Our emerging understanding of the roles of long non-coding RNAs in normal liver function, disease, and malignancy

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Summary
Long non-coding RNAs (lncRNAs) are important biological mediators that regulate numerous cellular processes. New experimental evidence suggests that lncRNAs play essential roles in liver development, normal liver physiology, fibrosis, and malignancy, including hepatocellular carcinoma and cholangiocarcinoma. In this review, we summarise our current understanding of the function of lncRNAs in the liver in both health and disease, as well as discuss approaches that could be used to target these non-coding transcripts for therapeutic purposes.

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Introduction
Seventy percent of the human genome is transcribed, yet only 2–3% of the genome encodes RNA transcripts that are translated into proteins.1 Most biomedical research has focused on protein-coding genes, because proteins are considered the primary molecular building blocks that control the structure and function of cells. Recent advances in genome-wide RNA sequencing approaches have revealed the abundance and diversity of non-coding (nc) RNAs, and these RNA transcripts are increasingly recognised for their diverse biological functions in eukaryotic cells.

ncRNAs are primarily classified by their mechanism of transcription and mode of action. Most ncRNAs, including ribosomal and transfer RNA molecules, are derived through transcription by RNA polymerase (RNA Pol) I and RNA Pol III. RNA Pol II also transcribes various classes of small ncRNAs including microRNAs (miRNAs), small nucleolar RNAs, and piwi-interacting RNAs, which have been reviewed in detail previously.3 The functions of longer RNA Pol II-transcribed ncRNAs, commonly referred to as long non-coding RNAs (lncRNAs) have more recently piqued the interest of researchers.3

What constitutes an lncRNA is still a matter of debate. However, lncRNAs are commonly defined as ncRNA transcripts that are greater than 200 nucleotides in length, which separates them from small ncRNAs such as miRNAs. Similar to messenger (m) RNAs, lncRNAs are 5′-capped and commonly contain a poly-adenylated tail at their 3′-end.4 Interestingly, a few lncRNAs have been described that contain an RNase P-catalysed triple-helical structure at their 3′-ends instead of a poly-adenylated tail, presumably increasing transcript stability.5

In comparison to mRNAs, lncRNAs tend to be shorter transcripts and contain fewer exons.6 The half-lives of lncRNAs are variable, but overall lncRNAs tend to be less stable than mRNAs.7,8 Decreased stability of lncRNAs helps to explain why transcription at genes encoding lncRNAs is closer in level to transcription at protein-coding genes, while there is a greater difference in expression of mature mRNAs and lncRNAs, as measured by RNA sequencing.9

Many lncRNAs include one or multiple open reading frame (ORF) regions for protein synthesis.10 Yet, possible ORFs in lncRNA transcripts are often not translated or, if translated, the resulting protein product is unstable and rapidly subjected to degradation; therefore these protein products are not thought to play a substantial biological role.4

The liver is an essential organ in the gastrointestinal system, which mediates numerous digestive and metabolic functions. Recent work from many research laboratories has led to an increased understanding of the role of lncRNAs in liver development, physiology, and pathology. We will review the identification and classification of lncRNAs, general mechanisms of action, our current understanding of lncRNA activities in the context of the liver, and the potential of lncRNAs as therapeutic targets in the treatment of liver disease.

Identification
Early efforts to identify and characterise lncRNAs employed sequencing of expressed cDNA libraries and expression sequence tags (ESTs).11 However, because of the inherent laboriousness of this strategy, few lncRNAs were characterised.
Subsequent approaches such as chromatin profiling by immunoprecipitation followed by high-throughput sequencing drastically improved the lncRNA discovery process. Regions containing specific histone marks, such as trimethylation of histone 3 lysine 4 (H3K4me3) and histone 3 lysine 36 (H3K36me3), define areas of active transcriptional initiation and elongation and were employed to identify lncRNA transcripts that do not overlap with protein-coding genes.12

With the advent of next-generation RNA-sequencing, lncRNA identification underwent a significant revolution.13 With this approach, short RNA-seq reads were used to assemble a transcriptome model by mapping reads to a reference genome to identify exons and splice junctions between exons. The assembled transcriptome was then further subjected to a multitude of algorithms to filter out transcripts that have coding potential.14 This computational strategy involved significantly less manual work than earlier approaches. However, this method relies heavily on the error-prone de novo construction of transcript sequences from short reads, which can lead to the assembly of incomplete transcripts. With this limitation in mind, defining the 5’ and 3’ ends of the lncRNA through approaches such as rapid amplification of cDNA ends (RACE),15 and cloning the full-length lncRNA transcript in a specific cell type of interest are good starting points to define the exons of poorly characterised lncRNAs.

Another alternative method that employs long-read sequencing is increasingly being utilised to sequence full-length transcripts.16 The promise of this newer method is the increased accuracy of the exons and splice junctions contained in each transcript, but this comes at a cost of an increased rate of sequencing errors and decreased genome coverage when performed at the same price point as short-read sequencing.

With notable exceptions, most lncRNAs do not possess cross-species sequence homology.17 However, this does not by any means suggest that lncRNAs are not or cannot be evolutionarily conserved. In contrast to the conservation of protein-coding genes, which rely heavily on the sequence homology to maintain amino acid sequence, lncRNA conservation may be enforced at the level of the RNA structure, function, and or syntenic expression pattern. The last criterion, which relies on conserved location in the genome relative to proximal genes, is often used to identify human orthologs of lncRNAs that are initially found in model organisms (e.g. mice).18 Even taking these additional criteria into account, it is estimated that only a little over one-third of human lncRNAs have orthologous transcripts in mice.19 Although these multiple levels need to be considered when addressing lncRNA conservation, it offers a far better insight into the mechanisms of lncRNA evolution across different organisms.

