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Long Noncoding RNAs and Human Liver Disease

Johanna K. DiStefano[1] and Glenn S. Gerhard[2]

[1]Diabetes and Fibrotic Disease Research Unit, Translational Genomics Research Institute, Phoenix, Arizona 85004, USA; email: jdistefano@tgen.org

[2]Lewis Katz School of Medicine, Temple University, Philadelphia, Pennsylvania 19140, USA; email: gsgerhard@temple.edu

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Abstract

Long noncoding RNAs (lncRNAs) are pervasively transcribed in the genome, exhibit a diverse range of biological functions, and exert effects through a variety of mechanisms. The sheer number of lncRNAs in the human genome has raised important questions about their potential biological significance and roles in human health and disease. Technological and computational advances have enabled functional annotation of a large number of lncRNAs. Though the number of publications related to lncRNAs has escalated in recent years, relatively few have focused on those involved in hepatic physiology and pathology. We provide an overview of evolving lncRNA classification systems and characteristics and highlight important advances in our understanding of the contribution of lncRNAs to liver disease, with a focus on nonalcoholic steatohepatitis, hepatocellular carcinoma, and cholestatic liver disease.

1. INTRODUCTION

While most of the human genome is transcribed into RNA (1), only 1–3% of the transcribed sequence corresponds to protein-coding genes (2, 3). The remainder of the transcribed human genome comprises an array of functionally significant elements, including nonprotein-coding transcripts (1, 2), such as ribosomal RNA, transfer RNA, small nuclear RNA, small nucleolar RNA microRNAs (miRNAs), piwi-interacting RNAs, small interfering RNAs (siRNAs), promoter-associated RNAs, enhancer RNAs (eRNAs), and others, as reviewed in Reference 4. Construction of a comprehensive consensus human transcriptome containing the entire set of noncoding and coding RNA transcripts identified nearly 60,000 long noncoding RNAs (lncRNAs), which represent almost 70% of expressed genes (5). The vast number of lncRNAs in the human genome raises important questions about their potential biological relevance, the significance of the substantial number and diversity of lncRNAs, and the role of these molecules in human health and disease. At this time, our understanding of lncRNAs as a group, including lncRNA characteristics and classification strategies, is growing but is not yet complete.

LncRNAs comprise a heterogeneous group of noncoding RNAs loosely classified on the basis of a transcript length >200 nucleotides (3). LncRNAs bind to DNA, RNA, and protein, often through complex three-dimensional interactions and configurations, and participate in a wide range of biological activities, including regulation of protein complexes (6), modulation of gene expression (7), recruitment of histone modifiers to chromatin (8, 9), chromosome inactivation (10), pluripotency and differentiation (11), and regulation of alternative splicing (12). The evidence accumulated to date supports a role for lncRNAs as important regulators of biological pathways underlying processes related to the pathogenesis and progression of human disease. However, while many studies have characterized the functionality of lncRNAs using in vitro models, relatively few have used in vivo approaches to obtain a comprehensive, context-specific annotation of specific transcripts. Furthermore, even though the number of publications related to lncRNAs has increased exponentially in recent years, relatively few have focused on lncRNAs involved in human hepatic physiology and pathology. Here we seek to (*a*) provide an up-to-date overview of evolving lncRNA classification systems and characteristics and (*b*) highlight important advances in our understanding of the contribution of lncRNAs to liver disease, with a focus on hepatocellular carcinoma (HCC), nonalcoholic steatohepatitis (NASH), and cholestatic liver disease.

2. A BRIEF OVERVIEW OF lncRNAs

2.1. Classification of lncRNAs

The concept of lncRNAs as a group is an evolving one. The initial classification of lncRNAs, reflected in the name, was based on length and absence of protein-coding potential (3, 13). As our knowledge of the characteristics and functions of this diverse group of molecules has expanded, different classification systems have emerged to meet these new levels of understanding (14). LncRNAs are commonly defined according to genomic localization and context (13); this classification scheme includes genes that are intergenic [long intergenic noncoding RNAs (lincRNAs)], bidirectional, intronic, sense, and antisense (15). Different classes appear to be enriched for certain features; for example, antisense lncRNA regions have been reported to contain more translated open reading frames (ORFs) than do lincRNAs or host noncoding RNAs (16). For the most part, however, classification by genomic context reveals little about the behavior or biological function of lncRNAs.

A classification system based on level of conservation and specific lncRNA features has also been proposed. In this schema, Class I lncRNAs exhibit conserved exonic structure and multiple regions of sequence homology; Class II lncRNAs are conserved with respect to transcription

and specific RNA elements; and Class III lncRNAs retain greater conservation relative to position, promoter sequences, and transcription within a specific region, with limited sequence or gene structure conservation (17). Class I and II lncRNAs are enriched in the cytoplasm and nucleus, respectively, and differences in conserved functionality, proximity to protein-coding genes, expression levels, and overlap with transposable elements and tissue-specific expression are observed among the three classes (17).

Mukherjee et al. (18) applied a strategy based on RNA processing features to group ~15,000 transcripts in HEK293 cells. The classes varied with respect to expression patterns, gene age, fitness signatures, and response to RNA regulatory pathways and comprised both mRNAs and lncRNAs within individual classes to varying degrees. The individual classes were distinguished by the type of RNA metabolism, evolutionary conservation patterns, and sensitivity to cellular regulatory pathways. These findings are among the first to suggest that a conceptual approach to RNA classification is warranted, as critical insights into lncRNA functionality may be provided. Other investigators have suggested that lncRNA localization patterns may reflect common mechanistic roles, thereby serving as a type of functional classification (19). As attempts to define and classify lncRNAs are predicated on the current knowledge of these molecules, it is likely that classification strategies will become more refined, similar to those for protein-coding genes, as our understanding of lncRNA function grows.

2.2. Characteristics of lncRNAs

Many lncRNAs are transcribed by RNA polymerase II, utilize the same consensus splicing signals as protein-coding genes, and are posttranscriptionally modified at the 5′ and 3′ ends (13). Like mRNAs, lincRNAs are commonly coexpressed with neighboring genes (20) and show variable regulation (21) and expression (22). Some studies have reported similar decay rates for lncRNAs and mRNAs (21, 23, 24), although other studies observed significantly shorter half-lives for lncRNAs (18, 25), with average degradation rates up to 9.6 times higher than those for mRNAs (18). These studies used different methods and in vitro systems to measure RNA stability, which may explain the discrepant findings; however, the main message from all of the studies suggests that regulation of transcript stability is as common for lncRNAs as it is for mRNAs. Despite these similarities, a number of characteristics distinguish lncRNAs from protein-coding genes, many of which are useful for understanding the unique regulatory roles played by lncRNAs.

