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# BIOCONJUGATION OF OLIGONUCLEOTIDES FOR TREATING LIVER FIBROSIS

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## Abstract

Liver fibrosis results from chronic liver injury due to hepatitis B and C, excessive alcohol ingestion, and metal ion overload. Fibrosis culminates in cirrhosis and results in liver failure. Therefore, a potent antifibrotic therapy is in urgent need to reverse scarring and eliminate progression to cirrhosis. Although activated hepatic stellate cells (HSCs) remains the principle cell type responsible for liver fibrosis, perivascular fibroblasts of portal and central veins as well as periductular fibroblasts are other sources of fibrogenic cells. This review will critically discuss various treatment strategies for liver fibrosis, including prevention of liver injury, reduction of inflammation, inhibition of HSC activation, degradation of scar matrix, and inhibition of aberrant collagen synthesis. Oligonucleotides (ODNs) are short, single-stranded nucleic acids, which disrupt expression of target protein by binding to complementary mRNA or forming triplex with genomic DNA. Triplex forming oligonucleotides (TFOs) provide an attractive strategy for treating liver fibrosis. A series of TFOs have been developed for inhibiting the transcription of  $\alpha 1(I)$  collagen gene, which opens a new area for antifibrotic drugs. There will be in depth discussion on the use of TFOs and how different bioconjugation strategies can be utilized for their site-specific delivery to HSCs or hepatocytes for enhanced antifibrotic activities. Various insights developed in individual strategy and the need for multipronged approaches will also be discussed.

## 1. INTRODUCTION

Fibrosis is characterized by an excessive production of extracellular matrix (ECM) components, in the interstitial space of an organ which if not controlled can result in organ dysfunctional (1). Liver fibrosis occurs in response to a variety of insults, including viral hepatitis (especially hepatitis B and C), excessive alcohol ingestion, drugs, metabolic diseases due to overload of iron or copper, autoimmune attack of hepatocytes or bile duct epithelium, or congenital abnormalities. Cirrhosis is an advanced stage of liver fibrosis, characterized by the formation of regenerative nodules of liver parenchyma separated by fibrotic septa. Cirrhosis is one of the leading causes of death in the United States, accounting for more than 25,000 deaths in 2000 (2). Major clinical complications of cirrhosis include ascites, renal failure, hepatic encephalopathy, and variceal bleeding. (3). Furthermore, cirrhosis is largely associated with primary liver cancer, with a further increase in the relative mortality rate (4,5). Cirrhosis is divided into compensated and decompensated cirrhosis. Patients with compensated cirrhosis may remain free of major complications for several years, while decompensated cirrhosis is associated with short survival (6).

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Regardless of extensive efforts, liver transplantation is currently the only curative approach for treating cirrhosis (7). However, the limited number of donor organs available and the condition of the potential recipient limit the applicability of this technique. Furthermore, in patients with hepatitis C virus (HCV)-induced cirrhosis, viral infection recurrence after transplantation leads to aggressive chronic hepatitis and progression to cirrhosis. Traditionally, liver fibrosis is considered reversible. However, reversibility of advanced liver fibrosis/cirrhosis may also be possible. In current view, death of parenchymal cells (hepatocytes) followed by inflammatory response in the injured liver initiates the liver repair process; besides the recruited leukocytes, Kupffer cells (KCs) as well as sinusoidal endothelial cells (SEC) are involved in the inflammation; and activated hepatic stellate cells (HSCs) are the key fibrogenic cells responsible for the excessive production of fibrillar collagens (Type I and III collagens) and other sources of fibrogenic cells are also possible (Figure 1 & 2). The interaction of these cells as well as many others results in the progression of liver fibrosis after chronic liver injury. Therefore, there is a considerable interest in developing antifibrotic strategies for treating liver fibrosis. Better understanding of pathophysiology of liver fibrosis is essential for developing antifibrotic treatment strategies. Current efforts in developing antifibrotic drugs are heavily relying on these basic understandings. Activated HSCs become the main focus. Different treatment strategies in development are discussed.

Emergence of various gene silencing technologies has boosted the possible use of nucleic acids (plasmid DNA, antisense oligonucleotides (ODNs), triplex forming oligonucleotides (TFOs), small interference RNA (siRNA)) for treating liver fibrosis. TFOs may confer some advantages over others due to their act on DNA rather than mRNA. Introduction of exogenous genes or specifically gene silencing provide attractive ways to regulate the function of key modulators in liver fibrosis. Therefore, efforts in developing TFOs as gene silencing therapeutics will be summarized with implications for treating liver fibrosis. However, there are many barriers for the application of nucleic acids *in vivo*, such as instability against nucleases, poor cellular uptake, and non-specific biodistribution. Therefore, the lack of efficient and targeted delivery after systemic administration of nucleic acids has impaired their use as therapeutics. Even though studies have shown that liver is the disposition site for phosphorothioate modified ODNs (PS-ODNs) after their systemic administration, there have still not been many studies for chemically modified as well as G-rich ODNs, which may have different biodistribution profiles. In addition, due to the potent gene silencing ability of ODNs and TFOs, a site-specific delivery is required to avoid toxic side effects in the body and maximize their efficiency *in vivo*.

Among different delivery strategies for ODN-based therapeutics, bioconjugation on ODNs is very attractive compared to other delivery strategies, such as cationic liposomes and cationic polymers, especially for the conditions of liver fibrosis. The pharmacological activity and pharmacokinetics of ODNs can be significantly improved by simple conjugation chemistry with lipids, sugars polyethylene glycol (PEG) and peptides among others. Moreover, conjugation with polymers due to their versatility and biocompatibility presents a beautiful scene in ODN delivery, in which many different components aimed at overcoming barriers for *in vivo* delivery of ODNs can be assembled. Carriers with high efficiency and safety can be constructed with a multicomponent polymer for ODNs. Several promising strategies for delivery of nucleic acids to the liver will be discussed.

Naturally, the liver is involved in the disposition of a variety of macromolecules including ODNs by phagocytotic processes as well as receptor-mediated endocytosis and followed by their degradation in KCs, HSCs, SECs and hepatocytes. For targeting different liver cells, receptor-mediated endocytosis in various cell types has been utilized extensively (8). On hepatocytes, asialoglycoprotein receptor (ASGPR) recognizes galactose- and N-acetyl-galactosamine (GalNAc)-terminated glycoproteins (<10 nm); on KCs, there is a mannose/

GalNAc/fucose receptor. In addition, a galactose-particle receptor also exists on KCs, recognizing particles exposing galactose larger than 10nm. Moreover, KCs exhibit receptors (scavenger receptors) for positively charged as well as negatively charged particles. Furthermore, these scavenger receptors are also present on SECs as well as HSCs. Therefore, modifications of cargoes with these different ligands present attractive specific targeting strategies for drug delivery to different liver cell types for varieties of purposes. For example, glycoproteins can be recognized by sugar-specific receptors or scavenger receptors in various cell types in the body and have been extensively used for drug delivery to the liver. Onto these glycoproteins, ODNs can be conjugated for their delivery to the liver cells. Specifically, a summary of targeted delivery of drugs to HSCs for treating liver fibrosis will be presented. We proposed a targeted delivery system for TFOs specific for  $\alpha 1(I)$  collagen to HSCs for treating liver fibrosis. Finally, we conclude with an outlook for the future and the strategies holding promise for treating liver fibrosis.

## 2. STRUCTURE AND FUNCTION OF THE LIVER

In the liver, there is rapid exchange of blood constituents with the tissue, which is related to both the impressive blood flow (25% of the cardiac output) as well as the fenestrated endothelium that allows direct contact of blood components with the very large surface of the villous plasma membranes of hepatocytes. It performs a large number of tasks that impact our body systems, such as detoxifying, manufacturing proteins, synthesizing, storing, and processing of fats, metabolizing and storing of carbohydrates, forming and secreting bile and eliminating the potentially harmful biochemical products from the body.

### 2.1. Types and Functions of Liver Cells

Liver contains several cell types, including hepatocytes, SECs, KCs, and HSCs, which contribute to 78%, 2.8%, 2.1%, and 1.4% of total liver volume and 92.5%, 3.3%, 2.5%, and 1.7% of the total liver cell volume, respectively (8). However, other cell types including pit cells, monocytes, T- and B-lymphocytes, bile duct epithelial cells (cholangiocytes), vascular smooth muscle cells, second-layer cells and portal fibroblasts are also involved in the different liver functions and may play important roles in liver fibrosis.

**2.1.1. Hepatocytes**—Hepatocytes are the major liver epithelial cells responsible for most of liver functions. They are metabolically active cells and contain a vast array of organelles. Their strategic position between two different environments, the blood and the bile, makes hepatocytes unique compared to other parenchymal cells. The hepatocyte plasma membrane is differentiated into sinusoidal, contiguous, and canalicular domains, whose relative surface areas are 77%, 15%, and 13%, respectively. The sinusoidal domain is the site of the sodium pump and organic ion and drug transporters. Receptors for glycoproteins, immunoglobulin A, asialoglycoprotein, various peptides and hormones, and growth factors are also located in this domain.

**2.1.2. Sinusoidal Endothelial Cells**—Liver SECs are regarded as: (a) “selective sieves” for substances passing from the blood to hepatocytes and HSCs, and vice versa, (b) a “scavenger system” which clears the blood from many different macromolecular waste products that originate from turnover processes in different tissues. These cells express a variety of adhesion molecules and have a capacity to secrete cytokines. In addition, SECs are also responsible for producing type IV collagen in normal liver (9).

The main structural characteristic of SECs is the fenestrae of 50–150 nm in diameter. Fenestrae filter fluids, solutes and particles that are exchanged between the sinusoidal lumen and the space of Disse, allowing only particles smaller than the fenestrae to reach the parenchymal cells or to leave the space of Disse. Molecules with a molecular weight exceeding 250 kD

cannot pass through the pores (10), and therefore do not interact with hepatocytes. Furthermore, the fenestrae controls the interchange between blood and perisinusoidal space, and its diameter is influenced by endobiotics (hormones and neurotransmitters) and xenobiotics (ethanol and drugs) (10).

**2.1.3. Kupffer Cells**—KCs are part of the reticuloendothelial system (RES) and represent 80–90% of all the resident liver macrophages (8). They attach to the sinusoidal wall, but possibly migrating along the lumen (11). KCs are the first cells in the liver to be exposed to materials absorbed from the gastrointestinal tract. Their ability to eliminate and detoxify microorganisms, endotoxins, degenerated cells, immune complexes, and toxic agents (e.g., ethanol) is an important physiological function. Upon activation, they secrete a number of products with potent biologic effects, including oxygen-derived free radicals, proteases and cytokines with influence on parenchymal and other sinusoidal lining cells (8).

**2.1.4. Hepatic Stellate Cells**—HSCs are also called para- or perisinusoidal cells, Ito cells, fat storing cells, vitamin A storing cells, lipocytes, and arachnocytes. HSCs occupy a perisinusoidal location, with cytoplasmic extensions wrapped around the sinusoidal endothelial lining, comparable to pericytes in other locations and they have spindle-shaped cell bodies with oval or elongated nuclei. They have moderately developed rough endoplasmic reticulum, juxtanuclear small Golgi complex, and prominent dendritic cytoplasmic processes. These subendothelial processes extend beneath endothelial cells and wrap around sinusoids. A single HSC usually surrounds more than two nearby sinusoids. On the luminal side, multiple processes extend across the space of Disse to make contact with hepatocytes and this intimate contact between HSCs and neighboring cell types may facilitate intercellular transport of soluble mediators and cytokines. In addition, HSCs have direct connections with nerve ending, which may be important for neurally mediated vasoregulation.

HSCs are the principle ECM producing cells in the liver (12). They play an important role in the maintenance of the ECM by synthesis and secretion of its normal components and their degradation by proteases. During the fibrotic process they can be transformed to myofibroblasts to increase the portal resistance in periportal areas and slow down portal blood flow (10). Besides, HSCs also have other major functions in a normal liver: (a) control of microvascular tone; (b) storage of retinoids; and (c) a role in the control of regeneration in the normal liver and in response to necrosis. In addition, HSCs represent an important source of cytokines in the liver, including acidic fibroblast growth factor (aFGF), connective tissue growth factor (CTGF), cytokine-induced neutrophil chemoattractant (CINC), endothelin-1 (ET-1), epidermal growth factor (EGF), insulin growth factors I and II (IGF-I and II) and their receptors, interleukin-6 (IL-6), interleukin-10 (IL-10), intercellular adhesion molecule 1 (ICAM-1), neural cell adhesion molecule (NCAM), platelet-derived growth factor (PDGF), platelet-activating factor (PAF), transforming growth factor  $\alpha$  (TGF- $\alpha$ ), transforming growth factor  $\beta$  (TGF- $\beta$ ), vascular cell adhesion molecule (VCAM) and others (13).

## 2.2. Pathogenesis of Liver Fibrosis

The abnormal deposition of ECM is the common characteristic of liver fibrosis regardless of etiology of the liver injury. Liver fibrosis results from the interplay among the injury, inflammation and fibrogenesis of the liver, which implicates sophisticated interactions among different liver cell types (14,15). Normally, liver injury leads to damage of hepatocytes, which is followed by activation of KCs and infiltration of inflammatory cells. Reactive oxygen species (ROS), inflammatory cytokines and growth factors are released, leading to activation of HSCs, which are responsible for the producing excess amount of ECM and progression of liver fibrosis (Figure 1).

**2.2.1. Injury to Parenchymal Cells**—Injury to hepatocytes is the common consequence of many liver diseases including alcoholic hepatitis, nonalcoholic steatohepatitis (NASH), viral hepatitis, and cholestatic liver diseases (16,17). However, intermediates that lead to hepatocyte injury are different. Retention of bile salts is a major cause of liver damage in cholestatic liver disease (17), while viral infections are associated with cytotoxic T lymphocytes (18). T lymphocytes have been shown to induce KCs to release TNF- $\alpha$  (19) or directly destroy hepatocytes (20). Excessive alcoholic ingestion increases oxidative stress by producing ROS, which directly mediate damage to hepatocytes (21). On the other hand, in alcoholic liver injury, ischemia/reperfusion and viral infections, interaction between KCs and lipopolysaccharide (LPS) is the initiating event leading to hepatotoxicity (22–24). Therefore, activation of KCs may happen before or after hepatocyte damage. Activated KCs are a major source of inflammatory mediators and demonstrate increased cytotoxicity and chemotaxis (25–28). Proinflammatory cytokines such as interferon  $\gamma$  (IFN- $\gamma$ ) followed by tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) are released by both KCs and NK lymphocytes (29,30), which leads to expression of ICAM-1 in ECs, allowing the recruitment and sinusoidal transmigration of inflammatory cells including T lymphocytes, macrophages, neutrophils, NK cells and mast cells among others (31).

Liver injury leads to death of hepatocytes either by necrosis or apoptosis (32). Necrosis is most often the consequence of metabolic injury leading to ATP depletion (33), while apoptosis may predominate in cholestatic liver disease (34) as well as NASH (35). Controversies still exist regarding which mode of death predominates in various forms of liver injury. These two events may represent alternate outcomes of cell death mediated by mitochondrial permeabilization (32). For apoptosis of hepatocytes, there are two mechanisms. The extrinsic pathway of apoptosis is signaled through cell surface death receptors, including Fas, TNF- $\alpha$ -receptor-1, and TNF- $\alpha$ -related-apoptosis-inducing-ligand (TRAIL) receptors 1 and 2 (36). Examples of this extrinsic pathway of apoptosis include autoimmune hepatitis, viral hepatitis, chronic alcohol consumption, D-galactosamine (GalN) plus LPS-induced acute liver injury, and ischemia/reperfusion-associated liver injury (37). In contrast, the intrinsic pathway of apoptosis is based on the damage or dysfunction of intracellular organelles, such as lysosomes, endoplasmic reticulum, nucleus and mitochondria (38). It is often seen in drug toxicity or hepatotoxin-induced liver injury, such as acetaminophen overdose and alcohol toxicity (37, 39).

Death of hepatocytes amplifies the inflammation by release of ROS and fibrogenic mediators, recruiting inflammatory cells or directly activating HSCs to synthesize ECM (40). Apoptosis of hepatocytes has now been considered as the nexus of liver fibrosis (41–43). When the magnitude of apoptosis overcomes the capacity to clear cellular debris, apoptotic bodies undergo spontaneous disruption and release their contents, which elicits an inflammatory response (44). Furthermore, engulfment of apoptotic bodies by KCs can induce expression of death ligands, especially Fas, thereby accelerating apoptosis of hepatocytes (45). Phagocytosis of apoptotic bodies by quiescent HSCs has been shown to stimulate their activation via increased intracellular oxidative stress (41,43,46). Other cells including epithelial cells and fibroblasts may also have phagocytic functions (47,48). One of the important consequences when cells phagocytose apoptotic bodies is the generation of TGF- $\beta$  (49,50), which is the most important fibrogenic mediator in liver fibrosis and leads to up-regulation of collagen gene expression in activated HSCs.

## **2.2.2. Hepatic Stellate Cells, the Principle Fibrogenic Cells**

**2.2.2.1. Initiation of HSC activation:** The consequence of liver damage is the generation of large amount of soluble mediators and increased oxidative stress, which result in activation of HSCs. In some liver diseases, some reagents, such as alcohol metabolites, ferritin, and bile

acids may also directly act on HSCs leading to their activation without induction of inflammatory responses (51–53). Initiation of activation represents the rapid early changes in gene expression and phenotype responsive to liver injury (54). HSCs transdifferentiate into myofibroblast-like cells, acquiring contractile, proinflammatory, and fibrogenic properties (54,55) under the direction of growth factors, oxidants, and additional stimuli released from injured hepatocytes and cholangiocytes, KCs, SECs or other recruited inflammatory cells (56–58). Hepatocytes and KCs promote HSC activation by producing ROS leading to oxidative stress (59,60). Cytokines released by damaged neighboring cells activate HSCs. These include TGF- $\beta$ 1 (61), PDGF (62) and ET-1 (63), which stimulate transcription factors such as Sp1, c-myc, nuclear factor  $\kappa$ B (NF- $\kappa$ B), c-jun/AP1, STAT-1, and SMAD proteins that regulate gene expression in activated HSCs. Injury to SECs stimulates the production of type IV collagen, fibronectin, proteoglycans, and urokinase-type plasminogen activator (uPA), which contribute to activation of HSCs through the activation of latent TGF- $\beta$ 1 (64–67).

**2.2.2.2. Perpetuation of HSC activation:** Amplification of activated phenotype of HSCs is called perpetuation, which involves several discrete changes in cell behavior (54): i) proliferation, ii) chemotaxis, iii) fibrogenesis, iv) contractility, v) matrix degradation, vi) retinoid loss and vii) chemoattractant and cytokine release.

Loss of intracellular vitamin A is a notable feature of HSC activation. However, its relationship with HSC activation remains unknown. Minor metabolites of retinoic acid (RA), 9-cis RA and 9, 13-di-cis RA can stimulate the activation of latent TGF- $\beta$ 1 implying a direct link to fibrogenesis (68). Activated HSCs have increased expression of cell membrane receptors including integrins and receptor tyrosine kinases (RTKs) (69). Increased cytokine effects and remodeling of ECM perpetuate the activation of HSCs (70). Activated HSCs also secrete TGF- $\beta$ 1 and have up-regulated level of its receptors, which perpetuates their own activation through autocrine loops (71,72). It is suggested that TGF- $\beta$ 1 is not required for initiating HSC activation (73), which emphasizes the fibrogenic property of TGF- $\beta$ 1.

HSCs accumulate at the injured sites via migration and proliferation. PDGF is the most powerful growth factor for HSCs, but EGF, FGF, or IGF also results in increased HSC proliferation (62). The expression of these growth factors has been markedly increased in hepatic tissue after both acute and chronic liver injury (74,75). PDGF and monocyte chemoattractant protein-1 (MCP-1) are the chemoattractants for activated HSCs (76–78).

Vasoactive substances also regulate HSC growth. Vasoconstrictors including thrombin (79), arginine-vasopressin (80) and angiotensin-II (AngII) (81), exert a mitogenic effect on activated HSCs, whereas vasodilators tend to inhibit cell proliferation, which include prostaglandin E<sub>2</sub> (82) and nitric oxide (83). The role of ET-1, a vasoconstrictor, in liver fibrosis remains disputed. Both profibrogenic and antifibrogenic action of ET-1 have been suggested in the process of liver fibrosis (63,84–86). It induces fibrogenic gene expression in quiescent HSCs, but inhibits proliferation of activated HSCs. ET-1 stimulates the contractility of activated HSCs, which represents an important mechanism underlying increased portal resistance during liver injury (63).

**2.2.2.3. Activation of HSCs and inflammation:** HSCs play an active role in hepatic inflammation (87). Activated HSCs migrate in response to cytokines released by monocytes and secrete a number of proinflammatory cytokines and chemokines including colony-stimulating factor and MCP-1 that could participate in the activation of lymphocytes and the recruitment of white blood cells (neutrophil and monocyte), thus amplifying the inflammatory response (78,88). HSCs can take up and process antigens and, under stimulation with cytokines, express the cell machinery required for antigen presentation and thereby modulate the growth of lymphocytes (89). A direct link between the inflammatory and fibrogenic properties of HSCs

is that they express cluster of differentiation (CD)-40, a receptor whose ligand is present on immune effector cells (90). Therefore, a loop in which inflammatory and fibrogenic cells stimulate each other is likely to occur (91), which results in amplified fibrogenesis.

**2.2.2.4. Survival of activated HSCs:** Apoptosis represents a default pathway for HSCs, and thus appropriate anti-apoptotic signals are needed for their survival (92). The survival of activated HSCs is dependent on soluble growth factors including IGF-1 (93–95), cytokines including TNF- $\alpha$  and TGF- $\beta$  (96), and components of the fibrotic ECM (97). Unlike IGF-1, these cytokines may act via the Fas/Fas-ligand (Fas-L) system (96). In contrast to IGF-1, PDGF has relatively little anti-apoptotic activity. The separate regulation of proliferation and survival in HSCs provides a further control on HSC numbers. Indeed, the expression of a single growth factor with both mitogenic and antiapoptotic activities for HSCs might lead to an uncontrolled and damaging increase in cell numbers during injury. TIMP-1 may also act as a survival factor and is known to be antiapoptotic for HSCs in an autocrine manner, which is independent of its ability to inhibit MMP activity (98).

**2.2.2.5. Increased production of ECM:** Progression of liver fibrosis is a remodeling process of ECM, by degradation of normal ECM and substitution with scar matrix (99,100). Activated HSCs are the principle fibrogenic cells responsible for producing interstitial collagens type I and III, which are the two main components of ECM in liver fibrosis (12,101–103). Matrix metalloproteinases (MMPs), which are responsible for degradation of ECM components, include MMP-1 in human and MMP-13 in rats and mice (104–106) as well as MMP-2 and MMP-14. HSCs and KCs are the major sources of MMPs (104,107,108). MMP inhibitors, tissue inhibitors of metalloproteinases (TIMP) 1 and 2 are also expressed by HSCs (30,109). Activated HSCs produce MMP-2 that degrades basement-membrane ECM (especially type IV collagen) (110). Degradation of normal ECM hastens its replacement by fibril-forming collagen. In addition, degradation of type IV collagen has been shown to facilitate HSC activation (25,100). This combination of enzymes and their inhibitors provides a mechanism for HSCs to degrade normal and fibrotic matrix in the remodeling process and deposit excess amount of ECM during progression of liver fibrosis.

