Title page

Title Regulation of microRNAs and their role in liver development, regeneration and disease

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Running title miRNAs in liver development, regeneration and disease

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Abbreviations ALD, alcoholic liver disease; ALT, alanine aminotransferase; CC, cholangiocarcinoma; EMT, epithelial-mesenchymal transition; ENCODE, Encyclopedia of DNA Elements; GO, gene ontology; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IFN, interferon; KEGG, Kyoto Encyclopedia of Genes and Genomes; lncRNA, long non-coding RNA; LPC, liver progenitor cell; miRNA, microRNA; mRNA, messenger RNA; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PH, partial hepatectomy; piRNA, piwi-associated RNA; pri-miRNA, primary microRNA; RISC, RNA-induced silencing complex; rRNA, ribosomal RNA; siRNA, small interfering RNA; snoRNA, small nucleolar RNA; snRNA, small nuclear RNA; tRNA, transfer RNA; UTR, untranslated region.

Keywords microRNA; liver; development; regeneration; disease

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Abstract

Since their discovery more than a decade ago microRNAs have been demonstrated to have profound effects on almost every aspect of biology. Numerous studies in recent years have shown that microRNAs have important roles in development and in the etiology and progression of disease. This review is focused on microRNAs and the roles they play in liver development, regeneration and liver disease; particularly chronic liver diseases such as alcoholic liver disease, non-alcoholic fatty liver disease, non-alcoholic steatohepatitis, viral hepatitis and primary liver cancer. The key microRNAs identified in liver development and chronic liver disease will be discussed together with, where possible, the target messenger RNAs that these microRNAs regulate to profoundly alter these processes.

1. Introduction

It has been estimated that approximately 97% of the human genome consists of non-protein coding DNA (Frith et al., 2005). Although originally considered to be "junk DNA", the Encyclopedia of DNA Elements (ENCODE) project; a consortium with the central aim to define all functional elements encoded in the human genome, concluded that the vast majority (~80%) of the human genome is not only transcribed, but has biochemical function (Bernstein et al., 2012). More recently, the role of non-coding RNAs has become a major focus of research efforts, effectively laying to rest the dogma that RNA functions only in a structural capacity or as an intermediary between DNA sequence and translated protein.

Many types of non-coding RNAs have now been described including infrastructural RNAs (ribosomal (rRNA), transfer (tRNA), small nuclear (snRNA) and small nucleolar (snoRNA) RNAs) as well as regulatory RNAs. Regulatory non-coding RNAs can be further divided into long non-coding (lncRNAs) and small non-coding RNAs, which include small interfering (siRNA), piwi-associated (piRNA) and micro (miRNA) RNAs. There are many excellent reviews covering the multitude of non-coding RNAs in detail (Mattick and Makunin, 2006; Ponting et al., 2009; Siomi and Siomi, 2009; Costa, 2010). The purpose of this review is to examine the function of miRNAs, and in particular their role in liver development, regeneration and disease. To assist the reader to identify miRNAs involved in these processes we have summarized the miRNAs that were reported in at least two or more studies in Figure 1.

The liver is an excellent system in which to study miRNAs in development, as liver organogenesis has been well characterised, and extensive analyses have defined the transcriptional changes that occur during cellular differentiation of hepatic progenitors and

through different stages of embryogenesis to form the functional adult liver (reviewed in Si-Tayeb et al., 2010). The liver is also remarkable in regard to its ability to regenerate following surgical resection of up to 70% of the liver mass (Steer, 1995), allowing the study of *in vivo* organ growth and cell repopulation in the adult. Furthermore, recent research has ascribed a role for miRNAs in all aspects of liver disease, from initiation, progression, diagnosis and treatment. Advantageously, there are many established models of acute and chronic liver injury that are routinely used to study a host of liver pathologies, and the role that miRNAs may play. Whilst numerous etiologies may underlie liver disease, this review will cover four of the most chronic liver diseases, namely alcoholic liver disease (ALD), non-alcoholic fatty liver disease (NAFLD), viral hepatitis, and primary liver cancer.

As interest in non-coding RNAs intensifies, particularly in the miRNA field, there is an increasing number of publications citing large-scale array analyses with little or no target gene validation or link to biological significance. For miRNA data to be of value for future investigators, a thorough understanding of the function of dysregulated miRNAs and the target messenger RNA/s (mRNA/s) they regulate is essential. Ideally, where possible, studies should demonstrate a cause and effect of regulated target mRNAs, for example siRNA silencing of target gene/s, to validate the biological relevance of changes in miRNAs and the targets they control. Therefore to assist the reader to identify meaningful data amongst a vast repository of published literature, this review will primarily focus on papers for which validation of potential targets of differentially regulated miRNAs has been performed.

2. MicroRNA

The first miRNA (*lin-4*) was identified in 1993 in *C. elegans* as a regulator of developmental timing (Lee et al., 1993; Wightman et al., 1993). However, it was not until 2000 with the discovery that *let-7* miRNA was highly conserved between invertebrates and vertebrates (Pasquinelli et al., 2000; Reinhart et al., 2000) that research into miRNAs really took off. MiRNAs represent the most abundant class of small endogenous non-protein coding RNAs, and function as post-transcriptional regulators of gene expression.

The biogenesis of miRNAs is a multi-step process that was first elucidated in *C. elegans* by several research groups (for review see Bartel, 2004). Briefly, miRNA genes are transcribed into hairpin stem-loop structures known as primary miRNA (pri-miRNA) by RNA polymerase II. Pri-miRNAs are then processed within the nucleus by the Drosha-DGCR8 complex to form precursor miRNAs, which are transported to the cytoplasm by Exportin 5. Further processing

by the Dicer1-TARBP2 complex generates the mature miRNA duplexes that are typically 18-25 nucleotides in length. Finally, the single active strand of the mature miRNA is loaded into the RNA-induced silencing complex (RISC) which delivers the miRNA to its target mRNA where it binds with imperfect complementarity, typically in the 3' untranslated region (UTR) (Bartel, 2009).

The binding of miRNAs to their target mRNAs induces gene silencing through destabilisation and degradation of the mRNA and/or via translational repression (reviewed in Valencia-Sanchez et al., 2006). Due to the imperfect nature of miRNA-mRNA binding, a single miRNA species is able to target multiple mRNAs (target multiplicity). Furthermore, a single mRNA can be targeted and regulated by several different miRNAs (miRNA redundancy). It has been estimated that approximately 30% of all human genes are under the control of miRNAs (Lewis et al., 2005). It is therefore not surprising that miRNAs are now implicated in the control of almost all known biological processes.

3. Liver development

The liver develops from the definitive endodermal epithelium of the embryonic foregut. While the liver serves as the initial site of hematopoiesis, hepatic specification only commences at mouse gestation day E8.5, corresponding to approximately three weeks of human gestation. Hepatic progenitor cells are hepatoblasts; bipotential cells which are able to differentiate to form cholangiocytes (bile duct epithelial cells) and hepatocytes, the two main cell types of the liver. Differentiation of hepatoblasts begins at approximately E13.5 in the mouse (~six weeks in humans), and continues until birth. Within the adult liver, a small population of cells resembling embryonic hepatoblasts persist within the Canals of Hering - the terminal bile ductules. These cells are known as liver progenitor cells (LPCs), and play an important role in the liver's response to chronic injury.

3.1 MicroRNAs in liver development

There have been three independent studies concerned with identifying miRNAs that are involved in liver development. Tzur et al. (2009) compared embryonic and adult livers, and an embryo with its liver removed to identify panels of miRNAs specifically expressed at different developmental stages. MiRNAs highly expressed in embryonic compared to adult liver included miRs-18a, 92a, 409-3p, 451, and 483-3p, and miRNAs enriched in adult compared to embryonic liver included miRs-22, 23b, 99a, 125b, 192, and *let-7a*, *b* and *c* (Fig. 1). Additionally the authors linked miRNA profiles with gene expression data and found an

inverse correlation between the expression of selected miRNAs and their predicted targets. Furthermore they showed that the type I receptor gene *TGFβR1* is negatively regulated by *let-7c* during development (Tzur et al., 2009). To assist the reader, Table 1 summarises the miRNAs with validated targets that are included in this review.

Lui and colleagues (Liu et al., 2010a) took a different approach to profile miRNAs in foetal and adult liver tissue as well as a chorionic villus. Expression profiles of the 42 miRNAs identified revealed a number of miRNAs differentially expressed between the foetal and adult liver samples, in particular miRs-122, 148a, 192, 194, 451, 21 and *let-7a* were all reduced in the adult liver compared to foetal liver. As expected, bioinformatic analyses revealed numerous potential targets for identified miRNAs, including multiple genes involved in proliferation and growth such as *PinX1* (Liu et al., 2010a).

It is difficult to compare these studies directly since they assessed liver tissues from markedly different developmental stages (7-10 weeks (Tzur et al., 2009) compared with 18-35 weeks (Liu et al., 2010a)). Furthermore, the studies used different techniques to assess the miRNA profile. Nevertheless, both human studies identified several miRNAs, namely miRs-122, 192, 194, 451, and to a lesser extent 483-3p, which were detected in embryonic/foetal liver. Of these however, only miR-451 was highly expressed in the embryonic/foetal liver compared to adult liver in both studies.

More recently, using next-generation sequencing to compare miRNA gene expression profiles of E8.5 foregut endoderm, E14.5 hepatoblasts and adult rodent liver, Wei et al. (2013a) identified two miRNAs, miR-302b and 20a, which were both highly enriched in the endoderm yet rapidly down-regulated during liver development. Interestingly, these miRNAs share a common target (*Tgfbr2*), which could regulate transforming growth factor beta (TGFβ) signalling.

Many miRNAs exhibit tissue and developmental timing specificity. MiR-122 is a liver specific miRNA accounting for ~70% of all expressed miRNAs in the liver whilst undetectable in other tissue types assessed (Lagos-Quintana et al., 2002). Furthermore, miR-122 is specifically switched on during embryogenesis, and is maintained in adulthood (Chang et al., 2004). Although many studies have investigated the role of miR-122 in different liver pathologies**,** in particular viral hepatitis (see section 5.3), little has been published on the role of this miRNA in liver development. One study by Xu et al. (2010) identified a link between transcription factors

enriched in the liver and the expression of miR-122 in developing embryos. Furthermore, a number of miR-122 targets were identified which are known to be involved in regulating cellular proliferation and differentiation during development. Using miRNA overexpression and knockdown experiments, the authors demonstrated that one of these targets, *Cutl1*, is negatively regulated by miR-122 during liver development (Xu et al., 2010) (Table 1).