2.2.1. Low abundance. Most lncRNAs exhibit lower expression levels compared with protein-coding transcripts (13). A tenfold-lower median maximal expression was observed for lncRNAs relative to protein-coding genes across 24 human samples and cell lines (20), while other data indicated that 80% of detected lncRNAs were present at <1 copy per cell (26). Similar results were obtained in a large-scale cap analysis of gene expression followed by sequencing data across 550 tissues and cell types (27).

2.2.2. High tissue-specific expression. Compared with protein-coding genes, lncRNAs show stronger tissue-specific patterns of expression (20, 26). Cabili et al. (20) found that 78% of interrogated lncRNAs were tissue specific, compared with only 19% of mRNAs. Among 15 different cell lines, 29% of all expressed lncRNAs were detected in just one cell line; in contrast, only 7% of protein-coding genes were cell line specific (26).

2.2.3. Reduced splicing efficiency. Results from several studies suggest that lncRNAs are inefficiently spliced compared with protein-coding genes (18, 21, 28). Introns of lncRNAs were

nearly 20 times more likely to have slow intron-incision rates, which are indicative of low splicing efficiency, compared with those from protein-coding genes (18). Features such as distance of introns from transcription start sites and transcription end sites, guanine-cytosine content of introns, and splice site strength were correlated with splicing speed (18), and the number of pyrimidines and branch point differences within internal 3′ splice sites may contribute to splicing differences between lincRNAs and mRNAs (21). Efficiently spliced lncRNAs were more likely to be functional, indicating that efficient splicing may be a critical step in the processing of a subset of lincRNAs with important roles within the cell.

2.2.4. Reduced primary sequence conservation.

In general, primary sequence is less evolutionarily conserved in lncRNAs than in protein-coding genes (20, 29), as evidenced by the number of mouse lncRNA versus mRNA orthologs (**Figure 1**) (30). In lncRNAs that are conserved, features such as longer length, a greater number of exons, and higher and wider expression patterns distinguish them from nonconserved genes (31). Large-scale analysis of RNA-sequencing data across 17 species found that lincRNAs evolve rapidly, with only a small number having sequence-specific orthologs in distantly related species (32). Despite this, evidence for homologs of numerous human lincRNAs in other species was observed, with only short regions of primary sequence conservation being shared (32). This observation is consistent with the presence of conserved exons, transcription initiation regions, regulatory regions, and nuclear localization signals in some lncRNAs (33). Similar levels of conservation of transcription factor binding sites
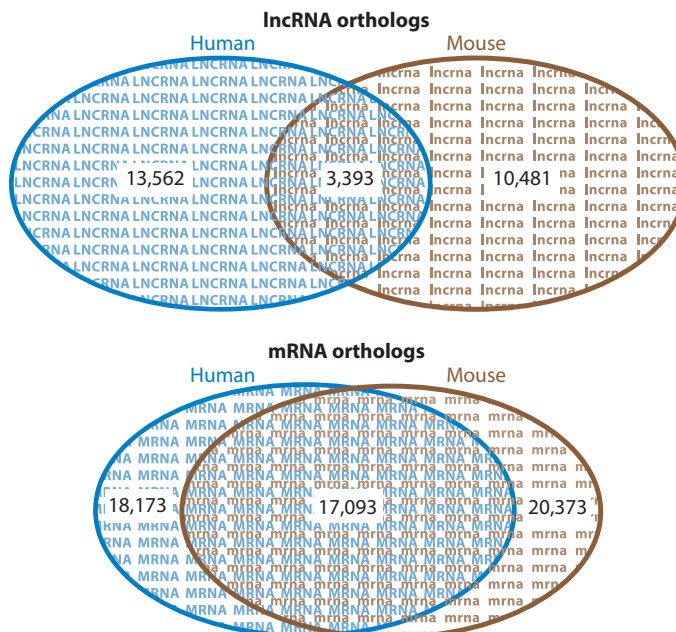

Figure 1

Comparison of the number of human-mouse orthologs for lncRNAs based on GENCODE-annotated human and mouse lncRNAs (30) and mRNAs based on mouse protein-coding genes in homology classes with human genes; 83.9% of mRNAs are orthologous with human, while only 25% of lncRNAs have mouse orthologs. Some data for this figure were retrieved from the Mouse Genome Database, Mouse Genome Informatics, The Jackson Laboratory, Bar Harbor, Maine (**http://www.informatics.jax.org/homology. shtml**, accessed February 20, 2021).

were observed in promoters of lincRNAs and mRNAs (87.4% and 97.8%, respectively), although the average conservation of some transcription factors was actually higher in lincRNA promoters (21). The number of conserved transcription factor binding sites was associated with increased expression and decreased tissue specificity, and lincRNAs with a greater number of conserved sites were more likely to be functional (21, 32, 33). Ribosomal profiling showed that lncRNA regions conserved in humans and mice contain almost three times as many ORFs with evidence for translation than nonconserved ones, and conserved regions in lincRNAs were significantly enriched in protein–RNA interactions compared with nonconserved ones (16).

2.2.5. Different subcellular localization patterns. Several studies have reported that lncRNAs may show a greater bias for nuclear localization than do protein-coding genes (13, 18, 26). Using RNA fluorescence in situ hybridization (RNA-FISH) to explore 61 lncRNAs in three different human cell lines, Cabili et al. (22) observed diverse subcellular localization patterns, with approximately half of all transcripts showing nuclear enrichment. Localization patterns were similar among the different cell types. Of note, some highly correlated lncRNAs and mRNAs (including many sharing the same promoter) showed different localization patterns, suggesting distinct independent functions for coregulated lncRNA/mRNA neighbors (22).

2.2.6. Smaller transcript length and number of exons. On average, lincRNAs have a shorter length (∼1 kb versus ∼2.9 kb) and fewer exons (2.9 versus 10.7) than protein-coding transcripts (20). The reason for this difference is not clear, although it may reflect functional specificity of coding for proteins versus regulating expression.

