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EXPERT REVIEW

Delivery and Targeting of miRNAs for Treating Liver Fibrosis

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ABSTRACT Liver fibrosis is a pathological condition originating from liver damage that leads to excess accumulation of extracellular matrix (ECM) proteins in the liver. Viral infection, chronic injury, local inflammatory responses and oxidative stress are the major factors contributing to the onset and progression of liver fibrosis. Multiple cell types and various growth factors and inflammatory cytokines are involved in the induction and progression of this disease. Various strategies currently being tried to attenuate liver fibrosis include the inhibition of HSC activation or induction of their apoptosis, reduction of collagen production and deposition, decrease in inflammation, and liver transplantation. Liver fibrosis treatment approaches are mainly based on small drug molecules, antibodies, oligonucleotides (ODNs), siRNA and miRNAs. MicroRNAs (miRNA or miR) are endogenous noncoding RNA of ~22 nucleotides that regulate gene expression at post transcription level. There are several miRNAs having aberrant expressions and play a key role in the pathogenesis of liver fibrosis. Single miRNA can target multiple mRNAs, and we can predict its targets based on seed region pairing, thermodynamic stability of pairing and species conservation. For *in vivo* delivery, we need some additional chemical modification in their structure, and suitable delivery systems like micelles, liposomes and conjugation with targeting or stabilizing the moiety. Here, we discuss the role of miRNAs in fibrogenesis and current approaches of utilizing these miRNAs for treating liver fibrosis.

KEY WORDS epithelial to mesenchymal transition · extracellular matrix · liver fibrosis · microRNA

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ABBREVIATIONS

Ab	Antibodies
AP-1	Activator proteins
α-SMA	Alpha smooth muscle actin
CBDL	Common bile duct ligation
DCs	Dendritic cells
ECM	Extracellular matrix
EMT	Epithelial to mesenchymal transition
EXP 5	Exportin 5
ERK	Extracellular signal regulated kinase
Hh	Hedgehog
HCV	Hepatitis C virus
HSC	Hepatic stellate cells
ICAM-1	Intercellular adhesion molecule-1
KC	Kupffer cells
LNA	Locked nucleic acid
MMP	Matrix metalloprotease
miRNA	MicroRNA
MF	Myofibroblasts
NO	Nitric oxide
ODN	Oligodeoxynucleotide
2′-OMe	2′-methoxy
PI3-K	Phosphatidylinositol 3-kinase
PDGF	Platelet derived growth factor
PPAR-γ	Peroxisome proliferator-activated receptors
PS	Phosphothioated
RXR	Retinoid receptors
RISC	RNA induced silencing complex
ROS	Reactive oxygen species
siRNA	Small interfering RNA
SHh	Sonic hedgehog
shRNA	Small hairpin RNA
SMO	Smoothened
TFO	Triplex forming oligonucleotide
TGF-β	Transforming growth factor beta
TIMP	Tissue inhibitors of metalloprotease
TNF-α	Tumor necrosis factor -α

TRAIL	TNF related apoptosis inducing ligand
tTG	Tissue transglutaminase
UTR	Untranslated region
VEGF	Vascular endothelial growth factor

INTRODUCTION

Liver fibrosis is a histological change caused by liver inflammation. This pathological condition originates from liver damage and accompanied by excess accumulation of extracellular matrix (ECM) proteins. Causes of liver damage could be diverse including alcoholic abuse, hepatitis B virus (HBV) and hepatitis C virus (HCV) infection, metabolic disease, toxins, and ischemia reperfusion (IR) injury (1). Excess ECM production in the perisinusoidal space (or space of Disse) results in a physical barrier between sinusoidal lumen and hepatocytes. Blood flow to hepatocytes is reduced, and liver function gets altered which can advance to cirrhosis. Currently, liver transplantation is the only option available to treat cirrhosis, which itself suffers from complications such as a complex procedure and limited organ donors. In the current situation, reversal of liver fibrosis before reaching the advanced stage of cirrhosis is the only possible approach (2).

Cessation of the causative agent is the most effective approach to fibrosis regression. Removal of excess alcohol consumption, treating viral infection, removing toxins and cholestasis are of major importance. Treatment approaches include the inhibition of collagen synthesis and abrogation of ECM deposition, stimulation of matrix degradation, modulation of hepatic stellate cell (HSC) activation, induction of HSC apoptosis and modulation of immune responses at the affected site. Interruption of fibrotic pathways and regulation of gene expression is an attractive approach to treat liver fibrosis. Various gene silencing methods used for treating liver fibrosis include antisense oligodeoxynucleotides (ODNs), triplex forming oligonucleotides (TFOs), small interfering RNA (siRNA), and miRNAs. The use of transgene modulating molecule is a highly specific and powerful technique to inhibit aberrant protein production, as it works at mRNA levels rather than at protein levels (3). miRNAs are known to alter gene expression at post transcriptional level in many developmental and physiological processes, especially in cell death and proliferation. Their expression profiles are different at normal and disease conditions and can also serve as diagnostic and prognostic purposes (4). In this review, we will critically discuss the roles of miRNAs in liver fibrosis, current status and strategies for utilizing miRNAs as therapeutics to treat liver fibrosis.

PATHOGENESIS OF LIVER FIBROSIS

In normal injury healing mechanism, there is a balance between production and degradation of ECM, but this balance gets disturbed in liver fibrosis. The activation of tissue repair process following liver injury and complex cellular and molecular mechanisms of liver fibrosis have been well characterized. Local inflammatory responses and oxidative stress are the major contributing factors to onset and progression of liver fibrosis (5). Excessive production of ECM including collagen is the hallmark of fibrosis. ECM consists of collagen type I, II and V, laminin, proteoglycans and matricellular proteins. After chronic liver injury, there is excess ECM production compared to its degradation, and there is net deposition of ECM due to the reduced activity of matrix metalloprotease (MMPs) in the presence of active forms of tissue inhibitors of metalloprotease (TIMPs) (6). Hepatic fibrosis develops as a result of the progressive thickening of fibrotic septa and chemical cross-linking of collagen. Collagen is also known as an important mediator of cell survival and proliferation and promotes HSC proliferation in liver fibrosis (7). Collapse of hepatic parenchyma and its substitution with a collagen-rich ECM reduces blood supply and associated with the onset of cirrhosis.

Cirrhosis is the advanced stage of liver fibrosis and is characterized by the replacement of liver tissue by unresolvable scar and regenerative nodules. Cirrhosis leads to hepatocellular dysfunction and increased intrahepatic resistance to blood flow and results in portal hypertension. Ultimately, liver functions are lost, and there is fluid retention in the abdominal cavity called ascites or leads to visceral hemorrhage/encephalopathy (8). Since cirrhosis is irreversible end stage disease, its treatment usually focuses on preventing progression or liver transplantation.

Various cell types such as activated HSCs, portal fibroblasts, bone marrow-derived myofibroblast precursors and hepatocytes after epithelial to mesenchymal transition (EMT) are considered as sources of ECM in liver fibrosis. However, among all these, HSCs are the main ECM producing cells after liver injury. HSCs are present in the space of Disse in the liver and primarily store vitamin A in quiescent or normal state. Damaged hepatocytes and Kupffer cells (KCs) release inflammatory cytokines and induce HSCs into activated state. After activation, HSCs become proliferative, increase α-smooth muscle actin (α-SMA) expression, lose their vitamin A content, acquire a myofibroblast like phenotype (MF-HSC) losing their typical star-shape and start producing excess of ECM (9).

Hepatocytes form the main tissue of the liver and are responsible for metabolism. Upon injury, hepatocytes experience high oxidative stress and endoplasmic reticulum stress resulting in lysosomal activation and mitochondrial damage leading to apoptosis or necrosis. Myofibroblasts phagocyte apoptotic hepatocytes and get activated themselves via NADPH oxidase 2 (NOX2) and the JAK/STAT and PI3K/Akt pathways (10).

Portal fibroblasts are essential for organ integrity, and their elimination promotes tissue necrosis and inflammation. In liver fibrosis, portal fibroblasts get converted into activated myofibroblast and contribute to fibrous scar tissue (11). In portal fibrosis, biliary progenitors (also called activated cholangiocytes) proliferate and tend to form small clusters around the bile duct. Activation of Hh pathway promotes EMT in cholangiocytes and as a result they acquire migratory phenotype and increased expression of various mesenchymal markers (12). Upon activation, these cells secrete several chemo-attractants that attract and activate HSCs/myofibroblasts to proliferate and deposit ECM (13,14).

CD4+ T cells with Th2 and Th17 polarization are the major driver of fibrogenesis. Th2 produce inflammatory cytokines such as IL-4 and IL-13, which stimulate macrophages in the liver (15). Transforming growth factor (TGF)-β1 and interleukin (IL)-6 induce Th17 cells, and they start secretion of IL-17A; which acts itself as profibrotic for myofibroblasts and further stimulates inflammatory cells for TGF-β1 secretion (16). Regulatory T cells a have dual function in fibrogenesis; subsets produce TGF-β1 and promote fibrosis, whereas other release immunosuppressive cytokines IL-10 (17).

KCs, which are resident macrophages of the liver, release inflammatory cytokines upon liver injury, which activate HSCs. After activation HSCs starts producing cytokines, which stimulate macrophages. On the other hand macrophages produce profibrotic mediators that directly activate fibroblasts, including TGF-β1 and platelet-derived growth factor (PDGF). They also propagate antigen-specific T-cell responses; secrete MMPs and TIMPs. As antifibrotic, macrophages remove dead cells and debris by phagocytosis and dampen proinflammatory and profibrotic signals and thus play a significant role in the resolution of fibrosis (18). Dendritic cells (DCs) are also implicated in inducing inflammation during fibrosis. DCs proliferate during liver fibrosis and start secreting several proinflammatory cytokines including tumor necrosis factor α (TNFα) and activate natural killer (NK) cells, cytotoxic T cells, and even HSCs (19).

