HANNA KANKKONEN

Gene Therapy in the Treatment of Familial Hypercholesterolemia

Evaluation and Development of Viral Vectors and Gene Transfer Techniques

Doctoral dissertation

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ABSTRACT

Sustained production of therapeutic protein is fundamental for long-term amelioration of familial hypercholesterolemia (FH), an inherited autosomal disease caused by a mutation in the low-density lipoprotein receptor (LDLR) gene. Moloney murine leukemia virus (MMLV) –based retroviral vectors and lentiviral vectors are capable of integrating into the host cell genome which makes them an attractive tool for stable gene transfer. In this study we evaluated MMLV retroviral vectors and lentiviral vectors, based on human immunodeficiency virus – 1 (HIV-1) and feline immunodeficiency virus (FIV), for their potential as gene delivery tools in conjunction with vascular ex vivo gene transfer and direct in vivo gene transfer to liver and brain.

We developed an ex vivo gene transfer technique into rabbit arterial wall using autologous smooth muscle cells (SMCs) and demonstrated high efficiency local gene transfer in arteries. In addition to targeting local vascular phenomena during surgical procedures, we showed that genetically engineered SMCs can be used as a means of systemic delivery of gene products. Ex vivo gene therapy for liver disorders such as FH has proven laborious, time consuming and inefficient. In order to circumvent the adversity of ex vivo gene transfer and also the need for liver resection of standard MMLV-retroviral vectors, we generated HIV-1 –based lentiviral vectors encoding rabbit LDLR or green fluorescent protein under the control of a liver-specific promoter (LSP), and evaluated their efficacy and safety in vitro and in vivo. After demonstrating the functionality of the vectors in cell culture, we injected $1 \times 10^9$ infectious virus particles into the portal vein of Watanabe heritable hyperlipidemic (WHHL) rabbits. In addition, we injected another group of WHHL rabbits with MMLV retroviral vectors encoding for human LDLR (LTR-LDLR) or a marker gene. From liver biopsy and tissue samples we showed stable transgene expression and normal liver function and morphology with no sign of major infection or inflammatory changes. LSP-LDLR and LTR-LDLR treatment of WHHL rabbits had a therapeutic effect as it resulted in decreased cholesterol levels in comparison with the control rabbits. Furthermore, the rabbits treated with LDLR exhibited prolonged lifespan. Veterinarian pathological examination of the rabbits in all study groups revealed symptoms mainly related to atherosclerosis. No obvious gene transfer related pathological findings were detected.

For improved vector safety, we constructed a doxycycline (Dox)–regulated self-inactivating HIV-1 lentiviral vector system. Following the verification of the system functionality in vitro, we assessed their applicability in vivo. We demonstrated that the dose-dependent and repeatedly inducible vector system was capable of efficient expression and explicit regulation of the transgene in rat brain in vivo.

In conclusion, this study demonstrates the safety and potential usefulness of HIV-1 lentiviral vectors, and also of the standard MMLV-retroviral vectors, in the treatment of familial hypercholesterolemia. Constructing the vector systems towards targeted and regulated expression of a carefully chosen therapeutic gene from a distinct target organ, together with accurate choice of gene delivery approach, have beneficial effects on the safety and efficacy of gene therapy applications.

National Library of Medicine Classification: WD 200.5.H8, QZ 50, QW 168.5.R18
Medical Subject Headings: hypercholesterolemia, familial / therapy; arteriosclerosis; gene therapy; gene transfer techniques; genetic vectors; retroviridae / genetics; lentivirus / genetics; receptors, LDL / genetics; myocytes, smooth muscle; transcription, genetic; promoter regions (genetics); liver; rabbits; rats
Any powerful idea is absolutely fascinating and absolutely useless until we choose to use it.

— Richard Bach
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Lempäälä, November 2004

