

ANNALS OF HEPATOLOGY

Volume **1** Number **4** October-December **2002**

Artículo:

**Genomic medicine in Mexico.
Applications of gene therapy for
cirrhosis reversion**

Copyright © 2002:
Mexican Association of Hepatology

**Otras secciones de
este sitio:**

-  **Índice de este número**
-  **Más revistas**
-  **Búsqueda**

***Others sections in
this web site:***

-  ***Contents of this number***
-  ***More journals***
-  ***Search***



Medigraphic.com

Concise Review

Genomic medicine in Mexico. Applications of gene therapy for cirrhosis reversion

Dr. Juan Armendariz-Borunda¹

Abstract

Genomic medicine represents a powerful armamentarium to tackle down most of chronic diseases which have not, so far, defeated. Thus, this new and powerful biotechnologic set of weapons enable us to make use of molecular diagnostic to detect silent diseases, otherwise undetectable by conventional analysis. Moreover, elucidation of the complete and final draft of the human genome code will allow, although not in this decade, the design of specific farmaco-genetic treatments for patients on basis of their individual genetic code. Regarding new medical treatments, gene therapy as emerged as a true hope for treatment of many chronic diseases. 636 FDA-approved clinical protocols are currently undergoing and sooner than later we'll be witness of the results.

Key words: Cirrhosis, gene transfer, safety, fibrosis reversion.

Introduction

Gene therapy can be defined as the introduction of any given nucleic acid inside eucariotic cells (but not germinal cells) with the purpose of altering the course of a medical condition or correcting a metabolic or genetic disorder. Originally, gene therapy was conceived specifically to treat monogenic disorders (a single gene defect), but it is clear now that a single gene can be considered as a new "pharmaceutical agent" to treat many types of disorders. In the last 10 years, the initial idea of gene therapy has come true with 636 clinical protocols worldwide and 3,496 patients involved the majority being in the United

States.¹ Although expectations have exceeded the initial success of this relatively new field, very important information from clinical and pre-clinical protocols has been obtained. Although gene therapy can be considered as a science in its infancy, it is very important to emphasize that with the recent technological advances, gene therapy will be used to treat a wide variety of illnesses² fact that has been reflected in the promising results showed in patients affected by X-linked Severe Combined Immunodeficiency Syndrome (SCID) and Hemophilia B.³⁻⁵

There are a number of hurdles limiting success of gene therapy. The most difficult obstacle to resolve has been the inability to efficiently transfer the "therapeutic" gene to a given target cell or tissue, so that an appropriate quantity of gene product takes place (usually a protein), and correct the disease. This is in part because cells and/or organs have developed powerful mechanisms to avoid internal accumulation of strange genetic material.²

Most efforts to develop systems of gene therapy in human have only been focused to somatic cells, since many ethical considerations have been raised with regard to the possibility of introducing exogenous genetic material inside germinal cells. Nonetheless, recent publications have focused in transfer of genes to uterus of animals with the aim of correcting embryo abnormalities.^{6,7} These findings require further considerations.

Basically, methods for clinical transfer of genes in human cells are based on 3 general strategies: *ex vivo*, *in vivo* and *in situ* strategies. *Ex-vivo* methods are, for instance, used to obtain, mainly blood white cells from the patient, harvested and stimulated to grow in culture, then genetic material is introduced and the cells are kept in culture. These cells now expressing the appropriate gene are injected back in the affected host. In contrast, *in vivo* techniques avoid this previous process of several stages introducing the therapeutic gene directly into the bloodstream of the host, reaching by this way the specific organ.² *In situ* delivery of genes is used when a given gene shuttled by an appropriate vector is directly injected, for example, in solid tumours (prostate cancer).

The liver as target organ

The liver possesses a variety of characteristics that makes it attractive for gene therapy. As a tissue where es-

¹ Institute of Molecular Biology in Medicine and Gene Therapy, CUCS, University of Guadalajara, Apdo. Postal 2-123, Guadalajara, Jal, México 44281,

Address for correspondence:
Dr. Juan Armendariz-Borunda,
Director, Institute of Molecular Biology in Medicine and Gene Therapy, CUCS, University of Guadalajara, Apdo. Postal 2-123, Guadalajara, Jal, MEXICO 44281
Phone/FAX: (52) (33) 3617-4159
E-mail: armendbo@cucs.udg.mx

sential metabolic routes take place, the liver is involved in many congenital metabolic illnesses. Due to its particular position with regard to bloodstream circulation, liver can function as a secretory gland for systemic liberation of therapeutic proteins. Due to the peculiar structure of hepatic endothelium, hepatic parenchyma is accessible to big such molecules as fragments of DNA or recombinant virus circulating in the bloodstream.

