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REVIEW

Progress of Targeting Transforming Growth Factor- β 1 Small Interfering RNA in Liver Fibrosis[△]

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Liver fibrosis is a common pathological consequence of a variety of chronic stimuli, including viral, autoimmune, drug-induced, cholestatic and metabolic diseases. Fibrosis is driven by a dynamic process involving increased synthesis of matrix components and a failure of physiological mechanisms of matrix turnover. Activation of hepatic stellate cells (HSCs) remains a central event in fibrosis. HSCs are the main source of extracellular matrix (ECM). Transforming growth factor-beta (TGF- β), which is the fibrogenic master cytokine, can induce the activation of HSCs to produce a large amount of ECM, and is capable of inducing apoptosis of liver cells. RNA interference (RNAi) is a novel gene disruption technology. Studies have shown that small interfering RNA (siRNA) targeting TGF- β 1 may inhibit the activation and proliferation of HSCs, suppress ECM synthesis and block liver fibrosis. TGF- β 1 siRNA-mediated gene silencing therapy provides a new avenue for liver fibrosis. This review summarizes recent progresses in research on HSCs, TGF- β 1 and TGF- β 1 siRNA in liver fibrosis.

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LIVER fibrosis is the abnormal accumulation of extracellular matrix (ECM) and fibroplasia, which produces scar tissue and damages the normal function of the liver. It is characterized by fibrous tissue hyperplasia, disordered lobular structure, deformation or inflammation of the bile duct.^{1,2} Liver fibrosis is a necessary stage of the development of a variety of chronic liver diseases to cirrhosis and even liver cancer. A variety of cytokines such as transforming growth factor-beta (TGF- β),

platelet-derived growth factor, connective tissue growth factor, vascular endothelial growth factor, etc. are secreted after liver injury. The damage of various cytokines stimulates the proliferation of hepatic stellate cells (HSCs) significantly, and inhibiting the activation of HSCs is an important treatment strategy for liver fibrosis.

RNA interference (RNAi), an effective means to study gene function, is specific degradation induced by double-stranded RNA (dsRNA) homologous messenger RNA (mRNA), then function loss of gene expression which leads to gene silencing.^{3,4} The dsRNA will format to many small fragments after cleavage, known as small interfering RNA (siRNA), which will lose function after complementary binding with homologous sequences to the mRNA because

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it cannot be translated to produce proteins, which is gene "silencing".⁵ Studies have shown that siRNA interference targeting gene expression of HSCs plays an important role in mitigating the process of liver fibrosis.^{6,7}

HEPATIC STELLATE CELLS

HSCs were first identified in 1876 by a German researcher Kupffer through gold chloride staining, and called Sternzellen. After that, Ito cells were proved to be Sternzellen. HSCs are a type of liver non-parenchymal cells, located within the space of Disse (designated space between hepatocytes and sinusoidal endothelial cells), also known as sinus cells, fat-storing cells, Ito cells, fat cells, stromal cells, or vitamin A storage cells.⁸ Under normal circumstances, HSCs account for 5%-15% of liver cells, and their main functions are to store vitamin A, synthesize and secrete a variety of collagenase and a small amount of ECM, release TGF- β_1 and hepatocyte growth factor, and participate in liver metabolism.⁹

HSCs change from quiescent to activated state along with morphological changes from the state of containing vitamin A to releasing vitamin A under the action of various stimuli. This process is called activation of HSCs. HSCs activation process mainly consists of two stages: the start-up phase and continuous phase.¹⁰ In the early start-up phase, HSCs go through gene expression and phenotypic change. HSCs activation initiates liver fibrosis, mainly due to the paracrine stimulation and cytokine stimulation. Continuous stage associates with paracrine, autocrine stimulation and remodeling of ECM, those incentives maintain HSCs activation phenotype and lead to liver fibrosis. Activated HSCs demonstrate the following biologically characteristics: (1) similar morphology with myofibroblasts; (2) phenotypic changes and expression of α -smooth muscle actin, vimentin and binding protein; (3) proliferative activity is significantly enhanced; (4) cytoplasmic lipid droplets and vitamin A decrease or even disappearance; (5) an increase in expression and secretion of pro-inflammatory and cytokine-induced fibrosis and ECM; (6) an increase in synthesis and secretion of tissue inhibitor of matrix metalloproteinase (TIMP); (7) enhanced shrinkage. Activated HSCs not only involve in the formation of liver fibrosis and liver rebuilding structures through proliferating and secreting ECM but also increase sinusoidal pressure through cell contraction. Activated HSCs have four main destinations: (1) phenotypic transformation; (2) apoptosis; (3) immune clearance; (4) aging.¹¹ Experiments have shown that the apoptosis of activated HSCs is related to slow-down or even reverse of liver fibrosis.

