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REVIEW ARTICLE

Interplays of liver fibrosis-associated microRNAs: Molecular mechanisms and implications in diagnosis and therapy

Hong Li [a], Tingli Liu [a], Yongchun Yang [b], William C. Cho [c], Robin J. Flynn [d,e], Majid Fasihi Harandi [f], Houhui Song [b,**], Xuenong Luo [a,***], Yadong Zheng [b,*]

[a] State Key Laboratory of Veterinary Etiological Biology, Key Laboratory of Veterinary Parasitology of Gansu Province, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Lanzhou, Gansu 730046, China
[b] Key Laboratory of Applied Technology on Green-Eco-Healthy Animal Husbandry of Zhejiang Province, Zhejiang Provincial Engineering Laboratory for Animal Health Inspection & Internet Technology, Zhejiang International Science and Technology Cooperation Base for Veterinary Medicine and Health Management, China-Australia Joint Laboratory for Animal Health Big Data Analytics, College of Animal Science and Technology & College of Veterinary Medicine of Zhejiang A&F University, Hangzhou, Zhejiang 311300, China
[c] Department of Clinical Oncology, Queen Elizabeth Hospital, Hong Kong SAR 999077, China
[d] Department of Infection Biology, Institute of Infection and Global Health, University of Liverpool, Liverpool L3 5RF, UK
[e] Graduate Studies Office, Department of Research, Innovation and Graduate Studies, Waterford Institute of Technology, X91 K0EK, Ireland
[f] Research Center for Hydatid Disease in Iran, Kerman University of Medical Sciences, Kerman 7616914115, Iran

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Abstract microRNAs (miRNAs) are a class of non-coding functional small RNA composed of 21–23 nucleotides, having multiple associations with liver fibrosis. Fibrosis-associated miRNAs are roughly classified into pro-fibrosis or anti-fibrosis types. The former is capable of activating hepatic stellate cells (HSCs) by modulating pro-fibrotic signaling pathways, mainly including TGF-β/SMAD, WNT/β-catenin, and Hedgehog; the latter is responsible for maintenance of the quiescent phenotype of normal HSCs, phenotypic reversion of activated HSCs (aHSCs),

* Corresponding author. 666 Wusu Street, Lin'an District, Hangzhou, Zhejiang 311300, China.
** Corresponding author. 666 Wusu Street, Lin'an District, Hangzhou, Zhejiang 311300, China.
*** Corresponding author. 1 Xujiaping, Yanchangbu, Lanzhou, Gansu 730046, China.
E-mail addresses: songhh@zafu.edu.cn (H. Song), luoxuenong@caas.cn (X. Luo), zhengyadong@zafu.edu.cn (Y. Zheng).
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inhibition of HSCs proliferation and suppression of the extracellular matrix-associated gene expression. Moreover, several miRNAs are involved in regulation of liver fibrosis via alternative mechanisms, such as interacting between hepatocytes and other liver cells via exosomes and increasing autophagy of aHSCs. Thus, understanding the role of these miRNAs may provide new avenues for the development of novel interventions against hepatic fibrosis.

Introduction

Liver fibrosis is characterized by excessive deposition of extracellular matrix, which destroys the physiological architecture of the liver.[1] At the early stage of fibrosis, quiescent HSCs (qHSCs) differentiate into activated HSCs (aHSCs) that lose intracellular lipid droplets and acquire a myofibroblast phenotype, which is characterized by increased expression of α-smooth muscle actin (α-SMA or ACTA2), desmin (DES) and type I collagen (COL I).[2,3] Thereafter the ongoing accumulation of collagen forms fibrotic scars that destroy the liver parenchyma and vascular structure, leading to the loss of cells, organ functionality, and eventually liver failure.[4] Liver fibrosis is secondary to chronic liver damage and inflammation. Common causes include parasitic, viral and autoimmune hepatitis, alcohol consumption, non-alcoholic steatohepatitis (NASH), and metabolic diseases that lead to copper or other iron overloads, toxins, and biliary tract obstruction.[5] For example, hepatitis by both hepatitis B virus (HBV) and hepatitis C virus (HCV) induces continuous hepatic inflammation and progressive liver injury that ultimately lead to liver fibrosis.[6] Schistosomiasis is another major chronic disease causing liver fibrosis due to *Schistosoma* egg deposition in the periportal zones, which induces a granulomatous reaction.[7] Under different mechanism, excessive alcohol, mostly in the form of acetaldehyde, can enhance HSC activation and thus lead to liver fibrosis.[8] All these causative factors overlap in their initiation of chronic inflammation and an abnormal wound healing response, then inducing the accumulation of extracellular matrix (ECM) components (Fig. 1).[9–11]

Recent accumulating studies have found that one of the molecular mechanisms underlying liver fibrosis is associated with non-coding RNAs (ncRNAs). microRNAs (miRNAs) are small ncRNAs that function as guide molecules in RNA silencing. Targeting most protein-coding transcripts, miRNAs are involved in nearly all physiological and pathological processes in animals.[12] miRNAs are easily detectable in various biological fluids, including blood, saliva, and urine, because they are stable outside of cells by either being incorporated into circulating exosomes or binding to proteins,[13–15] rendering them good candidates for the development of biomarkers and therapeutic targets for control of diseases. Recently, multiple lines of evidence suggest that aberrant miRNA expression has been shown to be closely related with the occurrence and development of liver fibrosis. In this review, we summarize the roles of miRNAs in HSC activation, HSC proliferation, apoptosis and senescence, and ECM deposition and discuss their potentials as diagnostic and therapeutic targets for liver fibrosis.

miRNAs involved in the regulation of HSC activation via various signaling pathways

HSCs are considered to be the main cell type involved in liver fibrosis. By inhibiting the activation, proliferation, phenotypic transformation and migration of HSCs in the damaged liver, the progression of liver fibrosis can be reduced. There is a plethora of miRNAs that are involved in the regulation of biological activities of HSCs, rendering them to be a promising target for the diagnosis and antifibrotic drug development.