The last decade has witnessed a booming in the implementation of a number of protocols to transfer genes into normal or diseased livers.^{8,9} Either using simple methods as direct injection of plasmidic DNA, naked or complexed with different ligands, or injecting into bloodstream sophisticated recombinant viral vectors accurately released to hepatic lobes, investigators have tried repeatedly to carry out a level of expression of transgenes in an efficient and stable way in a great number of hepatocytes.^{10,11}

Therapeutic strategies for hepatic fibrosis

In theory, effective anti-fibrotic treatments should satisfy several important criteria. First, any therapy should have sound biological basis, but also, the potential treatment should have advantageous pharmacodynamics, meaning that levels of active compounds should reach the liver in high concentrations and induce little extra-hepatic effects. Finally and ideally, a therapeutic agent should remain in the micro-environment of the target tissue for long periods, perhaps for an indefinite period in the case of enduring injury.

Current therapies

There are no established and definitive therapies for hepatic fibrosis. However, recent insights into the molecular pathogenesis of hepatic fibrosis and the role of activated hepatic stellate cells provides hope for future development of successful therapy. Although a comprehensive analysis of such strategies is not the goal of this review, the reader can gain access to several extensive papers on this issue.¹²

Advances in gene vector technology

A major impediment to the development of gene therapy is the availability of effective vector systems for gene delivery. An ideal vector would be one that grows to high titers, efficiently targets the appropriate cell types, and persists *in vivo* without toxic or immunological side effects. Development of this ideal vector remains an elusive goal, and it is not likely there will be a single vector suitable for all applications. Nonetheless, a variety of interesting and clever developments are continuously reported by different Gene Therapy groups around the world. Certainly, a rapid and fast progress is being made in this biotechnological area.

So far, retroviruses continue to be widely studied, due to a lack of immunogenicity, ease of use, and a unique ability to integrate into host cell chromosomes. Most applications of retroviruses are for hematopoietic diseases, where the different subfamilies of retroviruses (onco-, lenti- and foamy retroviruses) display varying abilities to integrate into stem cells. The best example of successful human gene therapy (up to date) continues to be the treatment of several young children affected with Severe Combined Immunodeficiency Syndrome SCID-XI.³

On the other side, lentiviral vectors are becoming increasingly safe and easy to use. Inducible, multicomponent packaging cell lines now allow for the production of high titer preparations with no detectable replication-competent recombinants.¹³ The lentiviral systems have now increased cloning capacity and display good efficiency for cell integration in non-dividing cells, particularly in hematopoietic stem cells in both adults and *in utero*.

Foamy viruses are receiving increased attention, as they display the largest cloning capacity of any retroviral subfamily, while efficiently integrating into hemopoietic stem cells.¹⁴

Adeno-associated virus (AAV) vectors are rapidly becoming one of the most extensively used systems. These vectors can be grown to relatively high titers and display few immunological side effects. AAV has raised hopes for an eventual cure for hemophilia, due to its efficient expression in liver and muscle according to recent evidence provided by the groups of Mark Kay and Katherine High.^{4,15} Initial trial using muscle delivery produced low levels of circulating Factor IX, and new trials using liver delivery are now undergoing.

However, and despite early promises that AAV completely avoids cytotoxic T-cell responses, it is becoming clear that some combinations of transgenes and promoters can lead to serious problems. Undoubtedly, this is a hurdle to be aware of in the future.