Hanna Kankkonen

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### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>AAV</td>
<td>Adeno-associated virus</td>
</tr>
<tr>
<td>ABP</td>
<td>α1-microglobulin/bikunin promoter</td>
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<tr>
<td>ACAT</td>
<td>Acyl coenzyme A:cholesterol</td>
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<tr>
<td>Ad</td>
<td>Adenovirus</td>
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<tr>
<td>ADA</td>
<td>Adenosine deaminase</td>
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<tr>
<td>ADH6</td>
<td>Alcohol dehydrogenase-6</td>
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<tr>
<td>AFOS</td>
<td>Alkaline phosphatase</td>
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<tr>
<td>AIDS</td>
<td>Acquired immune deficiency syndrome</td>
</tr>
<tr>
<td>ALAT</td>
<td>Alanine aminotransferase</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>apo</td>
<td>Apolipoprotein</td>
</tr>
<tr>
<td>apobec-1</td>
<td>ApoB mRNA editing polypeptide 1</td>
</tr>
<tr>
<td>ASAT</td>
<td>Aspartyl aminotransferase</td>
</tr>
<tr>
<td>ASGP</td>
<td>Asialoglycoprotein</td>
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<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CHD</td>
<td>Coronary heart disease</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalo virus</td>
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<tr>
<td>cppt</td>
<td>Central polypurine tract</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
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<tr>
<td>Dil</td>
<td>1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxiribonucleic acid</td>
</tr>
<tr>
<td>Dox</td>
<td>Doxycyclin</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
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<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EIAV</td>
<td>Equine infectious anemia virus</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immuno sorbent assay</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FLDB</td>
<td>Familial ligand-defective apo B</td>
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<tr>
<td>FH</td>
<td>Familial hypercholesterolemia</td>
</tr>
<tr>
<td>FH-NK</td>
<td>FH – North Karelia</td>
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<tr>
<td>FIV</td>
<td>Feline immunodeficiency virus</td>
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<tr>
<td>FIX</td>
<td>Clotting factor IX</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>hAAT</td>
<td>Human α1-antitrypsin</td>
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<tr>
<td>HAEC</td>
<td>Human amniotic epithelial cells</td>
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<tr>
<td>HBV</td>
<td>Hepatitis B virus</td>
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<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
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<td>HERV</td>
<td>Human endogenous retrovirus</td>
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<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<tr>
<td>HLP</td>
<td>Hyperlipoproteinemia</td>
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<tr>
<td>HMG-CoA</td>
<td>3-hydroxy-3-methylglutaryl coenzyme A</td>
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<td>HSV</td>
<td>Herpes Simplex virus</td>
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<tr>
<td>HTG</td>
<td>Hypertriglyceridemia</td>
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<tr>
<td>ICC</td>
<td>Immunocytochemistry</td>
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<tr>
<td>IDL</td>
<td>Intermediated density lipoprotein</td>
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<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
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<tr>
<td>INSIG</td>
<td>Insulin-induced gene</td>
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<tr>
<td>IRES</td>
<td>Internal ribosomal entry site</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>kDa</td>
<td>Kilo Dalton</td>
</tr>
<tr>
<td>lacZ</td>
<td>β-galactosidase</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
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<tr>
<td>LDLR</td>
<td>LDL receptor</td>
</tr>
<tr>
<td>LPDS</td>
<td>Lipoprotein deficient serum</td>
</tr>
<tr>
<td>LRP</td>
<td>LDLR-related protein</td>
</tr>
<tr>
<td>LSP</td>
<td>Liver specific promoter</td>
</tr>
<tr>
<td>LTR</td>
<td>Long terminal repeat</td>
</tr>
<tr>
<td>MLV</td>
<td>Murine leukemia virus</td>
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<tr>
<td>MMLV</td>
<td>Maloney murine leukemia virus</td>
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<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>NZW</td>
<td>New Zealand White</td>
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<tr>
<td>ORF</td>
<td>Open reading frame</td>
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<tr>
<td>OTC</td>
<td>Ornithine transcarbamylase</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PEPCK</td>
<td>Phosphoenolpyruvate carboxykinase</td>
</tr>
<tr>
<td>RCV</td>
<td>Replication competent virus</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RRV</td>
<td>Ross river virus</td>
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<tr>
<td>RRV-G</td>
<td>Ross River virus glycoproteins</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>rtTA</td>
<td>Reverse tetracycline-transactivator</td>
</tr>
<tr>
<td>SAE</td>
<td>Severe adverse event</td>
</tr>
<tr>
<td>SCAP</td>
<td>SREBP cleavage-activating protein</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe combined immunodeficiency disease</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>SIN</td>
<td>Self-inactivating</td>
</tr>
<tr>
<td>SIV</td>
<td>Simian immunodeficiency virus</td>
</tr>
<tr>
<td>SMC</td>
<td>Smooth muscle cell</td>
</tr>
<tr>
<td>SRE</td>
<td>Sterol regulatory element</td>
</tr>
<tr>
<td>SREBP</td>
<td>SRE binding proteins</td>
</tr>
<tr>
<td>SV40</td>
<td>Simian virus 40</td>
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<tr>
<td>SV-F</td>
<td>Sendai virus glycoprotein F</td>
</tr>
<tr>
<td>TBG</td>
<td>Thyroid hormone-binding globulin</td>
</tr>
<tr>
<td>Tc</td>
<td>Tetracycline</td>
</tr>
<tr>
<td>tetO</td>
<td>Tc resistance operator</td>
</tr>
<tr>
<td>tetR</td>
<td>Tc resistance repressor protein</td>
</tr>
<tr>
<td>TG</td>
<td>Triglyceride</td>
</tr>
<tr>
<td>tTA</td>
<td>Tc-regulated transactivator</td>
</tr>
<tr>
<td>tTS</td>
<td>Tc-responsive transcriptional silencer</td>
</tr>
<tr>
<td>TU</td>
<td>Transducing unit</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very low-density lipoprotein</td>
</tr>
<tr>
<td>VLDLR</td>
<td>VLDL receptor</td>
</tr>
<tr>
<td>VSV-G</td>
<td>Vesicular Stomatitis virus G-glycoprotein</td>
</tr>
<tr>
<td>WHHL</td>
<td>Watanabe Heritable Hyperlipidemic</td>
</tr>
<tr>
<td>WPRE</td>
<td>Woodchuck hepatitis virus post transcriptional element</td>
</tr>
</tbody>
</table>
LIST OF ORIGINAL PUBLICATIONS

This study is based on the following original articles, which are referred to in the text body by the corresponding Roman numerals (I – IV):


# TABLE OF CONTENTS

1. INTRODUCTION ...............................................................................................15

2. REVIEW OF THE LITERATURE ..............................................................................17

## 2.1. Cholesterol metabolism .............................................................................17
  2.1.1. LDL-cholesterol and atherosclerosis.................................................... 19

## 2.2. Familial hypercholesterolemia .....................................................................19
  2.2.1. Clinical features ............................................................................... 19
  2.2.2. Phenotypic variation ........................................................................... 20
  2.2.3. Prevalence of FH ............................................................................... 21
  2.2.4. Molecular bases ................................................................................. 21
    2.2.4.1. LDL receptor ......................................................................... 21
    2.2.4.2. Ligands for LDLR................................................................. 22
    2.2.4.3. Receptor-mediated endocytosis of LDL..................................... 24
    2.2.4.4. LDL uptake via LDLR independent pathway............................... 25
    2.2.4.5. Regulation of cellular cholesterol levels.................................... 25
  2.2.5. Gene defects ................................................................................... 26
    2.2.5.1. Gene defects associated with monogenic hypercholesterolemia . 26
    2.2.5.2. Functional defects of LDLR ...................................................... 26
    2.2.5.3. FH in Finland ........................................................................ 27
  2.2.6. Diagnosis and current treatment methods ........................................... 28
  2.2.7. Animal models of FH ........................................................................ 29

## 2.3. Gene therapy ............................................................................................30
  2.3.1. Definition......................................................................................... 30
  2.3.2. Gene transfer vectors ........................................................................ 30
    2.3.2.1. Viral gene transfer vectors ...................................................... 31
      2.3.2.1.1. MLV retroviral vectors.................................................. 31
      2.3.2.1.2. Lentiviral vectors ......................................................... 34
      2.3.2.1.3. AAV vectors................................................................ 37
      2.3.2.1.4. Adenoviral vectors ...................................................... 38
      2.3.2.1.5. Other viral vectors ..................................................... 39
    2.3.2.2. Non-viral gene transfer vectors ............................................... 42
  2.3.3. Development of targeted and regulated gene transfer vectors ............... 43
    2.3.3.1. Targeted vectors .................................................................... 44
      2.3.3.1.1. Targeting cell surface receptors .................................... 44
      2.3.3.1.2. Transcriptional targeting ............................................. 45
      2.3.3.1.3. Gene targeting ........................................................... 46
    2.3.3.2. Transcriptionally regulated vectors ......................................... 47
      2.3.3.2.1. TetON, TetOFF .......................................................... 47
  2.3.4. Gene transfer techniques .................................................................. 49
    2.3.4.1. Vascular gene transfer ........................................................... 49
    2.3.4.2. Liver-directed gene transfer ..................................................... 51
      2.3.4.2.1. Delivery route ................................................................ 51
      2.3.4.2.2. Vectors for liver-directed gene transfer .......................... 52
2.3.5. Apolipoproteins in gene therapy for hyperlipidemia.............................. 53
  2.3.5.1. Apo E .............................................................................. 53
  2.3.5.2. ApoA-I, apoA-I_Milano .................................................. 55
  2.3.5.3. Apobec-1 ..................................................................... 56
2.3.6. Human gene therapy ........................................................................ 56
2.3.7. FH gene therapy ........................................................................ 57
  2.3.7.1. Ex vivo approach .......................................................... 57
  2.3.7.2. In vivo approach ........................................................... 58

