Molecular therapy and prevention of liver diseases

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Abstract

Molecular analyses have become an integral part of biomedical research as well as clinical medicine. The definition of the molecular and genetic basis of many human diseases has led to a better understanding of their pathogenesis and has in addition offered new perspectives for their diagnosis, therapy and prevention. Genetically, liver diseases can be classified as hereditary monogenic, acquired monogenic, complex genetic and diseases. Based on this classification, gene therapy is based on six concepts: gene repair, gene substitution, cell therapy, block of gene expression or function, DNA vaccination as well as gene augmentation. While recent developments are promising, various delivery, targeting and safety issues need to be addressed before gene therapy will enter clinical practice. In the future, molecular diagnosis and therapy liver diseases will be part of our patient management and complement existing diagnostic, therapeutic and preventive strategies.

Key words: gene repair, gene replacement, gene augmentation, block of gene expression or function, ribozymes, antisense oligonucleotides, small interfering RNA, interfering peptides or proteins, suicide genes, cytokine genes, antiangiogenesis genes, immunization, cytolytic viruses, immune therapy, DNA vaccination.

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Introduction

Molecular biology and recombinant DNA technology increasingly contribute to the diagnosis, therapy and prevention of human diseases. Molecular methods allow the early and/or specific detection of inherited, infectious and malignant liver diseases. In addition, such analyses increasingly lead to a better understanding of the pathogenesis of the various liver diseases which in turn had an impact on patient management, including the presymptomatic identification of patients at risk, the correct staging of the disease and the follow-up of patients undergoing therapy. Thus, molecular biology is increasingly becoming an integral part of basic as well as clinical hepatology. In the following we will briefly review current concepts and potential applications of gene therapy for the treatment or prevention of various liver diseases.

Genetic classification of liver diseases

Genetically, human diseases can be classified into three major categories [1-4]: (1) Hereditary monogenic diseases that are caused by a single gene defect and inherited by the classical Mendelian rules. There are more than 4,000 monogenic diseases described. For an increasing number of these diseases the genetic basis is being identified; (2) Acquired monogenic diseases are infections as well as malignancies that are caused by the mutation or epigenetic modification of a single gene; (3) Complex genetic diseases are associated with mutations of several genes that are acquired and frequently accumulated during life-time. Several common human diseases belong to this category, such as most malignancies.

Gene therapy is defined as the introduction of genetic material into human cells with a therapeutic or preventive benefit. In a broader definition, cell or organ transplantation are included. In the following we will discuss the basic concepts of gene therapy [1-7] as well as some therapeutic and preventive applications for liver diseases.

Table 1. **Concepts of gene therapy**

Diseases	Gene Therapy
Hereditary monogenic diseases	Gene repair
	Gene replacement
	Cell therapy or organ transplantation
Acquired monogenic diseases	Block of gene expression
	DNA vaccination
Complex genetic diseases	Gene augmentation
	DNA vaccination

Table 2. **Hereditary monogenic liver diseases (selection)**

Gene	Disease
UDP-glucuronosyl transferase	Crigler Najjar syndrome type I
Alpha-1-antitrypsin	Liver cirrhosis, emphysema
CF transmembrane regulator	Mucoviscidosis, cystic fibrosis
Factor VIII	Hemophilia A
Factor IX	Hemophilia B
Fumarylacetoacetate hydrolase	Tyrosinemia type 1
LDL receptor	Familial hypercholesterolemia
Ornithine transcarbamylase	Hyperammonemia

Molecular therapy of liver diseases

Based on the genetic classification of diseases detailed above, the principle of gene therapy involves 6 therapeutic concepts (*Tab. 1*): gene repair, gene substitution and cell therapy for hereditary monogenic diseases, block of gene expression and DNA vaccination for acquired monogenic diseases and gene augmentation and DNA vaccination for complex genetic diseases. For clinical applications, gene therapy is explored with the aim to either provide novel therapeutic strategies for diseases for which there is no treatment available or to replace and in some cases complement existing treatment modalities, thereby increasing therapeutic efficacy and/or reduce adverse events.

Gene repair

An increasing number liver diseases has been molecularly defined as a defect of a single gene (*Tab. 2*). In this context, one therapeutic concept is the *in vitro* or *in vivo* repair of the defective gene. Indeed, in the Gunn rat model of the Crigler-Najjar syndrome type I Kren et al. [8] were able to partially correct the genetic defect underlying the UDP-glucuronosyl transferase deficiency by the intravenous injection of a cyclic normal/wild-type chimeric oligonucleotide. While these findings have not been independently confirmed or extended to other hereditary monogenic (liver) diseases, the data suggest that it is in principle possible to repair a gene defect *in vivo*. Further, it has been shown that cellular RNA species can be modified by trans-splicing group I ribozymes. Such ribozymes may in principle allow to treat a variety of inherited diseases at the RNA level [9-11].

Gene substitution

The targeted substitution of a defective cellular gene by the normal/wild-type homologue with production of the physiological gene product is another approach to correct a hereditary or acquired monogenic gene defect. Indeed, in an animal model of hereditary tyrosinemia type 1 (HT1), a liver disease caused by a deficiency of fumarylacetoacetate hydrolase (FAH), multiple injections of a retroviral vector carrying the FAH gene resulted in a gene transfer efficiency of >90% of hepatocytes and the restoration of a normal liver function [12,13]. In patients, examples for gene substitution are the partial correction of severe hemophilia A by the *ex vivo* transduction of autologous skin fibroblasts with the normal/wild-type factor VIII gene, followed by laparoscopic implantation of the genetically modified fibroblasts into the omentum majus [14] or of hemophilia B by adenovirus-associated vector (AAV)-based gene transfer [15].

In rare situations in which a hepatocelluar carcinoma (HCC) is caused by the mutation of a tumor suppressor gene, e.g., the p53 gene, the substitution of the mutated by the normal/wild-type gene *in vitro* can reduce the number of tumor cell colonies and restore cisplatin sensitivity [16,17].