**2.2.3. Existence of Other Fibrogenic Cells—**In most cases, activated HSCs are responsible for liver fibrosis (111) and they may take part in the repair process by migration and proliferation (112,113). However, HSCs themselves are not homogenous cell type and different subtypes are present in the liver parenchyma (114). On the other hand, other potential fibrogenic cell types in liver fibrosis should also be taken into account (Figure 2). Portal myofibroblasts, second-layer cells located around centrilobular veins (CLVs), vascular smooth muscle cells and cells of bone marrow origin have been shown to exhibit fibrogenic potential, probably by modulation to myofibroblastic cells (115–120). In addition, it is not clear what type of fibroblastic cells is involved in fibrosis induced by the intraperitoneal injection of pig serum in rats (121). Compared with activated HSCs, little is known about the factors leading to their proliferation and matrix synthesis (117). For these liver fibrogenic cells, differences may exist in the mechanisms of differentiation, activation and deactivation.

Relative importance of different fibrogenic cells in liver fibrogenesis depends on the origin of liver injury, since they are distributed differently in the hepatic lobule: HSCs are located along the sinusoids in the space of Disse; whereas the portal fibroblasts are in the portal tract connective tissue around portal structures. As a consequence, the distribution of fibrous ECM varies in different liver injuries. In chronic viral hepatitis and chronic cholestatic disorders, the fibrotic ECM is initially located around portal tracts, while in alcohol-induced liver diseases, it is located in the pericentral and perisinusoidal areas (103). However, in one disease status, different types of fibrogenic cells may be involved. For example, in biliary liver fibrosis, the

involvement of portal fibroblasts around biliary structures have been demonstrated, at least for the early stages of fibrosis and HSCs may be involved later (118,122,123).

Phenotypic and functional properties of hepatic myofibroblasts are similar to those of activated HSCs. However, several phenotypic markers can be used to distinguish them, including selective expression of fibulin-2 and IL-6 by hepatic myofibroblasts and protease P100 and reelin by activated HSCs (124–126). With increased amount of evidence for existence of hepatic myofibroblasts involved in liver fibrosis, the concern is that liver fibroblastic cells other than typical HSCs might have been analyzed since myofibroblasts proliferate better than HSCs *in vitro* (127). Therefore, clear classification of the liver fibrogenic cells is necessary and care should be taken when extrapolating data from *in vitro* studies to pathological situations.

**2.2.4. Deposition of Extracellular Matrix**—In normal liver, ECM may be interstitial or basement membrane-like (59). Interstitial ECM is in the connective tissue of the fibrous external capsule, septa, periductal and perivascular areas and portal tracts, rich in collagen types I, III, V and fibronectin, while basement membrane-like ECM is rich in types IV, VI together with laminin and fibronectin in the space of Disse. Fibroblasts are the cells essentially responsible for ECM production in interstitial space, while the ECM in the space of Disse is mainly dependent on HSCs.

In an acute liver injury, hepatocytes regenerate and replace the necrotic or apoptotic ones in response to inflammatory response, leading to limited deposition of ECM, especially type III collagen (128). However, this process is restricted in chronic injuries to the liver. In advanced liver fibrosis/cirrhosis, fibrotic septa separate regenerative nodules in liver parenchyma, which leads to liver dysfunction. Although these major morphological changes represent the most commonly observed form of scarring, it is actually the early deposition of fibrillar ECM in the subendothelial space of Disse that is more directly responsible for the progressive reduction of liver function (129).

Regardless of etiology of liver injury, ECM deposition is the common characteristic at the fibrotic stage, which changes both the quantity and composition of normal ECM (130). In normal liver, collagens (Types I, III, V, and XI) are largely confined to the capsule, the area around large vessels and the portal triad, with only scattered fibrils containing types I and III in the subendothelial space (128). In advanced fibrosis, the liver contains approximately 6 times more ECM, including collagens (I, III, and IV), fibronectin, undulin, elastin, laminin, haluronan, dermatan and proteoglycans (131–133). Among them, type I collagen increases the most (131,134–136). Excessive deposition of ECM results from both increased synthesis and decreased degradation of collagens (99). Expression of type I collagen is regulated at both the transcriptional and posttranscriptional levels (137). Posttranscriptional regulation results in up to 20-fold increase in the half life of collagen  $\alpha 1(I)$  mRNA (138,139). The production of type I collagen can be increased ~70 fold by activated HSCs (137). In addition, the degradation of ECM is down-regulated. Although mRNA for interstitial collagenases, such as MMP-1 and MMP-13 remains unaltered as fibrosis develops (105,106,140), their activities are inhibited by TIMP-1 and TIMP-2 (104–106). Furthermore, MMPs are released as inactive pro-enzymes, which needs cleavage of the inhibitory N-terminal peptide to confer activity (141). The means of pro-enzyme activation varies between different MMPs and the protease plasmin is required for efficient activation of pro-MMP-1 (142). Plasmin synthesis can be inhibited by plasminogen activator inhibitor-1 (PAI-1), which is produced by activated HSCs (143,144).

ECM has been shown to actively participate in the progression of liver fibrosis by providing survival factors for activated HSCs. It is a reservoir for growth factors and MMPs (145). Replacement of the low-density matrix of basement membrane by high-density interstitial matrix leads to capillarization of the sinusoids with formation of a continuous endothelium.

This contributes to alterations in the phenotype of HSCs, sinusoidal endothelial cells, and hepatocytes (146–149). ECM rich in fibril-forming collagen accelerates activation of HSCs through interactions with integrins, the classic ECM receptors and RTKs such as discoidin domain receptor (DDR) (69,150).

**2.2.5. Different Patterns of Fibrosis**—Different liver diseases induce different types of liver injuries, resulting in different patterns of liver fibrosis during disease progression (151). This includes portal-portal septa surrounding liver nodules in which the central vein and its connections with the portal tract are preserved until late stages; portal-central (vein) septa, central-central septa and that in which the deposition of fibrillar matrix is concentrated around the sinusoids and groups of hepatocytes.

In both alcohol-induced liver disease and NASH, early changes are concentrated in the area of CLV: (i) around the sinusoids with capillarization of the sinusoids, (ii) around groups of hepatocytes, and (iii) around the central vein (117). The fibrogenic cells in the center of the lobule are recruited: *in situ* activated HSCs, second-layer myofibroblasts around the CLV, and smooth muscle cells from the CLV wall (125). Infection with HCV can cause different injuries (152). In some cases, it may have portal inflammation, with additional damage to bile ducts and portal vessels. Interface hepatitis is defined as inflammation and damage to the hepatocyte plates surrounding the portal tract, whereas lobular hepatitis is characterized by hepatocyte damage with or without inflammatory cells, either dispersed throughout the lobules or confluent, leading to bridging necrosis (often from central vein to portal tract and from central vein to central vein). Therefore, ECM deposition is contributed by the fibrogenic myofibroblasts located in portal spaces and the fibrogenic cells recruited at the interface between the fibrous septa and the parenchyma. HSCs activated *in situ* may also be involved in interface hepatitis damage repair and/or may migrate towards the portal tract (78). In primary biliary cirrhosis (PBC), T lymphocytes and cytokines mediate persistent bile duct damage (115). Biliary cells secrete fibrogenic mediators activating neighboring portal myofibroblasts to secrete ECM. At the end, perisinusoidal HSCs become activated and fibrotic bands develop.

**2.2.6. Resolution of Liver Fibrosis**—In contrast with the traditional view, evidences indicate that even advanced fibrosis/cirrhosis is reversible (153–155). Spontaneous resolution of liver fibrosis has been observed in humans after successful treatment of the underlying diseases (153,156–161). However, resolution of advanced liver fibrosis is not always possible and may be limited due to ECM cross-linking and resistance to apoptosis of activated HSCs (130,162). To understand the mechanisms of resolution of liver fibrosis, establishing and analyzing a model of spontaneous recovery from liver fibrosis is needed. Elimination of activated HSCs plays key role in resolution of liver fibrosis (163). However, since it is not easy to clearly define the fate of activated HSCs during resolution of fibrosis in human (164), most of conclusions are based on studies in animal models of liver fibrosis.

Apoptosis is one mechanism for diminishment of activated HSCs. In both bile duct ligated (93) and carbon tetrachloride (CCl<sub>4</sub>)-induced liver fibrosis models (165,166), apoptosis of activated HSCs has been observed in resolution process, which decreases the production of both ECM and TIMPs. Apoptosis is a default pathway for HSCs. When partial degradation of fibrillar collagen occurs, the altered interaction between activated HSCs and ECM favors apoptosis (154). Death ligands such as Fas-L, and TRAIL-2 and -5, and their receptors are up-regulated in activated HSCs (166–168). The low-affinity NGF receptor/P75, another member of the TNF receptor superfamily may also be involved in apoptosis of activated HSCs (169). In addition, mast cells, which accumulate during fibrotic injury in areas adjacent to fibrosis, are a potent source of NGF (170), suggesting that incoming inflammatory and immune cells may also modulate HSC numbers through the control of apoptosis. Another possibility for the decreased number of activated HSCs during recovery from liver fibrosis is the conversion of

activated HSCs to quiescent status (171,172). A basement membrane-like ECM has been suggested to induce the deactivation of HSCs (171). However, this process is trivial, because there is no evidence of increased number of perisinusoidal cells after resolution (165).

Accompanying with the loss of activated HSCs is the increase in collagenolytic activity (130). During fibrosis resolution, the activity of MMPs was found to increase due to a rapid decrease in TIMP-1 and -2 expression (153,165). Since expression of MMP-13 in rats is constant despite reduction in HSC numbers (92), other cell types including KCs or inflammatory cells are the potential sources of MMP-13 and other types of MMPs including MMP-2 and MMP-14 might also contribute the increased collagenase activity. In addition, IL-10 produced by HSCs themselves down-regulates inflammation and increases interstitial collagenase activity, which also contribute to the resolution of liver fibrosis (173,174).

### 3. THERAPEUTIC APPROACHES TO LIVER FIBROSIS

Substantial progress has been made in understanding the pathogenesis of liver fibrosis, which has yielded potential new therapeutic targets. Antifibrotic therapies to regulate fibrosis independent of liver injury are likely to benefit all patients with fibrotic liver (158). The hope is that if antifibrotic therapy can reconstitute the normal microenvironment of liver, normal function can be restored and clinical manifestations may regress. Current and evolving approaches primarily target the activated HSCs to inhibit their activation, proliferation, and products (175). Gene therapy and gene silencing technologies may provide more opportunities. Although experimental studies have revealed targets to prevent fibrosis progression in rodents, the efficacy of most treatments has not been proven in humans (176).

Treatment strategies for liver fibrosis includes i) prevention of liver injury, ii) reduction of inflammation or host immune response, iii) inhibition of HSC activation, iv) degradation of scar matrix, v) induction of HSC apoptosis, vi) stimulation of liver regeneration, vii) removal of initial fibrotic stimulus, and viii) inhibition of collagen synthesis (Figure 3).

#### 3.1. Cure the Primary Disease

The most effective way to treat liver fibrosis is to clear underlying causes of liver diseases. Treatment of chronic HCV infection is of particular interest since it is the most common cause of cirrhosis in Western countries. IFN- $\alpha$  has been suggested to have antifibrotic effect in addition to its antiviral effect (177,178). Using IFN- $\alpha$  plus ribavirin (RBV), the persistent clearance of viral infection can be achieved with the resolution of hepatic inflammation and liver fibrosis/cirrhosis (179–182). Furthermore, Pegylated IFN may provide a sustained virologic response and has been approved for treating chronic HCV infection (183,184). Even though the treatment is better tolerated in patients with chronic hepatitis C and up to moderate fibrosis (185), it is poorly tolerated by patients with advanced-stage recurrent HCV (186). In CCl<sub>4</sub>-induced rat liver fibrosis, pegylated IFN- $\alpha$ -2b plus ursodeoxycholic acid (UDCA) has been shown to improve regression of liver fibrosis (187).

#### 3.2. Inhibition of Hepatocyte Apoptosis

There is direct link between hepatocyte apoptosis and liver fibrogenesis. Fas-deficient lymphoproliferation (lpr) mice show decreased inflammation and fibrosis following bile duct ligation (43). IDN-6556, a general inhibitor of caspases has been shown to reduce hepatocyte apoptosis and fibrosis (188,189) and is currently undergoing phase II clinical trials (190).

Nucleic acid-based gene silencing to interrupt signaling pathways (e.g., Fas/Fas-L system and other intracellular mediators) in hepatocyte apoptosis is a promising strategy for treating liver fibrosis. An antisense ODN, specific for mouse Fas receptor, reduced Fas mRNA and protein expression by 90%, and therefore prevented hepatocyte apoptosis in the liver (191). In another

study, using Fas-specific siRNA, long-term inhibition of Fas expression was achieved and liver fibrosis induced by repeated concanavalin A administration is inhibited (192). Bcl-2 family is the regulators of apoptosis, which block cytochrome C release during apoptosis signaling (193). Bid, which can promote apoptosis, is one member of this family. Using an ODN against Bid caused an 80% decrease in Bid expression and protected hepatocytes from Fas-mediated apoptosis in mice (194). siRNA against caspase 8 has been successfully used to prevent hepatocyte apoptosis in mice (195). However, the effect of agents on liver fibrosis and safety still remains to be confirmed. In addition, administration of molecules interfering with hepatocyte apoptotic pathways carry a high risk of carcinogenesis, particularly at the cirrhotic stage. Therefore, this option should be considered at early stages of chronic liver diseases.

### 3.3. Reduction of Inflammation and Immune Response

Corticosteroids are indicated for treating hepatic fibrosis in patients with autoimmune hepatitis and acute alcoholic hepatitis (156). In autoimmune hepatitis, patients responding to corticosteroids progress more slowly to fibrosis and cirrhosis (196). However, it is not effective in autoimmune cholestatic diseases such as primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC) (197,198). Malotilate is another agent with anti-inflammatory effects that is under investigation for treating liver fibrosis. It has been shown to prevent collagen deposition by inhibiting cytochrome P450 activity in animal models (199). However, this drug failed to improve liver histology and survival of patients with PBC or alcoholic liver disease (200).

Proinflammatory cytokines and their receptors present targets for inhibiting liver inflammation and therefore liver fibrosis. Antagonists of IL-1 receptor (201) or soluble TNF- $\alpha$  receptors (202) are associated with diminished hepatocyte necrosis and inflammation in the liver. IL-10 has been shown to have a negative auto-regulatory effect on collagen production by HSCs (173). IL-10 deficient mice have been shown to develop greater inflammation and fibrosis than wild-type mice (174,203). Even though a pilot study on IL-10 had suggested its therapeutic potential in patients with chronic hepatitis C, who did not respond to interferon-based therapy (204), the subsequently long-term study showed no beneficial effects (205).

### 3.4. HSC as a Target of Antifibrotic Drugs

**3.4.1. Modulation of Key Cytokine Production and Signaling Pathways**—The most extensively studied strategy relates to the inhibition of TGF- $\beta$  signaling pathways. TGF- $\beta$  favors the transition of HSCs to myofibroblast-like cells, stimulates the synthesis of ECM proteins, and inhibits its degradation (206). TGF- $\beta$  is synthesized in a latent form linked to a glycoprotein (latent TGF- $\beta$  binding protein, LTBP), which serves as an anchor in the ECM. Proteolytic cleavage of LTBP is a prerequisite for the release and generation of bioactive TGF- $\beta$ . Binding of active TGF- $\beta$  to its receptors results in the phosphorylation and activation of the Smad family of intracellular signaling proteins. Phosphorylated Smad2 and Smad3 recruit the common mediator Smad4 and translocate into the nucleus, regulating gene transcription. On the other hand, several nuclear oncoproteins, such as Ski and SnoN, as well as Smad7, repress the activity of Smad proteins and limit the biological actions of TGF- $\beta$  (207).

Strategies aimed at disrupting TGF- $\beta$  synthesis or signaling pathways have decreased liver fibrosis in experimental models. Camostat mesilate, a serine protease inhibitor that reduces the cleavage of LTBP and thus the release of active TGF- $\beta$  attenuated serum-induced liver fibrosis in rats (208). Approaches used to prevent the binding of TGF- $\beta$  to its receptors include the use of a dominant-negative type II TGF- $\beta$  receptor, the expression of the ectodomain of type II receptor fused to the Fc portion of human IgG, the expression of a truncated type II receptor, and the construction of a soluble type II receptor (209–212). These strategies utilized gene delivery to express specific protein products and showed successful prevention of liver fibrosis.

Inhibition of intracellular signaling steps in TGF- $\beta$  can also prevent liver fibrosis. Using an adenovirus expressing Smad 7, HSC activation and experimental fibrogenesis were inhibited (213). Directly silencing of TGF- $\beta$ 1 is another way to interrupt its signaling pathway and thus prevent progression of liver fibrosis. Kim et al. (214) used siRNA, which is against TGF- $\beta$ 1 mRNA and expressed from a plasmid vector, to silence TGF- $\beta$ 1 expression in CCl<sub>4</sub>-treated mice. TGF- $\beta$ 1 gene silencing was accompanied with reduced production of type I collagen and prevented progression of liver fibrogenesis. However, there is concern about the safety of a prolonged inhibition of TGF- $\beta$  activity in humans, because this cytokine plays a role in defense against cancer, modulates the immune response, and inhibits inflammation.

TNF- $\alpha$  is mainly produced by activated KCs during liver injury and play a crucial role in HSC activation and hepatocyte regeneration besides its involvement in hepatocyte apoptosis. An antisense ODN against TNF- $\alpha$  has been shown to prevent liver damage induced by ethanol and LPS with the help of a liposomal formulation (215,216).

CTGF is a highly profibrogenic molecule implicated in liver fibrogenesis. Intraportal vein injection of CTGF siRNA into CCl<sub>4</sub>-treated rats markedly attenuated the induction of CTGF, type I and III collagen, TGF- $\beta$ 1 genes, as well as activation of HSCs (217). The staging of liver fibrosis was also significantly decreased.

IFN- $\alpha$  and IFN- $\gamma$  markedly decreases HSC activation and collagen synthesis (177,218) and attenuates ECM deposition in experimental liver fibrosis (219–221). Studies in patients with chronic hepatitis C suggest that IFN- $\alpha$  may inhibit the progression of liver fibrosis irrespective of virological response (222,223). A randomized open-labeled multicenter trial demonstrated the antifibrotic activity of IFN- $\gamma$  in HBV infection (224). Recently, it was demonstrated that IFN- $\gamma$  displays antifibrotic effects via STAT-1 phosphorylation, up-regulation of Smad7 expression and impaired TGF- $\beta$  signaling (225). However, high dose or repeated administration of these cytokines needed for treating liver fibrosis may cause systemic toxicity.

In activated HSC, the most potent proliferative factor is PDGF that binds to its tyrosine kinase receptor and is up-regulated upon HSC activation. Glivec, a targeted tyrosine kinase receptor antagonist has attenuated the fibrotic response in fibrotic animal models (162). Pentoxifylline, a phosphodiesterase inhibitor, decreases HSC proliferation *in vitro* and *in vivo* by inhibiting PDGF-related signaling (226–228). Amiloride, an Na<sup>+</sup>/H<sup>+</sup> exchanger inhibitor, decreases PDGF-induced proliferation and modulates the fibrogenic effect of oxidative stress in HSCs (229,230) and has also been shown to be effective in experimental liver fibrosis (231). S-farnesylthiosalicylic acid, a ras antagonist, inhibits proliferation and migration of HSCs and reduces thioacetamide-induced liver fibrosis in rats (232). The semisynthetic analogue of fumagillin, TNP-470, inhibits HSC proliferation by blocking the cell cycle transition from G1 to S, prevents HSC activation, and attenuates the progression of liver fibrosis (233).

**3.4.2. Reduction of Oxidative Stress**—Oxidative stress stimulates HSC activation, and thus reducing oxidative stress is a possible antifibrotic target. They are particularly effective for alcohol-induced liver fibrosis, in which oxidative stress plays a key role (234), but have also shown some benefit in other experimental models such as CCl<sub>4</sub> and iron overload (235, 236). Antioxidant compounds not only inhibit the activation of both HSCs and KCs, but also protect hepatocytes from undergoing apoptosis.

Vitamin E ( $\alpha$ -tocopherol) decreases lipid peroxidation and has beneficial effects in CCl<sub>4</sub>- and iron overload-induced liver fibrosis (237). However, its efficacy in human liver diseases has not been confirmed (238,239). Phosphatidylcholine (PC), a polyunsaturated phospholipid extract from soybeans has been studied as a hepatoprotective and antifibrogenic substance (240) and probably reduces oxidative stress (241) and therefore inhibits the activation of HSCs

(242). A silybin-PC-Vitamin E complex has been demonstrated to be effective in prevention of liver fibrosis in experimental models (243). S-adenosyl-L-methionine (SAME) is a substrate of glutathione synthesis that has hepatoprotective and antioxidant properties (244). It attenuates liver fibrosis in alcohol, biliary obstruction, and CCl<sub>4</sub> models (245,246). SAME is currently used in several human liver diseases, such as alcoholic liver disease, primary biliary cirrhosis, drug-induced liver disease, and cholestasis of pregnancy. In alcoholic patients, it improves survival and delays the need for liver transplantation (247). Other substances with antioxidant properties that also inhibit experimental liver fibrosis include retinoids (retinyl palmitate) and natural phenolic compounds (resveratrol and quercetin) (248,249). Dietary saturated fatty acids have been shown to attenuate liver fibrosis *in vivo* (250). This dietary supplementation reduces endotoxemia, lipid peroxidation, and TNF- $\alpha$  level in rats with ethanol-induced liver disease. Another dietary supplement with antifibrotic action is zinc, a molecule involved in collagen synthesis and degradation (251,252).

**3.4.3. Modulation of Vasoactive Substances**—Vasoactive substances regulate HSC growth. These include vasoconstrictors (AngII, aldosterone, and ET-1) and vasodilators (prostaglandins and nitric oxide). AngII mediates key biological actions in liver fibrosis, including myofibroblast proliferation, infiltration of inflammatory cells and collagen synthesis. Activated HSCs secrete AngII, which induces contraction and proliferation of activated HSCs via AngII type 1 (AT-1) receptor through the activation of NADPH oxidase (81,253), while AngII has anti-fibrogenic effect by interaction with AT-2 receptors in oxidative stress-induced liver fibrosis (254). AT-1 receptor antagonists including captopril, candesartan and losartan inhibit liver fibrosis in bile duct-ligated and CCl<sub>4</sub> treated rats (255–257). In human studies, it is suggested that AT-1 receptor antagonist can decrease the number of activated HSCs (258). A controlled pilot study in hepatitis C showed that losartan reduces liver fibrosis as compared to untreated controls (259).