After liver injury, qHSCs are activated and transdifferentiated into fibroblast-like myofibroblasts.[16,17] On the onset of fibrosis, HSC activation plays a key role, leading to the expression of α-SMA, and the excessive accumulation of COL I and ECM proteins in the liver.[18] Numerous miRNAs are involved in the regulation of HSC activation via various signaling pathways, including transforming growth factor (TGF)-β, WNT/β-catenin, PTEN/PI3K/Akt, Hedgehog/NF-κB, PPAR-γ and NOTCH signaling pathways (Fig. 2 and Table 1).

TGF-β signaling pathway

Cells respond to the external stimuli through a series of complex and dynamic signaling pathways, including TGF signaling pathway. All the members of the TGF-β family are secreted dimeric polypeptides, including TGF-β, activins, bone morphogenetic protein (BMP), growth/differentiation factor (GDF), and Mullerian inhibiting substance (MIS). TGF-β1 secreted by immune cells, stellate cells and epithelial cells is the most potent pro-fibrogenic cytokine in liver, involved in regulating the activation of HSCs and production of excessive extracellular matrix during liver fibrosis. SMAD proteins act the downstream of TGF-β1 as essential transcription factors in the signaling cascades. SMAD3 and SMAD4 are pro-fibrotic, and SMAD4 interacts with SMAD2/3 to participate in the transcription of pro-fibrotic target genes.[19] Among them, miR-31 was stimulated by SMAD3, upregulated in HSCs treated with TGF-β and shown to be involved in HSC activation possibly through targeting *FIH1*, a suppressor of hypoxia-inducible factor (HIF).[20] Similarly, studies have shown that miR-98,[21] miR-130b-5p[22] and let-7a[23] positively regulate the activation of HSCs through the TGF-β/SMAD2/3 signaling pathway. Conversely, miR-122

and miR-146a negatively regulate the activation of HSC and inhibit the epithelial—mesenchymal transition (EMT) of HSCs induced by TGF-β1/SMAD4[24;25]. Unlike SMAD3 and SMAD4, SMAD7 is anti-fibrotic and negatively regulates TGF-β signaling responses. In HSCs, miR-30 blunted the profibrogenic TGF-β signaling by suppressing Krüppel-like factor 11, a negative regulator of SMAD7.[26] Moreover, miR-17-5p and miR-503 were also reported to promote HSC activation and liver fibrosis via downregulation of SMAD7.[27,28]

Canonical TGF-β signaling occurs when the TGF-β ligand binds to TGF-β receptor II (TGFBR II), which then recruits and phosphorylates TGFBR I. In turn, phosphorylated TGFBR I phosphorylates the associated SMAD2 and SMAD3, which recruit SMAD4 before translocating into the nucleus where it regulates the transcription of TGF-β-targeted genes.[29,30] In the signaling process, miRNAs are involved in regulating TGFBRs to mediate the activation of HSCs. For instance, miR-6133-5p and miR-20a-5p affected the fibrotic functions of HSCs by directly targeting *TGFBR2*.[31,32] Unlikely, miR-199a-3p induced by Twist1 indirectly promoted the TGF-β pathway by inhibiting the expression of connective tissue growth factor (*CTGF*), which negatively regulated the expression of TGFBR I, thereby mediating the activation of HSCs.[33] In a CCl_4-induced liver fibrosis model, miR-148a was downregulated, whereas ubiquitin-specific protease 4 (*USP4*) was upregulated. Overexpression of miR-148a attenuated USP4, α-SMA, and p-SMAD2, suggesting that miR-148a suppress the activation of HSCs and EMT by targeting *USP4* under the mechanism where USP4 acts to stabilize TGFBR I (Fig. 2)[34]. These results demonstrate that miRNAs regulate the activation of HSCs via TGFBR under multiple different mechanisms.

The liver is a target organ of various parasitic infections. Schistosomiasis caused by *Schistosoma japonicum* is a prevalent chronic infectious disease that can lead to substantial pathologic liver fibrosis by an accumulation of the eggs.[35] It was demonstrated that the parasite-derived sja-miR-71a was highly expressed in *S. japonicum* egg-associated extracellular vesicles (EVs) and could inhibit activation of host HSCs by directly targeting semaphorin 4D (*SEMA4D*), which increases the TGF-β1 level. In addition, suppression of liver fibrosis by sja-miR-71a was also partly mediated by regulating the Th1, Th2, Th17 and Treg cellular balance by inhibition of *SEMA4D*.[36] It was also found that the host miR-130a-3p was significantly decreased both in the sera of patients with cirrhosis and in the liver of mice infected with *S. japonicum*. Overexpression of miR-130a-3p not only inhibited the activation