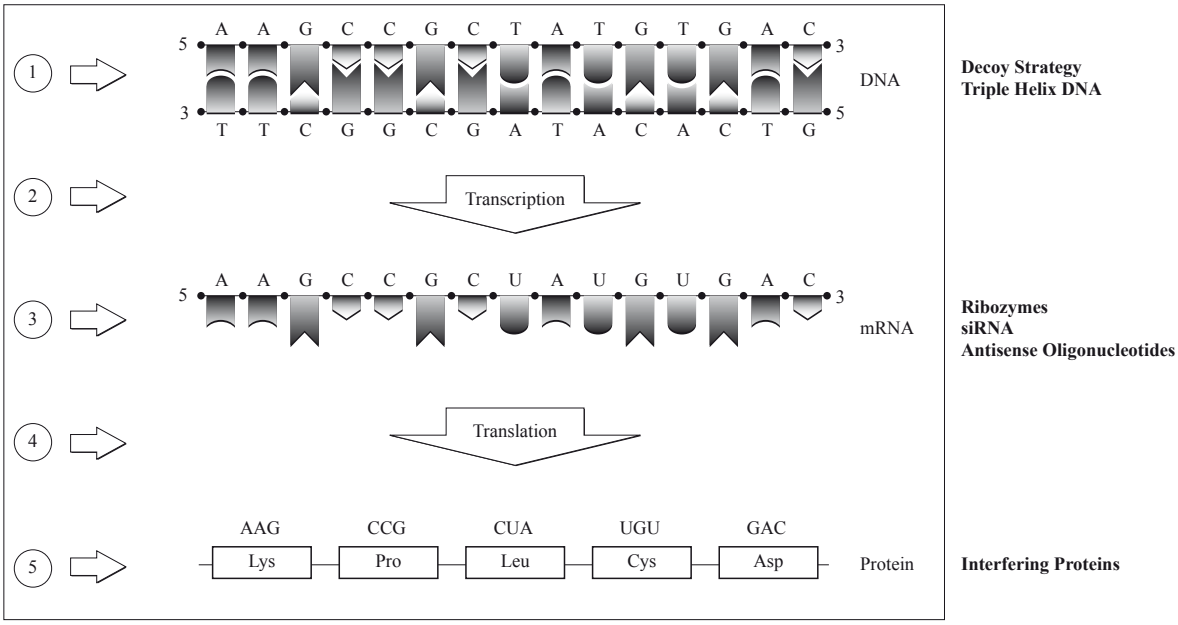
Cell therapy

Allogeneic or *ex vivo* genetically modified autologous hepatocyte transplantation is a promising strategy to treat hereditary monogenic liver diseases. In patients with familial hypercholesterolemia (FH) that is caused by various mutations in the low density lipoprotein (LDL) receptor gene [18], apart from orthotopic liver transplantation [19,20], liver-directed gene therapy has been performed in a pilot study in five patients [21,22]. Autologous liver cells, prepared from a surgical biopsy, were transduced *ex vivo* with a recombinant retrovirus expressing the normal LDL receptor. These *ex vivo* genetically modified hepatocytes were transplanted by portal infusion and resulted in significant and prolonged reductions in LDL cholesterol in 3/5 patients for at least four months, demonstrating the feasibility of engrafting a limited number of *ex vivo* transduced hepatocytes. Also, allogeneic hepatocyte transplantation has been successfully used in patients to partially correct Crigler-Najjar syndrome type I [23] and glycogen storage disease type I [24].

Block of gene expression or function

For diseases caused by the expression of an acquired gene or the overexpression of an endogenous gene, blocking gene expression can be an effective therapeutic approach. Several strategies can be employed (*Fig. 1*): interference with the transcription of genes by binding of transcription factors to nucleic acids introduced into or synthesized in the cells (decoy

Figure 1. **Strategies aimed at blocking gene expression**



strategy) [25,26], by binding of single-stranded nucleic acids to double-stranded DNA, forming a triple helix structure [25,26], hybridization of RNA molecules possessing endonuclease activity (ribozymes) to RNA, resulting in its sequence-specific cleavage [27,28], RNA interference (RNAi) by small inhibiting RNA (siRNA) or microRNA (miRNA) [29-32], block of translation by antisense oligonucleotides [25,26,33,34] and the intracellular synthesis of peptides or proteins, interfering with their normal counterpart, termed dominant negative (DN) mutant strategy [35]. These different strategies have been applied to a number of malignant and infectious diseases. In particular ribozymes, siRNAs, antisense oligonucleotides and DN mutants have been experimentally explored to treat hepatitis B virus (HBV) and hepatitis C virus (HCV) infections.

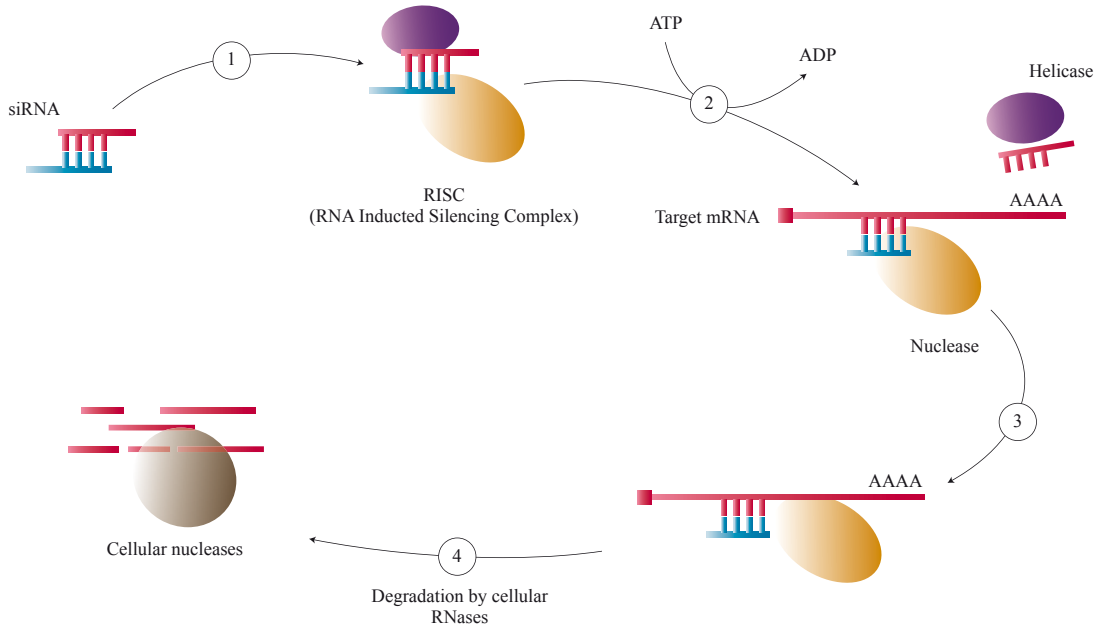
Ribozymes. Ribozymes ('ribonucleic acid enzymes') were originally discovered as naturally occurring RNA molecules that catalyze the sequence-specific cleavage of RNA and RNA splicing reactions [27,28]. This catalytic activity is the major attraction of the ribozyme concept since one ribozyme can cleave many target RNAs. Ribozymes that cleave RNA are being developed as inhibitors of gene expression and viral replication. Several studies have clearly demonstrated that hammerhead ribozymes can specifically cleave HBV RNA [36,37] or HCV RNA [38,39] *in vitro*. *In vivo*, however, an efficient ribozyme-mediated cleavage of HBV RNA could not be demonstrated to date. For HCV infection, the elimination of HCV RNA in infected hepatocytes by ribozymes has also been reported [38,40].