Besides tissue specificity, miRNAs also exhibit cell-specific expression. In one study, *in situ* hybridization on frozen liver sections was performed to identify miRNAs expressed in particular cell populations within the developing liver including hepatocytes, hepatoblasts of the developing ductal plate and mature cholangiocytes, as well as cells within the periportal space and scattered blood cells throughout the lobule (Hand et al., 2009a). With a specific focus on miRNAs that regulate development of the ductal plate, which gives rise to the mature bile ducts, it was revealed that miRs-30a and 30c are biliary specific in both mouse and human (Hand et al., 2009a). In support of this, miR-30a silencing in zebrafish resulted in an impairment of biliary function. Although the authors identified potential targets of miR-30a in a mouse embryonic LPC line, including *Ak1*, an enzyme involved in cellular energy homeostasis, and *Tnrc6a*, which encodes a component of the RISC complex, neither have been specifically linked to biliary function.

The importance of miRNAs in development has been emphasised by studies showing that animals devoid of *Dicer1*, resulting in loss of mature miRNAs, die early during embryogenesis (Bernstein et al., 2003). However, a separate study by Hand and colleagues (2009b) found that transgenic mice specifically lacking *Dicer1* in alpha-fetoprotein (Afp)-expressing hepatoblast-derived hepatocytes develop normally. This was despite large-scale changes in liver gene expression, including the increased expression of selected miRNA targeted mRNAs that were experimentally validated. Although liver damage accompanied by hepatic apoptosis and inflammation was eventually observed in older animals (2-4 months), it was concluded by the authors that mature miRNAs are not required for hepatic function (Hand et al., 2009b).

As noted by the authors, a limitation of this study for assessing the role of miRNAs in liver development is that the *Dicer1* transcript was not substantially depleted until birth, therefore the processing of some mature miRNA/s would likely have continued throughout development. Furthermore, due to the transgenic model used (AlfpCre), *Dicer1* was deleted only in hepatocytes derived from Afp-expressing hepatoblasts and not in other cell types within the liver including the hepatoblasts themselves or cholangiocytes, which is expected to be

significant. Therefore studies in which *Dicer1* is deleted earlier in development and/or in other hepatic cell types need to be performed before the role of miRNAs in hepatic development can be clearly defined. For instance, one study that targeted Dicer *in vitro* using shRNA in hepatic stellate cells observed a reduced proliferation rate as well as down-regulation of fibrosis-related genes including type I collagen (*Col1A1*), α-smooth muscle actin (*αSMA*), and tissue inhibitor of metalloproteinases (*TIMP*) (Yu et al., 2014), however these changes have yet to be documented *in vivo*.

Whilst *in vivo* studies enable the most relevant assessment of biological processes with regard to translation as an endpoint, *in vitro* models can make significant contributions; especially where information on direct cellular and/or molecular interactions is sought. Often these studies involve the differentiation of cultured cell lines. LPC lines can be derived from normal foetal and adult liver, as well as regenerating liver, and these can be successfully differentiated *in vitro* to form hepatocytes and cholangiocytes (Fougère-Deschatrette et al., 2006). Identifying miRNA expression changes that occur during the differentiation of progenitor cells into hepatocytes provides an insight into the molecular mechanisms controlling this developmental event. Using this approach, Chen et al. (2010) identified several miRNAs in the differentiated cells that showed similar expression patterns to freshly isolated hepatocytes, yet were significantly different to the undifferentiated LPCs. These miRNAs included miRs-122 and 101 that increased, and 146a, 146b and 142-5p that decreased, upon differentiation (Fig. 1).

Studies have also investigated changes in miRNAs during differentiation of other progenitor cell types into hepatocytes, including human embryonic stem cells (Kim et al., 2011) and human mesenchymal stem cells derived from the umbilical cord (Cui et al., 2012). Up-regulation of miRs-10a, 122, and 143 and down-regulation of miRs-20b, 30a, and 146a were observed in two out of the three studies, however no miRNA was consistently altered in all three studies, suggesting that the cell type from which they were derived may influence the miRNA expression profile of the differentiated cells. A more recent study has shown that the over-expression of miR-122 could indirectly promote the hepatic differentiation of mouse embryonic stem cells (ESCs) via indirect induction of two liver-specific transcription factors, FoxA1 and HNF4α (Deng et al., 2014).

4. MicroRNAs in liver regeneration

The liver is one of the few organs with the ability to regenerate following loss through trauma or surgical resection. Partial hepatectomy (PH), removing up to two-thirds of the liver mass in

rodents, is commonly used to study liver regeneration. Following two-thirds PH, replacement of liver mass is achieved by proliferation of mature hepatocytes which each undergo an average of 1.4 rounds of replication to re-establish normal liver weight within 5–7 days (8–15 days in humans) (reviewed in Michalopoulos, 2007). Numerous publications since 2010 have focused on the role of individual miRNAs in regulating hepatocyte proliferation during liver regeneration (Fig. 1) including miRs-21, 127 and 26a (Marquez et al., 2010; Ng et al., 2012; Pan et al., 2012; Zhou et al., 2012), or during the termination of liver regrowth in which miR-34a increases and miR-23b decreases (Chen et al., 2011a; Yuan et al., 2011), following PH.

As reported previously (Raschzok et al., 2012), one the of the complicating factors in identifying consistent changes in miRNAs during liver regeneration has been that individual groups use different models e.g. mouse, rat or human, and look at different times points during the regenerative process. Despite this, several reports have provided valuable insight into the changes in individual miRNAs during liver regeneration. Several groups have performed array-based studies to assess global miRNA changes during the highly proliferative phase of liver regeneration. All demonstrated that miRNA expression patterns changed considerably during liver regeneration, and putative targets for some of these regulated miRNAs have been identified (Song et al., 2010; Chen et al., 2011b; Raschzok et al., 2011; Shu et al., 2011). Shu and colleagues (2011) observed that miRNA regulation post-PH followed a biphasic pattern where miRNAs were up-regulated within the first 3-18 hours following PH before a general down-regulation of as much as 70% of miRNAs by 24 hours post PH. Additionally, increased expression of genes associated with miRNA biogenesis such as *Tarbp2* and *Dicer1* correlated with the decreased expression of some miRNAs, indicating that a negative feed-back loop exists between miRNAs and their regulatory genes.

Several studies have reported the induction of miR-21 during the first 24 hours of liver regeneration following PH thus making miR-21 the most consistently altered miRNA during the early stages of regeneration (Castro et al., 2010; Marquez et al., 2010; Song et al., 2010; Shu et al., 2011; Dippold et al., 2012; Ng et al., 2012; Schug et al., 2013). Consistent with this, several miR-21 targets have been reported that are involved in hepatocyte proliferation. These include *Btg2* which inhibits FoxM1b that is required for hepatocyte proliferation (Song et al., 2010); *RhoB* leading to increases in Cyclin D1 (Ng et al., 2012) and *Peli1*, a ubiquitin ligase involved in NFκB signalling, which may be part of a negative feedback loop required to inactivate NFκB in latter stages of liver regeneration (Marquez et al., 2010). Interestingly, miR-21 abundance is further enhanced by the bile-acid, ursodeoxycholic acid (UDCA) and by

ethanol following PH in rats (Castro et al., 2010; Dippold et al., 2012) however, despite elevated miR-21 levels, the presence of ethanol blocked hepatocyte proliferation (Dippold et al., 2012).

Several publications have reported increases in other miRNAs during liver regeneration but none as consistently altered as miR-21. These include miR-34a which targets *Met* and *Inhbb* possibly for termination of regeneration (Chen et al., 2011a) and miR-221 which targets the cyclin-dependent kinase (CDK) inhibitors *Cdkn1b* (p27), and *Cdkn1c* (p57), and Aryl hydrocarbon nuclear translocator (*Arnt)* (Yuan et al., 2013). Many miRNAs are also repressed during liver regeneration. Several papers have reported the repression of miR-26a that targets the cell-cycle genes, Cyclin D2 (*Ccnd2*) and Cyclin E2 (*Ccne2*), which are involved in proliferation in the remnant liver following PH (Chen et al., 2011b; Raschzok et al., 2011; Zhou et al., 2012). Indeed a nice correlation between down-regulated miRNAs and highly up-regulated genes was identified, for example decreased miR-26a correlated well with elevated *Ccne2* (Chen et al., 2011b). Interestingly, as noted below, increased expression of miR-26a can suppress tumour growth arising from c-MYC over-expression (Kota et al., 2009), highlighting miR-26a as an important regulator of hepatocyte proliferation.

Repression of miR-378 was also seen during the first 18 hours after PH and miR-378 inhibited *Odc1* (Table 1), which controls of DNA synthesis in hepatocytes (Song et al., 2010). Other studies have reported decreases in miRs-22a, 30b, *let-7* family members, and, 122a, and 150 within 12-48 hours after PH (Chen et al., 2011b; Yu et al., 2013). Of note miR-150 represses the hepatocyte growth factor, VEGF-A, thus increases in VEGF-A via reduced miR-150 levels could be important for the regenerative response (Yu et al., 2013). Finally, in accordance with the other studies, Raschzok et al. (2011) confirmed that changes in miRNA expression were most evident during the peak of DNA replication, occurring approximately 24 hours following PH. By coupling miRNA expression data with proteomic analysis 23 putative targets of 13 down-regulated miRNAs (including miR-26a and *let-7e*) were identified, of which many were associated with regulation of cell metabolism including pyruvate carboxylase and glutamate dehydrogenase as well as genes involved in protein translation e.g. eukaryotic translation elongation factor 1 alpha and cell shape e.g. keratins (Raschzok et al., 2011).

It is worth noting that some caution is required while interpreting these types of studies. As mentioned above, miR-21 is consistently increased during liver regeneration, however a recent elegant study that examined the miRNA load on RISC complexes found that while miR-21 was

increased following PH, its load on RISC in fact decreased (Schug et al., 2013). Thus simply determining the abundance of a particular miRNA and a correlated change of a potential target may not be sufficient to account for the changes seen in liver regeneration. Furthermore, other miRNAs with an altered RISC load may be more biologically relevant.

5. MicroRNAs and liver disease

Together with generation of bile and secreted blood proteins including albumin and coagulation factors, the liver is responsible for removal of toxic substances from the circulation. Loss of liver function leads to hepatic encephalopathy followed by progression to coma, seizures, cerebral oedema, and death (reviewed in Butterworth, 2003). Encephalopathy is frequently observed in chronic liver disease, which result from a number of etiologies including excessive alcohol consumption, obesity or viral hepatitis. Characterized initially by hepatic steatosis (fatty liver), chronic liver disease may progress to steatohepatitis, liver fibrosis and eventually liver cirrhosis, a major risk factor for the development of liver cancer (El-Serag and Rudolph, 2007).