3. AIMS OF THE STUDY .............................................................................. 62

4. MATERIALS AND METHODS ................................................................... 63

5. RESULTS AND DISCUSSION .................................................................... 68
  5.1. Article I ...................................................................................... 68
    5.1.1. Adventitial ex vivo gene transfer resulted in efficient gene expression .... 68
    5.1.2. Gene expression was transient ............................................ 69
    5.1.3. Extrahepatic apo E expression resulted in elevated plasma cholesterol levels ... 69
  5.2. Articles II and III .......................................................................... 70
    5.2.1. Liver-specific lentiviral vectors were functional in vitro ............... 70
    5.2.2. Liver-directed gene transfer resulted in a long-term therapeutic effect and stable transgene expression in WHHL rabbits ......................... 71
    5.2.3. Transgene expression was liver-specific ................................ 73
    5.2.4. Liver-directed in vivo gene therapy proved safe ......................... 74
      5.2.4.1. Animal well-being and survival ........................................ 74
      5.2.4.2. Liver function and histology after gene transfer .................. 74
      5.2.4.3. Pathology, SAE ........................................................... 74
      5.2.4.4. Biodistribution ............................................................ 76
      5.2.4.5. Provirus integration and possibility of insertional mutagenesis .... 76
  5.3. Article IV .................................................................................. 77
    5.3.1. Dox-regulated HIV-1 vector system showed tight control of gene expression .... 77
  5.4. Aspects of the future development of gene delivery strategies ............ 78

6. SUMMARY AND CONCLUSIONS .............................................................. 79

7. REFERENCES ...................................................................................... 80

Appendix: Original publications (I–IV)
1. INTRODUCTION

The development of safe, efficient and versatile gene therapy tools and methodology for the treatment of various diseases caused by a single gene defect or more complex multifactorial diseases has been the aspiration of numerous studies ever since the early history of gene therapy. Familial hypercholesterolemia (FH) was among the first monogenic diseases treated with gene therapy. This metabolic disorder is caused by the inherited deficiency of the low density lipoprotein (LDL) –receptor (LDLR); and in the homozygous form is a lethal disorder obstinate to other treatments, such as LDL apheresis and liver transplantation (Goldstein et al., 2002). The first protocol for FH gene therapy, initially utilized in animal models (Wilson et al., 1990; Chowdhury et al., 1991; Raper et al., 1992), and later in humans (Grossman et al., 1994; Grossman et al., 1995), was an ex vivo approach in which recombinant amphotropic retroviral vectors carrying the LDLR gene were used to transduce autologous hepatocytes which were subsequently transplanted back into the liver via the portal circulation. The method was both laborious and time consuming, and resulted in only a modest lipid-lowering effect. Even though further developments have been reported in ex vivo gene transfer methods (Nguyen et al., 2002; Giannini et al., 2003), the direct in vivo approach offers an attractive alternative for liver gene therapy (Ylä-Herttuala and Martin, 2000). In addition to being less invasive, in vivo gene therapy benefits from recent advances in targeted vector development (Lundstrom, 2003).

In vivo targeting to the liver has been performed by injecting DNA or viral vectors into the liver parenchyma (Kuriyama et al., 2000), splenic capsule (Chen et al., 2000), hepatic artery (Raper et al., 2002), or portal vein (Kozarsky et al., 1994; Pakkanen et al., 1999b). In the study of Pakkanen et al. (Pakkanen et al., 1999b) a maximum of 35% reduction in total plasma cholesterol levels was achieved 2 – 3 months after Moloney murine leukaemia virus (MMLV) –based retrovirus-mediated in vivo gene transfer via portal vein when a combination of 10% liver resection and thymidine kinase – ganciclovir treatment was used to stimulate hepatocyte proliferation prior to gene transfer. In contrast to MMLV-retroviruses, lentiviruses are capable of integrating into the chromosomes of non-dividing hepatocytes leading to long-term transgene expression without the need for target cell proliferation induction (Kafri et al., 1997). Hence, they are considered a valuable candidate for liver gene therapy.

In this study we aimed to evaluate the potential of stable gene expression from MMLV-based retroviral vectors and human immunodeficiency -1 (HIV-1) –based and feline immunodeficiency (FIV) –based lentiviral vectors to treat FH in a hypercholesterolemic rabbit model after vascular ex vivo gene transfer of human apolipoprotein (apo) E3 and after in vivo injection of LDLR gene into the liver via the portal vein. To improve safety and to overcome the constraint of non-specific and uncontrolled gene expression, a third-generation self-inactivating (SIN) HIV-1 lentiviral vector containing several enhancer and liver-specific promoter elements was produced for targeted transgene expression, and a doxycycline (Dox)-regulated SIN lentiviral vector system was constructed for regulated expression of the transgene.
Our results suggest that adventitial *ex vivo* gene therapy may be utilized for targeting local vascular phenomena during surgical procedures as well as for systemic delivery of gene products. Our results also indicate that MMLV-retroviral vectors as well as third-generation liver-specific lentiviral vectors have potential for the safe and long-term amending of LDLR deficiency in Watanabe Heritable Hyperlipidemic (WHHL) rabbits, an animal model for human FH. Furthermore, liver-specific lentiviral vectors resulted in efficient gene expression in the liver without the need for liver resection. Dox-regulated lentiviral vectors show potential for improving the safety of *in vivo* gene therapy.
2. REVIEW OF THE LITERATURE

2.1. Cholesterol metabolism

Cholesterol is an important structural component of cell membranes and a precursor molecule for the synthesis of steroid hormones, bile acids and vitamin D (Tabas, 2002). The cellular requirement for cholesterol is satisfied either by de novo synthesis within the cell (Fig. 1) (Liscum, 2002), or by being supplied from extra-cellular sources. Cholesterol accumulating within the cell above the amount capable of being utilized by the cell is esterified with a long-chain fatty acid and stored within the cytoplasm as cholesteryl ester droplets. Efflux of excess cholesterol from the peripheral tissues occurs via reverse cholesterol transport, a pathway necessary to maintain cellular cholesterol homeostasis (Groen et al., 2004).