Intra-hepatic inflammatory responses after liver injury play a critical role in the development of liver fibrosis. The process involves the recruitment of various cell populations in the liver microenvironment including sinusoidal endothelial cells, KCs, and even HSCs (Fig. 1). Leukocytes are recruited at the site of hepatic injury. After reaching the affected site they start adhering to blood vessels and transmigrate using various adhesion molecules of the integrin family, e.g. β2 integrins, and immunoglobulin gene superfamily, e.g. intercellular adhesion molecule-1 (ICAM-1). Infiltrated leukocytes and KCs secrete compounds and which can directly activate HSCs (20). Monocytes and macrophages also produce nitric oxide (NO) and inflammatory cytokines such as TNFα, which is responsible for HSC activation and excess collagen synthesis (21). KCs express TNF related apoptosis inducing ligand (TRAIL) with Fas ligand and mediate apoptosis in the liver, which further contribute to liver inflammation and fibrosis. KCs as well as hepatocytes also secrete IL-8; which is a potent chemokine responsible for recruiting neutrophils, and T cells into inflammatory sites. IL-8 is secreted by cooperative interaction of nuclear factor κβ (NF-κβ), activator protein 1 (AP −1), and IL −6 (22,23).

A number of growth factors and cytokines are known to promote liver fibrosis. After liver injury, damaged hepatocytes and other surrounding cells in the liver secrete multiple signaling molecules and inflammatory cytokines including Hh ligands, TGF-β1, vascular endothelial growth factor (VEGF), PDGF-β, ILs and TNF-α. Hh pathway plays an important role in the construction and remodeling of injured tissues and found to be active in liver fibrosis. In the presence of Hh, a cell surface transmembrane protein called Smoothened (SMO) gets accumulated and inhibits the proteolytic cleavage of Gli proteins from microtubules. Decreased degradation of Gli enables them to accumulate in the cytoplasm and then translocate into the nucleus and allows transcription of growth factor family proteins. Activation of Hh pathway plays a key role in the transition of quiescent HSCs into myofibroblast and controls this mechanism via regulating their metabolism (24,25).

TGF-β plays a critical role in the progression of liver fibrosis. TGF-β is produced by KCs and HSCs, or establishes its autocrine and paracrine loop production and upregulate collagen I and II protein expression. TGF-β mediates fibrosis via Smad3 and Smad4 proteins, while this signaling is intervened by Smad7. Some studies also conclude that TGF-β enhance collagen production via reactive oxygen intermediates in general, and H_2O_2 in particular. IL-6 is overexpressed by HSCs in injured liver and implied to upregulate TGF-β expression and accordingly enhances its fibrogenic action (26–28).

VEGF is a well-characterized angiogenesis modulator, which is known to be upregulated during HSC activation and stimulates cell proliferation, migration, and collagen production (29). Liver sinusoidal endothelial cells (LSECs) are known to secrete TGF-β and PDGF. Increased VEGF level in liver fibrosis promotes the growth of LSECs and thereby, increases hepatic levels of TGF-β1 (30). PDGF is a dimeric protein that is one of the most potent mitogens for HSCs. PDGF signals via tyrosine kinase receptors PDGFR-α and PDGFR-β and sequentially activate Raf-1, MEK and extracellular-signal regulated kinase (ERK). Nuclear translocation of ERK phosphorylate transcription factors Elk-1, SAP, and triggers proliferative response. Activated PDGF receptors also trigger phosphatidylinositol 3-kinase (PI3-K) which is involved in inflammation and fibrosis (31–33). PDGF also induces liver fibrosis by expanding the population of collagen-producing cells and aid in TGF-β-stimulated ECM production.

MMPs are the family of endopeptidases, which are capable of tissue remodeling and degradation of all types of ECM proteins. MMPs are secreted by different cell types including fibroblasts, osteoblasts, endothelial cells, macrophages, neutrophils, and lymphocytes in response to hormones and cytokines (34). In the liver, HSCs are the key source of MMPs, and their level is regulated by a family of endogenous proteinase inhibitors known as TIMPs. In normal liver, the ratio between MMPs and TIMPs plays an important role in ECM turnover. In liver fibrosis, MMP level is down due to HSC activation and net increase in TIMPs, resulting in higher ECM production but low degradation. Moreover, TIMP-1 also has an antiapoptotic effect on HSCs through BCl-2 pathway and promotes their survival (35). TGF-β is also known to induce TIMP-1 expression, and results in reduced collagen degradation (36).

NF-κB is a heterodimer of p50 and p65 proteins and regulates inflammation, wound-healing response, and cell survival in various tissues (37). NF-κB is an important mediator responsible for the activation and survival of HSCs in liver fibrosis. It maintains high Bcl-2 (pro-survival) expression and decrease BAX and PUMA (pro-apoptotic) expression in HSCs and prevents their apoptosis. Tissue transglutaminase (tTG) is an enzyme that catalyzes protein cross-linking in liver fibrosis. NF-kB increases tTG gene expression in liver fibrosis and this enzyme stabilizes the fibrotic bands during hepatic fibrogenesis (38).

CURRENT TREATMENT OF LIVER FIBROSIS

Removing the primary cause is the most effective approach to treat liver fibrosis. Alcohol abstinence in patients with alcohol-induced liver fibrosis is recommended. In chronic hepatitis C virus (HCV) infection, treatment with antiviral drugs (i.e. ribavirin) to clear viral infection is recommended treatment. In obstructive cholestasis, removal of obstructive agent or surgery is the main option. This section will discuss various treatment strategies currently being explored including the inhibition of HSC activation, induction of HSC apoptosis, reduction of collagen production and deposition, decrease in inflammation, and liver transplantation (Table I). These treatments are based on small drug molecules, antibodies, oligonucleotides (ODNs), siRNA and miRNAs.

Small Drug Molecules

Drugs currently being investigated for treating liver fibrosis are Hh inhibitors, TGF-β inhibitors, angiotensin inhibitors, endothelin inhibitors, PDGFR inhibitors, anti-inflammatory, and peroxisome proliferator-activated receptor (PPAR) agonists. Hh signaling plays a critical role in cellular proliferation, migration, differentiation, and the growth of HSCs. In the liver, Hh ligands, such as Sonic Hh (SHh) and Indian Hh (IHh) are expressed by hepatocytes, bile ductular cells, and HSCs. Quiescent HSCs respond to these ligands and transform into myofibroblasts and start producing excess ECM (63). Hh inhibitors such as cyclopamine and vismodegib (GDC-0449) have been found effective in treating early stage liver fibrosis (Fig. 2) in common bile duct ligated (CBDL) rats (39,40). Realizing the role of activated HSCs in ECM production and liver fibrosis, various strategies to inhibit their activation or promote apoptosis are being explored. Small molecules such as silymarin, phosphatidylcholine, vitamin E, and S-adenosyl-L-methionine inhibit HSC activation/ proliferation. Inhibiting key signal transduction pathways involved in liver fibrogenesis can also be used to treat liver fibrosis. Disrupting TGF-β pathway using gene therapy results in decreased collagen production and retard the fibrosis progression.

Certain growth factors such as insulin like growth factor (IGF), hepatocyte growth factor (HGF), and cardiotrophin exert their antifibrotic effect by preventing hepatocyte death by decreasing oxidative stress. Other treatments such as the phosphodiesterase inhibitor (pentoxifylline), Na^+/H^+ pump inhibitor (amiloride), and Ras antagonist (S-farnesylthiosalicylic acid) have also been proven to be effective in rodent model. PPAR-γ agonist keeps HSCs in a quiescent state by blocking the profibrotic effects in the liver. Loss of these receptors is reported and leads to trans-differentiation of HSCs from the retinoid storing state to the ECM-producing myofibroblasts. Thiazolidinediones are PPAR-γ agonist that exerts beneficial effects in experimental liver fibrosis. Angiotensin-II mediates and exacerbates liver fibrosis through HSC activation and by stimulating TGF-β1 via angiotensin type-1 (AT_1) receptors. Inhibition of the renin-angiotensin system can also be used as a strategy to treat liver fibrosis. Blockade of endothelin-1 type A receptors and administration of vasodilators (prostaglandin E2 and nitric oxide donors) has also been reported to exert antifibrotic activity in rodents (64,65).

Inflammation has a major contribution in the progression of liver fibrosis; therefore various anti-inflammatory drugs are being explored to retard the progression of liver fibrosis. Mainly, corticosteroids, colchicine, and vitamin D are shown to be effective in treating hepatic fibrosis in animal models (41). Due to excess deposition of ECM, antifibrotic drugs are unable to efficiently taken up by the liver and activated HSCs and may produce unwanted side effects. 5-methyl-1-phenylpyridine-2-one (commonly known as pirfenidone) is a small molecule that has well-established antifibrotic and anti-inflammatory properties. Pirfenidone has shown to reduce the production of fibrogenic mediators such as TGF-β and inflammatory cytokines such as TNF-α and IL-1β in a variety of fibrotic animal models (48).

Ursodeoxycholic acid (UDCA), also known as ursodiol, is one of the secondary bile acids which lower the progression

Table I Current Anti-fibrotic Treatments

Small drug molecules	Mechanism	Limitations	References
Cyclopamine, GDC-0449	Hh inhibitor, inhibits HSC activation	Short half-life	(39,40)
Colchicine	Anti-inflammatory	Highly toxic	(41,42)
Silymarin	Decrease NF-kβ activity, inhibit HSC activation/ proliferation	Short half-life, low bio availability	(43,44)
Rosiglitazone	PPAR-γ agonist, anti-inflammatory	Poor water solubility, Short half life	(45)
Pentoxyfyline	phosphodiesterase (PDE) inhibitor, antioxidant, anti-inflammatory	Poor bio-availability	(46,47),
Pirfenidone	Decrease TGF-β1	Short half-life	(48)
Amiloride	Na^+/H^+ pump inhibitor, PDGFR inhibitor, decrease oxidative stress		(49,50)
Imatinib	PDGFR inhibitor	Poor water solubility	(51,52)
Ursodeoxycholic acid	Reduce cytotoxicity bile acids, and reduce inflammatory cytokine	Not consistent in treating fibrosis	(53–55)
Farnesylthiosalicylic acid	RAS inhibitor		(56)
Cytokines			
Cardiotrophin-1	reduced hepatocellular injury and oxidative stress		(57)
Antibodies	Neutralize TIMP-1	Poor *in vivo* delivery	(58)
	Neutralize TGF-β1	Stability, poor *in vivo* delivery	(59)
	Neutralize PDGF-β		(60)
Peptides and hormones			
P11 and P12	Decrease TGF-β1 activity	Poor *in vivo* delivery	(61)
Relaxin	Decrease TIMP-1	Poor *in vivo* delivery	(62)

rate of liver fibrosis in early stage. UDCA binds to hepatocytes and exerts cytoprotective effect and reduces local inflammation. It is not an anti-fibrotic agent in the liver; rather it may impede progression of fibrosis in primary biliary

