Special Section on Drug Delivery Technologies—Minireview

Targeted Drug Delivery to Hepatic Stellate Cells for the Treatment of Liver Fibrosis

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ABSTRACT

Liver fibrosis is caused by excessive accumulation of extracellular matrix during chronic liver injuries. Although clinical evidence suggests that liver fibrosis can be reversed, there is no standard therapy for liver fibrosis. Moreover, there is a lack of diagnostic tools to detect early-stage liver fibrosis. Activation of hepatic stellate cells (HSCs) is the key step during liver fibrogenesis, and its mechanism has been extensively studied by various cell culture and animal models. Targeted delivery of therapeutic agents to activated HSCs is therefore critical for the successful treatment of liver fibrosis. A number of protein markers have been found to be overexpressed in activated HSCs, and their ligands have been used to specifically deliver various antifibrotic agents. In this review, we summarize these HSC-specific protein markers and their ligands for targeted delivery of antifibrotic agents.

Introduction

Liver fibrosis is a worldwide health problem and characterized by excessive accumulation of extracellular matrix (ECM) after chronic liver injuries. If detected early, liver fibrosis can be reversed by removing the underlying etiologies, followed by treatments to attenuate liver injuries. Otherwise, liver fibrosis will advance to liver cirrhosis, which is irreversible and one of the leading causes of mortality and morbidity in the world (Lozano et al., 2012; Yoon et al., 2016). Currently, there is no standard therapy for liver fibrosis, and there are no noninvasive diagnostic tools to detect early-stage liver fibrosis.

Activation of quiescent hepatic stellate cells (HSCs) in the liver is the key milestone during liver fibrogenesis. HSCs can be activated by various conditions including viral infection, nonalcoholic fatty liver disease, alcoholic steatohepatitis, toxins, and autoimmune and biliary diseases. After activation, quiescent HSCs migrate to the injury site, differentiate into myofibroblasts, and secrete large amounts of ECM as well as proinflammatory cytokines (Hernandez-Gea and Friedman, 2011). The composition of ECM in the liver is shifted from type IV collagen to type I and type III collagen during liver fibrogenesis.

Activated HSCs are the major cells in fibrotic liver to secrete excessive ECM (Kisseleva, 2017). As a result, activated HSCs are the target cells for antifibrotic agents. HSCs interact intensively with other cells in the liver, such as Kupffer cells, hepatocytes, endothelial cells, and immune cells by autocrine or paracrine functions of various cytokines and chemokines (Schuppan et al., 2018).

A number of the mediators, such as the transforming growth factor β1 (TGF-β1), insulin-like growth factor I (IGFI), platelet-derived growth factor (PDGF), reactive oxygen species, and endothelin-1 can activate quiescent HSCs. Among them, TGF-β1 is the most crucial cytokine for liver fibrosis. It binds to TGF-β receptors and regulates the synthesis and degradation of type I collagen, which contains two α1 (I) and one α2 (I) chains and is the major component of the ECM in fibrotic liver (Verrecchia and Mauviel, 2007).

ABBREVIATIONS: BDL, bile duct ligated; CCl$_4$, carbon tetrachloride; CD44, cluster of differentiation 44; ECM, extracellular matrix; GFAP, glial fibrillary acidic protein; HA, hyaluronic acid; HSA, human serum albumin; HSCs, hepatic stellate cells; IFNγ, interferon-gamma; IGFI, insulin-like growth factor I; IGFIIR, insulin-like growth factor II receptor; LDLR, low-density lipoprotein receptor; M6P, mannose-6-phosphate; M6PR, mannose-6-phosphate receptor; NAFLD, nonalcoholic fatty liver disease; PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth factor receptor; PEG, polyethylene glycol; RBP, retinol-binding protein; RGD, arginylglycylaspartic acid; TFO, triplex-forming oligonucleotide; TGF, transforming growth factor; TRAIL, TNF-related apoptosis-inducing ligand; TRAILR, TNF-related apoptosis-inducing ligand receptor; scAb, single-chain antibody; SELEX, systematic evolution of ligands by exponential enrichment; siRNA, small interfering RNA; SYN, synaptophysin; Y27632, 4-[(1R)-1-aminoethyl]-N-pyridin-4-ylcyclohexane-1-carboxamide.

PDGF, which is primarily secreted by Kupffer cells, is considered to be the second most important mitogen for the activation of HSCs. The PDGF family consists of PDGF-A, PDGF-B, PDGF-C, and PDGF-D. PDGF-D binds to PDGF receptor β (PGFGRβ) and induces autophosphorylation and activates the downstream signaling molecules such as protein kinase B (PKB/Akt), mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase p38 (JNK-p38), and extracellular signal–regulated kinases 1/2 (ERK1/2) (Borkham-Kamphorst et al., 2007).