Through these efforts, a large collection of annotated lncRNAs have been described in an increasing number of organisms and are accessible through a multitude of sources, including NONCODE,20 GENCODE,21 RNAcentral,22 and LNCipedia23 databases. Fig. 1 shows the number of genes encoding lncRNAs and the number of different lncRNA transcripts annotated in different species. The increased number of lncRNAs in humans and mice compared to other vertebrates likely reflects the greater depth of sequencing analysis in these two species.

**Diverse genomic origins of lncRNAs**

LncRNAs are classified according to their sites of transcription relative to annotated protein-coding genes24 (Fig. 2). Transcripts that do not overlap with known protein-coding or small RNA-coding genes are termed long intervening non-coding (linc) RNAs.12,27 LincRNAs are also often described as intergenic non-coding RNAs in the literature. This category of lncRNAs was easier to identify because of their distance from known genes, and many of the earliest described lncRNAs, including H19,28 X-inactive specific transcript (XIST),26,27 and HOX transcript antisense RNA (HOTAIR),28 fall into this category.

Natural antisense transcripts (NATs) are widely expressed in the human genome. NATs are the result of transcription on the opposite strand to the sense protein-coding gene such that the non-coding transcript is the reverse complement of a region of the protein-coding transcript.29 NATs are understood to function predominantly in cis (i.e. acting in proximity to where they are transcribed) by regulating the sense protein-coding transcript.30 One mechanism proposed to explain how NATs regulate gene expression is through the formation of duplexes in which the sequence complementarity between NATs and the sense transcripts interferes with the recruitment of the splicing machinery, resulting in skipped exons or incomplete splicing through a process called RNA masking.31 In an alternative mechanism, the RNA duplex may serve as an immediate substrate for either RNA editing32 or promote degradation through the RNA interference pathway.33

**Key points**

- Long non-coding RNAs (lncRNAs) are emerging as critical biological mediators in the normal functioning of the liver.
- Aberrant expression of lncRNAs is associated with metabolic diseases, fibrosis, and malignancies involving the liver.
- LncRNAs exert their pathobiological effects through a multitude of mechanisms.
- Liver-specific targeting of lncRNAs is a promising novel treatment modality.
- LncRNAs have potential as biomarkers in liver disease.

Fig. 1. Quantifications of lncRNA genes and their transcripts in multiple species as deposited in the release of NONCODE v.5 database. Transcripts consider the number of lncRNA isoforms identified for each lncRNA gene.
Divergent lncRNAs are transcribed head-to-head from protein-coding genes so that the transcription start sites of the lncRNA and protein-coding genes are in close proximity and are oriented in opposite directions. Transcription of the divergent lncRNA can be facilitated by a shared bi-directional promoter that induces the expression of both lncRNA and protein-coding genes. A criterion that is increasingly being used to define this class of lncRNAs is whether the lncRNA transcription starts within 300–500 base pairs of the transcription start site of a protein-coding gene.

Enhancer RNAs (eRNA) are another novel class of RNA species and are exclusively expressed at enhancer regions. Most eRNA transcripts are divergently transcribed (2D-eRNA) from enhancers, are composed of single exons, and are non-polyadenylated. Because of the relatively open chromatin structure at enhancers, these eRNAs are considered to be the indirect effect of noisy transcriptional activity mediated by Pol II-containing transcriptional machinery as enhancers loop into proximity with promoters. This transcriptional activity is primarily associated with divergently transcribed eRNAs that are not polyadenylated and has been exploited to identify novel enhancer regions by transcriptional profiling of eRNAs. Whether or not these divergent, non-polyadenylated eRNA transcripts have functional activity has been the subject of multiple studies. However, it is also imaginable that the process of eRNA transcription at the enhancer region could regulate enhancer activity by itself, through recruitment of activating transcription factors. A less abundant class of eRNAs is unidirectional and polyadenylated (1D-eRNA). These unidirectional eRNAs have similar features to lncRNAs but are encoded by genomic regions associated with an increased abundance of H3K4me1 relative to H3K4me3 when compared to regions encoding lncRNAs. The functional distinction between a non-coding transcript labelled as a unidirectional eRNA and one labelled as an lncRNA is still not well understood, and likely represents different RNA species across the same continuum.

Mechanisms of action: Transcription or transcription, that is the question

Perhaps the most studied question at the core of lncRNA biology is whether the non-coding transcript or the act of its transcription confers function to the lncRNA locus. In this line, evidence for each scenario is rapidly mounting for individual lncRNAs.

For some lncRNAs, genetic manipulation of the lncRNA locus in mouse models suggests that the mere act of transcription or related processes that include splicing allow cross-regulation of lncRNAs with that of nearby protein-coding genes. However, the use of genetically manipulated models to study lncRNA function should be carefully interpreted, as complete or partial deletion of regulatory regions (i.e. promoters or enhancers) that control transcription of lncRNAs may adversely affect the expression of nearby genes. In contrast, other studies point to the direct role of transcripts, often exerted in trans, as opposed to the act of transcription, as described below. We will briefly review the range of functions attributed to lncRNA transcripts before discussing how specific lncRNAs function in the liver.

Interactions between lncRNAs and chromatin

Perhaps the earliest and the best-known lncRNA that directly interacts with chromosomes to exert its function is XIST. One X chromosome is transcriptionally silenced in somatic cells of female mammals early in development in a process known as X-chromosome inactivation. X-chromosome inactivation begins with the expression of the XIST transcript and distribution of the transcript along the entire X chromosome. XIST assists the formation of silent heterochromatin through the recruitment of two polycomb repressive complexes (PRCs) known as PRC1 and PRC2.

