 randomly selected liver samples, including normal \( n = 8 \), NAFLD \( n = 8 \), and NASH \( n = 17 \) samples. We tested the expression of lnc18q22.2 in five hepatocyte cell lines (HCC-derived HepG2 [ATCC, HB-8065], Hep3B [ATCC, HB-8064], Huh7 [JCRB Cell Bank, JCRB0403]), in the nontumorous cell line IHH,(25) and in RNA isolated from three different batches of primary human hepatocytes (Tebu-Bio, Heerhugowaard, The Netherlands). Moreover, the log2 ratio of...
cytoplasmic and nuclear fractions of lnc18q22.2 was estimated in HepG2 cell line.

**lnc18q22.2 COEXPRESSION NETWORK ANALYSIS**

To predict a function for lnc18q22.2, we performed guilt-by-association analysis using data from 37,776 genes on the Illumina microarray. We assessed whether lnc18q22.2 expression correlated with the expression of genes in *cis* (within 5 Mb distance) and in *trans* (genome-wide) using Spearman’s correlation test. An FDR of 0.05 was used to correct for multiple testing. All significant correlations (FDR < 0.05) were analyzed for pathway analysis using the DAVID database (https://david.ncifcrf.gov).

**lnc18q22.2 KNOCKDOWN**

To knock down lnc18q22.2, we designed two short hairpin RNA (shRNA) cassettes for cloning into the lentiviral pLKO TRC vector. The cassettes were specifically designed using the full lnc18q22.2 sequence and targeted only lnc18q22.2 and not the overlapping transcript. For this purpose, we used the small interfering RNA selection program (http://sirna.wi.mit.edu/) and designed two shRNAs and one mock shRNA (see Supporting Information). Upon annealing of the oligos, the shRNAs were cloned into the pLKO TRC vector and lentiviral particles were produced as described.

**Cell Survival Counts**

Cells (HepG2, Hep3B, Huh7, IHH, HEK293T, and HeLa) were seeded in triplicate into 12-well plates for counting surviving cells and transduced with lentiviral particles as described above. Surviving cells were counted after 3, 6, and 9 days of puromycin selection.

**The Effect of lnc18q22.2 Knockdown on Apoptosis (Western Blot)**

The cleavage of poly(adenosine diphosphate-ribose) polymerase 1 (PARP-1) was assessed by western blotting as an indicator of apoptosis. Total cell lysates were extracted from cells treated with virus particles expressing shRNA1, shRNA2, or control shRNAs. Protein concentration was determined by bicinchoninic acid assay (Pierce, Rockford, IL), and proteins were separated by 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred to polyvinylidene fluoride membranes. The membranes were probed with cleaved PARP-1 antibody (1:1,000; Cell Signaling Technology) detecting both full size (116 kDa) and cleaved (89 kDa) PARP protein fragments. Anti-beta-actin antibody (1:1,000) was used as a control. Membranes were incubated overnight at 4°C, followed by 1-hour incubation with horseradish peroxidase–labeled secondary antibody. The Femto kit (Thermo Fisher Scientific) was used for detection, and the signal was quantified using the ChemiDoc XRS gel documentation system (Bio-Rad Laboratories, Hercules, CA). Experiments were performed in triplicate for all three time points (1, 2, and 3 days after virus transduction). The results were similar at all time points; therefore, only the results from day 3 are presented.

**The Effect of lnc18q22.2 Knockdown on Apoptosis and Necrosis (Nuclear Staining)**

IHH and Huh7 cells were incubated in virus media for 48 hours and analyzed for necrosis and apoptosis. Sytox green staining (Invitrogen; S7020) was used to detect necrotic nuclei (1:40,000 dilution, for 15 minutes), and acridine orange staining (Sigma; A8097) was used to detect apoptotic nuclei (1:4,000 dilution, for 15 minutes). Fluorescent nuclei were visualized using an EVOS FL Cell Imaging System (Advanced Microscopy Group, Bothell, WA). Pictures were taken at three time points (1–3 days after virus transduction), in triplicate.