ET-1 is another vasoconstrictor that merits study. Both ET-1 and its receptors are markedly induced in activated HSCs during liver fibrosis (260,261). ET-1 displays dual pro- and antifibrogenic effects in the liver according to receptor subtypes. In early phases of hepatic fibrogenesis, the expression of ET-A receptors is predominant over ET-B receptors; therefore, ET-1 could play a profibrogenic role (262). In contrast, as liver fibrosis progresses, there is a marked up-regulation of ET-B receptors in the diseased liver, which could prevent progression of liver fibrosis by inhibiting HSC proliferation and collagen synthesis, in which a mechanism involving the sequential generation of sphingosine-1-phosphate (S1P), cyclooxygenase-2 (COX-2)-derived prostaglandins, and elevation of cAMP is suggested (84,85,263,264). In light of these results, selective ET-A antagonists may be a proper strategy to treat liver fibrosis. A report has shown that the administration of a selective ET-A antagonist markedly prevents liver fibrosis development in bile duct-ligated rats (265), whereas treatment with a non-selective antagonist accelerates liver fibrosis in CCl<sub>4</sub> treated rats (266). Several vasodilators, including prostaglandin E<sub>1</sub> (PGE1) (267,268), PGE2 (269) and nitric oxide donors (83), also inhibit proliferation of HSCs and exert antifibrotic activity. Enisoprost, a PGE1 analog was found to suppress type I collagen gene expression (267). The hepatoprotective effect of PGE2 has been related to its antioxidative capacity (269). Because the intrahepatic synthesis of nitric oxide is markedly decreased in advanced human and rat liver fibrosis, it is suggested that nitric oxide also plays a role in the progression of liver fibrosis (270). Other vasoactive substances with some antifibrotic potential are octreotide and adenosine (271,272).

**3.4.4. Induction of HSC/Hepatic Myofibroblast Apoptosis**—The apoptosis of activated HSCs is a key step in the resolution of liver fibrosis, which is accompanied by a restoration of the collagenolytic capacities of MMP-1 and MMP-2 in the liver, subsequent to a decrease in TIMP-1 and TIMP-2 expression, which allows progressive matrix degradation. Gliotoxin, which provokes selective apoptosis of HSCs in cell culture and *in vivo*, leading to

reduced fibrosis in both CCl<sub>4</sub>- and thioacetamide-induced fibrosis (273,274). A COX-2 derived prostaglandin, 15-deoxy  $\Delta^{12,14}$  prostaglandin J<sub>2</sub> (15-D-PGJ<sub>2</sub>) induces apoptosis of hepatic myofibroblasts, which might involve oxidative stress (275). Sphingomyelinase metabolites including ceramide, sphingosine, and S1P also induce apoptosis of hepatic myofibroblasts (276). However, the cell selectivity is a major issue of a proapoptotic strategy, which may result in life-threatening side effects, such as severe or fulminant hepatitis.

**3.4.5. Inhibition of Other Signaling Pathways**—Leptin is a 16kDa protein hormone produced by activated HSCs and may have direct effect on matrix synthesis via up-regulation of TGF- $\beta$  in SECs and KCs (277–279). In mice with leptin deficiency or bearing mutations in leptin receptor, liver fibrosis is reduced, supporting a profibrogenic role of leptin (277,278). These observations suggest that antagonists of leptin receptors should be investigated as antifibrotic agents.

Adiponectin, a major insulin-sensitizing hormone, has also antifibrogenic role by suppressing proliferation, migration and matrix synthesis of activated HSCs. In mice knocked-out for adiponectin enhanced liver fibrosis following treatment with CCl<sub>4</sub> was observed, whereas pretreatment with an adenovirus encoding adiponectin prevents liver fibrogenesis (280). Adiponectin has been suggested to counteract progression of fibrosis in advanced stages since its serum level is elevated in patients with cirrhosis (281). Therefore, agonists of adiponectin receptors may have antifibrotic properties.

The cannabinoid (CB)  $\Delta^9$ -tetra-hydrocannabinol (THC) exerts a wide array of effects via two G protein-coupled receptors, CB1 and CB2. However, the cannabinoid system may be important in liver fibrogenesis. CB1 and CB2 receptors are up-regulated in cirrhotic liver and interestingly, they display opposite effects on liver fibrogenesis (282,283). Activation of CB-2 receptor induces antifibrogenic effects by growth inhibition and apoptosis of activated HSCs (282). It is further suggested that growth inhibition involves COX-2, and apoptosis results from oxidative stress. On the other hand, activation of CB1 receptors promotes progression of fibrosis. A CB1 receptor antagonist SR141716A inhibits progression of fibrosis after chronic liver injury (283). These promising results obviously warrant investigation of the effects of pharmacological antagonists of CB1 receptors and of selective agonists of CB2 receptors.

### 3.5. Inhibition of ECM Deposition

Because the collagen biosynthetic pathway has multiple steps involving transcription, mRNA stability, translation, as well as co-translational and posttranslational modifications, there are many potential targets for intervention.

Prolyl-4 hydroxylase catalyzes the synthesis of hydroxyproline residues, which are critical for the stability of the collagen triple helix. Inhibitors of prolyl-4 hydroxylase include HOE077 and S4682. In rat models of liver fibrosis induced by a choline-deficient L-amino acid-defined diet, CCl<sub>4</sub> and pig serum, administration of HOE077 inhibited hydroxyproline content and histological liver fibrosis (284–286). However, HOE077 inhibits HSC activation *in vitro* and *in vivo*, which might be the predominant mechanism for its antifibrotic effect (287). S4682 decreases liver fibrosis in the CCl<sub>4</sub> rat model, with a concomitant decrease in morbidity as documented by improved liver function tests and decreased ascites formation (288). The antimicrobial compound halofuginone, a potent inhibitor of  $\alpha 1(I)$  collagen gene expression, prevents the development of liver cirrhosis in dimethyl nitrosamine (DMN)-treated rats (289).

Type I collagen is the major structural protein of the ECM during liver fibrosis, which makes it an ideal target of silencing technologies. Type I collagen consists of two  $\alpha 1(I)$  and one  $\alpha 2(I)$  polypeptide chains encoded by the  $\alpha 1(I)$  and  $\alpha 2(I)$  genes, respectively, and synthesized at

a 2:1 ratio. As mentioned before, the enhanced expression of type I collagen by activated HSCs is regulated at both the transcriptional and posttranscriptional levels (137). The production and stability of  $\alpha 1(I)$  collagen mRNA are highly increased in liver fibrosis. Wu et al. designed several sequences of antisense ODN against  $\alpha 1(I)$  and  $\alpha 2(I)$  mRNA and tested their silencing efficiencies in cell culture using asialoorosomucoid (AsOR) modified poly (L-lysine) (PLL) as a carrier (290). The  $\alpha 1(I)$  and  $\alpha 2(I)$  mRNA levels were reduced by 67% and 73%, respectively, which led to significant reduction in collagen synthesis. In another study by Laptev et al. (291), a series of antisense ODNs were tested to define the best target sites within an RNA transcript of collagen for effective inhibition of expression. Mouse NIH 3T3 fibroblasts were stably transfected with a human  $\alpha 1(I)$  collagen gene so that the cells simultaneously synthesized full-length mouse  $\alpha 1(I)$  collagen chains and internally deleted human  $\alpha 1(I)$  collagen chains. It was demonstrated that antisense ODNs could be designed to specifically inhibit production of  $\alpha 1(I)$  collagen from the human gene without affecting the levels of  $\alpha 1(I)$  collagen chains from the mouse endogenous gene. The most effective ODNs reduced the human  $\alpha 1(I)$  collagen chains to 37–67% of the controls. Moreover, the most effective ODNs were those targeted to sequences that were predicted to form clustered double-stranded structures in RNA transcripts.

In type  $\alpha 1(I)$  procollagen promoters, two highly conserved polypurine-polypyrimidine tracts are present contiguous in the region from –140 to –200 (292). The polypurine sequence at –141 to –170, which we called C1, is localized on the non-coding strand, whereas the polypurine sequence from –171 to –200, which is denoted as C2, is present on the coding strand. As illustrated in Figure 4(A), several transcription factors are known to bind to the promoter sequence, which includes C1 and C2 regions (293). These regions play a key role for the cis-acting elements, and hence makes them an ideal target for developing antigene-based antifibrotic agents (293,294). Different TFO sequences have been designed to target these regions as shown in Figure 4B (292,295,296). These TFOs formed stable triplex with high efficiency with target double-stranded DNA (dsDNA) and demonstrated inhibition of the  $\alpha 1(I)$  procollagen promoter activity, which was cloned into a plasmid vector. Since the production of type I collagen is the end step in the process of liver fibrosis, using antifibrotic agents directly targeting to this step has many advantages. We are currently evaluating the therapeutic application of TFO-based agents in liver fibrosis.

Little progress has been made to date in modulating collagen degradation. Potential targets include the modulation of MMP activity and its regulators such as plasminogen activator or TIMPs. uPA, an initiator of the matrix proteolysis cascade, has been examined in a rat model of cirrhosis (297). A single intravenous administration of a replication-deficient adenoviral vector encoding a non-secreted form of human uPA resulted in high production of functional uPA protein in the liver. This led to the induction of MMP expression and reversal of fibrosis with subsequent hepatocyte regeneration and improved liver function. In another study, a single intravenous injection of an adenoviral vector expressing human MMP-1 reversed stable liver fibrosis with a return to normal liver histology in thioacetamide-induced cirrhosis in rats (298).

#### 4. TRIPLEX FORMING OLIGONUCLEOTIDES AS POTENTIAL ANTIFIBROTIC AGENTS

Advances in ODN-based therapeutics offer great hope in sequence-specific inhibition of aberrant gene expression. ODNs (299) as well as more recently developed siRNA/siRNAs (300) silence gene expression at translation levels via inducing degradation of target mRNA or inhibiting translation process, whereas those act at transcriptional levels by TFOs (301–306), called anti-gene strategy. In this section, some relevant issues regarding the application of TFOs for gene silencing and will be discussed.

Transcriptional inhibition via triplex formation presents an attractive application of TFOs and has several advantages over other gene silencing technologies (307,308). There are only two copies (two alleles) of the target gene, whereas there may be thousands of copies of an mRNA. In addition, blocking translation of mRNA does not prevent mRNA from re-production. In contrast, inhibition of gene transcription is expected to bring down the mRNA concentration in a more efficient and long-lasting way, depending on the residence time of TFO on its target sequence and its life-time determined by its nuclease sensitivity.

However, besides the delivery issue, which is common for most ODN-based therapeutics, the disadvantages of TFO-based therapeutics include instability of triplex, limited availability of target sequences and accessibility of the target sequence in the nucleus. Chemical modifications on TFOs provide promising strategies to assure their potential applications *in vivo*. TFOs have to get into nucleus and bind to their target DNA in the chromatin structures. The binding of TFOs may be affected by many cellular factors. Detection of triplex formation *in vivo* is another important issue for developing TFOs as therapeutics since observed gene regulation may not be always correlated with triplex formation. Therefore, many studies are focusing on developing molecular tools for detection of triplex formation inside living cells and it is worth a summary on the progress in this field.

#### 4.1. Rules for Triplex Formation

Since bases already engaged in Watson-Crick hydrogen bonding, only purines are able to further establish two Hoogsteen (or reverse-Hoogsteen) hydrogen bonds in the major groove of DNA, the optimal target sequences require consecutive purines on the same strand for stable binding (307,309). Therefore, triplex formation must follow precise rules to minimize energy, which on the other hand bring its specificity. It is suggested that at least 15–20 bases are needed for a TFO sequence to bind its target site with sufficient affinity to have biochemical effects.

Even though TFO can only bind to polypurine strand of target DNA, TFO itself can be consisted of purines and pyrimidines, depending on the nature of the target sequences (Figure 5). The (C, T)-motif involves the formation of C•G×C and T•A×T base triplets (• stands for Watson-Crick hydrogen bond; × stands for Hoogsteen/reverse-Hoogsteen hydrogen bond), upon binding of a (C, T)-containing TFO with a parallel orientation with respect to the purine strand (Hoogsteen hydrogen bonds) (310). The (G, A)-motif involves the formation of C•G×G and T•A×A triplets, upon binding of a (G, A)-containing TFO in an antiparallel orientation with respect to the purine strand (reverse-Hoogsteen hydrogen bonds) (311). In addition, a (G, T)-motif TFO is also permitted. The (G, T)-motif involves binding of a (G, T)-containing TFO, whose orientation depends on both the number of GpT or TpG steps in the third strand and on the length of G and T tracts. Because of these intrinsic rules for TFOs, the repertoire of their target sequences in the genome is limited to only polypurine/polypyrimidine sequences.

The stability of a triplex is challenged by many factors. First, the stability of TFO against nucleases is a concern, which is universal for all ODN-based technologies. Triplex formation involves conformational changes on TFO sequence and some distortion of the underlying dsDNA (312,313), which leads to the intrinsic instability of triplex structures. Triplex formation with (C, T)-motif TFOs is pH dependent because cytosines must be protonated at the N3 position in slightly acidic media (pH<6) to form two hydrogen bonds with G (314). Triplex formation is dependent on ionic strength. Typically, 5–10 mM of Mg<sup>2+</sup> ions are required for forming stable triplex (301). This is because triplex formation involves the binding of a negatively charged third strand to a double negatively charged duplex and neutralization of charge repulsion can be achieved with Mg<sup>2+</sup>. However, this concentration of Mg<sup>2+</sup> is much higher than that available inside cells (315). TFO can be made unavailable to target DNA by sequestering in self-associated stable structures. G-rich TFOs can easily form intra- or inter-molecular four-stranded structures involving G-quartets (316). Other intermolecular structures,

such as parallel homoduplexes involving AA, GG and GA base pairs can also be formed (317). In addition, monovalent cations ( $\text{Na}^+$ ,  $\text{K}^+$ ) at physiological concentrations (140 mM) favor G-quartet formation and thus inhibit triplex formation (318). All these factors impose kinetic barriers on triplex formation and reduce the stability of triplexes once formed, which results in that most triplexes, even under optimal conditions *in vitro*, are less stable than the corresponding dsDNA.

However, for a DNA target sequence, many different TFO sequences can be designed binding to different regions along the target DNA sequence. The triplex formation ability can be very different depending on the selection of TFO sequences. As shown in Figure 6, with different starting position, TFOs targeting  $\alpha 1(\text{I})$  collagen promoter region can have varied triplex forming kinetics based on the electrophoretic mobility assays (292). Therefore, careful design and selection of TFO sequences for further studies are necessary for achieving high bioactivity.

#### 4.2. Chemical Modifications on TFOs

Considerable efforts have been made to extend the repertoire of potential target DNA sequences and increase the triplex stability while keeping its specificity via chemical modification on TFOs (307,319). To extend the repertoire of target DNA sequences, most efforts focus on synthesis of new nucleobase analogues to avoid the requirement of a polypurine target sequence for TFO binding, i.e., to recognize a pyrimidine which interrupts an oligopurine sequence. For a TA base pair inversion, which means a thymidine interrupting an oligopurine sequence, an additional problem is the projection into the major groove by the 5-methyl group of thymine resulting in steric hindrance for TFO binding to the major groove, which further disturbs the triplex structures. A nucleoside analogue with a phenylimidazole derivative was shown to tolerate TA interruption and form stable triplexes (320). Two other nucleobase analogues for exact recognition of thymine in the TA base pair were also reported (321,322). To recognize cytosine in the GC base pair, a novel thymine nucleobase analogue  $^4\text{H}_\text{T}$ , which lacks a carbonyl oxygen at the 4-position, was reported (323). Several N4-substituted cytosine derivatives with side-chain extensions were shown to form hydrogen bonds with both the cytosine and the guanine in the inverted GC base pair and therefore enhance the stability of triplex structures (324). Furthermore, this approach is attractive because the formation of hydrogen bonds with both bases should enhance the specificity with the intended base pair.

To overcome pH dependence of the (C, T) motif, a cytosine analogue, 5-methylcytosine was shown to have potential use (325). Other analogues for substitution of cytosine include 8-oxoadenine (326), pseudoisocytidine (327), and a 6-keto derivative of 5-methylcytidine (328) and 2-aminopyridine (329,330). However, much work still needs to be done on nucleobase analogues regarding their biological applications.

Different backbone modifications have been investigated to enhance both the nuclease resistance and binding affinity of TFOs (Figure 7). Phosphorothioate (PS) modification is the most extensively studied chemical modification on ODN and is known to be nuclease stable. However, triplexes with PS-TFO are as stable as native phosphodiester (PO) TFOs in the purine motif and is greatly reduced in the pyrimidine motif (307,331). Replacement of the phosphate linkage with methylthiourea improves TFO binding (332), which is because this modification brings positive charges to the backbone of a TFO, and thus partially minimizes the charge repulsion. Similarly, a positive charge on a thymidine analogue (333), or attachment of positively charged moieties to TFOs (334,335) have also enhanced triplex stability. Replacement of a non-bridging oxygen atom in the backbone with a charged amine enhanced the stability of triplexes, which also partially resulted from reduction of the likelihood of self-structure formation of purine TFOs in physiological  $\text{K}^+$  concentration (336–338).

Phosphoramidates (PN) analogues containing N3'→P5' phosphoramidate linkages stabilize triplexes formed with (C, T)- and (G, T)-motif TFOs in a parallel orientation with respect to the oligopurine sequence, but not in an antiparallel orientation (339). A conformational change to N type in the sugar moiety might also contribute to this enhanced triplex-forming ability (319). It is suggested that the formation of a duplex or triplex is entropically unfavorable due to the restriction in conformational freedom of the furanose ring. Therefore, “pre-organization” of the sugar conformation in a suitable form to prevent the loss of entropy would be a promising strategy for stabilizing triplex structures.

Locked nucleic acid (LNA) is designed to have this “pre-organization” by introducing a bridge between the O2' and C4' atoms, the sugar conformation of which is restricted to the N type (319). As a result, triplex can be formed 300-fold more stable compared with the corresponding one without modification. Substitution of deoxyribose with ribose in the third strand of the triplex and this resulted in stabilization of triplexes for the (C, U) or (G, U)-motif with a parallel orientation of the third strand with respect to the purine target sequence (340–342). This inspired the use of RNA analogues, i.e., 2'-O-alkyl analogues (342,343). The beneficial effects are probably due to the shift in the conformational equilibrium of the sugar to C3' *endo* configuration. This conformation imposes minimal distortion on the underlying duplex (313) and thus provide “pre-organization”, which has entropic benefits (344). In 2'-O-aminoethyl (2'-AE) modification, both a positive charge and a “pre-organized” sugar conformation can be provided (343). TFOs carrying this substitution show enhanced kinetics of triplex formation and greater stability of the resultant complex at physiological pH and low Mg<sup>2+</sup> concentration

To minimize the G-quartet formation, G in the sequence was substituted with 6-thioguanine (345) or 7-deazaguanine (346). However, these modifications reduce the overall binding affinity. TFOs can be conjugated to a secondary structure that will impair the formation of self-associated structures but not triplex formation. For example, clamp or circular ODNs are less prone to self-association (347). An additional ODN helper forming a short duplex can be added at either the 3' or 5' end of the TFO, thereby reducing its propensity to self-association (348). Furthermore, a short hairpin structure has been designed incorporated at one end of TFO to prevent self-association (349).

The two most radical modifications are morpholino analogue, in which the sugars are replaced with morpholine rings, and peptide nucleic acids (PNAs). Replacement of ribose with a morpholino analogue reduces the Mg<sup>2+</sup> dependence (350). In PNA modification, the deoxyribose phosphate backbone is replaced by a homomorphous, achiral and uncharged backbone based on N-(2-amino-ethyl)glycine (351). PNAs are not degraded by nucleases or proteases. PNAs hybridize virtually independently of the salt concentration. However, binding of PNAs to their target sequences is dependent on pH, with more stable binding occurring at the neutral to slightly acidic pH. In addition, for PNA, the anti-parallel binding is favored over the parallel one. Furthermore, PNA binding is more affected by base mismatches than native DNA, which means more specificity of PNA. One of the striking properties of PNAs is that they lead to strand displacement in double-stranded DNA, forming a 2:1 complex with the purine target sequence, in which the complementary pyrimidine sequence is displaced, thus resulting in the formation of a P-loop structure (352). In these triplexes, one PNA strand hybridizes to DNA through standard Watson-Crick base pairing rules, while the other PNA strand binds to DNA through Hoogsteen hydrogen bonds.

Conjugation of TFOs with intercalating agents, which may interact with DNA target leading to either covalent modification or cleavage of backbone, can definitely increase binding affinity and obtain more durable biological effect. For example, psoralen- and orthophenanthroline-TFO derivatives are remarkable by inducing triplex-mediated cross-linking and cleavage reactions, respectively, on a duplex target, such that a durable biological effect can be obtained

(353). Intercalating agents can be conjugated to the 5'- or 3'-end or to internal positions, even though the preferential site of intercalation is formed at the triplex-duplex junction (307). Incorporation of acridine internally into TFOs, not only increased the stability of triplexes, but also enabled triplex formation with target DNA containing base pair inversions (354). However, only some of the chemicals have been found to specifically stabilize triplexes other than dsDNA (307).

### 4.3. Gene Regulation by Triplex Formation

**4.3.1. Transcriptional Inhibition by Triplex Formation**—TFOs bind to target DNA and lead to specific gene silencing. Transcription inhibition by triplex formation was first shown by Morgan and Wells (355). Binding of TFOs to target DNA sequences can compete with protein binding, therefore triplex formation can be designed to interfere with gene transcription at different levels depending on the target sites. Triplex formation has been shown to disturb chromatin structure (356), binding of transcription factors (308,357) and RNA polymerase binding (358,359). In addition, elongation process of DNA polymerase and RNA polymerase has also been shown to be interfered by triplex formation (331,360,361). Studies have shown that durable inhibition of transcription elongation can be further achieved by attachment of intercalating agents (e.g., acridine and psoralen) on TFOs (362,363). Since PNA binding to duplex DNA leads to strand displacement, they could stop RNA polymerases only when hybridized to the transcribed strand (364–366). Furthermore, PNA binding to DNA target is promoted by transcription process, leading to the concept of suicidal transcription (367).