A host of lncRNAs have also been described that function as more targeted activators or suppressors of gene expression through modification of chromatin or DNA. HOTTIP (HOX transcript at the distal tip), for instance, functions through the adaptor protein WDR5 to recruit histone methyltransferases to trimethylate the fourth lysine residue of histone 3 protein (H3K4me3), promoting gene expression. In contrast, immunoprecipitation of the PRC2 silencing complex provided the first indications that lncRNAs can direct regulatory complexes to specific loci in the genome to repress gene expression.

Structural lncRNAs

NEAT1 (nuclear enriched abundant transcript 1) was first described as an abundant nuclear lncRNA, Together, with other ncRNAs, NEAT1 plays a structural role to promote the formation of membrane-less nuclear bodies named paraspeckles. Paraspeckles contain both proteins and RNAs and are thought to
regulate gene expression through the retention of mRNAs in the nucleus, as recently reviewed.52,53 Similarly, another structural lncRNA MALAT1 (metastasis-associated lung adenocarcinoma transcript 1) is associated with nuclear speckles,54 which contain splicing factors.55 Both NEAT1 and MALAT1 are present at hundreds of transcriptionally active regions and act as structural components of these two nuclear bodies.56

Unexpectedly, a crosstalk between NEAT1 and mitochondria was recently described.57 In response to mitochondrial stress, the transcription factor ATF2 is activated, inducing expression of NEAT1, leading to a change in nuclear paraspeckle morphology and an increase in the number of paraspeckles that retain mitochondrial mRNAs. This response results in decreased production of mitochondria and mitochondria-dependent apoptosis proteins. Thus, NEAT1 affects mitochondrial function and dynamics through changes in the expression of mitochondrial proteins.

**LncRNAs as miRNA sponges**

One mechanism by which lncRNAs can regulate gene expression post-transcriptionally is to serve as miRNA sponges.58 Because this class of lncRNAs competes with mRNAs for miRNA binding, these lncRNAs also belong to the category of competing endogenous RNAs (ceRNAs) and have been shown to regulate a vast number of genes and related pathways.59 However, for an lncRNA to function as a miRNA sponge, it must be expressed at a high enough level to compete with mRNAs that contain the same miRNA seed sequence. LncRNAs are usually expressed at lower levels than mRNAs, and it is not clear how the stoichiometry could support this competing function for many examples of ceRNAs, even if an lncRNA contains multiple seed sequences for the same miRNA.60

**LncRNAs in early liver development**

Organ development and cell fate determination are driven by the coordinated regulation of hundreds to thousands of genes.61 Owing to their ability to modulate gene regulatory pathways and the tight control of their expression in differentiation, lncRNAs have been shown to play important roles in development.62

Foetal liver development is a complex process that comprises multiple differentiation stages from cells derived from different lineages.63 Lineage studies indicate that hepatocytes and cholangiocytes are derived from the mesendoderm lineage that in turn differentiates into the definitive endodermal (DE) layer of the early embryo. DE then organises in a tube along the anterior-posterior axis of the embryo, which ultimately forms the primitive digestive tract, including the foregut, midgut and hindgut. The foregut is the progenitor region for many internal organs including the liver, gastrointestinal system, lungs and thyroid.64

Differential expression analysis of lncRNAs during DE differentiation led to the identification of the DE-specific lncRNA DEANR1 (definitive endoderm-associated lncRNA).65 Activin signalling drives embryonic stem cell differentiation towards DE through activation of the transforming growth factor-β (TGF-β) receptor signalling pathway and phosphorylation of the co-activators SMAD2 and SMAD3 (SMAD2/3). DEANR1 recruits SMAD2/3 to the FOXA2 promoter to induce FOXA2 expression and promote DE differentiation.

Combined analysis of SMAD3 gene occupancy and expression profiling of DE differentiation led to the identification of DIGIT (divergent to goosecoid, induced by TGF-β family signalling).66 The transcription of DIGIT, which is divergently transcribed from the gene encoding goosecoid (GSC), is induced during DE differentiation in both human and mouse models of embryonic stem cell differentiation. DIGIT interacts with BRD3 at sites of histone 3 lysine 18 acetylation (H3K18ac), regulating gene expression and promoting DE differentiation.67

**LncRNAs in normal liver function and lipid processing**

The liver is responsible for homeostasis and regulation of lipid metabolism in mammals, and disruption of normal lipid metabolism can lead to hepatic steatosis.68 Many lncRNAs have been described that affect lipid metabolism and are reviewed elsewhere.69,70 We will focus our discussion on lncRNAs that have been shown to regulate key steps in lipid metabolism (Fig. 3).

**LncRNAs in lipid metabolism**

Cholesterol is imported into the liver from LDL lipoproteins via LDL receptors on hepatocytes. This influx is counterbalanced by the efflux of cholesterol through ATP-binding cassette transporter A1 (ABCA1), which delivers cholesterol to HDL lipoprotein.71 Intracellular cholesterol is processed by cholesterol 7α-hydroxylase (also called cytochrome P450 family 7 subfamily A member 1 [CYP7A1]) to form 7α-hydroxycholesterol,72 which is then modified by cytochrome P450 family 8 subfamily b member 1 (CYP8B1) to eventually form cholic acid.73 Cholic acid, in addition to chenodeoxycholic acid, is then conjugated to taurine or glycine for excretion in the bile.74 CYP8B1 activity shifts the ratio of cholic acid:chenodeoxycholic acid in favour of cholic acid, which has a weaker stimulatory effect on the farnesoid X receptor (FXR),75 a sensor of bile acid composition.76–79

Lnc-HC was discovered as an upregulated transcript in the livers of rats with high-fat diet-induced metabolic syndrome.80 Although Lnc-HC is not highly evolutionarily conserved, syntenic transcripts have been found in other organisms. The study of protein interactors of this lncRNA revealed that Lnc-HC interacts with hnRNPA2B1 and forms a ribonucleoprotein complex. Analysis of data from RNA-immunoprecipitation showed that transcripts encoding CYP7A1 and ABCA1 are present in the Lnc-HC-hnRNPA2B1 complex. These two genes have previously been shown to function as key regulators of lipid and specifically cholesterol catabolism.81 In vitro experiments using hepatocytes indicated that interaction with the Lnc-HC-hnRNPA2B1 complex induces nuclear retention and eventual degradation of CYP7A1 and ABCA1 transcripts, culminating in cholesterol accumulation in hepatocytes.