2.2.7. Differences in core promoter sequence. LincRNA promoters exhibit depletion of transcription factor binding compared with mRNAs with similar expression levels, although enrichment for certain transcription factors, such as the GATA family, JUN, and FOS, has been observed (21). Active lincRNA promoters were depleted for histone marks, with the exception of H3K9me3, a modification associated with transcriptional repression, which was more common in lincRNA promoters. LincRNAs with H3K9me3 showed greater tissue specificity compared with lincRNAs without H3K9me3, despite comparable levels of expression (21). The authors posited that lncRNAs may generally exist in a repressed transcriptional state and become activated only in response to certain stimuli or at precise developmental stages, most likely in a tissue-specific context. A subsequent paper by the same group used the massively parallel reporter assay to further delineate sequence properties of lincRNAs, eRNAs, mRNAs, and divergent lncRNAs and mRNAs in three different cell lines (27). The number of overlapping transcription factor motifs was associated with higher expression and lower cell type specificity, while fewer overlapping motifs were observed in RNAs with higher tissue-specific expression compared with ubiquitously expressed transcripts.

LncRNA characteristics continue to be defined. The work by Mele et al. (21) suggests that posttranscriptional regulation of lincRNAs is highly variable and describes a distribution with inefficiently spliced, lowly expressed lincRNAs with poorly conserved promoter transcription factor binding sites at one end of a spectrum and highly regulated, efficiently spliced lincRNAs with conserved exon-intron junctions and promoter transcription factor binding sites at the other. Differences in other characteristics, such as subcellular localization, transcriptional and posttranscriptional regulation, cell type specificity, and level of expression, belie the idea of a one-size-fits-all lncRNA. Furthermore, the cellular, spatial, or temporal context in which an lncRNA is characterized is likely to have distinct implications for any conclusions that can be drawn. Recent work by Carlevaro-Fita (34) also suggests that cancer-related lncRNAs have

different properties than lncRNAs not associated with cancer. While we are just beginning to understand the deep complexity of lncRNAs, the current acceleration in the pace of lncRNA research is expected to lead to deeper layers of insight in the near future.

2.3. Understanding the Biology of lncRNAs

Characterization of lncRNA function remains a challenging prospect, in part due to the wide range of biological roles performed by these transcripts combined with limitations of existing techniques (35). Moreover, an individual lncRNA may function in a number of different, potentially incongruous, ways depending on spatial or temporal context (4). LncRNAs are widely known to regulate gene expression and do so through a number of diverse mechanisms, including transcriptional regulation, chromatin modification, transcription factor trapping, and methylation (4). In addition, hundreds of publications have reported evidence for lncRNA-mediated regulation of gene expression through miRNA sponging, although the high ratio, in general, of miRNAs to lncRNAs suggests that levels of the latter would need to be abundant enough to mediate miRNA repression (36). Instead, such interactions may reflect differences in spatial expression (37), and experiments that take into consideration subcellular expression patterns may untangle the role of lncRNAs in miRNA repression. LncRNAs can regulate expression through DNA regulatory elements, through the lncRNA itself, or through the act of transcription (35), and these effects can be exerted locally (*cis*) or distantly (*trans*). In some cases, lncRNAs with ORFs encoding peptides with biological roles have been identified (38). Clues to lncRNA functionality can be found in subcellular localization; as noted by Schmitt & Chang (39), nuclear lncRNAs play roles in chromatin interactions, regulation of transcriptional programs, and RNA processing, while those located in the cytoplasm influence mRNA stability, translation, and signaling pathways.

At this time, no general consensus approach to functional characterization of lncRNAs exists. Thorough functional characterization of any given lncRNA is expected to include multiple converging lines of experimental findings involving the delineation of molecular pathways by which the lncRNA exerts effects (35) and consideration of specific developmental and disease contexts (38). For example, many studies have used a loss-of-function approach for studying phenotypes associated with lncRNAs. In one of the first of such studies, Guttman et al. (11) performed an unbiased loss-of-function analysis of lincRNAs expressed in mouse embryonic stem cells; the main findings of this work demonstrated strong evidence of lincRNA functionality and showed that lincRNAs largely affected gene expression through *trans*-acting mechanisms. Knockdown of many lincRNAs caused an exit from the pluripotent state, upregulation of lineage commitment programs, or induction of transcriptional programs associated with specific early differentiation lineages, suggesting that these transcripts function to maintain pluripotency and repress differentiation. Similarly, lncRNAs have been shown to exhibit dynamic expression patterns over different developmental time points across a variety of organ types and species lineages (40).

Sauvageau et al. (41) investigated the functional relevance of lncRNAs across different physiological conditions. Out of 18 lncRNA knockout mouse strains, three (*Fendrr*, *Peril*, and *Mdgt*) exhibited peri- and postnatal lethal phenotypes, while two others (*linc-Pint* and *link-Brn1b*) were associated with developmental defects. Not only did this work reveal important insights about these five lncRNAs, but it also provided a framework in which lncRNA functionality might be explored in vivo.

Some studies have applied multiple genetic approaches to characterize individual lncRNAs in vivo. For example, three distinct genetic mouse models, comprising loss of function, overexpression, and rescue, were implemented to assess the potential role of the *Firre* lncRNA within a hematopoietic context (42). Deletion of the *Firre* locus did not affect viability or fertility in mice,

nor did *Firre* exhibit a local *cis*-regulatory effect in nine different biological contexts. Instead, *Firre* was found to yield cell-specific defects during hematopoiesis, potentiate the innate immune response, and restore gene expression through a *trans*-acting mechanism (42). While this work thoroughly characterized the role of *Firre* in hematopoiesis, the authors indicated that this lncRNA may likely have other roles that vary by biological or disease-related context. Such a caveat is likely applicable to most lncRNAs of interest and should be kept in mind when making general conclusions about a specific transcript.

Genetic models such as those discussed above are important for delineating lncRNA functionality. However, there is not yet an efficient in vivo strategy to assess loss-of-function effects of lncRNAs, particularly those localized to the nucleus (43). Furthermore, a number of challenges limit the usefulness of conventional mouse models, including the amount of time, cost, expertise, and labor it takes to perform genetic studies and the weak conservation of trait-associated human lncRNAs, which limits the degree to which findings in mice can be extrapolated to humans. The development of humanized mouse models, such as the TK-NOG mouse, in which mouse liver is reconstituted with human hepatocytes (44), may circumvent some of the issues related to investigating human lncRNAs in mice (45).

