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TOPIC HIGHLIGHT

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microRNAs: Fad or future of liver disease

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Abstract

microRNAs (miRs) are small non-coding RNAs that regulate both mRNA and protein expression of target genes, which results in alterations in mRNA stability or translation inhibition. miRs influence at least one third of all human transcripts and are known regulators of various important cellular growth and differentiation factors. miRs have recently emerged as key regulatory molecules in chronic liver disease. This review details recent contributions to the field of miRs that influence liver development and the broad spectrum of disease, from non-alcoholic fatty liver disease to fibrosis/cirrhosis, with particular emphasis on hepatic stellate cells and potential use of miRs as therapeutic tools.

Key words: Liver; Fibrosis; microRNA; mRNA; Hepatic stellate cells

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INTRODUCTION

Post-transcriptional regulation of gene expression involves numerous modifications to the mRNA, including alterations at both 5' and 3' ends in addition to splicing of the transcripts. The 3' untranslated regions (UTRs) of mRNAs contain key stability elements that are subject to various regulatory proteins, as well as microRNA (miR) binding sites. miRs are small non-coding RNAs that alter gene expression through perfect complementarity of the miR to the target sequence, which results in mRNA instability and subsequent endonucleolytic cleavage or alternate 5' modifications[1]. Imperfect base pairing with the 3' UTR of target mRNA results in gene translation inhibition[2]. Research focusing on miR modes of action has primarily examined interactions with the 3' UTR of target genes; however, recent studies have expanded our knowledge and have demonstrated that miRs can interact with the 5' UTR as well as interact with DNA methylation machinery and affect chromatin status[3]. Since their discovery in 1993, miRs have been described in all multicellular organisms

and are associated with a vast breadth of biological functions including proliferation, cellular differentiation and immunity, as well as tissue remodeling and various human disease states, notably cancer[4]. Although much remains to be learned, miRs have already been shown to influence the expression of many mRNAs and proteins that are important in liver homeostasis in health and disease (Table 1). Emerging roles for miRs in liver development and disease are discussed in this review, as well as their potential for clinical application.

miRs IN LIVER DEVELOPMENT AND DISEASE

Liver development

miRs, while currently being exploited to understand disease pathologies, have been less appreciated in the role of functional gene repression during cellular growth and liver development. miRs, specifically let 7 and lin4, control larval development transition in *Caenorhabditis elegans*, as well as apoptotic pathways and growth in *Drosophila*[5]. Recent studies have elegantly demonstrated that miRs regulate differentiation of human cells and development of whole organ systems, which shows tissue-specific expression. miR 122 accounts for approximately 70% of the total liver miR population; however, specific roles for this miR outside of disease-associated functions [e.g. hepatitis C virus (HCV) and hepatocellular carcinoma (HCC)] are less clear, especially with regard to normal liver development. Xu *et al*[6] have shown that miR 122 is strongly upregulated during liver embryonic development with expression correlative to liver enriched transcription factors (LETFs) C/EBPα, HNF1α and HNF3 isoforms, which are all necessary for proper hepatocyte development and function. Additionally, the transcriptional repressor CUTL1, which is known to promote proliferation and suppress differentiation, is gradually repressed by miR 122 during development. *In vitro* miR 122 knockdown experiments have shown restoration of CUTL1 expression and of downstream target genes, which emphasizes that, during development, miR 122, as an effector molecule of LETFs, regulates a delicate balance between differentiation and proliferation[6]. Quantitative analysis of miRs in fetal *vs* adult livers has demonstrated that miR expression levels are higher in the fetal period and expression levels during this time are dynamic[5]. Additionally, recent studies have demonstrated that 162 miRs exhibit tissue-specific distribution in mice[7]. Endoderm-derived liver, jejunum and pancreas display differential miR enrichment, with miRs 122, 21, 101b, 107, 192 and 221 among those with the highest reads per million concentrations. Additionally, mapping of transcriptional start sites has demonstrated that divergent sites are used by miRs in endoderm-derived liver tissue as opposed to stem cells. Maternal influences on miR expression have also been identified and may be affected by diet. For example, studies by Zhang *et al*[8] have shown that, as a result of maternal high-fat diet, expression of miR let 7c is decreased in offspring, along with 23 other miRs including 122 and 194. let 7c was identified by Tzur *et al*[9] as being highly expressed in adult compared to embryonic liver, and that profibrotic transforming growth factor-β receptor 1 is a target of this miR. The miR 30 family has recently been shown to be crucial for liver development, because it is upregulated during later stages that influence known regulators of hepatic function, including epidermal growth factor receptor. GW182, a crucial component in miR-mediated gene repression, is also a target of this miR family, which indicates a more global role for miRs in earlier stages of epigenetic silencing *via* the RNA-induced silencing complex[10]. Functional analyses in zebrafish larvae have demonstrated that biliary morphogenesis is under the regulation of miR 30a, as knockdown studies have resulted in defective bile duct excretion[11]. The liver, as a vital multifunctional organ, must maintain proper epigenetic programming to ensure xenobiotic and bile metabolism, and vitamin and glucose storage among a plethora of other tasks, which emphasizes the importance of proper cellular differentiation and development.

