REVIEW ARTICLE

MicroRNAs: the fine modulators of liver development and function

Yemiao Chen^{1,2*} and Catherine M. Verfaillie^{2*}

1 Southwest Hospital, and Key Laboratory of Tumor Immunopathology of the Ministry of Education of China, Institute of Pathology and Southwest Cancer Center, Third Military Medical University, Chongqing, China

2 Department of Development and Regeneration, Stem Cell Institute Leuven, Cluster Stem Cell Biology and Embryology, KU Leuven Medical School, KU Leuven, Leuven, Belgium

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Correspondence

Yemiao Chen, Principal Investigator, Institute of Pathology and Southwest Cancer Center, Key Laboratory of Tumor Immunopathology of the Ministry of Education of China, Southwest Hospital, Third Military Medical University, Shapingba, Gaotanyan zhengjie 30, Chongqing, 400038, China
Tel: +86 18580770267
Fax: +86 23 68754431
e-mail: yemiao.chen@126.com

*Both authors contributed equally to this work.

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Abstract

MicroRNAs are a class of small non-coding RNAs involved in the transcriptional and post-transcriptional regulation of gene expression. The function of miRNAs in liver disease including hepatocellular carcinoma (HCC), hepatitis, and alcoholic liver disease, have been widely studied and extensively reviewed. Increasing evidence demonstrates that miRNAs also play a critical role in normal liver development and in the fine-tuning of fundamental biological liver processes. In this review, we highlight the most recent findings on the role of miRNAs in liver specification and differentiation, liver cell development, as well as in the many metabolic functions of the liver, including glucose, lipid, iron and drug metabolism. These findings demonstrate an important role of miRNAs in normal liver development and function. Further researches will be needed to fully understand how miRNAs regulate liver generation and metabolic function, which should then lead to greater insights in liver biology and perhaps open up the possibility to correct errors that cause liver diseases or metabolic disorders.

The liver is the largest gland in mammals serving as an endocrine and exocrine organ with numerous functions, including carbohydrate, lipid and amino-acid metabolism, urea synthesis, detoxification of drugs and toxic endogenous compounds, bile production and plasma protein secretion (1). As the liver is such a vital organ to life, its dysfunction or failure is associated with high levels of morbidity and mortality. Hence, gaining insights in how the different cell components of the liver develop and how its vast metabolic functions are developed and maintained is of great importance.

The liver consists of different types of cells, such as hepatocytes, cholangiocytes, liver sinusoidal endothelial cells (LSECs), hepatic stellate cells (HSCs) and kupffer cells (KCs). Less is known regarding the developmental processes underlying the generation of the mesodermal LSEC and HSC compartment, while the development of the endodermal compartments of the liver are intensively studied. There are four major steps in hepatocyte (endodermal origin) formation: formation of definitive endoderm (DE); hepatic specification; formation of

hepatoblasts and maturation of hepatocytes (2). Extensive and intensive studies have demonstrated that liver development is a tightly regulated, complicated process.

miRNAs are reported to be involved in almost every aspect of biology, including cell differentiation, proliferation, metabolism and apoptosis, in tumour formation, as well as in mobile genetic element stability and viral infection (3). It is estimated that at least one-third of human genes is regulated by miRNAs (4). Thus, it is not surprising that many organ development and differentiation processes are tightly regulated by miRNAs. Indeed, increasing evidences demonstrate that miRNAs play important roles in regulating liver development and homeostasis, as well as in many kinds of liver diseases.

The critical roles of miRNAs in liver disease have been extensively reviewed (5–9), especially in liver cancer (10–13), liver fibrosis (14), alcoholic liver disease (15), hepatitis (16–18), as well as induced liver injury (19). However, few articles have specifically focused on the roles of miRNAs during liver development and in fundamental liver functions. In this review, we will

highlight studies of miRNAs that regulate normal liver development and liver function, and propose some open questions in this field, which may further our understanding of liver development and function, and contribute to potential therapeutic approaches.

Global impact of microRNAs on Liver development

DICER1 and DGCR8 are two key factors in the miRNA-signalling pathway. Constitutive disruption of either of them will lead to a global loss of miRNAs (20). Loss of DICER in mice causes early embryonic lethality at E7.5 because of the defects in proliferation and incapability of maintaining pluripotency in extraembryonic tissues (21, 22). Hepatoblast/hepatocyte-specific *DICER1* and *DGCR8* conditional knock-out (cKO) mice were, respectively, generated to elucidate the global function of miRNAs in liver development.

Dicer1 knock out and liver development

Hand *et al.* generated hepatoblast-specific *DICER1* conditional knock-out mice by crossing the *Dicer1^{flox/flox}* mice with *AfpCre* mice (23), wherein DICER1 was deleted in hepatoblast-derived cells in *AfpCre; Dicer1^{flox/flox}* mutants. *Afp; Dicer1* cKO mice were born alive and showed no phenotype at birth compared to wild-type (WT) mice, even if there was efficient disruption of DICER1 in hepatocytes. The liver enriched miRNAs, such as miR-122, miR-192 and miR-194 were significantly down-regulated. Between 2–4 months of age mutant mice exhibited progressive hepatocyte damage with prominent steatosis, depletion of glycogen storage and increased levels of serum alanine aminotransferase and aspartate aminotransferase. Some proliferation-promoting and hepatoblast-specific genes were also increased. This resulted in increased hepatocyte proliferative activity and increased liver mass, as well as overwhelming apoptosis (23). Sekine *et al.* also generated a mouse model wherein *Dicer* was specifically eliminated from mature hepatocytes by mating *Dicer^{flox/flox}* mice with *AlbCre* mice (24). As was seen for *AfpCre; Dicer^{flox/flox}* mice, increased growth-promoting gene expression and robust expression of fetal stage-specific genes was seen, and hepatocytes from *AlbCre; Dicer^{flox/flox}* were susceptible to apoptosis. At 3 weeks, livers contained chiefly hepatocytes without DICER expression, however, mice surviving long-term had livers consisting of DICER expressing hepatocytes that had escaped the Cre-mediated deletion. Nevertheless, the remaining DICER negative hepatocytes lead, as in the *AfpCre; Dicer^{flox/flox}* mice, to hepatocarcinomas in two-thirds of animals at 1 year (24). These results suggest that DICER and miRNAs play critical roles in hepatocyte maturation, regeneration, hepatic gene regulation and tumour suppression in the liver.