Small interfering RNA. RNAi is a recently discovered basic intracellular mechanism [29-32] that has been explored also for the inhibition of HBV and HCV infection. For HBV, inhibition of viral gene expression and replication has been shown *in vitro* [41-43] and in different mouse models *in vivo* [44-48]. For HCV, inhibition of viral gene expression and replication has been shown *in vitro* in the replicon system [49-51]. While effective in blocking viral gene expression and replication, *in vivo* oversaturation of celluar miRNA/short hairpin RNA (shRNA) pathways can result in lethal hepatotoxicity [48]. For future RNAi-based strategies in animals or humans, these findings indicate that the control of intracellular shRNA expression levels through optimizing shRNA dose and sequence will be key to reduce the risk of oversaturating endogenous small RNA pathways.

Antisense oligonucleotides. Antisense nucleic acids are designed to specifically bind to RNA or mRNA, resulting in the formation of RNA-DNA (antisense oligodeoxynucleotides) or RNA-RNA hybrids (antisense oligoribonucleotides) with an arrest of RNA replication, reverse transcription or mRNA translation [25,26,33,34,52]. Antisense effects can be potentiated by degradation of RNA in RNA-DNA hybrids by cellular RNases H. While conceptually simple, it is clear now that not all desired as well as undesired effects are caused by the target sequence specific antisense action of the oligonucleotides or the cellular enzymes mentioned above [53,54].

The antisense strategy has been successfully applied *in vitro* to HBV infection, [55-58] as demonstrated in *Fig. 2*, and to HCV infection [59-64]. In addition, studies in nude mice[65], in the duck hepatitis B virus (DHBV) [66] and the woodchuck hepatitis virus (WHV) model of HBV infection [67] demonstrated the *in vivo* applicability of this approach. While no toxic effects have been observed in these experiments, the contribution of non-antisense effects to the inhibition of viral replication or gene expression has not been systematically assessed in most studies. Independent of the antisense or non-antisense mechanism of the biological effects, an *in vitro* screening procedure for the identification of functionally active oligonucleotides

***Figure 2*. Principle of RNA interference [31]**



[53,68] should greatly facilitate the design of oligonucleotide based antiviral therapies.

Interfering peptides or proteins. The intracellular synthesis of interfering peptides or proteins, including single chain or whole non-secreted antibodies, is aimed at the specific interference with the assembly or function of viral structural or non-structural proteins and represents a type of intracellular immunization [69]. This approach has been shown for block mammalian and avian hepadnavirus gene expression and replication *in vitro*. For example, the fusion of different polypeptides of various lengths to the carboxy-terminus of the viral core protein yields DN mutants [70-73]. These DN mutants are species-specific and suppress viral replication by at least 90% at an effector to target ratio of 1:10. Moreover, the non-secretory form of the hepatitis B e antigen (HBeAg) was shown to effectively inhibit viral replication and may indeed act as a natural regulator of HBV propagation [74-76]. The potential advantage of DN mutants over ribozymes or antisense oligonucleotides is their relative independence from viral sequence variations, minimizing the risk of selecting or accumulating 'therapy escape' mutants.

DNA vaccination

A novel approach is DNA vaccination resulting in the manipulation of the immune system by introduction of expression vectors into muscle cells or dendritic cells and long lasting cellular and humoral immune responses.The direct gene transfer into muscle [77] represents an exciting new development and elegant application of gene therapy [78,79]. The therapeutic DNA vaccine acts by the intracellular plasmid-derived synthesis of a viral protein which enters the cell's MHC class I pathway [78]. Only proteins that originate within the cell can be processed by MHC class I molecules that carry fragments of the protein to the cell surface. There they stimulate CD8+ cytotoxic T cells, resulting in cell-mediated immunity. In principle, this strategy is applicable to the treatment of acquired genetic diseases, associated with the expression of disease-specific antigens serving as targets for CD8+ cytotoxic T cells.

Therapeutic DNA vaccination has been experimentally explored for HBV [80-84] as well as HCV infection [85,86] and holds great promise as an effective molecular therapy for these viral diseases. In this context, the coexpression of HBsAg and interleukin-2 was shown to greatly increase humoral as well as cellular immune response [87].

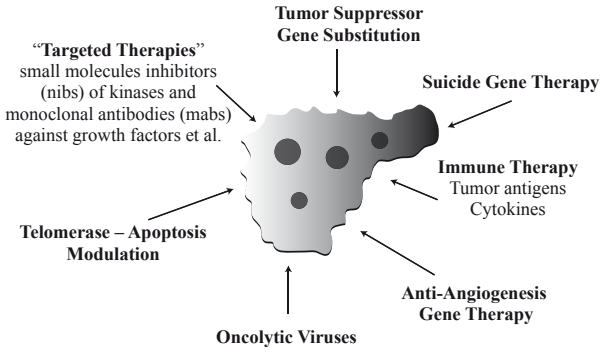
Further, DNA-based tumor vaccination against HCC may be possible, for example, by intramuscular introduction of a plasmid expressing HCC-specific antigens or antigens that are highly overexpressed in HCC cells, such as AF-20 antigen, insulin receptor substrate-1 [88], alpha-fetoprotein [89], aspartyl asparaginyl hydroxylase, mutated p53 protein and others. Potential limitations of this strategy include the regulation of the immune response as well as the low level expression of the targeted antigen in non-malignant cells [90], rendering them susceptible to immune mediated elimination as well.

Gene augmentation

Complex genetic diseases are among the most prevalent clinical problems. In this situation, gene augmentation is aimed at the local expression of a therapeutic gene product that is physiologically not expressed or expressed at therapeutically insufficient levels. This strategy is explored among others for the treatment of HCC.

Suicide gene therapy. An interesting strategy to treat HCCs is genetic prodrug activation therapy *via* the introduction of a 'suicide gene' into malignant cells followed by the admini-

***Figure 3*. HCC treatment: experimental strategies, incl. gene therapy**



stration of the prodrug. This concept has been experimentally explored in HCC cells *in vitro* and *in vivo*, e.g., for the HSV-tk gene [91-95], the gene encoding cytosine deaminase (CD) that converts the prodrug 5-fluorocytosine to 5-fluorouracil which inhibits RNA and DNA synthesis during the S-phase of the cell cycle [96], the gene encoding purine nucleoside phosphorylase that converts purine analogs into freely diffusible toxic metabolites [97,98] as well as the gene encoding cytochrome p450 4B1 [99]. A significant bystander effect of cell killing caused by suicide gene expression could be demonstrated *in vitro* and *in vivo*, based on cell-cell contact rather than release of cytotoxic substances from the transduced cells [100]. At the same time, the bystander effect may also affect non-malignant dividing cells in the target tissue, potentially limiting the application of this strategy.