A wide range of therapeutic agents including small molecules, proteins, small interfering RNA (siRNAs), microRNA (miRNAs), and plasmids have been investigated for the treatment of liver fibrosis (Tu et al., 2014; Wang et al., 2015; Yoon et al., 2016; Liu et al., 2017; Middleton et al., 2018). However, antifibrotic agents cannot easily reach HSCs in fibrotic liver because HSCs only account for 5% to 8% of total liver cells (Geerts, 2001). In addition, accumulated ECM and closure of endothelial fenestrae inhibit the delivery of antifibrotic agents to the liver (Garcia-Banuelos et al., 2002). Delivery of antifibrotic agents to HSCs is also reduced by the reduced space of Disse (Varin and Huet, 1985). Targeted delivery of therapeutic agents to HSCs is thus critical for the successful treatment of liver fibrosis.

A number of receptors—including type VI collagen receptor, retinol-binding protein (RBP) receptor, platelet-derived growth factor receptor (PDGFR), synaptophysin, insulin-like growth factor-II receptor (IGFIIR), low-density lipoprotein receptor (LDLR), and cluster of differentiation 44 (CD44)—are overexpressed in activated HSCs (Fig. 1), and their ligands have been used to specifically deliver various antifibrotic agents (Table 1). Several articles have reviewed the mechanisms of liver fibrogenesis, therapeutic targets for liver fibrosis, nanoscale delivery systems for antifibrotic agents, and some targeting ligands for fibrotic liver (Li and Wang, 2009; Schon et al., 2016; Schuppan et al., 2018). In this review, we summarize all the possible HSC-specific markers that can be potentially exploited for targeted delivery of antifibrotic agents to fibrotic liver. We hope this review article will serve as a one-stop reference for scientists who are interested in improving the specificity of their antifibrotic agents to the liver.
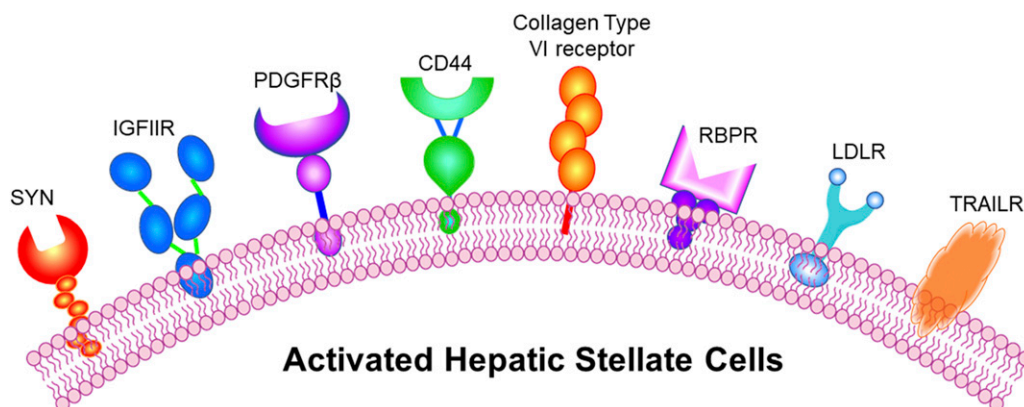
Type VI Collagen Receptor

Type VI collagen is a minor but important matrix protein in the liver. In a normal liver, type VI collagen is mainly distributed in the portal areas and at the membranes of endothelial cells, hepatocytes, and HSCs. The expression of type VI collagen is markedly elevated during liver fibrogenesis (Schuppan et al., 1985; Loreal et al., 1992; Takahara et al., 1995; Beljaars et al., 2000). Type VI collagen is one of the most important matrix proteins involved in cell adhesion to the surrounding matrix. It also modulates the homeostasis of matrix by maintaining the interaction between matrix molecules and the cells (Li et al., 2015). Type VI collagen receptor contains three α chains and is mainly expressed on activated HSCs but not in normal liver cells (Popov and Schuppan, 2009). Type VI collage receptor has thus been exploited for targeted delivery of antifibrotic agents to HSCs (Beljaars et al., 2000; Du et al., 2007).

A cyclic arginylglycylaspartic acid (RGD) peptide C*GRGDSPC* (* denotes the cyclizing cysteine residue) was discovered as a ligand for the type VI collagen receptor (Marcelino and McDevitt, 1995). The cyclic peptide was conjugated to human serum albumin (HSA) in a 10:1 molar ratio (Fig. 2). In vitro studies showed that the cyclic peptide-modified HSA would specifically bind to activated rat HSCs and enter the cells via internalization. A biodistribution study in rats with induced liver fibrosis demonstrated high accumulation of the peptide-modified HSA in activated HSCs (Beljaars et al., 2000). The cyclic RGD peptide was also attached to a biodegradable polymersome encapsulating the antifibrotic agent oxymatrine. The polymersome significantly inhibited the proliferation of activated HSCs and reduced the expressions of α smooth muscle actin and collagen in the cells. The peptide-modified polymersome exhibited higher antifibrotic activity in bile duct-ligated (BDL) rats than did the unmodified polymersome (Yang et al., 2014).