The mechanism of action of TFOs inside the live cells remains to be confirmed. Very few studies provides unambiguous evidence that transcriptional inhibition is due to triplex formation (307). Furthermore, sequence-specific effects can also be obtained by mechanisms other than triplex formation. For example, G-rich TFO can adopt structural conformations, which can be recognized by specific proteins (368–370). An oligothymidylate–acridine conjugate directed to the SV40 replication origin proved to be efficient at inhibiting viral replication in CV1 cells (371), but this effect may be due to the mechanisms other than triple helix formation on the target sequence. In lymphocytes, HIV-1 transcription was inhibited by (G, T)-rich 38-mer TFOs directed against the transcription initiation or nuclear factor Sp1 binding sites (372). This effect may be due to a direct interaction between the HIV-1 integrase and TFOs (307). In many studies, gene inhibition of the *c-myc* oncogene by TFOs is ascribed to triplex formation (373–375). However, other mechanism may also be possible since titration of the transcription activator CNBP by the purine-rich ODN could fully account for the observed decrease in *c-myc* transcription (376,377). In addition, if two potential targets are constituted by both genomic DNA and the corresponding mRNA, then TFO may exert its effect by Watson–Crick base pairing to the RNA target (364,378–380). The respective contribution of antigene, antisense or other effects is not easy to evaluate.

Because mechanisms other than triplex formation may also be involved in TFO-induced gene silencing, controls of studies regarding TFO induced gene silencing are very important for elucidating action of TFOs. Scrambled or mutant ODNs and irrelevant targets sequences are generally used as controls and definitive demonstration of a triplex-mediated mechanism is generally lacking (307). However, the best control experiment consists of using the same TFO but a mutated target sequence affecting triplex stability but not gene expression (381). One such example is the studies of transcription inhibition of the gene coding for the alpha subunit of interleukin-2 receptor (IL-2R $\alpha$ ). In this study, 15 mer-(C, T) TFOs with acridine or psoralen modification were used for triplex formation with genomic DNA; intracellular covalent triplex was measured in parallel to determine whether there is any correlation between triplex formation and transcription inhibition (381–383). A control plasmid mutated in the triplex site

was unable to bind TFO and there was little effect on the transcription activity and regulatory protein binding.

**4.3.2. G-rich Oligonucleotides—**For (G, A)-motif TFOs, there are very high chances to have continuous guanine in the sequence. G-quartets are structural motifs formed by the planar arrangement of four guanine bases that interact with each of the two adjacent guanines through Hoogsteen-like hydrogen bonds (384). ODNs possessing contiguous G bases are assumed to have a tendency to form thermodynamically stable quadruplex structures, i.e., through vertical stacking of two or more G-quartets, which may confer them biological activities other than triplex formation induced gene regulation (385). In fact, these quadruplex structures have been observed involved in various biological processes in important genomic locations such as chromosomal telomeres, gene promoter regions, immunoglobulin switch regions and recombination sites, its function in cells is not well understood (384–386). In the regulatory region of genes, they are suggested to act as transcriptional regulators that may block or enhance DNA synthesis (387,388). The potential of quadruplex DNA as targets for therapeutic intervention and as therapeutics is suggested (320).

An aptamer is RNA or DNA molecule that binds to a specific molecular target and the binding is related its quadruplex structures. The sequence GGTGGTGTGGTTGG was the DNA aptamer with the highest affinity for thrombin (320). This DNA aptamer significantly increases the thrombin catalyzed clotting times of both purified fibrinogen and human plasma. Specific 16-mer and 17-mer ODNs with sequences consistent with the formation of quadruplex structures have been found to be potent inhibitors of HIV-1 integrase (389). Wyatt et al. reported that a certain G4 forming ODN bound to HIV-1 gp120 protein inhibiting in this way both cell-to-cell and virus-to-cell infection (194).

Several studies have shown that G-rich ODNs exhibit antiproliferative activities. For example, the G-quadruplex ODN (GQ-ODN) T40214, which forms a G-quadruplex structure intracellularly, has been shown to be potent inhibitors of Stat3 DNA binding activity, inhibit IL-6-stimulated Stat3 and suppress the Stat3-mediated upregulation of Bcl-XL and Mcl-1 gene expression (390). Another group has shown that G-rich ODNs have antiproliferative activity against a number of cancer cell lines (391). Again, the antiproliferative activity of these G-rich ODNs is associated with their ability to form stable G-quartet-containing structures and their potential binding to a specific cellular protein, nucleolin.

**4.3.3. Accessibility of Genomic DNA to TFOs in Cells—**TFOs have to reach the nucleus and compete with all nuclear proteins for binding to genomic DNA. Compared to antisense ODNs, an additional restriction to potential target sequences for triplex formation is linked to chromatin structure, a parameter which is largely unknown for most of potential target sequences. Endogenous gene silencing by TFOs has been demonstrated in many studies (372,374,375,392,393), which suggests the accessibility of nuclear DNA for TFOs. However, in these studies, triplex formation with DNA in native chromatin structure was not unambiguous confirmed. In addition, some *in vitro* experiments indicate that chromatin structure is incompatible with triplex formation (356,394,395). Therefore, to clarify the accessibility of target sites in the context of chromatin is crucial for developing antigene strategies.

To determine the accessibility of DNA in intact chromatin structure, most studies are using permeabilized cells or physical transfection technologies to enhance TFO accumulation in intact nuclei. These studies confirm the accessibility of target sequences in intact chromatin structures. The accessibility is dependent on the sites of target sequences and TFOs used. In a pioneer study, Giovannangeli et al. demonstrated triplex formation of psoralen modified 15-mer PO- or PN-TFOs with genomic DNA within permeabilized cells chronically infected with

viral HIV-1 (396). PN-TFO showed higher efficiency in triplex formation than PO analogue. In another study, endogenous gene sequence of the chemokine receptor CCR5 was a target (397). A 12-mer (G, A)-motif TFO with G substituted by 8-aza-7-deazaguanine was modified with a phenylacetate mustard on the 5' end for alkylation with DNA sequence. TFO accessibility to target DNA in the cells permeabilized by streptolysin O treatment was revealed by showing that up to 24% of covalently modified target. Site-specific mutations induced by TFO-psoralen conjugates in the *Hprt* gene of CHO cells after electroporation have further confirmed the DNA accessibility in the cell nucleus (353).

The cellular environment has great effects on triplex formation. However, targeting of chromosomal sites by TFOs is limited by nuclear environment rather than cellular context (398). A LNA modified TFO was used to study the effect of nuclear environment on the triplex formation efficiency (398). When the target sequence was located in transcription region, the triplex formation efficiency varied from one gene to another and was not correlated with the status of gene expression. However, different degrees of triplex formation were observed when HIV-1 genome was integrated into different sites of the host genome (396). Since transcriptional activation can change chromatin organization including nucleosome disruption or displacement in several systems (399,400), the transcription status of target DNA sequences must affect the triplex formation efficiency. Therefore, it is suggested that transcriptionally active genes might be accessible to triplex-mediated regulation, especially when the target sequences are located in the same DNA domain as transcription factor binding sites. It is still debated whether actively transcribed gene sequences can have higher accessibility to TFOs. Induction of transcription activation was shown to enhance triplex formation (398,401). However, there was little effect on triplex formation when the cells were treated with phorbol-12 myristate-13 acetate (PMA), which is known to induce transcription activation of HIV-1 genes (396). Oh et al. did not see noticeably effect of transcription activation on triplex formation (402). This apparent contradiction may be because i) beyond certain transcription activity, an additional increase may have no effect on targeting efficiency by TFOs; and ii) difference exists for TFO targeting sites including promoter region or in transcription region (398).

**4.3.4. Detection and Quantification of Triplex Formation**—Most studies to demonstrate the efficiency and specificity of triplex formation are relying on more traditional molecular techniques including electrophoretic mobility shift assay (EMSA) (292), restriction enzyme protection assay (403,404) and dimethyl sulfate (DMS) footprinting (348). However, these methods are not quantitative. New methods have been developed based on PCR, including competitive PCR (396), linear amplified primer extension (382), single-strand ligation PCR (402), and real-time PCR-based methods (398,405,406). Currently, interests are focused on the detection and quantification of triplex formation in the native chromatin structures rather than on isolated DNA targets and plasmids. Direct demonstrations of triplex formation in cell nuclei and/or permeabilized cells have been reported (353,406,407). In many studies, several detection strategies are used in combination.

Psoralen is a bifunctional photoactive agent that has been used as probes of nucleic acid structure and function, and is minimally harmful to cellular constituents (408). Conjugation of psoralen to the 5' end of a TFO allows photo-induced cross-linking reaction of the psoralen at the specific sequence where the TFO binds to duplex DNA. To detect the triplex formation, psoralen conjugated TFO has been used extensively (382,406,409). Upon UV irradiation, psoralen introduces a covalent crosslink into the target DNA sequence which can effectively block transcription (272,382,409,410). This characteristic of psoralen is the basis of many PCR-based quantification methods.

**4.3.4.1. Electrophoretic mobility shift assay:** EMSA is used extensively for studying the kinetics of triplex formation with synthetic duplex DNA targets (292,296,352). In this method, either one strand of synthetic duplex DNA targets (411) or two strands (296) can be radiolabeled for detecting triplex formation using autoradiography after polyarylamide gel electrophoresis (PAGE) depending on the purpose of study. For example, the interaction between psoralen and duplex DNA after triplex formation was studied in details by Takasugi et al with changing of radiolabeled strand in the duplex DNA (411). Initial selection of potent TFO sequences as well as studies of effect of different factors on triplex formation (412) can be easily achieved by this method. In a study, the triplex formation ability of daunomycin-conjugated TFOs was studied using EMSA (413). Triplex formation using LNA modified TFOs has also been studied by EMSA (414).

**4.3.4.2. Restriction enzyme protection assay:** In restriction enzyme protection assay (REPA), a restriction enzyme is selected to have a recognition site within the triplex formation sequence. Formation of triplex structure at this recognition site prevents the restriction digestion by enzymes, thus giving different digestion pattern from those without triplex structures upon separation by gel electrophoresis (403). By utilizing this method, the efficiency and specificity of triplex formation can be established, mostly in targets cloned in plasmids (381,404,409). It is suggested that without psoralen modification on TFOs, the protection from restriction enzymes by triplex formation was only transient since the triplex formation is kinetically in equilibrium with duplex DNA targets (404). Using REPA, the persistence of preformed triplexes between a psoralen modified TFO with a plasmid after transfection into cells for 72 h has been established (409). When this method is used to detect the triplex structures isolated from cells, Southern blot is often used in combination for detecting expected DNA fragments to increase the sensitivity of detection (381,409). This is especially true for the targets in the genome context extracted from nuclei or cells, since the amount of the expected fragmental DNA is extremely low. In one study, the triplex forming sequence was in HIV-1 genome, which was integrated into cellular genome (396). TFOs used were PN analogue with psoralen modification. After forming triplex, the DNA was digested with *DraI* and the DNA fragments were detected by DNA probes. The specificity of triplex formation was confirmed by the lack of protection of the two neighboring *DraI* sites. Up to 30% of the target sequence in the nuclei of permeabilized cells was shown to be protected from digestion. Macris and Glazer also showed around 30% of triplex formation in permeabilized cells at target site which is also integrated into genome of human 293 cells by REPA (401). Similar *DraI* protection assay was also used in another study with LNA modified TFOs by Brunet et al (398). However, only triplex formation with naked genome DNA was reported.

**4.3.4.3. Competitive PCR:** Giovannangeli et al applied competitive PCR to quantify the triplex formation of TFO modified with psoralen with isolated genomic DNA (396). In this method, a competitor DNA fragment was constructed for quantifying unknown amount of genomic DNA by co-amplification with PCR. For quantification purpose, two sets of primers were used as well, one to amplify a fragment (denoted as  $R_T$ ) overlapping the triplex formation site and another for a fragment (denoted as  $R_C$ ) outside the triplex formation region. Each primer set consists of (i) two external primers ( $P_{E1}$  and  $P_{E2}$ ) to amplify fragment T or C, and (ii) one internal primer ( $P_I$ ). The internal primer was designed to containing identical sequence of  $P_{E1}$  at the 5' end, linked to a sequence complementary to a region within T or C at its 3' end. Competitors were obtained by successive PCR amplification of isolated genomic DNA first using primers  $P_I$  and  $P_{E2}$ , and then using the external primers  $P_{E1}$  and  $P_{E2}$ . Therefore, the competitor DNA fragments were similar to fragment T or C, but lacking several base pairs at one extremity.

For quantification, a fixed amount of genomic DNA of interest is treated with TFOs modified with psoralen and then mixed with increasing amounts of competitor for each primer set

(396). The two fragments in the same test tube were then co-amplified using one external primer set. The ratio between the two DNA fragments (competitor DNA fragment and T or C) remains unchanged during the amplification process based on the competitive PCR principle.

Therefore, the amount of fragment  $R_T$  or  $R_C$  can be calculated. For the same genomic DNA sample treated with TFOs, amplification of  $R_T$  will be inhibited and that of  $R_C$  will not. The difference between the amounts of  $R_T$  and  $R_C$  is the amount of genomic DNA containing triplex-induced psoralen modification. This method was used to quantify triplex formation with isolated genomic DNA and showed similar results as *DraI* protection assay (396), even though slightly lower degree of triplex formation was determined. This may be explained by the fact that the PCR-based assay measures the amount of triplex-induced cross-links, whereas the *DraI* cleavage assay reflects the amount of cross-links and mono-adducts.

**4.3.4.4. Linear amplified primer extension:** Linear amplified primer extension relies on the radiolabeled primers and is also based on the principle that TFO directed psoralen modification of target DNA sequences can block DNA polymerases. With covalent TFO bound on the target DNA sequences, the primer extension will give truncated product compared to no modification of target DNA sequences (402). On a plasmid, Guieysse et al. studied the triplex formation when transfected into cells using this method (382). After triplex formation either *in vitro* or inside cells, the plasmid was digested downstream of the triplex formation region and then subjected to primer extension. The cross-link by psoralen may lead to underestimation of the expected truncated product, since it favors the renaturation of two strands of DNA during primer hybridization. This is confirmed by the fact that when under conditions favoring cross-link formation, such as irradiation at  $\lambda > 310$  nm, 65% of truncated product formed and 85% for condition favoring monoadduct formation (irradiation at  $\lambda > 390$ ) (382).

**4.3.4.5. Single-strand ligation PCR:** Oh et al. developed a single-stranded ligation PCR for quantifying triplex formation (402). In this method, a ligation ODN was ligated to PCR intermediate product to enable simultaneous amplification of the DNA fragments, which resulted from a prior primer extension for DNA samples with or without TFO-directed crosslinking by psoralen. Genomic DNA of interest was first digested downstream of triplex formation region to release a free end. The DNA was then amplified by primer extension using a 5'-end biotinylated primer to produce either a truncated product or a 'full length' product. The biotinylated PCR products were captured on streptavidin-coated magnetic beads. An ODN was ligated to the 3' end of the product. This ligation product was subjected to exponential PCR using a primer partially nested relative to the biotinylated primer and a ligation primer complementary to ligation ODN. Finally, a primer extension with radiolabeled primer was performed for detection on PAGE. Fraction of triplex formation on genomic DNA in live cells was determined to be 15% (402).

**4.3.4.6. Real-time PCR-based strategies:** More recently, many studies have developed different real-time PCR-based methods for quantifying triplex formation, with focus on genomic DNA in native chromatin structures and in live cells. Real-time PCR can be more quantitative and time-saving. Similar to the idea in the competitive PCR, by comparing the PCR amplification of two regions, one overlapping the triplex formation region (T) and another outside the triplex formation region (C), the degree of triplex formation can be determined. Normally a standard curve (Ct versus amount of DNA of interest) is drawn for quantification (398). The level of inhibition in the PCR amplification directly reflected the level of triplex-induced adducts. The degree of triplex formation was 0% to 50% in permeabilized cells depending on the genes of interest and gene expression status (398). Besch et al. developed a real-time PCR-based method in combination with DNA capture using magnetic beads (405, 406). In this method, TFO was modified with psoralen at one end and biotin at the other end for capture. The idea is that only genomic DNA bound with biotin modified TFOs could be captured with streptavidin-coated magnetic beads. The captured genomic DNA is then

subjected to real-time PCR and the number of captured DNA sequences was determined by comparing to standard curve. However, in live cells, less than 1% of triplex formation was detected (406). This degree of triplex formation was very low, which may be due to many factors including the target gene of interest, cell types and sequences of TFOs. We proposed a much simpler real-time PCR-based strategy for detection of triplex formation in HSCs cells as schematic illustration in Figure 8 (unpublished data). An explicit purification step is added into the procedure aimed at removing unreacted TFO molecules.

## 5. SITE-SPECIFIC DELIVERY OF OLIGONUCLEOTICES

Barriers to systemic delivery of ODNs have been extensively described in many excellent reviews (415–417). These barriers include limited stability, poor cellular uptake, unfavorable subcellular trafficking, lack of nuclear targeting especially for antigene strategies, and non-specific tissue distribution (Figure 9). Synthetic carriers including polymers or lipids are attractive owing to the flexibility in design, ability to be chemically or biochemically functionalized and tunable toxicity properties. These properties of synthetic carriers in combination with increased understanding of pathophysiology in ODN delivery are important in turning ODNs into therapeutics.

### 5.1. Cellular Uptake and Subcellular Trafficking

Cellular uptake of naked ODNs is an active endocytotic process via adsorption and varies in efficiency depending on the cell type, cellular conditions, concentration as well as chemistry and length of ODNs (418–421). The uptake mechanism appears to be at least partially concentration dependent and that below a concentration of 1  $\mu$ M, the uptake of ODNs is predominantly via a receptor-like mechanism, while at higher concentrations a fluid-phase endocytosis mechanism appears to predominate. These processes lead to ODNs ending up endosome/lysosomes, with only small fraction diffusing into cytoplasm as well as nuclei. Degradation of ODNs has largely been observed, which results in insufficient concentrations for achieving biological effects. Nuclear translocation seems not be a rate-limiting step, since both microinjection and permeabilization lead to readily nuclear localization of ODNs (422–425). However, studies on the mechanisms involved are still needed, even though it has been suggested that nuclear entry can be accomplished by translocation across the nuclear pores (416). Regardless of these, subcellular distribution of PS-ODNs has been mostly studied in liver cells after parenteral administration in rats (426), since the subcellular location of ODNs are important for their function. Furthermore, the subcellular distribution can be modulated accordingly for the purpose of gene silencing by delivery strategies. It was found that in liver cells, including hepatocytes, KCs and SECs, cytosol associated with highest PS-ODNs (426). One interesting observation was that at low dose nuclear association was not seen in hepatocytes, while it could be observed with much high dose. Subcellular distribution of ODNs in hepatocytes is species dependent (426). Much higher nuclear association was observed in human hepatocytes compared to those of rats and mice.

Cho et al. (427) has reported the cellular uptake as well as subcellular distribution of a 29mer purine-rich TFO molecule in different cell types. In this study, TFO molecule was labeled with  $^{111}\text{In}$ . The total cellular uptake ranged from 20% to 55% of dose depending on the cell types at 24h incubation at 37 °C. In addition, cancer cell lines had higher TFO uptake than normal cell lines. For TFOs, they have to enter nuclei to bind to their target DNA sequences in the chromatin structures. Therefore, nuclear localization of the TFO was also determined. The wide variation in nuclear accumulation was also observed in different cell types ranging from 7% to 30% of the total cellular uptake. We have also studied the cellular uptake and subcellular distribution of a 25mer purine-rich TFO targeting to rat  $\alpha 1(\text{I})$  collagen promoter (428). Around 12% of the dose was found to be associated with cells in HSC-T6 cell line, in which 30% was associated with nuclear structures. We further studied the intracellular

distribution *in vivo* by isolating nuclear structures from freshly isolated liver cells after i.v. injection of radiolabeled TFOs, and found that 2–6% of total liver uptake was associated with nuclear structures (Figure 10C&D) (429).

Chemical modifications have been introduced with intention to increase the stability and binding affinity of ODNs and may also have effect on their cellular uptake. While PS-ODN has been shown to have similar uptake mechanism as native ODNs (430), methylphosphonate (MP) ODNs are uncharged molecules that enter cells via passive diffusion (417), although the rate of diffusion across the lipid bilayer membrane is extremely slow (431).

To achieve the efficient cellular uptake of ODNs for therapeutic effects, cationic polymers such as polyethyleneimine (PEI) have been used to enhance the cellular uptake and endosomal escape of ODNs (432,433). By forming complexes with cationic polymers, the internalization usually follows clathrin-mediated endocytosis pathway, which means the deposition of complexes inside acidic endosome/lysosome vesicles; therefore enhancing endosomal escape is necessary for avoiding degradation of nucleic acids and achieving high biological effects. Non-cationic polymers with endosomal lysis ability have also been developed for improved intracellular delivery of ODNs (434).

To avoid entrapment in endosome/lysosomes, other endocytosis mechanisms (caveolae-mediated endocytosis or macropinocytosis) not involving formation of endosome/lysosomes or nonendocytic delivery have been exploited for nucleic acid delivery (433). A class of cationic peptides, the protein transduction domains (PTDs), such as the TAT, penetratin, and VP22 peptides, may have the ability to be taken up by cells without endocytosis events (435–439). Therefore, incorporation of the TAT peptide into delivery systems has been shown to enhance the transfection efficiency (440,441), even though Kang et al. (442) did not achieve any improvements on existing carriers by incorporation of TAT peptide.

Even though the translocation of naked ODNs into nuclei is not a problem, it is needed to clarify whether the delivery system associated with ODNs can get entry into nuclei or ODNs need to dissociate from the delivery system prior to their nuclear translocation. Cationic lipids have been observed to be dissociated from ODNs and they were not associated with nuclear membranes (443–445), while PEI/ODN complexes could reach the nuclei where ODNs were dissociated (446,447). However, there are reports suggesting the dissociation of ODNs from the complexes before nuclear entry of ODNs (432). In fact, unpacking of ODNs is required for their biological activities inside the cells. Synthetic carriers can be tuned to have appropriate balance in the packing and unpacking of nucleic acids (448,449). Displacement of nucleic acids by intracellular polyanions is likely to result in their unpacking for cationic lipids or polymers (449). The reducing environment or acidic conditions inside endosome/lysosomes within the cells have been exploited to provide extracellular stability to the nucleic acids, while the carrier molecules should be eliminated from the cell after internalization (450–452). For example, degradable PEI has been synthesized using low molecular weight PEI (1.8kDa), which has much lower cytotoxicity and nucleic acid condensing capability compared to large molecular weight PEI (25kDa) (452).

## 5.2. Biodistribution

For systemic delivery, ODNs still need to overcome many other biological barriers including rapid elimination from the circulation, interactions with biological fluids and extracellular matrix, nonspecific accumulation in organs, and rapid degradation. Biodistribution of naked ODNs has been studied intensively in animals and humans, especially for PS-ODNs due to their relatively high stability compared to native phosphodiester-ODNs (PO-ODNs) (453–455). The pharmacokinetics of PS-ODN is generally sequence independent (453). Following intravenous administration, PS-ODNs are rapidly cleared from plasma with their distribution

half-lives of about 30–80 min and much slower elimination half-lives from 5 to 75 h depending on the isotope used to label the ODN, which might actually be related to the slow clearance of radiolabel from the distributed tissue as well as the low-molecular-weight metabolites of ODNs (453). In addition, it has been shown that plasma clearance rates are largely species independent in rat, rabbit, dog and monkey. ODNs are widely distributed to most peripheral tissues, with liver and kidney, spleen and lymph nodes accumulating the most. The residence time of PS-ODNs in tissues has been shown to be relatively long around days (453).