**Lipoproteins**

Both de novo synthesized cholesterol and cholesterol derived from the diet are transported in the plasma predominantly as cholesteryl esters associated with lipoprotein particles. Lipoprotein particles are spherical with a central core of nonpolar lipids (primarily triglycerides and cholesteryl esters) and a surface monolayer of polar lipids (primarily phospholipids) and noncovalently bound apoproteins (Havel and Kane, 2002). Lipoproteins are classified by the type and ratio of protein and lipids that they contain, which determines their size and density (Table 1).

**Cholesterol absorption from diet: chylomicron pathway**

Dietary triglycerides and cholesterol are transported from the site of absorption (small intestine) to liver and peripheral tissues within chylomicrons (Fielding and Fielding, 2002). In the capillaries of adipose tissue and muscle, lipoprotein lipase catalyzes the hydrolysis of the triglycerides resulting in the formation of chylomicron remnants. Because of lipolysis, the surface of the particles is reorganized to consist primarily of apo B-48, apo E, and the apo Cs. These partially triglyceride-depleted, cholesterol-enriched particles are rapidly and efficiently cleared from the circulation by the liver, through the interaction of apo E with LDLR family (Mahley and Rall, Jr., 2002) (see Chapter 2.2.4.2).
TABLE 1. Composition of the major human plasma lipoproteins

<table>
<thead>
<tr>
<th>Lipoprotein</th>
<th>Source</th>
<th>Apolipoproteins</th>
<th>% Protein</th>
<th>Core lipids (TG/CE)</th>
<th>Diameter (nm)</th>
<th>Density (g/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chylomicrons</td>
<td>Intestine</td>
<td>A (I,II,IV), B-48, C (I,II,III), E</td>
<td>2</td>
<td>86 / 3</td>
<td>75 – 100</td>
<td>&lt;0.95</td>
</tr>
<tr>
<td>VLDL</td>
<td>Liver</td>
<td>B-100, C (I,II,III), E</td>
<td>8</td>
<td>55 / 12</td>
<td>30 – 80</td>
<td>0.93 – 1.006</td>
</tr>
<tr>
<td>IDL</td>
<td>VLDL</td>
<td>B-100, C (I,II,III), E</td>
<td>19</td>
<td>23 / 29</td>
<td>25 – 35</td>
<td>1.006 – 1.019</td>
</tr>
<tr>
<td>LDL</td>
<td>VLDL</td>
<td>B-100</td>
<td>22</td>
<td>6 / 42</td>
<td>18 – 25</td>
<td>1.019 – 1.063</td>
</tr>
</tbody>
</table>

**VLDL and LDL pathway**
Cholesterol synthesized by the liver, or dietary cholesterol reaching the liver via chylomicron pathway, is packaged by hepatocytes into very low density lipoprotein (VLDLs) and secreted into the blood (Fielding and Firlding, 2002). In the circulation VLDL undergoes a lipolytic cascade and is either directly cleared by the liver or converted to progressively smaller and cholesterol-enriched intermediate density lipoproteins (IDLs) and finally LDLs. In humans, about half of the VLDL remnants are eventually converted to LDL. During lipolysis, VLDL remnants become relatively depleted in all protein components except apo B-100, leaving apo B-100 as the exclusive protein component of LDL. LDLs are taken up by cells via LDL receptor-mediated endocytosis (see Chapter 2.2.4.3). The uptake of LDL occurs predominantly in liver (75%), adrenals and adipose tissue.

**Reverse cholesterol transport: HDL pathway**
Reverse cholesterol transport allows excess peripheral cholesterol to be returned to the liver for excretion into the bile (Groen et al., 2004). Lipid-poor apo A-I promotes efflux of cholesterol and phospholipids found in plasma membranes via interaction with the adenosine triphosphate–binding cassette protein A1. The lecithin-cholesterol acyltransferase enzyme converts unesterified cholesterol into cholesteryl ester within high-density lipoproteins (HDLs). Selective uptake of high density lipoprotein (HDL) cholesteryl esters by the liver is mediated by the scavenger receptor class BI. In exchange for triglycerides, the cholesteryl esters can be transferred from HDL to VLDLs and LDLs via the action of HDL-associated cholesteryl ester transfer protein. Ultimately, cholesterol is excreted in the bile as free cholesterol or as bile salts after conversion to bile acids in the liver. From the intestine cholesterol can either be reabsorbed or excreted in the feces. On average, about half of all cholesterol entering the intestine is absorbed, but the fractional absorption rate varies significantly among individuals.
2.1.1. LDL-cholesterol and atherosclerosis

The presence of LDLR and reverse (HDL) cholesterol transport pathways allows for a sensitive regulation of cellular cholesterol content throughout the body. Unfortunately, this regulation is not always flawless. Absorption of excess cholesterol can potentially increase the amount of cholesterol stored in the liver. This, in turn, can result in increased VLDL secretion, and subsequent LDL formation, and also down regulation of hepatic LDLR activity (Turley, 2004). Such events will potentially increase plasma LDL-cholesterol levels. Defects in the cholesterol metabolism can further induce accumulation of cholesterol in the periphery causing cardiovascular disease.

Atherosclerosis is a life-long process that begins with innocuous fatty streaks in childhood (Ylä-Herttuala et al., 1986; Stary, 2000) and progresses through several stages of plaque formation and gradual narrowing of the large arteries to subsequent heart attacks, strokes and peripheral vascular disease (Lusis, 2000). As a major cholesterol-carrying lipoprotein in human plasma, LDL plays a major role in the development of atherosclerosis and coronary heart disease (CHD). The central process in the development of atherosclerosis is the infiltration of atherogenic cholesterol-rich lipoproteins, including LDL and VLDL, into the artery wall where subendothelial macrophages are activated by oxidized LDL particles via the scavenger receptor pathway (Steinberg, 2002). Total cholesterol levels of >6mmol/l in the circulation are associated with substantially increased risk of CHD. According to the current hypotheses, a priming factor in the development of atherosclerotic plaques is endothelial dysfunction due to oxidative stress, inflammation, infectious micro-organisms, or shear stress (Ross, 1999).

Generally atherogenesis is considered a polygenic disease and numerous candidate genes are proposed (Novelli et al., 2003; Chaer et al., 2004). In addition to environmental factors such as hypertension, diabetes, cigarette smoking and obesity, gene mutations affecting any of the metabolic pathways involved in the development of atherosclerosis may contribute to the risk of CHD. Not all cardiovascular diseases are polygenic in nature, and among patients with CHD onset before the age of 55, about 5% of cases are attributable to heterozygous FH.

