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**New strategies of gene therapy for hepatic fibrosis**

Salazar-Montes A *et al.* Gene therapy for cirrhosis

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**Abstract**

The liver is the largest organ of the body, which may suffer acute or chronic injury induced by many factors leading to cirrhosis and hepatocarcinoma. Cirrhosis is the irreversible end result of fibrous scarring and hepatocellular regeneration, characterized by diffuse disorganization of the normal hepatic structure, regenerative nodules and fibrotic tissue. Cirrhosis is associated with a high co-morbidity and mortality disease without effective treatment and efforts are continuously aimed at developing new therapeutic strategies to guarantee recovery. Liver-based gene therapy has been used with the aim of down-regulate a specific gene, to block the expression of deleterious genes, to delivery therapeutic genes, to prevent allograft rejection and to augment liver regeneration through viral and non-viral vectors, where viral vectors have shown to be more efficient. This review provides an overview of the main strategies used in liver-gene therapy represented by non-viral vectors, different kinds of viral vectors, novel administration methods like hydrodynamic injection, hybrids of two viral vectors and blocking molecules with the hope to translate basic findings from laboratory bench to patient´s bed-side.

**Key words:** Gene therapy; Hepatic fibrosis; Viral vectors; Non-viral vectors

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**Core tip:** Cirrhosis is the irreversible end result of fibrous scarring and hepatocellular regeneration. Cirrhosis is a disease without effective treatment and efforts are continuously aimed at developing new therapeutic strategies to accomplish healing. Liver-based gene therapy has been used to improve liver function using viral and non-viral vectors. This review provides an overview of the main strategies used in liver-gene therapy hoping to find a niche of application in a given clinical scenario.

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**INTRODUCTION**

The liver is the largest organ in the body. The main function of the liver is to take up nutrients, to store them and to provide nutrients to other organs. Cirrhosis is associated with high morbidity and mortality, and is induced by many factors, including chronic hepatitis virus infections, alcohol and drug abuse[1].

During acute injury, the changes in liver architecture are transient and reversible. With chronic injury, there is progressive substitution of the liver parenchyma by scar tissue[2]. Despite ongoing injury, the liver has a remarkable regenerative capacity, and, as a result, patients often progress slowly to cirrhosis over decades. Substantial improvements in the treatment of chronic liver disease have accelerated interest in uncovering the mechanisms underlying hepatic fibrosis and its resolution[3].

On this setting, this review will deal with targeted gene delivery using viral and non-viral “shuttle” vectors as a relatively novel technology that has the potential to treat both genetic and acquired disorders. Thus, liver in mammals is an organ that can be targeted for gene transfer applications because its blood-supply can be accessed reliably with current technology. In addition, hepatocytes are long-lived cells that can sustain gene expression from episomal vectors[4]. The potential application of gene therapy protocols to human cirrhosis will depend on the successful and tissue-specific delivery of therapeutic genes to livers affected with extensive fibrosis.

In this context, experimental protocols of gene therapy directed to treat extensive

liver fibrosis have been designed to deliver specific genes to fibrotic organs. These protocols are mainly based in the use of non-viral and viral shuttle vectors. Here we describe the most important protocols published by several authors.

**NON-VIRAL VECTORS**

Represented by all those delivery methods where virus are not used. Among these methods, the reader can found plasmids, liposomes, conjugation with inert polymers of high molecular weight as diethylaminoethyl (DEAE) dextran, polyethylene glycol, polymers for the folding of DNA, among others.

***DNA plasmids***

Plasmids are attractive vectors for direct injection into organs and tissues. Despite the relatively low expression achieved after a single plasmid administration, this expression is enough to reach physiological and therapeutics levels of the desired protein. Additionally, improvements in techniques and plasmid formulations have been performed to increase transfection[5].

Augmentation of liver regeneration (ALR) is a novel cytokine, which stimulates hepatic cell proliferation and is able to block acute liver failure by inhibition of hepatic natural killer cell activity in acute liver injury[6]. ALR is an important regulator of liver regeneration with trophic effects on regenerating liver and potent anti-hepatitis effects. In this context, Qing *et al*[7] investigated the effect of ALR recombinant plasmid on rat hepatic fibrosis. The histological examination showed less hepatic fibrosis in ALR group respect to the control group with also reduction on serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and expression of Col-I, Col III and tissue inhibitor of metalloproteinase-1 (TIMP-1) suggesting that ALR recombinant plasmid enhance hepatic regeneration of injured liver cells.

Transforming growth factor-beta 1 (TGF-β1) is the most prominent cytokine implicated in hepatic fibrosis. TGF-β1 stimulates production of extracellular matrix in hepatic stellate cells in the liver. It has been reported that blockage of TGF-β1 signaling prevents hepatic fibrosis. In this context, Nakamuta*et al*[8] evaluated the effect of transfection of a plasmid containing the soluble receptor type II TGF-β1 cDNA into skeletal muscle in an experimental model of dimethylnitrosamine (DMN) induced fibrosis in rats. This treatment decreased significantly DMN-induced hepatic fibrosis, hydroxyproline content, collagen and alfa-smooth muscle actin (α-SMA) expression. The authors suggested that this strategy may be useful for gene therapy of hepatic fibrosis.

Activation of metalloproteinases has also been evaluated. The delivery of an antisense molecule for the TIMP-1 into a plasmid to rats with hepatic fibrosis induced by pig serum injection, resulted in an increased activity of interstitial collagenase rendering degradation of collagen[9].

***Hydrodynamic administration***

A promising method of gene transfer in large animals is the hydrodynamic gene transfer (HGT), which consists in the application of controlled hydrodynamic pressure in capillaries to enhance endothelial and parenchymal cell permeability. It was used for first time in the late 1990s by Budker *et al*[10], demonstrating a successful gene transfer to rats skeletal muscle by a rapid injection of plasmid DNA solution into the femoral artery.

The first clinical trial to test the hydrodynamic gene delivery in humans was reported at the 9th Annual meeting of the American Society of Gene Therapy[11]. The most successful application of hydrodynamic delivery is observed in hepatocytes in rodents. This procedure involves a tail vein injection in few seconds of 8%-10% vol/body weight of physiological solution. The high DNA solution in the tail vein enters directly into the inferior vena cava and drives the injected solution into the liver in retrograde fashion[12,13].