Nonalcoholic fatty liver disease/nonalcoholic steatohepatitis

Nonalcoholic fatty liver disease (NAFLD) is characterized by hepatic fat accumulation without significant alcohol consumption or other underlying etiology[12]. NAFLD incidence is rising as a result of the increased population of obese individuals worldwide, with severity of the disease ranging from steatosis/steatohepatitis (NASH) to cirrhosis. Although most patients with NAFLD are asymptomatic, clinical manifestations of the disease are well described and include hyperechoic appearance of the liver on ultrasound, dyslipidemia, systemic arterial hypertension, and insulin resistance. Although disease pathogenesis is not completely understood, recently, a role for miRs in NAFLD has been established[13]. Lipid droplet accumulation in hepatocytes as a marker of steatosis is associated with various liver diseases including NAFLD. Recent high-content screening studies have shown that 11 miRs are able to alter lipid droplet formation/retention[14]. Specifically, miR 181d decreased droplets by approximately 60%, which subsequently reduced cellular triglycerides and cholesterol. Peroxisome proliferator-activated receptor (PPAR)α, a major contributor to NAFLD pathogenesis, has recently been identified as a target of miR 10b, which has been shown to regulate steatosis level in L02 cells (steatotic hepatocyte model)[15]. Additionally, post-transcriptional regulation of PPARα by miR 10b is maintained by a single binding site, as demonstrated by mutation analyses. NASH can progress to end-stage cirrhosis in approximately 15% of patients, with the mechanism that underlies disease progression being undefined. Recent studies in humans have demonstrated that miR expression profiles are altered in NASH. Unsurprisingly, miR 122 is significantly underexpressed (63%) in NASH subjects compared to those without the metabolic syndrome[16]. Overexpression of miR 122 in HepG2 cells results in a significant decrease in sterol regulatory element binding proteins (SREBP1-c, SREBP2), FAS and 3-hydroxy-3-methyl-glutaryl-CoA reductase; all of which are key lipogenic genes in human NASH. Lesser known

Table 1 Differentially expressed microRNAs in liver development and disease

Liver status	Upregulated miRs	Downregulated miRs	Ref.
Development	122, 92a, 483, 486-5p, 30 family	22, let 7 family, 199a, 21	[6,21]
NASH/NAFLD	34a, 146b, 200a, 224, 222, 10b, 22, 33, 31, 29c	122, 181d, 132, 150, 28-3p, 511, 517a, 671-3p, 99b, 433	[14-16]
HCV	122, let 7f, 155, 146, 296, 351, 128, 296, 141	196, 15a, 15b, 17, 106a, 106b, 181a, 29a, 29b, 93, 310, 30a, 30c, 24, 221, 222	[24,26,27]
ALD	705, 1224, 212	182, 183, 199a-3p, 199, NSC/NPC: 9, 21, 153, 335	[18,34]
Fibrosis	125-5p, 199b, 221, 302c, 223, 34c, 24	29a, 29b, 29c, 30b, 30c, 183, 96, 877, 341, 193, 132	[40,42]
Regeneration	21, 130a, 181b, 20a, 20b	378, 689, 7	[48,49]

NSC: Neural stem cell; NPC: Neural progenitor cell; miRs: microRNAs; HCV: Hepatitis C virus; ALD: Alcoholic liver disease; NAFLD: Nonalcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis.

miRs 34a and 146b are significantly overexpressed (99 and 80%, respectively) in NASH. In a smaller sample study (n = 12/group), seven miRs have been found to be differentially expressed, including 132, 150, 197 and 99; the latter two are significantly associated with pericellular fibrosis[17]. It is of particular importance to note that previous studies have shown that miR signatures change in accordance with disease stage and source of hepatic injury (ethanol or high fat diet)[18,19]. These studies emphasize the importance of avoiding generalization when examining miRs as potential biomarkers, because miR expression patterns of end-stage disease may be similar but expression profiles that lead to organ dysfunction appear to be divergent.