2.2. Familial hypercholesterolemia

2.2.1. Clinical features

FH was the first genetic disease of lipid metabolism to be clinically and molecularly characterized (Goldstein and Brown, 1974). It is a dominantly inherited autosomal disease characterized by extremely high levels of plasma cholesterol and LDL, which contribute to the formation of cutaneous and tendon xanthomas, arcus corneae and premature cardiovascular disease. Clinically identified FH usually results from defects in the LDLR gene (see Chapter 2.2.4). Homozygous deficient patients with two abnormal LDLR genes, either identical or different mutant genes, typically exhibit life-threatening coronary atherosclerosis and subsequent myocardial infarction before age 30 (Goldstein et al., 2002).
Plasma lipids
Deficiency of LDLR in FH patients results in accelerated cholesterol synthesis in cells and delayed clearance of LDL from the blood circulation (Goldstein et al., 2002). The mean plasma cholesterol level in FH heterozygotes is on average 9mmol/l. However, great biochemical and clinical variability is present among FH heterozygotes, even within a single family. In FH homozygotes the mean value of plasma cholesterol ranges from 15mmol/l to 30mmol/l. The concentration of LDL cholesterol found in homozygotes is about two to three times that found in heterozygotes, and six times higher than in normal subjects whereas HDL cholesterol levels are slightly lower in FH patients than in normal subjects. The mean value of plasma triglycerides in FH heterozygotes or homozygotes is not significantly different from that of the general population.

2.2.2. Phenotypic variation

Mutation heterogeneity of the LDLR (see Chapter 2.2.5.2) causes phenotypic variation in FH homozygotes. The severity of the homozygous disease can be classified according to the amount of functional LDLR activity. In the most severe form, less than 2% of normal receptor activity is detected in the patients’ cultured fibroblasts, coronary deaths are most frequent, and untreated patients rarely survive beyond the second decade of life (Goldstein et al., 2002).

In FH heterozygotes such genotype–phenotype correlation is less clear. In heterozygous patients, clinical expression of coronary disease is influenced by, besides the LDLR gene defect, behavioral, environmental and genetic factors (Jansen et al., 2002). Age, male gender, cigarette smoking, hypertension, western-type diet, severe maternal hypercholesterolemia and infection or inflammation have been associated with increased risk of lethal cardiovascular heart disease in FH heterozygotes (Pimstone et al., 1998; Kontula et al., 1999; Vuorio et al., 2001). While a common polymorphism of apo E (see Chapter 2.2.4.2) accounts for considerable variability in plasma LDL levels and therefore affects the risk of CHD (Eichner et al., 2002), the role of apo E polymorphism as a risk factor of CHD in FH is, to some extent, controversial. Apo E4 phenotype has in some studies been associated with elevated plasma LDL levels (Eto et al., 1988) and lower HDL cholesterol levels (Wiegman et al., 2003b), and apo E2 allele with increased plasma triglyceride levels (Hopkins et al., 1991; Vuorio et al., 1997c) in FH patients. However, many others claim that there is no statistically significant difference in plasma LDL cholesterol levels or coronary disease in the frequency of any of the apo E2, apo E3, or apo E4 alleles between heterozygotes and the control population (Berglund et al., 1993; Ferrieres et al., 1995; Mozas et al., 2003). Further, a high level of lipoprotein (a), an LDL particle bearing an additional apo (a) protein attached by a disulfide bond to the apo B component, has been suggested to be a potential factor affecting the incidence of myocardial infarctions in FH heterozygotes (Seed et al., 1990; Wiklund et al., 1990; Wiegman et al., 2003a). However, contradictory results have also been presented (Mbewu et al., 1991; Carmena et al., 1996; Mozas et al., 2003). Moreover, several other common genetic variables have been associated with the lipid phenotype and the risk of CHD in FH heterozygotes (Tai et al., 2003; Bertolini et al., 2004).
2.2.3. Prevalence of FH

FH is the most common and most severe form of monogenic hypercholesterolemia. In most countries the prevalence of the heterozygous form of FH, in which a defective gene for the LDLR is inherited from one parent and a normal gene from another, is 1:500 and that of the homozygous form of FH is 1:1,000,000 individuals, which renders FH probably the most common disease caused by a single-gene mutation in humans (Goldstein et al., 2002). It has been estimated that worldwide there are 10,000,000 people with FH of whom less than 10% are diagnosed, and less than 25% treated with LDL-lowering drugs (Civeira, 2004). In many cases the diagnosis is missed until a dramatic clinical event occurs.

In a small number of genetically relatively isolated communities the prevalence of heterozygous FH is much higher than in most populations. In these populations, few DLR mutations predominate. Increased prevalence of FH due to the founder effect can be detected in such populations as Ashkenazi Jews, Afrikaans-speaking white South Africans, French Canadians, Lebanese Christian Arabs, Icelanders and Finns (Austin et al., 2004).

2.2.4. Molecular bases

2.2.4.1. LDL receptor

FH is caused by a mutation in the gene coding for the LDLR. The 45kb human LDLR gene contains 18 exons and 17 introns (Südhof et al., 1985) and is mapped to chromosome 19 (Francke et al., 1984) in bands p13.1-13.3 (Lindgren et al., 1985) (Fig. 2). The 5.3kb human LDLR mRNA codes for a ubiquitous 115-kDa transmembrane glycoprotein of 839 amino acids (Yamamoto et al., 1984). The translation of LDLR mRNA into the polypeptide chain for the receptor protein takes place on the surface-bound ribosomes of the rough endoplasmic reticulum (ER) (Hobbs et al., 1990; Goldstein et al., 2002). The precursor-protein with added immature O-linked carbohydrate chains is transported from the rough ER to the Golgi complex, where the O-linked sugar chains are elongated. The signal sequence encoded by exon 1 is cleaved from the protein during translocation into the ER, and about 45 min after synthesis, LDL receptors appear on the cell surface, where they gather in clathrin-coated pits (see Chapter 2.2.4.3).

The LDLR protein consists of five distinguishable domains (Figs. 2 and 3). The 292 amino acid ligand-binding domain at the amino-terminal end of the receptor is assembled from seven imperfect cysteine-rich tandem repeats of the 40 amino acids involved in apo B
and apo E binding. The epidermal growth factor (EGF) precursor homology domain, encoded by exons 7 to 14, is responsible for the acid-dependent dissociation of the receptor from its ligand, its subsequent recycling and also the correct positioning of the ligand-binding domain on the cell surface. It consists of a β-propeller region containing the consensus sequence Tyr-Trp-Thr-Asp flanked with EGF repeats (Jeon et al., 2001). The serine/threonine-linked (O-linked) sugar domain located just outside the plasma membrane serves as an attachment domain for numerous O-linked carbohydrates. The functional importance of this glycosylated domain is unclear; however, one role of glycosylation is thought to be the stabilization of the receptor proteins (Kingsley et al., 1986). The hydrophobic membrane-spanning domain anchors the receptor to the cell surface. The short stretch of 50 amino acids in the cytoplasmic domain is involved in the targeting of the receptor protein to coated pits and the receptor basolateral targeting in the liver.