Huang *et al*[14] investigated the effect of recombinant interleukin-10 (*rIL-10*) gene by HGT on liver fibrosis progression induced by intraperitoneal administration of porcine serum (PS) in rats. Plasmid expressing rIL-10 was transferred into rats by HGT and the major organ expressing rIL-10 was evaluated by immunohistochemistry and reverse transcription (RT)-PCR. Results showed the major expression of rIL-10 in the liver after HGT. The *rIL-10* gene treatment attenuated liver inflammation and fibrosis in PS-induced fibrotic rats decreasing collagen deposition and expression of α-SMA.

To test the efficiency between plasmid versus foamy virus (FV) to liver gene delivery, Zacharoulis *et al*[15] applied HGT in 4 juvenile pigs, to deliver the same plasmid backbone than naked FV vector particle to compare both vectors. Gene transfer efficiency and persistence of expression was assayed by PCR at 1 wk and 1 mo, respectively after the infusions. HGT was well tolerated and no adverse reactions were observed. Plasmid injections resulted in no detectable DNA sequences at 1 wk. After 1 mo, 13% of liver sections analyzed were positive for plasmid DNA. When FV vectors were infused under identical conditions, 64.3% of liver samples were positive for vector sequences. These results indicate that the relative mild pressure obtained by hydrodynamic injection and the flooding of the liver was adequate for the entry of plasmids in hepatocytes and medium-term therapeutic levels of gene expression can be obtained with FV vectors. This effect could be attributed to the potential of HGT procedure and to the FV vectors natural affinity for hepatocytes[15].

High efficiency and simplicity of hydrodynamic injection have raised interest among medical community toward its possible application in patients. The major focus has centered on injection volume reduction maintaining an adequate pressure for gene transfer. A proposed strategy to reduce injection volume is to inject directly into the vasculature of target tissues. Zhang *et al*[16] have reduced the volume to < 1.5% of body weight in rats by targeting to liver through the hepatic vein with successful results. These results point out the possibility of liver gene delivery to hepatocytes in a 70 Kg man at a volume of 500-700 mL, a volume that might be clinically acceptable.

***Liposomes***

Many investigators are focused on the production of effective non-viral vectors gene therapeutic systems. These synthetic systems are largely based on polycationic structures, due to their ability to interact with negatively charged nucleic acids[17].

Within this group, liposomes are considered as a novel strategy for delivery of drugs and genes to cells. Use of liposome formulations for gene delivery *in vivo* is valuable for gene therapy and would avoid several problems associated with viral delivery[18]. Ueki *et al*[19] transfected the human hepatocyte growth factor(*HGF*) gene incorporated in liposomes into skeletal muscles in rats with cirrhosis induced by dimethylnitrosamine. HGF is a potent mitogen for hepatocytes with anti-apoptotic activity and essential for hepatic regeneration. The vector used consisted in liposomes containing the hemagglutinating virus of Japan with mixed liposomes (HVJ liposomes). This strategy induced a high plasma level of HGF, which binds and induces tyrosine phosphorylation of the HGF/c-Met receptor and suppression of TGF-β1 inhibiting fibrogenesis and hepatocyte apoptosis resulting in fibrosis resolution.

**VIRAL VECTORS FOR GENE DELIVERY**

Viral vectors are the most used by biologists to deliver genes into living organisms. Delivery of genes by a virus is termed transduction and the infected cells are described as transduced. Comparing the different vectors for gene therapy, viral vectors can ensure that nearly 100% of cells are infected without severely affecting cell viability. The first modified virus for gene therapy was constructed in 1970 by Paul Berg. He modified the simian virus-40 (SV40) virus by addition of the bacteriophage lambda DNA and infected successfully monkey kidney cells *in vitro*[20]. Since then, use of viral vectors has been reported by several authors in experimental and clinical protocols. For liver fibrosis, at this time, there is no clinical protocol for its treatment. However, several important strategies of gene therapy in animal models have shed light on this issue and created promissory hopes for using it in the not too distant future in humans. Here we described some of the main importance.

***Adenoviruses-based shuttle vectors***

Adenoviruses have shown to be the most efficient vector in fibrotic liver models overpassing hurdles and showing high hepatic tropism. In this context, several authors have used specific strain of adenovirus to deliver therapeutic genes with promising results. Arias *et al*[21] cloned the adenoviral construct Ad5- cytomegalovirus (CMV)-AS-TGF-β1 expressing an antisense complementary to the 3'-portion of rat *TGF-β1* mRNA and a control virus expressing the reporter gene green fluorescent protein (GFP). Both transgenes were driven by the human CMV promoter and are fused to the SV40 early mRNA polyadenylation signal. The authors found that transduction with Ad5-CMV-AS-TGF-β1 induced significantly more mRNA production than the endogenous gene. In cirrhotic rats with ligature of the common bile duct (BDL), the adenoviral vector abrogated production of collagen and α-smooth muscle actin but had no significant impact on serum levels of AST, ALT, or bilirubin. Authors concluded that transfer of the TGF-β1 antisense was sufficient to abolish ongoing liver fibrogenesis but did not interfere with the injury *per se*[21].

A different strategy was used by Salgado *et al*[22] where an adenoviral vector carrying a modified cDNA coding for a non-secreted form of human urokinase plasminogen activator (Ad-ΔhuPA) was used to administrate rats with liver fibrosis. The non-secreted uPA was chosen to diminish the risk of bleeding, which is an important problem in cirrhotic animals that may have preexisting coagulopathy and because huPA is known as a potent activator of latent hepatic collagenases, which in turn would promote the degradation of extracellular matrix deposited in cirrhotic livers. Salgado *et al*[22] demonstrated that a single application of Ad-ΔhuPA through the iliac vein of severely cirrhotic rats induced profound beneficial changes such as a significant reversion in carbon tetrachloride (CCl4)-induced hepatic fibrosis. Salgado *et al*[22] showed that Ad-ΔhuPA treated rats had an enormous improvement at day 10 *via* reduction of α-SMA, increase of MMP-2 and stimulation of liver regeneration with 40% more presence of PCNA. HGF expression was increased which correlated with its cognate receptor c-Met*.* Furthermore, an improvement in functional hepatic tests was evident diminishing ALT, AST and ALP levels.