Adenovirus have gone through enormous swings of popularity, with recent failures in clinical trials raising serious safety concerns, although the overall opinion about the use of these very useful vectors is becoming more benevolent. Ad vectors now have claim participation of many groups around the world involved in vector engineering.¹ An established fact is that Ad vectors have a huge number of different serotypes and this has been instrumental to make great progress in applying particular serotypes and altered surface proteins (working as ligands) to target or avoid specific cell types. Fully-deleted or "guttled" Ad virus can be grown to extremely high titers and overcome most immunological problems, although it has been recently shown that particular combinations of promoter elements, transgenes and certain pathological conditions are capable of eliciting residual immunological abnormalities.^{16,17} Naked plasmids, the simplest of vectors, are being delivered in increasingly creative ways and in some cases can equal the transduc-

tion levels of viruses as the group of Leaf Huang have demonstrated.¹⁸ An advantage of viral delivery systems was once thought to be an increased ability to target and transduce particular cell types compared with non-viral systems. However, growing safety concerns related to some viruses, combined with a rising ability to deliver and target non-viral vectors (mainly through combination with ligand molecules), may eventually make this advantage increasingly obsolete.

It is difficult to predict what future advances might bring, but it is a safe bet that the vector technology developed in a very short term, will set us even closer to the reality of human gene therapy. Moreover, the choice of adequate vectors carrying therapeutic genes will depend on each individual gene therapist's needs. As already stated, design and engineering of a universal and completely safe vector has yet to be implemented.

Gene therapy in hepatic fibrosis

There is a number of recent publications that have used gene therapy applications to ameliorate different experimental cirrhosis induced by several etiologic agents. Thus, Telomerase gene delivery has been invoked to inhibit and even to protect mice against CCl₄-induced liver fibrosis.¹⁹ A different group has reported that gene transfer of the neuronal Nitric Oxide Synthase isoform to cirrhotic rat liver ameliorates portal hypertension.²⁰ In another interesting report, Uesugi and colleagues proposed that NF- κ B inhibition by means of adenoviral gene delivery of I κ B superrepressor reduces early alcohol-induced liver injury in rats.²¹ Moreover, an adenoviral vector encoding for Hyper-IL-6 (a superagonistic designer cytokine consisting of human IL-6 linked by a flexible peptide chain to the secreted form of the IL-6 receptor) was able to maintain liver function, prevent the progression of liver necrosis and induced liver regeneration in a mouse model of acute liver failure induced by D-galactosamine administration.²²

Key facts

Despite of the strategies employed to alleviate experimental chronic liver diseases, it becomes even more evident that ideal strategies for treatment of hepatic cirrhosis should include fibrogenesis prevention, induction of liver cells proliferation, formation of new hepatic vessels and re-organization of the hepatic architecture.

Along this reasoning, a number of cytokines and small peptides have important effects on the metabolism of hepatic stellate cells and strongly influence their *in vivo* behavior. Thus, although a growing list of antagonists to a wide range of pro-fibrogenic cytokines and many of them have been assayed both *in vitro* and *in vivo* experimental models, few have proven to be really effective in treating human fibrosis and cirrhosis.

TGF- β is the most potent cytokine for inducing fibrogenesis in a wide variety of diseases states including patients with alcoholic and viral induced cirrhosis. TGF- β produces fibrosis because of its multiple and pleiotropic effects including the activation of hepatic stellate cells, the production of ECM proteins including collagen type I, the inhibition of production of collagenases or metalloproteases, and the stimulation of the production of inhibitors of metalloproteases such as TIMPs. Thus, TGF- β is a logical target to accomplish fibrogenesis down-regulation in the context of new biotechnological innovations.

In this line of evidence there is a recent report using strategies of gene delivery to obliterate the onset of experimental hepatic cirrhosis.

Qi et al²³ used adenovirus-mediated gene transfer to express a truncated type II TGF- β receptor (AdCATbeta-TR) in the liver. The truncated receptor theoretically inhibits TGF- β activity, by competing with binding of the cytokine to endogenous TGF- β receptors. In other words, the over-expressed truncated receptor would bind most of hepatic TGF- β acting as a dominant-negative receptor and quench intracellular signaling, resulting in the prevention of fibrosis. In this study, hepatic fibrosis was induced in rats by repeated injections of dimethylnitrosamine and the recombinant adenovector containing the cDNA coding for the truncated receptor was administered via portal vein. This approach ensured direct delivery to the liver, as these vectors show preferential tropism to the hepatic gland, and high-level of hepatic expression of the transgene was accomplished. Noteworthy, recombinant adenovirus was administered before initiation of liver injury with dimethylnitrosamine. Transgene expression driven by adenovirus injection was noted over 1 to 2 weeks, yielding a high level of truncated type II TGF- β receptor in the liver during the 3-week period of dimethylnitrosamine administration. At the end of 3 weeks, microscopic analysis of hepatic collagen and quantitative determinations of hydroxyproline content indicated that the recombinant AdCATbeta-TR adenovirus and its consequent expressed protein inhibited the wound healing response to dimethylnitrosamine. Furthermore, animals which received AdCATbeta-TR had low serum levels of hyaluronic acid and functional hepatic tests (transaminases), besides these animals had an improved survival rate compared with controls. Also, it could be noted how the TGF- β receptor construct used in this study inhibited fibrosis by decreasing the pro-fibrogenic effect of TGF- β on hepatic stellate cells.