LDLR sequence and structure are highly conserved. The cDNA sequence of the mouse LDLR (Polvino et al., 1992) and hamster LDLR (Bishop, 1992) shows 78% homology with the human gene. The most conserved domain is the cytoplasmic domain, which shows homologies of up to 90% between humans and other species (Russell et al., 1983; Yamamoto et al., 1986; Lee et al., 1989; Mehta et al., 1991; Hoffer et al., 1993; Hummel et al., 2003).

2.2.4.2. Ligands for LDLR

LDLR belongs to the LDLR gene family, a multifunctional and evolutionarily conserved group of cell-surface receptors, which, in addition to other roles in various cellular processes including signal transduction, mediate the cellular uptake of a diverse spectrum of extra-cellular ligands, including lipoproteins (Schneider and Nimpf, 2003). The primary role of LDLR is to remove cholesterol carrying lipoproteins, particularly LDL arising from the lipolysis of VLDL, from plasma circulation (Brown and Goldstein, 1986). The majority of the LDL is removed from circulation by the liver through the LDLR-mediated endocytosis (see Chapter 2.2.4.3), and, to a lesser extent, via other receptor and non receptor-mediated pathways (see Chapter 2.2.4.4). The LDLR binds with high affinity both apo B, a high molecular mass (550kDa) protein, containing LDL-particles, and apo E containing VLDL, IDL and chylomicron remnant particles (Rudenko and Deisenhofer, 2003). Multiple copies of lipoproteins containing apo E bind to LDLR with up to 20-fold higher affinity than
LDL containing only one copy of apo B (Mahley, 1988). Binding involves Ca\(^{2+}\) -dependent proper folding and disulphide bond formation within the conserved acidic residues of the LDLR ligand binding domain characterized by a tryptophan and a stacking of histidine residues (Prevost and Raussens, 2004). It is suggested that the resulting hydrophobic concave face on the opposite side of the Ca\(^{2+}\) cage interacts with the basic amino acids of the lipoproteins.

Four other mammalian receptors of the LDLR family have been recognized to bind apo B or apo E containing lipoproteins with high affinity. These are the VLDL receptor (VLDLR) (Takahashi et al., 1992), apo E receptor 2 (Kim et al., 1996), the LDLR related protein (LRP) (Beisiegel et al., 1989; Kowal et al., 1989), and megalin (LRP-2) (Willnow et al., 1992; Stefansson et al., 1995).

Apolipoprotein E

Human apo E regulates multiple metabolic pathways and plays an important role in the transportation and redistribution of lipoproteins by mediating the binding of lipoproteins containing apo E, i.e. chylomicron and its remnant, VLDL, IDL and a subclass of HDL, with different families of receptors including the LDLR family and cell-surface heparan sulfate proteoglycans (Mahley and Huang, 1999). The 34kDa apo E protein contains two independently folded functional domains separated by thrombin cleavage (Weisgraber, 1994). The 22-kDa N-terminal domain contains the receptor-binding region. The 10-kDa C-terminal domain is responsible for lipoprotein binding resulting in conformational change and greater positive electrostatic potential (Raussens et al., 2003), which probably explains the prerequisite of lipid association for high affinity binding to the LDLR (Innerarity et al., 1983).

Human apo E gene is composed of 299 amino acids and is located on chromosome 19 (Olaisen et al., 1982; Das et al., 1985). It exists in three common isoforms, apo E2, apo E3, and apo E4, arising from polymorphism of the apo E gene (Zannis and Breslow, 1981; Davignon et al., 1988). This polymorphism has profound effects on the biological functions of apo E and is associated with variations in plasma cholesterol level. The apo E2 allele exhibits defective binding to the LDLR and is associated with type III hyperlipoproteinemia (HLP) (Kypreos et al., 2003), while apo E4 allele is associated with high plasma cholesterol level, with an increased risk for CHD (Chen et al., 2003) and Alzheimer’s disease (Weisgraber and Mahley, 1996).

Apo E is synthesized mainly in the liver hepatocytes and brain, but also in other tissues including the kidney and adrenal glands; and by many different cell types including parenchymal cells, differentiated macrophages, monocytes, astrocytic glial cells, ovarian granulosa cells, smooth muscle cells and keratinocytes (Mahley, 1988). Apo E synthesized locally by monocytes and macrophages in vessels appears to protect against the development of atherosclerosis (Bellosta et al., 1995; Shimano et al., 1995; Fazio et al., 1997; Hasty et al., 1999). This is possibly achieved by the modulation of cholesterol efflux and cholesterol ester hydrolysis (Lin et al., 1999; Langer et al., 2000), the restriction of platelet aggregation (Riddell et al., 1997; Riddell et al., 1999), inhibiting smooth muscle cell (SMC) proliferation (Ishigami et al., 2000), the prevention of oxidation (Miyata and Smith, 1996; Tangirala et al., 2001) and through anti-inflammatory actions (Stannard et al., 2001).
2.2.4.3. Receptor-mediated endocytosis of LDL

The process of LDLR-mediated endocytosis (Fig. 4) involves the recognition and binding of an LDL particle from the extracellular membrane and internalization of the receptor-ligand complex in endocytic vesicles assembled from clathrin-coated pits (Defesche, 2004). Following uncoating of the vesicle, the ligand/receptor complex is transported intracellularly to endosomes where the ligand is released. The acidic environment in the endosome makes it possible for the β-propeller region of the EGF precursor domain to function as an alternate substrate for the ligand-binding domain, thereby promoting the release of pH-regulated LDLR from its lipoprotein ligand (Rudenko et al., 2002). Thereafter, the receptor is rapidly recycled back to the cell surface for several subsequent rounds of receptor-mediated endocytosis, and the ligand, LDL, is delivered to a lysosome and degraded by acid hydrolytic enzymes and proteases. The resulting unesterified cholesterol crosses the lysosomal membrane and enters the cellular compartment (Simons and Ikonen, 2000), where it is used for the synthesis of membranes, steroid hormones, and bile acids, and also as a regulator of intracellular cholesterol homeostasis (see Chapter 2.2.4.5). Excess intracellular cholesterol is re-esterified by acyl-CoA-cholesterol acyltransferase (ACAT), for intracellular storage.