***Metalloproteinases gene delivery in adenovirus for liver fibrosis***

Metalloproteinases (MMPs) play a crucial role in the pathogenesis of liver fibrosis and may represent an important therapy target in the design of anti-fibrotic strategies for chronic liver diseases. In this context, several types of MMPs can digest fibrillar collagens. The most potent MMPs against this kind of collagens are: metalloproteinase-1 (MMP1) metalloproteinase-8 (MMP8) and metalloptroteinase-13 (MMP13).

Delivery of collagenases has been reported in experimental models of cirrhosis by several authors.

Iimuro *et al*[23] delivered in an adenoviral vector a cDNA encoding for human pro-MMP-1 into established liver fibrosis in rats supposing that the manipulation of the imbalance between collagenases and their inhibitors (TIMPs) might attenuate liver fibrosis. After Ad-MMP-1 transduction, they found that liver fibrosis was significantly attenuated indicated by Masson´s trichrome staining. Notably, the area of α-SMA positive cells (a marker of activated HSC) dramatically decreased. Active MMP1 protein was detected by western-blot indicating that expressed pro-MMP-1 protein was activated *in vivo*. Hepatocytes proliferation was also induced by MMP-1 expression in the liver. Degradation of fibrillar collagens could affect the interaction between ECM and hepatocytes and this modification possibly stimulates several growth factors bound to extracellular matrix improving liver function.

Meanwhile, Kim *et al*[24] delivered to cirrhotic mice induced by CCl4 intoxication, a plasmid containing an internal ribosome entry site, the gene of the green fluorescent reporter protein and *MMP-13* gene into a vector cationic polymer which has a relatively high transfection efficiencies and prolonged gene expression. Intravenous injection of pMMP3 in the vector cationic polymer increased 25 times *MMP-13* mRNA in liver tissue slowing liver fibrosis, reducing collagen I deposition and restoring plasma AST levels when compared the control group mice treated with empty vector.

Considering all the experimental data reported by several authors, gene delivery of collagenases seems promising for the treatment of advanced cirrhosis in humans.

However, persistent over-expression of collagenases in the liver might digest normal architectures in addition to pathologically deposited ECM. Therefore, precise controlled delivery of active interstitial MMPs may be necessary for developing a treatment for clinical use. In this context, our group cloned MMP-8, a neutrophil collagenase, which degrades type I collagen preferably under the transcriptional control of a phosphoenol pyruvate carboxykinase (*PEPCK*)-gene promoter which contains the regulatory sites for hormonal regulation of expression in the liver. Experiments were conducted in HepG2 to demonstrate that addition of glucagon resulted in MMP-8 overexpression compared to the control using a plasmid without *PEPCK* gene promoter. These results showed that expression of any therapeutic gene like MMP-8 could be controlled at will allowing to modulate extracellular matrix quantity according to body needs[25].

In a different approach, Siller-López *et al*[26] showed that adenoviral administration containing the *MMP-8* gene promoted *in situ* degradation of extracellular matrix proteins in liver fibrosis induced by CCL4 intoxication and bile duct ligation in rats, releasing hepatic growth factors, and freeing up space for hepatic cell proliferation. Furthermore, they used a single application of 3 × 1011 VP/kg of AdMMP8 *via* the iliac vein in severely cirrhotic rats, obtaining *in situ* production of the cognate protein. AdMMP8-treated rats had a variable, yet remarkable, degree of hepatic fibrosis resolution by day 14 after adenovirus vector administration, the authors proposed that degradation of fibrotic tissue could also be taking place *via* activation of latent tissue gelatinases.

Because the systemic administration of adenovirus allows at least some of them to be introduced in different organs to liver, Liu *et al*[27] cloned a cDNA of the truncated active *MMP-8* in a hepatitis B virus vector. This vector was fused with an adenovirus to create a chimeric vector with the aim to increase liver tropism and transduction efficiency at the same time. Rats with thioacetamide-induced liver cirrhosis were injected with this vector to evaluate therapeutic efficacy. They observed beneficial effects of this vector on hepatic fibrosis and hepatocyte regeneration.

The imbalance between MMPs and TIMPs is considered a crucial parameter of deposition and breakdown of the extracellular matrix. TIMP-1, the most important endogenous inhibitor of MMPs, plays a crucial role in the pathogenesis of liver fibrosis and may represent an important therapeutic target in the design of anti-fibrotic strategies for chronic liver disease. TIMP-1 expression is up-regulated in cirrhotic rats compared with normal liver. TIMP-1 binds to MMPs and inhibits their activity. Roderfeld *et al*[28]had already shown *in vitro* that an inactive MMP-9 (MMP-9-H401A) binds to TIMP-1 inactivating it. Thus, they investigated the anti-fibrotic potential effect of WT-MMP-9 and MMP-9 mutants delivered by adenovirus vector to cirrhotic mice in a CCl4 model. They showed that inactive MMP-9 mutants delivered by adenovirus inhibited hepatic fibrogenesis, collagen-1 gene expression and hepatic stellate cells activation with TIMP-1 decrease. This was the first work using an inactivated enzyme acting as a TIMP-1 scavenger as a therapeutic agent against fibrosis and in summary, the authors concluded that application of *MMP-9* mutants as TIMP-1 scavengers opens a new avenue for therapeutic treatment of hepatic fibrosis.

***Delivery of additional therapeutic genes with adenovirus***

Several authors have reported that liver fibrogenesis involves a disturbance in mineral physiological concentrations in particular zinc. The availability of zinc affects the activities of the zinc-dependent enzymes like MMPs. In this context, there are several studies that have shown the beneficial effect of zinc supplementation on liver fibrosis. Metallothionein is a protein involved in the regulation of zinc homeostasis. For this reason, Jiang *et al*[29]delivered adenovirus containing the human MT-II gene (*Ad-MT2A*) through intravenous injection to study the effect on liver fibrosis induced by CCl4 in mice. Ad-MT2A reversed fibrosis along with increased hepatocyte regeneration. MT was associated with increased activities of liver collagenases. This study indicates that MT makes an important contribution in the resolution of chemical-induced hepatic fibrosis and could be a therapeutical outcome in patients with liver fibrosis of certain etiologies.