Careful analysis of the DICER negative cells in *AlbCre; Dicer^{flox/flox}* mice at 3 weeks of age demonstrated

that miRNAs are also involved in establishing/maintaining liver zonation (25). Although the perivenous location marker gene glutamine synthetase (GS) was found perivenously in *AlbCre; Dicer^{flox/flox}* mice, its expression extended beyond its normal boundaries. By contrast, some periportal marker genes completely lost their localized expression patterns and were diffusely expressed throughout the entire lobule (25). Thus, *Dicer* is required for the establishment of proper liver zonation. However, no direct evidence shows specific miRNAs are involved in regulating liver zonation. Thus, further exploration is needed to study the function of specific miRNAs in mediating liver zonation.

DGCR8 knock-out and liver development

No studies have been performed wherein DGCR8 has specifically been eliminated from hepatocytes. However, studies have been done in DGCR8 vascular smooth muscle specific cKO mice. The mutant mice had hepatomegaly and liver haemorrhage compared with control mice. Surprisingly, no defects in hepatocytes were seen as expected (26). To fully elucidate the role of DGCR8 in liver/hepatocyte development, a DGCR8 knock-out liver model should be generated in the future.

MicroRNAs and liver derived cell development

Definitive endoderm formation

Fu *et al.* evaluated dynamic miRNA changes during DE formation using murine embryonic stem cells (mESCs) as a model (27). They found a progressively increasing number of miRNAs to be up and down-regulated, following addition of Wnt3-a and Activin-A on days 1–5. Amongst them were miR-196a, miR-196b and miR-24-2*, also known to be up-regulated during Na-Butyrate mediated DE specification from human (h)ESCs (28), as well as miR-222, miR-338-5p and miR-340-3p (Fig. 1a). Forced expression of a combination of miRNAs (miR-181c/338-5p/222/196a/196b/let-7e), enhanced Activin-A mediated DE formation, at least in part by modifying the expression of histone deacetylase 9 (*HDAC9*), which is involved in histone acetylation of promoter regions of DE specific genes. A similar study was performed by Hinton *et al.* using two hESC lines (29). In a third study on hESC, Kim *et al.* compared hESC, DE and ESC-derived 'mature hepatocytes' (30). One mouse ESC (TCL-1) and 5 different human ESC lines (HES-1, HES-2, H9, CyT49 and CHA4) were used in the four studies, with little concordance between the results. As shown in Figure 1a, no miRNA was consistently up-regulated in DE cells compared with undifferentiated ESCs; one miRNA, miR-375, was significantly up-regulated in DE derived from four of six lines; and six miRNAs (miR-10a/24/218/371-3p/371-5p/373) were significantly up-regulated in two of the six DE progeny. For down-regulated miRNAs, miR-520 family was

Fig. 1. miRNAs involved in definitive endoderm (DE) formation (a), and hepatoblast migration and maturation (b). The top up/down regulated miRNAs when embryonic stem cells (ESC) are induced to differentiate into DE cells were assessed in the mESC line TCL-1, and the hESC lines HES1, HES2, H9, CyT49 and CHA4 (a). The expression levels of miR-30 family members decreased during the hepatic endoderm differentiation into hepatoblasts, accordingly their target gene *SNAIL1* was increased during the process. While miR-20a (targeting *TGFBR2*), miR-302b (targeting *TGFBR2* and *KAT2B*), and miR-495 and miR-218 (targeting *HNF6* and *OC2*) showed opposite expression patterns (b). References are listed in the figure.

significantly down-regulated in DE from three cell lines, and miR-204/525-5p in two (28–30). Different cell lines and differentiation protocol used in these studies may be the reason for the variety of the up/down-regulated miRNAs. Further analysis remains to be done to uncover which of the up/down regulated miRNAs contribute to DE formation.

Hepatoblast migration and maturation

During the delamination of hepatoblasts from the liver diverticulum and invasion in the STM, hepatoblasts exhibit morphological changes reminiscent of epithelium to mesenchymal transition (EMT). Although the precise factors that govern this process are not fully known, it is in general believed that TGF β plays an important role in EMT during development (31). Using the murine AML-12 hepatocyte cell line, Zhang *et al.* demonstrated that levels of miR-30b, miR-30c, miR-30d, miR-30e but not miR-30a support increased expression of snail homolog 1 (*SNAIL1*) (32), which resulted in the down-regulation of E-cadherin, the marker gene of epithelial cells (Fig. 1b). miR-30 family were also reported to suppress the EMT processes in

prostate cancer (33), which suggests miR-30 may be a therapeutic target as a tumour suppressor. By microarray profiling analysis, Wei *et al.* found that expression of miR-302b and miR-20a were higher in mouse E8.5 foregut (containing liver progenitor cells) than in E14.5 hepatoblasts (34). Target analysis demonstrated that miR-302b reduces the expression of transforming growth factor beta receptor II (*TGFBR2*, a type II receptor required for TGF β ligand signalling) and K (Lysine) acetyltransferase 2B (*KAT2B*, a histone acetyltransferase interacting directly with the intracellular TGF β signalling component – SMAD3) (34). These studies suggest that migration from the liver primordium into the STM may be governed by TGF- β mediated EMT, and that this might be regulated by miR-30, miR-302 and miR-20.