Viral infection

Along with NASH, HCV is one of the most common global causes of hepatic fibrosis and cirrhosis. According to the World Health Organization, over 180 million people are infected with the single-stranded RNA virus, with > 70% of that population at risk for developing cirrhosis[20]. Pegylated interferons in conjunction with ribavirin have improved treatment of chronic hepatitis C; however, a large percentage of the infected population remains unresponsive to available regimens, which emphasizes the need to better understand the mechanisms of cellular infection, for development of novel drug therapies. Numerous studies have emphasized a role for miR 122 in several aspects of hepatic function and disease, including HCV replication[21]. Plasma levels of miR 122 are elevated in hepatitis B virus (HBV)- and HCV-infected patients, as well as in models of alcohol and drug-induced liver damage reinforcing a role for miRs as biomarkers[22]. However, recent studies by Ura *et al*[23] have shown that miR expression patterns are divergent in HBV- and HCV-infected livers. Additionally, elegant studies by Hou *et al*[24] have demonstrated that miR 196 directly acts on the 3'UTR of Bach1, the transcriptional repressor of heme oxygenase 1, a critical cytoprotective enzyme. This miR also inhibits HCV expression (both genotype 1b and 2a) in human hepatocytes *in vitro*, which shows clinical promise. Accordingly, parallel miR and mRNA expression profiling studies in genotype-1b-expressing cells have shown numerous anti-correlated miR/mRNA pairs affected by the presence HCV[25]. Among the noted anti-correlated pairs that have been detected in a human cell line that expresses the HCV Con1 replicon, miR 130a/b is correlated with decreased PPARγ expression, which is a known regulator of the quiescent hepatic stellate cell (HSC) profile. Additional miR/mRNA profiling in HCV-infected hepatoma cells has shown a total of 108 differentially expressed miRs, and among this pool, miRs 24, 149, 638 and 1182 are predicted to control HCV entry, replication and viral propagation[26]. Scagnolari *et al*[27] have examined miR expression in peripheral blood mononuclear cells from healthy and chronic-HCV-infected patients and have found that miRs 1, 30, 128, 196 and 296 are differentially expressed and that treatment with interferon (IFN)α induces all aforementioned miRs. Although limited by small sample size, baseline levels of miRs do not appear to differ significantly between responders and non-responders; however, expression of miRs 128 and 196 appears higher in the responsive population. Additionally, Pedersen *et al*[28] have demonstrated this same set of miRs, which possess sequence-predicted targets within HCV genomic RNA, and are induced by IFNβ stimulation. The tumor suppressor gene DLC-1 (deleted in liver cancer-1) is also a target of miR repression[29]. Levels of DLC-1 are decreased in HCV-infected cells, which are correlated with the increase in miR 141. Depletion of this miR inhibits viral replication, while addition of exogenous miR 141 increases replication rates, which indicates that miR regulation of tumor suppressor genes can influence HCV infection and play a role in HCC development (for a comprehensive review of miRs in HCC see[30,31]).

Alcoholic liver disease

Alcoholic liver disease (ALD) is a major healthcare burden worldwide and a leading cause of liver-related mortality. It is well known that ethanol consumption alters methylation status and other epigenetic factors, including histone tail signatures, signal transduction pathways and transcription factor expression. Ethanol metabolism affects chromatin condensation, which results from direct DNA methylation or histone protein alterations (acetylation, methylation or phosphorylation)[32]. Additionally, DNA methylation is connected to direct interference by small RNAs[33]. Therefore, it is plausible that, as a result of epigenetic remodeling, ethanol exposure can also alter miR expression. Ethanol has been shown to repress miRs 9, 21, 153 and 335 in neural stem cells and neural progenitor cells that alter cellular development and maturation[34]. Additionally, miR 21 influences PTEN/PI3K signaling, which ultimately contributes to the fibrotic response, and both miRs 9 and 335 are predicted (by TargetScan) to bind to targets