Otherwise, Marquez-Aguirre *et al*[30] constructed a recombinant adenovirus containing the truncated receptor for TGFβ1 (Ad-TβRII∆cyt). They administrated a single injection of Ad-TβRII∆cyt (5 × 1011 vp/kg) *via* iliac vein in rats with TAA-induced cirrhosis. This single injection diminished significantly hepatic fibrosis and expression of fibrogenic genes like collagen α1, TGF-β1, PAI-1, and MMP-2. Ad-TβRII∆cyt also increased the expression of anti-fibrotic transcriptional factor SnoN in sinusoidal cells. There was also a significant difference in serum levels of AST and total bilirubin between cirrhotic rats and cirrhotic rats transduced with TβRII∆cyt. The results suggested that delivery of TβRII∆cyt in an adenovirus is effective to express this therapeutic gene. Blocking of TGF-β1 signal with Ad-TβRII∆cyt could up-regulates the transcriptional repressor SnoN, which antagonizes TGF-β1 signaling (TGF-β/Smad-pathway inhibitor) and down-regulated profibrogenic genes expression[30].

Increased intrahepatic vascular tone in cirrhosis has been attributed to a decrease of hepatic nitric oxide (NO) secondary to alterations in the post-translational regulation of the enzyme eNOS[31]. Low activity of superoxide dismutase contributes to a reduction of NO bioavailability in cirrhotic livers. Then, [Laviña](http://www.ncbi.nlm.nih.gov/pubmed?term=lavi%2525c3%2525b1a%252520b%25255bauthor%25255d&cauthor=true&cauthor_uid=18829979) *et al*[32] investigated if the removal of NO by a superoxide dismutase could improve endothelial dysfunction and reduce portal pressure in cirrhotic rats. To achieve this, they delivered an adenoviral vector expressing extracellular superoxide dismutase (SOD) (AdECSOD) or beta-galactosidase (Ad-βgal) by tail vein to CCl4-induced cirrhotic rats. This transduction to fibrotic livers reduced significantly O2- levels, increasing cGMP and decreasing liver nitrotyrosinated proteins which are associated with a significant improvement in vasodilatation. Portal pressure was also significantly decreased in comparison with control rats. The authors suggest that scavenging of O2- might be a good therapeutic strategy in the management of portal hypertension in cirrhosis[32].

On the other hand, the bone morphogenic protein 7, a member of the TGF β1 superfamily, has been reported to counteract the profibrogenic actions of TGF β1 Kinoshita *et al*[33] examined if adenovirus-mediated overexpression of bone morphogenetic protein-7 (BMP-7) administrated by tail vain, antagonized the effect of TGFβ1 in an experimental model of fibrosis induced by thioacetamide in rats. They found that hydroxyproline content and Sirius red stained areas were significantly reduced compared to control.

***Dual delivery of therapeutic genes***

[Qiu](http://www.ncbi.nlm.nih.gov/pubmed?term=qiu%252520h%25255bauthor%25255d&cauthor=true&cauthor_uid=22399249) *et al*[34] used adenovirus to deliver dual gene transfer, human IL-10 (hIL-10) and human hepatocyte growth factor (hHGF) to rats with liver fibrosis induced by CCl4. This strategy protected hepatocytes from damage by reducing hepatocyte degeneration, hepatic fibrosis, and intra-hepatic inflammatory cell infiltration, thereby preserving liver function. The authors concluded that this liver protection could be consequence of the regulation of immune response due for IL-10 and that this dual gene expression vector constitutes one of the most promising current strategies for liver gene therapy.

Meanwhile, Lin *et al*[35] used a combinatorial delivery of urokinase-type plasminogen activator (uPA) and *HGF* genes to investigate the effect of these two genes on hepatic fibrosis. Ad vectors expressing uPA (Ad-uPA), HGF (Ad-HGF) or uPA + HGF (Ad-uPA + HGF) were generated and injected into rats with hepatic fibrosis. Extracellular matrix and collagen type I and type III expression in the fibrotic liver, decreased significantly more in bi-genes transduction group respect to the individual AdHGF and AduPA groups, indicating that combinatorial genes delivery exert more effect on reversion of hepatic fibrosis than mono-gene therapy probably by a synergic effect of these two genes on hepatic fibrosis resolution.

A different strategy was devised by Ozawa *et al*[36] where they used the combination of truncated type II TGF-β1 receptor (*TβTR*) gene and *HGF* gene in an adenoviral vector (AdTβTR + AdHGF) to analyze the effect on liver fibrosis induced by chronic administration of dimethylnitrosamine in rats. The body and rats-liver weight treated with the combination and hepatocyte proliferation increased, while the grading of fibrosis was significantly less compared with an irrelevant vector AdLacZ or the single administration of either AdTβTR or AdHGF supporting the premise that the combination of two therapeutics genes for liver fibrosis treatment is more effective than individual delivery.

***Adeno-associated vectors***

Such as adenovirus, adeno-associated viral (AAV) vectors have been shown to be efficient in experimental cirrhosis models. They have high cellular tropism, can achieve long-term gene expression and they are now feasible for use in human gene therapy, because they do not awaken an exacerbated cellular immune response. For these reasons AAV has emerged as an attractive vector for gene therapy. Production and purification of AAV has been improved in past years, and now is possible to produce high yields of vector free of contaminating cellular and helper virus proteins. Eventually, tissue specific vectors to evade the immune response are being manufactured[37].

Some experiments have been focused to demonstrate that AAV can efficiently transduce livers with fibrosis. Sobrevals *et al*[38] compared the ability of AAV to transduce normal and cirrhotic rat livers. They injected AAV serotype-1 (AAV1) encoding for the reporter luciferase gene (*AAV1Luc*) through the hepatic artery, portal vein, into the biliary tree of normal and cirrhotic rats. They found that AAV1Luc allows long-term and constant luciferase expression in rat livers. Interestingly, intra-portal administration leads to higher expression levels in healthy than in cirrhotic livers, whereas the opposite occurs when using intra-arterial injection. Intra-hepatic administration leads to similar transgene expression in both animal groups, whereas intra-biliary infusion is the least effective route. After 70% partial hepatectomy, luciferase expression decreased in the regenerating liver, suggesting lack of efficient integration of *AAV1* DNA into the host genome. Transgene expression was found mainly in hepatocytes.