PO-ODNs have been shown to have less than 20% of hepatic uptake, while PS-ODNs have 40% of hepatic uptake (456). In addition, PS-ODNs, due to the extensive binding to the plasma proteins, accumulate much less in the kidney compared to PO-ODNs. Takakura et al. (457) examined the hepatic disposition characteristics of 20 mer PO-ODNs and the partially (PS<sub>3</sub>-ODNs, in which three internucleotide linkage at the 3'-end are phosphorothioated) and fully phosphorothioated derivatives in the isolated rat liver perfusion system after bolus injection into the portal vein. The magnitude of the hepatic interaction of ODNs increased as the extent of PS modification in the molecules increased: About 20%, 36% and 52% of the injected dose was taken up by the liver during a single passage after bolus injection of PO, PS<sub>3</sub> and PS, respectively. Urinary excretion is mainly dependent on the molecular weight of a compound, while its apparent charge affects both the hepatic uptake and renal excretion. Therefore, the disposition of ODNs at the whole body level can essentially be represented by hepatic uptake and urinary clearances. More favorable pharmacokinetic characteristics can be achieved using 2'-ribose alkoxy modification of PS-ODNs (453). This modification gives much longer elimination half-lives due to the improved stability of these derivatives.

Fibrosis results in the deposition of excess ECM proteins in the subendothelial space of Disse and loss of sinusoidal fenestrae. This, in turn, results in decrease free exchange flow between hepatocytes and sinusoidal blood. Furthermore, most biodistribution studies used PS-ODNs, which are not G-rich. PS-ODNs containing four or more consecutive guanine residues have tendency to form G-quartet structures and may show non-specific antiproliferative activity due to their binding the cellular nucleolin. Our studies have shown that in the DMN-induced liver fibrotic rats, the hepatic accumulation of a G-rich TFO decreased from 43% to 33% at 30 min and 38% to 27% at 4 h post-injection (429).

Suborgan distribution of ODNs focus on liver tissue in most studies, since liver is the major tissue to take up ODNs after intravenous administration. In the liver, most of ODNs could be detected in nonparenchymal cells (NPCs) including KCs and SECs; lower levels were localized in the cytoplasm of hepatocytes (458,459). Graham et al. (460) reported that almost 80% of injected PS-ODNs were taken up by KCs and SECs equivalently using nonlabeling method, capillary gel electrophoresis (CGE). The remaining 20% of the dose was taken up by the hepatocytes. On the other hand, Bijsterbosch et al. (461) studied suborgan distribution of a parenterally administered <sup>3</sup>H labeled PS-ODN specific for murine ICAM-1. They found PS-ODNs to be taken up by SECs and hepatocytes, with 56.1% and 39.6%, respectively, while KCs accumulated only 4.3% of the hepatic uptake. However, the concern with using radiolabeled ODNs in suborgan fractionation is that the redistribution or contamination often occurs during tissue homogenization (462). Furthermore, suggestions that PS-ODNs are taken up in liver principally by scavenger receptors on endothelial cells and that these compounds are not localized to KCs, are controversial since in a scavenger receptor knockout mice, PS-ODNs gave a similar distribution as to wild-type mice (463). In addition, in all these studies, the authors did not isolate HSCs and thus the reported uptake of PS-ODNs by KCs and SECs may be overestimated. We first showed that in the liver HSCs, KCs and SECs showed the highest uptake accounting for approximate 65–70% of the liver uptake (Figure 10B). At 1 mg/kg dose, the TFO concentration at different cells was in the following order: 1342, 1433 and 100 ng/mg of cell protein for HSCs, KCs & SECs, and hepatocytes, respectively (Figure 910)

(429). Again, the scavenger receptors were also suggested to be involved in the cellular uptake of ODNs in liver.

### 5.3. Bioconjugation

The *in vivo* use of cationic polymers and cationic liposomes has many problems. The electrostatic nature of the self-assembly between cationic vectors and anionic nucleic acids has the potential to be unstable in an environment with high ionic strength, such as blood, leading to rapid aggregation. Furthermore, these charged complexes may fail to reach their target cells intact, owing to nonspecific interactions with serum proteins, ECM proteins and cell surface proteins. The reticuloendothelial system (RES) of organs, such as liver, spleen, kidney and lungs represent most likely effective clearance sites, following systemic administration of ODN complexes. Accordingly, directing ODNs to non-RES organs still remains a challenge. One example comes from a study of the disposition characteristics of glycosylated poly(L-lysine) (Gal-PLL)/ODN complexes targeting hepatocytes in mice in relation to their physicochemical properties (456). Although the uptake of Gal-PLL/PS-ODN complexes by hepatocytes was significantly higher than that of naked PS-ODNs, the specificity was not completely achieved. Due to the negative zeta potential ( $-30 \sim -40$  mV) and wide particle size distribution ( $150 \pm 70$  nm), a part of the complexes is likely to be recognized by KCs and by SECs via scavenger receptors. Thus, formulations are typically prepared with a minimum of net charge and are modified with hydrophilic, neutral polymers (PEG) (464–466), N-(2-hydroxypropyl) methacrylamine polymer (pHPMA) (467,468)) that help to shield the charges and confer long circulation time in blood.

Delivery of ODNs relying on bioconjugation rather than complex formation provides an attractive strategy to avoid some problems associated with complexes. Bioconjugates are smaller in size compared to the particulate systems (such as liposomes, polyplexes and nanoparticles). Even though relatively low loading capacity may be one disadvantage compared to particulate systems, this will not be a problem for high potent nucleic acid-based therapeutics. However, one of the advantages is that bioconjugates can easily leave the systemic circulation. Consequently, they can be used to target drugs to cells that are not in direct contact with the blood, including hepatocytes and HSCs. Construction of a delivery system with multifunctional components greatly depends on the bioconjugate chemistry. Modification of existing carrier molecules as well as direct conjugation on ODNs has been developed. The most common strategy for active targeting of delivery systems is to exploit a unique cell surface receptor to achieve receptor-mediated endocytosis. A ligand (a small molecule, protein or antibody) is incorporated within the delivery system. For liver targeting, asialoglycoprotein, galactose, mannose, and many other carbohydrate derivatives are extensively used ligands. To achieve efficient cellular uptake and subcellular distribution, peptides including protein transduction domains (PTDs), fusogenic peptides and nuclear targeting signal have also been conjugated with either carrier or ODN itself. PEGylation of ODNs can elongate their blood circulation time. Further modification of PEGylated ODNs with targeting ligands presents a targeted delivery strategy. In addition, in most cases, conjugation on ODNs confers some stability against nucleases. Polymers such as synthetic polymers, antibodies, and serum proteins may present an ideal platform for construction of multicomponent carriers for delivery of ODNs.

**5.3.1. Conjugation with Lipophilic Molecules**—The hydrophilic character and anionic backbone of the ODNs reduces their uptake by cells as well as pharmacokinetics after systemic delivery. Therefore, various lipophilic molecules have been conjugated to ODNs to confer them more 'drug-like' properties (469). Many lipophilic molecules have been used to modify ODNs (470,471). However, among them, cholesterol is perhaps the best characterized and extensively studied *in vivo* (472–474). Unlike other lipophilic molecules, it has been shown

that cholesteryl conjugation confers ODNs enhanced binding to lipoproteins and thereby improved cellular association and transport especially into liver tissue. Therefore, liver targeting is the interest for most of studies involving cholesteryl conjugated ODNs.

Cholesterol can be conjugated to ODNs at 3'- or 5'-end. Conjugation with cholesterol can be integrated into the synthesis of ODNs using nucleoside derivatives (472,475,476). However, cholesterol can also be conjugated to post-synthetic ODNs with functionalities at ends. Alefelder et al. (477) reported modification of postsynthetic PS-ODNs with either  $\alpha$ -(bromoacetamido)-3-cholesterol or 2-(5'-nitropyridyl)-3-cholesterol disulfide. We conjugated thiocholesterol to TFOs containing sulfhydryl group at 3'-end via activation by bis-(5-nitro-2-pyridyl)-disulfide (Figure 11A) (428). The disulfide bond formation between cholesterol and TFO has advantage over other non-cleavable bonds in releasing TFO molecules within target cells.

Cholesterol conjugation did not affect the binding affinity of ODNs to their targets (469). Under certain conditions, cholesterol conjugation to ODNs could even increase the binding affinity to mRNA targets as well as forming more stable triplex structures, possibly through intercholesteryl hydrophobic interaction (478). In the mean time, the sequence specificity is also increased. The stability against nuclease attack is also increased by 3'-cholesteryl conjugation, but not by 5'-end conjugation (470). This is because the 3'-hydroxyl group is involved in the nucleophilic attack of the adjacent phosphate bond by the exonuclease enzyme.

Cellular uptake of a 3'-cholesterol-conjugated ODN was studied using a real-time confocal laser microscopy (479). It was observed that cytosolic uptake of cholesterol conjugate was increased 5-fold of that of PS-ODNs and 2-fold in nuclear uptake. Positions of conjugation on cholesterol (3, 7, or 22 positions of cholesterol attached to 3'-end of ODNs) have effects on the cellular uptake due to different hydrophobicity of the conjugates (480). It was found that cholesterol-derivatized ODNs at position 3 or 7 gave more favorable cellular binding and subcellular distribution. Furthermore, it is suggested that the interaction with specific proteins on cell surfaces contribute to the enhanced entry of cholesterol conjugates into cells (470).

The increased stability and enhanced cellular uptake result in the increased bioactivity of cholesterol conjugate. A PS-ODN specific for the 3' untranslated region of ICAM-1 mRNA was incorporated with a cholesterol at the 5'-end and shown to have increased antisense effect in cell culture (476). By conjugation with cholesterol at the 5'-end, a PS-ODN against mRNA multiple drug resistance-1 (MDR-1) gene has also shown increased silencing effects *in vitro* (481). Furthermore, it was found that conjugating cholesterol to both ends of a PS-ODN could confer more activity as compared to only one-end modification (482).

Cholesterol conjugation to PS-ODNs resulted in increased retention in plasma as well as their accumulation in the liver (470,483). When a 3'-cholesteryl-conjugated PS-ODN was administrated into rats intravenously, 63.7% of the dose was found in the liver, which was almost 2-fold higher compared to naked PS-ODNs (484). The plasma half life was 49.9 min, which is at the level of naked PS-ODNs. SECs, KCs and hepatocytes accounted for 45.7%, 33.0% and 21.3% of the liver uptake, respectively. A 3',5'-bis-cholesteryl-conjugated ODN with two cholesteryl moieties increased hepatic uptake to 83% (485). The hepatic uptake of occurred mainly by SECs (51.9% of the liver uptake). Hepatocytes and KCs were responsible for 24.9% and 23.3%, respectively. The increased retention in plasma is possibly due to the increased serum protein binding (486). However, it is not clear whether the increased hepatic uptake is due to an active transport of the lipophilic conjugates or whether the effects were simply due to the changes in lipophilicity. Nonetheless, this prompts using of cholesterol-conjugated ODNs for treating liver related diseases. The therapeutic effect of 5'-cholesterol-

conjugated PS-ODNs against ICAM-1 mRNA in mouse liver *in vivo* was significantly increased over unmodified PS-ODNs because of the increased hepatic uptake (470).

We have also conjugated cholesterol to a TFO molecule against  $\alpha 1(I)$  collagen gene promoter at 3' end via disulfide bond formation (428). Around 4-fold increase in the cellular uptake and enhanced bioactivity in inhibition of  $\alpha 1(I)$  collagen gene transcription were observed in HSC-T6 cell culture. After i.v. injection into rats, significant increase in hepatic uptake both in normal rats and fibrotic rats were achieved (Figure 11B). However, no significant change in the suborgan distribution in comparison with free TFOs was seen after separation of different liver cells (35%, 49% and 16% for hepatocytes, HSCs and KCs & SECs, respectively).

**5.3.2. PEGylation of Oligonucleotides**—PEG plays important roles in the pharmacokinetic profiles of small molecules as well as macromolecules (487). PEG is nontoxic and very soluble, reduces immunogenic reactions. By direct conjugation of PEG to ODNs, many advantages have been implicated: longer half-life of ODNs, higher stability against exonucleases and increased cellular uptake. In some cases, the solubility of ODNs can be increased, especially for some chemically modified ODNs. Furthermore, short PEG conjugation on ODNs is used as a spacer for additional conjugation to happen, since PEG itself can be made bifunctional. The higher molecular weight of PEG conjugates guarantees the longer circulation time by decreasing renal clearance. For PEG, the high water coordination of the polymer increases its hydrodynamic volume up to 3–5 times that of a globular protein having the same molecular weight and a glomerular filtration threshold is around 30–45 kDa.

PEG can be introduced to an ODN at the 3' end or 5' end or internal positions in the solid-phase synthesis of ODNs (488–490). However, these synthesis strategies do not allow easy upscaling. Subsequently, the use of the liquid-phase approach utilizing the PEG polymer as support became possible (491,492) and has been used for the large scale synthesis of PEGylated ODNs. PEG is used as a soluble, inert synthetic support, onto which nucleosides are added sequentially using phosphoramidite-based procedure. Normally, PEG is monomethoxy at one end (mPEG). The hydroxyl group at the distal end of mPEG is functionalized with the first nucleoside using similar chemistry for elongation of ODN chain, which results in the formation of a phosphate bond between PEG and ODN. PEG with a molecular mass between 5 and 20 kDa has been successfully incorporated with ODNs of up to 20 monomers. Normally, a phosphate bond between PEG and ODN is formed. However, different chemistries in the linker between ODN and PEG can also be introduced (493–495), which may confer better properties to the conjugates. PEG can be introduced after the synthesis of ODNs, which contain reactive groups, such as amines or thiols. For example, ODNs can be easily introduced with a 5' or 3' aminoethyl functionality. The formation of carbamate between ODN and PEG enables the release ability of ODN (494).

PEGylation of ODNs can increase their stability. PEG coupling to both the 3' and 5' terminal positions of an antisense ODN produced more than 10-fold increase in exonuclease stability, while having no effect on the ability of hybridization (488). Furthermore, the cellular uptake of ODNs can also benefit from PEGylation (496). This is possibly due to the introduction of hydrophobicity and shielding of negative charges of ODNs by PEG. Rapozzi et al. demonstrated that PEGylation (9 kDa) of a 13-mer (A, G) motif TFO targeting the 5' flanking region of the human bcr/abl oncogene could enhance cellular uptake (497). The triplex formation ability of the TFO was not affected *in vitro*. The stability was increased while PEGylated TFO less inclined to self-associate into multistrand structures. Therefore, the PEGylated TFO showed enhanced downregulation of the transcription of bcr/abl mRNA. In contrast, Manoharan et al. demonstrated that conjugation of ODNs against human ICAM-1 to a series of PEG esters of average molecular weight 550, 2200, and 5000 interfered with the cellular permeation of ODNs *in vitro* (470).

Different structures of PEG conjugated can confer different biological properties of PEGylated ODNs. Bonora et al. studied two different conjugates of an anti-HIV 12-mer ODN with a linear PEG and a branched PEG (498). It was found that only the conjugate with linear PEG showed anti-HIV activity. Furthermore, the number and attachment sites (at the 5', 3' or both 5' and 3' termini) of the conjugated PEG greatly influence the hydrophobicity of the conjugates (488).

Prolonged plasma retention is one of the major advantages of PEGylated ODNs compared to naked ODNs *in vivo*. Watson et al. (499) studied the pharmacokinetic parameters and *in vivo* activity of a PEGylated (40kDa) 2'F pyrimidine 2'OH purine RNA anti-human L-selectin aptamer. They found that the pharmacokinetic parameters were significantly improved by PEGylation. However, further improvement of *in vivo* activity is still needed. An aptamer directed against TGF- $\beta$ 2 was also PEGylated (20 kDa and 40 kDa) (500). Following single bilateral subconjunctival administration to Dutch-belted rabbits, the PEGylated aptamer showed prolonged residence in the subconjunctival space and aqueous compartment ( $t_{\max}$  = 6 and 12 h, respectively, in plasma) compared to naked ones. These properties warranted the further pharmacological evaluation of these conjugates.

An 18-mer PS-ODN targeting the initiation codon region of the anti-apoptotic gene Bcl-2 mRNA was conjugated to a PEG (20 kDa) at 5' end via releasable linkers (501). ODNs could be continuously released from the conjugate. Surprisingly, the researchers found that in 518A2 melanoma cells *in vitro*, PEGylation resulted in greatly diminished cellular uptake and therefore, bioactivity in cell cultures. In contrast, they demonstrated the pharmacokinetic profile of the conjugate was significantly improved (e.g., the dramatically increased AUC). However, only similar anti-tumor effects were observed for the conjugate and naked ODNs. In addition, this anti-tumor activity is observed accompanied with minimal immunostimulation, which may be due to the shielding effects of PEG for CpG motifs embedded in the ODN sequence.

However, in most cases, PEGylated ODNs themselves are used in combination with other delivery carriers, which further enhances the bioactivity. They can form micellar structures by forming complexes with cationic polymers such as PEI (502). The ODN/PEI polyelectrolyte complex forms an inner core while PEG chains surround it as a shell. Different linkage (glutathione-sensitive SS linkage, pH-sensitive linkage and thiomaleimide linkage) in the conjugates also substantially affected biological effects of the micelles (451,503). The use of PEGylated ODNs in combination with lipids could significantly improve the stability of complexes against serum (504). Furthermore, different targeting ligands such as folic acids can be attached at the distal terminus of PEG to have receptor-mediated targeted delivery of ODNs (503,505,506).

**5.3.3. Carbohydrate Conjugates for Targeted Delivery**—Delivery of ODNs to tissues does not mean that ODNs have been localized to target cells within tissues (453). ODNs can also be found at extracellular spaces. Therefore, to have efficient cellular uptake is one of the objectives for delivery of ODNs. Furthermore, specifically for liver tissue, ODNs nonspecifically distribute more to NPC compared to hepatocytes. Therefore, targeted delivery of ODNs to hepatocytes becomes the focus of most studies using direct carbohydrate conjugates with ODNs.

Carbohydrate conjugates, termed 'glycotargeting', provide novel strategies for targeted delivery, since different liver cells may express specific receptors recognizing different carbohydrate clusters. Glycotargeting exploits the highly specific interactions of endogenous lectins with carbohydrates (often with multiple carbohydrates). In particular, small molecule drugs including ODNs, no matter how heavily glycosylated will always have the potential to

pass into the kidneys, through glomerular filtration, and be rapidly cleared. Consequently, carbohydrate in combination with PEGylation provides an attractive alternative option.

The ASGPR is expressed in liver by hepatocytes, which recognizes specifically glycoproteins that possess exposed terminal galactose or lactose residues (507,508). In the case of lactose, the glucose moiety functions as a tether and the galactose serves as the ligand. The ligands are internalized via receptor-mediated endocytosis (509) and transferred to lysosomes (510). Therefore, galactose and its derivatives have been exploited for targeted delivery of ODNs by direct conjugation with them.

Cluster of galactose or its derivatives is believed to have more binding affinity to ASGPR than monovalent galactosides, which led to the term 'cluster effect' (511,512). The binding strength of a cluster ligand depended mainly on two factors: (1) the maximum spatial inter-galactose distances and (2) the flexibility of the arm connecting galactosyl residues and the branch points (513,514). A glycotriptide, *N*-acetylgalactosamine neoglycopeptide, Tyr-Glu-Glu-(aminohexyl GalNAc)<sub>3</sub>, YEE(ahGalNAc)<sub>3</sub>, was shown to bind to Gal/GalNAc receptor on hepatocytes with K<sub>d</sub> of subnanomoles (515). Hangeland et al. (516) conjugated a 7-mer MP-ODN with YEE(ahGalNAc)<sub>3</sub> via a metabolically stable thioether in HepG2 cells. The cellular uptake was ligand-specific and was increased 20- to 40-fold. However, the bioactivity was not tested. Subsequent studies of this glycoconjugate in mice also showed that hepatic uptake of this conjugate was 70% of the injected dose (517). In another study, when a PS-ODN was conjugated to this ligand, >90% inhibition of the integrated HBV via expression in hepatoma cells could be achieved (518).

A tetra-antennary cluster galactoside is N<sub>2</sub>-(N<sub>2</sub>-(N<sub>2</sub>,N<sub>6</sub>-bis[N-[p-(β-D-galactopyranosyloxy)-anilino]thiocarbamyl]-L-lysyl)-N<sub>6</sub>-(N-[p-(β-D-galactopyranosyloxy)-anilino]thiocarbamyl]-L-lysyl)-N<sub>6</sub>-(N-[p-(β-D-galactopyranosyloxy)-anilino]thiocarbamyl)]-L-lysine (L3G4) with K<sub>d</sub> of 6.5 nM (519,520). When attached to a 20-mer PO-ODN with a 3'-capped amine (L3G4-ODN), it was shown that in rats hepatic uptake was greatly enhanced from 19% for unconjugated ODN to 77% of the injected dose for L3G4-ODN. Furthermore, the uptake of L3G4-ODN could be mainly contributed by hepatocytes (75% of the total liver uptake).

Maier et al. (521) designed and synthesized another cluster galactoside based on structural features of known high affinity ligands for the ASGPR. It involves three β-aminogalactosyl residues attached to a rigid cholane scaffold via ε-aminocapramide linkers. The rigid steroid scaffold conferred more affinity upon binding to the receptor via a proper preorganization of the galactose residues. This ligand has been conjugated with different ODNs. However, no biological testing is available.

**5.3.4. Conjugation of Peptides**—Peptide conjugates of ODNs can be used to change pharmacokinetics as well as enhance cellular uptake and nuclear targeting. The hybridization properties of ODNs may also be improved in some cases. In contrast to the use of complexes, such covalent conjugates of peptides and ODN are discrete chemical entities of known stoichiometry. The efficiency of such peptides is generally high and, hence, very low concentrations of the peptides are needed to observe any biological effects.

**5.3.4.1. Conjugation with Cell Penetrating Peptides:** Cell penetrating peptides (CPPs), also called PTDs are small motifs in proteins having been shown to carry nucleic acids such as ODNs, siRNAs, and even plasmid DNA across cell membranes (522–524). Representatives of these peptides are the TAT protein from HIV-1 virus (525), *Drosophila* homeotic Antennapedia protein (Antp, marketed as penetratin) (526), and the herpes simplex virus type-1 (HSV-1) VP-22 transcription factor (527). The domains responsible for the transduction have been identified for these CPPs and the commonality between each PTD is the presence of basic

amino acids (arginine and lysine), which might be important for penetration of the membrane (524). Other peptides with similar properties include transportan (528) and MPG (529) and have also been developed for delivery of ODNs. One of the advantages of CPPs is that they are taken up by cells efficiently via caveolae-mediated endocytosis as well as macropinocytosis, which do not lead to lysosomal degradation of cargos, or nonendocytic pathways (433), even though the mechanisms are still controversial and may depend on cell type, conjugated cargo and other factors (530). Therefore, these peptides provide an efficient strategy for enhancing cellular uptake of ODNs and improving their intracellular delivery to cytosol as well. In most cases, the covalent linkage between CPPs and ODNs is via disulfide bond formation, which facilitates the release of cargoes inside cells.