Other signals that govern this initial migration step include hepatocyte nuclear factor 6 (HNF6, also known as Onecut-1 or OC1) and OC2 (35, 36). miR-495 and miR-218 [also identified to be up-regulated during DE specification(27)] actively regulate expression of *OC1* and *OC2* (37). miR-495 and miR-218 are expressed at low levels in e8.5 endodermal epithelium and in e9.5 in hepatic buds emerging from the endoderm, but are

found at higher concentrations in fetal liver at e14.5 and e16.5, at which time migration of hepatoblasts is no longer occurring (Fig. 1b) (37).

Hepatocyte maturation

Hepatoblasts are bipotential cells, which differentiate into hepatocytes to form the liver parenchyma, and into cholangiocytes to form biliary ducts (2). Bipotent murine embryonic liver (BMEL) cells, which can differentiate into hepatocytes and BECs in different culture systems, are frequently used to study the mechanism whereby hepatocytes and cholangiocytes are specified (38). Using the BMEL cell system, Laudadio *et al.* found that miR-122, the liver specific and most abundant miRNA, which accounts for about 70% of the total liver miRNA population (39), directly or indirectly stimulates the expression levels of 24 hepatocyte-specific genes. Forced expression of miR-122 in transgenic mice promoted *HNF6* expression. Moreover, *HNF6* stimulated the expression of miR-122 by directly binding to its promoter during liver development (40). This data indicates that a positive feed-back loop between miR-122 and *HNF6* regulates proper hepatocyte-specific gene expression. Deng *et al.* demonstrated that forced expression of miR-122, during differentiation of mESCs to hepatocytes from day 12 onwards, significantly increased the levels of hepatocyte indocyanine green (ICG) uptake, glycogen staining, urea synthesis, ammonia elimination, albumin expression and cytochrome P450 (CYP) metabolism (41). They further showed that high levels of miR-122 may indirectly lead to up-regulation of forkhead box A1 (*FOXA1*) and hepatocyte nuclear factor 4, alpha (*HNF4A*), two liver-specific transcription factors. miR-122, *FOXA1* and *HNF4A* also form a posi-

tive feedback loop to promote hepatocyte maturation. miR-122 is down-regulated upon transformation and re-expression of miR-122 in the human liver cancer cell line HepG2 suppressed cellular proliferation and enhanced hepatocyte functions (42). These studies thus suggest that miR-122 is a key regulator to maintain the balance between hepatocyte proliferation and differentiation. Furthermore, miR-122 showed down-regulation in HCC cells, restoration of miR-122 can suppress cell proliferation and tumour metastasis (43). A list of miRNAs has potential clinical implications related to the processes of liver development and metabolism can be found in Table 1.

Gailhouse *et al.* cultured E-cadherin positive cells isolated from mouse fetal liver to gain insight in maturation of hepatocytes (44). They found that forced-expression of miR-148a also enhanced the expression level of some hepatic marker genes. Target analysis identified that miR-148a directly inhibits the expression of DNA (cytosine-5-)-methyltransferase 1 (*DNMT1*), the major DNA methylation enzyme, which prevents cell maturation.

Other mature hepatic genes include transporters for bile salts and phospholipids, such as ATP-binding cassette sub-family B member 11 (*ABCB11*) and ATPase aminophospholipid transporter class I type 8B member 1 (*ATP8B1*) (45,46). Allen *et al.* identified *ABCB11* and *ATP8B1* as two direct targets of miR-33 (47). Forced expression of miR-33 significantly decreased biliary output, while silencing of miR-33 increased sterols in bile and enhanced reverse cholesterol transport. Notably, silencing of miR-33 can rescue simvastatin and lithogenic diet induced cholestasis and liver damage (47), which suggests miR-33 is a potential therapeutic target for cholesterol metabolism related diseases.

Table 1. Potential clinical significance of some miRNAs related to liver development and metabolism

miRNAs	Process	Potential clinical significance	Reference
miR-30 family	Hepatoblast migration and maturation	Therapeutic target to suppress EMT	(32)
miR-122	Hepatocyte maturation	Biomarker for liver injury, therapeutic target to suppress cell proliferation	(41, 42)
miR-33	Hepatocyte maturation	Biomarker and therapeutic target for cholestasis and statin- and diet-induced hepatotoxicity	(47)
miR-23b cluster	BEC maturation	Biomarker for bile duct injury	(53)
miR-27a/b	HSC activation and proliferation	Biomarker and therapeutic target for liver fibrosis and other diseases	(59)
miR-16/19b/29b/146a/150/194/195/335	HSC activation and proliferation	Biomarker and therapeutic target for liver fibrosis and other diseases	(56,58,60,62, 63,124–126)
miR-34a/181a, miR-103/107, miR-122, miR-802	Glucose and insulin metabolism	Therapeutic target for obesity related diseases	(71–73,82,83)
Let-7, miR-29a/b, miR-143	Glucose and insulin metabolism	Therapeutic target for diabetes	(76,80,81)
miR-122	Lipid metabolism	Therapeutic target for lipid metabolic disease	(85,86)
miR-27b	Lipid metabolism	Biomarker and therapeutic target for dyslipidaemia and atherosclerosis	(94)
miR-122	Iron metabolism	Biomarker and therapeutic target for hereditary hemochromatosis	(103)
miR-93/214/699, miR-27b	Detoxification	Therapeutic target for anti-aging	(120,121)