Through the years, different protocols using AAV have been developed for the treatment of hepatic fibrosis. In 2005 Chen *et al* [39] constructed a recombinant AAV vector encoding human IFN-gamma (rAAV-IFN-γ) and took the primary rat hepatic stellate cells and carbon tetrachloride-injury induced rats as the experimental hepatic fibrosis model *in vitro* and in vivo. Histological examination revealed that rAAV-IFN-γ could inhibit the progression of hepatic fibrosis, hydroxyproline content and serum AST and ALT levels were decreased compared with fibrosis control group. Messenger RNA expression of *TIMP-1*, *TGF-β1* and *MMP-13* genes were decreased.

In the same year, Tsui *et al*[40], demonstrated that rAAV exhibit high efficiency in transduction of a homeostatic gene, heme oxygenase-1 (HO-1), to activated stellate cells, where the binding of rAAVs to HSCs increased significantly after serum-stimulated activation compared with quiescent status. Portal injection of rAAVs to normal or CCl4-induced liver fibrosis showed a distinct distribution of rAAV binding. The majority of injected rAAVs bound to the cells in fibrotic areas that were associated with higher expression levels of fibroblast growth factor receptor-1alpha at 2 h after administration. Isolation of different types of cells from CCl4-induced fibrotic livers showed predominant expression of transgene in stellate cells after rAAV/HO-1 administration on day 3 and remained stable for 12 wk. In addition, HO-1-transduced stellate cells showed reduced transcript levels of type 1 collagen and impaired proliferative ability compared with controls.

At the end of 2007 Suzumura *et al*[41], constructed an AAV vector expressing HGF (AAV5-HGF) and examined its effect in two mouse hepatic fibrosis models: CCl4 administration and BDL in Balb/c mice. Mice that received AAV5-HGF achieved stable HGF expression both in the serum and liver for at least 12 wk. In both models, significant improvement of the liver fibrosis was found in all mice receiving AAV5-HGF based on Azan-Mallory staining. Suppression of HSCs was confirmed by immunohistochemistry. Expression of TGF-β1, collagen I and α-SMA mRNAs were significantly suppressed in the liver of AAV5-HGF transduced mice damaged with CCl4 or BDL. Expression of the inhibitor of matrix metalloproteinases TIMP-1 was significantly suppressed in livers of AAV5-HGF-transduced mice treated in both animal models[41].

In 2009 with the aim to investigate the effects of TGFβ3 on rat hepatic fibrosis, Liu *et al*[42] cloned the *TGFβ3* cDNA into rAAV2 vector. TGFβ3 is an anti-fibrotic cytokine that inhibits collagen production. Rats were randomly divided into four groups: normal control group, model group, negative control group and TGFβ3 group. Hepatic fibrosis was induced by hypodermic injection of 40% CCl4. Recombinant AAV2-TGFβ3 viral particles were injected *via* caudal vein one week before CCl4 treatment. Rats were sacrificed 8 wk after CCl4 treatment, global histological change was observed after HE staining indicating that collagen fibers were reduced in the TGFβ3 group. Masson staining showed that collagen fibers deposited around the blood vessels, portal area and Disse space in liver tissues of TGFβ3 group were significantly decreased.

New approaches have been tested with novel administration ways, less invasive and easier to perform. For example, Hao *et al*[43] used the BMP-7 that is a potent antagonist of TGF-β1 and an antifibrotic factor. In this study, they generated a recombinant AAV carrying BMP-7 (AAV-BMP-7) and tested its ability to suppress CCl4-induced hepatic fibrosis in mice. The results showed that ectopic expression of BMP-7 in gastrointestinal (GI) mucosa due to AAV-BMP-7 administration led to long-term elevation of serum BMP-7 concentrations and resulted in drastic amelioration of CCl4-induced hepatic fibrosis in BALB/c mice. Immunostaining for α-SMA and desmin demonstrated that AAV-BMP-7 inhibited HSCs activation and promoted hepatocyte proliferation. The authors suggested that oral AAV-BMP-7 could be developed into a safe, simple, and effective therapy for hepatic fibrosis.

**NOVEL VIRAL VECTORS FOR GENE THERAPY**

Even though some serotypes of Ads and AAVs such as Ad-5 or AAV-5/8 have high hepatic tropism, scientists continue the search for the best vector. Several investigations involve the usage of others recombinant viruses. In 2006, Merle *et al*[44], proved the principle of a lentiviral gene transfer in the Long-Evans cinnamon (LEC) rat, an animal model of Wilson disease. Rats were treated either by systemic application of lentiviral vectors or by intrasplenic transplantation of LEC-rat hepatocytes lentivirally transduced with *ATP7B* gene. *ATP7B* gene codes for a copper transport protein that plays a key role in incorporating copper into ceruloplasmin and moving excess copper out of the liver. *ATP7B* gene expression was analyzed by RT-PCR and its hepatic expression was detected at different time-points post-treatment and lasted for up to 24 wk (end of experiment). Liver copper levels were lowered in all treatment groups compared to untreated LEC rats. Twenty-four weeks after treatment, the area of the examined liver-tissue sections occupied by fibrosis was significantly minor, only with small fibrous septa in rats treated with cell therapy compared with untreated rats.

In the same manner Hamada *et al*[45], assessed the usefulness of oncostatin M (*OSM*) gene therapy in liver regeneration. They examined whether the introduction of OSM cDNA enhances regeneration of livers damaged by DMN in rats. They enclosed the cDNA of OSM in hemagglutinating virus of Japan envelope into the spleen resulting in the exclusive expression of OSM protein in Kupffer cells of the liver, which was accompanied by increases in body weight, liver weight, and serum albumin levels and reduction of serum liver injury parameters (bilirrubin, AST and ALT) and a serum fibrosis parameter (hyaluronic acid). Histological examination showed that *OSM* gene therapy reduced centrilobular necrosis and inflammatory cell infiltration and augmented hepatocyte proliferation. Apoptosis of hepatocytes and fibrosis were suppressed by *OSM* gene therapy.

Different sort of vectors were tested such as SV40, an icosahedral papovavirus, which has recently been modified to serve as a gene delivery vector. Recombinant SV40 vectors (rSV40) are good candidates for gene transfer, as they display some unique features: they are non-replicative vectors, easy-to-make, and can be produced in high titers. They also efficiently transduce both resting and dividing cells, deliver persistent transgene expression to a wide range of cell types and are non-immunogenic.