Immune therapy. In the process of malignant transformation new antigenic surface proteins can be expressed (tumor antigens) or oncofetal proteins can be re-expressed, e.g., alpha fetoprotein (AFP).

AFP-specific immune therapy has been explored in mice and humans. Vaccination with an AFP-expressing DNA construct resulted in tumor rejection and prolonged survival in a mouse model [89]. Also in patients AFP-specific T cells could be detected [101,102]. Since AFP is not only expressed by tumor cells but also by regenerating liver cells and in liver cirrhosis immunization against AFP carries the risk of autoimmune hepatitis, as has been experimentally shown in mice [90].

Immune therapy with antigen presenting cells (APC) is another strategy that has been explored using dendritic cells (DC) exposed to tumor lysates, peptides or *ex vivo* transduced with tumor antigen expressing DNA constructs. While this strategy is conceptually very interesting, to date there are no data available that demonstrate its clinical efficacy [103].

Cytokine gene therapy has been explored using tumor necrosis factor (TNF)-alpha, GM-CSF, interferon-alpha or interferon-gamma, interleukin (IL)-2, -4, -6, -7, -12 and -18, B7-1 as well as CD40 ligand. Complete regression of a HCC was demonstrated *in vivo* by TNF-alpha [104], IL-2 [105], IL-12 [106] and an activatable interferon regulatory factor-1 in mice [107]. Gene transfer was achieved *in vivo* by delivering retroviral [104] or adenoviral vectors [105] systemically, directly into the tumor or into the peritoneal cavity. A pilot study in patients with gastrointestinal tumors exploring the intratumoral injection of an adenoviral IL-12 expression construct showed only marginal efficacy, however [108].

Antiangiogenic gene therapy. This concept has been experimentally explored in a HCC mouse model using the angiostatin gene. Angiostatin gene transfer resulted in reduced tumor volume and vascular density [109].

Oncolytic viruses. This new and elegant approach uses p53 mutations for selective, adenovirus-mediated lysis of tumor cells. Thus, an adenovirus mutant was engineered that replicates selectively in p53-deficient human tumor cells [110-112]. Other examples are the adenoviral introduction of Smac that antagonizes the inhibitor of apoptosis proteins in HCC tumor cells and enhances tumor cell death [113] and tumor-specific replication-restricted adenoviral vectors [114]. Further, the intravascular administration of a replication-competent genetically engineered herpes simplex virus (HSV)-1 resulted in oncolysis of a diffuse HCC [115]. More efficient HSV-1-based vectors have been developed [116].

Molecular prevention of liver diseases

DNA-based prophylactic vaccination against HBV infection, for example, is possible by intramuscular introduction of a plasmid expressing hepatitis B surface antigen (HBsAg). HBsAg is taken up by cells *via* phagocytosis or endocytosis, processed through the major histocompatibility complex (MHC) class II system and primarily stimulates an antibody response through CD4+ helper T cells with the production of anti-HBs [78,79,117-120]. While the DNA-based vaccination against HBV infection induces anti-HBs antibodies and prevents HBV infection, DNA-based vaccination against HCV infection of chimpanzees has been shown not to prevent infection but to result in the resolution of acute HCV infection through an effective vaccine-induced cellular immune response [121,122].

Conclusions

Molecular analyses have become an integral part of biomedical research as well as clinical medicine. The definition of the genetic basis of many human diseases has led to a better understanding of their pathogenesis and has in addition offered new perspectives for their diagnosis, therapy and prevention. Genetically, human diseases can be classified as hereditary monogenic, acquired monogenic and complex genetic diseases. Based on this classification, gene therapy is based on four concepts: gene repair, gene substitution, cell or organ transplanation, block of gene expression or function, gene augmentation and DNA vaccination. While the recent developments in gene therapy for liver diseases are promising, various delivery, targeting and safety issues need to be addressed before these strategies will enter clinical practice. Nevertheless, gene therapy will become part of the management of patients with liver diseases, complementing existing diagnostic, therapeutic and preventive strategies.