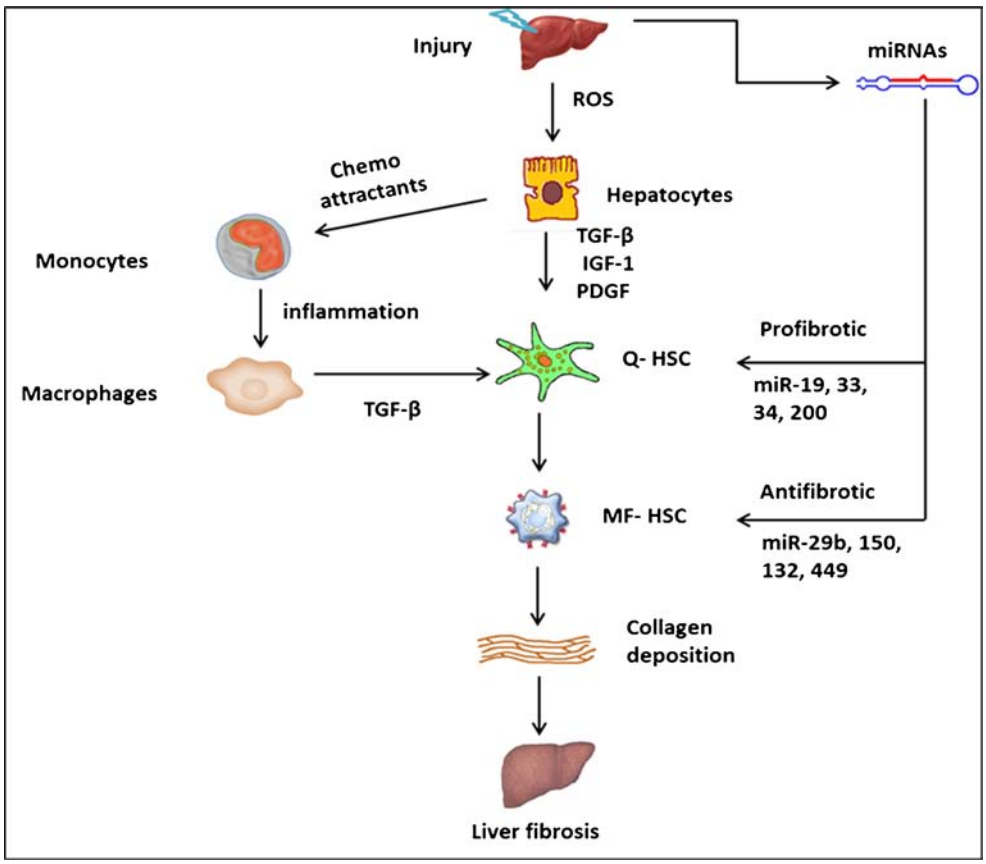

Fig. 1 Pathology of liver fibrosis and role of miRNAs.

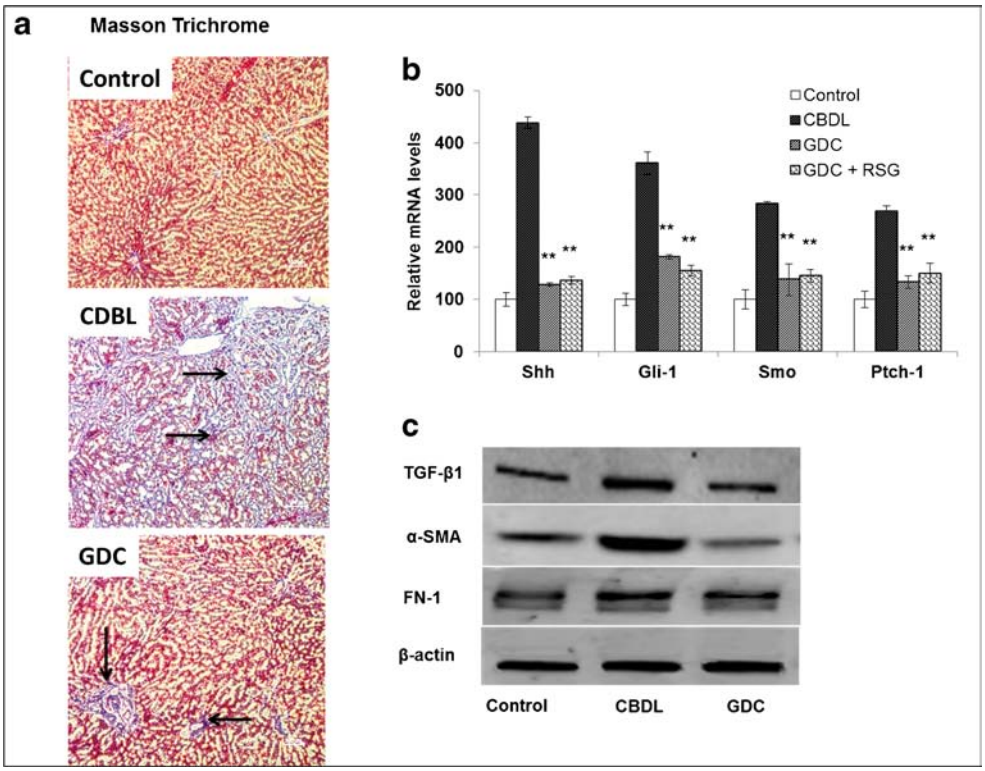

Fig. 2 Effect of treatment with nanoparticles containing GDC-0449 on liver fibrosis in experimental animals. (**a**) Masson's trichrome staining shows increased collagen deposition in CBDL rats which is reduced after systemic treatment with GDC nanoparticles. (**b** and **c**) reduction in expression levels of Hh ligands after GDC nanoparticle treatment.

cirrhosis via effects on biliary ductal inflammation. A peptide hormone relaxin is known to decrease TIMP-1 and TIMP-2 expression in HSCs (62). Decreased expression of TIMP results in degradation of ECM and reduction in deposition of interstitial collagen. PDGF-β is a profibrotic stimulus and potential inducer of HSC trans-differentiation. PDGF-β overexpression causes liver fibrosis via TGF-β1 independent mechanism. Small molecules imatinib and nilotinib block the tyrosine kinase activity of PDGF receptors (51,52). These molecules also bind to the ATP-binding pocket of Abelson kinase (c-Abl) which is an important downstream signaling molecule of TGF-β signaling thus blocks two major profibrotic pathways.

A number of approaches and drug molecules have been applied to treat liver fibrosis. The main limitations of these drugs are either low uptake by activated HSCs or unwanted side effects. Targeted delivery to HSCs by different researchers has generated some encouraging results, but that has to be optimized to be an approved therapy.

Antibodies

Antibodies (Abs) against specific fibrosis causing disease have been studied by various researchers. Abs against TIMP-1, TGF-β1, and PDGF-β has been studied for reversing liver fibrosis in animal models (58–60). Simtuzumab is a humanized monoclonal antifibrotic antibody. It binds to lysyl oxidase-like 2 (LOXL-2) enzyme, which promotes crosslinking of type-1 collagen. This Ab acts as an immunomodulator for treating liver fibrosis and is being currently tested in clinical trials (66).

Antisense Oligodeoxynucleotides

Antisense ODNs inhibit gene expression at post-transcriptional levels. ODNs bind to their target mRNA by the reverse complementarity and inhibit translation either by steric blocking of mRNA sequences important for translation or degrade mRNA by RNase H, which is an endonuclease present in abundance in cytoplasm and cleaves only mRNA component of RNA:DNA hybrids. Therefore, each ODN can hybridize and degrade multiple RNA molecules (67). Efficiency of ODNs to hybrid with mRNA depends on their physicochemical and thermodynamic properties. Minimum 12–15 bases are required to make stable duplex with mRNA, and for the practical purpose ODNs are typically synthesized from 13 to 35 nucleotides (nt) in length. Chemical modification of ODNs can be used for enhancing their stability, efficiency, and target specificity. Phosphorothioated (PS) ODNs show enhanced stability without affecting RNase H activation efficiency. Modification with methylphosphonate (MP) significantly reduces enzyme activation. These observations lead to synthesize ODNs containing nuclease-resistant MP modifications at the 3′ and 5′ ends while six to eight unmodified or PS-modified linkages in the middle portion (68,69).

Antigene Therapy

ODNs can form triple helices with genomic DNA and inhibit gene expression at transcription level, which is advantageous as it blocks repopulation of mRNA. Furthermore, mRNA can have variant isoforms and inhibition of translation by ODNs may not be fully efficient. Triplex formation is sequence-specific and polypurine:polypyrimidine portion of DNA favors stable hybrid formation. Triplex forming oligonucleotides (TFOs) are of typically 10–30 nt in length and bind to the major groove of duplex DNA. TFO can be both polypurine or polypyrimidine molecules, and they bind to the purine-rich strand of their target. Inhibition of gene transcription through TFO depends on its residence time on its target sequence and its stability against nuclease (70). Some of the limitations of TFO are its inability to target gene sequence in condensed chromatin structure, and the need of TFO translocation to the nucleus. TFO approach has been used and shown to treat liver fibrosis in our laboratory. Panakanti *et al.* used TFO against collagen type α1 (I) and demonstrated inhibition of liver fibrosis induced by common bile duct ligation (CBDL) in rats (71). For site-specific delivery of TFOs to HSCs after systemic administration, Yang *et al.* conjugated mannose 6-phosphate (M6P) to poly(*N*-(2-hydroxypropyl) methacrylamide (HPMA) polymer and then to TFO via GFLG linker. Compared to free TFOs, M6P conjugated TFO significantly accumulated in the liver and was mainly taken up by HSCs having upregulated M6P receptors in liver fibrotic animals (72).