5.1 Alcoholic liver disease

Excessive alcohol consumption is a major risk factor for the development of ALD. Whilst the mechanism of how alcohol induces liver damage is complex, chronic inflammation of the liver, most likely a result of oxidative stress and reactive oxygen species produced during alcohol metabolism by specific enzymes in the liver, appears to be key. Direct damage of liver cells, in addition to the production of an inflammatory cascade involving many inflammatory mediators may contribute to alcoholic hepatitis, and eventual ALD (reviewed in Szabo, 2007). Epidemiological data suggests that 10-35% of alcoholics will develop alcoholic hepatitis, regardless of the amount of alcohol consumed (reviewed in Mandayam et al., 2004). This suggests that there are additional genetic, epigenetic and/or environmental factors that can modulate ALD risk. Published data of miRNA expression profiles in ALD is limited, however there are a few studies that have focused on the expression of liver miRNAs following chronic ethanol feeding of rodents, or treatment of cultured cells with ethanol.

The Lieber-DeCarli diet is a commonly used model of chronic ethanol feeding in rodents. There are two studies in which the authors subjected mice to an ethanol diet for 4-5 weeks before profiling miRNA expression in the liver (Dolganiuc et al., 2009; Bala and Szabo, 2011). Dolganuic et al. (2009) found 2% of all liver miRNAs were altered following ethanol feeding; up-regulated miRNAs included miRs-320, 486, 705 and 1224, and down-regulated miRNAs

included miRs-27b, 182, 183, 200a, 214, 199a-3p, and 322. Whilst the authors did not include any target prediction or validation experiments, some of the dysregulated miRNAs have published targets involved in proliferation and modulation of alcohol-induced liver injury. In the second study, miRs-155 and 132 were up-regulated in the liver of alcohol-fed mice (Bala and Szabo, 2011). MiR-155 was consistently increased in both isolated hepatocytes and Kupffer cells (liver macrophages). Furthermore, isolated hepatocytes also showed decreased miR-125b expression, whereas an increase in miR-132 was only seen in isolated Kupffer cells. This finding was complemented by a subsequent study by Bala et al. (2011) in which a correlation was identified between increased miR-155 expression and increased TNFα production in macrophages treated with alcohol (Table 1). The authors also suggested increased NF-κB activation as a mechanism for miR-155 up-regulation following ethanol exposure.

A third study did not use the Lieber-DeCarli diet but administered ethanol to mice by gastric intubation and profiled plasma miRNAs to evaluate the possibility of using miRNAs as non-invasive biomarkers of liver injury (Zhang et al., 2010c). Plasma miR-122 was significantly elevated within 0.5 hours of ethanol administration, and correlated with an increase in alanine aminotransferase (ALT) activity, a traditional marker of hepatocyte injury (Ozer et al., 2008). Most notably, miR-122 plasma abundance was increased following alcohol exposure before any histopathological findings of liver damage were apparent (Zhang et al., 2010c). This suggests that circulating miRNAs could be developed as early diagnostic markers of liver injury resulting from alcohol abuse.

Comprehensive studies published by Yin et al. (2012) and Meng et al. (2012), specifically focused on potential targets of regulated miRNAs in an attempt to provide insight into how miRNAs may contribute to toxicity in ALD. In the first study, miR-217 was increased in both cultured hepatocytes exposed to ethanol, and in livers from ethanol fed mice, where it caused excess fat accumulation (Yin et al., 2012). Over expressed miR-217 resulted in a decrease in the NAD^+-dependent deacetylase Sirtuin-1 (*Sirt1*), as well as downstream effectors encoding lipogenic or fatty acid oxidation enzymes (Yin et al., 2012). This study provides a crucial link between ethanol exposure and fat accumulation that may lead to hepatic steatosis. In the second study, *Caspase-2* (*CASP2*) in addition to *SIRT1* were identified as direct targets of miR-34a, which increased following four weeks of ethanol administration to mice (Meng et al., 2012). Furthermore, the abundance of two matrix metalloproteases (MMP-2 and 9) was increased in ethanol-exposed mouse liver tissue, and following transfection of normal human hepatocytes with a miR-34a precursor. MMPs are proteolytic enzymes involved in cell remodeling,

suggesting their regulation by miR-34a may be involved in hepatic remodeling in ALD.

A recently published study on human ALD patients identified six miRNAs that could regulate as many as 79 downstream targets (Liu et al., 2013). While the authors did not provide any validation in this study, six miRNAs, namely miR-570, 122, 34b, 29c, 922 and 185, are implicated to regulate genes with diverse biological functions such as the immune response, the inflammatory response and glutathione metabolism. Intriguingly, with the exception of miR-122, there appears to be little overlap between the miRNAs identified in these human samples with those from rodent experiments. However as this study had a sample size of only ten subjects per group, additional human studies may yield more consistent results.

5.2 Non-alcoholic Fatty Liver Disease

Like ALD, NAFLD is a chronic liver disease characterised by hepatic steatosis, however this occurs in the absence of excessive alcohol consumption. Obesity, insulin resistance and type II diabetes have been recognised as major risk factors for NAFLD, with prevalence increasing from ~30% in the general population to ~74% amongst diabetics, and up to 90% in the morbidly obese (reviewed in Baffy et al., 2012). NAFLD patients are at risk of developing non-alcoholic steatohepatitis (NASH), which increases their risk of progression through hepatic fibrosis and cirrhosis to liver cancer.

As with other conditions, changes in the abundance of miRNAs has been linked to NAFLD. Comprehensive studies by several groups have focused on the identification of miRNA target genes involved in the pathogenesis of NAFLD/NASH in human patients. MiR-34a, which was dysregulated in some cases of ALD (Meng et al., 2012), was also increased in the liver of patients with NASH (Fig. 1), and correlated with a decrease in its target *SIRT1* (Min et al., 2012; Castro et al., 2013; Derdak et al., 2013). Decreased SIRT1 results in dephosphorylation of AMP kinase and increases the active form of HMG-CoA reductase, a key regulator of cholesterol synthesis that has been linked to NAFLD (Caballero et al., 2009). A miR-34a associated reduction in SIRT1 is also linked to reduced hepatic malonyl-coA levels which promotes β-oxidation of fatty acids to reduce hepatic steatosis (Derdak et al., 2013). Finally, in morbidly obese patients increases in miR-34a, acetylated-p53 levels, and apoptosis correlated with disease severity, while SIRT1 abundance was diminished in the NAFLD liver (Castro et al., 2013). Interestingly, feeding rats with UDCA resulted in inhibition of the miR-34a/SIRT1/p53 pathway suggesting p53 increases miR-34a abundance to repress SIRT1 in a positive feedback loop (Castro et al., 2013). In addition to miR-34a, miR-296-5p was also

decreased in NASH patients (Cazanave et al., 2011), and studies in a human hepatoma cell line identified *PUMA* as a direct target (Table 1). PUMA is a protein that is likely involved in apoptotic damage in fatty liver disease, evidenced by miR-296-5p inhibition experiments in which cells became sensitised to lipoapoptosis. A recent study by Sharma et al. (2013) which assessed RNA expression in the visceral adipose tissue of morbidly obese patients found that several enzymes involved in miRNA processing namely, Dicer1, Drosha and DGCR8, are elevated in patients with NASH compared to the non-NASH NAFLD cohort. This highlights a novel mechanism of miRNA regulation that may contribute to pathogenesis in patients with NAFLD.

Genetic and dietary rodent models of obesity have been developed to mimic the development of NAFLD. These models have been used to profile changes in miRNA expression, and identify associated gene targets that may play a role in the progression of obesity to NAFLD, and subsequent development of NASH. Additionally, cellular models of steatosis provide an excellent system in which to study the effects of single miRNAs on gene expression.

In an extensive study by Wang et al. (2009), miRNAs were profiled in the livers of mice fed a choline-deficient, low methionine, amino acid-defined diet that is known to promote NASH that will progress to hepatocarcinogenesis. Many significantly altered miRNAs were identified, including up-regulation of miRs-155, 221, 223, 342-3p and 34a and down-regulation of miRs-323-5p and *let-7a*. This study also characterised the regulation of miR-155, including induction of miR-155 expression by the transcription factor NF-κB, as mentioned previously, and subsequent down-regulation of the transcription factor *C/EBPβ* by miR-155 binding to it's 3' UTR. Lipoprotein lipase (*Lpl*), a regulator of lipid metabolism, was identified as a target of miR-467b, which was decreased in the livers of mice fed a high fat diet and in cultured hepatocytes (Ahn et al., 2011).

Development of an *in vitro* steatotic cell model by overloading a human hepatocyte cell line with fat resulting in a high concentration of free fatty acids was utilised by one group to identify miRNAs dysregulated under conditions mimicking NAFLD (Zheng et al., 2010). Numerous miRNAs were altered under conditions of fat-overload, with miRs-10b, 22, 29b-1 and 107 showing the greatest changes. *PPARα*, a transcription factor and lipid regulator in the liver, was verified as a direct target of miR-10b using miRNA overexpression and knockdown in steatotic L02 cells (Zheng et al., 2010) (Table 1). Consistently, a recent paper by Bechmann et al. (2013), confirmed the regulation of PPARα by miR-10b, which lies downstream of

Kruppel-like factor 6 (KLF6). Interestingly however, using *in vivo* studies the authors found that mice lacking KLF6 were protected from hepatic steatosis when fed a high fat diet for 16 weeks, which was accompanied by a decrease in PPARα activity. Furthermore, miR-10b was significantly decreased and negatively correlated with KLF6 expression in NAFLD patients with concomitant inflammation (Bechmann et al., 2013).

There is evidence to suggest that NAFLD, like ALD, is a complex disease regulated by a number of genetic and environmental factors. This was highlighted by two studies, the first of which showed variation in the miRNA expression profiles of rats fed three different diets (high fat, high sugar or both) to induce NAFLD (Alisi et al., 2011). In particular, miRs-21, 24, 99a and 140-3p exhibit markedly different expression patterns in each diet. In the second study, C57BL/6J and DBA/2J mice fed the same lipogenic methyl-deficient diet capable of inducing a NASH-like pathology exhibited marked strain-specific differences in the severity of NASH-symptoms (Pogribny et al., 2010). Disease severity correlated directly with a greater up-regulation of fibrosis-related genes. Furthermore, whilst similar miRNAs (including miRs-29c, 34a, 122, 155, and 200b) were dysregulated in the livers of both mouse strains, the magnitude of the changes were often greater in the strain with the more severe phenotype (Pogribny et al., 2010). The authors also correlated expression patterns of miRNAs with their published targets in mouse liver and in cultured hepatocytes, including miR-155 with *Cebpβ* and *Socs1*, and miR-200b with *Zeb1* and its downstream target E-cadherin.