Some researchers have suggested that RNA imaging experiments serve as a first step in lncRNA functional characterization, as knowledge of subcellular localization might provide a framework in which to develop mechanistic hypotheses (19). Single cell quantitation of lncRNAs using a technique such as small molecular RNA-FISH allows assessment of the number and location of lncRNA molecules as well as variability in lncRNA abundance across a population of cells. Efforts to identify functional RNA sequences and domains, such as RNA-mediated localization signals, scaffolding motifs, protein-guidance cues, and catalytic domains, will be critical for a more nuanced understanding of lncRNA functionality (35).

Use of CRISPR (clustered regularly interspaced short palindromic repeats) technology is becoming a common strategy for large-scale identification of functional lncRNAs. CRISPR-mediated interference (CRISPRi), composed of a catalytically inactive CRISPR effector protein, (d)Cas9, fused to a repressive Krüppel-associated box domain and targeted by a single guide RNA, was used to identify nearly 500 lncRNAs that modify robust cell growth (46). Eighty-nine percent of lncRNA gene hits modified growth exclusively in a single cell line and no hits were common to all seven cell lines tested. Interestingly, lncRNA abundance in a cell type was not correlated with cellular phenotype. The specificity of lncRNA function appears to be related to differences in transcriptional networks across cell types. These results underscore the role of cellular context in determining lncRNA function. Other studies have used CRISPR technology to identify functional lncRNAs regulated by the oncogene *MYC* (47) and identify contributors to cytarabine (ara-C) resistance in acute myeloid leukemia cell lines (48). The major strength of CRISPR genome editing is in providing a large-scale, systematic approach to identify loci that are important for a particular phenotype. Direct evidence for the function of a particular lncRNA or information with respect to underlying mechanisms or related pathways is not available with this technique.

3. lncRNAs AND LIVER DISEASE

LncRNAs are emerging as important contributors to biological processes underlying the pathophysiology of human disease (49–51). Several manually curated databases provide updated information on lncRNA-disease associations: At the time of this writing, the LncRNADisease database v2.0 (**http://rnanut.net/lncrnadisease**) reports 2,297 lncRNA causative associations; the Lnc2Cancer database v3.0 (**http://www.bio-bigdata.com/lnc2cancer**) lists 2,659 lncRNAs associated with 216 human cancer subtypes; and the Mammalian ncRNA-Disease Repository

v3.1 (**http://rna-society.org/mndr**) lists almost 40,000 human lncRNA-disease associations. Here we focus on lncRNA involvement in three specific hepatic diseases: NASH, HCC, and cholestatic liver disease.

3.1. Nonalcoholic Steatohepatitis

Nonalcoholic fatty liver disease (NAFLD) describes a chronic, progressive hepatic condition that develops as a result of excessive triacylglycerol deposition in hepatocytes (52). NAFLD encompasses a histological spectrum with simple steatosis at one end and NASH, often accompanied by fibrosis, at the other (53, 54). NAFLD is the most common chronic liver condition in Western populations (55, 56), and the global prevalence of NAFLD is growing (57, 58). In the United States, NASH is the major cause of chronic liver disease and is projected to soon become the most common indication for liver transplantation (59).

Experimental studies linking aberrant lncRNA function with NASH pathogenesis are emerging in the literature. Many studies have reported associations between lncRNA expression and NAFLD, but few of these have provided evidence in support of causality. In this section, we summarize the major findings from in vivo functional studies. Most of these studies evaluated lncRNA candidates in mice treated with carbon tetrachloride (CCl_4) or bile duct ligation (BDL), both of which produce hepatic injury resembling NASH fibrosis, although neither of these models fully recapitulates human NASH.

3.1.1. Alu-mediated p21 transcriptional regulator.

Alu-mediated p21 transcriptional regulator (*APTR*) was first identified in a search for human lncRNAs involved in cell proliferation (60) and was later found to be significantly upregulated in fibrotic livers of CCl_4 and BDL mice and humans with hepatic fibrosis (61). Knockdown of *APTR* in CCl_4-treated mice ameliorated hepatic fibrosis and decreased levels of profibrotic markers (61). *APTR* silencing in primary hepatic stellate cells (HSCs), the main fibrogenic cell type of the liver, reduced levels of fibrotic proteins. Serum *APTR* levels were fourfold higher in cirrhotic patients compared with individuals with normal histology and twofold higher in patients with decompensated cirrhosis compared with those with compensated cirrhosis; these results provide preliminary support that *APTR* levels may have diagnostic value. In general, lncRNAs are detectable in serum and plasma in humans and remain stable enough for molecular analysis (62); these are important considerations given the lack of accurate noninvasive markers of NASH fibrosis. Correspondence between mouse and human findings, independent of the underlying etiology of liver fibrosis, is a promising aspect of this work, although specific spatiotemporal mechanisms by which *APTR* might contribute to fibrogenesis await characterization.

3.1.2. Homeobox transcript antisense RNA.

Homeobox (*HOX*) transcript antisense RNA (*HOTAIR*) is a lincRNA that is widely upregulated in a number of different cancers (63). A role for *HOTAIR* in liver fibrosis was first suggested when *HOTAIR* expression was found to be elevated in CCl_4-treated mice compared with control animals (64). In that study, *HOTAIR* expression was also increased in primary HSCs and hepatocytes from CCl_4-treated mice as well as in primary HSCs from healthy mice following transactivation in culture. *HOTAIR* knockdown suppressed CCl_4-induced hepatic injury and reduced accumulation of collagen and alpha-smooth muscle actin (α-SMA) in vivo and in vitro and also inhibited HSC proliferation and cell cycle. Mechanistically, *HOTAIR* knockdown was found to restore miR-29b levels, which repressed DNA methyltransferase 3b (*DNMT3b*), leading to reduced methylation of phosphatase and tensin homolog (*PTEN*) and a subsequent increase in *PTEN* levels. *PTEN* inhibited features of HSC activation, including

cell proliferation, collagen, and α-SMA expression, consistent with fibrogenesis. Overexpression of *HOTAIR* reversed these effects.