LncLSTR (lncRNA liver-specific triglyceride regulator) is a murine lncRNA whose depletion results in reduced plasma triglyceride levels.82 LncLSTR interacts with TDP-43 and interferes with the ability of TDP-43 to suppress the expression of CYP8B1, which is an essential enzyme in cholesterol metabolism. Thus, depletion of lncLSTR leads to decreased expression of CYP8B1, which affects bile acid composition.83,84 Activation of apolipoprotein C2 (APOC2) and the reduction in plasma triglyceride levels observed with depletion of lncLSTR were attenuated with depletion of FXR.76–79 Together these results suggest that lncLSTR modulates bile acid composition to regulate APOC2 expression, via FXR,85 and to control serum triglyceride levels.

Steroid response binding proteins (SREBPs) are a family of transcription factors that bind sterol response elements (SREs)
in promoters to regulate the expression of genes involved in lipid metabolism. SREBPs are found as transmembrane proteins in the endoplasmic reticulum and Golgi; protein cleavage releases the DNA-binding domain which then acts as a transcription factor. In the past few years, several lncRNAs that modulate the activity of SREBPs have been characterised. Overexpression of MALAT1 in HepG2 cells, for example, increases the stability of the mRNA encoding SREBP-1c, resulting in increased intracellular lipid droplets. siRNA-mediated knockdown of MALAT1 in the ob/ob mouse model of obesity results in a decrease in hepatic lipid content. These experiments also revealed that depletion of MALAT1 in the ob/ob mouse results in improved insulin sensitivity and glucose tolerance.

In a similar mechanism, H19 – through interaction with PTBP1 – stabilises the SREBP-1c transcript and may also promote nuclear localisation of the SREBP-1c protein. Both H19 and its binding protein are increased by fatty acids in hepatocytes and mouse fatty liver models. Data also suggest that H19 is induced in patients with type 2 diabetes. Interestingly, H19 is increased in mouse models of diabetes and temporary fasting. Mechanistically, it is proposed that H19 also interacts with S-adenosylhomocysteine hydrolase to suppress DNA methylation of the gene encoding hepatocyte nuclear factor-4α (HNF4α), resulting in increased expression of this transcriptional regulator of gluconeogenesis.

Liver X receptors (LXRs) are key transcription factors that control cholesterol homeostasis. Activation of LXRs results in decreased cholesterol content in the liver. LeXis (liver-expressed LXR-induced sequence) was originally characterised in cells treated with the LXR agonist GW3965. Detailed analysis of the LeXis interactome suggests that LeXis regulates cholesterol anabolic pathways through interaction with the transcriptional coactivator RALY. In adenovirus-transduced mouse models, the induction of LeXis is associated with reduced serum cholesterol levels.
**LncRNAs that modulate the transfer of lipid molecules**

Lipoproteins are a class of lipid-binding proteins that are involved in transfer of lipid molecules in the body. Apolipoprotein A1 (APOA1) is synthesised in both the liver and intestine and is the primary protein found in HDL complexes, which are responsible for the transport of excess cholesterol to the liver (as previously reviewed). Apolipoprotein A4 (APOA4), is produced in the intestinal epithelium upon lipid absorption and is involved in a number of different physiological functions including lipid absorption, metabolism, and platelet aggregation.

APOA1-AS is a transcript antisense to the gene encoding APOA1, which negatively regulates APOA1 transcription in both in vivo and in vitro liver models. Although not much is known about the functional mechanisms of this lncRNA, it is proposed that APOA1-AS recruits various histone-modifying enzymes to the APOA1 locus, which in turn regulate APOA1 expression.

Similar to APOA1-AS, APOA4-AS belongs to the class of NAT lncRNAs. Expression of APOA4-AS is elevated in fatty liver disease, and depletion of this lncRNA in the ob/ob mouse model results in reduced expression of APOA4 and decreased serum triglyceride levels. APOA4-AS interacts with an RNA-stabilising protein named human antigen R (HuR) in a sequence-dependent manner. The depletion of HuR protein is associated with reduced APOA4-AS and APOA4 transcript levels and suggests that interaction with HuR may help promote stability of both APOA4-AS and APOA4 mRNA.

**LncRNAs in liver injury and fibrosis**

Microarray profiling of lncRNAs in the whole mouse liver uncovered multiple transcripts whose expression changed significantly as a result of liver injury induced by 3 weeks of carbon tetrachloride (CCL4) treatment. Among these, Gm2199 was shown to be repressed with CCL4-induced damage. Treatment of hepatocytes with CCL4 caused a significant reduction in proliferation, which was restored by ectopic expression of Gm2199. As for the mechanism, the authors provide evidence that Gm2199 achieves these effects through increased expression and activation of mitogen-activated protein kinase ERK1/2. In vivo studies corroborated these results by showing that increased expression of Gm2199 protects cells from the adverse effects of CCL4 treatment. Although a human lncRNA orthologous to Gm2199 has not yet been described and thus Gm2199 may not have relevance for human disease, this study demonstrates how an lncRNA can affect intricate molecular pathways involved in liver injury.