Despite the complexity of NAFLD/NASH development, a ‘molecular signature’ of dysregulated miRNAs was identified that could distinguish steatosis from steatohepatitis in a rat model (Jin et al., 2012). This signature included 14 up-regulated miRNAs such as miR-29c, 34a, 146 and 210, and six down-regulated miRNAs including miR-33, 145 and 196b. It was hypothesised that these miRNAs play a pivotal role in the transition from one disease state to the next, and thus may be potential targets for preventing this form of chronic liver disease progression.

Several studies have also investigated the potential use of miRNAs as biomarkers for detecting early stage cases of NAFLD or their use as therapeutic targets to treat the disease (reviewed in Ceccarelli et al., 2013). MiR-15b was up-regulated in the livers of a rat model of NAFLD and also in the sera of a cohort of 69 patients with fatty liver compared with control subjects (Zhang et al., 2013). In a more comprehensive study with 403 participants, several miRNAs, namely miRs-21, 34a, 122 and 451, were higher in patients with NAFLD than those without (Yamada

et al., 2013). Not surprisingly since it is a liver-specific miRNA, the serum level of miR-122 positively correlated with the severity of liver steatosis (Yamada et al., 2013).

5.3 Viral hepatitis

Chronic viral hepatitis remains one of the most significant causes of chronic liver disease and primary liver cancer, with approximately 80% of hepatocellular carcinoma cases associated with chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infections (El-Serag, 2012). Treatment options for chronic HBV/HCV infection include interferon (IFN) therapy and administration of drugs such as Ribavirin, however successful clearance of virus is typically observed in only 40% of cases (Walsh and Alexander, 2001). Alternative therapies are thus needed to decrease the burden of HBV and HCV infection worldwide. Recent reports have shown antiviral effects of some mammalian miRNAs by direct targeting of HBV and HCV viral genes (Russo and Potenza, 2011). MiRNAs therefore offer great potential for the development of novel therapeutics for viral hepatitis, however a comprehensive understanding of how host miRNA expression is affected by viral infection is first required.

In a study comparing miRNA expression levels in livers of control subjects and patients with chronic HBV or HCV infection, it was found that eight down-regulated miRNAs, including miRs-26a, 29c, 219 and 320 (Fig. 1), could distinguish 'normal' liver from virally infected liver (HBV or HCV) using hierarchical cluster analysis (Ura et al., 2009). Despite having distinct virological features, the clinical manifestation of chronic HBV and HCV infections are indistinguishable (Geller, 2002). Interestingly, in the above-mentioned study the authors were able to show that miRNA profiles could differentiate HBV and HCV-infected liver. In this comparison, 13 miRNAs showed decreased expression in the HCV liver whereas six miRNAs showed decreased expression in the HBV liver. Furthermore, global gene expression analysis in each sample was used to correlate altered miRNAs with their potential targets. This revealed antigen presentation, lipid metabolism, immune response and cell cycle pathway activation in HCV infection, and cell death, DNA damage and signal transduction pathway activation in HBV infection (Ura et al., 2009). Recently, in a study of ten patients chronically infected with HCV, it was found that 22 miRNAs were increased, including miR-144, 486-3p, and 200c, and 35 miRNAs were decreased, including miR-802, 556-3p, and 615-5p, more than 2-fold (Ramachandran et al., 2013). Gene ontology (GO) analysis identified several dysregulated miRNA clusters affecting diverse pathways such as phospholipase C signalling (miRs-200c, 20b, and 31), response to growth factors and hormones (miRs-141, 107, and 200c), and cell proliferation (miRs-20b, 10b, and 141). Specifically, increased expression of miR-200c in

HCV liver can lead to down-regulation of its target FAP-1, and consequent increase in c-Src to promote fibrosis (Ramachandran et al., 2013).

Differential miRNA profiles between HBV and HCV infections can be explained by the fact that viral proteins can directly alter the expression of host miRNAs. In separate studies, Dicer and Drosha, two core elements of the miRNA biogenesis pathway, were negatively regulated by the HCV core protein and HBV protein X (HBx) respectively (Chen et al., 2008; Ren et al., 2012). Inhibition of Dicer or Drosha would decrease the expression of mature miRNAs, significantly impacting gene regulation. The expression of the HBx has also been inversely correlated with *let-7a* expression in a human hepatoma cell line, resulting in up-regulation of its target, *STAT3* (Table 1), and increased cell proliferation (Wang et al., 2010). Furthermore, HBx up-regulated miR-29a, which induced hepatoma cell migration via decreased expression of the tumour suppressor *PTEN* and downstream phosphorylation of *AKT*, a regulator of cell growth, survival, and migration (Kong et al., 2011). HBx was also shown to decrease miR-132 abundance in HCC cells via methylation of the miR-132 promoter, leading to inhibition of AKT signalling (Wei et al., 2013b). Reduced miR-132 abundance was also observed in HBV-related HCC tumour tissue compared with adjacent non-tumour tissue, and the levels of miR-132 in patient serum and tumour tissue were significantly correlated (Wei et al., 2013b). Taken together, these results indicate an oncogenic role for dysregulated miRNA in viral infection.

Interestingly, HCV has recently been reported to promote hepatic steatosis via induction of miR-27. By comparing HBV and HCV infected hepatoma cells it was demonstrated that HCV infection produced higher levels of miR-27a, and this promoted an increase in cellular lipid (Shirasaki et al., 2013). MiR-27a was shown to target several genes involved in lipid metabolism including the transcription factor RXRα and the lipid transporter ATP-binding cassette subfamily A member 1 (ABCA1). Furthermore, miR-27a enhanced IFN signaling *in vitro*, and patients who expressed high levels of miR-27a in the liver showed a more favorable response to IFN-based therapy (Shirasaki et al., 2013). Similarly, Singaravelu et al. (2014) showed that HCV infection of cells both *in vitro* and *in vivo* increased cellular abundance of miR-27b and decreased expression of its target PPARα, resulting in lipid accumulation and hepatic steatosis. These studies indicate that miRNAs may be mediators of HCV induced hepatic steatosis.

In addition to modulation of host cell properties, host miRNAs can directly regulate viral replication of both HBV and HCV. For example, miRs-372 and 373 that are up-regulated in

HBV-infected liver can stimulate the production of HBV DNA and proteins via down-regulation of the transcription factor Nuclear factor I/B (*NFIB*) (Guo et al., 2011). NFIB has previously been implicated in the regulation of several viruses such as feline leukemia, human papilloma, and mouse mammary tumour viruses. In another study that assessed the effect of miRNA mimics on HBV replication, miR-1 was shown to indirectly increase HBV transcription, gene expression and progeny secretion through modulation of several host genes including farnesoid X receptor α (*FXRA*), which enhanced HBV promoter activity (Zhang et al., 2011). It has also been shown that HBV significantly increases miR-501 abundance in hepatoma cells, and suppression of miR-501 reduced HBV replication via repression of HBXIP, a negative regulator of HBV replication, identifying miR-501 as a promoter of viral replication (Jin et al., 2013). Likewise, HBx expression in similar cells reduced miRs-15 and 16 abundance, which could prevent HBV replication by targeting viral HBx and HBV polymerase protein (HBp) (Wang et al., 2013). Conversely, HBV infection of hepatoma cells can also induce the miR-17-92 cluster (miRs-17-5p, 18a, 19a, 19b, 20a, and 92a-1) in a c-MYC dependent manner to restrict HBV replication (Jung et al., 2013). Similarly for HCV, miR-141 could down-regulate expression of the tumour suppressor *DLC-1*, increasing HCV replication and cell proliferation in primary human hepatocytes (Banaudha et al., 2011). Furthermore, using miRNA mimics and inhibitors, four miRNAs (miRs-24, 149-3p, 638 and 1181) dysregulated in a HCV infected human hepatoma cell line were able to influence HCV entry or HCV RNA abundance, indicative of viral replication (Liu et al., 2010b). Collectively, these studies highlight the intricate interplay between HBV and HCV and the host cellular microRNAs and the regulatory affects they exert on viral replication.

As a liver specific miRNA, considerable attention has focused on the role of miR-122 in regulating chronic liver disease, particularly in HBV/HCV infection. A search of the literature revealed more than 100 publications linked to the search terms “miR-122 and HBV or HCV”. Subsequent to the discovery that HBV and HCV can induce markedly different host miRNA expression profiles, a striking difference in the role of miR-122 in HBV and HCV infection has been observed. Jopling et al. (2005) showed that cellular HCV RNA decreased when miR-122 was inactivated. Since then, several published studies have revealed a requirement of miR-122 and/or its precursor molecule for HCV replication and stability (Jangra et al., 2010; Cox et al., 2013; Mortimer and Doudna, 2013) and successfully validated an anti-miR-122 therapy for suppression of viremia in chronically HCV infected primates (Lanford et al., 2010). Moreover, a study in which the serum from 102 HCV patients were compared to healthy controls identified a 23-fold increase of miR-122, indicating that it may be a useful biomarker to detect

chronic HCV infections (van der Meer et al., 2013). Conversely, miR-122 was found to be significantly down-regulated in the livers of chronic HBV infected patients, inversely correlating with viral load (Wang et al., 2012). Further investigation disclosed that loss of miR-122 enhances HBV transcription through a complex signalling pathway involving up-regulation of Cyclin G1 (*CCNG1*) which binds p53, thus preventing p53 mediated repression of a HBV enhancer element (Wang et al., 2012). Consistent with these findings, miR-122 is decreased in HBV-associated but not HCV-associated HCC (Spaniel et al., 2013). The mechanism of miR-122 down-regulation is mediated, at least in part, by the binding of HBx to PPARγ leading to inhibition of miR-122 transcription (Song et al., 2013).

At first glance miR-122 appears to be a good candidate for development of a miRNA-based therapy for modulating viral replication in both HCV and HBV infected patients. However, a separate study revealed a subset of chronic hepatitis C individuals with decreased levels of miR-122 that would be poor candidates for miR-122-based therapy (Sarasin-Filipowicz et al., 2009). Thus a more thorough understanding of the role of miRNAs in viral infection is required before successful miRNA therapies can be developed.