This study will have important implications when researchers try to translate these findings to a clinical scenario on cirrhosis therapy. The use of an adenoviral vector is enticing for a number of reasons. Our own observations (see ahead) correlate well with this study done by Qi and collaborators in the sense that adenovectors had been shown to readily transduce hepatic cells in normal livers from multiple species. However, a very important fact con-

cerning the ability of adenoviral vectors (of first, second or third generations) transducing cirrhotic livers, in this case murine livers, had not been resolved at that point. Now, another advantage consisted in the homing cell for adenovirus. Different evidence, including ours, pointed out that most of Adenoviral particles would be internalized mainly by hepatocytes followed by non-parenchymal cells. That is, Ad-vectors will be delivering the putative therapeutic gene, in a preferential manner to the whole liver after systemic injection. This specific organ delivery may reduce systemic toxicity. On the other hand, a disadvantage of many adenoviral carriers is that gene expression and the corresponding protein function does not last too long. Different researchers have reported expression lasting from two-three weeks to two-three months. As we have shown, this fact will mostly depend on the quality and titration of each batch of Adenoviral vectors.^{24,25}

Another important drawback when using Ad-vectors as shuttle vectors for targeted gene delivery is the elicited immune response by the host. This important response manifested by the formation of abundant circulating antibodies against the Adenoviral backbone precludes, at high extent, a second application of a potential "therapeutic vector". However, there is hope in the near future to solve this problem, since the up-coming of new-generation vectors, "gutted" Ad vectors will greatly help to solve this immunity problem.

Coming back to the work of Qi and col, it is important to remember that the use of this anti-fibrotic strategy was judged on its administration from the earliest stages of liver injury. This approach is useful in understanding the biology of compounds in the wound healing process, but it does not accurately model their proposed use in the clinical scenario. There is a very simple reason for this: the vast majority of patients with liver disease seek medical care after cirrhosis is already established. In other words, a preventive strategy for cirrhosis by using this bio-technological approach would be extremely difficult to accomplish.

In a separate investigation, researchers from Japan²⁶ also took a Gene Therapy approach, though the mechanism for delivering the putative therapeutic gene was different. In this study, the investigators injected a mixture of haemagglutinating virus of Japan (HVJ), liposomes and a plasmid containing the cDNA for human Hepatocyte Growth Factor (HGF) into the gluteus muscle of dimethylnitrosamine-cirrhotic rats. In these experiments they administered dimethylnitrosamine alone for 3 weeks to induce fibrosis followed by liposome-HGF treatment together with additional dimethylnitrosamine for 3 more weeks. Plasma HGF was increased in animals receiving the liposome-HGF mixture, demonstrating a successful expression of the foreign gene. Morphometric analysis of livers at the end of the study showed that HGF-treated rats had approximately 50% less fibrosis than control rats. The over-expression of HGF caused liver TGF β inhibition as well as a diminished hepatocyte apoptosis. More-

over, cirrhotic rats over-expressing human HGF had an improved survival rate as compared with their control counterparts.

Comparing the two studies shown before, it is clearly understood how the truncated TGF β receptor construct can inhibit fibrosis by a preventive effect decreasing the pro-fibrogenic effect of the cytokine on liver cells, more importantly on hepatic stellate cells. However, the mechanisms by which HGF acts to modify the wound-healing response to dimethylnitrosamine, remain unsettled. Specifically, there is no rationale and explanations concerning degradation of excessively deposited extracellular matrix.

On the other hand, the use of liposomes fused to any molecule is rather circumstantial since this delivery is non-specific.

In conclusion, a major clinical question remains unanswered concerning the experimental approach used in the two previous treatments whether they are able to reverse established fibrosis or cirrhosis.