In the adult, hepatocytes are quiescent, except following liver injury where proliferation of hepatocytes occurs to replace lost cells, or during transformation to HCC (48–50). Numerous miRNAs have been shown to play a role in controlling hepatocyte proliferation during liver regeneration, including miR-21/23b/221, among others (Table 2). Jung *et al.* identified a group of miRNAs, including miR-106a/106b/17/93/301a/130b that are expressed significantly more in HCC and hESCs

than in mature quiescent hepatocytes (51). These miRNAs promote hepatocyte proliferation/transformation by regulating phosphatase and tensin homolog (PTEN) and TGF β tumour suppressor pathways (51).

These studies all point to the critical role miRNAs play in maintaining mature hepatocytes (Table 2), even if many mechanistic studies are yet to be performed to fully understand the mechanisms underlying the miRNA-mediated hepatocyte homeostasis.

Table 2. MicroRNAs Involved in Hepatocyte, BEC and HSC development

miRNAs	Tissue/cell type	Target genes	Function	References
Hepatocyte development				
miR-122	BMEL	–	Forms a positive feedback loop with HNF6 to regulate hepatocyte differentiation	(40)
	CCL-9.1	–	Regulates hepatic differentiation and maturation	(41)
	C57BL/6J mouse liver and human/HepG2, Huh-7	CUL1	Regulates hepatocyte proliferation and differentiation	(42)
miR-148a	Mouse/Fetal Hepatoblast and human/HepG2, Hep3B, Huh-7	DNMT1	Promotes hepatic differentiation	(44)
miR-33	C57BL/6 mouse liver and human/Huh-7	ABCB11 ATP8B1	Regulates hepatocyte biliary transportation	(47)
Hepatocyte proliferation during liver regeneration				
miR-21	Mouse and rat liver	PEL1 BTG2	Promotes hepatocyte proliferation	(127–130)
miR-23b	Rat liver and BRL-3A	SMAD3	Promotes hepatocyte proliferation	(131)
miR-26a	Mouse liver	CCND2 CCNE2	Inhibits hepatocyte proliferation	(132)
miR-33	Mouse liver and A549, Huh-7	CDK6	Inhibits hepatocyte proliferation	(122)
		CCND1		
miR-34a	Rat liver	INHBB MET	Inhibits hepatocyte proliferation	(133)
miR-127	Rat liver	BCL6 SETD8	Inhibits hepatocyte proliferation	(134)
miR-221	Mouse liver	P27 P57 ARNT	Promotes hepatocyte proliferation	(135)
BEC development				
miR-23b, miR-27b, miR-24-1	HBC-3	SMAD3, 4, 5	Repressing bile duct gene expression	(53)
miR-30a	Zebra fish	AK1 TNRC6a	Required for biliary development	(54)
HSC development				
miR-27a, b	Rat HSC	RXR α	Promotes HSC proliferation and inhibits lipid accumulation	(59)
miR-29b	Human, mouse HSC	COL1A1	Inhibits HSC activation and proliferation	(60,125,126)
miR-150	Human HSC	COL1A1 COL4A4 c-MYB	Inhibits HSC activation and proliferation	(62,63)
miR-194	Human HSC	RAC1	Inhibits HSC activation and proliferation	(63)
miR-15b	Rat HSC	BCL2	Promotes HSC apoptosis	(136)
miR-16	Rat HSC	CD-1 BCL2	Inhibits HSC proliferation and promoting apoptosis	(136)
miR-19b	Human and rat HSC	TGFBR1	Inhibits HSC activation and proliferation	(56)
miR-146a	Rat HSC	SMAD4	Inhibits HSC proliferation and promotes apoptosis	(137)
miR-195	Human HSC	Cyclin E1	Inhibits HSC activation and proliferation	(58)
miR-335	Rat HSC	TNC	Inhibits HSC proliferation and migration	(124)

BEC maturation

Cholangiocytes, or bile duct epithelial cells (BECs) are, like hepatocytes, of endodermal origin. BECs line the interlobular and extrahepatic bile ducts. As is true for hepatocytes, multiple studies have assessed the role of miRNAs in BEC diseases, such as cholangiocarcinoma, polycystic liver disease and primary biliary cirrhosis [reviewed in (52)], although much less is known regarding the role miRNAs during BEC development. BEC specification from hepatoblasts is mediated by both NOTCH and TGF β signalling. Studies using the murine fetal hepatoblast cell line, HBC-3, showed that TGF β receptors and SMADs (key signal transduction molecules for TGF β) are down-regulated during differentiation towards hepatocytes but induced during BEC differentiation (53). miR-23b (aside from miR-27b and miR-24-1) was identified as directly targeting *SMAD 3*, 4 and 5. Knockdown of miR-23b under hepatocytic differentiation conditions of HBC-3 cells promoted expression of bile duct genes, while forced expression of miR-23b under bile duct differentiation conditions repressed the process. In situ hybridization analysis confirmed miR-23b to only be expressed in developing hepatocytes, but not in the ductal plates (53). These results suggest that miR-23b is involved in regulating lineage specification of hepatocytes and cholangiocytes by influencing the TGF β signalling pathway. On the contrary, the miR-30 family members, miR-30a and miR-30c were identified specifically expressed in ductal plates. Knock-down of miR-30a in the Zebrafish larva caused defective biliary development (54). Together, these studies also exemplify the importance of miRNA mediated regulation of hepatocyte vs. BEC specification, even if many more studies will be needed to fully understand the mechanisms involved in miRNA-mediated BEC development (Table 2).