Similar findings were reported by Bian et al. (65), who demonstrated that *HOTAIR* regulates expression of maternally expressed gene 3 (*MEG3*) by sequestering miR-148b, which relieves inhibition of DNA methyltransferase 1 (*DNMT1*) expression and enhances methylation of *MEG3*; these results are in line with an earlier study showing increased *DNMT1* expression and *MEG3* promoter methylation in livers of CCl_4-treated mice and human fibrotic liver tissue (66). In addition, *HOTAIR* was shown not only to enhance polycomb repressive complex 2 (*PRC2*) occupancy and histone H3K27me3 repressive marks in the *MEG3* promoter but also to recruit *PRC2* to the *MEG3* promoter through formation of an RNA/DNA hybrid. These results are consistent with previous work demonstrating that *HOTAIR* regulates gene expression through interaction with *PRC2* and increased trimethylation of H3K27 (67). *HOTAIR* is localized to both the cytoplasm and the nucleus (68), concordant with the dual roles identified in this study.

3.1.3. Liver fibrosis-associated lncRNA 1.
Liver fibrosis-associated lncRNA 1 (*LFAR1*) was first identified in a microarray analysis to profile lncRNAs in CCl_4-treated mice, with increased expression occurring in HSCs (69). *LFAR1* depletion in CCl_4-treated and BDL mice improved hepatic fibrosis and corresponded with reduced levels of hepatic hydroxyproline content; alanine transaminase; aspartate transaminase; and profibrogenic, proinflammation, and proapoptosis gene expression. In mechanistic studies, the authors demonstrated that (*a*) *lnc-FAR1* promotes association of Smad2/3 with TgfβR1, which then phosphorylates Smad2/3 in the cytoplasm, and (*b*) *lnc-FAR1* binds directly to Smad2/3 to regulate transcription of a number of genes, leading to activation of the Tgfβ and Notch pathways. *LFAR1* was also found to promote intrahepatic cholangiocarcinoma proliferation and invasion through a similar pathway (70). Furthermore, *LFAR1* knockdown in vivo ameliorated proinflammatory M1 macrophage activation and NLRP3 inflammasome-mediated pyroptosis induced by CCl_4 and BDL (71), suggesting an additional mechanism by which the lncRNA might affect fibrogenesis. Despite these promising findings, it is not clear if there is a human ortholog of *LFAR1*.

3.1.4. Metastasis-associated lung adenocarcinoma transcript 1.
Metastasis-associated lung adenocarcinoma transcript 1 (*MALAT1*) promotes cell proliferation, migration, and invasion in several different human cancers, including HCC (72). Hepatic *MALAT1* expression was significantly upregulated in CCl_4-treated mice and in HSCs and hepatocytes isolated from CCl_4-treated animals, and *MALAT1* knockdown in these mice resulted in decreased collagen accumulation (73). Mechanistically, *MALAT1* sequestered miR-101b, leading to activation of RAS-related C3 botulinum substrate 1 (Rac1) and promoting proliferation, cell cycle progression, and activation of HSCs. Levels of *MALAT1* and *Rac1* were increased in patients with liver cirrhosis, suggesting that the same network may play a role in human fibrosis (73), while other studies have shown that hepatic *MALAT1* levels are higher in NASH patients with fibrosis (74), increase with NAFLD severity (75), and may promote NAFLD progression through regulation of Janus kinase–signal transducer and activator of transcription signaling (75).

3.1.5. Nuclear-enriched abundant transcript 1.
Emerging work suggests that nuclear-enriched abundant transcript 1 (*NEAT1*) accelerates the progression of liver fibrosis (76) and is associated with cell proliferation, invasion, and migration in HCC (77). *NEAT1* expression was elevated in whole livers and primary HSCs derived from CCl_4-treated mice, while *NEAT1* knockdown attenuated CCl_4-induced liver fibrosis in these animals and reduced proliferation and markers of fibrosis in primary HSCs (76). In both HSCs and hepatocytes, *NEAT1* effects were

mediated by Krüppel-like factor 6 (*KLF6*) through a mechanism involving miR-122, and levels of *NEAT1* and *KLF6* were increased, while miR-122 levels were decreased in human cirrhotic liver tissues (77). We also observed elevated levels of *NEAT1*, although not *KLF6*, in liver tissue from NASH patients with advanced fibrosis (74). Of note, hepatic *NEAT1* expression was increased in high fat diet–induced animal models of NAFLD (78–80), suggesting that aberrant expression occurs early in NAFLD pathogenesis, but these biological effects may be mediated by alternative signaling pathways.

As described above, most of the lncRNAs that have been implicated in NASH have been identified using animal models of hepatic fibrosis, largely CCl_4-induced fibrosis. CCl_4 is a common method for inducing liver fibrosis, and like fibrogenesis attributed to NAFLD in humans, it causes HSC activation, dysregulated extracellular matrix production and degradation, and progressive hepatic fibrosis (81). However, CCl_4 causes hepatic inflammation, and the inherent toxicity of the compound alters liver physiology in a way that does not recapitulate NAFLD fibrogenesis in humans (82). Despite these limitations, the replication of findings between CCl_4-treated animals and patients with hepatic fibrosis in a number of studies warrants further investigation of these lncRNAs in NASH, especially *APTR*, *HOTAIR*, *MALAT1*, and *NEAT1*.

3.2. Hepatocellular Carcinoma

HCC is the most common form of liver cancer (83) and the fastest rising cause of cancer-related death in the United States (84). The major risk factors for HCC initiation are viral infection, NAFLD/NASH, and chronic alcohol consumption (85). Although diagnostic and treatment options have improved in recent years, the five-year survival rate for advanced HCC remains bleak (86). In general, HCC is a difficult disease to diagnose and treat, in part because the molecular mechanisms underlying the malignant transformation of hepatocytes remain only partially understood (87).

The investigation of lncRNAs in the initiation, progression, metastasis, and development of chemoresistance of HCC has steadily accelerated within the past decade. While numerous publications have reported dysregulated lncRNA expression in HCC (88–98), relatively few studies have focused on functional characterization of HCC-associated lncRNAs, in part because many of these lncRNAs are specific to humans and not readily amenable to in vivo experiments. In this section, we focus on two well-characterized lncRNAs in HCC, highly upregulated in liver cancer (*HULC*) and *HOTAIR*.