Our group has precisely undertaken this final approach. We have induced experimental liver cirrhosis in rats resembling alcohol-induced and Hepatitis C-induced human cirrhosis. Also, by virtue of ligation and sectioning of the common bile-duct in the rat resembling secondary biliary cirrhosis in humans, we have studied the effect of two innovative and bio-technological approaches of Gene Therapy on the reversion of established fibrosis/cirrhosis.^{25,27}

Transduction of cirrhotic livers with Adenovectors to search for toxicity, bio-distribution and safety

It has been shown that vector types are efficient at delivering genes to normal livers.⁹ Similarly, the use of viral and non-viral vectors for gene delivery to functionally compromised livers has been instrumental to establish "proof of concept" in several experimental models. Various strategies, including ours, involving adenoviral vectors have been used.²⁷

However, a key issue concerning toxicology, bio-distribution and safety when using adenoviral vectors in cirrhotic animals, had remained unresolved. Part of our studies,²⁵ were designed to evaluate bio-distribution, liver toxicity and efficiency of transduction of a reporter gene in hepatic cells of cirrhotic Wistar rats. We also wanted to delimit the "therapeutic window" for potential and safe use of these vectors in a given clinical setting. Animals with decreased liver function may have increased sensitivity to hepatotoxic effects of adenoviral vector administration, though we found elevations in transaminases to be transitory. Moreover, Ad vector expression was exclusively restricted to hepatic gland and importantly, no expression found in testis. Therefore, we were able to use these Ad vectors to search for "proof of principle" in cirrhotic animals.

Use of Ad-vectors containing MMP8 gene as therapeutic agent in hepatic cirrhosis

The present work focused on restoration of normal liver architecture with consequent recovery of liver function of cirrhotic animals by adenoviral administration carrying a gene coding for matrix metalloprotease 8 (MMP8), a neutrophil collagenase which preferential substrate is type I collagen. The rationale for using MMP8 consisted in promotion of *in situ* degradation of ECM proteins, release of hepatic growth factors, and free-up space for hepatic cells proliferation. We constructed an Ad vector (AdMMP8) of clinical-grade which was proven to efficiently transduce cultured cells and induced MMP8 production with properties of the natural occurring protein, which degraded efficiently *in vitro* collagen type I and was inhibited by EDTA, TIMP-1 and 1,10-phenanthroline. Furthermore, and since we had shown that cirrhotic livers transfection by Ad-vectors was dose-dependent and dosing in the range of 10^{11} viral particles/kg was the most effective in inducing a therapeutic effect, we decided to use a single application of 3×10^{11} vp/kg of AdMMP8 via iliac vein in severely cirrhotic rats which resulted in amelioration of the cirrhotic process. It is also important to notice that we used two different experimental cirrhosis models to validate the proof of concept of our rationale.^{25,28}

Our *in vivo* studies to establish efficacy of this characterized vector were carried out in CCl_4 - and BDL-cirrhotic rats. Transduction with 3×10^{11} viral particles/kg resulted in hepatic detection of both mRNA and protein MMP-8. A consistent response in fibrosis reversal was observed in CCl_4 rats. Liver fibrosis in BDL cirrhotic animals was decreased in 45% along with diminished hydroxyproline content after AdMMP-8 treatment. Gene treatment in both models correlated with improvement in ascitis, functional hepatic tests and gastric varices indicating a diminished intrahepatic blood pressure in animals injected with AdMMP-8. Therefore, therapy with MMP-8 gene possess promise to be used in a clinical setting.

Hepatic cirrhosis gene therapy using human urokinase plasminogen activator gene as a therapeutic agent

A different strategy we designed consisted in the use of an adenoviral vector bearing a modified cDNA coding for a non-secreted form of human urokinase Plasminogen Activator (Ad- Δ huPA) to deliver the gene *in vivo*.²⁷ The non-secreted uPA was chosen to diminish the risk of bleeding, which would be particularly problematic in cirrhotic animals that may have preexisting coagulopathy. An important fact to notice is that CCl_4 was injected in rats for six weeks (three times a week) and then 6×10^{11} viral particles/kg Ad- Δ huPA were administered via iliac vein and CCl_4 Continued being administered until rat sacrifice and further analysis. Thus, all changes took place

even in the presence of the hepatotoxin. Human uPA expression was readily detected by ELISA and immunohistochemistry in liver homogenates and cells from treated animals and no systemic toxicity was apparent from vector or protein effects. VEGF gene and protein expression was found to be increased in the treated cirrhotic animals and correlated with angiogenesis.