RNA interference (RNAi) is a specific regulatory pathway that results in gene silencing at post-transcriptional level (73). Mechanistically, double-stranded small RNAs (siRNA) get incorporated into the RNA-induced silencing complex (RISC), where guide strand is used as a template to recognize complementary or near-complementary region of target mRNA by RISC. When RISC finds its complementary strand gene expression is suppressed either by degrading or blocking translation of target mRNA. Two proteins, Dicers and Argonaute (Ago) have been identified as essential for RNAi or as components of the RISC. Dicers are ~200 kDa proteins complex and contain ATPase/RNA helicase, and Piwi–Ago–Zwille (PAZ) domains, two catalytic RNase III domains, and a C-terminal dsRNA binding domain (dsRBD). Among the two Dcrs, Dcr-1 process precursor molecules into siRNAs and miRNAs, while Dcr-2 function in downstream steps of RNAi. Ago is a ~100 kDa protein consistently found in all RISC and microRNA ribonucleoprotein complex (miRNP) has characteristic PAZ and P-element induced wimpy testis (PIWI) domains. Among all Ago proteins, only Ago2 is believed to be responsible for mRNA degradation and gene silencing effect. Ago2 has three functional domains, PAZ, middle (MID) and PIWI. PIWI has an RNase H domain and performs the cleavage of the target mRNA substrates. Guide-strand 5′ monophosphate group tucks in between the MID and PIWI domains. Meanwhile, PAZ domain specifically recognizes the guide-strand 3′ dinucleotide overhang. This positioning exposes the guide-strand's "seed region" to complementary target mRNA for base pairing. Next base pairing at 10–11 nt correctly orients the scissile phosphate between them for cleavage by PIWI domain (74,75).

siRNA is a chemically synthesized short (usually 21 bp), double-stranded RNA having well-defined structure with a phosphorylated 5′ end and hydroxylated 3′ ends with two overhanging nt. siRNA get incorporated into RISC and its guide-strand binds with perfect complementary to the target mRNA and degrade it. Within the RISC, mRNA cleavage ATP independent and specific between residues base paired to nt 10 and 11 of the siRNA. After degradation, cleaved mRNA is released, and a new cycle of target mRNA degradation is started using the same guide strand in the RISC. Therefore, one siRNA molecule once associated with RISC can degrade several molecules of target mRNAs. Despite very attractive traits, there are some inherent problems associated with siRNA delivery: (1) being negatively charged macromolecules poor penetration into the cell membrane; (2) being a nucleotide, its highly susceptible to degradation by RNases; (3) they can cause sequence dependent/independent off target effects; (4) ineffective where target mRNA has mutated sequence; and (5) silencing effect is of short duration (76). To improve stability and reduce off-targeting siRNAs can be covalently linked with functional or targeting molecules via either cleavable or non-cleavable bond. Zhu *et al.* conjugated 3′-sense strand of TGF-β1 siRNA to M6P and galactose via poly(ethylene glycol) (PEG) spacer to enhance cellular uptake by HSCs and hepatocytes, respectively (77).

Short hairpin RNA (shRNA) makes a tight hairpin turn and has been developed as an alternative RNAi molecule. An external expression vector bearing a short double-stranded DNA transcribes shRNA in the nucleus. Depending on the promoter driving their expression, shRNAs are transcribed by either RNA polymerase II or III. The shRNA transcript is then processed by Drosha, an RNase III endonuclease, which results in pre-shRNA and exported to the cytoplasm, wherein it is processed by Dicer (another RNase III enzyme) and forms siRNA, which is then incorporated into RISC. Depending on the type of expression vector shRNA can be constantly produced in the host cells leading to more durable gene silencing compared to siRNA (78). Moreover, a shRNA technique is cost effective as an expression vector cost less than the bulk manufacturing of siRNA. Cheng *et al.* used two siRNAs targeting 769 and 1033 start sites of rat TGF-β1 mRNA and then converted into shRNA by cloning to enhance TGF-β1 gene silencing (79).

MICRORNAS

miRNAs are small regulatory non-coding RNA molecules of approximately 21–23 nt in length, which can modulate gene expression at post transcriptional level. miRNAs are known to affect cell proliferation, apoptosis, inflammation, oxidative stress,

and metabolism. Aberrant miRNA expression can be a key pathogenic factor in liver fibrosis. Therefore, there is an urgent need to understand the mechanisms involved in miRNA dysregulation to treat liver diseases (80). Unlike siRNAs, miRNA do not require perfect base pairing of the seed region with mRNA for its degradation, and therefore, one miRNA can target several mRNAs. This is advantageous in term of efficiency especially when target mRNA has mutation or alternative isoforms, but at the same time it can induce off target effects. This section will discuss the biogenesis, target prediction, possible chemical modification and some important deregulated miRNA in liver fibrosis (81).

Biogenesis and Mechanism of Action of miRNAs

More than 4000 miRNAs are known so far in the genomes of over 80 species. Multiple steps are involved in the process from 200 nt long primary transcripts translated from miRNA genes to form mature 19–25 nt long miRNA. Majority of primary transcripts of miRNA originate from splicing of introns of other protein-coding genes, but miRNAs from independent gene are also known. The primary transcript is in the form of hairpin conformation and called pri-miRNAs. This pri-miRNA contains a 5-cap structure and a poly-A tail similar to mRNA. These structures are recognized by RNAs III (Drosha) and cleaved into 70–100 nucleotide precursor miRNA called pre-miRNAs (82). Pre-miRNA transcripts are recognized by Exportin-5 (Exp5) in the nucleus and transported to the cytoplasm. Exp5 is a nucleocytoplasmic factor and a member of karyopherin family that require GTP-bound form of Ran GTPase in RNA binding and nuclear export. Exp5 recognizes double-stranded RNA binding domain and double-stranded RNAs as well as RanGTP, which is present in high levels in the nucleus. This trimetic complex of RanGTP: Exp5: pre-miRNA then translocate in the cytoplasm, where RNA and Ran are released upon GTP hydrolysis. Exp5 is then recycled to the nucleus by diffusion through the nuclear pore complex for another round of transport. Expression level of Exp5 is a key factor and can affect the RNA-mediated gene silencing. In cytoplasmic Dicer enzyme recognizes pre-miRNA and cleaves the hairpin loop to produce mature miRNA duplex. Depending on the thermodynamic stability of the ends of the duplex, one strand functions as a mature miRNA and another strand is degraded. Within the original duplex, a strand with hydrogen bonding at its 5′ end is stabilized and selectively becomes the mature miRNA. Usually, miRNA strand having U residue at 5′ end forms less stable base pair (U:G or U:A) compared to G:C base pair and most likely the selection criterion for mature miRNA (83).

Despite various proposed modes of action, most studied and recognized mode of gene silencing by miRNAs is by inhibiting translation. Mature miRNA strand incorporates into RISC. RISC complex recognizes its target mRNA, based on complementarity of different degrees as described. Perfect complementarity between a miRNA-target mRNA pair leads to mRNA degradation, whereas imperfection in pairing leads to several alternative mechanisms of repression including:

1. Promoting ribosome drop-off
2. Degradation of the nascent polypeptide
3. Sequestration of mRNAs in P-bodies or stress granules
4. Inhibition of translation initiation
5. Inhibition of translation at a step after translation initiation, and
6. Deadenylation of mRNA

In animals, miRNAs repress target protein expression by repressing mRNA translation either at the initiation stage or during the elongation phase (84).

Thermodynamic Properties of miRNAs

Thermodynamic stability of miRNAs correlates with their ability to induce RNA interference. In miRNA molecule, pair mismatches, gaps, and bulges generate low internal energy and important for duplex unwinding, strand selection and RNAi pathway. When pre-miRNA is processed to mature miRNA, strand instability and sequence asymmetry play a major contribution in the selection of sense strand and entry into RISC. miRNA loading into Ago is divided into two different steps, physical association and activation. Activation is the rate-limiting step of unwinding process and facilitated by thermodynamic instability rather than structure of the duplexes (85). Normally, the first base pair of the 5′ antisense terminus is most-destabilizing elements within the pre-miRNA precursors and is required for the RNAi processing. On average, the 5′ region of the antisense strand is less stable than the 5′ terminus of the sense strand. While unwinding step by RISC helicase, strand with 5′ low internal stability is selectively processed and retained by the RISC. Thus, selective modification of the guide strand utilized for loading into RISC for higher efficacy. Elmen *et al.* designed locked nucleic acid (LNA) at 5′ terminus of siRNA sense strand and increased loading of the antisense strand (86). Nucleotides 2–8 from 5′ terminal (known as a seed region) overlap with the 5′-end. Gene silencing efficiency of miRNA is mediated both by stability between seed and target pair and stability of 5′ end. Seed-target duplex stability is the function of the nucleotide sequence, whereas the stability in the 5′-terminal is a structural feature (87). miRNA gene silencing can be correlated, with the correlation score 'miScore' and calculated as follows:

$$\text{miScore} = T_{m2-8} - 0.5 \text{ X } miT_{m\ 1-5} \quad (1)$$

Where, $T_{m\ 2-8}$ is the melting temperature (T_m) of the seed-target duplex (positions 2–8), miT_{m1-5} is T_m value of positions 1–5 of 5′-end of miRNA duplex and 0.5 is a multiplier

coefficient as the contribution of $miT_{m\ 1–5}$ might be half of $T_{m\ 2–8}$. Thus, for efficient gene silencing miRNA should have an unstable 5′-end 5-bp duplex and a stable 7-bp seed-target duplex.

Antisense strand thermodynamic stability in the position 9–14 from 5′ terminal also affects the functional ability of miRNA. For efficient gene silencing, multiple turnovers of the target mRNA release from RISC is a necessary step. Antisense strand 9–14 positions bases have low internal stability compared to sense strand bases. Low internal stability of this region in the stands may facilitate cleavage of target mRNA for release, and allows the RISC to find the next target mRNA (88). However, the thermodynamic stability between seed region and target mRNA also reduces the off-target effects (89). G:U base pair in the seed region of miRNA are known to decrease miRNA silencing efficacy significantly, although they are thermodynamically similar to A:U base pair. G:U wobble base pairs within miRNA behave like mismatch and reduce RISC loading and unwinding (90). Sequence analyses of miRNAs from different species show that a U or an A at the 5′ position of the antisense strand. The reason is simply that the mid domain of Ago2 has a greater affinity toward ATP or UTP than CMP or GMP. Therefore, A/U nucleotide 5′ terminal influences the incorporation of the guide strand into RISC strongly (91).