Another cyclic RGD peptide, C*GRGDSPK*, was also exploited as a targeting ligand for the type VI collagen receptor. The peptide preferentially binds to activated HSCs rather than hepatocytes. An interferon-α1b loaded liposome was coupled with the RGD peptide to achieve targeted delivery to fibrotic liver via recognition of type VI collagen receptors on HSCs. In rats with BDL-induced liver fibrosis, the RGD peptide-coupled liposome exhibited 10-fold higher accumulation in HSCs than the unmodified liposome. Moreover, the RGD peptide-modified liposome demonstrated significant higher antifibrotic activity in the rats with liver fibrosis (Du et al., 2007).

Fig. 1. Receptors overexpressed in activated HSCs: synaptaphysin (SYN), insulin growth factor receptor 2 receptor (IGFIIR), platelet-derived growth factor receptor (PDGFR), cluster of differentiation 44 (CD44), collagen type VI receptor, retinol-binding protein (BBP) receptor, low-density lipoprotein receptor (LDLR), and TNF-related apoptosis-inducing ligand receptor (TRAILR).

TABLE 1
HSC-specific markers for targeted delivery of antifibrotic agents

HSC-Specific Marker	Targeting Ligand	Carrier	Drug	Reference
Type VI collagen receptor	Cyclic RGD peptide (C*GRGDSPC*)	Liposome	IFN-α1b	Du et al., 2007
		Polymersome	Oxymatrine	Yang et al., 2014
		Conjugate	HSA	Beljaars et al., 2000
RBP receptor	Vitamin A	Liposome	siRNA	Sato et al., 2008
		Nanoparticle	Oligonucleotide	Zhang et al., 2015
PDGFR-β	Cyclic peptide (C*SRNLIDC*)	Conjugate	HSA	Beljaars et al., 2003
		Liposome	IFNγ	Li et al., 2012
Synaptophysin	scAb	Conjugate	Tributyltin	Douglass et al., 2008a
IGFIIR	M6P	Conjugate	Y27632	Bansal et al., 2011b
	Peptide	Nanocomplex	siRNA	Zhao et al., 2018
	Aptamer	Conjugate	siRNA	Chen et al., 2017
LDLR	Cholesterol	Conjugate	Oligonucleotide	Cheng et al., 2006
		Nanocomplex	siRNA	Shukla et al., 2013
CD44	Hyaluronic acid	Nanoparticle	Curcumin	Chen et al., 2016
		Micelle	Losartan	Thomas et al., 2015
		Nanocomplex	siRNA	Park et al., 2011
		Conjugate	TRAIL	Yang et al., 2015

Retinol-Binding Protein (RBP) Receptor

A key role of quiescent HSCs in the liver is to store up to 80% of vitamin A as retinyl palmitate in lipid droplets. Vitamin A binds to RBP in the systematic circulation and is taken up by HSCs via RBP receptors (Higashi et al., 2005). In addition, vitamin A is taken up more efficiently by activated HSCs rather than quiescent HSCs (Senoo et al., 2007; Sato et al., 2008). As a result, vitamin A and RBP have been used as ligands for HSC-specific delivery.

In one study, the domain III of an albumin was fused to RBP for HSC-specific delivery. The fusion protein can be efficiently taken up by HSCs in vitro and in vivo. More importantly, the fusion protein ameliorated liver fibrosis in rats induced by carbon tetrachloride (CCl$_4$) or BLD (Park et al., 2012; Lee et al., 2015). Using a similar concept, Zhang et al. (2015) developed a retinol-modified nanoparticle encapsulating an antifibrotic antisense oligonucleotide. The nanoparticle binds to RBP in the serum and is selectively taken up by HSCs via the RBP receptor. The nanoparticles successfully suppressed the expression of type I collagen in the liver and reversed BDL- and CCl$_4$-induced liver fibrosis in mice (Zhang et al., 2015).

In a pioneering in vivo study using siRNA for liver fibrosis therapy, a gp46 siRNA was encapsulated in a liposome coupled with vitamin A. The functionalized liposome efficiently silenced gp46, inhibited collagen secretion in HSCs, and reversed CCl$_4$- and BDL-induced liver fibrosis in rats. This was one of the first animal studies demonstrating the therapeutic potential of siRNAs in an animal model (Sato et al., 2008).

Similar strategies have been adopted by scientists to deliver various antifibrotic agents to HSCs (Sauvant et al., 2011; Duong et al., 2015; Lee et al., 2015; Pan et al., 2016). A phase 1b/2 clinical trial was recently conducted to evaluate the safety and tolerability of a vitamin A–coupled lipid nanoparticle containing siRNA targeting heat shocking protein 47 (HSP47) in patients with moderate to extensive liver fibrosis (ClinicalTrials.gov Identifier: NCT02227459).

Platelet-Derived Growth Factor Receptor-β (PDGFR-β)

Platelet-derived growth factor (PDGF) plays a critical role in the initiation and progression of liver fibrogenesis (Beljaars et al., 2003). It binds to PDGFR and regulates the migration, proliferation, and survival of HSCs. PDGFR is not only up-regulated in activated HSCs in animals but also is up-regulated in human fibrotic livers (Campbell et al., 2005; Borkham-Kamphorst and Weiskirchen, 2016). Particularly, PDGFR-β is dramatically overexpressed on activated HSCs, and its expression is much higher than that on other PDGFR-β-positive cells (Friedman, 2003). Therefore, several PDGFR-β-targeted delivery systems have been investigated for antifibrotic agents (Bansal et al., 2011a,b; Li et al., 2012).