5.4 Primary liver cancer

Liver cancer is the sixth most commonly diagnosed cancer, and the third leading cause of cancer related death worldwide (Jemal et al., 2011). Hepatocellular carcinoma (HCC) accounts for the majority (85-90%) of primary liver cancers in adults, with the remainder attributed to cholangiocarcinoma (CC) as well as other rare entities (El-Serag and Rudolph, 2007). Chronic liver diseases such as chronic viral hepatitis as well as alcohol abuse, metabolic disorders and aflatoxin B exposure are major etiologic risk factors for the development of primary liver cancer, in particular HCC. Notably, in the Western world the sharp increase in the incidence of NAFLD and NASH is predicted to supersede alcohol as the most prevalent risk factor for liver cancer within the next decade. Curative treatment of liver cancer remains challenging as traditional chemo- and radiation therapies are largely ineffective against advanced HCC. Surgical intervention by means of liver resection or transplantation as well as radio-frequency ablation for eligible patients therefore offers the most effective treatment option for liver cancer. However, in the majority of patients diagnosis occurs at advanced stages leading to ineligibility for liver transplant. Furthermore, high recurrence rates and frequent metastases result in poor patient outcomes (reviewed in Rahbari et al., 2011). These observations clearly indicate that liver cancer is a substantial health care problem and the development of novel molecular approaches to treat HCC is therefore a growing field of interest.

Hepatocarcinogenesis is a multi-stage process that results from sequential accumulation of (epi)-genetic events in three core cellular processes, i.e. cell fate, cell survival, and genome maintenance (Vogelstein et al., 2013). MiRNAs are implicated in all of these processes and, like protein-coding genes, can function as oncogenes or tumour suppressors depending on the mRNAs they target. MiRNAs are frequently located in cancer associated genomic regions or fragile sites, and dysregulated miRNA expression has been reported in many cancers, including liver cancer (reviewed in Calin and Croce, 2006; Zhang et al., 2007). A number of studies have assessed the role of miRNAs with regulatory effects on key pro-oncogenic processes in the context of primary liver cancer. Down-regulation of several miRNAs in HCC cells including miRs-26a, 101, 22, 99a, and 129-5p (Fig. 1) has been reported with subsequent up-regulation of their respective targets, *Ccnd2* and *Ccne2*, *MCL1*, *HDAC4*, *IGF-1R* and *mTOR*, and Valosin-containing protein (*VCP*) (Kota et al., 2009; Su et al., 2009; Zhang et al., 2010b; Li et al., 2011; Liu et al., 2012b) (Table 1). Each of these targets has been reported to regulate different aspects of tumour growth including cell cycle progression e.g. Cyclin D2, and apoptotic resistance e.g. Mcl-1. Similarly, increased miR-25 abundance was observed in CC lines and patient samples, and correlated with apoptotic resistance via down-regulation of the TRAIL receptor, Death Receptor-4 (*TRAIL-DR4*) (Razumilava et al., 2012). Additionally, many recent studies have compared miRNA expression profiles in liver cancer to "normal" liver, and there are several excellent reviews that describe these data comprehensively (Gramantieri et al., 2008; Varnholt, 2008; Isomoto, 2009). Therefore this review will focus mainly on more recent studies that have analysed miRNAs, in particular those with translational implications such as predictors of patient outcome. These include miRNAs involved in early diagnosis and tumour classification, disease progression, chemotherapy resistance, tumour metastasis, and recurrence of HCC and CC.

Recently, global analyses of miRNAs by next-generation sequencing identified nine miRNAs (miRs-122, 99a, 101, 192, 199a/b-3p, and several *let-7* family members) with differential expression between normal human liver, hepatitis, and HCC accounting for ~88.2% of the "miRNome" in the liver (Hou et al., 2011). The study also demonstrated that low miR-199a/b-3p expression was associated with poorer outcome for HCC patients. Moreover, subsequent targeting of miR-199a/b-3p using adeno-associated virus (AAV) 8 inhibited tumour growth via interacting with PAK4/Raf/MEK/ERK pathway.

Given the prominent role of miR-122 in liver homeostasis, several studies have demonstrated the relevance of this miRNA in liver cancer development (Gramantieri et al., 2007; Zhang et al., 2009). The deletion of murine miR-122 not only leads to hepatic steatosis and inflammation but also to HCC development by activating oncogenic pathways e.g. TGFβ, MAPK and PTEN, as well as increased infiltration of inflammatory cells that produce pro-inflammatory cytokines, including IL-6 and TNF (Hsu et al., 2012; Tsai et al., 2012). Furthermore, in a clinical context, HCCs with low miR-122 displayed a particularly poor prognosis and showed enrichment of gene sets commonly associated with cancer progression (Coulouarn et al., 2009). Functionally, loss of miR-122 resulted in an increase of cell migration and invasion indicating that miR-122 is a marker of hepatocyte-specific differentiation and an important determinant in the control of metastasis.

Another global miRNA analysis performed on a large cohort of 104 HCC, 90 adjacent cirrhotic livers, 21 normal livers, and 35 HCC cell lines identified a set of 12 dysregulated miRNAs (including miRs-21, 221, 222, 34a, 519a, 93, 96, and *let-7c*) that were associated with liver cancer progression, where miR-221/222 were the most up-regulated (Pineau et al., 2010). Moreover, miR-221/222 enhanced cell proliferation via targeting *CDKN1B* (p27) (Pineau et al., 2010), and induced TRAIL resistance and enhanced cellular migration through the activation of the AKT pathway as well as metalloproteinases by targeting the *PTEN* and *TIMP3* tumour suppressors (Garofalo et al., 2009). Interestingly, as in liver regeneration, miR-21 is also highly increased in HCC and affected cell proliferation, migration, and invasion by targeting the PTEN/mTOR pathway (Meng et al., 2007). Besides the above mentioned studies several other miRNAs have been demonstrated to harbour both tumour suppressive (e.g. miRs-1, 26, 29, 34a, 195, and 223) and oncogenic activity (e.g. miRs-9, 181, and 224) in HCC underlining the relevance of miRNAs as targets for diagnostic or therapeutic interventions in liver cancer (see review by Huang and He, 2011).

MiRNAs show great potential as biomarkers since they are secreted into the circulation through vesicles (microvesicles or exosomes) and can be detected in almost all body fluids (Borel et al., 2012). Consequently, several studies have assessed the potential of miRNAs to aid diagnosis of primary liver cancer, including improving the sensitivity and specificity of HCC detection by evaluating tissue and plasma miRNA expression (Tomimaru et al., 2012). Using a cohort of 96 HCCs Liu et al. (2012a) demonstrated that the combined use of miRs-15b and 130 yielded 98.2% sensitivity and 91.5% specificity, and detection sensitivity remained high (96.7%) in a subgroup of HCCs with low circulating Afp, a commonly used serum biomarker for HCC (<20

ng/mL). Furthermore, these miRNAs successfully identified Afp negative, early-stage HCC cases indicating their potential as complementary diagnostic tools. In another screen of sera from more than 500 patients, six miRNAs were identified that were significantly increased in HCC vs control samples (Li et al., 2010). Among these, miRs-375 and 92a were also enriched in patients infected with HBV. Moreover, a combination of three of these miRNAs (miRs-25, 375, and *let-7f*) accurately distinguished HCC from control patients.

Similarly, using a cohort of 934 patient plasma samples Zhou et al. (2011) identified a miRNA panel (miRs-122, 192, 21, 223, 26a, 27a, and 801) with high diagnostic accuracy for HCC irrespective of disease status. However, it was particularly useful for the diagnosis of early-stage HBV-related HCCs, and could discriminate HCC from healthy, chronic HBV and cirrhotic patients. Other miRNAs associated with HCC included miRs-222, 223, 21, and miR-122 (Qi et al., 2011; Xu et al., 2011). Another recent study showed that circulating miR-21 was significantly increased in patients with HCC compared to patients with chronic hepatitis or healthy individuals (Tomimaru et al., 2012), and miR-21 expression was reduced following surgical resection of HCC. Finally, combination of miR-21 expression levels with traditional markers of HCC such as Afp increased the sensitivity and specificity of detection thus improving the diagnostic power. Together, these data provide compelling evidence for the feasibility of utilising circulating miRNAs as biomarkers for HCC diagnosis.

Prognostic classification using expression profiles has a long-standing history in HCC (Marquardt et al., 2012). In recent years, the power of miRNAs for classifying liver cancers has been repeatedly demonstrated. Profiling miRNA by microarray has revealed subclasses associated with clinico-pathological features as well as mutations in several oncogenic pathways such as β-Catenin and HNF1A (Ladeiro et al., 2008). A recent study that investigated the heterogeneity of miRNA profiles of 89 HCCs identified three clinically distinct subclasses of HCCs that showed enrichment of specific miRNAs (Toffanin et al., 2011). The same study also identified an oncogenic role for miR-517a *in vitro* and *in vivo*, potentially via activation of NF-κB or MAPK/ERK signalling. Other miRNA profiling of paired tumour and non-tumourous adjacent liver tissue from 73 HCC patients identified 13 and 56 recurrence-related miRNAs, respectively (Sato et al., 2011). Importantly, while the number of miRNAs associated with recurrence was significantly higher in non-tumourous tissue and predicted late recurrence, the tumour-derived miRNAs gave superior accuracy in prediction of early recurrence.

Given the described changes of several miRNAs during hepatocarcinogenesis, it is not surprising that miRNAs have emerged as attractive drugable targets in liver cancer (Szabo et al., 2012). Kota et al. (2009) demonstrated that induction of miR-26a expression in hepatoma cells leads to a cell-cycle arrest by directly targeting of Cyclin D2 and E2. Subsequently, systemic *in vivo* delivery of this miRNA by AAVs in a mouse model of HCC resulted in reduced tumourgenicity without overt cellular toxicity in normal cells. Recent studies by two groups established the potential of targeting miR-221 as a cancer therapeutic (Park et al., 2011; Callegari et al., 2012). First, Park et al. (2011) demonstrated that anti-miR-221 could effectively reduce *in vivo* miR-221 levels and lead to subsequent inhibition of tumour cell proliferation as well as increased apoptosis. Callegari et al. (2012) then utilised a transgenic mouse model with liver-specific constitutive overexpression of miR-221 and resultant concomitant inhibition of its target protein-coding genes (i.e., CDK inhibitors *Cdkn1b* (p27), and *Cdkn1c* (p57), and B-cell lymphoma 2-modifying factor (*Bmf*)). The miR-221 transgenic mouse develops spontaneous nodular liver lesions, and consistent with the previous study, *in vivo* delivery of anti-miR-221 oligonucleotides led to a significant reduction of tumourgenicity in the same model.