References

1. Anderson WF. Human gene therapy. Science, 1992; 256: 808-13.
2. Friedmann T. Progress toward human gene therapy. Science, 1989; 244: 1275-81.
3. Gutierrez AA, Lemoine NR, Sikora K. Gene therapy for cancer. Lancet, 1992; 339: 715-21.
4. Miller AD. Human gene therapy comes of age. Nature, 1992; 357: 455-60.
5. Morgan RA, Anderson WF. Human gene therapy. Annu Rev Biochem, 1993; 62: 191-217.
6. Mulligan RC. The basic science of gene therapy. Science, 1993; 260: 926-32.
7. Rosenberg SA. Gene therapy for cancer. JAMA, 1992; 268: 2416-9.
8. Kren BT, Parashar B, Bandyopadhyay P, Chowdhury NR, Chowdhury JR, Steer CJ. Correction of the UDP-glucuronosyltransferase gene defect in the gunn rat model of Crigler-Najjar syndrome type I with a chimeric oligonucleotide. Proc Natl Acad Sci USA, 1999; 96: 10349-54.
9. Sarver N, Cairns S. Ribozyme trans-splicing and RNA tagging: Following the messenger. Nature Med, 1996; 2: 641-2.
10. Jones JT, Lee SW, Sullenger BA. Tagging ribozyme reaction sites to follow trans-splicing in mammalian cells. Nature Med, 1996; 2: 643-8.
11. Lan N, Howrey RP, Lee S-W, Smith CA, Sullenger BA. Ribozyme-mediated repair of sickle ß-globin mRNAs in erythrocyte precursors. Science, 1998; 280: 1593-6.
12. Overturf K, Al-Dhalimy M, Tanguay R, Brantly M, Ou C-N, Finegold M, Grompe M. Hepatocytes corrected by gene therapy are selected *in vivo* in a murine model of hereditary tyrosinaemia type I. Nat Genet, 1996; 12: 266-73.
13. Grompe M. The pathophysiology and treatment of hereditary tyrosinemia type 1. Semin Liver Dis, 2001; 21: 563-71.
14. Roth DA, Tawa NE, Jr., O'Brien JM, Treco DA, Selden RF. Nonviral transfer of the gene encoding coagulation factor VIII in patients with severe hemophilia A. N Engl J Med, 2001; 344: 1735-42.
15. Kay MA, Manno CS, Ragni MV, Larson PJ, Couto LB, McClelland A, Glader B, Chew AJ, Tai SJ, Herzog RW, Arruda V, Johnson F, Scallan C, Skarsgard E, Flake AW, High KA. Evidence for gene transfer and expression of factor IX in haemophilia B patients treated with an AAV vector. Nat Genet, 2000; 24: 257-61.
16. Xu GW, Sun ZT, Forrester K, Wang XW, Coursen J, Harris CC. Tissue-specific growth suppression and chemosensitivity promotion in human hepatocellular carcinoma cells by retroviral-mediated transfer of the wild-type p53 gene. Hepatology, 1996; 24: 1264-8.
17. Zender L, Kock R, Eckhard M, Frericks B, Gosling T, Gebhardt T, Drobek S, Galanski M, Kuhnel F, Manns M, Kubicka S. Gene therapy by intrahepatic and intratumoral trafficking of p53-VP22 induces regression of liver tumors. Gastroenterology, 2002; 123: 608-18.
18. Brown MS, Goldstein JL. A receptor-mediated pathway for cholesterol homeostasis. Science, 1986; 232: 34-7.
19. Bilheimer DW, Goldstein JL, Grundy SM, Starzl TE. Liver transplantation to provide low density lipoprotein receptors and lower plasma cholesterol in a child with homozygous familial hypercholesterolemia. N Engl J Med, 1984; 311: 1658-64.
20. Hoeg JM, Starzl TE, Brewer HB. Liver transplantation for treatment of cardiovascular disease: comparison with medication and plasma exchange in homozygous familial hypercholesterolemia. Am J Card, 1987; 59: 705-7.
21. Grossman M, Raper SE, Kozarsky K, Stein EA, Engelhardt JF, Müller D, Lupien PJ, Wilson JM. Successful ex vivo gene therapy directed to liver in a patient with familial hypercholesterolaemia. Nat Genet, 1994; 6: 335-41.
22. Grossman M, Rader DJ, Muller DWM, Kolansky DM, Kozarsky K, Clark III BJ, Stein EA, Lupien PJ, Brewer HB, Raper SE, Wilson JM. A pilot study of *ex vivo* gene therapy for homozygous familial hypercholesterolaemia. Nature Medicine, 1995; 1: 1148-54.
23. Fox IJ, Chowdhury JR, Kaufman SS, Goertzen TC, Chowdhury NR, Warkentin PI, Dorko K, Sauter BV, Strom SC. Treatment of the Crigler-Najjar syndrome type I with hepatocyte transplantation. N Engl J Med, 1998; 338: 1422-6.
24. Muraca M, Gerunda G, Neri D, Vilei MT, Granato A, Feltracco P, Meroni M, Giron G, Burlina AB. Hepatocyte transplantation as a treatment for glycogen storage disease type 1a. Lancet, 2002; 359: 317-318.
25. Helene C. Rational design of sequence-specific oncogene inhibitors based on antisense and antigene oligonucleotides. Eur J Cancer, 1991; 27: 1466-71.
26. Helene C, Toulme JJ. Specific regulation of gene expression by antisense, sense and antigene nucleic acids. Biochem Biophys Acta, 1990; 1049: 99-125.
27. Haseloff J, Gerlach WL. Simple RNA enzymes with new and highly specific endoribonuclease activities. Nature, 1988; 334: 585-91.
28. Thompson JD, Macejak D, Couture L, Stinchcomb DT. Ribozymes in gene therapy. Nature Med, 1995; 1: 277-8.
29. Dykxhoorn DM, Lieberman J. The silent revolution: RNA interference as basic biology, research tool, and therapeutic. Annu Rev Med, 2005; 56: 401-23.
30. Williams BR. Targeting specific cell types with silencing RNA. N Engl J Med, 2005; 353: 1410-1.
31. Kitabwalla M, Ruprecht RM. RNA interference – a new weapon against HIV and beyond. N Engl J Med, 2002; 347: 1364-7.
32. Taylor JA, Naoumov NV. The potential of RNA interference as a tool in the management of viral hepatitis. J Hepatol, 2005; 42: 139-44.
33. Calabretta B. Inhibition of protooncogene expression by antisense oligodeoxynucleotides: biological and therapeutic implications. Cancer Res, 1991; 51: 4505-10.
34. Tseng BY, Brown, KD. Antisense oligonucleotide technology in the development of cancer therapeutics. Cancer Gene Ther, 1994; 1: 65-71.
35. Herschkowitz I. Functional inactivation of genes by dominant negative mutations. Nature, 1987; 329: 219-22.
36. von Weizsäcker F, Blum HE, Wands JR. Cleavage of hepatitis B virus RNA by three ribozymes transcribed from a single DNA template. Biochem Biophys Res Commun, 1992; 189: 743-8.
37. Beck J, Nassal M. Efficient hammerhead ribozyme-mediated cleavage of the structured hepatitis B virus encapsidation signal in vitro and in cell extracts, but not in intact cells. Nucl Acids Res, 1995; 23: 4954-62.
38. Lieber A, He C-Y, Polyak SJ, Gretch DR, Barr D, Kay MA. Elimination of hepatitis C virus RNA in infected human hepatocytes by adenovirus-mediated expression of ribozymes. J Virol, 1996; 70: 8782-91.
39. Sakamoto N, Wu CH, Wu GY. Intracellular cleavage of hepatitis C virus RNA and inhibition of viral protein translation by hammerhead ribozymes. J Clin Invest, 1996; 98: 2720-8.
40. Welch PJ, Tritz R, Yei S, Leavitt M, Yu M, Barber J. A potential therapeutic application of hairpin ribozymes – *in vitro* and *in vivo* studies of gene therapy for hepatitis C virus infection. Gene Therapy, 1996; 3: 994-1001.
41. Shlomai A, Shaul Y. Inhibition of hepatitis B virus expression and replication by RNA interference. Hepatology, 2003; 37: 764-70.
42. Ying C, De Clercq E, Neyts J. Selective inhibition of hepatitis B virus replication by RNA interference. Biochem Biophys Res Commun, 2003; 309: 482-4.
43. Konishi M, Wu CH, Wu GY. Inhibition of HBV replication by siRNA in a stable HBV-producing cell line. Hepatology, 2003; 38: 842-50.
44. Giladi H, Ketzinel-Gilad M, Rivkin L, Felig Y, Nussbaum O, Galun E. Small interfering RNA inhibits hepatitis B virus replication in mice. Mol Ther, 2003; 8: 769-76.
45. McCaffrey AP, Nakai H, Pandey K, Huang Z, Salazar FH, Xu H, Wieland SF, Marion PL, Kay MA. Inhibition of hepatitis B virus in mice by RNA interference. Nat Biotechnol, 2003; 21: 639-44.
46. Klein C, Bock CT, Wedemeyer H, Wustefeld T, Locarnini S, Dienes HP, Kubicka S, Manns MP, Trautwein C. Inhibition of hepatitis B virus replication *in vivo* by nucleoside analogues and siRNA. Gastroenterology, 2003; 125: 9-18.
47. Uprichard SL, Boyd B, Althage A, Chisari FV. Clearance of hepatitis B virus from the liver of transgenic mice by short hairpin RNAs. Proc Natl Acad Sci USA, 2005; 102: 773-8.
48. Grimm D, Streetz KL, Jopling CL, Storm TA, Pandey K, Davis CR, Marion P, Salazar F, Kay MA. Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. Nature, 2006; 441: 537-41.
49. Randall G, Grakoui A, Rice CM. Clearance of replicating hepa-

titis C virus replicon RNAs in cell culture by small interfering RNAs. Proc Natl Acad Sci USA, 2003; 100: 235-40.