3.2.1. *HULC.* *HULC* was first identified as a spliced and polyadenylated transcript that was highly expressed in HCC (99). An absence of ORFs with a significant number of amino acids or a detectable protein product led the authors to suggest that *HULC* was a noncoding transcript. *HULC* was found to localize to the cytoplasm and copurify with ribosomes of carcinoma cells, and while the lncRNA showed conservation in primates, no homologs were detected in the mouse or rat genome (99). A cAMP response element binding site in the *HULC* proximal promoter region was found to be critical for transcriptional activity in liver cancer (100). In addition to upregulation in HCC, elevated *HULC* levels have been associated with clinical-stage intrahepatic metastases, HCC recurrence, and postoperative survival (99, 101–104).

A number of studies have offered mechanistic insight into *HULC* function. *HULC* was found to upregulate peroxisome proliferator-activated receptor alpha, which activates the acyl-CoA synthetase subunit (*ACSL1*) to promote lipogenesis and alter lipid metabolism in hepatoma cells (105). Of interest, overexpression of *ACSL1* resulted in excessive cholesterol levels, which enhanced cell proliferation, while treatment with cholesterol induced *HULC* expression. Treatment of BALC/c

athymic nude mice with *ACSL1* siRNA abrogated *HULC*-mediated proliferation of hepatoma cells in these animals (105).

HULC expression levels were found to be positively correlated with high mobility group A2 (*HMGA2*), a known oncogene, in HCC (106). The authors demonstrated that overexpression of *HULC* enhanced proliferation of hepatoma cells, while inhibition of *HMGA2* and overexpression of miR-186, a microRNA that targets *HMGA2*, suppressed it. Interestingly, *HULC* also interacted with miR-186, suggesting that elevated *HULC* levels might effectively sequester miR-186, leading to the derepression of *HMGA2* and resulting in enhanced tumorigenesis. These findings were re-iterated in a tumor xenograft model in which *HULC* and *HMGA2* levels were elevated while those of miR-186 were reduced. In these animals, *HULC* overexpression was associated with increased tumor weight and volume, consistent with other reports (107), which was mitigated by *HMGA2* silencing. Results from this comprehensive study support a mechanism by which *HULC* promotes hepatocarcinogenesis through an axis involving *HMGA2* and miR-186.

Y-box protein 1 (YB-1), a member of the cold-shock protein family, was identified as a *HULC* binding partner using a combination of RNA pull-down and mass spectrometry (104). Despite the specific interaction between *HULC* and YB-1, modulation of *HULC* expression had no effect on YB-1 protein levels. Because the interaction between *HULC* and YB-1 was localized predominantly to the cytoplasm, where YB-1 is known to regulate mRNA translation following phosphorylation, the authors hypothesized that *HULC* might modulate phosphorylation of YB-1. Indeed, overexpression or knockdown of *HULC* increased or reduced the phosphorylation of YB-1, respectively, and appeared to do so in a dose-dependent manner. *HULC* was also found to modulate the phosphorylation of a YB-1 interaction protein, extracellular signal-regulated kinase, resulting in the release of YB-1 from YB-1–mRNA complexes; disinhibiting translation of tumor-associated mRNAs such as cyclin D1, cyclin E1, and matrix metalloproteinase 3; and leading to enhanced cell proliferation. While these findings support an alternative mechanism by which *HULC* might promote hepatic tumorigenesis, it will be important to confirm this pathway in vivo.

HULC has been shown to interact with *MALAT1*, which is also upregulated in human HCC, to promote growth of liver cancer stem cells (108). Mechanistically, increased *HULC* and *MALAT1* levels led to the recruitment of key transcription factors to the promoter of telomere repeat-binding factor 2 (*TRF2*), and together, the two lncRNAs and *TRF2* formed a complex on the telomeric region, which had the effect of protecting the telomere and enhancing its elongation (108). Using a xenograft tumor model, *HULC* and *MALAT1* increased tumor weight, which was attenuated by *TRF2* knockdown.

In addition to these studies, *HULC* has been found to trigger autophagy through sirtuin 1–mediated mechanisms in HCC (107, 109), further supporting a biological role for *HULC*, which may represent a potential target for the development of agents with which to treat the HCC.

Circulating *HULC* levels are elevated in HCC patients, reflect expression levels in the cancer, and are associated with tumor aggressiveness and progression (99, 102, 103). *HULC* was also detected more frequently in HCC patients with hepatitis B virus (HBV) versus those without HBV (90% versus 25%) (102). Receiver operating characteristic curve analysis for *HULC* was 0.78 (103). While the prognostic power of *HULC* requires further substantiation by longitudinal analysis in prospective studies, these reports provide a significant step toward establishing the utility of *HULC* expression as a prognostic indicator for HCC.

3.2.2. *HOTAIR*. While functional characterization studies for *HULC* have been comprehensive, emerging evidence also tentatively supports a functional role for *HOTAIR* in HCC. *HOTAIR* was first identified in primary human fibroblasts in a screen of HOX loci (67) and later found to be highly expressed in HCC tumors (110, 111). Patients with elevated *HOTAIR* levels had a higher

recurrence of HCC following liver transplantation, shorter recurrence-free survival, and greater risk of metastasis (110, 111). Functionally, *HOTAIR* knockdown was associated with decreased cell viability, proliferation, and invasion; increased tumor necrosis factor alpha–mediated apoptosis; pronounced sensitivity to chemotherapeutic agents; and reduced levels of genes associated with cell motility and metastasis (110–113).

An early study to profile changes in mRNA expression following *HOTAIR* knockdown in hepatoma cells identified RNA binding motif protein 38 (*RBM38*) as a key *HOTAIR*-regulated gene (112). In HCC patients, *RBM38* levels were also elevated in tumors relative to adjacent nontumor paired samples. In addition to increasing *RBM38* mRNA and protein levels, *HOTAIR* knockdown corresponded with reduced HCC cell migration and invasion, which was rescued by *RBM38* downregulation. Other studies have demonstrated an array of functional roles for *HOTAIR* in HCC cell models, including activation of autophagy in HCC cell lines (93), G0/G1 cell cycle arrest (114), and downregulated expression of Wnt and β-catenin (115). Combined, these studies suggest that like *HULC*, *HOTAIR* likely contributes to HCC pathogenesis through multiple signaling pathways.