HSC and LSEC development

HSCs are derived from mesoderm, even if little is known on how HSCs are specified and differentiate. Hence, no data is available on miRNAs that play a role in HSC generation. However, much is known regarding the mechanisms underlying HSC activation into proliferative, fibrogenic and contractile myofibroblasts (55), including miRNAs that modify TGF β signalling (56,57) and IFN signalling (58) in HSCs. Down-regulation of miR-27a and 27b induced retinoid X receptor- α expression and induced a quiescent state of activated HSCs, including restoration of cytoplasmic lipid droplets and decreased cell proliferation (59). Forced expression of miR-29 in HSCs inhibits collagen type I production by targeting *COL1A1* (60,61), while forced expression of miR-150 inhibits both collagen type I and type IV by directly binding to *SP1* and *COL4A4* (62). Venugopal *et al.* found that forced expression of miRNA-150 and miRNA-194 inhibited HSC activation and ECM

production by inhibiting *c-MYB* and ras-related C3 botulinum toxin substrate 1 (*RAC1*) expression respectively (63). A list of miRNAs known to play a role in the HSC quiescence vs. activation can be found in Table 2.

Whether miRNAs play a role in the development of LSECs is largely unknown. Some studies have identified miRNAs that play a role in LSEC based diseases, including alcoholic or drug induced liver disease and idiopathic portal hypertension among others (64–66).

MicroRNAs in liver metabolism

Glucose metabolism

Plasma glucose levels are dynamic, and are tightly controlled by insulin and glucagon. Insulin is secreted in response to high glucose levels by pancreatic β -cells and acts on hepatocytes to stimulate glucose uptake and store glucose as glycogen (67). On the contrary, glucagon is secreted by pancreatic α -cells and promotes the degradation of glycogen to glucose when blood glucose levels decrease (68).

As described above, global loss of miRNAs in the liver causes severe liver dysfunction (25). These studies also demonstrated that miRNAs are involved in the regulation of glucose metabolism. Conditional deletion of the miRNA processing enzyme DICER1 in mouse liver resulted in severe hypoglycaemia in the fasting state because of the depletion of glycogen. Loss of Dicer was associated with loss of miR-122/148a/192/194 expression (23). Which of these miRNAs are specifically involved in glucose homeostasis remains to be determined.

Besides the studies in DICER1 null hepatocytes, many reports have indeed shown a critical role of specific miRNAs in regulating glucose metabolism in the liver. Glucose-6-phosphatase (*G6PC*) hydrolyses glucose 6-phosphate to an inorganic phosphate and free glucose; therefore it is an important enzyme in glucose homeostasis. A maternal low-protein (LP) diet during pregnancy caused a higher expression level of *G6PC* in the liver of male but not female piglets. Further analysis showed two significantly up-regulated miRNAs (only in female piglets) *ssc-miR-339-5p* and *ssc-miR-532-3p*, which directly target *G6PC* (69). When blood glucose level is high, glucose will be stored as glycogen via gluconeogenesis mediated part by insulin signalling. Sirtuin-1 (*SIRT1*) is a positive regulator of insulin signalling in many cell types, including hepatocytes (70). Two miRNAs, miR-34a and miR-181a can target the 3'-UTR of *SIRT1* and inhibit its expression (71,72). Ectopic expression of miR-181a impaired hepatic insulin signalling glucose, while inhibition of miR-181a improved homeostasis in diet-induced obese mice (Fig. 2) (72). The liver specific miR-122 was also found involved in regulating insulin signalling. Yang *et al.* identified that miR-122 was down-regulated in high-fat-diet-fed mice or hepatocyte models of insulin resistance (73). Further

analysis showed miR-122 can directly target protein tyrosine phosphatase 1B (*PTP1B*), the inhibitor of hepatic insulin signalling, by dephosphorylating tyrosine residues in IR and IRS. The modified antisense agents or antagomir of miR-122 have already been used to against HCC (74) and/or HCV (75), however, the clinical trial for miR-122 on glucose metabolism related diseases should be further explored.

A recent study uncovered a link between the Lin28/let-7 pathway and glucose metabolism (76). Transgenic mice wherein both Lin28a and Lin28b were ectopically expressed had enhanced insulin sensitivity and glucose tolerance; while loss of Lin28a or forced expression of *let-7* resulted in enhanced insulin tolerance and glucose intolerance (76). These effects occurred partly because of *let-7*-mediated repression of the components of initial steps in the insulin-PI3K-mTOR pathway, including IGF1R, IR and IRS2. In addition to *let-7*, other miRNAs have been identified to regulate the initial steps in insulin-mediated glucose regulation, including miR-144, which targets *IRS1* in a Type 2 Diabetes mellitus (T2D) rat model (77), and miR-33 that targets *IRS2* in HepG2 and Huh7 cells (78).

miR-126 was found to target both *IRS1* and *PI3KR2*, a regulatory component of PI3K in the SK-Hep1 cells (79). A study in white adipocytes and liver of diabetic rats demonstrated that up-regulation of miR-29a/b inhibits AKT (a key enzyme in the insulin-signalling pathway) activation and leads to impaired glucose homeostasis (80). Another miRNA, miR-143 was identified as an indirect regulator of AKT (81). Forced expression in hepatocytes of miR-143 caused impaired insulin-stimulated AKT activation and glucose tolerance in *db/db* mice. Subsequent analysis revealed that oxysterol-binding-protein-related protein 8 (*ORP8*), which regulates AKT activation, is a direct target of miR-143 (81).