50. Kapadia SB, Brideau-Andersen A, Chisari FV. Interference of hepatitis C virus RNA replication by short interfering RNAs. Proc Natl Acad Sci USA, 2003; 100: 2014-8.

51. Wilson JA, Jayasena S, Khvorova A, Sabatinos S, Rodrigue-Gervais IG, Arya S, Sarangi F, Harris-Brandts M, Beaulieu S, Richardson CD. RNA interference blocks gene expression and RNA synthesis from hepatitis C replicons propagated in human liver cells. Proc Natl Acad Sci USA, 2003; 100: 2783-8.

52. Wagner RW. Gene inhibition using antisense oligodeoxynucleotides. Nature, 1994; 372: 333-5.

53. Branch AD. A hitchhiker's guide to antisense and nonantisense biochemical pathways. Hepatology, 1996; 24: 1517-29.

54. Dougherty WG, Parks TD. Transgenes and gene suppression: telling us something new? Curr Opin Cell Biol, 1995; 7: 399-405.

55. Goodarzi G, Gross SC, Tewari A, Watabe K. Antisense oligodeoxyribonucleotides inhibit the expression of the gene for hepatitis B virus surface antigen. J Gen Virol, 1990; 71: 3021-5.

56. Blum HE, Galun E, Weizsäcker F, Wands JR. Inhibition of hepatitis B virus by antisense oligodeoxynucleotides. Lancet, 1991; 337: 1230.

57. Wu GY, Wu CH. Specific inhibition of hepatitis B viral gene expression *in vitro* by targeted antisense oligonucleotides. J Biol Chem, 1992; 267: 12436-9.

58. Nakazono K, Ito Y, Wu CH, Wu GY. Inhibition of hepatitis B virus replication by targeted pretreatment of complexed antisense DNA *in vitro*. Hepatology, 1996; 23: 1297-303.

59. Wakita T, Wands JR. Specific inhibition of hepatitis C virus expression by antisense oligodeoxynucleotides. *In vitro* model for selection of target sequence. J Biol Chem, 1994; 269: 14205-10.

60. Mizutani T, Kato N, Hirota M, Sugiyama K, Murakami A, Shimotohno K. Inhibition of hepatitis C virus replication by antisense oligonucleotide in culture cells. Biochem Biophys Res Commun, 1995; 212: 906-11.

61. Alt M, Renz R, Hofschneider PH, Paumgartner G, Caselmann WH. Specific inhibition of hepatitis C viral gene expression by antisense phosphorothioate oligodeoxynucleotides. Hepatology, 1995; 22: 707-17.

62. Seki M, Honda Y. Phosphorothioate antisense oligodeoxynucleotides capable of inhibiting hepatitis C virus gene expression: in vitro translation assay. J Biochem, 1995; 118: 1199-204.

63. Hanecak R, Brown-Driver V, Fox MC, Azad RF, Furusako S, Nozaki C, Ford C, Sasmor H, Anderson KP. Antisense oligonucleotide inhibition of hepatitis C virus gene expression in transformed hepatocytes. J Virol, 1996; 70: 5203-12.

64. Vidalin O, Major ME, Rayner B, Imbach JL, Trepo C, Inchauspe G. *In vitro* inhibition of hepatitis C virus gene expression by chemically modified antisense oligodeoxynucleotides. Antimicrobial Agents & Chemotherapy, 1996; 40: 2337-44.

65. Yao Z, Zhou Y, Feng X, Chen C, Guo J. *In vivo* inhibition of hepatitis B viral gene expression by antisense phosphorothioate oligodeoxynucleotides in athymic nude mice. Journal of Viral Hepatitis, 1996; 3: 19-22.

66. Offensperger WB, Offensperger S, Walter E, Teubner K, Igloi G, Blum HE, Gerok W. *In vivo* inhibition of duck hepatitis B virus replication and gene expression by phosphorothioate modified antisense oligodeoxynucleotides. EMBO J, 1993; 12: 1257-62.

67. Bartholomew RM, Carmichael EP, Findeis MA, Wu CH, Wu GY. Targeted delivery of antisense DNA in woodchuck hepatitis virus-infected woodchucks. J Viral Hepatitis, 1995; 2: 273-8.

68. Tuerk C, Gold L. Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. Science, 1990; 249: 505-10.

69. Baltimore D. Intracellular immunization. Nature, 1988; 325: 395-6.

70. Delaney MA, Goyal S, Seeger C. Design of modified core genes that inhibit replication of woodchuck hepatitis virus. In: Hollinger FB, Lemon SM, Margolis H, editors. Viral hepatitis and liver disease. Baltimore, Maryland: Williams & Wilkins, 1991; 667-8.

71. Scaglioni PP, Melegari M, Wands JR. Characterization of hepatitis B virus core mutants that inhibit viral replication. Virology, 1994; 205: 112-20.

72. von Weizsäcker F, Wieland S, Blum HE. Inhibition of viral replication by genetically engineered mutants of the duck hepatitis B virus core protein. Hepatology, 1996; 24: 294-9.

73. Scaglioni P, Melegari M, Takahashi M, Chowdhury JR, Wands J. Use of dominant negative mutants of the hepadnaviral core protein as antiviral agents. Hepatology, 1996; 24: 1010-7.

74. Scaglioni PP, Melegari M, Wands JR. Posttranscriptional regulation of hepatitis B virus replication by the precore protein. J Virol, 1997; 71: 345-53.

75. Buckwold VE, Xu Z, Chen M, Yen TSB, Ou J-H. Effects of a naturally occurring mutation in the hepatitis B virus basal core promoter on precore gene expression and viral replication. J Virol, 1996; 70: 5845-51.

76. Guidotti LG, Matzke B, Pasquinelli C, Shoenberger JM, Rogler CE, Chisari FV. The hepatitis B virus (HBV) precore protein inhibits HBV replication in transgenic mice. Journal of Virology, 1996; 70: 7056-61.