In 2007 Vera *et al*[46], analyzed the efficacy of a rSV40 encoding IGF-I (rSVIGF-I) to prevent cirrhosis progression. The transgene expression luciferase was evaluated in mice. The results showed long-term hepatic expression of the transgene with luciferase expression increased significantly in CCl4-damaged livers and upon IGF-I administration. Thus, liver injury and IGF-I expression from rSVIGF-I should favor transgene expression. rSVIGF-I therapeutic efficacy was studied in rats where cirrhosis was induced by CCl4 inhalation during 36 wk. At the end of the study, hepatic levels of IGF-I and IGF-binding protein 3 were higher in rSVIGF-I-treated rats than in control cirrhotic animals.

This vector was also used in experiments performed by Sobrevals *et al*[47], where they found that injection through the hepatic artery with SV40 vector encoding for insulin growth factor (SVIGF-I) in cirrhotic rats increased hepatic levels of IGF-I, improved liver function tests, and reduced fibrosis in association with diminished of α-SMA expression, up-regulation of MMPs and decreased expression of tissue inhibitors of MMPs, TIMP-1 and TIMP-2. SVIGF-I therapy induced down-regulation of TGF-β1, amphiregulin, platelet-derived growth factor (PDGF), connective tissue growth factor (CTGF), vascular endothelium growth factor (VEGF) and induction of the antifibrogenic and cytoprotective HGF.

It´s well known that some virus have this high natural tropism for liver, one of them is the hepatitis B virus (HBV). However, HBV vectors have a limited insertion capacity and are replication-defective. Conversely, in an HBV infected cell vector replication may be rescued in trans by the resident virus, allowing conditional vector amplification and spreading. Capitalizing on a resident pathogen to help in its elimination and/or in treating its pathogenic consequences would provide a novel strategy. However, resident HBV may also reduce susceptibility to HBV vector super-infection. Thus, a size-compatible truncated MMP-8 (tMMP8) gene was cloned into an HBV vector, which was then used to generate a chimeric Ad-HBV shuttle vector that is not subject to super-infection exclusion. Rats with TAA-induced extensive liver fibrosis were injected with this chimera to evaluate therapeutic efficacy. The data demonstrate that infectious HBV vector particles can be obtained *via* trans-complementation by wild-type virus, and that tMMP8 HBV vector can efficiently be shuttled by an Ad vector into cirrhotic rat livers. There, it exerted a comparable beneficial effect on fibrosis and hepatocyte proliferation markers as a conventional full-length MMP-8 Ad vector[27].

HBV also contain numerous overlapping open reading frames and regulatory *cis*-elements, facts that have hampered early attempts to harness HBV into a gene-transfer vector by simple insertion of foreign sequences. HBV vectors obtained by this way selectively accumulate in the liver after inoculation into peripheral vessels, efficiently infected quiescent hepatocytes, and successfully transduced genes for GFP and type I interferon (IFN-γ) These data suggested that HBV-based vectors may also become useful against other liver diseases[27].

***Gene therapy to hepatic stellate cells***

HSCs are known to play a central role in hepatic fibrosis and their elimination is a crucial step towards the resolution and reversion of liver fibrosis. [Arabpour](http://www.ncbi.nlm.nih.gov/pubmed?term=arabpour%252520m%25255bauthor%25255d&cauthor=true&cauthor_uid=25088657) *et al*[48], investigated the potential application of a fused protein of an anti-epidermal growth factor receptor scFv antibody-TNF-α (scFv425- sTRAIL) delivery by an Ad vector on the targeted elimination of activated HSC in cell culture. Treatment with Ad-scFv425-sTRAIL induced a reduction of around 100% in HSC viability, reduction of 60% in ECM production (60% reduction), and a reduction of the caspase inhibition where no effect was observed on hepatic parenchymal cells. With these results the authors suggested that this strategy may represent a new therapeutic strategy against liver fibrosis.

In a different study, other authors developed a CCl4-induced micronodular cirrhosis model to study the effect of rAAV/HO-1 administration, where expression of HO-1 by rAAV/HO-1 significantly increased the HO enzymatic activities in a stable manner. The development of micronodular cirrhosis was significantly inhibited in rAAV/HO-1-transduced animals. Portal hypertension was markedly diminished in rAAV/HO-1-transduced animals as compared to controls, and no significant changes in systolic blood pressure were noted. These findings were accompanied with improved liver biochemistry, less infiltrating macrophages and less activated HSCs in rAAV/HO-1-transduced livers[49].

In a different study Reetz *et al*[50] described a novel method to delivery genes for HSC in fibrotic liver ablating the native tropism to liver. This paper is based in the concept that the expression of P75 neutrophin receptor (P75NTR) is increased in HSC in liver fibrosis where expression is low in quiescence HSCs and no expression is observed in heptocytes. Nerve growth factor (NGF) is a neutrophin which bind P75NTR. With this premise, NGF was conjugated to the Ad surface using an adapter derived from a single chain antibody *via* polientilenglicol. Ads carried the reporter gene *GFP*. This vector was injected systemically in mice and GFP expression was evaluated. The authors showed that the *GFP* expression was detected in liver and not detection was observed in other organs like lung and brain. Liver expression was selective and increased in HSC of liver fibrosis compared to normal. There was not expression in hepatocytes. Authors conclude that this strategy might provide an effective mechanism for direct therapeutic genes delivery to HCSs activated without affecting hepatocytes[50].

In order to reduce liver fibrosis, Narmada *et al*[51]delivered *HGF* gene specifically to activated HSC in fibrotic liver using vitamin A coupled liposomes by retrograde intrabiliary infusion to bypass capillarized hepatic sinusoids. HSC-targeted transgene enhanced the antifibrotic effect by reduction of α-SMA and collagen genes.

**BLOCKING MOLECULES FOR INHIBITION OF DELETERIOUS GENES**

Technology based in the delivery of short DNA or RNA is a revolutionized tool employed to silence the expression of specific genes in cells with no toxic response. These molecules are delivered to the cells or produced by them using expression cassettes, which are introduced into cells through viral vectors, plasmids or DNA constructs[52]. In this context, several molecules like decoy, antisense oligonucleotides, siRNA and miRNA have been investigated to evaluate their effect on expression inhibition of important genes for cirrhosis development.