The most extensive applied CPPs for delivery of ODNs are TAT and Antp peptides. The minimal TAT transduction domain is the basic residues 47–57 (435) and the third alpha helix (residues 43–58) of the Antp homeodomain are required for transduction (526). Astriab-Fisher et al. (531) used TAT and Antp conjugated to a PS-ODN targeting a site flanking the AUG of mRNA of the MDR1 gene, which encodes P-glycoprotein. Both peptides were able to efficiently deliver the ODN to cells in culture. Effective inhibition of P-glycoprotein expression was attained with submicromolar concentrations of the conjugates. One striking feature of the peptide-ODN conjugates is that they inhibited P-glycoprotein expression more effectively in the presence of serum than in the absence of serum. Another study from the same group also showed the successful transportation of 2'-O-methyl PS-ODNs conjugated to TAT and Antp peptides to the cytoplasm as well as nucleus, leading to an increased pharmacological activity without compromising the specificity of antisense ODNs (531). In addition, a *retro-inverso* form of Ant was a peptidomimetic modification to confer more stability but still retain its activity and can rapidly be internalized and distribute throughout the cytoplasm and even the cell nucleus (532). When a PNA specific for the AUG translation inhibition region of prepro-oxytocin mRNA was coupled to this *retro-inverso* peptide, the conjugate could be quickly taken up by cultured cerebral cortex neurons (533). PNA was also able to enter the cells, but more slowly. However, in this study, both PNA-peptide and PNA itself gave similar effects on gene inhibition.

Other peptides may also have similar properties. A chimeric peptide MPG consisting of a hydrophobic fusion domain derived from HIV gp41 and a hydrophilic nuclear localization signal derived from the SV40 T-antigen was constructed to have membrane translocation property (529,534). This peptide was conjugated to a PS-ODN via a disulfide bond and greatly increased the cellular uptake of the ODN (534). Moulton et al. (535) studied the effect of conjugation of arginine-rich peptides to PMO. Different factors including the number of arginines in the peptide, the choice of linker, the peptide conjugation position, the length of the PMO, and the cell culture conditions, were investigated to optimize the efficiency of peptide-PMOs. PMO was used to correct missplicing of exogenous luciferase pre-mRNA. A peptide containing 9 arginines was found to be best and even more efficient than Tat and Antp. Following study confirmed the effectiveness of the PMO conjugated with one arginine-rich peptide, (RXR)<sub>4</sub>XB, where R = arginine, X = 6-aminohexanoic acid and B = beta-alanine in correcting the endogenous gene mutation *in vitro* (536) as well as in human muscle explants (537).

Only few reports demonstrated the *in vivo* use of these peptide carriers for ODNs. A 21-mer PNA specific for the human type 1 galanin receptor was conjugated to cell permeation peptides via disulfide bonds and the bioactivity *in vivo* was demonstrated in rats (538). The peptides used in this study were transportan and Antp. The conjugates improved internalization and down-regulation of the human galanin receptor *in vitro* in Bowes cell line. After intrathecal administration of the conjugates, the galanin binding was decreased in the dorsal horn.

The stability of CPP-ODN conjugate is one concern for their *in vitro* and *in vivo* applications. A recent study investigated the effects of several structural features of arginine-rich CPPs on the metabolic stability of CPP conjugated to phosphorodiamidate morpholino oligonucleotides (PMOs) in human serum and in cells (539). Those structural features include amino acid configurations (d or l), incorporation of non-alpha-amino acids, peptide sequences, and types of linkages between CPPs and PMOs. It was found that d-configuration CPPs were completely stable, while l-CPPs were degraded in both serum and HeLa cells. Insertions of 6-amino hexanoic acid residues (X) into an R8 peptide failed to increase intracellular stability, while insertions of  $\beta$ -alanines (B) increased intracellular stability. An amide or a maleimide linkage was stable in both serum and cells; however, an unhindered disulfide linkage was not stable in either, which is contrary to other studies.

Furthermore, there are several concerns for covalent attachment of CPPs with ODNs. It is known that the dense cationic charge of CPPs is critical for their interaction with negative charged phospholipids and proteoglycans on the cell surface prior to the induction of cell internalization (540). Upon linkage of CPPs with 6–8 positive charges to ODNs with ~20 negative charges, the CPPs are almost neutralized and lose the ability to bind the cell surface (541). Furthermore, the high tendency of cationic peptides to condense ODNs makes covalent attachment of CPPs to ODNs difficult. In fact, CPP mediated delivery of ODNs has been unsuccessful in several instances (542–544), which suggests that further understanding of the mechanisms of the cellular uptake of CPPs and careful design of CPP-ODN conjugates are needed. Using uncharged ODNs such as PMO-ODN and PNA may avoid this problem.

**5.3.4.2. Fusogenic Peptide Conjugation:** Peptides derived from the N-terminal sequence of the influenza virus hemagglutinin subunit HA-2, or prepared synthetically, such as GALA or KALA are pH-sensitive fusogenic, which can use the acidic pH of the endosome to induce their rupture (545,546). These peptides change conformation at acidic pH and destabilize the endosomal membrane resulting in an increased cytoplasmic delivery of ODNs. Bongartz et al. (545) conjugated a peptide derived from HA-2 to an antisense PO-ODN targeting the AUG initiation site of the HIV TAT protein via a disulfide or thioether bond and observed as 5 to 10 fold improvement of the anti HIV activities, respectively. However, no sequence specificity was obtained and the fusogenic peptide possessed some antiviral activities on its own (IC<sub>50</sub>: 6  $\mu$ M). Very few following work exists regarding fusogenic peptide conjugated ODNs. Shadidi et al. (547) explored the potential of the cancer cell binding peptide LTVSPWY to specifically deliver antisense ODN. The PS-ODN against the ErbB2 receptor was conjugated to the peptide via a disulfide bond. The conjugate increased the internalization of PS-ODN by cancer cell line SKBR3 and resulted in ~60% inhibition of ErbB2, which was probably due to the fusogenic property of the LTVSPWY peptide.

**5.3.4.3. Conjugation with Nuclear Targeting Peptides:** Chaloin et al. (534) designed a series of peptides with a nuclear localization signal (PKKKRKV) fused with different peptides containing a hydrophobic motif. A PS-ODN was conjugated to these peptides through a disulfide bridge. The cellular up was found to be an efficient, with a little effect of temperature on internalization, suggesting the involvement of non-endocytic pathway in the cellular uptake. Another signal peptide KEDL was shown to carry ODNs to the endoplasmic reticulum and from there to the cytosol and the nucleus where their targets are located (548). When a 5', 3'-modified pentacosanucleotide, complementary to the translation initiation region of the gag mRNA of HIV was coupled to a bromoacetyldodecapeptide containing a KDEL signal sequence, the anti-HIV activity was improved. Furthermore, it was also found that a thioether bridge for the peptide conjugate had a higher activity over that with a disulfide bond.

**5.3.5. Polymer-based Conjugates—**Using polymer-based conjugates, it becomes possible to present viral mimetic properties to the carriers for ODNs such that several

functionalities can be integrated into a multi-component carrier with many objectives: i) targeting by receptor-mediated binding to surfaces of recipient cells and endosomal uptake into these cells; ii) provision of an activity for disruption of endosomal membranes and for the release of the drug carrier system into the cytoplasm; iii) a cleavable linkage for the release of the drug from the carrier system; iv) a prolonged circulation through protection by PEG against degradation activities; and v) attachment of a fluorescent reporter for the localization of the carrier system. For a polymeric carrier to be efficient in delivering ODNs to the target cells, it has to overcome many extracellular and intracellular barriers as mentioned above. Although polymeric carriers have witnessed great advancements, a polymeric carrier, that is capable of addressing all these challenges, is still yet to be achieved. Furthermore, regarding so many barriers in ODN delivery, different design elements in developing polymeric carriers may affect different barriers to varying extents, such that a polymeric carrier that is rationally designed to overcome certain barriers may alter the cellular processing of the carrier, dramatically increasing the importance of other barriers.

**5.3.5.1. Hydroxypropyl methacrylate (HPMA)-based polymers:** HPMA-based polymers have been extensively used as carriers for drug delivery including delivery of ODNs (549). HPMA homopolymer is biologically inert and exhibits no antibody response after intraperitoneal injection into mice (550). They can be easily modified to incorporate various functionalities by forming different HPMA copolymers and these copolymers may present some immunogenicity (550). Normally, HPMA polymers of molecular weight less than 50kDa are used to permit glomerular filtration and eventual excretion. Targeting to a specific cell population by attaching moieties such as carbohydrates or antibodies can be achieved. However, even though HPMA-based polymers have been studied *in vivo* for delivery of anticancer drugs, few studies on the delivery of ODNs using HPMA polymers are reported.

HPMA-based polymer-drug conjugates normally are lysosomotropic (551), leading to possible degradation of drugs, especially for nucleic acids. Jensen et al. (552) conjugated an antisense PS-ODN to HPMA copolymer via lysosomal cleavable glycyphenylalanylleucylglycine (GFLG) peptide linker and studied the subcellular fate of the conjugate in HepG2 cells (Figure 12A). Two different conjugates were synthesized. One is PS-ODN attached to the HPMA polymer via non-cleavable spacer, glycyglycine (GG), and another is PS-ODN attached to the HPMA polymer via GFLG peptide linker. In the latter conjugate, the HPMA polymer and the PS-ODN were labeled with different fluorescence dyes for separate tracking under confocal microscopy. With the non-cleavable spacer, the conjugate was entrapped in the vesicular compartments. In contrast, the conjugate with cleavable linker showed separation of two different colors after 2~4 h incubation, which suggested the release of PS-ODN from HPMA polymer, and significant amount of PS-ODN distributed into cytoplasm and nuclei compared to free PS-ODN. Furthermore, the biological activity of the PS-ODN was increased compared to free PS-ODN.

The versatility of HPMA polymers can be further demonstrated in another study. Wang et al. (553) synthesized a HPMA copolymer containing an activated sulfide group, onto which a PS-ODN modified with sulfhydryl group and/or Antp peptide, were attached via disulfide bond formation. Antp peptide was also included to increase cellular uptake (173). The successful synthesis of the conjugates including HPMA polymer-Antp, HPMA polymer-PS-ODN, and HPMA polymer-Antp-PS-ODN was demonstrated. However, the conjugate was found to be no improvement for cellular uptake of PS-ODN, which may be because the peptide formed an intermolecular electrolyte complex with ODNs leading to its incapability of translocating through the membranes.

**5.3.5.2. Poly(L-malic acid):** Lee et al developed poly( $\beta$ -L-malic acid) (PLMA) based copolymer, Polycyfin, as a platform for drug delivery (Figure 12B) (214). PLMA can be

prepared from the myxomycete *Physarum polycephalum* with high purity (554). PLMA resembles HPMA polymers in carrying abundant carboxyl groups. However, unlike HPMA polymers, it is biodegradable since it is a polyester. In addition, it is highly water-soluble. These properties make PLMAs ideal drug carriers.

Polycefin was synthesized as a multicomponent prototype for targeted delivery of ODNs to brain tumors (214), which contained: i) biodegradable, nonimmunogenic, nontoxic PLMA as backbone; ii) morpholino ODN targeting  $\alpha 4$  and  $\beta 1$  chains of laminin-8, which is specifically overexpressed in glial brain tumors via disulfide bonds; iii) monoclonal anti-transferrin receptor antibody for specific tissue targeting; iv) L-valine containing, pH-sensitive membrane disrupting unit(s), v) PEG. Polycefin was extensively characterized (214). The different functionalities were statistically distributed over PLMA backbone. On the basis of a Mw of 50kDa (approximately 500 malyl residues), Polycefin molecules contain on average 1 molecule of the monoclonal antibody (mAb), 11 molecules of either morpholino ODN, 22 molecules of mPEG (5kDa), 210–220 molecules of L-valine (membrane disrupting module), and 140–150 pending carboxylate residues. The composition accounted for a molecular mass of approximately 550kDa.

Polycefin was shown to be taken up by two human glioma cell lines U87MG and T98G cells via a receptor mediated endocytosis and was able to inhibit the synthesis of  $\alpha 4$  and  $\beta 1$  chains of laminin-8. After i.v. inject into mice, the accumulation of Polycefin in brain tumor tissue was via the antibody-targeted transferrin receptor-mediated pathway along with a less efficient mechanism of enhanced permeation and retention (EPR). Furthermore, Polycefin was shown to be nontoxic. The accumulation of Polycefin to other major tissues such as liver and kidney was to much less extent (555). And, it was preferably accumulated into xenografted human MDA-MB 468 breast tumor in mice after 60min (555).

**5.3.5.3. Proteins as carriers:** Besides as carriers, proteins can be served as ligands, such as glycoproteins and antibodies of specific receptors. Synthetic polypeptides have also been developed for delivery of ODNs.

ASGPs recognize their receptors (ASGPR) on hepatocytes. Rajur et al. conjugated ODNs to ASGP by a disulfide linkage (556). Six ODN molecules could be conjugated to each ASGP molecule. The ODN was designed to be complementary to the mRNA of the IL-6 signal transduction protein (gp130) and be delivered to HepG2 cells *in vitro*. These conjugates inhibited the cytokine-stimulated up-regulation of the acute-phase protein haptoglobin.

Glycoproteins are also attractive objects for targeting liver. Plasma proteins such as albumin can be randomly derivatized with sugar groups. For example, in bovine serum albumin (BSA), sugars can be reacted with  $\epsilon$ -NH<sub>2</sub> of lysines. Galactose, mannose and mannose 6-phosphate (M6P) have been used to modify BSA for drug delivery to hepatocytes, KCs and HSCs, respectively (557,558). The efficiency of endocytosis and further degradation of glycoproteins can be largely influenced by the number, density, and geometric clustering of the sugar groups on the protein molecules (559,560). Even though many drugs have been targeted to liver using glycoproteins, few reports focused on the delivery of ODNs as well as siRNAs.

Specific antibodies for receptors on target cell surface can be used as carrier molecules besides being used as targeting ligands. Walker et al. (561) used a transferring receptor antibody to deliver ODNs. A maleimide-derivatized IgG was reacted with a 5'-thiol-modified ODN. Human glioblastoma cell line U87-MG and the human endothelial cell line ECV304, which express the transferring receptor on their surfaces, bound and internalized the antibody-ODN conjugates three-fold more effectively than naked ODNs. In another study, a rat transferring receptor antibody (OX-26) was conjugated with ODNs and could enhance the cellular uptake

into an immortalized cell line called RBE4 as an *in vitro* model of the blood-brain barrier (562). The subcellular trafficking was studied. It was found that the OX-26 conjugates showed altered subcellular distribution from those for naked ODNs or a nonspecific IgG conjugate.

**5.3.5.4. Targeted drug delivery for treating liver fibrosis:** Antifibrotic drugs, which are used predominantly in animal models and cell cultures but not humans, are neither liver- nor fibrosis-specific. Therefore, their clinical application necessitated the development of site-directed delivery systems affecting quite selectively the effector cells during liver fibrosis. However, particles larger than 100–150 nm cannot pass the fenestrae in the endothelial lining. This is true for most particulate systems and they can only reach KCs, pit and SECs. This situation is even worse in the case of liver fibrosis, where the loss of fenestrae becomes apparent.

Efforts to targeted delivery to HSCs were first contributed by Beljaars et al. (558). In their study, a human serum albumin (HSA)-based bioconjugate modified with M6P was reported to be specifically taken up by activated HSCs in experimental liver fibrosis models. The M6P/insulin-like growth factor II (M6P/IGF-II) receptor, which is expressed in particular upon HSC during fibrosis, can be utilized for targeted delivery to activated HSCs (563,564). The M6P/IGF-II receptor has been characterized as a 275 kDa Mr receptor which bind M6P even in absence of cation is a membrane lectin involved both in lysosomal enzyme trafficking and in the endocytic pathway (565) and can bind diverse M6P- and non M6P-containing ligands such as TGF- $\beta$ , proliferin, IGF-II, RA and uPA receptor (566). In the fibrotic liver M6P/IGF-II receptors facilitate the activation of latent TGF- $\beta$  (567,568). In fact, Bonfils et al. (569) had conjugated 19mer ODNs to M6P-BSA via disulfide bond and demonstrated 2-fold higher cellular uptake by macrophages (J774 cells) as compared to fibroblast-like BHK cells and 30-fold higher than monocytes (U937 cells). However, these authors did not determine the biodistribution and uptake of M6P-BSA-ODN conjugate by different liver cells.

HSA modified with M6P (M6P-HSA) was synthesized with different numbers of M6P (2, 4, 10, and 28) (558). The hepatic uptake of M6P-HSA was found to increase with increasing numbers of M6P in the conjugates. With 2, 4 and 10, the conjugates accumulated for 9% or less of injected dose in fibrotic rat livers, while with 28, 59% was in the liver. Intrahepatic distribution of M6P-HSA was determined as well using double-immunostaining techniques (staining for HSA and HSCs). The number of M6P in the conjugates also affected their accumulation in HSCs, with increased substitution of M6P associated with an increased accumulation in HSCs and 70%  $\pm$  11% of the intrahepatic staining for M6P<sub>28</sub>-HSA was found in HSCs. In addition, M6P-BSA accumulates in slices of normal and cirrhotic human livers. After incubation of this neoglycoprotein with human tissue, the protein is found in nonparenchymal liver cells. Furthermore, an extensive study *in vitro* and in human liver slices confirmed the specific uptake of M6P<sub>28</sub>-HSA by activated HSCs, but not quiescent HSCs (570). In addition, a lysosomal pathway for cellular uptake was suggested for the conjugate. These studies created new opportunities for the pharmacological intervention of liver fibrosis, since no delivery strategies had been developed for HSCs before. Following these, many antifibrotic drugs have been delivered to HSCs by conjugation with M6P-BSA/HSA, such as glycyrrhizin (277), antiproliferative drugs including mycophenolic acid (571), pentoxifylline (571) and doxorubicin (571), apoptosis-inducing drug 15-D-PGJ2 (572), a tyrosine kinase inhibitor, 4-chloro-N-[4-methyl-3-(4-pyridin-3-yl-pyrimidin-2-ylamino)-phenyl]-benzamide (573) and 18 $\beta$ -glycyrrhizic acid (574). These attempts to selectively deliver antifibrotic drugs to HSCs in experimental liver fibrosis have demonstrated enhanced disposition in activated HSCs. However, the efficacy in preventing liver fibrosis still needs to be improved.

Few studies have attempted to delivery ODNs to activated HSCs. The high potency of ODN-based gene silencing therapeutics such as TFOs grants them high potential as antifibrotic agents. Based on the above mentioned delivery strategy for HSCs, we proposed M6P-BSA

conjugates with TFOs to treat liver fibrosis (Figure 13). Bovine serum albumin is used as the backbone, onto which targeting ligand M6P can be attached with the NH<sub>2</sub> of lysines. In addition, TFO molecules modified sulfhydryl functionalities can be conjugated with BSA via disulfide bond formation. Disulfide bonds enable the intracellular release of TFOs upon taken up by HSCs. TFOs used are specific for type  $\alpha 1(I)$  collagen gene promoter. Therefore, this conjugate has potential to be used as antifibrotic drugs.

## 6. CONCLUDING REMARKS

Thorough understanding of liver pathophysiology is essential to devise strategies for treating liver fibrosis. Injury to hepatocytes leads to the release of ROS, inflammatory cytokines and growth factor, which in turn activate HSCs. Therefore, prevention of apoptosis of hepatocytes and induction of apoptosis of activated HSCs are attracting more attention. Although many different organic compounds are being developed as antifibrotic drugs, none of them are liver- and fibrosis-specific. Therefore, strategies for site-specific delivery strategies are urgently needed.

ODN-based gene silencing technologies provide an efficient and specific way to interrupt the signaling pathways and prevent the production of key factors promoting the progression of liver fibrosis. However, ODNs are polyanions and poorly taken up by target cells. Moreover, deposition of excess scar matrix and the disappearance of sinusoidal gaps do not allow efficient delivery of liposomal and nanoparticulate systems. Further, complex formation with cationic lipids and polymers causes non-specific interaction with plasma proteins and non-target cells. Therefore, bioconjugation of ODNs with non-charged polymer containing ligands has great potential for enhance delivery of ODNs to specific liver cells. Since M6P receptors present on the surface of HSCs are upregulated during liver fibrosis, bioconjugation of TFOs specific for  $\alpha 1(I)$  collagen gene promoter with M6P-BSA is efficiently taken up by activated HSCs and M6P-BSA-TFO conjugates have potential for treating liver fibrosis.