Target Prediction of miRNA

Target gene identification of miRNAs is critical for functional analysis. Biological studies to predict targets are slow and complicated, because of multiple targeting capabilities. For fast prediction of miRNA functions/targets, numerous bioinformatic methods are being used. Some of these algorithms including miRanda, TargetScan, Pictar, TargetBoost, and PITA are the gold standard before starting any biological assay.

Within the RISC, miRNAs form specific base pairing with mRNA, which form the basis for in target recognition algorithms. Normally, 5′ region base pairing of the miRNA is considered more important compared to 3′ region. Bases of miRNA seed region form a perfect complement with 3′ region of mRNA. Most of the miRNA target prediction programs use the same general principles in the development of their algorithm and search for targets in the 3′-UTR region of various mRNAs. Base pairing at 3′ region of miRNA is considered less important for target prediction (92,93).

The RNAfold program predicts the minimum energy of secondary structures and thus pairing probabilities, and the RNAduplex program predicts possible hybridization sites within the duplex. One another fact these algorithms apply in target predictions is the degree of sequence conservation. It is believed that if 3′-UTR sequences of miRNA is conserved in orthologous species, their targets are also conserved. This phenomenon provides a useful filter in target prediction (94,95).

To begin target scanning one of the most popular algorithms miRanda first retrieves the mRNA's 3′-UTR sequences and finds out maximum complementarity alignments with 5′ UTR of mature miRNA. G:C and A:T are assigned + 5 score, while G:U is given +2. The gap opening is considered unsuitable for complementarity and given negative score (−8) and gap elongation if any is assigned −2. The complementarity scores up to certain regions depending on program and parameters from the miRNA 5′ end is multiplied by scaling factor of 2. No mismatching at positions 2 to 4 at the 5′ end is tolerated, and less than five mismatches between positions 3–12; and in the last five positions less than two mismatches of the alignment are considered for potential target. Based on the alignment and matching scores, algorithm creates a ranking of all non-overlapping hybridization down to some cut-off values. The key difference between most of the algorithms is the difference in weightage score of certain positions in the alignment (96). Another well-established algorithm TargetScan emphasizes on the seed pairing and scan for matching between 2 and 8 positions from the 5′ end of the miRNA for perfect Watson-Crick complementarity (G:C or A:U) (97).

Thermodynamic properties of the miRNA:mRNA duplex *in vivo* is another important factor which estimates free energy of RNA secondary structure. The stability of base-pairing depends on the G:C content of the nucleic acid sequence. However, vertical stacking of bases in a sequence-dependent manner also contributes to helix stability and thermodynamic properties of the sequence and is the function of its structure (98). Stability of the predicted miRNA:mRNA duplex, is estimated by calculating free energy of the duplex and checked against a threshold value, usually Gibb's free energy (G)<−17 kcal/mol or G<0–14 kcal/mol depending on the type of algorithm being used in miRanda. In TargetScan calculated Z score is used to rank the possible target for the miRNA:

$$Z = \sum_{K=1}^{n} e^{-G_k - /T} \tag{2}$$

Where,

n is the number of seed matches in the UTR
G_K is the free energy of the duplex for the k^{th} target site
T is the relative weighting of UTRs binding affinity and the total number of sites.

Identification of orthologous 3′ UTR mRNA sequences and checking if the potential miRNA target site is conserved in other species is another criterion of target prediction. In this method, conserved target site between orthologous species is

predicted and compared to the reference species. For example, if we want to predict target in human genome, the alignment of the target sites are generated in UTR of human with miRNA UTR of mouse or rat. The complementary pair's position of target sites between species must fall within ±10 residues in the aligned 3′ UTRs. Conserved target sites sequence between two species must also meet a certain threshold value (≥90% used for analysis in humans) (Fig. 3).

Based on the alignment score, free energy secondarily and after passing the conservation filter, predicted target sites for each miRNA are sorted. If more than single miRNA shows targetability, miRNA with highest complementary score and lowest free energy is considered appropriate. An important drawback of using this prediction is that miRNAs are present in different concentrations at different cell cycles. Many miRNAs may have single binding sites, but do not compete because they are never expressed at the same time, thus can result in false negative prediction. Algorithm PicTar considers co-expressed miRNAs for a queried 3′ UTR, because gene repression will be affected in direct relation with a number of binding miRNAs available at that time (99).

Seed region of miRNA is short and miRNA–mRNA duplexes may not be entirely complementary, which increases the complexity of gene regulation by miRNA and decrease the accuracy of target prediction. There are some reported cases where miRNAs regulate target gene independent of base pairing in the seed region. Algorithm program determines duplex stability by calculating the threshold free energy. The value of appropriate thresholds free energy is difficult to determine because it differs from organisms to organisms. At present, we have limited data sets of known free energy of miRNA–mRNA duplexes. Moreover, stable binding does not always represent the target genes.

DYSREGULATION OF MIRNAS IN LIVER FIBROSIS

Aberrant expression of several miRNAs has recently been implicated in liver fibrosis and carcinogenesis. Various genes including CTGF, TGF-β1, PDGF-β and TIMPs are markedly upregulated during hepatic fibrogenesis (Table II). All of these genes can be manipulated by different miRNAs, and therefore disease progression can be reversed.

Two different approaches are being used for delivery and targeting of miRNAs to different cells and organs. First is to increase the level of miRNAs in the target cells using miRNA mimic, and the second approach is to block upregulated miRNAs by using anti-miRNA, commonly known as antimiR.

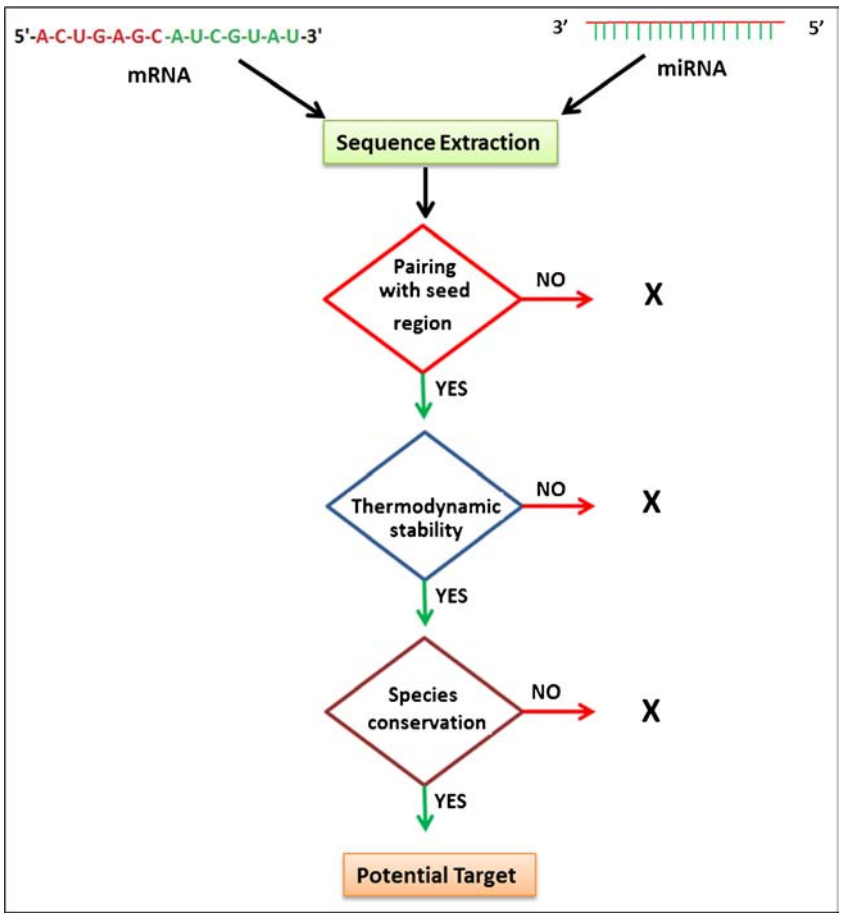

Fig. 3 Representative steps used for miRNA target prediction by computational analysis.

Table II Various Anti-fibrotic and Pro-fibrotic miRNAs Involved in Liver Fibrosis and Their Targets

Anti-fibrotic miRNA	Target gene	Reference
miR-29b	Akt, SP-1, collagen	(100)
miR-150	C-myb, collagen, α-SMA	(101)
miR132	MeCP2	(102)
miR- 449	YKL40, NOTCH-1	(103)
miR-122	P4HA1	(104)
miR-335	TNC	(105)
miR-15b and miR-16	Bcl-2	(106)
miR-126	CRK	(107)
miR-19b, miR-101b	TGF-β	(108,109)
miR-449a,miR-107	IL-6R, JAK-1	(110)
miR-200a	Nrf2	(111)
miR-214	CCN2	(112)
miR-483	PDGF-β and TIMP2	(113)
miR-195	cyclin E1	(114)
Profibrotic miRNA		
miR-33a	PPAR-α	(115)
miR-200c	FAP-1	(116)
miR-34a	ACSL1	(117)
miR-27a, 27b	RXR gene	(118)
miR-21	PTEN/Akt signaling	(119)
miR-221/222	CDKN1B, PPP2R2A	(120,121)
miR-93 and miR-106b	c-Myc	(122)
miR-181b	p27	(123)
miR-615	IGF-II	(124)

Anti-fibrotic miRNA

This approach uses synthetic ODNs that enhance the expression of a specific miRNA which possesses anti-fibrotic properties, but its expression is downregulated upon liver fibrosis. After entering the cells, one strand of this anti-fibrotic miRNA mimic is stabilized and get associated within the miRISC complex and inhibit the target mRNAs. Liver fibrosis leads to downregulation of several miRNAs which target several pro-fibrotic genes post-transcription level, and their overexpression decreases liver fibrosis (Fig. 4).

miR-29b

Several recent studies have shown that miR-29 levels get significantly decreased by NF-kB in liver fibrosis, and its downregulation is inversely related to the activation of HSCs. There are three members in its family: miR-29a, b, and c. all of these target fibrosis associated key genes such as Akt, SP-1, laminin, collagen I and IV. Some studies have shown that replenishment of miR-29 family members leads to a significant decrease in collagen I and IV as well as phospho-FADD, cleaved caspase-8, 3, Bax, Bcl-2, PARP, and NF-κB expression. These results indicate that miR-29a can decrease cholestatic liver injury and fibrosis (101).