***Decoy molecules***

The usage of decoy technology has been recently reported. A synthetic double-stranded oligodeoxynucleotide (ODN) containing the consensus binding sequence of the transcription factor Sp1, which regulates the inflammation-repair process and suppresses expression of several genes including TGF-β1, collagen type I (COL1A2), VEGF, to block its activity was used by Park *et al*[53] in a CCl4-liver fibrosis model. They injected 10 μg of this ODN through tail vein in mice. The decoy molecule for Sp1 reduced gene expression of TNF-α, IL-1β, IL-6, VEGF and MCP-1 and also decreased production of pro-fibrogenic proteins like fibronectin, α-SMA, TGFβ-1 and TIMP-1.

***Antisense oligonucleotides***

In 2005, Cheng *et al*[54], developed an anti-gene approach using a type alpha1 (I) promoter specific TFO to inhibit collagen gene expression. In this report, biodistribution and hepatic cellular and subcellular localization of the 25-mer antiparallel phosphorothioate triplex-forming oligonucleotide (TFO) were determined after intravenous injection int o rats. TFOs distributed to all the major organs, with higher uptake in the liver, kidney, and spleen. Competition studies with polyinosinic acid and dextran sulfate suggested the involvement of scavenger receptors in the hepatic uptake of TFO. Intrahepatic cellular distribution by Kupffer, endothelial, and HSCs accounted for almost 70% of the liver uptake of P-TFO, while only 30% was associated with hepatocytes. The level of liver nuclei-associated TFO was much lower relative to that found in the cytoplasm at 2 and 4 h post-injection. TFO, however, inhibited collagen expression as evidenced by sirius red staining of the liver section of fibrotic rats. In conclusion, systemic delivery of the TFO against type alpha1 (I) collagen gene promoter may be used for the treatment of liver fibrosis.

In the same year Jiang *et al*[9], constructed a rat antisense TIMP-1 recombinant plasmid that can be expressed in eukaryotic cells. The recombinant plasmid were encapsulated with glycosyl-poly-*L*-lysine and injected into rats suffering from pig serum-induced liver fibrosis. The expression of exogenous transfected plasmid was assessed by northern blot, RT-PCR, and Western blot and it was successfully expressed *in vivo* and could block the gene and protein expression of TIMP-1. Active and latent hepatic interstitial collagenase activities were elevated. Hepatic hydroxyproline content and the accumulation of collagen types I and III were lowered, and liver fibrosis was alleviated in the antisense TIMP-1 group as compared with the model group.

The usage of this kind of molecules had an important increment in recent years and a large number of molecules have been tested. At this time, Lu *et al*[55] constructed a rat antisense RNA of CTGF recombinant plasmid which could be expressed in eukaryotic cells. The recombinant plasmids were encapsulated with lipofectamine and then transduced into a CCl4 induced rat liver fibrosis model. The authors found that gene and protein expression of CTGF were significantly decreased in the fibrotic liver transfected with antisense-CTGF compared with the control group. Index fibrosis and collagens type I and type III, were also significantly minimized in this group.

***Small interference RNA***

Small interference RNA (siRNA) is a recent powerful tool for post-transcriptional gene silencing and has opened new avenues in gene therapy. The problems of lack of cell specificity *in vivo* and the subsequent occurrence of side effects, has hampered the development of hepatic fibrosis treatment. To overcome these shortcomings, several targeted strategies using siRNA for cirrhosis treatment have been developed. As an example, in 2007 George *et al*[56] used a CTGF siRNA to prevent the progression of NDMA-induced hepatic fibrosis. The serial administration of NDMA resulted in activation of HSCs, up-regulation of CTGF and TGF-β1 both at mRNA and protein levels and well-developed hepatic fibrosis. Immunostaining, western blot, and semiquantitative real-time RT-PCR studies showed down-regulation of CTGF and TGF-β1 after treatment with CTGF siRNA. These results demonstrated that CTGF gene silencing through siRNA reduced activation of hepatic stellate cells, prevented up-regulation of *CTGF* and *TGF-β1* gene expression and inhibited accumulation of liver connective tissue proteins.

Three years later, another working group also tested the anti-fibrogenesis property of a single intra-portal vein injection CTGF siRNA in a rat model of liver fibrosis. The authors observed that CTGF siRNA treatment in cirrhotic rats, protein expression of CTGF and α-SMA and the number of active HSC decreased compared to the model group. Attenuation of liver fibrosis was also observed[57].

In 2008, Chen *et al*[58], constructed a PDGFR-β siRNA expression plasmid and investigated its effect on the activation of HSCs. A hydrodynamics-based transfection method was used to deliver PDGFR-β subunit-siRNA to rats with hepatic fibrosis. PDGFR-β-siRNA significantly down-regulated PDGFR-β expression and suppressed HSCs activation and proliferation *in vitro*. The progression of fibrosis in the liver was significantly suppressed by PDGFR- β siRNA in two animal models of fibrosis: DMN intoxication and BDL. With these results, authors suggested that the plasmid could be delivered into activated HSCs by the hydrodynamics-based transfection method, and remarkably improve liver function in cirrhotic rats.

At the same year Cheng *et al*[59], designed a siRNA and short hairpin RNA (shRNA) targeting different regions of *TGF-β1* mRNA and they measured the silencing effect after transfection into immortalized rat liver HSC (HSC-T6). There was not only significant decrease in TGF-β1, TIMP-1, α-SMA and type I collagen after transfection with TGF-β1 siRNAs, but also synergism in gene silencing when siRNAs targeting two different start sites were used as a pool for transfection. The two siRNA sequences which efficiently inhibited *TGF-β1* gene expression were converted to shRNAs *via* cloning into the pSilencer1.0. In conclusion, both siRNA and shRNA showed sequence-specific and dose dependent *TGF-β1* gene silencing and have the potential to treat liver fibrosis.

***MicroRNAs***

MicroRNAs (miRNAs) are short, endogenous, noncoding RNA molecules that regulate gene expression at a post-translational level. miRNAs have been recognized in the regulation of physiological conditions. Moreover, awareness of the association between dysregulated miRNAs and human diseases is increasing, which consequently brings miRNAs into the frontline of novel therapeutic strategies.