The list of lncRNAs identified that modulate fibrosis, in general, continues to grow and was recently reviewed elsewhere. We will focus here on lncRNAs most relevant to liver fibrosis (Fig. 4). Hepatic stellate cells (HSCs) are the primary cell type responsible for liver fibrosis, and lncRNAs expressed in human HSCs were recently described, many of which are co-expressed with extracellular matrix genes. In mouse models of CCL4-induced liver fibrosis, lincRNA-p21 was found to be significantly decreased. Overexpression of lincRNA-p21 in primary HSCs resulted in increased expression of the p21 protein and reduced expression of α-smooth muscle actin (α-SMA [ACTA2]) and collagen type I (Col1A1), suggesting a role for lincRNA-p21 in suppressing HSC activation and transformation to myofibroblasts.

MEG3 (maternally expressed gene 3) is a maternally imprinted gene whose non-coding product has been described in multiple cancers. MEG3 expression is decreased in fibrotic liver tissue and activated in primary HSCs. Overexpression of this lncRNA through a viral induction system also showed antifibrotic activity in murine CCL4-induced liver fibrosis. In the immortalised human HSC line LX-2, MEG3 transcript levels are reduced in response to TGF-β treatment. The mechanism by which MEG3 confers antifibrotic activity is still largely unknown.
MEG3 is suggested to inhibit HSC activation by suppressing epithelial-mesenchymal transition through interactions with smoothened (SMO) protein. MEG3 has also been shown to induce cholestatic liver damage by accelerating the decay of small heterodimer partner (SHP) mRNA through interactions with PTBP1. In turn, attenuation of SHP protein, which is a key repressor in the bile acid biosynthesis pathway, results in liver injury.

The role of GAS5 (growth arrest-specific transcript 5) in liver fibrosis has also been described. Expression of GAS5 is decreased in human and mouse fibrotic liver tissues. Similarly, in vitro experiments employing activated HSCs also suggest that GAS5 expression is reduced compared to quiescent HSCs. Overexpression of this lncRNA results in decreased expression of COL1A1 and ACTA2 in HSCs in vitro and decreased collagen levels in CCl4-induced murine liver fibrosis models, as determined by measurement of hepatic hydroxyproline content. Mechanistically, GAS5 is considered to function as a ceRNA through interaction with miR-233. The authors demonstrated that sequestration of miR-233 by GAS5 results in increased expression of the miRNA target transcripts including the p27 gene, whose protein product is directly involved in HSC proliferation and activation.

H19 is induced in cholestatic liver fibrosis. Ectopic expression of H19 in the liver is associated with increased necrosis and fibrosis in the setting of bile duct ligation (BDL), and H19-deficient mice show reduced cholestatic liver injury and fibrosis in response to BDL. H19 exerts its function in this setting by binding and inhibiting the zinc finger E-box-binding homeobox 1 (ZEB1), whose activity suppresses activation of epithelial cell adhesion molecule (EpCAM). Ectopic expression of ZEB1 or depletion of EpCAM in H19-deficient mice reduced fibrosis, leading to a model where bile acids induce H19 expression in hepatocytes and cholangiocytes. H19 then interacts with ZEB1, preventing binding to the EpCAM promoter, leading to increased EpCAM expression and increased susceptibility to cholestatic liver fibrosis.

**LncRNAs in liver cancer**

LncRNAs are increasingly recognised as mediators of human cancers. In this regard, depending on the context, lncRNAs can function as either oncogenes or tumour suppressors. In the liver, lncRNAs have been identified that are associated with hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA) (Table 1), which comprise the majority of liver cancer cases, and were reviewed recently. Below, we describe oncogenic lncRNAs that are involved in promoting HCC and CCA; we then look at lncRNAs that function as tumour suppressors to hinder malignant growth.

**Oncogenic LncRNAs**

As the name suggests, MALAT1, also known as NEAT2 (nuclear-enriched abundant transcript 2), is an oncogenic lncRNA in human cancer. This lncRNA is highly conserved in mammals, and its role in numerous biological processes has been extensively studied. In HCC, MALAT1 is upregulated, and its depletion results in lower tumorigenicity, which is largely driven by reduced expression of the oncogenic splicing factor SRSF1. Furthermore, MALAT1 activates the mammalian target of rapamycin (mTOR) pathway through SRSF1-mediated splicing of S6 kinase 1 (S6K1) to an alternative isoform named Iso-2. The Iso-2 protein product has oncogenic potential through direct binding and activation of mTOR complex 1 (mTORC1).

We discussed the role of NEAT1 as a structural lncRNA previously. However, NEAT1 has also been implicated in multiple cancers, and its role and expression status in HCC have recently been described. Expression profiling of NEAT1 transcripts in normal liver and HCC tumour tissues and cell lines revealed that NEAT1 is induced in neoplastic cells. Fang et al.

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Table 1. LncRNAs associated with liver cancer.

| LncRNA          | Role          | Mechanism                                                                 | Ref     |
|-----------------|---------------|---------------------------------------------------------------------------|---------|
| **Hepatocellular carcinoma (HCC)** |               |                                                                           |         |
| MALAT1          | Oncogene      | Activation of oncogenic splicing factor SRSF1                              | 118     |
| NEAT1           | Oncogene      | Acts through the sponge activity to inhibit miR-129-5p.                   | 121     |
| HEIH            | Oncogene      | Interacts with PRC2 component EZH2                                        | 122     |
| HULC            | Oncogene      | Inhibition of PTEN tumour suppressor gene through the sponge-activity on miR-15a | 131     |
| HOTTIP          | Oncogene      | Regulates gene expression from the HOXA region                            | 134     |
| HOTAIR          | Oncogene      | Repressively alters the chromatin landscape of promoters through recruitment of PRC2 | 137     |
| DANCR/ANCR      | Oncogene      | Increased stability of HNRNPA1                                            | 143     |
| H19             | Mixed         | Conflicting studies suggest both oncogenic and tumour suppressor roles in HCC | 138-140 |
| MEG3            | Tumour suppressor | Increases expression of miR-122 through which pyruvate kinase muscle isozyme M2 (PKM2) is down-regulated | 153     |
| FENDRR          | Tumour suppressor | Acts as a miR-423-5p sponge to regulate GADD45B expression                | 155     |
| DILC            | Tumour suppressor | Interacts with the IL6 promoter to suppress its expression.              | 157     |
| GAS5            | Tumour suppressor | Functions as a sponge to bind miRNAs. Binds to miR-126-3p, whose ectopic expression has been shown to decrease cell proliferation in HCC. Binds to miR-21 to derepress the PTEN protein levels | 161     |
| **Cholangiocarcinoma (CCA)** |               |                                                                           |         |
| DANCR/ANCR      | Oncogene      | Associated with increased cellular proliferation and migration through interaction with EZH2 | 145     |