The cyclic peptide C*SRNLIDC* (* denotes the cyclizing cysteine residue) was designed based on the receptor-binding residues of the PDGF B-chain. The cyclic peptide-conjugated HSA highly accumulated in the liver of the rats with BDL-induced liver fibrosis. Moreover, an intrahepatic distribution study showed that the majority of the peptide-conjugate HSA is located in HSCs, indicating selective binding of the cyclic peptide to PDGFR-β on activated HSCs (Beljaars et al., 2003).

In another study, the cyclic peptide C*SRNLIDC* was fused to a single-chain antibody fragment targeting the knob of a recombinant adenovirus. After binding to the adenovirus, the fusion protein retargeted the adenovirus to activated HSCs and abolished the virus's natural tropism for hepatocytes
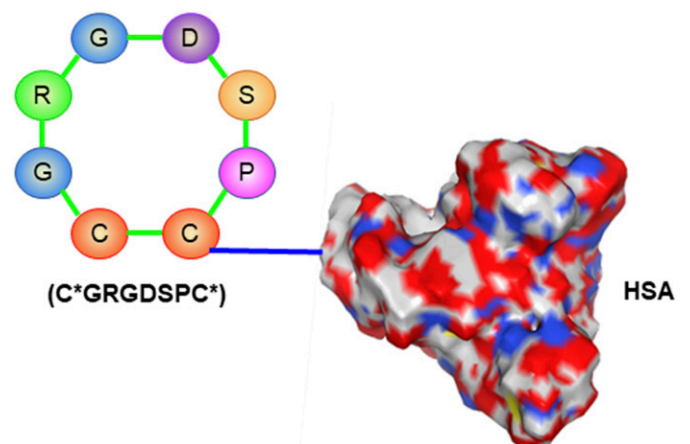

Fig. 2. Cyclic RGD peptide C*GRGDSPC* conjugated human serum albumin for type VI collagen receptor.

and Kupffer cells (Fig. 3). This assembly provides a platform to selectively deliver antifibrotic genes to activated HSCs without nonspecific uptake by neighboring cells, such as Kupffer cells and hepatocytes (Schoemaker et al., 2008).

The cyclic peptide C*SRNLIDC* was also conjugated to interferon-γ (IFNγ) using polyethylene glycol (PEG) as a linker. The peptide-conjugated IFNγ showed specific binding to PDGFR-β on cultured HSCs and inhibited their activation. It also significantly inhibited fibrogenesis in acute liver injury and in CCl4-induced liver fibrosis animal models without inducing IFNγ-related side effects. By contrast, unmodified IFNγ failed to inhibit fibrogenesis in both animal models (Bansal et al., 2011b).

Instead of direct conjugation to IFNγ, the cyclic peptide C*SRNLIDC* was also coupled to a liposome encapsulating IFNγ, leading to higher uptake in activated HSCs. Compared with free IFNγ and unmodified liposome encapsulating IFNγ, the cyclic peptide-coupled liposome demonstrated higher inhibitory effect on the proliferation of HSCs. In vivo studies in rats with thioacetamide-induced liver fibrosis showed a high accumulation of the peptide-coupled liposome in activated HSCs. Moreover, an enhanced antifibrotic effect of the peptide-coupled liposome was observed (Li et al., 2012).

Synaptophysin

Synaptophysin (SYN) is a membrane glycoprotein present in the presynaptic vesicles of neurons, adrenal medulla, and other neuroendocrine epithelial cells (Wiedenmann et al., 1986). Its main function involves exocytosis of neurotransmitter. HSCs also express several neuroectodermal differentiation markers such as glial fibrillary acidic protein (GFAP) and nestin (Cassiman et al., 1999). SYN was found present in HSCs in both human and rat liver biopsies. After only 36 hours of galactosamine intoxication, rat liver biopsies revealed significant SYN expression in comparison with normal rat liver biopsies. This result suggests that SYN is a potential marker for HSCs (Cassiman et al., 1999).

Researchers have used SYN as a marker to differentiate portal fibroblasts from HSCs of fibrotic livers of humans, rats, and mice (Iwaisako et al., 2014). However, studies have found that canine HSCs (quiescent and activated) are negative for SYN.

Ijzer et al. (2006) investigated the morphologic characteristics of canine HSCs and myofibroblasts. They reported that muscle-specific actin clone HHF35 (HHF35) and α smooth muscle actin were potent markers for canine HSCs and myofibroblasts, but liver sections were consistently negative for SYN and GFAP. SYN has been used as a diagnostic marker because of its variable expressions with respect to different liver stresses and injuries. In a study, rats with nonalcoholic fatty liver disease (NAFLD) showed high hepatic inflammation, necrosis and fatty infiltration. Apart from Sirius Red staining, which is a primary staining method for collagen fibers, SYN staining was used to evaluate HSC activation and the fibrogenesis. Xiao et al. (2014) reported distinct increase in SYN positive areas in a rat model of NAFLD and subsequent decline in SYN expression after therapeutic intervention with Epigallocatechin gallate.