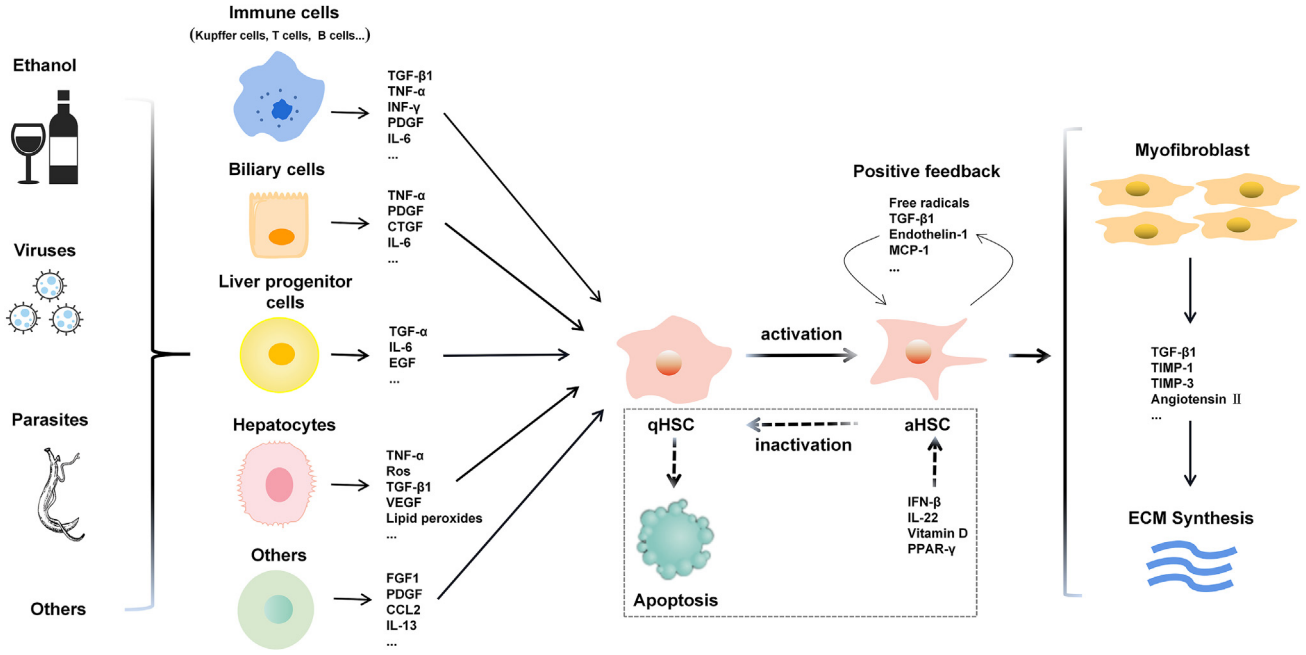

Figure 1 The role of HSCs in liver fibrosis. Upon liver injury caused by many factors such as alcohol consumption and viral and parasitic infections, Kupffer cells, T cells, hepatocytes, biliary cells and others are initiated to participate in inflammatory responses by releasing a plethora of cytokines and other active molecules. In this setting, if persistent liver injury occurs, hepatic stellate cells (HSCs) are continuously transdifferentiated from quiescent HSCs (qHSCs) to activated myofibroblast-like HSCs (aHSCs), which are capable of synthesizing a large amount of ECM proteins, ultimately leading to liver fibrosis. These aHSCs can promote their activation state via positive feedbacks by releasing MCP-1, endothelin-1 and so on. If the cause of the liver injury is removed, aHSCs are reversely inactivated and then subjected to apoptosis under the actions of IL-22, vitamin D and others, leading to the resolution of fibrosis. Abbreviations: CCL 2, C—C motif chemokine 2; CTGF, connective tissue growth factor; ET1, endothelin 1; FGF1, fibroblast growth factor 1; INF, interferon; MCP-1, monocyte chemoattractant protein 1; PDGF, platelet derived growth factor; PPAR-γ, peroxisome proliferator activated receptor gamma; ROS, reactive oxygen species; TGF-β 1, transforming growth factor beta 1; TNF-α, tumor necrosis factor alpha; TGF-α, transforming growth factor alpha; TIMP, tissue inhibitor of metalloproteinase; VEGF, vascular endothelial growth factor.

and proliferation of HSCs, but also induced the apoptosis of HSCs through targeting the expression of multiple genes, including *TGFBR1* and *TGFBR2*.[37] *Echinococcus granulosus*, another liver-residing parasite responsible for cystic echinococcosis in humans and animals, has been shown to manipulate the TGF-β signaling pathway to promote liver fibrosis through inhibition of miR-19.[38] Together these findings suggest a role of the miRNA-TGF-β axis in liver fibrosis caused by multiple agents including parasites.

WNT/β-catenin signaling pathway

The WNT/β-catenin pathway plays a role in almost every facet of liver biology. Thus, its aberrant activation is a hallmark of various hepatic pathology,[39] including fibrosis.[40] During the pathogenesis of NASH, miR-146a-5p inhibits the activation and proliferation of HSCs by targeting *WNT1* and *WNT5a*, key components of the WNT signaling pathway (Fig. 2)[15]. Similarly, sja-miR-1 was abundantly present in the HSCs in schistosomiasis patients. It was further shown that sja-miR-1 contributed to the parasite-induced hepatic fibrosis through activating the WNT/β-Catenin pathway by targeting secreted Frizzled related protein 1 (*SFRP1*),[41] which is very similar in structure to Frizzled receptors and can directly inhibit the WNT pathway by competitively binding to WNT or Frizzled receptors.[42]

Zinc finger E-box binding homeobox 1 (ZEB1), a transcriptional repressor, is a member of the zinc-finger family of proteins. ZEB1 plays a significant role in the activation of HSCs and fibrogenesis by positively regulating the WNT/β-catenin signaling pathway. Inhibition of miR-708 in the aHSCs and liver leads to an increase of ZEB1, while overexpression of miR-708 reduces HSC activation and proliferation via downregulation of *ZEB1*.[43] Moreover, miR-708 was also reported to directly inhibit the expression of transmembrane protein 88 (TMEM88), a potential 2-transmembrane protein that interacts with the PDZ domain of Dishevelled-1 (DVL-1), a key component in the WNT signaling pathway. TMEM88 inhibition led to a significant increase of the expression of α-SMA and COL I and ECM accumulation by disrupting the balance between matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs).[44] These results demonstrate that miR-708 is involved in the HSCs activation and ECM accumulation via the WNT/β-catenin signaling pathway by targeting *TMEM88*.