In HCC, lncRNAs have been described to function as both oncogenes and tumour suppressors. In CCA, DANCR/ANCR has been shown to function as an oncogene.
suggested that, mechanistically, NEAT1 exerts its oncogenic property as a ceRNA through binding and inhibition of miR-129-5p, whose main cellular targets include valosin-containing protein (VCP) and the NF-xB inhibitor protein (IxB).

HEIH (high expression in HCC) was originally identified in HBV-associated HCC tissues. RNA pulldown assays indicated that HEIH interacted with the PRC2 complex protein EZH2. shRNA-mediated depletion of HEIH caused increased expression of genes that are normally repressed by the PRC2 complex. HEIH promotes cell cycle progression and tumour growth via a pathway that is at least in part dependent on EZH2. This study also found that the expression level of HEIH in tumour tissue has potential as a prognostic marker in patients with HCC.

HULC (highly upregulated in liver cancer) is an oncogenic lncRNA whose role, among others, has been described in hepatic, gastric, and colorectal cancers. Abnormal expression of HULC is associated with poor prognosis of pancreatic cancers and hepatomas in the clinic. Measuring serum levels of this lncRNA has also been proposed as a biomarker in liver cancer. HULC influences many cell traits in cancer through multi-pronged metabolic and cellular signalling pathways that are all relevant in hepatic biology.

The transcription factor CREB and the HBV X protein (HBx) have both been well-studied in the context of liver cancer and are upstream regulators of HULC. HULC can act as a miRNA sponge. It contains binding sites for miR-372, which usually targets translation of the CREB-kinase PRKACB. PRKACB-mediated phosphorylation of CREB results in chromatin modifications that promote gene expression including that of HULC. Additionally, infection with HBV is associated with increased levels of HULC in HCC samples. Detailed analysis of HBV-induced HCCs has demonstrated that HBV promotes the expression of HULC. Interestingly, HULC has also been shown to accelerate liver cancer via inhibition of the PTEN tumour suppressor gene through sponge-activity on miR-15a.

HULC also promotes lipogenesis in HCC. Cholesterol molecules upregulate the expression of HULC by activating the retinoic receptor RXR-α. Increased expression of HULC is associated with increased methylation of the gene encoding miR-9. This results in decreased expression of miR-9 and increased expression of its target, the nuclear receptor peroxisome proliferator-activated receptor-α (PPAR-α). Increased levels of PPAR-α then induce transcription of long-chain-fatty-acid-CoA ligase 1 (ACSL1), to promote fatty acid synthesis.

HOTTIP is induced in HCC. Studies have shown that the HOTTIP gene is located near to the HOXA13 gene and controls the gene expression of the HOXA loci. The HOXA locus codes for a multitude of transcription factors that are involved in embryo-genesis, with aberrant expression of these factors implicated in cancers.

HOTAIR was originally identified through microarray tiling of the HOXC locus on chromosome 12. HOTAIR repressively alters the chromatin landscape of promoters through recruitment of PRC2. In a separate but related mechanism, HOTAIR recruits the lysine-specific demethylase 1 (LSD1) complex, which is composed of LSD1, REST and CoREST proteins, and mediates demethylation of activating histone marks (H3K4me3). The combined action of PRC2 and LSD1 complexes suppresses target gene expression. The role of HOTAIR in HCC has been extensively studied and reviewed elsewhere. Aberrant expression of HOTAIR is correlated with worse outcomes in patients with HCC.

H19 is discussed above as a factor that regulates lipid metabolism and liver fibrosis; its role in HCC is less clear. In one study, increased expression of H19 was identified in the setting of hypoxia and metastatic disease, suggesting possible oncogenic activity, while in HCC cell lines ectopic expression of H19 was shown to induce cell apoptosis and inhibit cell proliferation. Further data from mouse models found that expression of H19 from a modified H19 locus negatively correlates with tumour emergence in SV40-induced HCCs.

DANCR (differentiation antagonising non-protein coding RNA), also known as ANCR, has been implicated in many cancers. DANCR is highly expressed in HCC and promotes cellular proliferation and metastasis. In HepG2-induced xenograft mouse models of HCC, depletion of DANCR results in reduced tumour burden and decreased metastasis. The main mechanism through which DANCR exerts its oncogenic role is through interaction with the heterogenous nuclear protein ribonucleoprotein A1 (HNRNP A1), which regulates epithelial-mesenchymal transition in HCC. DANCR directly binds to the HNRNP A1 protein resulting in its increased stability. DANCR has also been shown to have sponge activity, binding to miR-140-3p to inhibit its ability to affect HNRNP A1 translation.