**RNA-seq of lnc18q22.2 Knockdown Cell Lines**

HepG2 and Hep3B were seeded into six-well plates (200,000 cells/well) and incubated with lentivirus particles for 48 hours once 80% confluence was reached. Green fluorescent protein (an in-house-generated plasmid similar to pRRLSIN.cPPT.PGK-GFP.WPRE from Addgene) was used to monitor the transduction efficiency in all cell lines. After the virus–particle–containing medium was removed (after 48 hours), fresh complete culture medium was added for 24 hours. After 72 hours, Hep3B and HepG2 cells were treated with puromycin (1 μg/mL) for 3 and 4 days, respectively, to produce stable cell lines, followed by RNA isolation. All experiments were performed in duplicate. Twelve samples in total were isolated for RNA sequencing, including six samples per cell line (HepG2, Hep3B): knockdown shRNA1, knockdown...
shRNA2, and control mock shRNA, each in duplicate. RNA was isolated using Qiazol reagent (Qiagen, Germantown, MD) and purified using the RNeasy Mini Kit (Qiagen). RNA concentration was measured by spectrophotometry, and complementary DNA was reverse-transcribed for quantitative RT-PCR. Before RNA-seq libraries were prepared, quantitative RT-PCR was performed to confirm Inc18q22.2 knockdown, and the quality of the isolated RNA was measured on a bioanalyzer (LabchipGX; PerkinElmer, Waltham, MA). Sample preparation was done using standard Illumina TruSeq mRNA-SamplePrep, and paired-end sequencing was performed on the Illumina HiSeq2500 sequencer. On average, ~18 million reads were produced per sample. All RNA-seq reads were aligned to the human genome (hg19) using STAR(31) and rlog-normalized using the R package “DESeq2.”(32) The same package was used to analyze differentially expressed genes between the two knockdown groups versus control. Differentially expressed genes with FDR < 0.01 were selected for pathway enrichment analysis. The DAVID database was also used for this purpose.(27)

**Results**

**Inc18q22.2: AN IncRNA ASSOCIATED WITH NASH**

Whole-genome gene expression oligonucleotide arrays have played a crucial role in quantitatively determining the levels of gene expression. Even though most of the currently available commercial microarrays are designed to capture all known protein-coding transcripts, they also include subsets of probes that capture transcripts of unknown function. To identify IncRNAs associated with NASH, we took advantage of microarray-based expression data of 82 liver samples and mapped 4,468 microarray probes to 4,383 lncRNAs located in intergenic regions, hereafter referred to as the in-house microarray data (Fig. 1). Spearman’s rank correlation analyses are reported at FDR < 0.05 significance.
FIG. 3

A

Chromosome 18

Transcript Structures

(q)RT-PCR
To verify transcript structure

shRNA knockdown

B

Product 1
(113 bp)

Product 2
(317 bp)

Product 3
(406 bp)

Sequence confirmed by Sanger sequencing

C

Normalized RNA reads of IncRNA 18q22.2

Tissue

Liver

Cell type

Normalized RNA reads of IncRNA 18q22.2

D

HepG2

Hep3B

HuH7

HepG1

Hep293T

E

Expression levels of IncRNA 18q22.2

Un Certified

DANOR

GIPS-AS

HepG2

Hep293T
SPECIFIC EXPRESSION AND TRANSCRIPT STRUCTURE OF Inc18q22.2 IN LIVER AND HEPATOCYTE CELL LINES

Previously Inc18q22.2 was annotated to chromosome region 18q22.2 and does not overlap with any established protein-coding genes except one putative transcript for the putative protein-coding gene RP11-4104.1 (Fig. 3A). The closest known protein-coding gene is the suppressor of cytokine signaling 6 (SOCS6), which is some 5 kb upstream (Supporting Fig. S3). Inc18q22.2 contains two exons, and annotations of the second exon are not consistent across different databases. The GENCODE database indicates a length of 287 bp for this second exon, whereas the liver tissue panel of the Human Body Map database annotation is longer, 537 bp, and fully overlaps with the microarray probe of Inc18q22.2 (Fig. 3A). We therefore further delineated the transcript structure of Inc18q22.2 in liver samples and validated the association of Inc18q22.2 with NASH. First, RNA-seq data from 2,432 samples from 30 different tissues and 67 different cell lines were extracted from the SRA, hereafter referred to as public SRA-seq data. The average intensity of SRA-seq reads from 63 liver samples confirmed the extended exon 2 seen in the Human Body Map database (Fig. 3A). Thus, the total length of the lncRNA transcript was estimated to be 633 bp. Second, RT-PCR experiments were performed to validate the transcript structure of Inc18q22.2 (Fig. 3A). The PCR products showed the expected length (Fig. 3B) and were validated by Sanger sequencing. Moreover, the expression level of Inc18q22.2 and its association with NASH were validated in 33 random samples by quantitative RT-PCR.