that influence the progression of ALD[35]. Previous studies have highlighted the importance of miRs in the gut-liver axis and have shown that ethanol exposure alters miR profiles in liver cells[36]. Specifically, endotoxemia and direct ethanol exposure modulate hepatic miRs 182, 183, 705, 1224 and 199a-3p[37]. Symptomatic of ALD, hepatic microcirculation is disrupted when increased portal pressure arises as a result of altered endothelin-1 (ET-1) expression, with subsequent increases in NADPH oxidase and hypoxia inducible factor-1α[38]. Recent studies have shown that miR 199 is involved in ET-1 expression in rat liver sinusoidal endothelial cells and human endothelial cells, by functioning as a negative regulator of ET-1 transcription and thus vascular tone in ALD[39]. For still unknown reasons, only 20%-30% of chronic heavy alcohol users develop hepatic fibrosis. Future studies of miRs involved in ALD may allow us to uncover additional factors that contribute to the disparity that exists in disease development.

Liver fibrosis

Commonalities of miR expression can also be seen among fibrotic disorders of different organ systems, including cardiac and renal fibrosis, with an overarching effect of the miR 29 family in regulation of effector cellular differentiation or in translation of extracellular matrix (ECM) components[35]. Additionally, miRs involved with translation of profibrotic transforming growth factor (TGF)β/SMAD signaling are shared amongst pathologies. Roderburg *et al*[40] have examined miR expression profiles in a CCl_4 rodent model of hepatic fibrosis, and microarray analyses have revealed 31 differentially expressed miRs; 10 of which are overexpressed in fibrotic tissue, including miRs 125-p, 199b, 221 and 302c. Marked downregulation has been observed in a pool of 21 miRs; specifically, miR 29 family members were significantly reduced. Additional experiments in an alternate model of fibrosis (bile duct ligation; BDL) have confirmed that miRs 29b/c are significantly reduced compared to sham controls. Downregulation of miRs 29a/b/c could also be observed in explanted liver samples with patients denoted by a Desmet fibrosis score of 2-4. Low plasma levels of miR 29a correlate with advanced stage liver fibrosis in human patients. The idea that low miR levels in the tissue correlate with low miR in the circulating plasma is in opposition to recent hypotheses that an inverse correlation between tissue and plasma miR concentrations exists as a byproduct of vesicles that release miRs[41]. Hepatic fibrosis is influenced by several epigenetic factors that control the wound-healing response. Recent studies by Mann *et al*[42] have shown that miR 132 is significantly decreased in fibrotic livers as demonstrated in two different models (BDL, CCl_4), and this downregulation influences HSC activation. Collectively, these studies have emphasized that, during development of fibrosis, there are important changes in miR expression that regulate wound-healing transcripts; however, effects exerted by miRs appear to act in concert with other epigenetic factors to direct disease progression.

miRs IN HSCs

HSCs are the main effector cell population in hepatic fibrosis as the primary source of type I collagen deposition following injury. Injury from several sources, including viral infection, obesity and alcohol consumption, causes activation of HSCs, in which quiescent lipid-rich cells transdifferentiate into fully activated myofibroblasts. The activated form of the HSC secretes profibrogenic mediators, including TGFβ, and generates ECM components including fibrillar collagens, fibronectin and laminin, which exacerbates the wound-healing process[38]. Recent studies have shown that miRs are involved in HSC transdifferentiation (Figure 1). Specifically, miRs 150, 187, 194 and 207 are significantly downregulated in HSCs isolated from BDL animals compared to sham controls, whereas let 7 family members are significantly upregulated[43]. Overexpression of miRs 150 and 194 in human HSCs (LX-2) results in proliferation inhibition as well as decreases in type I collagen and smooth muscle alpha actin (αSMA), which are hallmarks of HSC activation. Specific action of these two miRs includes inhibition of c-Myb and Ras-related C3 botulinum toxin substrate 1 (Rac-1), which both contribute to development and progression of fibrosis. Additional studies have examined differential expression in quiescent (day 2) and activated (day 14) rat HSCs, and have shown 12 upregulated and nine downregulated miRs, of which, 15b, 16, 122, 138, 140 and 143 have been validated[44,45]. Gene ontology has revealed specific linkages between the miR 15/16 family and anti-apoptotic pathways that are important in HSC activation. Administration of miR mimics induced Bcl-2 inhibition and subsequent apoptosis in the activated HSCs, an important aspect of potential therapeutic targeting. Additional studies by Guo *et al*[44] have further demonstrated that lentiviral delivery of miR 16 greatly reduces cyclin D1 levels in addition to inhibiting proliferation and increasing apoptosis in activated HSCs. More recent studies by Ogawa *et al*[46] have shown