Despite its unique expression patterns during liver fibrosis and NAFLD, SYN has not been thoroughly investigated as a ligand for targeted drug delivery. However, SYN is advantageous over other markers for targeted delivery of antifibrotic agents because it forms part of endocytosing vesicles, leading to increased chance of endocytosis of its ligand and associated cargos (Douglass et al., 2008a). A group of researchers discovered a single-chain antibody (scAb), C1-3, that binds to SYN on activated HSCs in fibrotic mouse liver. C1-3 scAb was generated by phage display biopanning against the peptide sequence that is part of the extracellular domain of SYN (Elrick et al., 2005). Studies have shown that C1-3 scAb binds specifically to activated HSCs but not hepatocytes. The C1-3 scAb-conjugated tributyl tin was delivered into HSCs and exert its activity (Douglass et al., 2008a). This demonstrated the feasibility of using C1-3 scAb as a targeting ligand for HSC-specific delivery. One potential limitation of this strategy
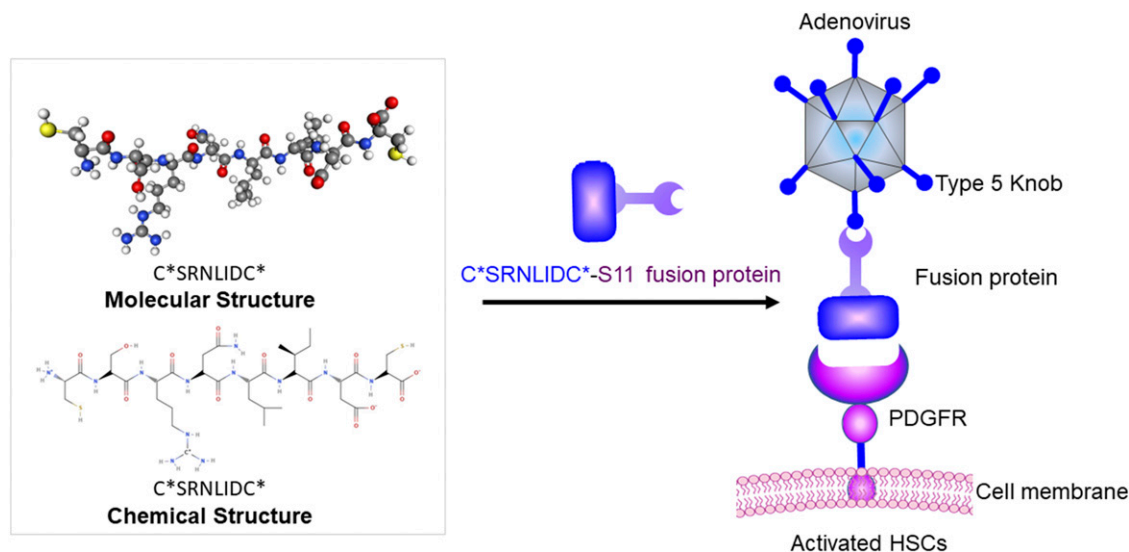
Fig. 3. A fusion protein to change the tropism of adenovirus to HSCs. The PDGFRβ-specific cyclic peptide C*SRNLIDC* was fused to a single-chain antibody fragment targeting the knob of a recombinant adenovirus. After binding to the adenovirus, the fusion protein retargets the adenovirus to activated HSCs and abolishes its natural tropism for hepatocytes and Kupffer cells.

is the nonspecific delivery to neuroendocrine and neural cells. However, there are no evidence showing that scAb can cross the blood–brain barrier, which addresses the nonspecific issue to some degree (Douglass et al., 2008b).

Insulin-Like Growth Factor II Receptor (IGFIIR)

Also known as the cation-independent mannose-6-phosphate receptor (M6PR), the 300 kDa IGFIIR is a member of the IGF signaling family (Morgan et al., 1987). The major functions of IGFIIR include regulating the activity of insulin like growth factor II (IGFII); transportation of M6P-tagged lysosomal enzymes from Golgi network and outside of cells to lysosomes; and activation of TGF-β. M6P-tagged proteins are transported to lysosomes for degradation by lysosomal acid hydrolases (Puxbaum et al., 2012). It is noteworthy that IGFIIR is unrelated to tyrosine kinase activity and proliferation (Sedlaczek et al., 2003). As a result, IGFIIR can be exploited as a surface maker for targeted drug delivery without inducing the proliferation of HSCs.