Another study exploiting obese mice, found that miR-103/107/802 were more highly expressed in obese than WT mice (82,83). Silencing of miR-103/107 improved glucose homeostasis and insulin sensitivity in the liver and adipose tissue. Conversely, forced expression of miR-103/107 in either liver or fat resulted in an increase of fasting blood-glucose and insulin levels. Caveolin-1 (*CAV1*), a critical regulator of the insulin receptor, was identified as a direct target gene of miR-

Fig. 2. miRNAs involved in glucose homeostasis. The initial steps of the insulin signalling pathway are tightly controlled by miRNAs, such as miR-103/107 targeting *CAV1*, miR-144 and miR-126 targeting *IRS1*, miR-122 targeting *PTP1B* which further inhibits IR and Lin28/let-7 regulating *IRS2* and *IGF1R*. The downstream PI3K/AKT signalling is also controlled by miRNAs, such as miR-126 targeting *PI3KR2*, miR-143 targeting *ORP8* to affect AKT and miR-29 directly targeting *AKT*. AKT controls glucose transportation by regulating GLUT4. In the glucose metabolic pathway, miR-339 and miR-532 regulate glucose synthesis by targeting *G6PC*; miR-181a and miR-34a regulate gluconeogenesis by targeting *SIRT1*. miR-802 inhibits *HNF1b* and its downstream genes related to glucose tolerance and insulin sensitivity. References are listed in the figure.

103/107. Silencing of miR-103/107 in CAV1 null mice did not improve insulin sensitivity (83). Moreover, Dicer has been reported as a direct target of miR-103/107 (84). Therefore, up-regulation of miR-103/107 resulting in a low level of *DICER* may aggravate the glucose tolerance impairment, as described in the conditional *DICER* knock-out mice (25). mi-802 had a similar effect on glucose homeostasis and insulin sensitivity as miR-103/107 in inducible overexpression or knock-down studies (82). Further analysis confirmed *HNF1 β* , a member of the homeodomain-containing superfamily of liver-enriched transcription factors as a direct target of miR-802. Inhibition of hepatic *HNF1 β* impaired insulin signalling and promoted hepatic gluconeogenesis (82).

Together, these results indicate that the glucose metabolism and the insulin signalling pathway in hepatocytes (and adipose cells) are tightly regulated by miRNAs (Fig. 2).

Lipid metabolism

MicroRNA-122 was the first miRNA identified to regulate lipid metabolism in 2006 (85). Esau *et al.* found that inhibition of miR-122 with antagomirs reduced plasma cholesterol levels and increased fatty-acid oxidation, which resulted in a decrease of hepatic fatty-acid and cholesterol synthesis rates (85). Down-regulation of miR-122 also decreased low density lipoprotein (LDL) and high density lipoprotein (HDL) fractions both in liver and blood (86). Interestingly, miR-370 was identified to have similar effects on lipid metabolism by

down-regulating miR-122 and carnitine palmitoyl transferase 1 α (*CPT1 α*), an enzyme that promotes fatty acid β -oxidation (87). miR-33 was also identified to regulate cholesterol homeostasis via targeting the sterol transporter proteins, *ABCA1*, *ABCG1* and *NPC1* (88), and fatty acids metabolism via targeting *CROT*, *CPT1 α* , *HADHB* and *AMPK α* (89). Scavenger receptor class B member 1 (SR-BI) that regulates selective uptake of high-density lipoprotein cholesterol (HDL-C) (a step of reverse cholesterol transport) (90) is inhibited by miR-185, miR-96 and miR-223, which repress selective HDL-C uptake (91). Angiopoietin-like 3 (*ANGPTL3*) and glycerol-3-phosphate acyltransferase (*GPAM*) are two genes implicated in the regulation of lipid metabolism (92,93). miR-27b targets these two genes and their expression levels are inversely altered in a mouse model of dyslipidaemia (94). More recently, miR-1 and miR-206 were identified to suppress lipogenesis via directly targeting liver X receptor alpha (*LXR α*), a nuclear hormone receptor critical for transcriptional control of lipid metabolism (95). These results indicate that as in glucose metabolism, miRNAs play important roles in regulating lipid metabolism and suggest miRNAs may be used as therapeutic targets for metabolic diseases (Fig. 3).

Proanthocyanidins, mainly present in many fruits including grapes, can improve human health via multiple activities (96). A grape seed proanthocyanidin extract (GSPE) was shown to regulate lipid metabolism and correct dyslipidaemia associated with dietary obesity in rats (97,98). GSPE was identified to promote hepatic cholesterol efflux to produce new HDL com-

Fig. 3. miRNAs involved in lipid metabolism. *Leishmania donovani* surface protein GP63 targets DICER1 to inhibit its downstream miRNAs, such as miR-122 which affects fatty-acid and HDL/LDL metabolism. miR-370 inhibits *CPT1 α* directly and miR-122 indirectly to regulate fatty-acid and HDL/LDL metabolism. Proanthocyanidins (GSPE) regulate fatty acid oxidation, HDL/LDL and cholesterol metabolism via inhibiting miR-122 and miR-33. miR-185, miR-96 and miR-223 inhibit *SRB1* to affect HDL-C uptake. miR-27b targets *ANGPTL3* and *GPAM* to regulate plasma lipid level. miR-1 and miR-206 can regulate cholesterol homeostasis and lipogenesis via inhibiting *LXR α* and its downstream genes. References are listed in the figure.

plexes by repressing miR-33 and reduce lipogenesis by repressing miR-122 *in vivo* and *in vitro* (99). Not only mammals, but also *Leishmania donovani*, can take advantage of miRNAs to regulate lipid metabolism. *L. donovani* infects liver and spleen and thus causes visceral leishmaniasis (100). High serum cholesterol causes resistance to *L. donovani* infection (101), while *L. donovani* infected liver contains low levels of cholesterol and altered expression of lipid metabolic genes, many of which are direct or indirect targets of miR-122. Glycoprotein GP63, a Zn-metalloprotease on the surface of *L. donovani*, targets DICER1 to prevent miRNA production, including miR-122 (102). Hence, *L. donovani* exploits the host liver metabolism to aid in infection by suppressing Dicer and down-streaming miRNAs that lower cholesterol levels (Fig. 3).