PI3K/AKT signaling pathway

The PI3K/AKT pathway is involved in regulating liver fibrosis possibly through tensin homolog (PTEN).[45] In liver diseases, PTEN expression is dysregulated and restoring PTEN expression is a promising strategy for the treatment of liver

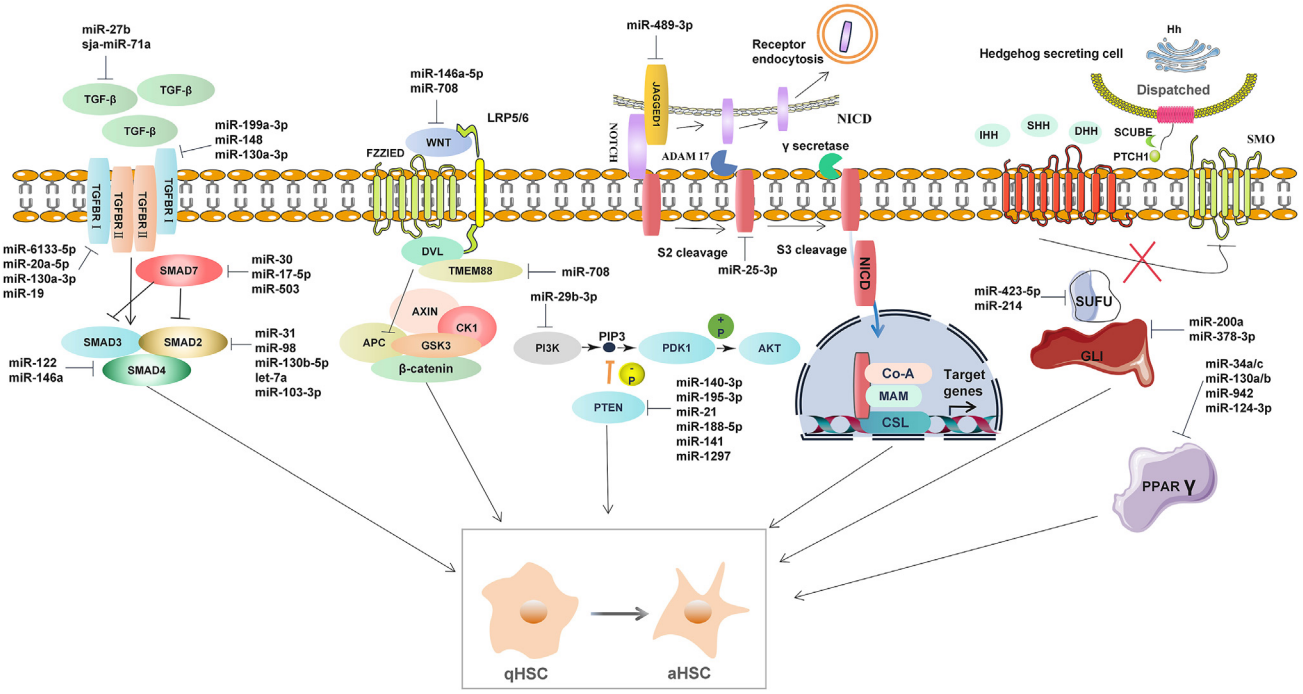

Figure 2 The role of miRNAs in HSC activation. A great number of miRNAs are involved in the regulation of HSC activation through multiple signaling pathways, including TGF-β, WNT/β-catenin, PI3K/AKT, NOTCH, Hedgehog and PPAR-γ. Abbreviations: ADAM10, a disintegrin and metallopeptidase domain 10; Akt, protein kinase B; CK1, casein kinase 1; Co-A, co-activator; CSL, CBF-1/Su(H)/LAG1; DHH, desert hedgehog; DVL, disheveled; Gli, glioma-associated oncogene; GSK, glycogen synthase kinase; Hh, hedgehog; IHH, Indian hedgehog; MAM, mastermind; NICD, Notch intracellular domain; PDK1, 3-phosphoinositide-dependent protein kinase 1; PI3K, phosphatidylinositol-3-kinase; PIP3, phosphoinositol-3′,4′,5′-trisphosphate; PPAR-γ, peroxisome proliferator activated receptor gamma; PTEN, phosphatase and tensin homolog; SHH, sonic hedgehog; Smo, smoothened receptor; Sufu, suppressor of fused homolog; TGF-β, transforming growth factor beta; TGFBR2, transforming growth factor beta receptor.

Table 1 miRNAs involved in the regulation of HSC activation via multiple signaling pathways

Signaling pathway	miRNAs	Target	Function	Ref
TGF-β	miR-27b	*KSRP*	Regulate HSC activation	140
	miR-31	*FIH1*	Promote fiber formation and HSC activation	20
	miR-98	*HLF*	Inhibit HSC activation	21
	miR-130b-5p	*SIRT4*	Regulate HSC activation, proliferation and apoptosis	22
	let-7a	*SMAD2/3*	Reduce cell viability and promote HSC apoptosis	23
	miR-122, miR-146a	*SMAD4*	Inhibit EMT of HSCs	24,25
	miR-30	*KLF11*	Inhibit HSC activation	26
	miR-17-5p, miR-503	*SMAD7*	Promote HSC activation	27,28
	miR-103-3p	*KLF4*	Promote HSC activation	80
	miR-6133-5p	*TGFBR2*	Affect the activation and fibrotic functions of HSCs	27
	miR-20a-5p	*TGFBR2*	Promote ECM production	32
	miR-199-3p	*CAV2*	Mediate HSC activation and TGF-β expression	33
	miR-148a	*USP4*	Suppress activation of HSC and EMT	34
	sja-miR-71a	*SEMA4D*	Inhibit HSC activation	36
	miR-130a-3p	*TGFBR1, TGFBR2*	Inhibit HSC activation and proliferation	37
	miR-19	*TGFBR2*	Increase the activation of HSCs and ECM production	38
WNT/β-catenin	miR-146a-5p	*WNT1, WNT5a*	Inhibit HSC activation and proliferation	15
	sja-miR-1	*SFRP1*	Increase the expression of α-SMA and Col I and promote HSC activation	41
	miR-708	*ZEB1*	Reduce HSC activation and proliferation	43
	miR-708	*TMEM88*	Promote HSC activation and enhance ECM accumulation	44
PI3K/AKT	miR-140-3p	*PTEN*	Improve HSC proliferation and expression of α-SMA	48
	miR-195-3p	*PTEN*	Promote HSC activation and proliferation and the expression of COL I and α-SMA	49
	miR-21	*PTEN*	Attenuate the liver fibrosis induced by arsenite	50
	miR-188-5p	*PTEN*	Inhibit the expression of pro-fibrotic and pro-inflammatory markers and proliferation of HSCs	51
	miR-141	*PTEN*	Inhibit HSC activation	52
	miR-1297	*PTEN*	Mediate cellular communication between HCs and HSCs, contributing to HSC activation and proliferation	53
Hedgehog	miR-423-5p, miR-214	*SUFU*	Promote HSC activation and cause the accumulation of extracellular matrix	57,58
	miR-378a-3p	*GLI3*	Suppress HSC activation and pre-fibrotic genes' expression	59
	miR-200a	*GLI3*	Inhibit HSC activation	141
PPAR-γ	miR-34a/c	*PPAR-γ*	Downregulate the expression of α-SMA	61
	miR-130a/b, miR-942	*PPAR-γ*	Enhance HSC activation	62,63
	miR-124-3p	*PPAR-γ*	Inhibit HSC activation	64
NOTCH	miR-489-3p	*JAG1*	Reduce the expression of pro-fibrosis markers and inhibit HSC activation	65
	miR-25-3P	*ADAM-17, FKBP14*	Reduce HSC activation	66