Chemotherapeutic resistance is another major challenge in the treatment of primary liver cancer and contributes to poor patient outcome. Resistance of liver cancer cell lines to cytotoxic compounds including IFN-α, 5-fluorouracil, and cisplatin has been associated with changes in miRs-146a, 193a-3p and 199a-5p abundance, respectively (Tomokuni et al., 2011; Ma et al., 2012; Xu et al., 2012). However, the proposed mechanism for how each miRNA confers resistance differs. For example, miRs-193a-3p and 146a suppress apoptosis via modulation of proapoptotic CASP2L through *SRSF2*, and *SMAD4* respectively, whereas decreased miR-199a-5p in resistant cells increases cell proliferation by activation of autophagy via *ATG7*. Importantly, in all three studies, the sensitivity to the respective chemotherapeutics could be altered *in vitro* by modulating miRNA abundance (Tomokuni et al., 2011; Ma et al., 2012; Xu et al., 2012). Ji and colleagues (2009) recently dissected the miRNA contribution for the response to adjuvant therapy with IFN-α thereby confirming the therapeutic potential of miRNA-guided treatment modalities in HCC. The authors profiled miRNAs from 445 HCC patients comprising several cohorts, and demonstrated that the miRNA profile in liver tissue is vastly different between men and women with HCC. Furthermore, the authors identified miR-26 as a good predictor for sensitivity to IFN-α treatment and, more recently, developed a simple and reliable diagnostic test (MIR26-DX) to select HCC patients for adjuvant IFN-α therapy

thereby setting a benchmark first step to successfully utilise miRNAs in the clinic (Ji et al., 2013).

Liver cancer has a strong tendency for metastasis and early tumour recurrence following surgical intervention, contributing significantly to poor patient outcome. Besides malignant transformation and promotion of HCC, several miRNAs have been implicated in promotion or repression of this process (reviewed in Zhang et al., 2010a; Giordano and Columbano, 2013). Several groups have investigated the role of miRNAs in metastasis of primary liver cancer. Not surprisingly, a prominent role for miR-122 was also confirmed in this process. Loss of miR-122 induced the generation of intrahepatic metastasis by promoting angiogenesis via regulation of *ADAM17* (Tsai et al., 2009). Li et al. (2009) correlated decreased miR-34a abundance with metastasis and invasion of HCC tumours and identified *c-MET* as a direct target of miR-34a, which can modulate cell migration and invasion through ERK1/2 signalling. A recent study further demonstrated that reduction of miR-26a was associated with HCC recurrence and metastasis (Yang et al., 2013). Xu and colleagues (2013) showed that HBx directly repressed miR-148a expression in a p53-dependent manner thereby promoting cancer growth and metastasis through targeting of hematopoietic pre-B cell leukemia transcription factor-interacting protein (*HPIP*). Accordingly, inhibition of *HPIP* expression by miR-148a, reduced AKT and ERK levels and subsequently mTOR through AKT/ERK signalling. The authors concluded that miR-148a activation or *HPIP* inhibition might be a useful strategy for cancer treatment. Another recently described tumour suppressive miRNA with anti-metastatic properties is miR-612 (Tao et al., 2013). MiR-612 exerted its function by regulating *AKT2* during epithelial-mesenchymal transition (EMT) and metastasis and showed an inverse correlation with tumour size, stage, EMT, and metastasis in HCC patients. Furthermore, miR-612 not only affected local invasion but also intravasation at distant sites indicating that this miRNA is involved throughout the entire metastatic process.

Budhu et al. (2008) generated a 20-miRNA signature from a cohort of 241 HCC patients that efficiently predicted the occurrence of venous metastases and this correlated with patient outcome. This included four increased miRNAs including miR-219-1, and sixteen decreased miRNAs including miRs-30c-1, 148a, and 34a (Fig. 1). A separate study also compared the miRNA profiles of primary HCC and venous metastasis for 20 matched patients (Wong et al., 2012). Interestingly, although non-tumourous livers exhibited distinct miRNA profiles compared to primary HCCs and venous metastases, no apparent difference in the profiles of primary HCCs and venous metastases was revealed. However, miRNA levels were markedly

reduced in venous metastases compared to primary HCCs, suggesting that miRNA deregulation occurs early in hepatocarcinogenesis and that the generation of metastasis is aggravated by a stepwise disruption of the deregulated miRNAs. Few studies have been performed that compared metastatic and non-metastatic CC, however in one study, miR-214 was decreased in intrahepatic metastatic CC tissues compared to non-metastatic tissues, and inhibition of miR-214 promoted metastasis of a CC cell line *in vitro* (Li et al., 2012). Subsequent up-regulation of *TWIST1*, a miR-214 target gene associated with EMT, and consequent decrease in E-cadherin, could explain the EMT phenotype and may be crucial for CC metastasis *in vivo*.

The ability to predict an individual's risk of liver tumour recurrence would be highly beneficial to aid candidate selection for transplant and in tailoring appropriate post-operative care for the individual. Altered expression of certain miRNAs in the cancerous liver has been linked to an increased risk of disease recurrence following resection. Specifically, three studies defined miRNA expression signatures, consisting of 7, 18 and 67 miRNAs respectively, from HCC tissue samples to distinguish patients with a high risk of recurrence from those with a low risk (Chung et al., 2010; Barry et al., 2012; Han et al., 2012). From these studies miRs-10b, 21, 34c, 155 and *let-7d* positively correlated with recurrence, whilst miRs-15b, 20a, 24, 122, 145 and 182 negatively correlated with recurrence. Further investigation revealed a role for miR-15b in cell proliferation and inhibition of apoptosis, with the anti-apoptotic gene *BCL-W* identified as a direct target of miR-15b (Chung et al., 2010) (Table 1). Finally, Han et al. (2012) also performed bioinformatics Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis which identified signal transduction, cell cycle, cell differentiation, cell proliferation, and apoptosis as the most significant GO categories for predicted miRNA targets, with MAPK signalling, pathways in cancer, Wnt signalling and insulin signalling as the most significant KEGG pathways for dysregulated miRNAs in HCC recurrence.

6. Conclusions and future directions

The literature covered by this review clearly demonstrates that miRNAs play an important and extensive role in liver development and regeneration as well as contributing to or preventing chronic liver disease. Furthermore, it appears possible to use miRNA expression data to distinguish different types of liver pathologies emanating from different etiologies. This strongly advocates miRNA profiling as a viable alternate means to diagnose liver disease and predict patient outcome, and further suggests that miRNA manipulation may be the basis of effective therapy for the treatment of liver disease. The use of anti-miR-122 to suppress HCV

viremia (Lanford et al., 2010) and the observation that adeno-associated viral delivery of miR-26a to the livers of mice can suppress tumour growth arising from c-MYC over-expression (Kota et al., 2009) are two encouraging examples that highlight the potential of miRNA-based therapy to treat liver disease. Indeed the efficacy of an anti-miR-122 therapeutic (miravirsen) for the treatment of genotype 1 HCV is currently in phase 2 clinical trials (Lindow and Kauppinen, 2012).

Whilst significant progress has been made in our understanding of the biological role of miRNAs, the complexity of miRNA regulation, including specific cell/tissue type and temporal expression, target multiplicity and miRNA redundancy, presents many challenges to the miRNA researcher. The purpose of this review was to examine the role of miRNAs in liver development, regeneration and disease. Remarkably, only a handful of miRNAs are consistently reported in these processes, including miRs-23, 29, 30, 122, 192 and members of the *let-7* family (Fig. 1). Not surprisingly, as a liver-specific miRNA, miR-122 featured prominently in all processes discussed except liver regeneration. Furthermore, it was the only miRNA to be consistently dysregulated in all four chronic liver diseases covered by this review. It is noteworthy that while some miRNAs featured prominently in one disease or process, e.g. miR-34a in NAFLD/NASH or miR-21 in primary liver cancer, they were not consistently reported in other conditions (Fig 1). Our criterion for inclusion in Figure 1 was that a miRNA be reported at least twice for a particular process/condition and the majority of miRNAs did not meet this criterion. Thus the conclusions that can be drawn from these types of studies are two-fold; that miRNAs regulating these processes are in fact quite unique with little overlap, or alternatively that the full complement of miRNAs participating in these processes is not yet known. It is likely however, that the above mentioned miRNAs are critical in liver development and disease.

As miRNAs exert their effect by targeting protein-coding mRNAs, verifying targets of dysregulated miRNAs is of utmost importance. Whilst a large body of research has been undertaken thus far, much of it highly descriptive, detailing numerous dysregulated miRNAs, many studies lack definitive evidence of functional relevance by showing that appropriate target mRNA/s are affected. Drawing meaningful conclusions from this body of work has also been complicated by the fact that research groups often use different model systems, time points and methodologies, and currently, the field lacks studies that verify the findings of other groups. Looking ahead, miRNA research in liver development and disease could be significantly enhanced by encouraging comprehensive target validation for all miRNA studies.

Importantly, this should be performed under well-defined conditions, for example cell/tissue type or disease model, as it is clear that these variables can affect outcome in regard to miRNA expression and target regulation.

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Table and Figure legends

Table 1. Changes in microRNA abundance and validated target genes during liver development, regeneration and disease.

Figure 1. MicroRNA regulation during liver development, regeneration and disease. Schematic illustration of the changes in microRNA abundance during liver development, regeneration and disease. MicroRNAs cited as being up- (red) or down- (green) regulated during differentiation of hepatic progenitors, embryogenesis, and regeneration, as well as in four of the most common types of chronic liver disease (alcoholic liver disease (ALD), non-alcoholic fatty liver disease/non-alcoholic steatohepatitis (NAFLD/NASH), viral hepatitis, and primary liver cancer are represented. A minimum of two reports showing dysregulation of a miRNA in the same direction was the criterion for inclusion.