While IGFIIR is expressed in quiescent HSCs, its expression is strongly upregulated (~20 folds) in activated HSCs relative to quiescent HSCs (Beljaars et al., 1999; Mousavi et al., 2013). The expression of IGFIIR is often positively correlated with chronic liver injury (Hellemans et al., 2004). Moreover, IGFIIR-mediated ligand internalization is 3 times faster than cellular endocytosis in activated HSCs (York et al., 1999). Therefore, IGFIIR is considered as a promising receptor for HSC-targeted drug delivery (Ye et al., 2005; Gary-Bobo et al., 2007; Mousavi et al., 2013). Additionally, upregulation of IGFIIR is also a signature feature of nephroblastoma, hepatocellular carcinoma and rhabdomyosarcoma (Sedlaczek et al., 2003).

Mannose 6 phosphate (M6P) is the natural ligand for IGFIIR. IGFIIR consists of two M6P binding extracellular domains. Generally, the affinity of M6P toward IGFIIR is low, but it can be enhanced by oligomerization of the receptor (Byrd and MacDonald, 2000). Dimerization of a ligand has also proven to increase its binding affinity toward IGFIIR (Chen et al., 2015). Researchers have conjugated multiple M6Ps to HSA to deliver triplex-forming oligonucleotide (TFO) to activated HSCs in rats. This delivery system demonstrated high specificity toward HSCs and efficiently delivered the therapeutic load to target cells. Moreover, in vivo studies also revealed that TFO was selectively distributed in HSCs of fibrotic rats (Ye et al., 2006).

Similarly, the Rho-kinase inhibitor (+)-(R)-trans-4-(1-aminoethyl)-N-(4-pyridyl) cyclohexanecarboxamide dihydrochloride (Y27632) was conjugated with M6P/HSA, and its in vivo biodistribution was studied (van Beuge et al., 2011a,b). In comparison with free Y27632, the Y27632-M6P/HSA conjugate demonstrated exclusive distribution in HSCs. The conjugated Y27632 significantly reduced the collagen deposition in the extracellular matrix in contrast to the free Y27632. M6P-GlcNAc is another such conjugate, which has shown high binding affinity toward IGFIIR (Liu et al., 2013). Despite the high binding affinity of the M6P conjugate toward IGFIIR, the greatest limitations remain the difficulty in conjugation and relatively low binding affinity (approximately K_D 23 μM) of M6P (Jeanjean et al., 2006).

Using a novel combinatorial biopanning strategy, Chen et al. (2015) discovered an IGFIIR-specific peptide, peptide-431. Peptide-431 demonstrated high specificity toward IGFIIR expressed on human and rat HSCs. It has been used as a promising ligand for antifibrotic siRNA nanocomplexes (Fig. 4).(Zhao et al., 2018) This novel peptide is a promising ligand for the delivery of a therapeutic payload to activated HSCs.

Chen et al. (2017) also discovered IGFIIR-specific aptamer using systematic evolution of ligands exponential enrichment (SELEX). The aptamer was annealed to the 3′ end of an siRNA and effectively delivered the siRNA to rat and human HSCs. The aptamer-siRNA chimera also exhibited high accumulation in the liver of the rats with CCl₄-induced liver fibrosis.

Low-Density Lipoprotein Receptor (LDLR)

The liver is the major organ for cholesterol homeostasis by controlling lipid synthesis and uptake in the body (van de Sluis et al., 2017). The expressions of LDLR and high-density lipoprotein receptor-scavenger receptor class B type1 (SR-B1) are high in the liver (Gao and Brigstock, 2003; Wolfrum et al., 2007). Clearance of plasma lipids in the liver is mediated by LDLR and LDLR-related protein 1 (van de Sluis et al., 2017).

LDLR plays a critical role in cholesterol metabolism via recognizing the apolipoprotein B100 of cholesterol-rich LDL and mediating a rapid endocytosis recycling process (Rudenko et al., 2002). LDLR binds to cholesterol-rich LDL at neutral pH through the "ligand-binding domain" with the help of Ca^{2+} but rapidly dissociates from these ligands at acidic pH in the endosome (Rudenko et al., 2002; Rudenko and Deisenhofer, 2003). LDLR also induces receptor-mediated endocytosis for very-low-density lipoprotein and chylomicron remnants through binding with apolipoprotein E (Rudenko et al., 2002).

Cholesterol was found accumulated in HSCs of the mice with CCl₄- and BDL-induced liver fibrosis. Accumulation of cholesterol in HSCs leads to sensitization of the cells to TGF-β and subsequently aggravates fibrogenesis (Teratani et al., 2012). Furthermore, the exacerbation of liver fibrosis and activation of HSCs also enhance the accumulation of cholesterol in HSCs, which is partially correlated with the increased expression of LDLR (Tomita et al., 2014).

A triplex-forming oligonucleotide targeting the genomic DNA of Type I collagen was conjugated with cholesterol and showed higher uptake in the liver of the rats with dimethylnirosamine-induced liver fibrosis (Cheng et al., 2006). In another study, cholesterol was used as a targeting ligand and was coupled to a streptavidin-based siRNA nanocomplex. The cholesterol-modified nanocomplex showed a higher uptake in HSC-T6 (LDLR+) cells rather than in Caco-2 (LDLR-) cells. Meanwhile, incubation of HSC-T6 cells with puromycin, an LDLR inhibitor, suppressed the cellular uptake of the cholesterol-modified nanocomplex (Shukla et al., 2013).