injury.[46,47] In TGF-β1-induced HSC-T6 cells, miR-140-3p-stimulated PTEN silencing improved cell proliferation and α-SMA expression, accompanied by decreased apoptosis, possibly via enhancing the p-AKT and p-mTOR levels (Fig. 2)[48]. Similarly, overexpression of miR-195-3p also resulted in a decrease of PTEN expression and subsequent activation and proliferation of HSCs and significant upregulation of COL I and α-SMA.[49] Conversely, knockout of miR-21 promoted the expression of PTEN, thus giving rise to attenuation of the liver fibrosis in animals exposed to arsenite. Therefore, it may be plausible to base the development of therapeutic interventions on the miR-21-PTEN-AKT axis for treatment of liver fibrosis.[50]

Activation of HSCs via the PI3K/Akt pathway, involving PTEN, also includes miR-188-5p,[51] miR-141[52] and miR-1297.[53] Of these, miR-1297 was shown to be enriched in exosomes derived from lipotoxic hepatocytes (HCs) and to mediate cellular communication between HCs and HSCs. The exosomal miR-1297 directly targeted *PTEN* and contributed to the activation and proliferation of HSCs via the PI3K/ATK pathway, thus leading to acceleration in the progression of liver fibrosis.[53]

Conversely, miR-29b is involved in negative regulation of the PI3K/ATK pathway. In aHSCs, the increased expression of miR-29b inhibited cell viability and colony formation and caused cell cycle arrest in a G1 phase by downregulating *CYCLIN D1* and *P21cip1*. miR-29b was further shown to prevent liver fibrogenesis by inhibiting HSC activation and inducing HSC apoptosis through inhibiting the PI3K/AKT pathway.[45] However, it remains unclear how the expression of miR-29b is regulated during the activation of HSCs.

Hedgehog signaling pathway

Hedgehog (Hh) signaling acts in both paracrine and autocrine manners, regulating the proliferation of Hh-responsive cells, such as HSCs and hepatic progenitor cells.[54] In the Hh pathway, some effector proteins act as a regulator in liver fibrosis, such as suppressor of fused (SUFU)[55] and GLI family zinc finger (GLI).[56] It was shown that miR-423-5p and miR-214 was significantly up-regulated during the activation of HSCs and caused the accumulation of extracellular matrix via *SUFU*, implicating a role of the Hh signaling pathway in hepatic fibrosis (Fig. 2)[57;58]. Similarly, in a mouse model of CCl_4-induced liver fibrosis, miR-378a-3p was upregulated and directly targeted *GLI3* to promote activation of HSCs and expression of pre-fibrotic genes while also downregulating glial fibrillary acidic protein (GFAP), a marker of qHSCs. During liver fibrosis, overexpression of miR-378a-3p is also associated with the dysregulation of the NF-κB pathway,[59] so understanding what interactions exit between the Hh signaling pathway and NF-κB-mediated inflammation will be useful for developing therapeutics against liver fibrosis.

PPAR-γ signaling pathway

PPAR-γ is a key factor in the inhibition of HSC activation and its expression is decreased during liver fibrosis.[60] Numerous studies have indicated that miRNAs can negatively regulate PPAR-γ to promote HSC activation, such as miR-34a/c, miR-130a/b, miR-942 and miR-124-3p.[61–63] Rosiglitazone (RGZ) inhibited the activation of HSCs and then alleviated hepatic fibrosis by the upregulation of miR-124-3p; the expression of PPAR-γ in this context was regulated via the miR-124-3p/HOTAIR axis (Fig. 2)[64]. Hence, the interventions against the PPAR-γ pathway may be a promising therapeutic strategy for hepatic fibrosis.

NOTCH signaling pathway

NOTCH signaling is essential for the activation of HSCs in the stationary phase. In a CCl_4-induced fibrosis model, miR-489-3p expression was significantly reduced, while the expression of jagged canonical NOTCH ligand 1 (JAG1) was increased. Overexpression of miR-489-3p reduced the expression of pro-fibrosis markers and inhibited the activation of HSCs by inhibiting the JAG1/NOTCH3 signaling pathway (Fig. 2)[65]. Similarly, miR-25-3p overexpression repressed NOTCH1-dependent HSC activation via downregulation of ADAM metalloprotease domain-17, matrix metalloproteinases-17 and the γ-secretase co-activator FK506 binding protein 14, simultaneously leading to inhibition of TGF-β and WNT signaling.[66] It is reasonable that miR-25-3p may be involved in regulation of HSC activation via multiple signaling pathways.