While the role of lncRNAs in liver cancers has mostly been described in HCCs, at least one lncRNA has been described in the context of CCA. CCA is an aggressive form of liver cancer whose malignant cells originate from bile ducts. DANCR is induced in CCA in addition to HCC. In vitro analysis of DANCR in CCA models shows that this lncRNA is associated with increased cell proliferation and migration through interaction with EZH2, which leads to epigenetic suppression of the fructose-bisphosphatase 1 (FBP1) gene. FBP1 is an enzyme that regulates gluconeogenesis and is implicated in many forms of cancer including HCCs. While DANCR appears to function as an oncogene in both HCC and CCA, the described mechanisms of action for DANCR in these 2 liver malignancies are currently different. Future studies will be required to understand if interactions with HNRNP A1 also play a role in CCA and if interactions with EZH2 also play a role in HCC.

Tumour suppressor lncRNAs

In addition to having roles in liver fibrosis as discussed before, MEG3 is also implicated in cervical, pancreatic, and many other cancers. Whereas other lncRNAs appear to promote oncogenesis, MEG3 is largely thought to function as a tumour suppressor gene. Compared to normal cells, malignant hepatocytes show a significant reduction in the expression of this lncRNA. Overexpression of MEG3 in both cancer cell lines and mouse models inhibits growth of liver cancer cells. The authors suggest that MEG3 increases expression of miR-122 through which pyruvate kinase muscle isozyme M2 (PKM2) is downregulated. PKM2 is an important metabolic enzyme in glycolysis that influences oncogenesis.

As a miRNA-sponge, FENDRR (foetal-lethal non-coding developmental regulatory RNA) has a unique role in HCC development. Compared to normal cells and tissues, FENDRR levels are drastically lower in HCC cell lines and tissues. The sponge activity of FENDRR is exerted through binding to miR-423-5p, which targets and degrades growth arrest and DNA-damage-inducible beta protein gene (GADD45B) transcripts. Lower expression of the IncRNA in HCC allows the miRNA to attenuate GADD45B protein levels, which results in unchecked proliferation and immune evasion of tumour cells. Forced
expression of FENDRR in mice resulted in decreased burden and proliferation of cancer cells.

Progression of liver cancer is thought to be mediated through a class of stem-like cancer cells termed liver cancer stem cells (LCSCs), which are capable of sustaining proliferation within the tumour. DILC (downregulated in liver cancer stem cells) has been identified to play crucial roles in these cells. Depletion of this lncRNA resulted in expansion of the LCSC population and progression of disease, whereas ectopic expression led to a reduction in the size of the LCSC population in the tumour. Mechanistically, DILC is thought to interact with the IL6 promoter leading to its inhibition. IL6 suppression disrupts the IL6/STAT3 autocrine signalling pathway, which is required for the maintenance of LCSCs.

GAS5 has been implicated in multiple forms of cancers, and is a well-known tumour suppressor gene. However, its multitude of roles in HCC have only recently been investigated. Wang et al. showed that GAS5 expression in HCC is lower than in normal cells and tissues, and depletion of GAS5 increased resistance to the chemotherapeutic agent doxorubicin. The authors found that GAS5 acts as a sponge to inhibit onco-gene miR-21, which targets the tumour suppressor gene PTEN. In contrast, Faranda et al. also reported that the GAS5 transcript functions as a sponge to bind miR-126-3p, whose ectopic expression has been shown to decrease cell proliferation in HCC model cell lines. Although the authors did not elaborate on the mechanistic function of this miRNA in the context of HCC, studies in endothelial cells suggest that miR-126 acts through the mechanistic function of this miRNA in the context of HCC, studies in endothelial cells suggest that miR-126 acts through the mechanism, cleaved RNAs are quickly degraded by various exonucleases.

Multiple ASOs are currently being employed clinically to target liver-based gene expression. Mipomersen, for example, targets apolipoprotein B-100, a component of LDL, to treat patients with familial hypercholesterolemia disorder. However, the clinical use of ASOs, including mipomersen, is not without caveats and drawbacks, as ASOs may elicit an interferon response and tissue toxicity. Patisiran is another successful example of an ASO approved for clinical use. Patisiran, a lipid nanoparticle-containing double-stranded RNA, is used to treat poly-neuropathy of hereditary transthyretin (TTR)-mediated amyloidosis. Partisiran binds to TTR mRNA and causes its degradation resulting in a reduction in serum and tissue deposition of TTR proteins. We envision IncRNAs can be targeted using similar approaches.

Conjugation of chemical groups to ASOs, as a method to increase half-life and tropism to a specific tissue or cell type, has also been examined. For example, conjugation of cholesterol molecules to ASOs enhances their absorption by hepatocytes. Hepatocytes express asialoglycoprotein receptors whose function is to clear aged circulating serum glycoproteins that have lost terminal sialic acid moieties in their glycann chain, resulting in exposure of N-acetylgalactosamine residues. For this reason, N-acetylgalactosamine-conjugated molecules have been under active investigation for hepatocyte-specific delivery of siRNAs.

The delivery of ASOs through lipid-containing vehicles is also under active consideration. Liposome-mediated delivery of ASOs also enhances the efficiency and tropism of liver cells. Sato et al. exploited vitamin A-coupled liposome systems to introduce ASOs (targeting the collagen chaperone gp46) to rat HSCs in order to inhibit cirrhosis. They found that liposome-mediated delivery of the ASO caused a marked reduction in liver fibrosis and increased survival in rat models. As an alternative ASO delivery method, lipid-like nanoparticles have also been used to introduce ASOs into the liver. Lipid-like nanoparticle-siRNAs against procollagen α-1 were primarily localised to non-parenchymal cells in the liver and caused a significant reduction in collagen expression and deposition of collagen in mouse liver tissue. With increased efficiency in the delivery of ASOs to specific cell types in the liver, we anticipate seeing the development of ASO-based drugs that target disease-relevant IncRNAs in the not too distant future.