77. Blau HM, Springer ML. Muscle mediated gene therapy. N Engl J Med, 1995; 333: 1554-6.

78. McDonnell WM, Askari FK. DNA vaccines. N Engl J Med, 1996; 334: 42-5.

79. Pardoll DM, Beckerleg AM. Exposing the immunology of naked DNA vaccines. Immunity, 1995; 3: 165-9.

80. Schirmbeck R, Bohm W, Ando K, Chisari FV, Reimann J. Nucleic acid vaccination primes hepatitis B virus surface antigen specific cytotoxic T lymphocytes in nonresponder mice. J Virol, 1995; 69: 5929-34.

81. Vitiello A, Ishioka G, Grey HM, Rose R, Farness P, LaFond R, Yuan L, Chisari FV, Furze J, Bartholomeuz R, et al. Development of a lipopeptide based therapeutic vaccine to treat chronic HBV infection. I. Induction of a primary cytotoxic T lymphocyte response in humans. J Clin Invest, 1995; 95: 341-9.

82. Kuhöber A, Pudollek HP, Reifenberg K, Chisari FV, Schlicht HJ, Reimann J, Schirmbeck R. DNA immunization induces antibody and cytotoxic T cell responses to hepatitis B core antigen in H-2b mice. J Immunol, 1996; 156: 3687-95.

83. Mancini M, Hadchouel M, Davis HL, Whalen RG, Tiollais P, Michel M-L. DNA-mediated immunization in a transgenic mouse model of the hepatitis B surface antigen chronic carrier state. Proc Natl Acad Sci USA, 1996; 93: 12496-501.

84. Davis HL, Schirmbeck R, Reimann J, Whalen RG. DNA--mediated immunization in mice induces a potent MHC class I--restricted cytotoxic T lymphocyte response to the hepatitis B envelope protein. Human Gene Therapy, 1995; 6: 1447-56.

85. Lagging LM, Meyer K, Hoft D, Houghton M, Belshe RB, Ray R. Immune responses to plasmid DNA encoding the hepatitis C virus core protein. J Virol, 1995; 69: 5859-63.

86. Major ME, Vitvitski L, Mink MA, Schleef M, Whalen RG, Trepo C, Inchauspe G. DNA based immunization with chimeric vectors for the induction of immune responses against the hepatitis C virus nucleocapsid. J Virol, 1995; 69: 5798-805.

87. Chow YH, Huang WL, Chi WK, Chu YD, Tao MH. Improvement of hepatitis B virus DNA vaccines by plasmids coexpressing hepatitis B surface antigen and interleukin-2. J Virol, 1997; 71: 169-78.

88. Wands JR, Lavaissiere L, Moradpour D, de la Monte S, Mohr L, Nicolau C, Tanaka S. Immunological approach to hepatocellular carcinoma. Journal of Viral Hepatitis, 1997; 4 (Suppl. 2): 60-74.

89. Grimm CF, Ortmann D, Mohr L, Michalak S, Krohne TU, Meckel S, Eisele S, Encke J, Blum HE, Geissler M. Mouse alpha-fetoprotein-specific DNA-based immunotherapy of hepatocellular carcinoma leads to tumor regression in mice. Gastroenterology, 2000; 119: 1104-12.

90. Geissler M, Mohr L, Weth R, Kohler G, Grimm CF, Krohne TU, von Weizsäcker F, Blum HE. Immunotherapy directed against alpha-fetoprotein results in autoimmune liver disease during liver regeneration in mice. Gastroenterology, 2001; 121: 931-9.

91. Ido A, Nakata K, Kato Y, Nakao K, Murata K, Fujita M, Ishii N, Tamaoki T, Shiku H, Nagataki S. Gene therapy for hepatoma cells using a retrovirus vector carrying herpes simplex virus thymidine kinase gene under the control of human alpha-fetoprotein gene promoter. Cancer Research, 1995; 55: 3105-9.

92. Kaneko S, Hallenbeck P, Kotani T, Nakabayashi H, McGarrity G, Tamaoki T, Anderson WF, Chiang YL. Adenovirus-mediated gene therapy of hepatocellular carcinoma using cancer-specific gene expression. Cancer Research, 1995; 55: 5283-7.

93. Qian C, Bilbao R, Bruna O, Prieto J. Induction of sensitivity to ganciclovir in human hepatocellular carcinoma cells by adenovirus mediated gene transfer of herpes simplex virus thymidine kinase. Hepatology, 1995; 22: 118-23.

94. Wills KN, Huang WM, Harris MP, Machemer T, Maneval DC, Gregory RJ. Gene therapy for hepatocellular carcinoma: chemosensitivity conferred by adenovirus-mediated transfer of the HSV-1 thymidine kinase gene. Cancer Gene Therapy, 1995; 2: 191-7.

95. Kanai F, Shiratori Y, Yoshida Y, Wakimoto H, Hamada H, Kanegae Y, Saito I, Nakabayashi H, Tamaoki T, Tanaka T, Lan K-H, Kato N, Shiina S, Omata M. Gene therapy for alpha-fetoprotein-producing human hepatoma cells by adenovirus-mediated transfer of the herpes simplex virus thymidine kinase gene. Hepatology, 1996; 23: 1359-68.

96. Kanai F, Lan KH, Shiratori Y, Tanaka T, Ohashi M, Okudaira T, Yoshida Y, Wakimoto H, Hamada H, Nakabayashi H, Tamaoki T, Omata M. In vivo gene therapy for alpha-fetoprotein-producing hepatocellular carcinoma by adenovirus-mediated transfer of cytosine deaminase gene. Cancer Res, 1997; 57: 461-5.

97. Mohr L, Shankara S, Yoon SK, Krohne TU, Geissler M, Roberts B, Blum HE, Wands JR. Gene therapy of hepatocellular carcinoma in vitro and in vivo in nude mice by adenoviral transfer of the Escherichia coli purine nucleoside phosphorylase gene. Hepatology, 2000; 31: 606-14.

98. Krohne TU, Shankara S, Geissler M, Roberts BL, Wands JR, Blum HE, Mohr L. Mechanisms of cell death induced by suicide genes encoding purine nucleoside phosphorylase and thymidine kinase in human hepatocellular carcinoma cells in vitro. Hepatology, 2001; 34: 511-8.

99. Mohr L, Rainov NG, Mohr UG, Wands JR. Rabbit cytochrome P450 4B1: A novel prodrug activating gene for pharmacogene therapy of hepatocellular carcinoma. Cancer Gene Ther, 2000; 7: 1008-14.