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Figure1

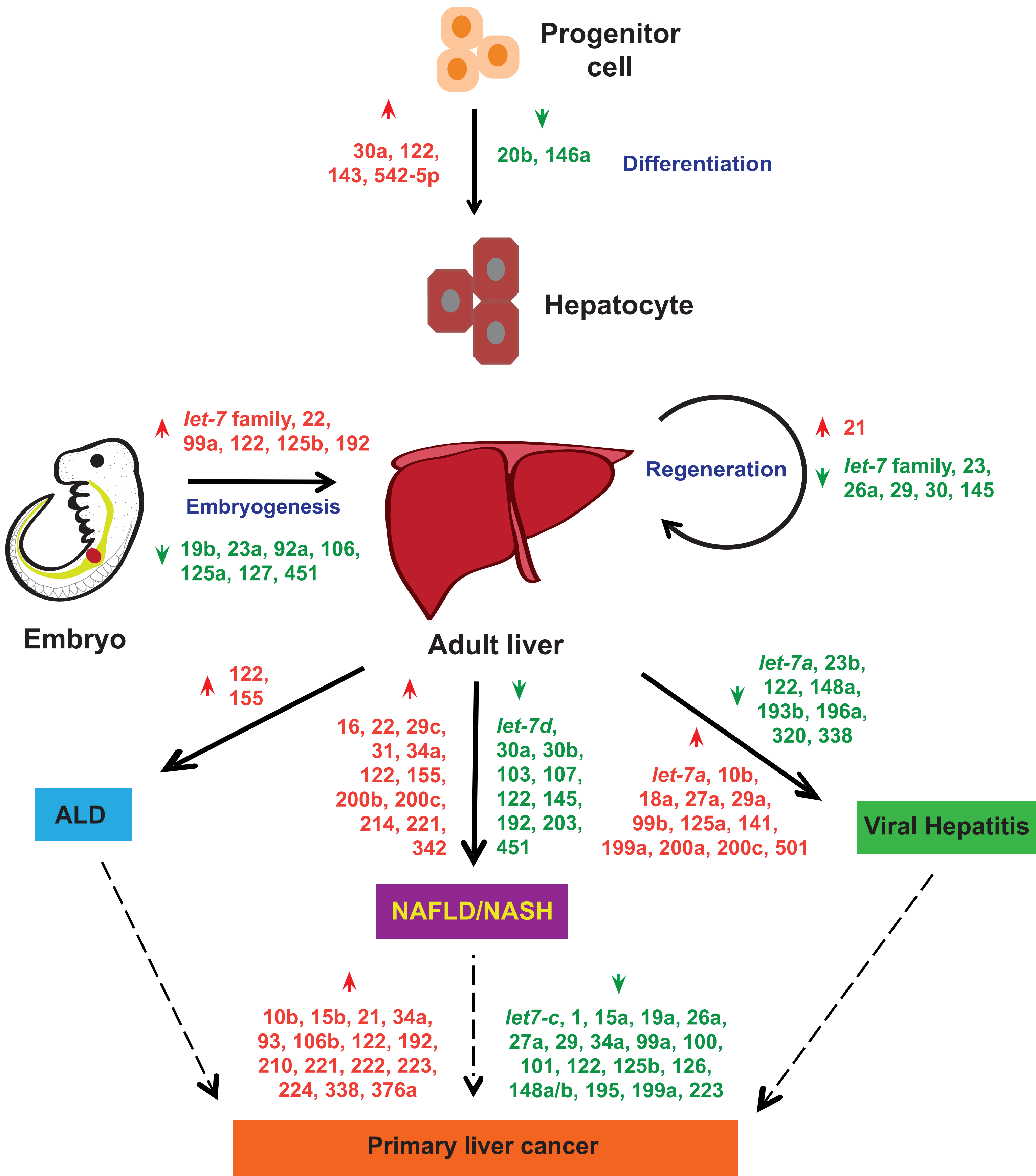

Table1

[a]miR	Δ	Process/disease etiology	Model/tissue/cell type	Method	Target/s	Target validation	Reference
let-7	↑	Regeneration	**Rat** liver 3-72h post PH	MA, qPCR	*Dicer1* *Tarbp2*	Inhibitor in Huh-7 cells with qPCR, W, LA	Shu et al. (2011)
let-7a	↓	Viral hepatitis (HBV)	**Human** HepG2 cells ± transduction HBV protein X, HCC T and NT liver	MA, qPCR	*STAT3*	Mimic/inhibitor with qPCR, W, β-Gal RA. Phenotype of target KD	Wang et al. (2010)
let-7c	↑	Development (embryo-adult)	**Human** embryonic (7-10w) and adult liver	MA, qPCR	*TGFβR1*	Mimic/inhibitor in Huh-7 cells with mRNA/protein quantification, LA	Tzur et al. (2009)
1	↑	Viral hepatitis (HBV)	Transfected miR mimic into ± constitutively expressing HBV **human** HepG2, and Huh-7 cell lines	qPCR for HBV	*HDAC4* *FXRA* *E2F5*	Mimic in HepG2, Huh-7 cells ± constitutively expressing HBV with qPCR, W, LA. Phenotype of target KD	Zhang et al. (2011)
10b	↑	NAFLD/NASH	Steatotic **human** L02 heps with high free fatty acid	MA, qPCR	*PPARα*	Mimic/inhibitor in L02 heps with qPCR, W, LA	Zheng et al. (2010)
15a	↓	Viral hepatitis (HBV)	**Human** HepG2 cells with overexpression or knockdown of miR-15a	Mimic/ inhibitor	*HBx* *HBp*	Mimic/inhibitor in HepG2 cells with LA, qPCR, W	Wang et al. (2013)
15b	↓	Cancer (HCC)	**Human** HCC tissue ± recurrence post resection	MA	*BCL-W*	Mimic/inhibitor in SNU-475 cells with W	Chung et al. (2010)
20a	↓	Development (embryo-adult)	**Mouse** foregut endoderm, hepatoblasts and adult liver	NGS	*Tgfbr2*	Inhibitor in HEK293T cells with LA and W	Wei et al. (2013a)
	↑	Viral hepatitis	**Human** HepAD38 cells ± HBV replication	qPCR, N	HBV DNA fragment	Inhibitor in HepAD38 cells with LA	Jung et al. (2013)
21	↑	Regeneration	Liver of **rats** ± Lieber-DeCarli diet for 5w, assessed 1-36h post PH	MA, qPCR	*Crebl2*	Precursor in HEK293 cells with LA	Dippold et al. (2012)
	↑	Regeneration (proliferation)	**Mouse** liver 1h-7d post PH	N	*Peli1*	Mimic in HEK293 cells with LA	Marquez et al. (2010)
	↑	Regeneration (proliferation)	**Mouse** liver 0-36h post PH	qPCR	*RhoB*	Mimic/inhibitor in Hepa1-6 cells with qPCR, W, LA	Ng et al. (2012)
	↑	Regeneration (proliferation)	**Mouse** liver 0-18h post PH	MA, qPCR	*Btg2*	Mimic/inhibitor in Hepa1-6 cells with qPCR, LA	Song et al. (2010)
	↑	Regeneration	**Rat** liver 3-72h post PH	MA, qPCR	*Dicer1*	Inhibitor in Huh-7 cells with qPCR W, LA	Shu et al. (2011)
	↑	Cancer (HCC)	**Human** T (HCC) and NT liver tissues and HCC cell lines	MA, qPCR, N	*PTEN*	Inhibitor in SK-HEP-1, SNU-182, HepG2, and PLC/PRF-5 cells with LA. Correlated target expression in tissue and cells	Meng et al. (2007)
22	↓	Cancer (HCC)	T (HCC) and NT **human** liver	qPCR	*HDAC4*	Mimic in Hep3B, SMMC-7721 cells with W, LA. Phenotype of target KD. Correlated target expression in tissue	Zhang et al. (2010b)
23b	↓	Regeneration (termination)	**Rat** liver 24-168h post PH	qPCR	*Smad3*	Mimic in BRL-3A cells with qPCR, W, LA	Yuan et al. (2011)
25	↑	Cancer (CC)	**Human** CC and benign cell lines. T (CC) and NT **human** liver	qPCR	*TRAIL-DR4*	Mimic/inhibitor in KMCH, H69, Mz-Cha-1 cells with W, LA, IF. Correlated target expression in tissue	Razumilava et al. (2012)
26a	↓	Regeneration (proliferation)	**Mouse** liver 24-168h post PH	qPCR	*Ccnd2* *Ccne2*	Mimic/inhibitor in mouse liver and Nctc-1469 cells with qPCR, W	Zhou et al. (2012)
	↓	Regeneration (proliferation)	**Rat** liver 24-72h post PH	MA, qPCR	*Ccne2*	Mimic in HepG2 cells with qPCR, W	Chen et al. (2011b)
	↓	Cancer (HCC)	**Mouse** liver ± specific overexpression of liver-tumour initiating MYC, panel of **human** HCC	N, qPCR	*Ccnd2* *Ccne2*	Mimic in HepG2 cells with W, LA	Kota et al. (2009)
	↓	Cancer (HCC)	**Human** T (HCC) and NT liver tissues, and ± metastasis	qPCR	*IL-6*	Mimic/Inhibitor in HCC-LM3, MHCC97-H, HepG2 and PLC cells with LA, qPCR, ELISA	Yang et al. (2013)
27a	↑	Viral hepatitis (HCV)	**Human** HCV- compared to HBV-infected liver	qPCR	*RXRα* *ABCA1*	Mimic//inhibitor in Huh-7.5 cells with LA, W	Shirasaki et al. (2013)
27b	↑	Viral hepatitis (HCV)	**Human** cells (Huh-7.5) and mouse liver tissues infected with HCV	qPCR	*PPARα*	Mimic in Huh-7 cells with qPCR	Singaravelu et al. (2014)
29/ 29a	↑	Regeneration	**Rat** liver 3-72h post PH	MA, qPCR	*Dicer1*	Inhibitor in Huh-7 cells with qPCR, W, LA	Shu et al. (2011)
	↑	Viral hepatitis (HBV)	Transgenic **mouse** liver, **human** HepG2 cells expressing HBV protein X	qPCR	*PTEN*	Mimic/inhibitor in HepG2, MHCC-97L cells with qPCR, W, LA. Phenotype of target KD. Correlated target expression in HCC	Kong et al. (2011)
30a	↑	Development (biliary)	**Mouse** and **human** embryonic (E12.5-18.5) and adult liver. Knockdown in **zebrafish**	MA, N, qPCR, ISH	*Ak1* *Tnrc6a*	Inhibitor in BMEL cells with MA, LA	Hand et al. (2009)
34a	↑	Regeneration (termination)	**Rat** liver 1-9d post PH	MA, qPCR	*Inhbb* *c-Met*	Mimic in BRL-3A cells with qPCR, W, LA. Phenotype of target KD	Chen et al. (2011a)
	↑	ALD	Liver of **mice** fed 22.7-35g/kg/d EtOH for 4w, **human** heps, cholangiocytes and HepG2 cells ± EtOH, **human** ALD and paired normal liver	MA, qPCR	*CASP2* *SIRT1* *MMP-2* *MMP-9*	Mimic in human heps with HPLC-Chip/MS analysis, qPCR, W, LA. Correlated target protein expression in heps ± EtOH	Meng et al. (2012)
	↑	NAFLD/NASH	**Human** liver biopsy from NASH, NAFLD, weight matched normal, and lean normal subjects	qPCR	*SIRT1*	Mimic/inhibitor in Huh-7 cells with qPCR, W	Min et al. (2012)