Cluster of Differentiation 44 (CD44)

CD44 is the major receptor for hyaluronic acid (HA), which is an important component of the extracellular matrix. CD44 was initially found overexpressed in liver biopsies

A

Peptide-431 dimer

B
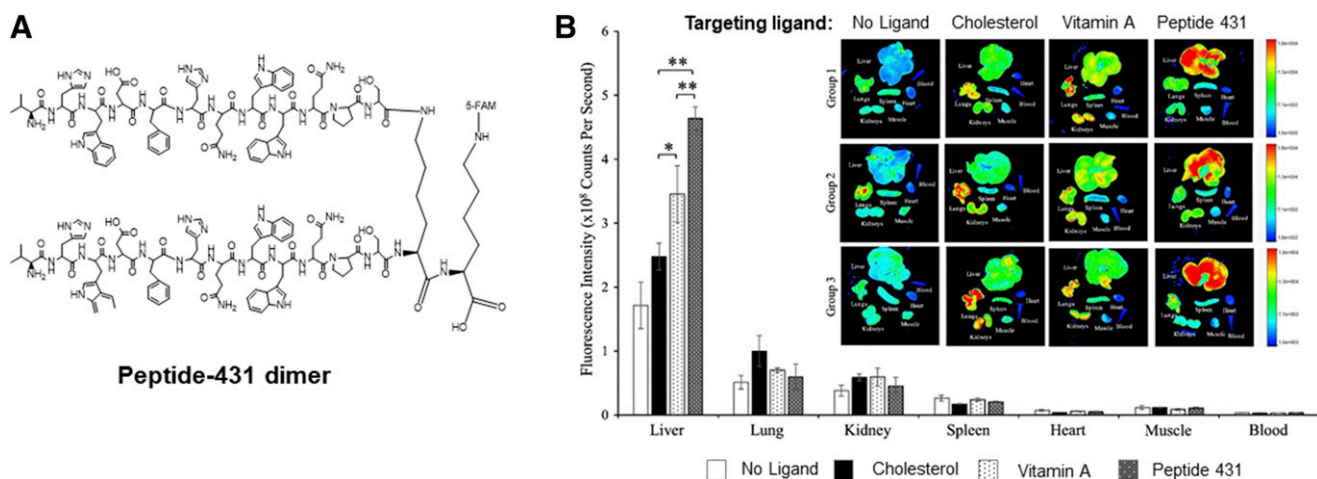
Fig. 4. Peptide-431, an IGF2R-specific peptide, enhances the accumulation of a siRNA nanocomplex in fibrotic liver. (A) Structure of the peptide-431 dimer. (B) Biodistribution of the siRNA nanocomplexes modified with different ligands including cholesterol, vitamin A, and peptide-431. Adapted from Zhao et al. (2018).

from patients with alcoholic liver disease (Urashima et al., 2000). Similar results were observed in rats with dimethylnitrosamine-induced liver fibrosis. CD44 was highly expressed in fibrotic liver but only weakly expressed in normal liver. Although HA was localized in HSCs and endothelial cells, CD44 was mainly detected in infiltrating lymphocytes, Kupffer cells, and some endothelial cells (Satoh et al., 2000). In another study, researchers specifically investigated the expression of CD44 in HSCs from normal and fibrotic rats. The CD44 splice variant, CD44v6, is highly expressed in activated HSCs and facilitates the migration of the HSCs (Kikuchi et al., 2005).

HA is a well-established ligand for CD44 and has been used for HSC-targeted delivery of antifibrotic agents. In one study, curcumin was encapsulated in HA-modified polylactide nanoparticles. The nanoparticle induced cell death of activated HSCs without affecting quiescent HSCs and hepatocytes (Chen et al., 2016). In another study, HA was conjugated to 5β-cholanic acid to form micelles and encapsulate losartan. The HA micelles showed specific accumulation in fibrotic liver and ameliorated liver fibrosis in mice (Thomas et al., 2015).

HA was also used for targeted delivery to cirrhotic livers in mice. Quantum dots (QDots) were conjugated to HA and demonstrated higher uptake by HSCs than by normal hepatocytes. Biodistribution study showed specific delivery of the QDots to HSCs in mice with liver cirrhosis (Kim et al., 2010). The same group then developed HA-coupled polyethyleneimine to deliver a TGF-β siRNA for the treatment of liver cirrhosis. The siRNA/HA-polyethyleneimine nanocomplex entered HSCs via CD44-mediated endocytosis and significantly attenuated liver cirrhosis (Park et al., 2011).