Iron metabolism

Liver is the central organ to control iron homeostasis through synthesis of the peptide hormone hepcidin (encoded by HAMP), the key factor of duodenal iron absorption and release. Specific depletion of miR-122 caused systemic iron deficiency in mice (103). This is consistent with the low expression levels of miR-122 in a mouse model of hereditary haemochromatosis and in liver biopsies of patients with hereditary haemochromatosis (103). At the molecular level, miR-122 inhibition increased mRNA levels of genes that control systemic iron levels, including haemochromatosis (*HFE*), haemojuvelin (*HJV*), bone morphogenetic protein receptor type 1A (*BMPRIa*) and *HAMP*. *HFE* and *HJV* have been confirmed to be direct targets of miR-122 (103).

Forced expression of miR-485-3p in HepG2 cells was associated with increased cellular iron levels, while inhibition of miR-485-3p decreased cellular iron content. Moreover, the only known mammalian cellular iron exporter – ferroportin (FPN), was shown to be targeted by miR-485-3p (104). Hence, iron cellular export may be a miRNA-mediated process. MicroRNAs may also be involved in protecting hepatocytes from oxidant injury via regulating iron reutilization as *let-7* directly targets the haeme-regulated transcriptional repressor basic leucine zipper transcription factor 1 (*BACH1*), resulting in a de-repression of the *BACH1* target haeme oxygenase (*HMOX1*) (105), the key enzyme in iron reutilization (106). The function of miRNAs in iron metabolism has also been demonstrated to regulate iron acquisition, iron storage and iron utilization by the studies in other cell types, as well as in several diseases (107,108).

Interestingly, iron in turn appears to affect miRNA processing via its physiological role as the functional component in haeme. Faller *et al.* described that DGCR8 is a haeme-binding protein, and haeme-free DGCR8 is less active than haeme-bound DGCR8 (109). Additionally, haeme-mediated regulation of DGCR8

depends on the oxidation state of iron in haeme (110). Therefore, both inadequate iron levels resulting in low haeme levels, and the wrong oxidation state of iron resulting in abnormal haeme, can decrease pri-miRNA processing. However, high haeme levels in cells could also affect miRNAs expression (111), but the molecular mechanism remains to be explored. The putative role of miRNAs in iron homeostasis in hepatocytes is summarized in Figure 4.

Detoxification

The liver is the main organ for detoxification of drugs and toxic endogenous compounds. The CYP superfamily is a large and diverse group of enzymes involved in drug metabolism and bioactivation, which are highly expressed in the liver (112).

To investigate which miRNAs regulate genes related to drugs and xenobiotic substances metabolism, screens have been done using cohorts of human liver tissue. Association analysis between the expression levels of CYP genes and preselected miRNAs revealed that *CYP1A1* might be regulated by miR-132/142-3p/21, *CYP2A6* by miR-142-3p/21, *CYP2C19* by miR-130b/185/34a, and *CYP2E1* by miR-10a/200c/let-7 g (113). These preliminary analyses suggest that miRNAs may directly regulate CYP expression levels in the liver, even if proof for this hypothesis is still lacking.

Some studies have identified miRNAs that indirectly affect CYP genes by targeting their upstream transcription factors. The metabolism of more than 50% of all clinically relevant drugs is catalyzed by CYP3A4 in human (114). The expression of CYP3A4 is predominantly regulated by pregnane X receptor (PXR). Takagi *et al.* found that miR-148a can directly target human PXR (115). Thus, miR-148a may indirectly modulate CYP3A4 levels in the liver. Another study in Zebrafish liver showed that PXR, a regulator of the *CYP3A65* gene, was targeted by dre-miR-27b (116). *CYP1A1*, a phase I enzyme in drug metabolism, was previously identified to be regulated by aryl hydrocarbon receptor nuclear translocator (ARNT), which is involved in biological responses to xenobiotic exposure and hypoxia. Oda *et al.* demonstrated that ARNT (protein level but not mRNA level) is regulated by miR-24. Forced expression of miR-24 in HuH7 and HepG2 significantly decreased the mRNA and protein levels of *CYP1A1* (117). A further study in HepG2 cells revealed that miR-24 together with miR-34a could directly target the liver enriched nuclear factor *HNF4 α* , decreasing levels of *CYP7A1* and *CYP8B1*, downstream genes of *HNF4A* (118). Some miRNAs that can directly regulate CYP genes in other tissue or cells are also summarized in Table 3. Together, these studies suggest that miRNAs are directly or indirectly involved in drug metabolism by regulating the expression of CYP superfamily genes, and miRNAs may act as potential therapeutic targets for drug-induced liver diseases.