Although most of our discussion has focused on in vivo depletion of IncRNAs, delivery of IncRNAs to specific cell types in the liver could also have significant therapeutic value for managing chronic liver disease and cancer. The methods of RNA delivery in general and IncRNA in particular, as well as methods to induce their expression in tissues, are still in their infancy. Adeno-associated virus-based delivery methods have been acclaimed as the current gold standard for tissue delivery of nucleic acids in model organisms. The rather obvious drawback of using this system is that it is largely limited by IncRNA size and concern over carcinogenicity of the virus-delivery method. Due to the unstable nature of RNAs and their large size, methods to directly deliver IncRNA molecules (as nanoparticles or other vehicles) to human tissues have not been well developed. With the development of safer and more effective delivery methods, the introduction of IncRNAs may have great potential in liver-based gene therapy.

**LncRNAs as biomarkers in diagnostic settings**

Because of their tissue-specific expression, IncRNAs are being investigated as biomarkers in clinical settings. In liver cancer, studies and meta-analyses to identify IncRNAs that could be used as biomarkers have already begun. Through analysis of data generated from the cancer genome atlas (TCGA) consortium, Li et al. constructed mRNA-lncRNA coexpression networks. The
expression pattern of coding and non-coding transcripts in HCC led to the identification of several lncRNAs that have potential as biomarkers for HCC.\textsuperscript{185} Although these lncRNAs were not able to predict patient outcomes, they hold promise in the diagnostic setting.

As described earlier, perhaps the most promising lncRNA biomarker in HCC is \textit{HULC}. \textit{HULC} transcripts are significantly elevated in the plasma of patients with HCC.\textsuperscript{126,127} Additionally, in comparison to serum derived from healthy individuals, higher levels of \textit{HULC} transcripts are found in the serum of patients with positive HBV status. Detection of lncRNAs such as \textit{HULC} in plasma suggests that lncRNAs have the potential to serve as blood-based biomarkers in the clinical setting.

**Conclusion**

lncRNAs play essential roles in early liver development, the metabolic function of the liver, liver fibrosis, and cancer. While lncRNAs have been identified that regulate each of these stages, these transcripts likely represent only a small fraction of the lncRNAs that control key processes from liver development to homeostasis to disease. As we gain a deeper understanding of the diversity and function of lncRNAs across the different cell types of the liver, we anticipate this knowledge will lead to the establishment of lncRNAs as therapeutic targets to treat diseases of, or regulated by, the liver; this knowledge could also be used to identify new biomarkers to track or predict disease progression.

**Abbreviations**

ACAT1, ATP-binding cassette transporter A1; ACTA2, a-SMA, a-smooth muscle actin; APO, apolipoprotein; ASO, antisense oligonucleotides; BDL, bile duct ligation; CCA, cholangiocarcinoma; CCL\textsubscript{4}, carbon tetrachloride; cER, competing endogenous RNA; COL1A1, collagen type I a 1; CYP, cytochrome P450; DE, definitive endoderm; DANCH, differentiation antagonising non-protein coding RNA; DEANR1, definitive endoderm-associated lncRNA1; DIGIT, divergent to goosose, induced by TGF-b family signalling; DILC, downregulated in liver cancer stem cells; EpCAM, epithelial cell adhesion molecule; eRNA, enhancer RNAs; EST, expression sequence tag; FBP1, fructose-bisphosphatase 1; FENDRR, foetal-lethal non-coding developmental regulatory RNA; FXR, farnesoid X receptor; GASS, growth arrest-specific transcript 5; H3K4me3, histone 3 lysine 4 trimethylation; H3K18ac, histone 3 lysine 18 acetylation; H3K36me3, histone 3 lysine 36 trimethylation; HCC, hepatocellular carcinoma; HEIH, high expression In HCC; HNRNPAL, heterogenous nuclear protein ribonucleoprotein A1; HOTAIR, HOX transcript antisense RNA; HOTTIP, HOXA transcript at the distal tip; HSC, hepatic stellate cells; HuR, human antigen R; HULC, highly upregulated in liver cancer; LCSC, liver cancer stem cell; LeXis, liver-expressed LXR-induced sequence; lincRNA, long intervening transcript; lncRNA, long non-coding RNA; LincSTR, IncRNA liver-specific triglyceride regulator; LSD1, lysine-specific demethylase 1; LXR, liver X receptors; MALAT1, metastasis-associated lung adenocarcinoma transcript 1; MEG3, maternally expressed gene 3; mTOR, mammalian target of rapamycin; NAT, natural antisense transcript; NEAT1, nuclear enriched abundant transcript 1; ORF, open reading frame; PKM2, pyruvate kinase muscle isozyme M2; PPAR-\alpha, peroxisome proliferator-activated receptor-\alpha; PRC, polycomb repressive complex; RACE, rapid amplification of cDNA ends; RNA Pol, RNA polymerase; S6K1, S6 kinase 1; SHP, small heterodimer partner; siRNA, small interfering RNA; SREBP, steroid response binding proteins; SREs, sterol response elements; TGF-\beta, transforming growth factor-\beta; TTR, transthyretin; XIST, X-inactive specific transcript; ZEB1, zinc finger E-box-binding homeobox 1.

**Financial support**

ACM is supported by NIH/NIKDD grant R01DK116999, NIH/NIH grant R01HD09277302 and a two Biomedical Scholars Award. Dr. Mullen also receives research support from the Chan Zuckerberg Initiative and has received research funding from Boehringer Ingelheim, Bristol-Myers Squibb, Takeda Pharmaceuticals and Roche Pharmaceuticals in the last 36 months.

**Conflict of interest**

The authors declare no conflicts of interest that pertain to this work.

Please refer to the accompanying ICMJE disclosure forms for further details.

**Authors’ contributions**

The authors contributed equally to the production of this manuscript.

**Supplementary data**

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhepr.2020.100177.

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Author names in bold designate shared co-first authorship

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