100. Kuriyama S, Nakatani T, Masui K, Sakamoto T, Tominaga K, Yoshikawa M, Fukui H, Ikenaka K, Tsujii T. Bystander effect caused by suicide gene expression indicates the feasibility of gene therapy for hepatocellular carcinoma. Hepatology, 1995; 22: 1838-46.

101. Butterfield LH. Immunotherapeutic strategies for hepatocellular carcinoma. Gastroenterology, 2004; 127: S232-41.

102. Butterfield LH, Meng WS, Koh A, Vollmer CM, Ribas A, Dissette VB, Faull K, Glaspy JA, McBride WH, Economou JS. T cell responses to HLA-A*0201-restricted peptides derived from human alpha fetoprotein. J Immunol, 2001; 166: 5300-8.

103. Iwashita Y, Tahara K, Goto S, Sasaki A, Kai S, Seike M, Chen CL, Kawano K, Kitano S. A phase I study of autologous dendritic cell-based immunotherapy for patients with unresectable primary liver cancer. Cancer Immunol Immunother, 2003; 52: 155-61.

104. Cao G, Kuriyama S, Du P, Sakamoto T, Kong X, Masui K, Qi Z. Complete regression of established murine hepatocellular carcinoma by in vivo tumor necrosis factor alpha gene transfer [see comments]. Gastroenterology, 1997; 112: 501-10.

105. Huang H, Chen SH, Kosai K, Finegold MJ, Woo SL. Gene therapy for hepatocellular carcinoma: long-term remission of primary and metastatic tumors in mice by interleukin-2 gene therapy *in vivo*. Gene Therapy, 1996; 3: 980-7.

106. Barajas M, Mazzolini G, Genove G, Bilbao R, Narvaiza I, Schmitz V, Sangro B, Melero I, Qian C, Prieto J. Gene therapy of orthotopic hepatocellular carcinoma in rats using adenovirus coding for interleukin 12. Hepatology, 2001; 33: 52-61.

107. Kroger A, Ortmann D, Krohne TU, Mohr L, Blum HE, Hauser H, Geissler M. Growth suppression of the hepatocellular carcinoma cell line Hepa1-6 by an activatable interferon regulatory factor-1 in mice. Cancer Res, 2001; 61: 2609-17.

108. Sangro B, Mazzolini G, Ruiz J, Herraiz M, Quiroga J, Herrero I, Benito A, Larrache J, Pueyo J, Subtil JC, Olague C, Sola J, Sadaba B, Lacasa C, Melero I, Qian C, Prieto J. Phase I trial of intratumoral injection of an adenovirus encoding interleukin-12 for advanced digestive tumors. J Clin Oncol, 2004; 22: 1389-97.

109. Ishikawa H, Nakao K, Matsumoto K, Ichikawa T, Hamasaki K, Nakata K, Eguchi K. Antiangiogenic gene therapy for hepatocellular carcinoma using angiostatin gene. Hepatology, 2003; 37: 696-704.

110. Heise C, Sampson-Johannes A, Williams A, McCormick F, von Hoff DD, Kirn DH. ONYX-015, an E1B gene-attenuated adenovirus, causes tumor-specific cytolysis and antitumoral efficacy that can be augmented by standard chemotherapeutic agents. Nature Med, 1997; 3: 639-45.

111. Lowe SW. Progress of the smart bomb cancer virus. Nature Med, 1997; 3: 606-8.

112. Habib N, Salama H, Abd El Latif Abu Median A, Isac Anis I, Abd Al Aziz RA, Sarraf C, Mitry R, Havlik R, Seth P, Hartwigsen J, Bhushan R, Nicholls J, Jensen S. Clinical trial of E1B-deleted adenovirus (dl1520) gene therapy for hepatocellular carcinoma. Cancer Gene Ther, 2002; 9: 254-9.

113. Pei Z, Chu L, Zou W, Zhang Z, Qiu S, Qi R, Gu J, Qian C, Liu X. An oncolytic adenoviral vector of Smac increases antitumor activity of TRAIL against HCC in human cells and in mice. Hepatology, 2004; 39: 1371-81.

114. Hallenbeck PL, Chang YN, Hay C, Golightly D, Stewart D, Lin J, Phipps S, Chiang YL. A novel tumor-specific replication-restricted adenoviral vector for gene therapy of hepatocellular carcinoma. Hum Gene Ther, 1999; 10: 1721-33.

115. Pawlik TM, Nakamura H, Yoon SS, Mullen JT, Chandrasekhar S, Chiocca EA, Tanabe KK. Oncolysis of diffuse hepatocellular carcinoma by intravascular administration of a replication-competent, genetically engineered herpesvirus. Cancer Res, 2000; 60: 2790-5.

116. Liu BL, Robinson M, Han ZQ, Branston RH, English C, Reay P, McGrath Y, Thomas SK, Thornton M, Bullock P, Love CA, Coffin RS. ICP34.5 deleted herpes simplex virus with enhanced oncolytic, immune stimulating, and anti-tumour properties. Gene Ther, 2003; 10: 292-303.

117. Davis HL, Michel ML, Whalen RG. DNA-based immunization induces continuous secretion of hepatitis B surface antigen and high levels of circulating antibody. Hum Mol Genet, 1993; 2: 1847-51.

118. Davis HL, McCluskie MJ, Gerin JL, Purcell RH. DNA vaccine for hepatitis B: Evidence for immunogenicity in chimpanzees and comparison with other vaccines. Proc Natl Acad Sci USA, 1996; 93: 7213-8.

119. Davis HL, Mancini M, Michel ML, Whalen RG. DNA--mediated immunization to hepatitis B surface antigen – longevity of primary response and effect of boost. Vaccine, 1996; 14: 910-5.

120. Michel ML, Davis HL, Schleef M, Mancini M, Tiollais P, Whalen RG. DNA-mediated immunization to the hepatitis B surface antigen in mice: aspects of the humoral response mimic hepatitis B viral infection in humans. Proc Natl Acad Sci USA, 1995; 92: 5307-11.

121. Forns X, Payette PJ, Ma X, Satterfield W, Eder G, Mushahwar IK, Govindarajan S, Davis HL, Emerson SU, Purcell RH, Bukh J. Vaccination of chimpanzees with plasmid DNA encoding the hepatitis C virus (HCV) envelope E2 protein modified the infection after challenge with homologous monoclonal HCV. Hepatology, 2000; 32: 618-25.

122. Folgori A, Capone S, Ruggeri L, Meola A, Sporeno E, Ercole BB, Pezzanera M, Tafi R, Arcuri M, Fattori E, Lahm A, Luzzago A, Vitelli A, Colloca S, Cortese R, Nicosia A. A T-cell HCV vaccine eliciting effective immunity against heterologous virus challenge in chimpanzees. Nat Med, 2006; 12: 190-7.