The relative expression of Inc18q22.2 assessed by quantitative RT-PCR was positively correlated with the intensity level of the microarray probe \( r = 0.74, P = 8.50 \times 10^{-7} \) (Supporting Fig. S4A), NASH grade \( r = 0.65, P = 4.55 \times 10^{-5} \), NAS \( r = 0.58, P = 8.64 \times 10^{-4} \), and lobular inflammation \( r = 0.62, P = 1.38 \times 10^{-3} \) (Supporting Fig. S4B).

Liver-specific expression of Inc18q22.2 had already been suggested in the Human Body Map catalog in 2011. Comparing the average expression level of Inc18q22.2 across 30 different tissues, and 67 different cell lines (public SRA-seq data) confirmed that Inc18q22.2 is predominantly expressed in liver tissue and in the HCC cell line HepG2 (Fig. 3C). Inc18q22.2 was not detected in whole blood \( (n = 1141) \) or plasma samples of four healthy volunteers and four NASH patients (Supporting Table S4). The liver-specific expression of Inc18q22.2 was further confirmed by quantitative RT-PCR analysis in five hepatocyte cell lines (HCC-derived Huh7, Hep3B, and HepG2 cells, nontumorous IHH cells, and primary human hepatocytes) and two nonhepatocyte cell lines (HEK293T and HeLa). Inc18q22.2 was expressed in all hepatocytes, especially HepG2, IHH, and primary human hepatocytes. No expression was detected in HEK293T cells, and very low expression was found in HeLa cells (Fig. 3D). Moreover, fractionation experiments showed that Inc18q22.2 was mainly present in the cytoplasm (Fig. 3E).

To evaluate the protein-coding potential of Inc18q22.2, we used the Coding Potential Calculator tool (http://cpc.cbi.pku.edu.cn/)(34) and the Coding Potential Assignment Tool (http://lilab.research.bcm.edu/cpat/index.php).(35) Neither tool detected an open reading frame for both the novel and annotated sequences of Inc18q22.2.
FIG. 4. Silencing of lnc18q22.2 expression in vitro and phenotypic characteristics of the cells. (A) Quantitative RT-PCR of lnc18q22.2 expression after shRNA-mediated knockdown in three HCC cell lines (HepG2, Hep3B, and Huh7) and one nontumorous cell line (IHH). Values are shown with shRNA mock–mediated lnc18q22.2 expression set to 1; mean ± standard error of the mean. (B) Cell proliferation after shRNA-mediated depletion of lnc18q22.2. Cell counts on surviving cells were obtained after 3 days (time point 1), 6 days (time point 2), and 9 days (time point 3) of puromycin selection. At baseline (0), 200,000 cells were seeded per well for all cells.
Taken together, these results validate the association of lnc18q22.2 with NASH and reveal its novel transcript structure, high expression in liver tissue, subcellular localization in the cytoplasm, and lack of coding potential.

**lnc18q22.2 IS CRUCIAL FOR GROWTH AND VIABILITY OF HEPATOCYTES**

To further elucidate the function of lnc18q22.2, two different shRNA cassettes were used to silence its expression in four different hepatocyte cell lines (HepG2, Hep3B, Huh7, and IHH) (Fig. 4A). Both shRNAs significantly down-regulated the expression of lnc18q22.2 in all four hepatocyte cell lines, with shRNA1 showing a stronger effect than shRNA2 (Fig. 4A). Notably, the silencing of lnc18q22.2 expression resulted in reduced growth in HepG2 and IHH cells (only shRNA1) and promoted cell death in Huh7 and Hep3B cells (only shRNA1) (Fig. 4B). Huh7 cells died within 2–4 days of shRNA1-mediated knockdown and within 4–6 days of shRNA2-mediated knockdown.
of shRNA2-mediated knockdown, whereas Hep3B cells died 6–8 days after shRNA1-mediated knockdown. HepG2 and IHH cells did not die, but cell growth in the shRNA1 knockdown was markedly reduced compared to controls. Furthermore, shRNA1 seemed to be more efficient at down-regulating the expression of lnc18q22.2 than shRNA2 (Fig. 4A). The downstream phenotype in shRNA1-mediated knockdown is consistently more severe than that in shRNA2.

To exclude any potential off-target effects of both shRNAs, we stably expressed both shRNAs in two control cell lines (HEK293T and HeLa cells) in which lnc18q22.2 was not expressed or showed a very low level of expression (Fig. 3D). No differences in cell viability and cell growth were seen in the HEK293T and HeLa cells (Fig. 4B).