TRANSFORMING GROWTH FACTOR- β

At molecular level, the mechanism of fibrosis is the result of the interaction of a variety of pro- and anti-fibrotic factors as well as inflammatory cytokines. Pro-fibrotic growth factors include platelet-derived growth factor, TGF- β , etc. TGF- β_1 is one of the most important pro-fibrotic cytokines, which involves in the regulation of cell proliferation, differentiation, apoptosis, autophagy and ECM synthesis.^{12,13} Studies have shown that siRNA-mediated TGF- β_1 gene silencing can inhibit activation, proliferation of HSCs and synthesis of ECM, thereby relieving liver fibrosis.

TGF, which was first introduced by Moses et al,¹⁴ was called transformation fibroblast stimulating factor to the cancer cell phenotype. Later it turned out that there are many branches in TGF family such as TGF- α and TGF- β , and the latter was first isolated in 1978 with six isoforms from β_1 to β_6 . TGF- β is a polypeptide composed by 112 amino acids, encoded by a gene located on the long arm of chromosome 19. TGF- β_{1-3} are present in humans, mammals and birds, while TGF- β_4 and TGF- β_5 in birds and amphibians. In humans, the predominant isoform is TGF- β_1 , which is synthesized primarily by platelets, macrophages/monocytes, lymphocytes, fibroblasts, epithelial cells and dendritic cells. The positive expression of TGF- β_1 mainly locates in the periportal and centrilobular vein around the cells in liver tissue. TGF- β_1 is a homodimer with a mass of 25 kDa. The amino acids sequences of TGF- β_1 proteins across different species are very stable, which leads to the conclusion that in the process of evolution, TGF- β has been only slightly altered, and that its function is similar in both humans and animals.¹⁵

There are three fundamental directions of TGF- β 's activities: (1) regulating cell proliferation, growth, differentiation and cells movement; (2) immunomodulatory effects; and (3) profibrogenic effects.¹⁵ TGF- β_1 is the most effective stimulated cytokine in HSCs activation, which plays a very important role in the development of liver fibrosis.¹⁶ The mechanism of TGF- β_1 leading to ECM accumulation is as follows: (1) directly increasing synthesis of ECM components as isoform I collagen; (2) inhibiting expression of tissue collagenases; (3) increasing synthesis of ECM-degrading enzyme inhibitors (such as plasminogen activator inhibitor-1, tissue inhibitor of metalloproteinase). TGF- β_1 plays a key regulatory role in the conversion process of HSCs through direct and indirect pathways.

TGF- β produces biological effect mainly through TGF- β -Smad signaling pathway. Current studies have found that there are 10 kinds of Smad protein family,

divided into three categories according to their roles in TGF- β signaling pathway: (1) receptor modulators of Smads (R-Smads), including Smad 1, 2, 3, 5 and 8. Smads 2 and 3, involved in the TGF- β signal transduction, are the target molecule effectors of TGF- β 's downstream receptor complex; (2) general Smads, i.e. Smad 4, combined with other Smads, are necessary transit molecules in TGF- β signal transduction; (3) inhibitory Smads (I-Smads), including Smads 6 and 7, can be competitive in binding with R-Smads, inhibiting TGF- β signal transduction molecules.^{17,18} TGF- β signaling activity works in the cells only when they have specific membrane receptors which appear as dimeric proteins. So far, nine different isoforms of molecules (receptors and proteins) having the ability to bind with TGF- β have been identified. The best known receptors are isoforms I, II, and III. These three versions of TGF- β receptor (TGF- β RI, TGF- β RRII, and TGF- β RIII) regulate the signaling activity of all TGF- β isoforms.

TGF- β_1 may promote HSCs activation via TGF- β /Smad pathway, and facilitate HSCs transformation into myofibroblast-like cells in order to induce contraction of activated HSCs. It can be activated and stimulate synthesis and deposition of ECM, including collagens I, III and IV.¹⁹ Therefore inhibiting or blocking the expression of TGF- β_1 will likely suppress the activation of HSCs and reduce formation and deposition of ECM, thereby preventing the development of liver fibrosis.

EFFECT OF TARGETING TRANSFORMING GROWTH FACTOR- β SMALL INTERFERING RNA ON LIVER FIBROSIS

Compared with normal tissue, the expression level of TGF- β is increased 6-8 times in hardened tissue, positively correlated with the degree of cirrhosis.²⁰ Huang et al²¹ established that in mice liver fibrosis model infected with *Schistosoma japonicum*, they observed the elevated expression of TGF- β_1 and connective tissue growth factor, egg granuloma gradually gathered in the portal district, and tree-shaped formation of liver fibrosis around the portal vein branches at 6, 8, 10 and 12 weeks after schistosomiasis infection. The expression of connective tissue growth factor mRNA was related with duration of infection, while the highest level of TGF- β_1 mRNA was observed at 10 weeks after infection, which had no correlation with duration of infection. Guo et al²² observed the role of TGF- β_1 in the process of liver fibrosis and found that along with the increasing severity of liver fibrosis, the expression of TGF- β_1 protein showed a rising trend, the positive rate was 58.33%, 78.00% and 88.88%, indicating

that TGF- β_1 may be involved in the formation of liver fibrosis. Liu et al²³ observed the biological effectiveness of the entirely exogenous TGF- β_1 by paracrine stimulation to quiescent state, intermediate activation and activation state of HSCs. They found that TGF- β_1 showed an significantly proliferation-inhibiting effects on stationary state of HSCs. However, the growth of HSCs in fully activated state had no visible change, but TGF- β_1 could promote the activation of fully activated phenotype of HSCs and collagen production, and distort its function. HSCs in intermediate activation state showed the most obvious pro-phenotype generation and collagen production; HSCs in activation state were accompanied with the disappeared reaction of the TGF- β_1 -mediated growth inhibition, in order to facilitate proliferation of HSCs in liver fibrosis and repair wound.