Others

miR-29 is one of the most studied miRNAs contributing to liver fibrosis. The expression of miR-29 is significantly reduced in the fibrotic liver and its downregulation alters HSC activation. In primary mouse HSCs, increased miR-29a promoted BRD4, enhancer of Zeste homolog 2 (EZH2), SNAI1 and PPRP-γ expression, thus inhibiting HSC activation. Consistent with this finding, *in vivo* models of fibrosis demonstrated that miR-29a overexpression reduced bile duct ligation-mediated fibrosis by upregulating *BRD4* and *SNAI1*.[67] miR-29 can also influence liver fibrosis through autophagy. Increased autophagy is normally observed in mice with liver fibrosis, and the inhibition of autophagy can reduce HSC activation and fibrogenesis.[68] In a bile duct ligation-induced model, the overexpression of miR-29a significantly inhibited autophagy and thus reduced liver damage and fibrosis. However, in cholestatic liver disease increased miR-29a inhibited HSC activation and liver fibrosis, possibly by means of the downregulation of ribonucleic acid requires kinase 1a, protein kinase-like endoplasmic reticulum kinase, CCAAT/enhancer binding protein homologous protein and spliced-X-box binding protein 1 (sXBP1).[69] These results suggest that miR-29a is involved in liver fibrosis via HSC activation by way of multiple signaling mediators in a number of disease settings.

miR-223 is also involved in the development of fibrosis and the activation of HSCs via negatively regulating NOD-like receptor 3 (*NLRP3*); in this setting miR-223 has been targeted to treat acute and chronic hepatitis.[70] miR-223 was found to be highly expressed in the exosomes derived from NK cells and was transferred to HSCs via these exosomes, where it suppressed autophagy through inhibition of *ATG7*, thereby attenuating TGF-β1-induced HSC activation.[71] Additionally, during spontaneous resolution of

liver inflammation (SRLI), neutrophil-derived miR-223 functioned as a silencer of NLRP3 in hepatic macrophages, which were polarized to a restorative phenotype that reduced the release of IL-10, thus mitigating fibrogenesis by impairing the activation of HSCs and collagen synthesis.[72]

miRNAs associated with HSC proliferation, apoptosis and senescence

Understanding the regulatory mechanisms of miRNAs involving in the proliferation or apoptosis of HSCs is essential because they may be used as the targets for potential therapeutic interventions. During liver fibrosis, a panel of miRNAs including miR-708,[43] miR-455-3p,[73] miR-193a/b-3p,[74] miR-150[75] and miR-194[76] were downregulated in HSCs and induced HSC proliferation and the high expression of COL I and α-SMA, leading to the occurrence of liver fibrosis. Conversely, there are a number of miRNAs that exhibit the induction of HSC proliferation. Studies have demonstrated that miR-29a-3p, miR-188-5p, miR-146b and miR-7-5p increase the expression of pro-fibrotic markers and HSC proliferation.[51,77–79] In addition, during liver fibrosis THP-1 macrophages interact with HSCs by their released exosomes, which contain miR-103-3p that promotes HSC proliferation by targeting *KLF4*.[80]

In addition to HSC proliferation, several miRNAs, including miR-29b,[45] miR-148a-3p,[81] miR-494-3p[82] and miR-150-5p,[83] can participate in liver fibrosis by regulating HSC apoptosis. Of them, miR-150-5p was notably increased in hepatocytes but decreased in HSCs during liver fibrosis in a CCl_4-induced model. It was further shown that miR-150-5p overexpression promoted HSC apoptosis and sensitized hepatocytes to apoptosis. Hepatocyte apoptosis and subsequent release of damage-associated patterns (DAMPs) not only directly activate HSCs but also lead to the recruitment and activation of both lymphocytes and macrophages that contribute to the promotion of HSC trans-differentiation and myofibroblast activation by producing pro-inflammatory and pro-fibrogenic cytokines.[84] Therefore, the interactions between HSCs and hepatocytes or macrophages will open a new window for studies of liver fibrosis in the future.

In the cirrhotic liver, HSCs in a senescence state remain non-proliferative, lack collagen-producing capacity, and produce more inflammatory cytokines.[85] Overexpression of miR-145 led to reduction in the expression of HSC activation markers α-SMA and COL I in HSCs. Moreover, silencing of ZEB2 promoted the senescence of aHSCs.[86] These results suggest that miR-145 as a suppressor of fibrosis participate in the senescence of aHSCs through the ZEB2-p53 pathway.

miRNAs in regulation of ECM deposition

Chronic liver diseases lead to hepatocyte damage and infiltration of immune cells that can cause the trans-differentiation of HSCs into collagen-producing myofibroblasts.[87] Following short-term injury, myofibroblasts are physiologically involved in tissue repair and are rapidly cleared by apoptosis or inactivation. However, an imbalance of pro-fibrogenic and anti-fibrogenic reactions causes persistent activation of proliferation and migration of myofibroblasts, which leads to disruption of the balance between ECM deposition and dissolution, ultimately triggering progressive liver fibrosis.[88] The most important ECM components include collagen, proteoglycans, laminin, fibronectin, and stromal cell proteins. In the normal liver, the low-density basement membrane-like matrix of the Disse space is mainly composed of collagens IV and VI, but after liver injury the matrix is destroyed and replaced by collagens I and III and fibronectin, thus leading to ECM deposition.[89] Recent studies showed that overexpression of miR-193a/b-3p and miR-101 inhibited expression of COL I and α-SMA in the *in vitro* model of liver fibrosis.[74,90] Conversely, up-regulated miR-181a suppressed the expression of augmenter of liver regeneration (ALR) and promoted the expression of COL I, α-SMA and RAC1 in HSCs.[91] These results reveal a crucial role of miRNAs in the ECM deposition, which implicates a promising strategy of alleviation of liver fibrosis via reversing ECM deposition as evidenced by targeted delivery of miR-29b and miR-122 to HSCs down-regulating COL I, α-SMA and metalloproteinase inhibitor 1 *in vitro* and in the rats with CCl_4-induced liver fibrosis.[92]

miRNAs in regulation of liver fibrosis under other mechanisms

In patients with liver cirrhosis, the degree of fibrosis was negatively correlated with the expression of hepatocyte vitamin D receptor (VDR) and autophagy flux but positively correlated with the expression of miR-125a. It was confirmed that the miR-125a/VDR axis reduced liver cell damage and liver fibrosis by regulating the autophagy rate of liver cells, providing a basis for early treatment of liver fibrosis.[93] Moreover, miR-322/424 levels were also increased in these patients and was demonstrated to target *Cullin2* to destabilize the VCBCR complex and increase the level of hypoxia-inducible factor-1α (HIF-1α), thus facilitating progression of liver fibrosis.[94] In other studies, the underlying mechanisms of miR-375,[95] miR-219,[96] miR-125b[97] miR-132,[98] miR-200b[99] and miR-29a[100] in liver fibrosis were investigated and demonstrated that they could improve liver fibrosis induced by CCl_4. Of them, miR-29a plays an important role in the resolution of fibrosis by inducing transformation of aHSCs into qHSCs partially through the negative regulation of ATPase H transport V1 subunit C1 (*ATP6V1C1*).[100]