To further characterize the cell death phenotype, we assessed cell viability in lnc18q22.2 knockdown in Huh7 cells (both shRNAs) and IHH cells (shRNA1). Cleavage of full size PARP-1 is a hallmark of apoptosis. We observed a significant reduction of full-size (intact) PARP-1 (Fig. 5A) and a significant increase in necrotic nuclei as visualized by using Sytox green staining (Fig. 5B). But knockdown did not increase the number of apoptotic nuclei as visualized by acridine orange staining (Supporting Fig. S6).

lnc18q22.2 INVOLVEMENT IN ESSENTIAL BIOLOGICAL PROCESSES IN HEPATOCYTES

To gain more insight into the potential role of lnc18q22.2 in hepatocyte cell viability, we performed various pathway analyses on genes coexpressed with lnc18q22.2 based on the in-house microarray data and using the RNA-seq data from the lnc18q22.2 knockdown cell lines.

First, we examined whether lnc18q22.2 affects nearby protein-coding genes (i.e., the cis-regulatory effect) by testing the correlation between linc18q22.2 expression and the expression of genes residing within 5 Mb, using the microarray data of the NASH cohort and the RNA-seq data of 63 liver samples and 34 HepG2 cell lines from the public SRA-seq data (Supporting Table S3). Analysis of the microarray data did not reveal any genes correlated with linc18q22.2 within 5 Mb. However, the putative protein-coding genes RP11–4104.1 and SOCS6 were associated with linc18q22.2 expression in the public sequencing data (Supporting Table S3 and Fig. S3). The function of RP11–4104.1 is unknown. Depletion of SOCS6 has been linked to suppression of programed cell death and apoptosis. However, we observed the opposite in our cellular models. The protein-coding genes nearby could not explain the observed effect of linc18q22.2 on cell viability.

At the genome-wide level, 1,985 genes were significantly coexpressed with linc18q22.2 at FDR < 0.05 in the in-house microarray data (Fig. 6A; Supporting Table S5). Among these, 984 positively coexpressed genes were enriched for the wound-healing pathway and the regulation of apoptosis and cell death pathways, whereas 1,001 negatively coexpressed genes were enriched for the oxidation reduction pathway (Fig. 6A).

We further performed RNA-seq experiments to profile gene expression in Hep3B and Hep2G cells after knockdown of linc18q22.2; unfortunately, not enough cells could be harvested from the Huh7 knockdown experiment for RNA-seq analysis. From the genes within the 5-Mb region, SOCS6 was down-regulated in both the HepG2 (log2 fold change = −0.41, FDR = 5.09 × 10−5) and Hep3B shRNA1 (log2 fold change = −0.51, FDR = 1.47 × 10−5) knockdown but not in the shRNA2 knockdown. No effect was observed for the putative protein-coding gene RP11–4104.1. At the genome-wide scale, we confined the pathway analysis to genes that were significantly affected at the FDR 0.01 level. In HepG2 cells, 4,045 genes were affected by shRNA1, 3,209 genes were affected by shRNA2, and 1,821 genes were affected by both shRNAs (Fig. 6B). Out of the genes affected by both shRNAs, 1,625 genes (89.2%) showed the same direction of regulation (Supporting Table S6). In Hep3B cells, 4,978 genes were differentially expressed in shRNA1 and 3,766 genes in shRNA2 at FDR 0.01, with 1,724 of the 2,063 shared genes (83.5%) affected in the same direction in both shRNAs (Fig. 6B; Supporting Table S7). Pathway analyses were performed on the 1,625 regulated genes in HepG2 cells and the 1,724 regulated genes in Hep3B cells that were consistently affected by shRNA1 and shRNA2. The down-regulated genes in the knockdown (i.e., those positively regulated by linc18q22.2) were consistently enriched for the pathways of cell death, apoptosis (enriched in antiapoptotic genes), and translation elongation. The up-regulated genes in knockdown (i.e., those negatively regulated by linc18q22.2) were mostly enriched for the oxidation reduction pathway (Fig. 6B). These results are in line with our coexpression analysis of the microarray data.
FIG. 6
(Fig. 6A). The same pathways were enriched in the 201 up-regulated and the 278 down-regulated genes in HepG2 and Hep3B cells (Fig. 6C; Supporting Tables S8 and S9). These genes were most enriched in the translation elongation pathway (FDR = 6 × 10⁻¹⁶) (Fig. 6C). These results indicate that lnc18q22.2 may be directly or indirectly involved in numerous essential biological processes in hepatocytes, including oxidation reduction, translation elongation, and regulation of cell death.