Fig. 4. miRNAs involved in liver iron metabolism. Low haeme levels regulate mature miRNA production by inhibiting Drosha/DGCR8 complex. Liver specific miR-122 inhibits hepcidin production by targeting *HFE* and *HJV*, the co-receptors in SMAD signalling pathways. Hepcidin inhibits iron transportation, thereby preventing excess iron absorption. miR-485-3p inhibits iron transporter *FPN*. Let-7 inhibits iron re-utilization from haeme by targeting *BACH1*. References are listed in the figure.

The glutathione S-transferases superfamily is another large family of enzymes that have important detoxification roles, especially related to oxidative stress (119). By screening liver miRNAs patterns in 4–33 month old mice, Maes *et al.* identified three miRNAs, miR-93, miR-214 and miR-699, which are up-regulated in aged liver and may contribute to oxidative defense by targeting various classes of glutathione S-transferases (120). Another comprehensive miRNA microarray study in Ames dwarf mice, a model mouse famous for its remarkable propensity to delay the onset of aging, identified miR-27b as a miRNA capable of suppressing ornithine decarboxylase (*ODC*) and spermidine synthase (*SRM*), two key enzymes involved in ammonia and glutamine metabolism (121). It is hypothesized that the high levels of miR-27b, and consequent inhibition of its targets in dwarf mice, may improve their detoxification ability, and ultimately contribute to their extended health span and longevity (121).

Conclusions

Extensive studies over the past decade have revealed that miRNAs play critical roles in regulating multiple aspects of liver development, including hepatic and biliary specification and differentiation, hepatocyte and HSC development, hepatic metabolic functions, as well as the establishment of liver zonation. The regulation shows several features: (i) one miRNA can be involved in multiple developmental aspects. For instance, miR-33 regu-

lates hepatocyte proliferation (122), lipid metabolism (88) and biliary secretion (47), while miR-122 regulates hepatocyte specification and proliferation (40, 42), lipid metabolism (85) and iron metabolism (103). (ii) Metabolic pathways can be sequentially regulated by different miRNAs. For instance, miR-339 and miR-532 inhibit *G6PC* to control glucose synthesis (69), then miR-29 (80) and miR-143 (81) (via targeting *ORP8*) inhibit AKT to control glucose translocation through GLUT4, and lastly various miRNAs inhibit the insulin pathway to influence glucose up-taking (Fig. 2). (iii) The roles of at least some of these miRNAs are conserved. miR-122 controls hepatocyte differentiation both in Zebrafish and mouse (40), and miR-29b suppresses HSCs activation by targeting *COL1A1* both in mouse and human (60,61).

Even if our understanding of the roles of miRNAs in liver development is improving, some important questions remain to be solved. (i) How do miRNAs regulate hepatocyte maturation? The *in vitro* generation of hepatocyte-like cells from progenitor or stem cells is widely being studied because of their potential application in ADME-Tox studies, hepatocyte transplantation, and drug development for liver diseases. However, generation *in vitro* of fully mature hepatocytes is yet to be achieved. Exploring the role of miRNAs important for supporting hepatocyte maturation may provide a very promising way to improve hepatocyte maturation. (ii) How do specific miRNAs regulate liver zonation? It is clear from hepatocyte specific

Table 3. miRNAs involved in liver detoxification

miRNAs	Tissue/cell type	Target genes	Indirectly regulating detoxification genes	References
miR-132 miR-142-3p miR-21	Human liver	CYP1A1	–	(113)
miR-142-3p miR-21	Human liver	CYP2A6	–	(113)
miR-130b miR-185 miR-34a	Human liver	CYP2C19	–	(113)
miR-10a miR-200c let-7 g	Human liver	CYP2E1	–	(113)
miR-148a	Human liver, HepG2, Huh-7, and HLE	PXR	CYP3A4	(115)
miR-27b	Zebra fish liver	PXR	CYP3A65	(116)
miR-24	Human liver, HepG2, and Huh-7	ARNT	CYP1A1	(117)
miR-24 miR-34a	HepG2 and HEK293	HNF4a	CYP7A1 CYP8B1	(118)
miR-892a	MCF7	CYP1A1	–	(138)
miR-27b	MCF7	CYP1B1	–	(139)
miR-27b	PANC1	CYP3A4	–	(140)
miR-126	F344 rat lung	CYP2A3	–	(141)
miR-378	HEK293	CYP2E1	–	(142)
miR-93	C57BL/6J mouse liver	MGST1	–	(120)
miR-214	C57BL/6J mouse liver	EEF1A1 AKR1C6 GSTZ1 LDHA ACSL1 GSTM1	–	(120)
miR-699	C57BL/6J mouse liver	GSTT1	–	(120)
miR-27b	Ames dwarf and control mouse live	ODC1 SRM	–	(121)

conditional Dicer knock-out mice that miRNAs are essential for the establishment of liver zonation. A further exploration of the molecular mechanism of specific miRNAs in this zonation process may guide us to generating zoned hepatocytes, which might be helpful for creating bioartificial liver devices and for ADME-Tox studies. Moreover, gaining knowledge in zonation process may also help us to find solutions against liver diseases originated from zonation-dysfunction. (iii) Further elucidation of the role of miRNAs in metabolic processes governed by the liver, might shed light on innovative therapies for metabolic disorders. For instance, initial studies have shown that ectopic expression of miR-122 may be a novel therapy for hyperlipidaemia in a diet-induced obesity mouse model (85) or in human patients (123). As miRNAs are also controlling blood glucose levels, insights in this role might lead to improved anti-diabetic therapies. Interestingly, a metabolic shift is almost always observed in preneoplastic and neoplastic cancer cells. Therefore, identifying the role of miRNAs as important regulators of metabolism might provide a clinical perspective against HCC.

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