![LDLR synthesis and receptor-mediated endocytosis of LDL](image)

**FIG. 4** LDLR synthesis and receptor-mediated endocytosis of LDL
2.2.4.4. LDL uptake via LDLR independent pathway

The liver takes up approximately 70% of LDL particles, mainly through LDLR–mediated endocytosis. The remainder are cleared by means of low affinity, non-specific mechanisms. The receptor-independent pathway, which is considerably less efficient than the receptor-dependent pathway, is alone responsible for the clearance of LDL in null-allele homozygous FH patients and for clearing about half of plasma LDL in heterozygous patients (Goldstein et al., 2001). In WHHL rabbits the receptor-independent LDL uptake is divided equally between the liver and the extrahepatic tissues (Pittman et al., 1982; Spady et al., 1987). Some of the LDLR-independent clearance of LDL occurs in splenic macrophages, hepatic Kupffer cells, histiocytes of the reticuloendothelial system and other scavenger cells in numerous organs either through receptor-mediated endocytosis (i.e. scavenger receptor, LRP) or by a mechanism resembling phagocytosis (Rhainds and Brissette, 1999; van Berkel et al., 2000; Rhainds and Brissette, 2004).

2.2.4.5. Regulation of cellular cholesterol levels

At the cellular level, de novo cholesterol synthesis and uptake of lipoprotein cholesterol are regulated at multiple steps through a negative feedback mechanism that responds to the free cholesterol derived from the lysosomal hydrolysis of LDL cholesterol esters (Goldstein et al., 2002). First, the elevation in intracellular cholesterol level decreases endogenous cholesterol production by suppressing activity and inducing rapid degradation of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate limiting enzyme in cholesterol biosynthesis (see Fig. 1). Second, cholesterol activates the storage of esterified cholesterol by inducing ACAT. Third, the cholesterol down-regulates LDLR expression, thereby preventing further LDL entry (Fig. 4).

To prevent cytotoxicity due to accumulation of excess cholesterol, liver X receptors and the farnesoid X receptor, together with other members of the nuclear receptor superfamily, promote the storage, transport, and catabolism of sterols and their metabolites (Ory, 2004). These metabolic receptors play a central role in bidirectional flux of cholesterol between the liver and peripheral tissues, and in hepatic excretion of cholesterol, dietary sterols, and sterol metabolites.

SREBP pathway

A family of membrane-bound transcription factors, sterol regulatory element (SRE)–binding proteins (SREBPs) are responsible for the sterol-mediated transcriptional regulation of the HMG-CoA reductase and LDLR genes, as well as of more than 30 other genes involved in the synthesis of cholesterol and fatty acids and their receptor-mediated uptake from plasma lipoproteins (Horton et al., 2003). When cellular cholesterol levels are normal, SREBP and SREBP cleavage-activating protein (SCAP), together with one of a pair of ER retention proteins called insulin-induced gene 1 (INSIG-1) and 2 (INSIG-2), form a macromolecular complex in the ER (Yabe et al., 2002; Yang et al., 2002a). This prevents the exit of SCAP-SREBP complexes from the ER, thereby reducing the ability of SREBPs to activate transcription of target genes. The block in SREBP export is achieved through sterol-induced binding of SCAP to the INSIG’s (Radhakrishnan et al., 2004). When cholesterol level in the ER is lowered, release of INSIG from the complex is stimulated allowing SCAP to escort SREBP to Golgi complex where the NH₂-terminal transcription factor of the
inactive SREBP precursors is released by a two-step proteolytic process. The released sterol-binding element then migrates to the nucleus and activates transcription by binding to nonpalindromic SREs in the promoter/enhancer regions of the cholesterol-regulated target genes.

In addition to binding to SCAP, INSIG’s regulate lipid synthesis by binding in a sterol-dependent fashion to HMG-CoA reductase. Sterol-stimulated binding of INSIG’s to HMG-CoA reductase leads to its ubiquitination and proteosomal degradation (Sever et al., 2003). Unlike the sterol-mediated regulation of HMG-CoA reductase and LDLR, ACAT activity is not regulated by the SREBP pathway. Instead, ACAT activity is allosterically regulated at the post-translational level by membrane cholesterol content in the ER (Chang et al., 1997).

2.2.5. Gene defects

2.2.5.1. Gene defects associated with monogenic hypercholesterolemia

FH is one of the genetically heterogeneous autosomal diseases that are characterized by an elevation of total plasma cholesterol associated with increased LDL particles (Pullinger et al., 2003). In addition to mutations in LDLR, other gene loci have been linked as causing a disorder clinically indistinguishable from FH. The molecular basis of FH is characterized by high allelic heterogeneity. More than 800 molecular defects have been identified in the LDLR (see Chapter 2.2.5.2). Furthermore, few specific mutations in the apo B-100 gene, and in the FH3 genomic locus at 1p34.1–p32 proprotein convertase subtilisin/kexin type 9 gene (Abifadel et al., 2003) that encodes for neural apoptosis regulated convertase, a newly identified human subtilase that contributes to cholesterol homeostasis (Seidah et al., 2003), have been characterized. Mutations in the apo B-100 gene induce familial ligand-defective apo B (FLDB) by failure of LDL binding to its receptor and by secondary plasma LDL-cholesterol elevation (Whitfield et al., 2004). Evidence for a milder biochemical phenotype in patients with FLDB than in patients with FH has been presented (Pimstone et al., 1997). In addition, autosomal recessive forms of hypercholesterolemia have been identified. In autosomal recessive hypercholesterolemia, hepatic LDL degradation is markedly reduced owing to disrupted internalization of LDLR (Wilund et al., 2002). In cholesterol 7α-hydroxylase deficiency the patients are hypertriglyceridemic as well as hypercholesterolemic because of decreased bile acid production (Pullinger et al., 2003). Patients with sitosterolemia show strikingly low rates of cholesterol synthesis but increased absorption of dietary sterols and a defective ability to secrete sterols into the bile, resulting in the accumulation of both animal and plant sterols in the blood and body tissues (Berge, 2003).

2.2.5.2. Functional defects of LDLR

The vast variety of LDLR mutations can either destroy or significantly impair the proper functioning of the receptor. The most frequent mutations, point mutations such as missense, non-sense and splice site mutations, have been described in all of the 18 exons. Deletions and duplications account for ~5% of the LDLR mutations found in a genetically heterogeneous population of FH patients (Austin et al., 2004). Many of the major rearrangements have occurred in recombination between Alu sequences that are more
frequently present in chromosome 19 and the LDLR gene than in the average region of the genome (Venter et al., 2001; Lander et al., 2001) and represent 65% of LDLR intronic sequence (Amsellem et al., 2002).

Five major classes of LDLR mutations have been defined on the basis of their functional consequences (Goldstein et al., 2001). The mutation classes, the nature of the mutations and affected domains are listed in Table 2. Besides the distinct mutation classes, many LDLR alleles are derived from more than a single class. In addition, deletions in the transcriptional regulatory elements in the promoter of the LDLR gene have been identified. These promoter mutations can decrease the LDLR transcriptional activity.