Discussion

In recent years, several lncRNAs have been described to play a functional role in the pathogenesis of different liver diseases.(31) These lncRNAs can serve as biomarkers for use in disease diagnosis, in disease prognosis, or in therapeutic response; and they may also represent direct targets for therapeutic intervention.(37,38) In the current study, we identified lnc18q22.2 as a novel liver-specific lncRNA with elevated expression in the liver of NASH patients. Silencing the expression of lnc18q22.2 resulted in either a lethal phenotype or decreased cell viability in four hepatocyte cell lines. Pathway analysis indicated that lnc18q22.2 might be involved in mRNA translation, cell death, apoptosis, and oxidative reduction. Elevated lnc18q22.2 expression was also observed in patients with steatohepatitis in a mixed patient cohort of both ASH and NASH but not in HCC patients. However, such analysis was performed in publically available data sets (with small sample size and/or without detailed phenotypic information); thus, we cannot support or rule out the role of lnc18q22.2 in ASH, NASH-associated HCC, or other liver diseases. This needs to be further investigated. Moreover, our data do not support the potential of lnc18q22.2 as a noninvasive biomarker for NASH as it was undetectable in circulation, either in plasma or whole blood of NASH patients or controls.

Our data show that genes negatively regulated by lnc18q22.2 are enriched in the process of oxidation reduction. This is consistent with the observation that NAFLD is often accompanied by increased oxidative stress. Reactive oxygen species attack cellular macromolecules such as DNA, lipids, and proteins and have been detected in the liver of NAFLD patients and animal models of NAFLD.(39,40) Expression of lnc18q22.2 was elevated in NASH patients, indicating a putative suppression effect on the genes involved in redox reactions. Additionally, the genes positively regulated by lnc18q22.2 were enriched with pathways in translational elongation. Our data show that lnc18q22.2 is predominantly present in the cytoplasm of HepG2 cells. Cytoplasmic lncRNAs have been described that facilitate mRNA decay, stabilize mRNAs, and promote or inhibit the translation of target mRNAs through extended base-pairing.(41-43) It is possible that lnc18q22.2 regulates the translation of target mRNAs, but this hypothesis needs further experimental validation.

One of the most enriched pathways we observed is the regulation of cell death and apoptosis, which is consistent with the observed cell viability phenotype after lnc18q22.2 knockdown. Hepatocyte apoptosis is a major feature in NASH. To execute both intrinsic (by activating death receptors) and extrinsic (by intracellular stress inducers) apoptosis, liver cells depend heavily on mitochondrial outer membrane permeabilization and its regulation by Bcl-2 proteins.(44-46) Several antiapoptosis genes(47) were down-regulated after lnc18q22.2 knockdown, including MCL1, BCL2L1, BCL2L2, BFAIR, CARD10, IGFIR, and MKL. In line with this, we observed a significant reduction of intact PARP-1 and a significant increase in the number of necrotic nuclei after lnc18q22.2 knockdown. However, we did not detect the appearance of cleaved PARP-1 and/or an increase in apoptotic nuclei. These results pointed to a necrosis-like phenotype, although we cannot exclude that this necrosis was preceded by apoptosis. Similarly, network analysis showed that the increased expression of lnc18q22.2 in NASH livers...
was coexpressed with the genes involved in the negative regulation of apoptosis. In contrast, cell death and fibrosis are increased in NASH patients. Our results suggest that elevated lnc18q22.2 expression might be a protective mechanism against liver damage by inhibiting apoptosis of liver cells. [48] However, the lnc18q22.2 role in NASH development still needs to be studied in vivo, and the primary targets of lnc18q22.2 remain unclear. We do not yet know whether lnc18q22.2 affects cell viability directly or through other pathways such as redox and fatty acid metabolic processes or through translation of target and apoptotic genes. In this study, we performed a cross-sectional transcriptome analysis. To further understand the role of lnc18q22.2 in NASH progression, a longitudinal study should be performed.

In conclusion, our study has identified a liver-specific IncRNA, lnc18q22.2, with elevated expression in the liver of NASH patients. lnc18q22.2 played a crucial role in hepatocyte viability and is likely to play a regulatory role by inhibiting hepatocyte apoptosis and necrosis. The pathway analysis in lnc18q22.2 knockdown cells implicated several biological mechanisms that are also involved in NASH. However, these potential mechanisms need to be studied and validated in vivo.

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Supporting Information

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep.29034/supplinfo.