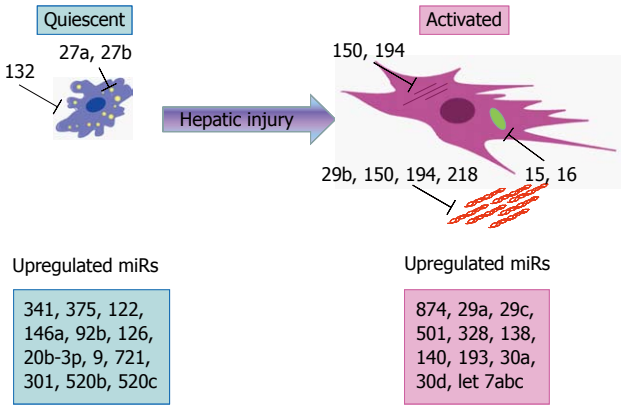

Figure 1 microRNAs involved in hepatic stellate cell transdifferentiation. Functional manipulation studies utilizing mimics and/or antagomirs have demonstrated that the miRs depicted in the above schematic regulate key genes/functions in hepatic stellate cells (HSCs). (Quiescent HSC: yellow circles represent cytoplasmic lipid droplets; activated HSC: purple lines indicate cytoskeletal protein smooth muscle alpha actin; green oval represents Bcl-2; red fibrils represent collagen). Additional profiling studies have shown upregulation of several microRNAs (miRs) in both phenotypes, some of which are already associated with hepatic disease (boxes contain a small fraction of published miRs). References[38-43,47] were used to generate the contents of this figure with design software BioDraw Ultra 12.0.

that TGFβ1 or IFNα stimulation validate *in silico* analyses of miR-pro-collagen [Colα1 (Ⅰ)] mRNA binding sites. miRs 29b, 143 and 218 demonstrate the highest degree of homology to the Colα1 (Ⅰ) 3'UTR and have been further analyzed. miR 29b directly bound to the 3'UTR suppresses type Ⅰ collagen at the mRNA and protein levels. Potential antifibrotic benefit to miR 29b has also been validated *in vivo* using a murine CCl_4 model of injury (see Liver fibrosis section). *In vitro* studies conducted by Roderburg *et al*[40] have also shown that overexpression of miR-29b in murine HSCs results in decreased collagen expression and that lipopolysaccharide and nuclear factor (NF)κB are involved in downregulation of this miR. The process of HSC activation includes the methyl CpG binding protein MeCP2 and constituents of the polycomb repressive complex. More recently, miR 132 has been shown to activate this epigenetic pathway because its downregulation, observed in fibrotic livers, permits translation of MeCP2, which is subsequently recruited to the 5' end of PPARγ, and through altered methylation patterns, it confers suppression of the quiescent profile[42]. Previously studies by Ji *et al*[47] showed miRs 27a and b are upregulated during activation and directly repress RXRalpha. Inactivation of these miRs in vitro showed partial reversion of the cell to a quiescent phenotype with restoration of lipid droplet accumulation. Improved understanding of the regulation of HSC activation by miRs will undoubtedly advance our knowledge of many liver etiologies.