Tumor Necrosis Factor–Related Apoptosis-Inducing Ligand Receptor (TRAILR)

The tumor necrosis factor receptor superfamily (TNFRSF) are cytokine receptors, including TRAILR1 (death receptor 4), TRAILR2 (death receptor 5), TRAILR3 (decoy receptor 1), and TRAILR4 (decoy receptor 1) (Locksley et al., 2001). TNF-related apoptosis-inducing ligand (TRAIL) is a known anticancer agent that induces apoptosis of cancer cells by

specifically targeting TRAILRs (Singh et al., 2017). It was recently found that some immune cells, such as natural killer cells, play important roles in preventing liver fibrosis by killing activated HSCs in a TRAIL-dependent manner. Once activated, HSCs are more susceptible to apoptotic factors, such as TRAIL.

TRAILR1/2 contain a death domain, which plays an essential role in the apoptosis of HSCs (Ashkenazi and Dixit, 1998; Singh et al., 2017). Moreover, TRAILR1/2 are highly expressed in activated HSCs (Taimr et al., 2003; Yang et al., 2015). For example, during the activation of human HSCs (LX-2), the mRNA expression of TRAILR1 and TRAILR2 was increased by 18-fold and 17.6 fold, respectively. Similar results were observed in murine HSCs (Taimr et al., 2003).

In addition to binding to TRAILR1/2 to induce apoptosis of HSCs, TRAIL also binds to TRAILR3/4, which are also expressed on human HSCs (Singh et al., 2017). Because they lack a death domain, TRAILR3/4 act as a decoy receptors to limit the engagement of TRAIL with TRAILR1/2 (Falschlehner et al., 2007). A recent study has shown that silencing the expression of TRAILR3/4 in human HSCs sensitizes the cells to apoptosis (Singh et al., 2017).

Despite its therapeutic potential, TRAIL's clinical applications are limited because of its very short half-life in the body. HA was conjugated to the N-terminal amino group of TRAIL to achieve targeted delivery to HSCs because CD44 is overexpressed in fibrotic livers. The TRAIL-HA conjugate showed similar biologic activity as native TRAIL but a higher accumulation in the liver. Moreover, the conjugate exhibited long circulation times in the blood for more than 4 days and reversed liver fibrosis in a rat model (Yang et al., 2015). In another study, PEG was conjugated to TRAIL to prolong its half-life. The PEG-conjugated TRAIL showed an extended half-life not only in rodents but also in nonhuman primates. PEG-TRAIL also attenuated CCl$_4$-induced liver fibrosis in rats (Oh et al., 2016).

Although TRAILR has yet to be exploited as a marker for HSC-specific drug delivery, it is possible to discover artificial ligands of TRAILR using in vitro selection procedures, such as phage display biopanning or SELEX. The artificial ligands can

then be conjugated to antifibrotic agents to increase their uptake by HSCs.

Conclusion and Perspective

Scientists have made good progress in understanding the mechanisms of liver fibrogenesis and have discovered numerous potential targets for antifibrotic therapy. The major hurdle in developing effective antifibrotic therapies is that antifibrotic agents cannot easily reach activated HSCs, which are the major players in liver fibrogenesis. HSCs only account for a very small percentage of total liver cells (Geerts, 2001). In addition, accumulated ECM and closure of endothelial fenestrae inhibit the delivery of antifibrotic agents to the fibrotic liver (Garcia-Banuelos et al., 2002).

Targeted delivery of antifibrotic agents to activated HSCs is therefore critical for the successful treatment of liver fibrosis. Despite great efforts in developing targeted delivery systems for liver fibrosis in preclinical studies, the importance of targeted drug delivery has not been fully realized in clinical trials. A number of clinical trials have been conducted for the treatment of liver fibrosis using small molecules, proteins, monoclonal antibodies, and nucleic acids. Among them, only one clinical trial (NCT02227459) used vitamin A as a targeting ligand for the antifibrotic siRNA.

A number of protein markers, including type VI collagen receptor, RBP receptor, PDGFR, synaptophysin, IGFIIR, LDLR, and CD44, are overexpressed in activated HSCs, and their ligands have been used to specifically deliver various antifibrotic agents in preclinical studies. Currently, most investigators focus on natural ligands, which are very limited and generally have low affinity for these receptors. In addition, some of the natural ligands may activate downstream signaling pathways after binding to their receptors on HSCs.

A potential solution for this dilemma is to use affinity selection technology such as phage display biopanning to discover peptide- or antibody-based ligands, or SELEX to discover aptamer-based ligands. It is noteworthy that the Nobel Prize in chemistry 2018 was awarded to two scientists for "the phage display of peptides and antibodies," supporting the great promise of affinity selection technology.

Compared with natural ligands, artificial ligands including peptides, antibody fragments, antibodies, and aptamers have higher affinity and are more flexible for chemical modification and coupling to antifibrotic agents or delivery systems. In particular, peptides and aptamers are attractive ligands because of their small size, ease of production, and nonimmunogenicity.

Considering the fact that targeted delivery of antifibrotic agents has shown promising results in numerous animal studies, we believe that incorporating HSC-specific ligands in antifibrotic agents could dramatically increase their success rate in clinical studies.

Authorship Contributions

Participated in research design: Chen, Cheng.

Wrote or contributed to the writing of the manuscript: Chen, Jain, Liu, Zhao, Cheng.

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