Ad- Δ huPA administration led to almost complete resolution of periportal and centrilobular fibrosis compared to a progressive fibrosis in control. 85% reduction in hepatic fibrosis at day 10 was noted, fact that correlated with increased expression of MMP-2. In addition, cirrhotic livers showed numerous α -SMA positive cells embedded in the perinodular fibrous tissue which increased two days after Ad- Δ huPA administration but decreased to 50% of the control cirrhotic livers by day 10.

In addition to resolution of fibrosis, liver regeneration was also observed in cirrhotic rats treated with Ad- Δ huPA. Two days after Ad- Δ huPA administration, liver sections contained a substantial increase in the number of mitotic figures, binucleated hepatocytes and cells expressing proliferative cell nuclear antigen (PCNA). Percentage of PCNA positive cells was still as high as 40% on day 8 and included periportal and lobular areas. The hepatocyte growth observed correlated with increased expression of hepatocyte growth factor (HGF) and its cognate receptor c-met measured by semiquantitative RT-PCR. Ad- Δ huPA gene therapy also led to improvement in hepatic function in cirrhotic animals. These important changes were accompanied by normalization of prothrombine time which was not observed in animals treated with Ad-GFP.

Thus, expression of human uPA in cirrhotic rat livers led to resolution of fibrosis and regeneration of functional hepatocytes suggesting that degradation of fibrosis together with up-regulation of c-met and activation of HGF may be the trigger for liver function recuperation in this model of cirrhosis.

Important physiologic implications in uPA treated animals concerning uncontrolled liver cell proliferation are present, since induction of hepatocyte growth without establishment of normal architecture is not likely to restore liver function. Noteworthy, we observed a re-arrangement of the hepatic architecture in the livers of Ad- Δ huPA-treated cirrhotic rats.

There is a great deal of data we need to recollect before we attempt to use these biotech strategies in human patients. We are aware of that. However, these and other equally innovative and cutting-edge technologies give us the notion that, we are certainly discovering a new brave scientific world.