miRs IN LIVER REGENERATION

As a result of hepatic injury, hepatocytes are capable of undergoing multiple divisions, and in the case of partial (2/3) hepatectomy (PH), liver mass is restored within 7 d[48]. Liver regeneration is a complex process that involves intricate cytokine and growth factor signaling influencing cell cycle progression. Recent studies have begun to examine the role of miRs in this process, to uncover underlying mechanisms that may be manipulated to ensure rapid tissue repair. miR-deficient mice (DGCR8 inactivated) that have undergone PH are clearly delayed in cell cycle progression; specifically G1 to S phase transition[48]. Examination of differentially expressed miRs in wild-type mice has revealed marked induction of miR 21 at 18 h post-PH, and downregulation of miR 378. These studies also have shown that miR 21 targets cell cycle inhibitor B cell translocation gene 2 (*Btg2*), which prevents DNA synthesis in hepatocytes, whereas miR 378 inhibits ornithine decarboxylase (*Odc1*), a promoter of DNA synthesis. Studies by Castro *et al*[49] have confirmed that miR 21 expression is increased following PH, and can be modulated by ursodeoxycholic acid (UDCA), a pro-survival agent in hepatic regeneration. miR profiles from rats that have undergone 70% PH have shown that miR 21 levels are highest at 24, 36 and 72 h post-hepatectomy. Inhibition of this miR reduces hepatocyte proliferation and increases levels of lactate dehydrogenase. UDCA treatments increase miR 21 as well as several others, including 143 and 151, which points towards a mechanism of action of the compound. As previously noted, miR 21 has been shown to repress PTEN signaling, the negative repressor of the AKT pathway that is necessary for hepatic regeneration. Additional experiments with miR 21 have shown that it is upregulated in early stages of regeneration and its overexpression inhibits NF-κB signaling[50]. Although the miR signature following 50% PH is slightly distinct from published data on 2/3 PH, miR 21 as well as miRs 22a, 26a, 30b and members of the let 7 family, which are already known to play a role in hepatic tissue development, are also differentially expressed[51]. Although key regulatory factors that govern organ regeneration are mostly unknown, recent studies in zebrafish have shown that suppression of miRs 133 and 203 is required for appendage regeneration; however, this process is mediated by stem cells in contrast to fully differentiated hepatocytes that are necessary to regenerate mammalian liver tissue. Enhanced knowledge of miRs that regulate hepatocyte proliferation following PH may also allow restoration of this function in diseased parenchymal cells[48].

CLINICAL APPLICATION OF miRs

Currently, there are no established FDA-approved treatments for hepatic fibrosis. Outside of surgical resection, full organ transplantation is an option for certain disease states; however, the current list of patients in need of transplant far exceeds the annual donor rate. As is always the case, the clinical applications of new discoveries, such as the existence and manifold roles of miRs in liver physiology and pathophysiology, lag behind the laboratory-based discoveries summarized above. Thus, currently, profiles of miRs and creation of miRs in various liver diseases, and the use of alterations in these as prognostic indicators or for categorization of liver diseases, have not been introduced into routine clinical practice. Nevertheless, it seems likely that, within the next 3-5 years, some centers and physicians will begin to use miR profiling to help categorize patients at higher or lower risk of adverse outcomes, especially from acute liver diseases, such as drug- or toxin-induced injury or fulminant liver failure of any cause. Another likely application will be the profiling of miR expression in hepatic tumors as an aid to prognosis, and perhaps, to help guide therapeutic decisions. However, it seems unlikely that such profiling will become generally available for widespread clinical application within the next 10 years, because many more studies with large numbers of subjects will be needed, with demonstration that such specialized profiling offers clear advantages in improving outcomes at reasonable and justifiable incremental costs.

What seems more likely is that therapy directed at influencing the hepatic levels of selected miRs such as miR 122, already shown to play an important role in affecting levels of serum cholesterol and levels of the HCV, both in cell culture[52,53] and chimpanzee models[54], will find a place in clinical therapeutics. Indeed, phase 2 clinical studies of locked nucleic acids that downregulate miR 122 expression are already in progress, and results of these are awaited with great interest. Similarly, therapeutic alteration of miR 196[24] and, in future, other miRs seems likely also to be pursued. However, we must also keep in mind

the high costs and lengthy duration usually needed for approvals of all new drugs. Also, the unwanted side effects of such treatments may limit their clinical application.

CONCLUSION

Thus, at this juncture, miRs are clearly more than just the latest fad. They are important and fundamental modulators of mRNA and protein expression. In future, purposeful modulation of these levels and effects will, more likely than not, become important in the therapeutic armamentarium of hepatic and other diseases. Although the clinicopathological and prognostic value of miRs remains highly anticipated, further exploration into systemic effects of miR modulation, both endogenously and exogenously, is needed, as well as a deeper understanding of miR biogenesis in both the development and progression of liver diseases.

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