In vivo studies have provided important insight into *HOTAIR* functionality. *HOTAIR* was shown to negatively regulate miR-218 expression in HCC, through a promoter regulatory axis involving EZH2-targeting miR-218 (116). In vitro, *HOTAIR* knockdown inhibited HCC cell viability and induced G1-phase arrest, while in a xenograft model, *HOTAIR* depletion suppressed tumorigenicity through disinhibition of miR-218 expression. The *Bmi-1* oncogene was identified as a functional target of miR-218, which was activated in *HOTAIR*-suppressed tumorigenesis. In primary human HCC specimens, *HOTAIR* and *Bmi-1* were upregulated, whereas miR-218 was downregulated in these tissues. Furthermore, *HOTAIR* was inversely associated with miR-218 expression and positively correlated with *Bmi-1* expression in these clinical tissues.

In an investigation of *HOTAIR*, forkhead box C1 (*FOXC1*), and miR-1, levels of *HOTAIR* and *FOXC1* were increased, while levels of miR-1 were decreased in HCC tissues and HepG2 cells compared with normal liver cells and adjacent nontumor tissues (117). Overexpression of *HOTAIR* in the immunodeficient nude mouse model (nu/nu) resulted in enhanced HCC cell proliferation and progression of tumor xenografts. Functional characterization studies showed that FOXC1 binds an upstream region of *HOTAIR* and activates its expression in HCC cells, while *HOTAIR* negatively regulates miR-1 expression. Results from this work suggested that *HOTAIR* is a *FOXC1*-activated driver of malignancy, which acts in part through the repression of miR-1.

Since its annotation in 2007, *HOTAIR* has emerged as a novel prognostic marker for HCC. While a number of studies have indicated multiple pathways by which *HOTAIR* may affect HCC cell proliferation and invasion, further investigation of the molecular mechanisms underlying dysregulated *HOTAIR* expression and the manner in which the lncRNA promotes HCC progression is necessary to nominate its use as a potential therapeutic target in the treatment of HCC.

3.3. Cholestatic Liver Disease

Cholestatic liver diseases, including primary sclerosing cholangitis (PSC) and primary biliary cirrhosis (PBC), encompass conditions in which normal bile flow from the liver is obstructed (85). If unresolved, intrahepatic accumulation of bile acids can lead to hepatocyte injury, macrophage infiltration, inflammation, fibrosis, and malignant proliferation of cholangiocytes. As in NASH, HSC activation plays a critical role in the progression of liver fibrosis in chronic cholestatic liver diseases (118).

Obstruction of the bile duct, drug-induced liver toxicity, pregnancy, and autoimmune disease are known to cause cholestasis (119), although the molecular mechanisms underlying the pathogenesis of cholestatic liver diseases continue to be characterized. LncRNAs have been

linked to cholestatic liver injury, including cholangiocarcinoma (120), but to date, the lncRNA that has been the best characterized in cholestasis is *H19*.

H19 encodes an imprinted, maternally expressed lncRNA (121) that is primarily expressed during embryonic development (122). This gene was first identified in fetal mouse and human liver (122), and its expression is repressed after birth (123). Although *H19* is nearly undetectable in adult human liver, its expression is elevated in hepatic fibrosis (124–127). *H19* expression is also induced in liver and gastric cancers (128, 129), has been shown to play a role in cell proliferation (130), and may contribute to the development of some cancers (131). In the liver, *H19* has an exclusively cytoplasmic localization (132).

Hepatic *H19* expression was observed to be highly induced in mice who developed severe cholestatic liver fibrosis due to overexpression of *Bcl2* (124). A subsequent study by this group reported that hepatic *H19* expression was significantly increased in BDL mice, a model of obstructive cholestatic injury in rodents, and in PSC and PBC liver tissue compared with normal adult liver (125). Hepatic overexpression of *H19* exacerbated liver injury in BDL mice compared with null-BDL animals, while *H19*-deficient mice showed a marked reduction in cholestatic liver fibrosis compared with control mice (125). *H19* was also found to decrease hepatic zinc finger E-box binding homeobox 1 (*ZEB1*) and increase epithelial cell adhesion molecule (*EpCAM*) expression in BDL mice; overexpression of *ZEB1* or knockdown *EpCAM* attenuated *H19*-induced fibrosis in these animals. Increased hepatic *H19* expression and association with fibrosis were also observed in the multidrug resistance 2 knockout (Mdr2$^{-/-}$) mouse, a model of PSC, although aberrant expression occurred only in female mice (133). *H19* knockdown in female Mdr2$^{-/-}$ mice improved hepatobiliary injury and liver fibrosis. Aberrant *H19* expression was associated with downregulation of the nuclear receptor small heterodimer partner (*SHP*), which was consistent with earlier findings (124). In addition, hepatic *H19* levels were significantly elevated in PSC patients.

In a study by Li et al. (133), *H19* was expressed mainly in cholangiocytes, but significant upregulation was observed in hepatocytes of mice with severe cholestatic liver injury, suggesting the possibility that *H19* is secreted by one cell type to be taken up by another. This finding was consistent with the detection of *H19* RNA in the interspace with neighboring cells under severe cholestatic conditions (132). Subsequently, *H19* was found to be transferred from cholangiocytes to hepatocytes by extracellular vesicles (EVs) (127). Cholangiocyte-derived EVs carrying *H19* from wild-type mice, but not *H19*$^{-/-}$ mice, were also able to suppress *SHP* expression in hepatocytes. Interestingly, circulating levels of exosomal *H19* gradually increase during hepatobiliary disease progression in Mdr2$^{-/-}$ mice, as well as in PSC patients with cirrhosis (127) and individuals with biliary atresia, a neonatal liver disease featuring cholestasis and severe liver fibrosis (134). Whether EV-mediated transfer of lncRNAs is a primary pathophysiological mechanism or may be useful as a potential biomarker of disease is not clear.

Treatment of young Mdr2$^{-/-}$ mice with serum-derived *H19*+ exosomes from aged Mdr2$^{-/-}$ mice with fibrosis resulted in liver fibrosis in the exposed animals (127). Furthermore, transplanted cholangiocyte-derived *H19*-enriched EVs were also shown to be rapidly and preferentially taken up by HSCs and were able to promote liver fibrosis in H19-deficient BDL mice (126), and EV-derived *H19* was similarly shown to enhance the activation of Kupffer cells (135).