miR-150

TGF-β1 suppresses miR-150, which target c-myb expression and inhibits the activation of HSCs and LX-2. c-myb is a proto-oncogene, and its activity level is increased in activated HSCs. In the influence of c-myb, HSCs gets activated and induces both collagen-I and α-SMA (125). Overexpression of miR-150 in HSCs resulted in the inhibition of cell proliferation and reduction in ECM proteins and α-SMA. In another study, miR-150 was shown to target and downregulate ECM protein transcription factor Sp-1, without affecting Sp-1's upstream mediators, such as Smad2 and p-Smad2 (101).

miR132

MeCP2 is a fibrosis promoting gene and regulates transdifferentiation of HSCs by inhibiting PPARγ transcription in hepatic fibrosis. miR-132 binds 3′-UTR of MeCP2 and inhibits its translation, and its expression is lost in HSCs upon activation. When miR-132 was incubated with myofibroblasts, it represses MeCP2 gene and hence activation (102).

miR-122

miR-122 is a liver-specific and most abundant miRNA found in the adult human liver and is downregulated in activated HSCs and fibrotic liver. miR-122 targets prolyl 4-hydroxylase (P4HA1) which is an enzyme that regulates the maturation of collagen. Overexpression of miR-122 inhibits the proliferation and activation of HSCs and collagen production (104).

miR-449

miR-449a is an anti-fibrotic miRNA, and its level is downregulated in HCV infection mediated liver fibrosis. miR-449a plays as an anti-fibrotic role by targeting YKL40, which is upregulated in fibrosis. HCV infection upregulates TNF-α, which eventually increases NF-κB activity leading to increased inflammatory response and promote cell proliferation in the liver. NOTCH-1 and YKL40 help TNF-α in induction and nuclear retention of NF-κB. Increased NF-κB transcription factor leads to increased inflammatory response and promotes cell proliferation. Overexpression of miR-449a resulted in downregulation of NOTCH-1, and thus decreases overall progression of liver fibrosis (103).

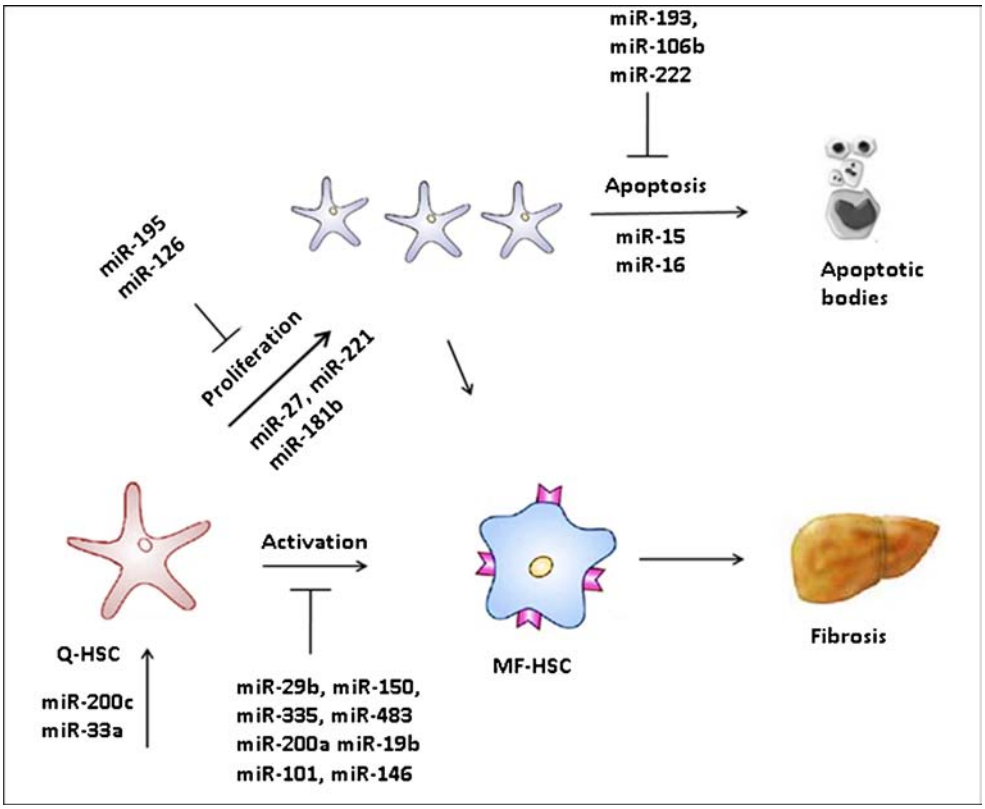

Fig. 4 Role of various miRNA in progression as well as attenuation of liver fibrosis.

miR-335

miR-335 is downregulated in activated HSCs compared to non-activated control. miR-335 targets tenascin-C (TN-C), extracellular matrix glycoprotein upregulated in liver fibrosis and promotes HSC migration and activation via integrin β1. Overexpression of miR-335 resulted in a decreased α-SMA and collagen type-I level by decreasing the expression of TNC (105).

miR-126

v-crk avian sarcoma virus CT10 (CRK) expression increases during HSCs activation and has a key role in signaling pathways regulating cell adhesion, proliferation, and migration (126). Gong *et al.* confirmed that due to overexpression of CRK, there was low expression of miR-126 in activated HSCs. miR-126 directly targets 3′-UTR of CRK, and controls the cell shape, cell to cell adhesion and locomotion of HSCs. Overexpression of miR-126 after transduction with lentiviral vector encoding miR-126 decreased α-SMA and collagen α(1) expression. Although miR-126 decreases HSC migration, it did not affect their proliferation (107).

miR-19b, miR-101 and miR-146a

Members of miR-17-92 family and miR-106b-25 clusters play an important role in liver fibrosis. Ashley M. Lakner reported miR-17-92 cluster (19a, 19b, 92a) expression levels were significantly low in activated HSCs upon transfection with miR-19b, which also resulted in significant reduction in TGF-β receptor 2 (TGFBRII) expression. miR-19b also decreased SMAD3 expression post transfection and inhibited TGF-β signaling. The overall impact of miR-19b regulated HSC transdifferentiation and decreased myofibroblast marker α-SMA expression (108). In a study by Tu *et al.*, significant down-regulation of miR-101a and miR-101b was observed in the carbon tetrachloride (CCl_4)-induced fibrotic liver. Using computational analysis, TGF-βRI mRNA and Krueppel-like factor 6 (KLF6) mRNA were found two conserved targets of miR-101. miR-101 delivery through lentivirus suppressed liver fibrosis by reducing target genes along with collagen, vimantin, SMA and snail (109). He *et al.* investigated antifibrotic role of miR-146a in HSCs and demonstrated that miR-146a targets SMAD4 and therefore, decreases profibrotic TGF-β pathway. miR-146a also reduces HSC proliferation and induces HSC apoptosis (127).

miR 107 and miR-449a

In a study, Sharma *et al.* reported down-regulation of miR-107 and miR-449a after HCV infection in patients. They demonstrated that miR-449a and miR-107 regulate IL-6-mediated chemokine (C-C motif) ligand 2 (CCL2) expression and STAT3 phosphorylation by targeting IL-6R and JAK1.

CCL2 expression in HSCs. This regulates HSC chemotaxis to the site of injury and promotes HCV-induced liver fibrosis. When hepatocytes were transfected with a construct containing IL-6R UTR or JAK1 UTR along with the vector expressing miR-449a or miR-107, reduced expression of IL-6R and JAK1 was observed at RNA and protein levels (110).

miR-200a

Oxidative stress is known to involve in the activation of HSCs, ECM production and induction of liver fibrosis. Nuclear factor-erythroid-2-related factor 2 (Nrf2) regulate transcripts of several antioxidant enzymes including NQO1 (NAD(P) H-quinone oxidoreductase, glutathione S-transferases (GSTs), and glucuronosyl transferases Kelch-like ECH-associated protein 1 (Keap1) negatively regulate (Nrf2) level and thus contribute to oxidative stress during liver fibrosis. In normal liver, miR-200 family target Keap-1 factor, and is often found down-regulated in liver fibrosis. Yang *et al.* demonstrated that miR-200a down-regulates Keap1 and ultimately results in Nrf2-dependent antioxidant pathways active in HSCs. miR-200a also decreased TGF-β induced α-SMA expression in HSCs (111).

miR-214

In fibrotic liver, connective tissue growth factor (CCN2) is activated by TGF-β1, and it stimulates connective tissue cell proliferation and ECM synthesis (12787797). In HSCs, CCN2 levels are high during liver fibrosis, and in inverse relation with miR-214. miR-214 is transferred to neighboring HSCs or hepatocytes via exosomes to keep checking on CCN2, and its level is downregulated during fibrosis. Chen *et al.* when transfected pre-activated mouse HSCs with pLmiR-214, decreased CCN2 mRNA and protein levels along with its downstream markers such as α-SMA or collagen α(I) were observed. Although collagen and SMA mRNA is not the direct target of miR-214, their levels decreased in CCN2-dependent manner (112).

miR-483

miR-483 is down-regulated in HSCs during their activation in liver fibrosis. Fuyuan *et al.* determined the role of miR-483 *in vivo* using pre-miR-483 overexpressing transgenic mice. Compared to normal mice, pre-miR-483 overexpressing transgenic mice inhibited CCl_4-induced liver fibrosis and showed low expression of collagen and α-SMA. In LX-2 cells, miR-483 decreased TGF-β induced PDGF-β and TIMP2 expression *in vitro* and in CCl_4 induced fibrotic mouse model. Both PDGF-β and TIMP2 are the direct targets of miR-483. Interestingly, overexpression of miR-483 induced carcinogenesis in mouse liver by suppressing cytokine signaling 3 (Socs3) (113).

miR-195

IFNs are immunomodulatory cytokines with antiviral, and cell growth suppression effects. IFN-α is known to have antifibrotic properties in the liver (128). Sekiya *et al.* reported that IFN-β induced miR-195 expression, and otherwise has low expression in normal mouse HSCs. miR-195 induction reduces cyclin E1 expression levels while increases p21, thereby inhibits cell proliferation by delaying their G1 to S phase. The direct interaction between miR-195 and cyclin E1 was studied by luciferase reporter activity by cloning two miR-195 target sites (497 bp) of the cyclin E1 3′-UTR from LX-2 cells. There was significant decrease in luciferase activity after transfection of LX-2 cells with miR-195 precursor. These results show the antifibrotic role of miR-195 and explain the antifibrotic mechanism of IFNs (114).

miR-15b and miR-16

Both of miR-15b and miR-16 are downregulated in HSCs upon activation. These miRNAs downregulate Bcl-2 gene expression and induce apoptosis of activated HSCs (106).