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## ABBREVIATION

2'-AE, 2'-O-aminoethyl  
aFGF, Acidic Fibroblast Growth Factor  
AngII, Angiotensin-II  
ASGP, Asialoglycoprotein  
ASGPR, Asialoglycoprotein Receptor  
AsOR, Asialoorosomucoid  
AT-1, Angiotensin-II Type 1 receptor  
AT-2, Angiotensin-II Type 2 receptor  
BSA, Bovine Serum Albumin  
CB, Cannabinoid  
CCl<sub>4</sub>, Carbon Tetrachloride  
CPPs, Cell Penetrating Peptides  
CD, Cluster of Differentiation  
CGE, Capillary Gel Electrophoresis  
CINC, Cytokine-Induced Neutrophil Chemoattractant  
CLV, Centrolobular Vein  
COX-2, Cyclooxygenase-2  
CTGF, Connective Tissue Growth Factor

DDR, Discoidin Domain Receptor  
 DMF, Dimethylformamide  
 DMN, Dimethyl Nitrosamine  
 DMS, Dimethyl Sulfate  
 15-D-PGJ2, 15-Deoxy  $\Delta^{12, 14}$  Prostaglandin J2  
 dsDNA, Double-Stranded DNA  
 ECM, Extracellular Matrix  
 EGF, Epidermal Growth Factor  
 EMSA, Electrophoretic Mobility Shift Assay  
 EPR, Enhanced Permeation and Retention  
 ET-1, Endothelin-1  
 Fas-L, Fas Ligand  
 FGF, Fibroblast Growth Factor  
 GalN, *D*-Galactosamine  
 GalNAc, *N*-Acetylgalactosamine  
 GLAP, Glial Fibrillary Acidic Protein  
 HCV, Hepatitis C Virus  
 HGF, Hepatocyte Growth Factor  
 HSA, Human Serum Albumin  
 HSC, Hepatic Stellate Cell  
 HSV-1, Herpes Simplex Virus Type-1  
 ICAM-1, Intracellular Adhesion Molecule-1  
 IGF-I, Insulin Growth Factors I  
 IGF-II, Insulin Growth Factors II  
 IFN-,  $\gamma$  Interferon  $\gamma$   
 IL-6, Interleukin-6  
 IL-10, Interleukin-10  
 IL-2R,  $\alpha$  Interleukin-2 Receptor  
 KC, Kupffer Cell  
 L3G4, N2-(N2-(N2,N6-bis[N-[p-( $\beta$ -D-galactopyranosyloxy)-anilino]thiocarbamyl]-L-lysyl)-N6-(N-[p-( $\beta$ -D-galactopyranosyloxy)-anilino]thiocarbamyl]-L-lysyl)-N6-(N-[p-( $\beta$ -D-galactopyranosyloxy)-anilino]thiocarbamyl)]-L-lysine  
 LNA, Locked Nucleic Acid  
 lpr, Lymphoproliferation  
 LPS, Lipopolysaccharide  
 LTBP, Latent TGF- $\beta$ -Binding Protein  
 mAb, Monoclonal Antibody  
 M6P, Mannose 6-phosphate  
 M6P/IGF-II, Mannose 6-phosphate/Insulin-like Growth Factor II  
 MCP-1, Monocyte Chemotactic Protein-1  
 2-MEA, 2-Mercaptoethylamine  
 MMP, Matrix Metalloproteinase  
 MP, Methylphosphonate  
 mPEG, Poly(ethylene glycol) with Monomethoxy at one end  
 mRNA, Messenger RNA  
 NASH, Nonalcoholic Steatohepatitis  
 NCAM, Neural Cell Adhesion Molecule  
 NF- $\kappa$ B, Nuclear Factor  $\kappa$ B  
 NGF, Nerve Growth Factor  
 NHS, *N*-Hydroxysuccinimidyl Ester  
 NK, Natural Killer  
 NPCs, Nonparenchymal cells  
 ODN, Oligonucleotide

PAF, Platelet-Activating Factor  
 PAGE, Polyacrylamide Gel Electrophoresis  
 PAI-1, Plasminogen Activator Inhibitor-1  
 PBC, Primary Biliary Cirrhosis  
 PC, Phosphatidylcholine  
 PDGF, Platelet-Derived Growth Factor  
 PEG, Poly(ethylene glycol)  
 PEI, Poly(ethyleneimine)  
 PGE, Prostaglandin E  
 PLL, Poly(L-lysine)  
 PLMA, poly( $\beta$ -L-malic acid)  
 PMA, Phorbol-12 myristate-13 acetate  
 PMOs, Phosphorodiamidate Morpholino Oligonucleotides  
 PN, Phosphoramidates  
 PNAs, Peptide nuclei acids  
 PO, Phosphodiester  
 PPAR, Peroxisome Proliferator-Activated Receptor  
 PS, Phosphorothioate  
 PTDs, Protein Transduction Domains  
 REPA, Restriction Enzyme Protection Assay  
 RA, Retinoic Acid  
 RBV, Ribavirin  
 RES, Reticuloendothelial System  
 ROS, Reactive Oxygen Species  
 RTK, Receptor Tyrosine Kinase  
 SAME, S-Adenosyl-L-Methionine  
 SEC, Sinusoidal Endothelial Cell  
 S1P, Sphingosine-1-phosphate  
 siRNA, Small Interference RNA  
 TFO, Triplex Forming Oligonucleotides  
 TGF-,  $\beta$  Transforming Growth Factor- $\beta$   
 THC,  $\Delta$ 9-Tetra-Hydrocannabinol  
 TIMP, Tissue Inhibitor of Metalloproteinase  
 TNF-,  $\alpha$  Tumor Necrosis Factor- $\alpha$   
 TRAIL-R, TNF- $\alpha$ -Related-Apoptosis-Inducing-Ligand Receptor  
 UDCA, Ursodeoxycholic Acid  
 uPA, Urokinase-Type Plasminogen Activator  
 VCAM, Vascular Cell Adhesion Molecule

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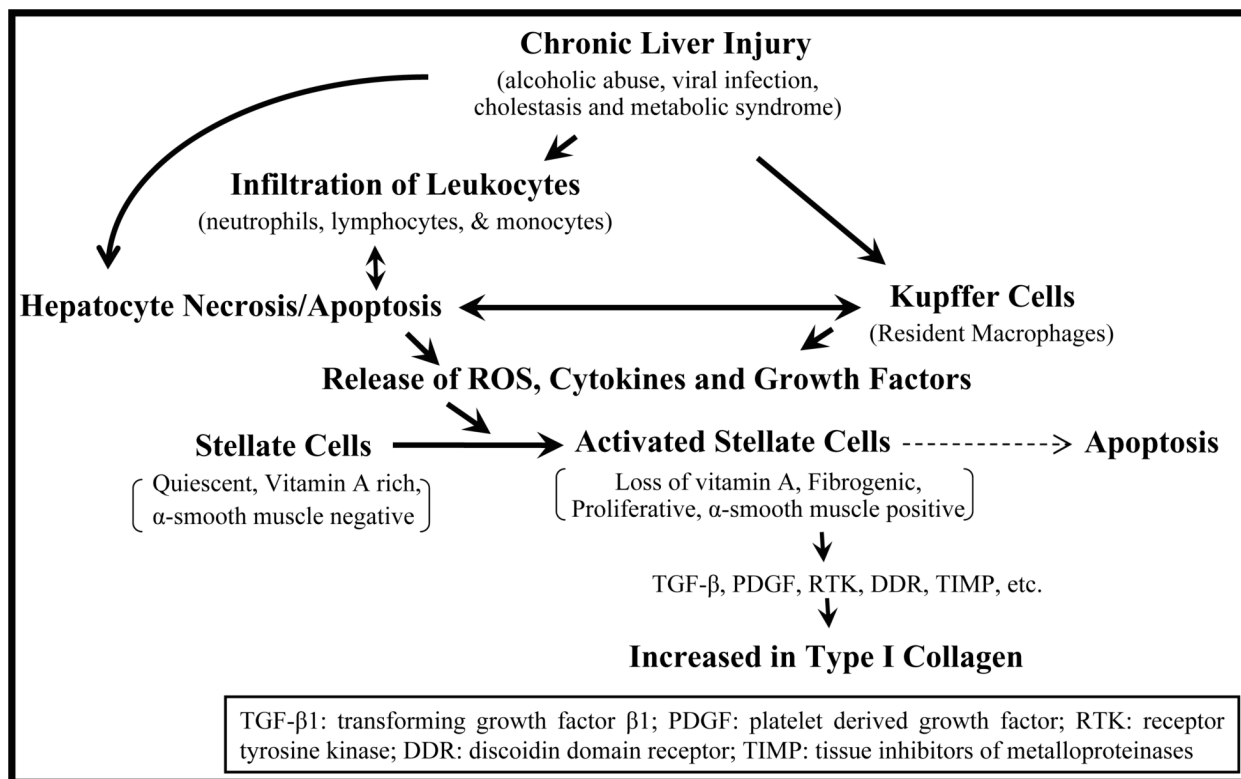
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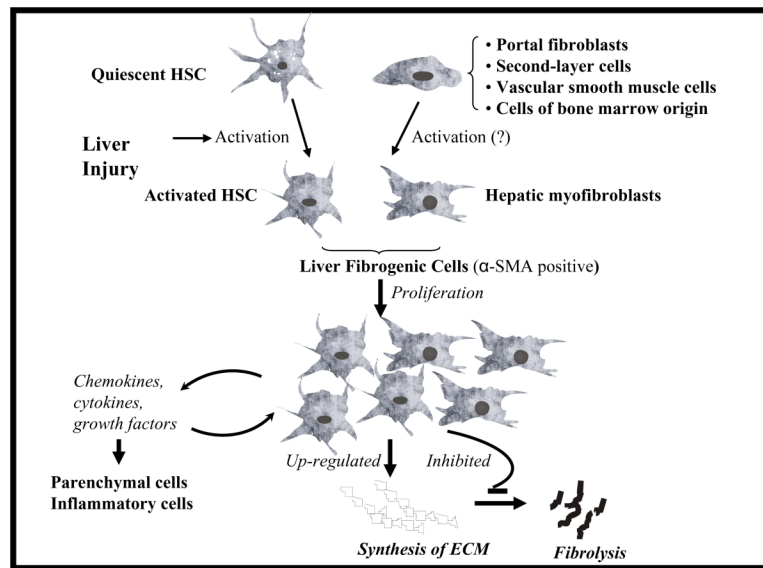
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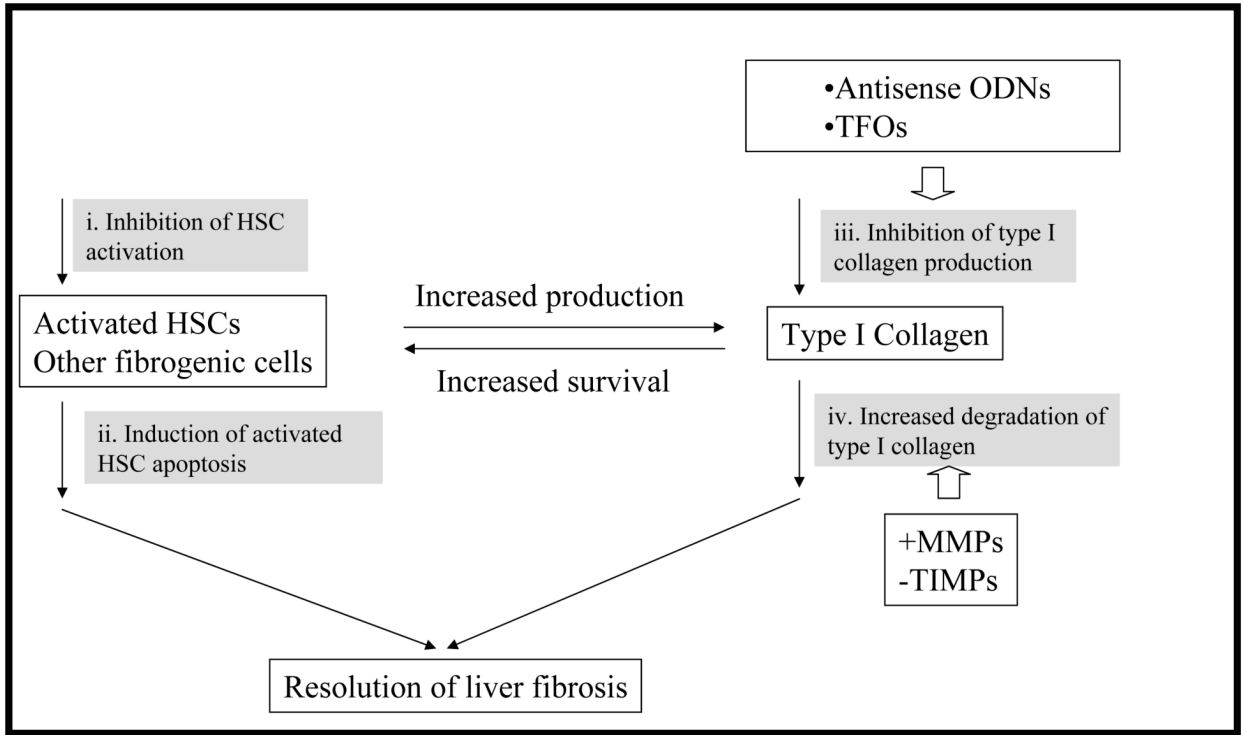
**Figure 1. Events leading to liver fibrosis**

During liver injury, infiltrating leukocytes (neutrophils, lymphocytes and monocytes) along with resident macrophages (Kupffer cells) release reactive oxygen species (ROS), growth factors and inflammatory cytokines, leading to activation of hepatic stellate cells (HSCs) into actively proliferating,  $\alpha$ -smooth muscle actin-containing myofibroblast-like cells. The activated HSCs are the source of cytokines, chemotatics, and also secrete large amounts of type I collagen and other extracellular matrix (ECM) components. Apoptosis of activated HSCs is implicated in the spontaneous resolution of liver fibrosis.



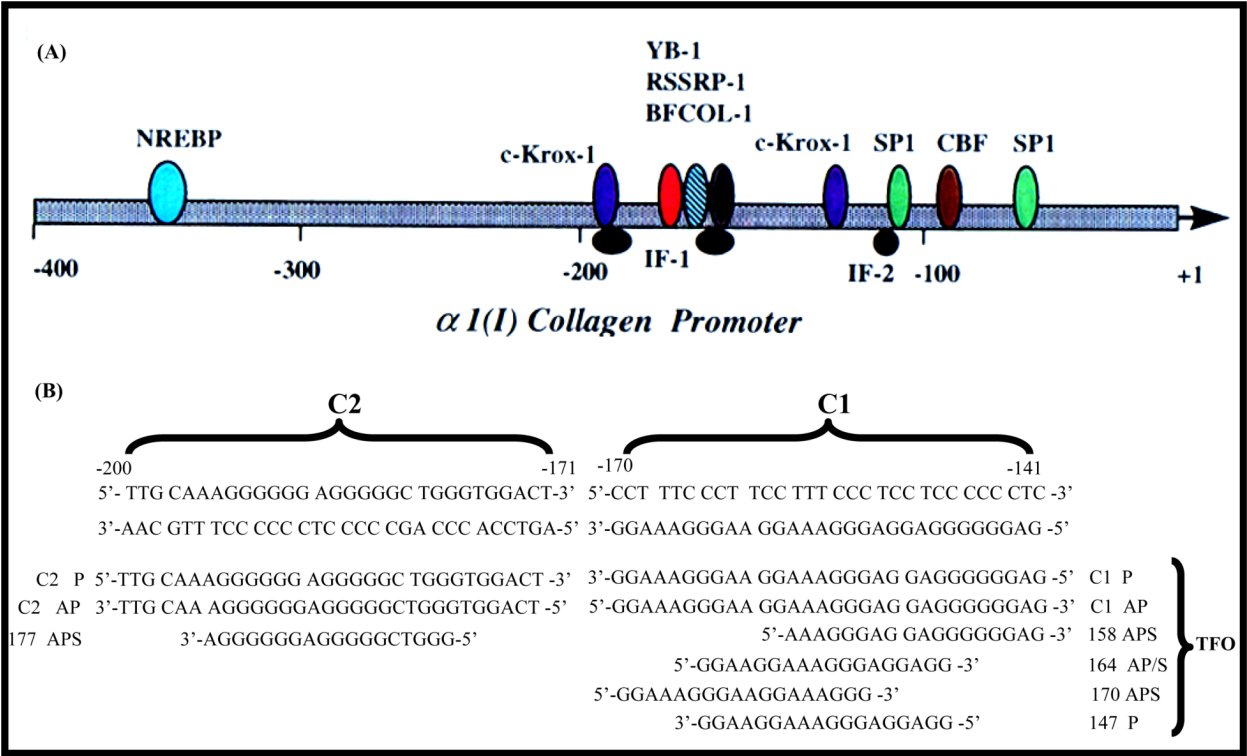
**Figure 2. Different liver fibrogenic cells**

Due to liver injury, hepatic stellate cells (HSCs) undergo transformational change into myofibroblast-like activated HSCs, which are depleted of vitamin A, but rich in  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA). There is considerable evidence supporting that HSCs are a major source of fibrogenic cells in the injured liver. However, contributions from other cell types including portal fibroblasts, second-layer cells located around centrilobular veins (CLVs), vascular smooth muscle cells and cells of bone marrow origin are also possible. These liver fibrogenic cells proliferate at the sites of liver injury, produce a variety of proinflammatory cytokines, chemokines and growth factors, synthesize extracellular matrix (ECM) proteins and inhibit their degradation, leading to fibrosis.

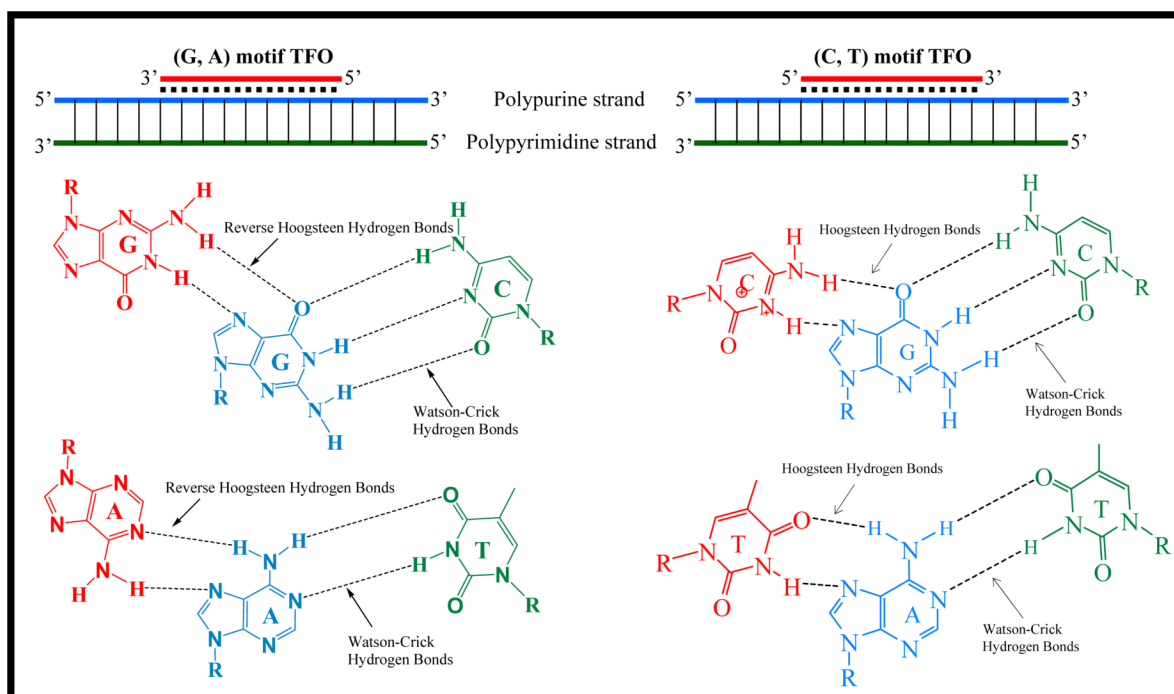


**Figure 3. Treatment strategies for liver fibrosis**

The common characteristics of liver fibrosis is that activated HSCs and other fibrogenic cells produce excess amount of type I collagen. Accumulation of type I collagen provides survival signal for activated HSCs. Therefore, strategies focusing on fibrogenic cells themselves as well as type I collagen regardless of etiology of liver injuries are attractive. Inhibition of the activation of HSCs (i) and induction of their apoptosis (ii) have attracted lots of attention. Gene silencing technologies have been utilized to directly inhibit the production of type I collagen (iii) at gene level. Inducing degradation of type I collagen (iv) can be realized by increasing the activity of matrix metalloproteinases (+MMPs) and inhibiting the activity of tissue inhibitor of metalloproteinases (–TIMPs). Decreased disposition of type I collagen and remodeling of extracellular matrix (ECM) accelerate apoptosis of activated HSCs.

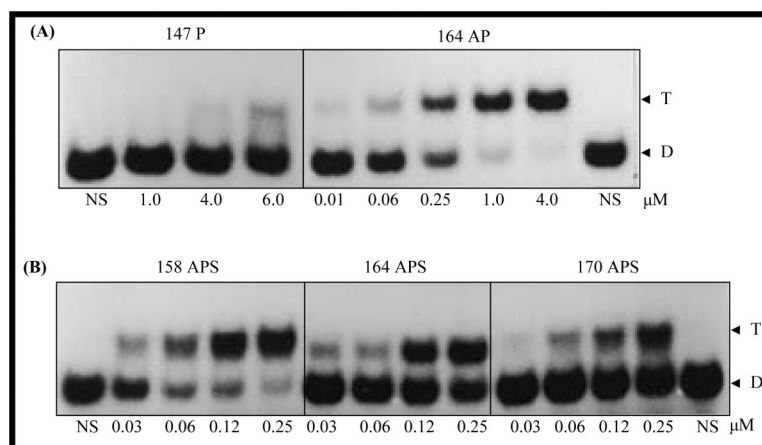


**Figure 4. Sequence of the rat  $\alpha 1(I)$  collagen promoter showing duplex targets C1 and C2 and the TFOs**  
(A) The schematic illustration of rat  $\alpha 1(I)$  collagen promoter region. Many transcription factors have been shown binding to this region and play key roles in regulation of the gene expression.  
(B) Phosphodiester and phosphorothioate triplex forming oligonucleotides (TFOs) are designed with different starting position corresponding to the type  $\alpha 1(I)$  collagen promoter sequence. These TFOs can be parallel or antiparallel to the target sequence. They are 18mer or 30mer. The ability of triplex formation can be different. P: parallel; AP: antiparallel; APS: antiparallel phosphorothioate. Reproduced with permission from Joseph et al. (1998) *Nucleic Acids Res.* **25**(11):2182–8.