siRNA is a powerful tool for gene silencing. Its adjustability and strong targeting could effectively inhibit the expression of target genes in order to reduce the corresponding protein levels and function.²⁴ Ellermeier et al²⁵ treated the mice model of tumor with TGF- β siRNA combining RIG-I signaling and observed antitumor efficacy against pancreatic cancer by breaking tumor-induced CD8(+) T cell suppression. Li et al²⁶ blocked the TGF- β signal pathway in T24 human bladder cancer cells with siRNA, and noted down-regulation of the expression of α_3 , β_1 and α_2 integrin subunits and the enhancement of the activity of matrix metalloproteinase 9 by exogenous TGF- β_1 . They suggested that inhibition of TGF- β_1 signaling pathway by siRNA could be beneficial in the treatment of patients with metastatic bladder cancer. Using a short hairpin RNA vector designated shTB1d, Hwang et al²⁷ significantly suppressed TGF- β_1 in both transcriptional and translational levels *in vitro* (in cultured cells) and *in vivo* (in fibrosis-induced mouse kidney), accompanied by the suppression of target genes (e.g., type I collagen and PAI-1) of TGF- β_1 . Furthermore, shTB1d suppressed the expression of TGF- β_1 and type I collagen in tubule interstitial cells until 7 days after unilateral ureteral obstruction-induced fibrosis, compared with the maintained expression level in none- or vector-treated mice, suggesting that the TGF- β_1 shRNA delays the process of renal fibrosis in unilateral ureteral obstruction-induced renal fibrosis mouse model. Their work would provide a valuable tool to prevent tubule interstitial fibrosis using RNA interference strategy. Similarly, taking HSCs as the core and designing siRNA to inhibit the expression of TGF- β_1 may reverse the progress of liver fibrosis. Lang et al²⁸ designed to investigate the anti-fibrotic effects using siRNA to target TGF- β_1 in liver fibrosis in rats, which were exposed to a high-fat diet and

carbon tetrachloride (CCL₄). They found that compared to the model group, the TGF- β_1 siRNA negative control group and the TGF- β_1 siRNA 0.125 mg/kg treatment group, the group treated with TGF- β_1 siRNA 0.25 mg/kg showed significant reduction of pathological changes, protein expression and the mRNA expression of TGF- β_1 , type I collagen and type III collagen. The possible mechanism is that through the down-regulation of TGF- β_1 expression, siRNA could inhibit HSCs activation, as well as the proliferation and collagen production, so that collagen deposition in the liver is reduced. Sun et al²⁹ investigated the changes in Smads 2, 3, 4 and 7 of the TGF- β_1 /Smad signaling pathways in CCL₄-induced liver fibrosis rats treated with TGF- β_1 siRNA. They found that siRNA-mediated silencing of TGF- β_1 in rats led to significantly reduced expression of Smads 2, 3 and 4, but significantly increased expression of Smad 7. TGF- β_1 regulation of Smad signaling molecules may contribute to liver fibrosis in rats and represent an intervention target of future therapeutic methods.

In conclusion, liver fibrosis is the result of the wound-healing response of the liver to repeated injury, resulting from chronic damage. Liver fibrosis is a dynamic process, however, it is difficult to reverse when cirrhosis is formed. Therefore, comprehending the pathogenesis of liver fibrosis, blocking or reversing liver fibrosis have great significance for the clinical treatment of chronic liver disease. siRNA is used not only to study gene function, but also as a highly efficient, specific and targeted treatment. As an effective treatment of liver fibrosis, studies of gene therapy in recent years have shown that siRNA-mediated TGF- β_1 gene silencing is a promising treatment of liver fibrosis. TGF- β_1 is considered the most critical cytokine to participate in liver fibrosis, targeting TGF- β_1 siRNA can inhibit expression, activation, proliferation of TGF- β_1 and synthesis of ECM, thus preventing the progress of liver fibrosis. However, long-term suppression of TGF- β_1 may be harmful to human health since it has a strong anti-proliferative and immunosuppressive effects simultaneously. In addition, rapid acceptance of the use of siRNAs has been accompanied by recognition of several hurdles for the technology, including lack of specificity. Off-target activity can complicate the interpretation of phenotypic effects in gene-silencing experiments and potentially lead to unwanted toxicities.³⁰ Design of efficient siRNA is key to achieving the target gene-specific silencing. Translation of the technology from *in vitro* and animal experiment to the clinical treatment of liver fibrosis is the direction of our efforts in the future.

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