Pro-fibrotic miRNAs

Aberrantly up-regulated miRNA can cause significant change in critical biological pathways. The inhibition of target miRNA is based on specific annealing with synthetic complementary sequence known as antagomir or anti-miR. An ant-miR is usually complementary to the specific miRNA target with either mispairing at Ago2 cleavage site or some base modification to inhibit Ago2 cleavage. Studies by various groups have shown that modulating miRNA by anti-miR can effectively regulate biological process in liver fibrosis and can produce beneficial therapeutic effects.

miR-33a

miR-33a with its host gene sterol regulatory element-binding protein 2 (SREBP2) is overexpressed in activated Lx-2 cells than in quiescent cells. PPAR-α is one of the predicted targets of miR-33a using bioinformatics analysis. Anti-miR-33a significantly increases target gene PPAR-α at mRNA and protein levels, suggesting that miR-33a modulates HSC functions by targeting PPAR-α. Also, miR-33a activates PI3K/Akt pathway and induces expression of ECM through HSCs (115).

miR-200c

HCV infection results in the up-regulation of miR-200c, which is in direct correlation with increasing growth factors and hormones. HCV infection results in the inhibition of Fas

associated phosphatase 1 (FAP-1) that regulates the function of oncogenic SRC kinase and upregulates different growth factors. Increased level of miR-200c decreases FAP-1 expression, and thus promotes fibrosis by modulating growth factor signaling Src activation (116).

miR-34a

miR-34a is upregulated in dinitrosamine (DMN) induced liver fibrosis. It targets and decreases the levels of acyl-CoA synthetase long chain member 1 (ACSL1) gene. ACSL1 regulates hepatocellular lipid metabolism and its low-level results in accumulation of high intracellular fat, which eventually leads to apoptosis, chronic liver injury and nonalcoholic liver fibrosis (117).

miR-27a, 27b

In one of the studies, miR-27a and 27b are found upregulated in HSCs. miR-27a, b directly target retenoid receptor X (RXR) gene and downregulate its expression in fibrosis. RXRα is involved in multiple signaling pathways related to cell proliferation and differentiation by forming a heterodimer with PPAR-γ. Transfection with anti-miR-27 partly reverses the phenotype of activated HSCs (118).

miR-21

miR-21 is upregulated in various organ fibrosis including liver fibrosis and plays an important role in PDGF-BB induced liver fibrosis. In a recent study, miR-21 was found to regulate PTEN/AKt pathway and promote liver fibrosis (119). miR-21 also targets Smad7, a negative regulator of TGF-β signaling. Overexpression of miR-21 enhances TGF-β signaling and leads to increased fibrogenesis (129).

miR-222

Ogawa *et al.* reported upregulation of miR-221/222 in human fibrotic patients and rodent fibrotic model. There was direct correlation between miR-221/222 expression level with that of collagen-1. miR-222 was found to target CDKN1B gene (120). Wen-Jun *et al.* reported that high expression level of miR-222 in activated HSCs, with direct correlation between miR-222 expression levels and biliary atresia (BA) disease. Pathology of early BA includes the absence of patent extrahepatic bile ducts (EHBD) with inflammation and fibrosis in the hepatic portal area and ultimately leading to cholestasis and liver fibrosis in infants. Increased miR-222 expression levels target PP2R2A gene and activate the Akt pathway. Phosphorylated Akt inhibits the release of mitochondrial cytochrome-c and active caspase-9, thus prevents apoptosis and stimulate proliferation of HSCs (121).

miR-93 and miR-106b

During chronic fibrosis, hepatocytes up-regulate c-Myc expression and consequently alters the levels of several miRNAs. Specifically, c-Myc up-regulate MCM7 gene, and miR-106b-25 cluster as this cluster is embedded in the intron of MCM7 gene. Pineau *et al.* reported up-regulation of miR-93 and miR-106b during the development of hepatic cirrhosis, which eventually leads to HCC. Target genes of this cluster induce cell proliferation or decreased apoptosis and metastasis in hepatocellular carcinoma (122).

miR-181

Wang *et al.* demonstrated significantly high levels of miR-181a and miR-181b in TGF-β1 treated HSC-T6 cells. miR-181b is a growth regulator and increases S phase of cell cycle, and thereby promotes HSC-T6 proliferation. Cyclin-dependent kinase inhibitor 1B (CDNK1B) gene encodes p27 protein that binds to and controls the cell cycle progression at G1 phase by preventing cyclin E-CDK2 or cyclin D-CDK4 complex activation. 3′-UTR mRNA of p27 encompasses binding site for miR-181a and miR-181b. Experimentally, miR-181b, but not miR-181a, targets p27 and downregulates its endogenous expression in HSC-T6 cells. Moreover, there is elevated miR-181b expression in the serum of cirrhosis patients. Therefore, miR-181b can also serve as a biomarker for liver fibrosis and cirrhosis (123).

miR-615

Tayebi *et al.* investigated role of miR-615 in hepatocellular carcinoma (HCC). miR-615 level was undetectable in healthy liver tissues, but its expression was up regulated significantly in HCC patient liver tissues. miR-615 represents a scenario where an anticancer miRNA only appears in cirrhotic and cancerous tissues. miR-615 showed a significant retardation in cellular proliferation and migration effect in HuH-7 and HepG2 cells. Using luciferase reporter assay 3′-UTR of IGF-II gene was found the direct target for miR-615-5p, which is a potent mitogen frequently overexpressed in HCC (124).

CHEMICAL MODIFICATION OF MIRNAS

miRNAs are not stable molecules and tend to hydrolyze in acidic or basic environment. Moreover, naked miRNAs are susceptible to degradation by various RNases present in the serum as well as in the intracellular environment and thus possess very short half-life *in vivo*. However, miRNA can be chemically modified to alter their properties such as nuclease

resistance, binding affinity, increased cellular uptake and decreased off-target effects (130). Resistance to degradation can be enhanced by chemical modifications in the backbone or in the sugar moiety. Type and site selection for modification can pose challenges while designing modified miRNAs. Some of the common modifications used for improving the stability and potency of miRNAs are:

1. Backbone modification includes PS by replacing non-bridging oxygen atom with the sulfur atom.
2. 2′-O-methyl- (2′-OMe) or 2′-O-methoxyethyl oligonucleotides (2′-O-MEO)
3. Locked nucleic acid (LNA) oligonucleotides
4. Peptide nucleic acid (PNA)
5. Fluorine derivatives (FANA and 2′-F)

Most of these modifications are preferred in the passenger strand or at 3′ end of the guide strand to avoid any functionality issue of miRNA. There are certain rules which are followed while modifying RNA. The 5′-end of the guide strand is critical for activity, hence cannot be modified and should have free hydroxyl or phosphate group. Modification on 3′-end of both strands is less prone to efficacy loss and can be modified by various means. 5′-end of the passenger strand can be modified with protecting functional groups to reduce enzymatic degradation. Ribose sugars of both strands can be modified at 2′ position with halogens or small hydrocarbons. Large molecules such as 2′-OMe can only be acceptable in a passenger strand. O-methyl groups at 2′ position of the ribosyl ring in the guide strand alter the thermodynamic properties of the duplex and reduce off-target effects. With this modification, the binding affinity (melting temperature or Tm) of the duplex becomes higher than one without modification (131). 3′-Exonucleases are the primary enzymes responsible for ODN degradation in the serum. 2′-OMe modified miRNA is less susceptible to degradation by these degrading enzymes. On the other hand, heavy 2′- OMe modification can abolish RNA potency completely. Pyrimidines in the guide strand can be halogenated for increasing potency of miRNA. The 2′-F modification allow more favorable interactions of miRNA with the RISC and dramatically improve the efficacy. The combined use of 2′-F pyrimidines with 2′-OMe purines can results in enhanced stability of RNA duplexes and improved performance *in-vivo* (132).

Nucleic acid phosphate bonds between the nucleotides are cleaved by both endo- and exonucleases. To stabilize RNA structure, PS backbone modification is most common and can be used in either strand. In this modification, a non-bridging oxygen atom in the phosphate backbone is replaced with a sulfur atom. This modification reduces the nuclease degradation of this bond. However, it has significant impact on miRNA efficacy, as it reduces binding affinity, lower Tm and increase toxicity as the number of PS is increased. Therefore, it is critical to place PS modification at selective locations only (133). Combining 2′-OMe nt and PS modification at selective molecule can be a better strategy to protect from exonuclease attack and without additional negative effects (134). An alternative backbone modification to PS is boranophosphate linkage that not only increases serum stability, but also substantially increases its potency. Mayumi *et al.* investigated the effect of the modification of anti-miR-21 and anti-miR-122 by 2′-OMe-4-thioribonucleoside in terms of potency and duration of activity *in vitro*. Moreover, after systemic administration modified miRNAs using a liposomal delivery system, there was an increase in the levels of target miRNA (135).

Locked nucleic acid (LNA) is a modified RNA nucleotide whose ribose moiety is modified with an extra bridge connecting the 2′-O of sugar is to the 4′-C via a methylene bridge and lock the structure into 3′ conformation. This modification stabilizes the molecule against nucleases, but more importantly it increases binding affinity by increasing T_m from 1 to 6°C per modification of miRNA duplex. Gunter *et al.* showed that 2′-OMe modified antimiR-21, but not 2′-DNA specifically inactivate target miRNA activity in HeLa cells (136). LNA can be used alone or with combination with other modifications to get the desired properties of miRNA molecule. Modifications resulting in increased binding affinity make a miRNA nonspecific to its target. Joacim *et al.* used LNA and PS modified antimiR-122 to silence miR-122 function in non-human primates. Results show that highly substituted LNA modification enabled to reduce the dose in antagonizing miR-122 *in vivo* (137).

In morpholino modification, a six membered morpholine ring replaces the sugar moiety to improve the physicochemical and binding affinity of oligomers. Morpholinos are non-toxic, charged neutral, stable against nucleases and increase binding affinity of oligomers to miRNAs. Wigard *et al.* used morpholino modified oligo complementary to miR-206 and induced complete or near complete loss of target miRNA (138).