A number of specific diets, such as the high-fat diet (HFD) and methionine- and choline-deficient (MCD) diet, can be used to induce the progression of nonalcoholic fatty liver disease (NAFLD) to hepatic fibrosis in the experimental animals. In HFD diet mice, miR-378 and miR-142-5p exert opposite effects. miR-378 positively regulated the NF-κB/TNF-α axis to trigger the development of NASH and fibrosis,[101] while miR-142-5p reduced fibrosis in the liver and relieved the progression of nonalcoholic steatohepatitis by inhibiting the JAK-STAT signaling pathway.[102] In mice fed a MCD diet, miR-29a suppressed CD36 to alleviate the induced steatosis and subsequent liver fibrosis.[103,104] However, in mice fed a methionine-choline-deficient and high-fat (MCDHF) diet, the expression of miR-26b-5p was reduced in liver, and this was negatively correlated with platelet-derived growth factor receptor-beta (PDGFR-β), fibrosis and

angiogenesis markers. Further experiments demonstrated that miR-26b-5p negatively regulated the expression of PDGFR-β to reduce liver fibrosis and angiogenesis.[105] These results suggest a role of miRNAs in the pathogenesis of diet-induced liver fibrosis.

miRNAs involved in lncRNAs-orchestrated liver fibrosis

Extensive studies demonstrate the link between long non-coding RNAs (lncRNAs) and liver fibrosis development, suggesting a potential value as regulators of liver fibrosis. Mechanically, miRNAs mediate lncRNAs to participate in the pathogenesis of liver fibrosis by way of competing endogenous RNA (ceRNA). For example, the up-regulation expression of lncRNAs, including ANXA2P2 (mouse Anxa6),[106] G protein-coupled receptor 137B (Gpr137b-ps),[107] small nucleolar RNA host gene 7 (SNHG7),[108] lncRNA-MBI-52,[109] nuclear paraspeckle assembly transcript 1 (Neat1)[110–112] and lncRNA X-inactive-specific transcript (XIST),[113] results in decreased expression of miR-9-5p, miR-200a-3p, miR-29b, miR-466g, miR-148a-3p, miR-22-3p, miR-129-5p, miR-139-5p and miR-539-3p, respectively, which in turnpromote HSCs activation and liver fibrosis in CCl_4-treated mice. Conversely, the lncRNAs activated by DNA damage (NORAD)[114] and NONRATT013819.2[115] suppressed HSC activation via the miR-495-3p/S1PR3 and miR-24-3p/lox axis, respectively.

It is well known that NF-κB signaling is involved in regulation of the activation of HSCs. More recently, lncRNAs GAS5[116] and metastasis associated lung adenocarcinoma transcript 1 (MALAT1)[117] were shown to exert regulatory effects on HSC activation by participating in the NF-κB signaling pathway through regulation of the miR-433-3p/TLR10 and miR-181a/TLR4 axis, respectively. These studies demonstrate that lncRNA/miRNA/mRNA interactions play a crucial role in regulating HSC activation and hepatic fibrogenesis.

miRNAs as potential diagnostic and therapeutic candidates

Reversing fibrosis has been becoming possible, however, it is generally irreversible in advanced cases. Therefore, early diagnosis is urgently required to develop specific therapies. Due to the disadvantages of the liver biopsy including invasiveness, sampling error, and poor repeatability, attentions have mainly been paid in recent years to non-invasive diagnostic approaches. The levels of miRNAs in serum are stable and reproducible and can be easily detected and quantified, rendering them promising non-invasive candidates for diagnosis of liver fibrosis. Serum exosomal miR-122, for example, was significantly decreased with the progression of liver fibrosis and may has the potential to serve as a biomarker for advanced liver fibrosis, especially in patients with non-viral etiologies of chronic liver disease.[118] Conversely, miR-155 was significantly upregulated in Child-Pugh C and was demonstrated to be significantly correlated with liver fibrosis, thus potentiating it as a non-invasive biomarker for the diagnosis and progression of hepatic fibrosis.[119] In patients with biliary atresia, miR-214 levels in both liver and sera were significantly higher in those who had severe liver fibrosis compared to people with no or mild fibrosis.[120] Similarly, in sera of NAFLD patients miR-193a-5p and miR-181a levels strongly correlate with fibrosis stages, and use of miR-181a combined with FIB-4 may increase the accuracy of each method alone,[121,122] rendering them potential clinically biomarkers of predicting fibrosis.

Increasing studies have demonstrated that miRNAs can also serve as potential biomarkers of liver fibrosis caused by different etiologies. The levels of three serum exomiRs (miR-92a-3p, miR-146a-5p and miR-532-5p) were able to distinguish patients with fibrosis grades I–III from those who have no fibrosis, being a supplementary tool for grading liver fibrosis in hepatosplenic schistosomiasis patients.[123] For hepatic fibrosis caused by viral infections, a panel of miRNAs, including serum miR-17, miR-448 and miR-34a, could be a non-invasive hallmark for assessment of liver fibrosis severity.[124–126]

At present new therapeutic techniques based on miRNA molecules are emerging as promising alternatives to conventional drug therapies; these can target miRNAs that are either upregulated or downregulated. Upregulated miRNAs can be reversed by miRNA sponges, miRNA masking, and anti-miRNA oligonucleotides, such as antagomirs that are a class of chemically modified oligonucleotides which specifically and efficiently block the functions of a given upregulated miRNA.[127] For downregulated miRNAs, their functions can be restored or augmented using miRNA mimics or plasmids expressing the same miRNAs.[128] In liver fibrosis, for instance, miR-98, which binds to the 3′-UTR of the *HIF-1α* mRNA, was decreased in aHSCs. miR-98 overexpression significantly attenuated CCl_4-induced hepatic fibrosis in mice after injection of ago-miR-98.[21] miR-494-3p, being downregulated in the alcoholic hepatitis (AH) mice model, can be targeted by transfection with the miR-494-3p mimic. This significantly prevented liver fibrosis by inhibiting proliferation and inducing apoptosis of HSCs through targeting TRAF3.[82]