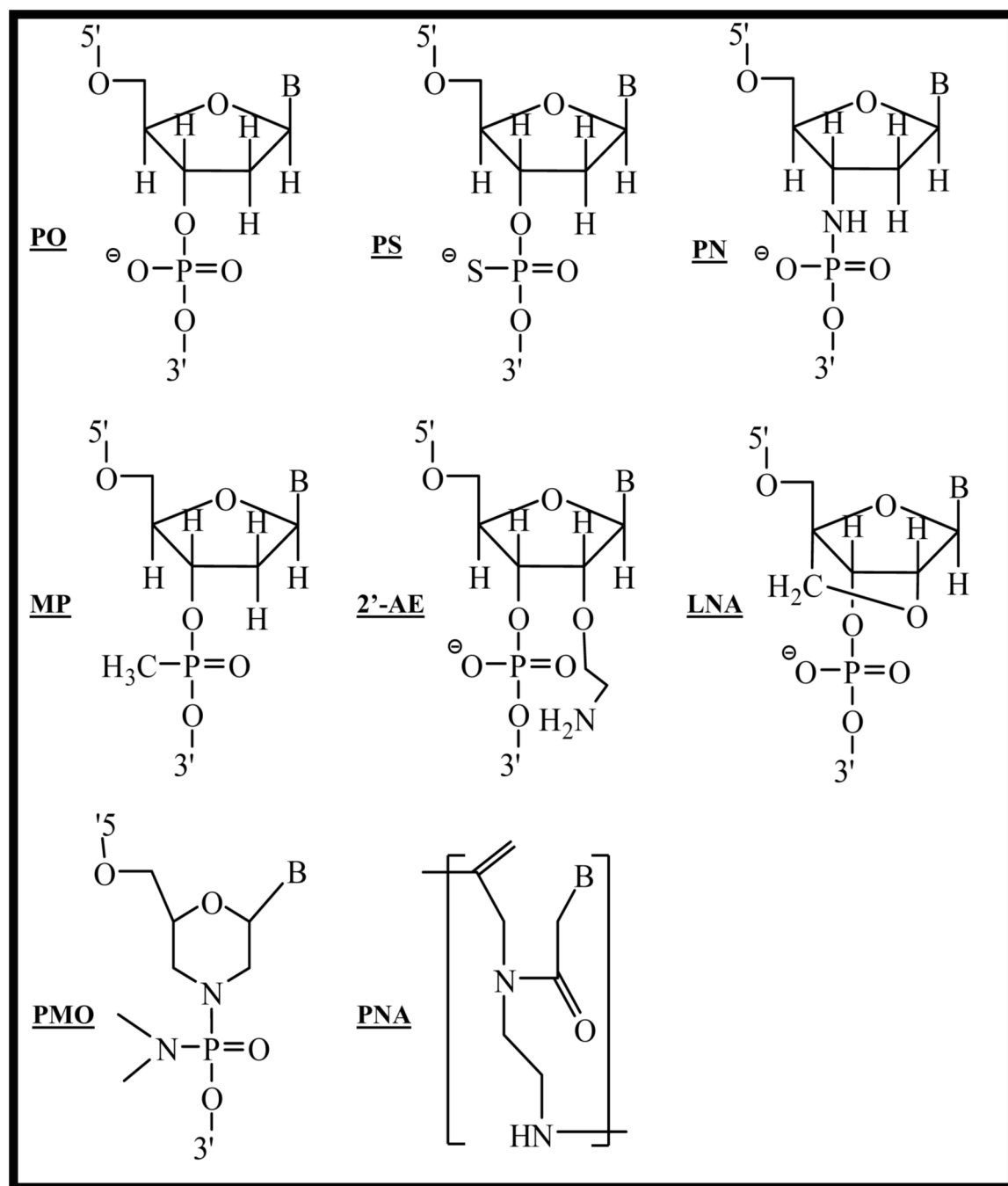
**Figure 5. Rules of triplex formation**

A third polynucleotide sequence can bind to double-stranded DNA at the major groove to form triplex structure via formation of Hoogsteen/reverse Hoogsteen hydrogen bonds. TFOs can only bind to polypurine strand of target DNA. TFOs can be either (G, A)-motif or (C, T)-motif. The (C, T)-motif involves the formation of C•G×C and T•A×T base triplets (• stands for Watson-Crick hydrogen bond; × stands for Hoogsteen hydrogen bond), upon binding of a (C, T)-containing TFO with a parallel orientation with respect to the purine strand (Hoogsteen hydrogen bonds). The (G, A)-motif involves the formation of C•G×G and T•A×A triplets, upon binding of a (G, A)-containing TFO in an antiparallel orientation with respect to the purine strand (reverse-Hoogsteen hydrogen bonds). A (G, T)-motif TFO is also permitted. The (G, T)-motif involves binding of a (G, T)-containing TFO, whose orientation depends on both the number of GpT or TpG steps in the third strand and on the length of G and T tracts.



**Figure 6. Electrophoretic mobility shift assays showing triplex formation with parallel and antiparallel phosphodiester and phosphorothioate triplex forming oligonucleotides (TFOs) with C1 duplex**

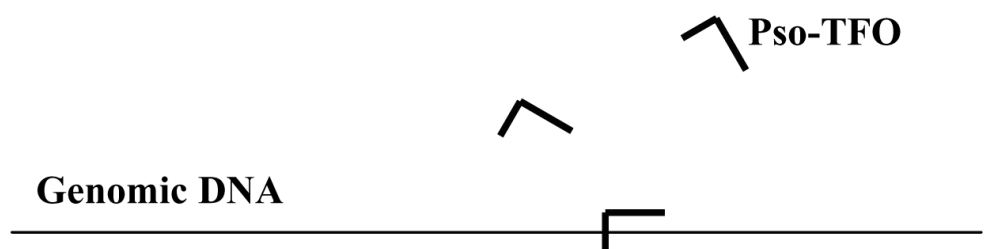
(A) Comparison between the TFOs with different directions relative to C1 duplex; (B) Comparison between TFOs with different start positions on the C1 duplex. C1 represents the sequence from -141 to -170 of  $\alpha 1(I)$  gene promoter, in which a polypurine sequence exists on the non-coding strand. Duplex concentration, 2 nM; TFO concentration ( $\mu\text{M}$ ) in each triplex forming reaction is shown below the corresponding lane. T, triplex; D, duplex; P, parallel; AP, antiparallel; APS, antiparallel phosphorothioate; 164 AP/APS, TFO sequence corresponding to the region from -164 to -147; 147 P, TFO sequence corresponding to the region from -147 to -164; 158 APS, TFO sequence corresponding the region from -158 to -141; 170 APS, TFO sequence corresponding to the region from -170 to -153. Reproduced with permission from Joseph et al. (1998) *Nucleic Acids Res.* **25**(11):2182–8.



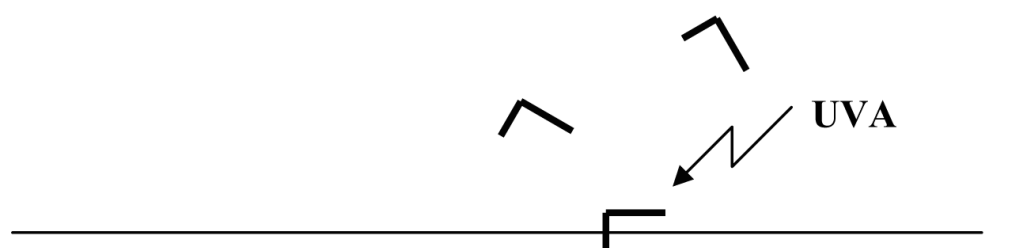
**Figure 7. Modifications of the TFOs**

Different modifications have been developed to increase the stability and binding affinity of TFOs. Modifications can be on backbone and sugar moieties. More radical modification is to substitute the whole sugar structure, such as phosphorodiamidate morpholino oligonucleotides (PMOs) and peptide nuclei acids (PNAs). PO: phosphodiester; PS: phosphorothioate modification; PN: N3'→P5' phosphoramidate modification; MP: methyl phosphodiester; 2'-AE: 2'-O-aminoethyl (2'-AE); LNA: Locked nucleic acid (LNA).

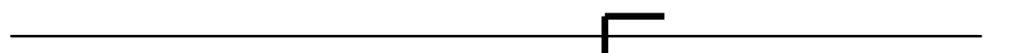
### (i) Triplex formation in buffer/in living cells



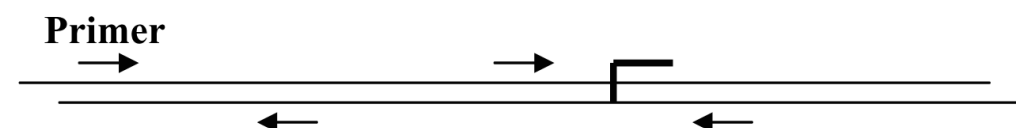
### (ii) UVA irradiation



### (iii) Gel elution and Extraction



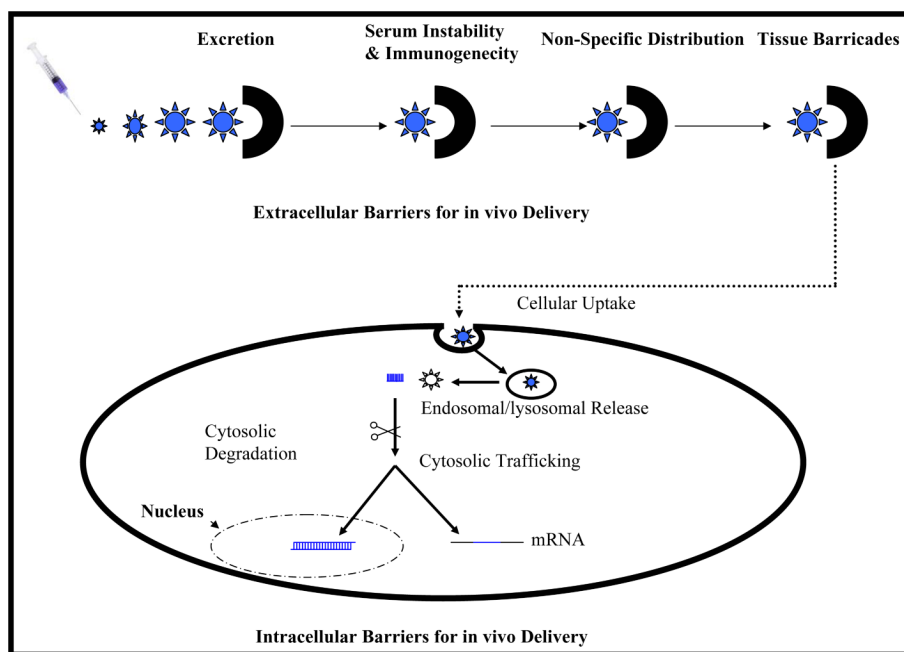
### (iv) Real-time PCR



#### Figure 8. Strategy for detecting triplex formation with genomic DNA using real-time PCR

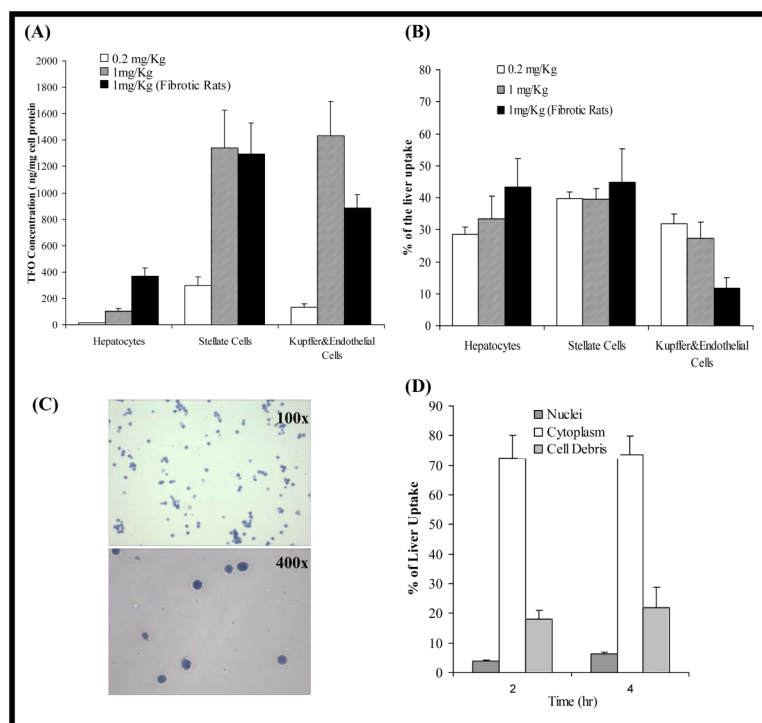
Triplex formation can be detected using a real-time PCR based method. TFO is modified with psoralen at the 3' end for forming covalent attachment at triplex formation site. The triplex formation can be done with isolated genomic DNA or genomic DNA in isolated nuclei or live cells. After triplex formation reaction (i) and UVA irradiation at 366nm for 10 at ~3.8 mW/cm<sup>2</sup> from 5 cm distance (ii), the DNA samples are subjected to a agarose gel electrophoresis (0.5%) and extracted for the gel (iii). The aliquots of extracted DNA samples are subjected to real-time PCR (iv). Two sets of primers are used: one set of primer for amplification of a control region and another for target region overlapping triplex forming site. The DNA with psoralen

covalent modification is not substrate of PCR reaction. Inhibition of PCR reaction at the triplex formation site relates to the triplex formation.



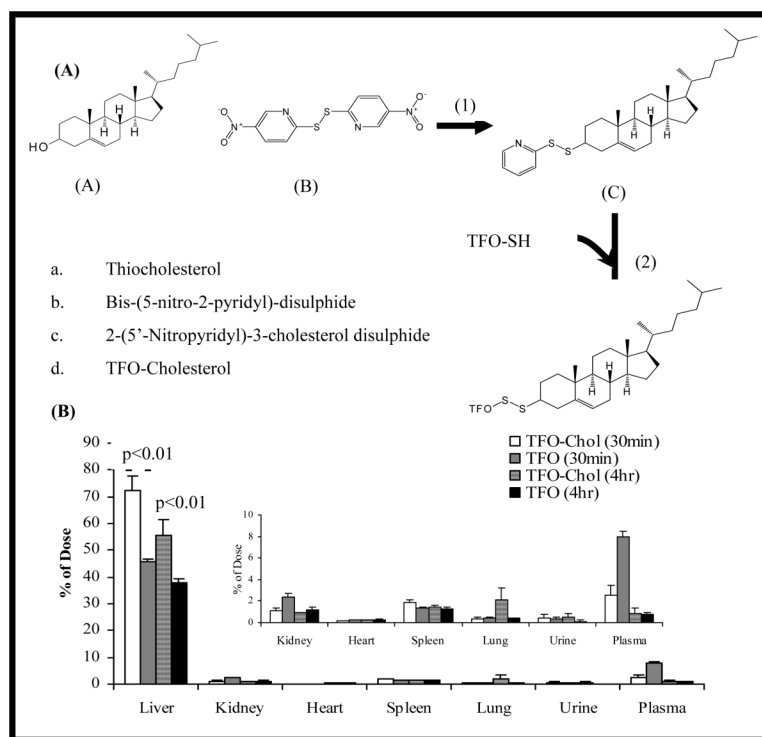
**Figure 9. Barriers to systemic delivery of ODNs**

For ODNs to reach target cells from the site of administration there are four major barriers to overcome: instability against nucleases, non-specific tissue distribution, poor cellular uptake, and uncontrolled subcellular trafficking. ODNs are polyanions and have a relatively small molecular weight with around 10kDa. They are subjected to quick urine excretion. After uptake by cells, they are inefficient to escape from endosome/lysosomes to cytosol. For antisense ODNs, they need to be in cytosol and bind to mRNA; while TFOs have to enter nucleus and bind to genomic DNA.

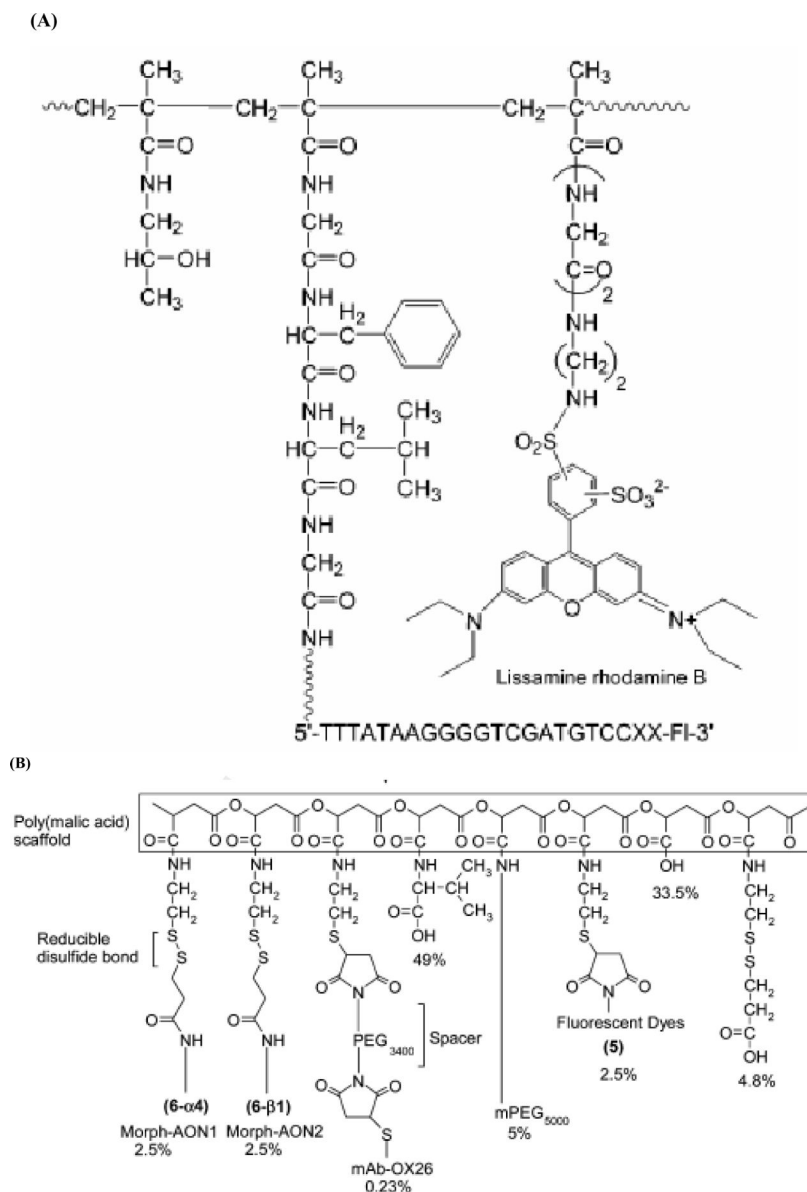


**Figure 10. Hepatic cellular localization and subcellular distribution of  $^{33}\text{P}$ -TFO after intravenous administration in rats at doses of 0.2 and 1 mg/kg**

Different liver cells were isolated at 30 min post-injection by liver perfusion with a mixture of 0.5 mg/ml collagenase and 0.1 mg/ml pronase and fractionation on Nycodenz gradient. Fibrotic rats were induced by dimethyl nitrosamine (DMN). The amount of the TFO in each cell type is given as ng/mg cell protein (A) and % of the total liver recovery (B). Subcellular distribution of  $^{33}\text{P}$ -TFO in the liver at 2 and 4 h after intravenous administration in rats at doses of 1 mg/kg. Highly purified nuclei were isolated from cytoplasm and cell debris using the sucrose gradient separation method. The purity and number of isolated nuclei were determined under microscopy by dilution in trypan blue solution (C). TFO distributions in the nuclei, cytoplasm and cell debris were given as % of the total liver recovery (D). Data are presented as the mean  $\pm$  SD (n = 4). Reproduced with permission from Cheng et al. (2005) *Mol Pharm.* 2(3):206–217.

**Figure 11.**

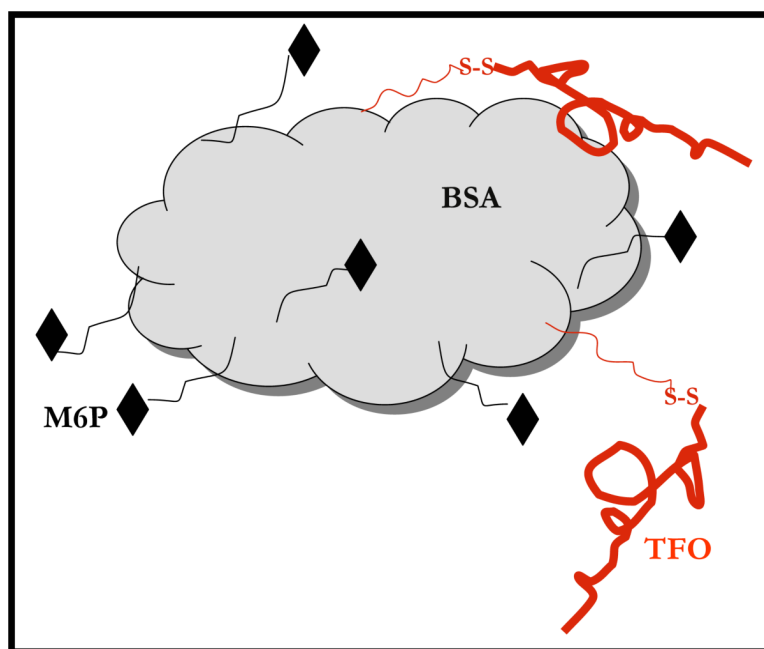
(A) Synthesis of TFO-Chol using thiocholesterol and bis-(5-nitro-2-pyridyl)-disulphide and bioconjugation via disulfide bond formation Reagent and conditions: (1) 5 ml of pyridine, stirring at room temperature for 2 h; and 2, 1500  $\mu$ L of dimethylformamide (DMF), stirring under  $N_2$  protection at 40°C for 24 h. (B) Biodistribution of TFO-Chol after systematic administration of a mixture of  $^{33}P$ -TFO-Chol and TFO-Chol or a mixture of  $^{33}P$ -TFO and TFO at a dose of 0.2 mg/kg. At 30 min and 4 h after injection, blood was collected by cardiac puncture, and urine was collected from the bladder. The rats were sacrificed, tissues were collected, washed, and weighted, 150 mg of tissue was digested, and radioactivity was determined using a scintillation counter. Data are represented as the mean  $\pm$  S.D. (n = 4). Reproduced with permission from Cheng et al. (2006) *J Pharmacol Exp Ther.* **317**(2):797–805.



**Figure 12. Synthetic polymer-based multicomponent carriers for delivery of ODNs**

(A) Hydroxypropyl methacrylate (HPMA)-based copolymers. A phosphorothioate oligonucleotides (PS-ODN) was covalently linked to the HPMA copolymer via the degradable GFLG linker. The ODNs was labeled with fluorescein on the 3' end. The polymer backbone was labeled with Lissamine rhodamine B to allow independent visualization of the polymer and the ODN. Reproduced with permission from Jensen et al. (2002) *Bioconj Chem.*, **13**(5), 975–984. (B) Polycetin, poly(β-L-malic acid) based copolymer. Percent values refer to the number of malyl moieties of PMLA that are conjugated with a given module (100% total malyl content). The distribution of conjugates along the scaffold is assumed to be random. Abbreviations are: PMLA, poly(β-L-malic acid); PMLA-NHS, N-hydroxysuccinimidyl ester at pending carboxyl groups of PMLA; mPEG5000, methoxy poly(ethylene glycol) (5kDa); 2-MEA, 2-mercaptoethylamine; mAb OX-26, mouse monoclonal antibody to rat transferrin receptor; maleimide-PEG3400-maleimide, a bifunctional maleimide derivative of PEG (3400 Da); MORPH-AON-1, morpholino antisense oligonucleotide to laminin α4 chain; MORPH-

AON-2, morpholino antisense oligonucleotide to laminin  $\beta$ 1 chain. Reproduced with permission from Lee et al. (2006) *Bioconjug Chem.*, **17**(2), 317–326.



**Figure 13. Schematic of a propose conjugate for site-specific delivery of TFOs to activated HSCs for treating liver fibrosis**

Bovine serum albumin is used as the backbone, onto which targeting ligands, mannose 6-phosphate (M6P) can be attached with the  $\text{NH}_2$  of lysines. In addition, TFO molecules modified sulfhydryl functionalities can be conjugated with BSA via disulfide bond formation. The M6P/insulin-like growth factor II (M6P/IGF-II) receptor, which is expressed in particular upon HSCs during fibrosis, can be utilized for targeted delivery to activated HSCs. Disulfide bonds enable the intracellular release of TFOs upon taken up by HSCs. TFOs used are specific for type  $\alpha 1(\text{I})$  collagen gene promoter and can inhibit the gene transcription. This conjugate has potential to be used as antifibrotic drugs.