This technology is effective and currently used as demonstrated by the work of Yang *et al*[60]. They investigated the antifibrotic effects of an artificial miRNA targeting CTGF using the ultrasound-targeted cationic liposome-bearing microbubble destruction gene delivery system. Plasmids carrying the most effective artificial miRNA sequences were delivered by ultrasound-targeted cationic liposome-bearing microbubble destruction gene delivery system to rats with hepatic fibrosis. The results showed that this method of gene delivery effectively transported the plasmids to the rat liver. The artificial miRNA reduced hepatic fibrosis pathological alterations as well as the protein and mRNA expressions of *CTGF* and *TGF-β1*. Furthermore, the *CTGF* gene silencing decreased the levels of type I collagen and α-SMA. These data suggest that delivery of an artificial miRNA targeted against CTGF using ultrasound-targeted cationic liposome-bearing microbubble destruction may be an efficacious therapeutic method to ameliorate hepatic fibrosis.

CONCLUSION

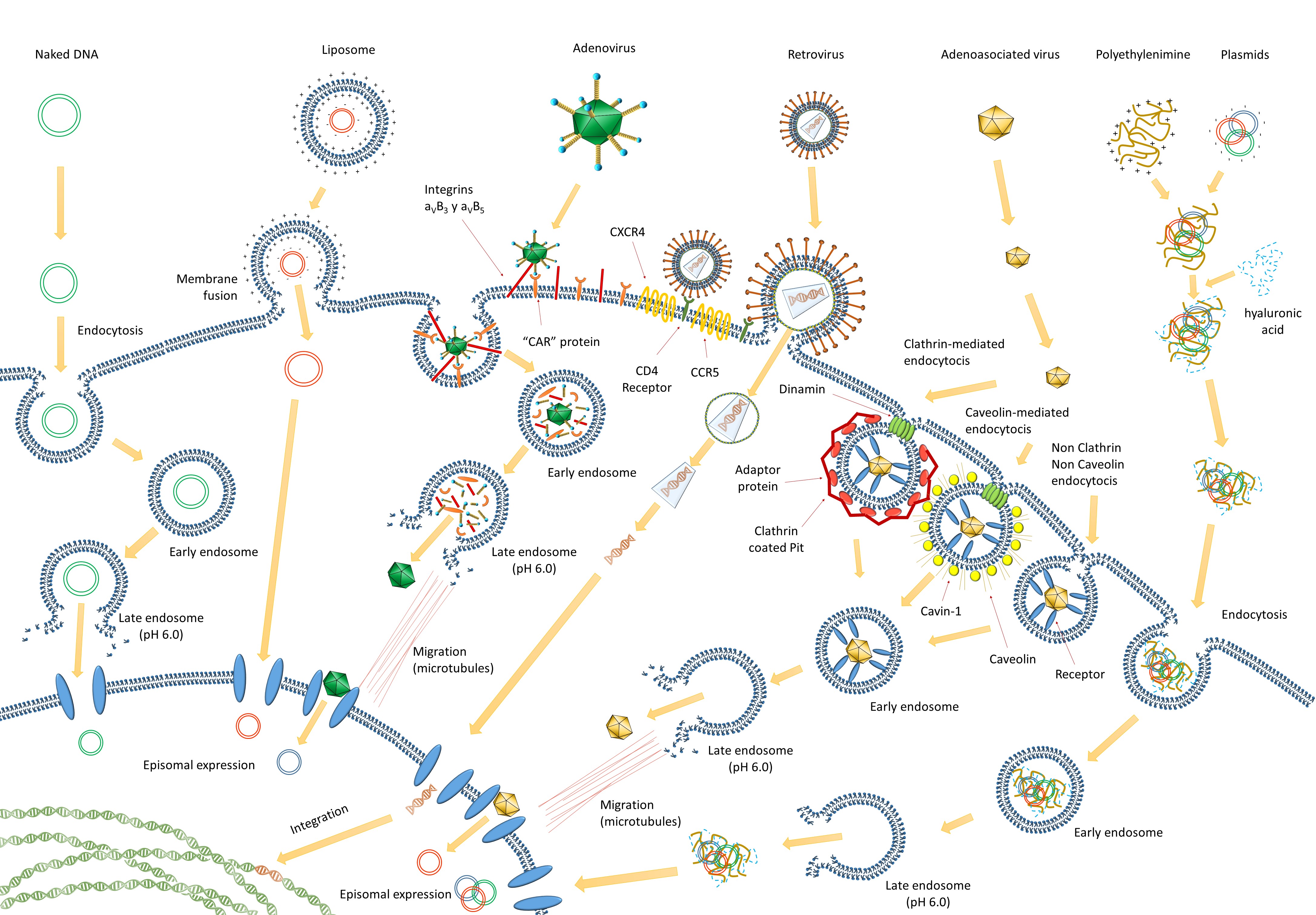
Gene therapy is at this moment, a novel alternative for the treatment of those diseases that currently have no satisfactory cure. In recent years, gene therapy has been directed to the treatment of mortal chronic degenerative diseases in order to offer the patient a better quality of life. For those diseases caused by lack of control in the expression of certain genes such hepatic fibrosis, delivery of genes that counteract this over-expression should be undoubtedly an excellent strategy to control it. That is why numerous articles are daily published in the experimental field of gene therapy addressing innovative strategies that provide information that hepatic fibrosis could be treated with gene therapy supporting its use in a near future in cirrhotic patients.

A large number of vectors used in gene therapy have been implemented, each vector has advantages and disadvantages. The use of gene therapy in humans has been controversial since that delivered genes could potentially be integrated into the cell genome causing an insertional mutation that can result in cancer, especially if the used vector is a virus. As mentioned earlier in this paper, adenovirus are most commonly viral vector used for therapeutic gene delivery to liver[61] with the advantage that they recognize the CAR receptor present in hepatocytes, do not integrate into the cell genome and the risk of insertional mutation is null[62]. Other vectors like liposomes, plasmids and recently adeno-associated viral vectors have lately been proved, however, the ideal vector to deliver genes in a diseased liver remains to be established. Some clinical trials using gene therapy against hepatocarcinoma and hepatitis C infection are being implemented[64] , but there are only experimental models directed to liver fibrosis at this moment. These experimental approaches have demonstrated to be effective to decrease and prevent experimental fibrosis. Their implementation in humans in the near future remains to be witnessed.

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**Figure 1 Several cell-targeted methods employing different vectors routinely used in gene therapy approaches.** Some of them remain episomally and their expression is transient. Some others enter to the nucleuos and integrate their genome in cellular genoma and its expression is permanent.