Combined, these data suggest that cholangiocytes are the primary source of hepatic and EV-derived *H19* under cholestatic and fibrotic conditions. In Mdr2$^{-/-}$ mice, cholangiocyte-derived EVs were preferentially taken up by HSCs (50–70%), compared with CD45+ immune cells (18%) and hepatocytes (27%), suggesting that HSCs are the major target cells for EVs (126). However, a study by Jiang et al. (132), who used a combined in situ hybridization and immunofluorescence colabeling technique, showed that *H19* was not detectable in cholangiocytes (CK19$^+$) or stellate cells (desmin+) in cholestatic livers from BDL, Mdr2$^{-/-}$, PSC, and PBC

Table 1   lncRNAs associated with liver diseases

lncRNA	Human ortholog[a]	Model	Direction	Pathway	Reference(s)
APTR	No	CCl$_4$ mice	Increased	p21	61
		BDL mice	Increased		61
		Human fibrotic liver	Increased		61
H19	Yes	BDL mice	Increased	*ZEB1/EpCAM*	125
		Mdr2$^{-/-}$ mice	Increased		133
		PBC	Increased		125
		PSC	Increased		125
HOTAIR	Yes	CCl$_4$ mice	Increased	miR-148b/*MEG3/DNMT1*	65
				miR-29b/*DNMT3b/PTEN*	64
		Human HCC	Increased	*RBM38*	110–112
HULC	No	Human HCC	Increased	*PPARA/ACSL1*	99, 101–105
LFAR1	No	CCl$_4$ mice	Increased	Smad2/3–TgfβR1	69
MALAT1	Yes	CCl$_4$ mice	Increased	miR-101b/*Rac1*	73
		Human HCC	Increased		72
		Human fibrotic liver	Increased	JAK/STAT	73–75
NEAT1	Yes	CCl$_4$ mice	Increased	miR-122/*KLF6*	76
		Human fibrotic liver	Increased		74, 76
		Human HCC	Increased		77

Abbreviations: *ACSL1*, acyl-CoA synthetase subunit; BDL, bile duct ligation; CCl$_4$, carbon tetrachloride; *DNMT1*, DNA methyltransferase 1; *DNMT3b*, DNA methyltransferase 3b; *EpCAM*, epithelial cell adhesion molecule; HCC, hepatocellular carcinoma; JAK, Janus kinase; *KLF6*, Krüppel-like factor 6; lncRNA, long noncoding RNA; Mdr2, multidrug resistance 2; *MEG3*, maternally expressed gene 3; PBC, primary biliary cirrhosis; *PPARA*, peroxisome proliferator-activated receptor alpha; PSC, primary sclerosing cholangitis; *PTEN*, phosphatase and tensin homolog; *Rac1*, RAS-related C3 botulinum substrate 1; *RBM38*, RNA binding motif protein 38; STAT, signal transducer and activator of transcription; ZEB1, zinc finger E-box binding homeobox 1.
[a]Based on the ortholog search program at Southern Medical University (**http://lncrna.smu.edu.cn/show/info1**).

livers and instead was partially colocalized with HNF4α+ hepatocytes, SOX9+ progenitor cells, and F4/80+ Kupffer cells in periportal areas. While the discrepancy between the studies might be due to contamination of CK19$^+$/*H19*$^-$ cholangiocytes from neighboring CK19$^-$/*H19*$^+$ cells (132), cholangiocytes purified using the sensitive methods of immunopurification and laser-capture microdissection yielded similar results (126).

4. CONCLUSIONS

Our understanding of lncRNAs has advanced rapidly in recent years and continues to expand at a brisk pace. Despite this momentum, much still remains to be discovered. For example, what fraction of lncRNAs in the human genome are functional, and to what extent do lncRNAs contribute to the pathogenesis of human diseases? Issues related to lncRNA annotation persist, largely because annotation efforts are hindered by the low expression of lncRNAs, a limited understanding of the lncRNA sequence-function relationship, and the weak level of conservation of lncRNAs among species (136). At this time, the biological significance of the vast majority of lncRNAs remains poorly understood, and even the term lncRNA itself needs revision to reflect the broad diversity of genes currently grouped under this designation. Improved methods for annotation, localization, and screening; better biological models; and more effective ways to investigate the therapeutic potential of lncRNAs are warranted (51). These factors likely contribute to the paucity of available information on lncRNAs, as summarized in **Table 1**.

An emerging aspect of lncRNA biology is the presence of lncRNAs in the circulation. Symptoms of liver diseases such as NASH and HCC are often silent, and diagnosis usually occurs only after significant disease advancement. While HCC can be detected using imaging modalities (137), the reference standard for the diagnosis and staging of liver fibrosis is histological examination of biopsied tissue, which is associated with several shortcomings, including patient discomfort, risk for complications, sampling error and bias, variability in histopathologic interpretation, and financial cost (138). Accurate, inexpensive, and noninvasive strategies to detect unsuspected liver disease would mitigate morbidity and mortality associated with NASH and HCC (139). Because lncRNAs are often cell type and tissue specific, and can be released into circulating blood where they exhibit stability, the potential application of these molecules as novel biomarkers of various human diseases, including NASH (140, 141) and HCC (98), may eventually yield significant clinical impact (142, 143). Already, prostate cancer associated 3, an lncRNA abundantly expressed in the vast majority of prostate cancers, is regarded as a highly accurate biomarker for the clinical diagnosis of prostate cancer (144, 145). To date, however, the investigation of circulating lncRNAs as biomarkers of liver disease has been limited, although the potential of these molecules to predict disease progression is high.

At this time, data from animal models and human patients, though still sparse, provide compelling evidence supporting an involvement of functionally relevant lncRNAs in liver diseases, though care must be taken to ensure the external validity of animal models. Given the number of as-yet-uncharacterized lncRNAs, much more research needs to be conducted to understand the molecular mechanisms by which lncRNAs contribute to liver diseases, the hepatic cell types and time points in disease pathogenesis when lncRNAs are activated or repressed, and the importance of the expression and molecular function of lncRNAs in hepatic physiology and pathology.

DISCLOSURE STATEMENT

ACKNOWLEDGMENTS

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