Although the prospect of microRNA-based gene therapy is promising, major challenges exist in ensuring safe and efficient delivery of miRNAs to liver. One major obstacle is that synthetic oligonucleotides are not stable in circulation and they can be targeted to be degradation. Another challenge of miRNA-based therapy is that one particular miRNA can simultaneously control multiple target genes and possess divergent functions that are cell-type dependent. Therefore, off-target effects of miRNA-based therapies may occur and must be a major consideration in any therapeutic development. Nevertheless, these challenges could be addressed by multiple chemical modifications that maximize the stability, delivery and cellular uptake efficiency of oligonucleotides *in vivo*. In addition, the mode of delivery is important in circumventing problems related to stability and tissue specificity of miRNAs. Viral vector delivery systems including lentivirus vector (LV), adenovirus (AD) and adeno-associated virus (AAV) vector seem the most reliable carriers for delivery of miRNA mimics as well as anti-miRNAs. Due to modifications in certain specific regions of the genome, these delivery vehicles show a high

infection rate and a high expression level of exogenous miRNAs.[129]

Among them, LV is an enveloped single-stranded RNA virus that produces its own reverse transcriptase and produces a double-stranded DNA provirus, which integrates into the cell genome.[130] LV delivery therefore enables continuous expression of the target gene for a prolonged period. It has been demonstrated that hydrodynamic tail-vein injections could effectively transport LV into the liver with the highest concentration. Injection of miR-122-, miR-130a-3p- or miR-200a-expressing LV significantly inhibited the activation and EMT of HSCs and suppressed the development of hepatic fibrosis.[24,37,131] The beneficial characteristics of using AD for liver gene delivery include marked hepatotropism, high expression, large packaging capacity, and low genotoxicity.[132] Nevertheless, the antiviral inflammatory responses result in the elimination of infected cells in a relatively short time, and therefore limit the applications.[132] In order to achieve long-term expression, a third generation of adenoviruses, free of viral proteins, has been developed,[133] but their delivery efficacy and safety need further investigations. AAV can be stably expressed in hosts with a low pathogenicity and immunogenicity, and is a promising vehicle for delivery of miRNAs to liver. A recent study showed that AAV8-mediated efficient and sustained inhibition of sja-miR-2162 led to significantly elevated expression of *TGFBR3* and thereby reduced collagen production and attenuated hepatic fibrosis.[134] Similarly, AAV-mediated hepatic delivery of miR-191-3p significantly attenuated cholestatic liver injury in a murine model of cholestasis.[135]

Despite a high degree of efficacy in terms of delivery, the lack of cell-type specificity and the risk to patients due to the possibility of mutations *in vivo* prevent viral-mediated miRNA therapy from being widely used. Attention has therefore been paid to non-viral vectors that have an improved safety profile, are less immunogenicity, and face fewer restrictions compared with viral vectors.[136] These include lipid nanoparticles (LNPs), lipid-calcium-phosphate nanoparticles (LCP NPs) and lipoplexes. A most recent study has shown that, when relaxin binds to the primary relaxin receptor, hepatic macrophages switch from a profibrogenic to pro-resolution phenotype, releasing exosomes that promote the relaxin-mediated quiescence of aHSCs through miR-30a-5p. Lipid nanoparticles containing relaxin and miR-30a-5p can be modified to have a surface that allows them to target aHSCs via aminoethyl anisamide. These nanoparticles can significantly reduce liver fibrosis and injury in a mouse model.[137] Furthermore, chitosan (CS) nanoparticles are also a promising vehicle for targeted delivery of miRNAs to liver. miR-4989-loaded CS nanoparticles have been demonstrated to be predominantly enriched in the liver and significantly downregulate miR-4989 target, *UBE2N*, at both mRNA and protein levels.[138] Despite the low degree of efficacy in terms of cell proliferation and apoptosis, poor knowledge on long-term effects and metabolic dynamics of CS nanoparticles in animals requires further evaluations.

Furthermore, hepatocytes consist up to 80% of the liver mass and mediate a broad range of interactions among different cells. A recent study demonstrated that *in vivo* knockdown of miR-221-3p by AAV TuD suppressed HSC activation and alleviated hepatotoxin-induced liver fibrosis in mice, which specifically targeted hepatocytes with a decreased profile of off target effects.[139] Therefore, attentions should be given to the interactions of hepatocytes with other liver cells, helping us understanding of the pathogenesis of liver fibrosis.

Conclusion

Our understanding of the biological role of miRNAs in liver fibrosis is rapidly increasing. Expanding studies have demonstrated that multiple miRNAs are involved in the process of liver fibrosis at many levels; this extends to regulating HSC activation, proliferation, cell cycle and apoptosis, and the expression of genes involved in collagen production and ECM deposition. Therefore, it is plausible to develop interventions that simultaneously target two or more biological processes in HSCs.

Although many challenges remain, deeper and comprehensive knowledge on miRNAs and their bioactivities in liver fibrosis will help improve our understanding of the pathogenesis of liver fibrosis and the design of miRNA-based therapies. Advances in technologies for studying miRNAs, based on improved sequencing technologies and miRNA mimics or inhibition screening, will be valuable resources for identifying novel diagnostic and therapeutic targets for liver fibrosis.

Author contributions

Hong Li, Tingli Liu and Yongchun Yang contributed to literature searching and wrote the manuscript. Yadong Zheng, William C. Cho, Robin J. Flynn, Majid Fasihi Harandi, Houhui Song and Xuenong Luo reviewed and revised the manuscript. All authors read and approved the final manuscript.

Conflict of interests

The authors have declared that no competing interest exists.

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