	↑	NAFLD/NASH	**Human** liver samples from NAFLD patients with steatosis and NASH	qPCR	*SIRT1*	Precursor in primary rat heps with W, LA	Castro et al. (2013)
	↓	Cancer (HCC)	**Human** HCC tissue ± metastasis	qPCR	*c-MET*	Mimic in HepG2 cells with qPCR, W. Correlated target protein expression in liver. Phenotype of target KD	Li et al. (2009)
92a-1	↑	Viral hepatitis	**Human** HepAD38 cells ± HBV replication	qPCR, N	HBV DNA fragment	Inhibitor in HepAD38 cells with LA	Jung et al. (2013)
99a	↓	Cancer (HCC)	T (HCC) and NT **human** liver	Deep seq., qPCR	*IGF-1R* *mTOR*	Mimic/inhibitor in HepG2, SMMC-7721, Huh-7, HL-7702 cells, SMMC-LTNM tumor mass, with LA, W. Correlated target expression in liver	Li et al. (2011)
101	↓	Cancer (HCC)	T (HCC) and NT **human** liver. **Mouse** and **human** liver/non-liver cells	MA, N	*MCL1*	Mimic in HEK293T cells with LA. Mimic/ inhibitor in HepG2 cells with qPCR, W	Su et al. (2009)
122/ 122a	↑	Development (embryo-adult)	**Mouse** embryonic (E12.5-18.5) and adult liver	N, qPCR	*Cutl1*	Mimic/inhibitor in human HCC cell lines with W. Correlated target protein expression during development	Xu et al. (2010)
	↓	Viral hepatitis (HBV)	Liver of chronically infected HBV **human** patients	AP-ISH, qPCR	*CCNG1*	Mimic/inhibitor in HepG2, Huh-7 cells with LA, W. Correlated target expression to HBV load in human liver	Wang et al. (2012)
	↓	Cancer (HCC)	**Human** HCC arisen on cirrhotic livers and HCC derived cell lines	MA, N, qPCR	*CCNG1*	Precursor in SNU-449 and Hep3B cells with LA, W. Correlated target expression in HCC	Gramantieri et al. (2007)
	↓	Cancer (HCC)	**Mice** with germline deletion of miR-122a, develop spontaneous HCC	Deletion	*KLF6*	Mimic in HEK293T cells with LA. Correlation of protein in knockout liver	Tsai et al. (2012)
	↓	Cancer (HCC)	**Human** HCC tumour and non-tumour pairs	N, qPCR	*SLC7A1* *AKT3* *ADAM17*	Mimic in HEK293T cells with LA	Tsai et al. (2009)
127	↓	Regeneration (proliferation)	**Rat** liver 0-168h post PH	MA, qPCR	*Bcl6* *Setd8*	Mimic/inhibitor in BRL-3A, Huh-7 cells with LA, qPCR, W. Phenotype of target KD	Pan et al. (2012)
129-5p	↓	Cancer (HCC)	**Human** T (HCC) and NT liver tissue	qPCR	*VCP*	Mimic/inhibitor in HepG2, MHCC-LM3, SK-HEP1 cells with LA. Correlated target expression in liver	Liu et al. (2012)
141	↑	Viral hepatitis (HCV)	Primary **human** heps infected with HCV	RPA-KA	*DLC1*	Mimic/inhibitor in primary heps with qPCR, W, LA	Banaudha et al. (2011)
146	↑	Cancer	**Human** PLC/PRF/5 hepatoma cells ± IFN-α resistance	MA, qPCR	*SMAD4*	Mimic/inhibitor in PLC/PRF/5 cells with W. Phenotype of target KD	Tomokuni et al. (2011)
148a	↓	Cancer (HCC)	**Human** T (HCC) and NT liver tissue ± HBV	qPCR	*HPIP*	Mimic/inhibitor in HepG2, BEL-7402, SMMC-7721, MHCC97-H and LO2 cells with WB, LA	Xu et al. (2013)
150	↓	Regeneration (proliferation)	Rodent liver 12-48h post PH	qPCR	*Vegfa*	Inhibitor in primary heps with qPCR, W.	Yu et al. (2013)
155	↑	ALD	RAW 264.7 **mouse** macrophages ± 50mM EtOH. Isolated Kupffer cells from **mice** fed Lieber-DeCarli diet (5% EtOH (v/v)) for 4w	qPCR	*TNFα*	Mimic/inhibitor in RAW 264.7 cells, and inhibitor in isolated Kupffer cells with TNFα production by ELISA	Bala et al. (2011)
	↑	NAFLD/NASH	Liver of **mice** fed choline-deficient, low methionine, amino acid-defined diet for 6-65w	MA, qPCR	*Cebpβ*	Mimic in HepG2, Hep3B cells with qPCR, W. Correlated expression in liver	Wang et al. (2009)
	↑	NAFLD/NASH	Liver of **mice** fed lipogenic, methyl-deficient diet for 12w	MA, qPCR	*Cebpβ* *Socs1*	Correlated protein expression with lipogenic diet by W. Mimic in primary heps with W	Pogribny et al. (2010)
193a-3p	↑	Cancer (HCC)	**Human** hepatoma cell lines sensitive (QGY-7703) or resistant (SMMC-7721) to 5-fluorouracil	Deep seq., qPCR	*SRSF2*	Correlated target mRNA/protein expression with sensitive/resistant cell lines. Mimic/ inhibitor in hepatoma cell lines with qPCR, W. Phenotype of target KD	Ma et al. (2012)
199a/b-3p		Cancer (HCC)	NT, viral infected and T (HCC) **human** liver	MPSS, qPCR	*PAK4*	Mimic/inhibitor in Hep3B cells with LA, W	Hou et al. 2011
199a-5p	↓	Cancer (HCC)	Blood from **human** patients with cisplatin treated unresectable/metastatic HCC. Hepatoma cell lines ± cisplatin	qPCR	*ATG7*	Mimic in HEK293T cells with LA. Mimic in Huh-7 cells with W	Xu et al. (2012)
200b	↑	NAFLD/NASH	Liver of **mice** fed lipogenic, methyl-deficient diet for 12w	MA, qPCR	*Zeb1*	Correlated target protein expression with lipogenic diet. Mimic in primary mouse heps with W	Pogribnyet al. (2010)
200c	↑	Viral hepatitis (HCV)	Chronic HCV infected **human** liver	MA	*FAP-1*	Mimic/inhibitor in normal human liver fibroblasts with qPCR, W	Ramachandran et al. (2013)
214	↓	Cancer (CC)	**Human** CC tissue ± metastasis	qPCR	*TWIST1*	Mimic in HEK293T cells with LA. Mimic in ICC-9810 cells with W	Li et al. (2012)
217	↑	ALD	**Mouse** AML-12 heps ± 25-100mM EtOH for 24h. Liver of **mice** fed low fat Lieber-DeCarli diet for 4w	qPCR	*Sirt1*	Mimic/inhibitor in AML-12 cells with LA, qPCR, W and FTAA	Yin et al. (2012)
221/ 222	↑	Regeneration	Primary heps and **mice** with overexpression of miR-221	Over-expression	*Arnt*	Mimic/inhibitor in primary heps with qPCR, W, LA. Correlated target protein in post PH tissue	Yuan et al. (2013)

	↑	Cancer (HCC)	**Human** T (HCC), cirrhotic and NT liver tissues and cell lines	MA, qPCR, N	*CDKN1B (p27)*	Mimic in HeLa and HEK293T cells with W, LA. Correlated target protein in tissue samples.	Pineau et al. (2010)
	↑	Cancer (HCC)	**Human** T (HCC) and NT liver tissue and cell lines	N, qPCR	*PTEN TIMP3*	Mimic/inhibitor in MEG01, H460, and Calu-1-lung cells with LA, W, qPCR	Garofolo et al. (2009)
296-5p	↓	NAFLD/NASH	**Human** livers – obese normal, simple steatosis and NASH	qPCR	*PUMA*	Mimic/inhibitor in Huh-7, KMCH cells with qPCR, WB, LA. Correlated target mRNA and protein in human livers – normal, simple steatosis and NASH	Cazanave et al. (2011)
302b	↓	Development (embryo-adult)	**Mouse** foregut endoderm, hepatoblasts and adult liver	NGS	*Tgfbr2*	Mimic in HEK293T cells with LA and W	Wei et al. (2013a)
372	↑	Viral hepatitis (HBV)	HBV infected **human** liver, HepG2 cells ± constitutive HBV production	MA, qPCR	*NFIB*	Cluster mimic in HepG2 cells with MA. Mimic in HeLa, HepG2 cells with W, LA	Guo et al. (2011)
373	↑	Viral hepatitis (HBV)	HBV infected **human** liver, HepG2 cells ± constitutive HBV production	MA, qPCR	*NFIB*	Cluster mimic in HepG2 cells with MA. Mimic in HeLa, HepG2 cells with W, LA	Guo et al. (2011)
378	↓	Regeneration (proliferation)	**Mouse** liver 0-18h post PH	MA, qPCR	*Odc1*	Mimic/inhibitor in Hepa1-6 cells with qPCR, LA	Song et al. (2010)
467b	↓	NAFLD/NASH	Liver of **mice** fed high fat diet for 8w. **Mouse** Hepa1-6 cells ± 50µM SFA for 24h	qPCR	*Lpl*	Mimic/inhibitor in Hepa1-6 cells with qPCR, W, LA. Correlated target mRNA with high fat diet and SFA treatment of heps	Ahn et al. (2011)
501	↑	Viral hepatitis (HBV)	**Human** HepG2 ± constitutive HBV production, **human** HBV related HCC tissue with high/low HBV replication	MA, qPCR	*HBXIP*	Inhibitor in HepG2.2.15 cells with qPCR and W	Jin et al. (2013)
612	↓	Cancer (HCC)	HCC tissues and paired lung metastases	MA, qPCR	*AKT2*	Mimic/inhibitor in HCCLM3 and HepG2 cells by W, LA	Tao et al. (2013)

[a]Abbreviations: ALD – alcoholic liver disease, AP-ISH – alkaline phosphatase *in situ* hybridization, β-Gal RA – β-Galactosidase reporter assay, CC – cholangiocarcinoma, Δ – change, d – days, Deep seq. – deep sequencing, ELISA – Enzyme-linked immunosorbent assay, EtOH – ethanol, FTAA – fluorometric target activity assay, h – hours, HBV/HCV – hepatitis B/C virus, HCC – hepatocellular carcinoma, heps – hepatocytes, HPLC-Chip/MS – high performance liquid chromatography on chip with mass spectrometry, IF – immunofluorescence, ISH – *in situ* hybridization, KD – knock down, LA – luciferase reporter assay, MA – microarray, miR – miRNA, MPSS – massively parallel signature sequencing, N – Northern blot, NAFLD – non-alcoholic fatty liver disease, NASH – non-alcoholic steatohepatitis, NGS – next generation sequencing, NT – non-tumourous, PH – partial hepatectomy, qPCR – quantitative reverse-transcriptase polymerase chain reaction, RPA-KA – RNA-primed array-based Klenow assay, SFA – saturated fatty acid, T – tumourous, v – volume, w – weeks, W – Western blot.