DELIVERY OF MIRNAS

Despite their therapeutic potential, *in vivo* applications of miRNAs are limited due to their anionic charge, poor stability and high molecular weight. These factors pose major obstacles to their therapeutic application. Several approaches have been explored for *in vivo* delivery and targeting of miRNAs to modulate gene expression. These include cationic polymers, nanoparticles, and bioconjugates. This section will discuss some of these approaches.

Non Targeted Particulate Systems

Nanocarriers can overcome aforementioned challenges associated with the *in vivo* delivery of miRNAs. These miRNA carrying nanoparticles offer several advantages including increased stability and reduced dose frequency, tunable small nano-scale size facilitates passive targeting, possesses favorable biodistribution, they can neutralize polyanionic charge of miRNAs and can facilitate its crossing through negatively charged cell membranes. Normally, these particles are non-immunogenic compared to viruses, allowing repeated treatment for chronic diseases, thus providing a platform for targeting and imaging. Several nano-delivery systems made of different materials with varying physiochemical properties have been pursued *in vivo* miRNA therapeutics. They include polymer/lipid based nanoparticles, lipid based liposomes and virus-like particles (139).

Ammar *et al.* designed nanoparticle complexes for the photo-activated miRNA delivery. miR-148b mimic was conjugated to the surface of silver nanoparticles via a photocleavable (PC) linker. Upon discrete photo trigger, miRNA was released and upregulate its level in cell culture to modulate osteogenesis (140). Shu-Hao *et al.* designed cationic lipid nanoparticles (LNP) for delivery of miR-122 for restoring deregulated gene expression in HCC cells. These LNP were safe upon systemic administration and did not cause innate inflammatory response or systemic toxicity in immune competent mice. In miR-122 knockout (KO) mice LNP-DP1 loaded with miR-122 when injected intravenously increased the expression level of miR-122 compared to the negative control and with a concomitant decrease in the protein levels of two of its validated targets namely, Adam10 and Mapre1. This delivery system was able to reduce angiogenesis in tumors and suppress tumor growth (141). Similarly, Pasqualino *et al.* developed stable nucleic acid lipid particles (SNALPs) that encapsulate miR-199b-5p and decreased medulloblastoma (MB) cancer stem cells (CSCs) through a decrease in CD133+/CD15+ cell population (142). Dipankar *et al.* developed a systemic miRNA delivery to pancreatic cancer by liposomes prepared using cationic lipid DOTAP and co-lipids cholesterol and DSPE-PEG-OMe. They have evaluated these formulations for the systemic miRNA delivery and demonstrated the growth inhibition of subcutaneous pancreatic cancer xenografts (143).

miRNAs can degrade mRNA even without perfect base pairing of the seed region and may often lead to off-target effects. Therefore, it is important to track miRNA containing formulation into the transfected cells. For this purpose, Gomes *et al.* developed nanoparticles composed of poly(lactide-co-glycolide) (PLGA) carrying MRI detectable PFCE for cell tracking and simultaneous delivery of miR-132 (144). Aramaki *et al.* developed liposomal system that can encapsulate ultrasound contrast gas and miRNA. They utilized these bubble liposomes (BLs) for miR-126 delivery for an ischemia-induced angiogenic response. BLs contains perfluoropropane gas and can be tracked using ultrasound detection method *in vivo* (145).

Polyethylenimine (PEI) is protonated at acidic pH and can form complexes with negatively charged ODNs. These polyplexes internalized via caveolae- or clathrin-dependent mechanism into cells and followed "proton sponge effect" and thereby facilitate RNAi release from endosomes. For this property, PEI has been extensively studied to deliver RNAi in the past decades, but high toxicity limits its utilization as a carrier system. Low molecular weight PEI is less toxic and thus Ahmed *et al.* delivered miR-145 and miR-33a after complex formation with low molecular weight PEI for antitumor effects (146). Pan *et al.* developed miRNA delivery system based on bacteriophage MS2 virus-like particles (VLPs). These particles were loaded with single-plasmid expression system for the production of VLPs containing pre-miR-146a or negative control RNA. HIV Tat47–57 was conjugated to MS2 VLPs by reacting amino group of MS2 VLP capsids and the cysteine of Tat47–57 peptide with the help of sulfosuccinimidyl 4-(*p*-maleimidophenyl) butyrate (sulfo-SMPB). These MS2 VLPs had high transfection efficacy and suppressed its target gene IRAK-1 significantly *in vivo* (147). Although transfection efficiency of virus-based systems is excellent, they are not considered safe for human use.

Targeted Particulate Systems

Transfection efficiency of polymeric carriers can be enhanced by optimizing particle size and surface properties and by attaching targeting moiety for a favorable biodistribution. Furthermore, targeted polymeric carrier system allows higher drug accumulation at the diseased site and higher receptors based uptake in appropriate cells and minimize side effects. Normally, targeting ligands have high affinity to specific cell receptors and are attached to the exterior surface of delivery carrier system or directly to the miRNA. Various types of ligands including functional peptides, antibodies (Ab) and aptamers have been explored for targeting miRNA carriers. Huang *et al.* developed a polycation-hyaluronic acid (LPH) based liposomal nanocarrier formulation conjugated with tumor targeting single-chain antibody fragment (scFv). These tumor targeted formulations were used for systemic delivery of miR-34a into experimental lung metastasis of murine B16F10 melanoma. Formulations containing miR-34a were more effective in inhibiting the tumor growth compared to non-targeted formulations (148). Wang and coworkers have developed PEGylated cyclic RGD (cRGD) peptide modified LPH NP formulation delivery of anti-miR-296. These formulations were used to target αvβ3 integrin present on endothelial cells of the tumor neo vasculature. cRGD modified LPH NPs have shown potential of delivering miRNA at target sites and

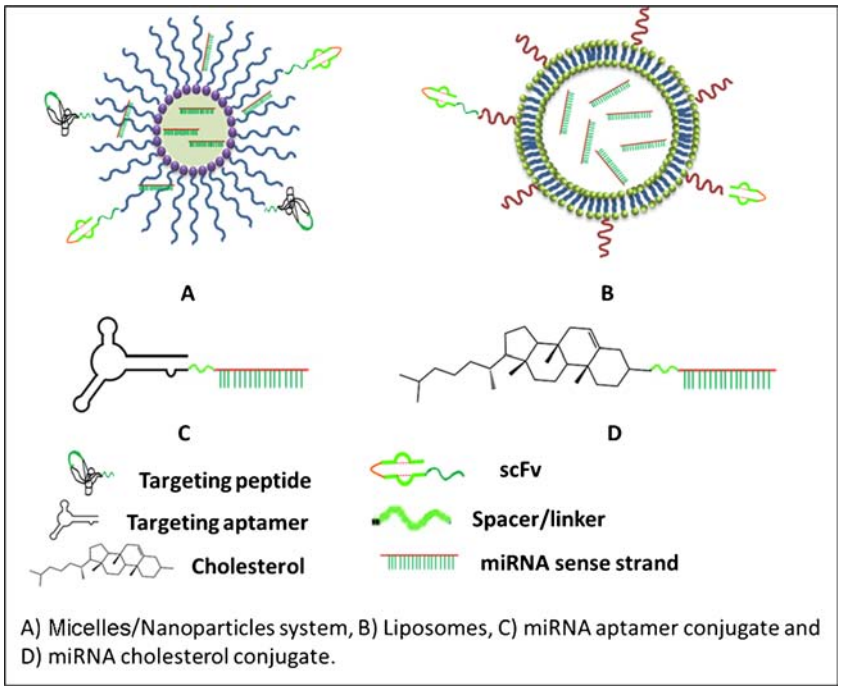

Fig. 5 Strategies used for delivery and targeting of miRNA *in vivo*.

produced significant anti-angiogenic effect (149). Hu *et al.* synthesized polyethylenimine-β-cyclodextrin (PEI-CD) by crosslinking β-CD with low molecular weight PEI (600 Da), and conjugated a tumor homing and penetrating bifunctional peptide CC9 (CRGDKGPDC). Formulation complexed with miR-34a was able to accumulate significantly at the tumor site and downregulated target genes, such as E2F3, Bcl-2, c-myc and cyclin D1 (150).

Non Particulate Systems

Esposito *et al.* used tyrosine kinase AXL receptor binding nucleic acid aptamer (GL21.T) as carriers for cell-targeted delivery of a miRNA. This aptamer itself antagonizes oncogenic receptors and also deliver tumor suppressor miRNA function in AXL-expressing tumors. Conjugation of miR-let7g to GL21.T ensures its specific delivery to target cells and decreases the tumor growth (Fig. 5) (151). Krutzfeldt *et al.* developed 2′-*O*Me PS modified, 3′-end cholesterol-conjugated single-stranded RNA analogues complementary to miR-122. There was a marked decrease in endogenous miR-122 levels after administration of this modified anti-miR into mice by intravenous injection, compared to unmodified single-stranded RNA (anti-miR-122), whereas non-conjugated, but partially or fully PS modified backbone, and 2′-*O*Me sugar modifications led to only incomplete effect (134). Neri *et al.* used similar modification for anti-miR-221/222 for treating prostate tumor in xenograft mouse model (152). Anti-tumor effect of cholesterol conjugated miR-199a/b-3p was determined by Jin *et al.* both *in vitro* and *in vivo* for treating hepatocellular carcinoma. After intratumoral injection of conjugated miR-199a/b-3p, its expression in tumor was elevated while there was dramatic repression of HCC growth (153).

CONCLUSIONS

Liver fibrosis is a formidable disease which is hard to treat and monitor in real clinical practice. Recent studies indicate that it can be reversible by removing causatives and the right treatment in early stage. Although small molecules are attractive in terms of ease in production and handling, their side effects and inconsistent anti-fibrotic results are big problems in securing the FDA approval. To design effective therapy of fibrosis, we need to understand complex pathways involved and to develop combination therapy. miRNAs contribute significantly in disease progression and regression and could be very helpful in controlling liver fibrosis. miRNAs have great therapeutics potential, but their unfavorable physicochemical properties hinders them for their use as therapeutics. We need to improve their stability without inducing any side effects. Suitable chemical modifications and novel formulation strategies are urgently needed to develop miRNAs into therapeutics.

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