References

1. Gene therapy clinical trials. The journal of gene medicine. www.wiley.co.uk/genmed.

2. Kay M, Liu D, Hoogerbrugge P. Gene Therapy. *Proc Natl Acad Sci USA* 1997; 94: 12744-12746.
3. Hacein-Bey- S, Le Deist F, Carlier F, Bouneaud C, Christophe Hue Ch, De Villartay J, Thrasher A, et. al. Sustained correction of X-linked severe combined immunodeficiency by *ex vivo* gene therapy. *N Eng J Med* April 2002; 346(16): 1185-1193.
4. Kay M, Manno C, Ragni M, Larson P, Couto L, McClelland A, Glader B, et al. Evidence for gene transfer and expression of factor IX in haemophilia B patients treated with an AAV vector. *Nature Genetics* 2000; 24: 257-261.
5. Hacein-Bey FF, Cavazzana-Calvo M. Gene therapy of severe combined immunodeficiencies. *Nat Rev Immunol* 2002; 2(8): 615-21.
6. Porada Ch, Tran N, Eglitis M, Moen R, Troutman L, Flake A, Zhao Y, et al. *In utero* gene therapy: Transfer and long-term expression of the bacterial neo gene in sheep after direct injection of retroviral vectors into preimmune fetuses. *Human Gene Therapy* 1998; 9: 1571-1585.
7. Zanjani E, Anderson W. Prospects for *in utero* human gene therapy. *Science* 1999; 285: 2084-2088.
8. Zern M, Kresina T. Hepatic drug delivery and gene therapy. *Hepatology* 1997; 25(2): 484-491.
9. Ferry N, Heard J. Liver-directed gene transfer vectors. *Human Gene Therapy* 1998; 9: 1975-1978.
10. Martinez-Fong D, Mullersman J, Purchio A, Armendariz-Borunda J, Martinez-Hernandez A. Nonenzymatic glycosylation of poly-L-lisine: A new tool for targeted gene delivery. *Hepatology* 1994; 20(6): 1602-1608.
11. De Godoy J, Malafosse R, Fabre M, Mitchell C, Mehtali M, Houssin D, Soubrane O. A preclinical model of hepatocyte gene transfer: the *in vivo*, *in situ* perfused rat liver. *Gene Therapy* 2000; 7: 1816-1823.
12. Bataller R, Brenner D. Hepatic stellate cells as a target for the treatment of liver fibrosis. *Seminars in Liver Disease* 2001; 21(3): 437-451.
13. Kafr T. Lentivirus vectors: difficulties and hopes before clinical trials. *Curr Opin Mol Ther Aug.* 2001; 3(4): 316-26.
14. Russell D, Miller AD. Foamy virus vectors. *Journal of Virology* 1996; 70(1): 217-222.
15. Olivarez E, Hollis R, Chalberg T, Meuse L, Kay M, Calos M. Site specific genomic integration produces therapeutic factor IX levels in mice. *Nature Biotechnology* 2001; 20: 1124-1128.
16. Stephen S, Ehrhardt, A, Giehm J, Meuse L, Pham T, Kay M. Transposition from a gutless adeno-transposon vector stabilizes transgene expression *in vivo*. *Nature Biotechnology* 2002; 20: 999-1005.
17. Hartigan-O'Connor D, Amalafitano A, Chamberlain J. Improved production of gutted adenovirus in cells expressing adenovirus preterminal protein and DNA polymerase. *Journal of Virology* 1999; 73(9): 7835-7841.
18. Liu F, Huang L. Development of non-viral vectors for systemic gene delivery. *Journal of Controlled Release* 2002; 78: 259-266.
19. Rudolph KL, Chang S, Millard M, Schreiber-Agus N, DePinho RA. Inhibition of experimental liver cirrhosis in mice by telomerase gene delivery. *Science* 2000; 287: 1253-1258.
20. Yu, Q., Shao, R., Quian, HS, George SE, Rockey DC. Gene transfer of the neuronal NO synthase isoform to cirrhotic rat liver ameliorates portal hypertension. *J Clin Invest* 2000; 105(6): 741-748.
21. Uesugi T, Froh M, Arteel GE, Bradford BU, Gabele E, Wheeler MD, Thurman RG. Delivery of Ikb superrepressor gene with adenovirus reduces early alcohol- induced liver injury in rats. *Hepatology* 2001; 34: 1149-1157.
22. Hecht N, Pappo O, Shouval D, Rose-John S, Galun E, Axelrod JH. Hyper-IL-6 gene therapy reverses fulminant hepatic failure. *Molecular Therapy* 2001; 3(5): 683-687.
23. Qi Z, Atsuchi N, Ooshima A, Takeshita A, Ueno H. Blockade of type beta transforming growth factor signaling prevents liver fibrosis and dysfunction in the rat. *Proc Natl Acad Sci USA* 1999; 96: 2345-2349.
24. Aguilar L, Aguilar-Cordova E, Ando D, Armendariz-Borunda J, Brud P, Butler E, Carter B, et al. A Prescription for gene therapy. *Molecular Therapy* 2000; 1(5): 385-388.
25. Garcia-Bañuelos J, Siller-Lopez F, Miranda A, Aguilar LK, Aguilar-Cordova E, Armendariz-Borunda J. Cirrhotic rat livers with extensive fibrosis can be safely transduced with clinical-grade adenoviral vectors. Evidence of cirrhosis reversion. *Gene Therapy* 2002; 9: 127-134.
26. Ueki T, Kaneda Y, Tsutsui H, Nakanishi K, Sawa Y, Morishita R, Matsumoto K, et al. Hepatocyte growth factor gene therapy of liver cirrhosis in rats. *Nat Med* 1999; 5: 226-230.
27. Salgado S, Garcia J, Vera J, Siller F, Bueno M, Miranda A, Segura A, et al. Liver cirrhosis is reverted by urokinase-type plasminogen activator gene therapy. *Molecular Therapy* 2000; 2(6): 545-551.
28. Siller F, Salgado S, Sandoval A, Salazar A, Garcia J, Vera J, Galvan FJ, Juan Armendariz-Borunda. Treatment with human metalloproteinase-8 gene delivery ameliorates experimental rat liver cirrhosis. *Gastroenterology*. In press.