**TABLE 2.** Major LDLR mutation classes based on functional defects

<table>
<thead>
<tr>
<th>Class of mutation</th>
<th>Nature of mutation</th>
<th>Affected domains</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><strong>Null alleles:</strong> failure to produce immunoprecipitable LDLR protein due to deletions in the promoter; production of truncated LDLR mRNA via splicing mutations or large deletions; reduced concentration of mRNA caused by nonsense and frameshift mutations; or accelerated degradation of the receptor protein.</td>
<td>LDLR promoter; all domains</td>
</tr>
<tr>
<td>2</td>
<td><strong>Transport-defective alleles:</strong> abnormal folding of the LDLR protein results in defects in the LDLR precursor transport from ER to Golgi complex.</td>
<td>Ligand binding domain; EGF precursor homology domain</td>
</tr>
<tr>
<td>3</td>
<td><strong>Binding-defective alleles:</strong> LDLR protein is synthesized and transported to the cell surface normally but fails to bind ligand (LDL).</td>
<td>Ligand binding domain; EGF precursor homology domain</td>
</tr>
<tr>
<td>4</td>
<td><strong>Internalization-defective alleles:</strong> LDLR is capable of binding the ligand but is not able to cluster on cell surface coated pits and therefore cannot transport the bound ligand into the cell.</td>
<td>Cytoplasmic domain; Membrane-spanning domain</td>
</tr>
<tr>
<td>5</td>
<td><strong>Recycling-defective alleles:</strong> LDLR is able to bind and internalize the ligand in coated pits, but fails to release the ligand in endosome or recycle back to the cell surface.</td>
<td>EGF precursor homology domain</td>
</tr>
</tbody>
</table>

**2.2.5.3. FH in Finland**

In the genetic isolate of the Finnish population (Peltonen et al., 1999), the majority of FH cases consist of a handful of founder gene mutations that occur rarely elsewhere in the world. Twenty-four different LDLR gene mutations have been identified in Finns to date (Vuorio et al., 2001; [http://www.ucl.ac.uk/fh/](http://www.ucl.ac.uk/fh/)). Two of these mutations, namely FH-Helsinki (Aalto-Setälä et al., 1989) and FH-North Karelia (FH-NK) (Koivisto et al., 1992) are responsible for over 65% of the Finnish FH cases. In the FH-Helsinki mutation, a 9.5kb deletion from intron 15 to exon 18 of the LDLR gene results in defects in receptor-mediated binding and internalization (Class 3/4 phenotype). The FH-Helsinki mutation is
most common in Central and Northern Finland, being responsible for over 50% of the variety of mutations, whereas the FH-NK mutation accounts for nearly 85% of the FH cases in North Karelia and represents the highest local enrichment of a single FH mutation (Vuorio et al., 2001). The ancestors of 18 families carrying the FH-NK mutation, a seven nucleotide deletion in the exon 6 of the LDLR gene resulting in defects in ligand binding (Class 3 phenotype), have been traced to a single couple who lived in the Polvijärvi region in the late 17th century (Vuorio et al., 1997c).

Other common LDLR gene mutations, FH-Turku and FH-Pori point mutations (Koivisto et al., 1995) are responsible for over 5% of FH cases in Finland, while in South-Western Finland they cover almost 25% of the mutation spectrum (Vuorio et al., 2001). A single nucleotide change in the FH-Turku mutation affects the sequence encoding the putative basolateral sorting signal of the LDL receptor protein and results in mistargeting of the mutant receptor to the apical surface in epithelial cells and reduced endocytosis of LDL from the basolateral/sinusoidal surface (Koivisto et al., 2001). FH-Pogosta mutation, a single nucleotide alteration in exon 12, is responsible for 2% of FH cases in Southern Finland and North Karelia (Vuorio et al., 2001). Of the other less common FH mutations in Finland, some represent a mild phenotype with moderately elevated cholesterol levels (Koivisto et al., 1993; Koivisto et al., 1997; Vuorio et al., 1997a).

2.2.6. Diagnosis and current treatment methods

Clinical diagnostic definition of FH is based on criteria for markedly elevated cholesterol levels together with the presence of tendon xanthomata in the patient or first degree relative, and family (or personal) history of premature coronary heart disease or elevated cholesterol (Civeira, 2004). Blood lipid levels, or more specifically LDL cholesterol levels, can also be used for diagnosing heterozygous FH already at birth (Vuorio et al., 1997b). Molecular PCR-based testing is feasible in populations where particular LDLR mutations are frequent; however, in most populations the multitude of LDLR mutations prevent direct DNA-based diagnosis unless a distinct mutation is presumed (Marks et al., 2003).

Strategies for treating patients with FH are directed at lowering the plasma level of LDL (Table 3). Therapeutic lifestyle changes are not sufficiently effective for the treatment of heterozygous or homozygous FH, whereas heterozygous FH patients respond well to statin (HMG-CoA reductase inhibitor) therapy (Rodenburg et al., 2004). However, statin treatment alone often fails to reduce plasma LDL levels sufficiently. Combination therapy of statins together with cholesterol absorption inhibitors or bile acid sequestrants can further reduce plasma LDL cholesterol levels (Civeira, 2004).

LDLR deficient homozygous FH show little response to statins or combination therapy even at high doses (Naoumova et al., 2004). Consequently, drug therapy alone is insufficient treatment for homozygous FH patients. The recommended current treatment method for homozygous FH, as well as for heterozygous patients suffering from CHD who do not sufficiently respond to the primary lipid lowering regimen, is LDL apheresis (Thompson, 2003). However, the procedure is time-consuming and expensive, and because of rapid re-accumulation of the LDL, the procedure must be repeated every 1 – 2 weeks to maintain the lowered LDL cholesterol levels. A more direct approach to correcting the hepatic LDLR deficiency is to transplant a liver that expresses normal levels of LDLR (Naoumova et al., 2004). However, as a major operation, liver transplantation carries a significant morbidity and mortality risk. Moreover, even when the transplantation is
successful, the undesirable co-treatment of lifelong immunosuppressant to prevent organ rejection is necessary. The latest addition to the subordinate repertoire of FH treatment methods is gene therapy (see Chapter 2.3.7).

**TABLE 3.** Treatment strategies for FH

<table>
<thead>
<tr>
<th>Treatment strategy</th>
<th>Mechanism of action</th>
<th>Response to treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy lifestyle</td>
<td>Improved carbohydrate metabolism, lower blood pressure, greater antioxidant protection, inflammatory and thrombo-genic process regulation</td>
<td>++ 18-21% -</td>
</tr>
<tr>
<td>Lipid-lowering drugs</td>
<td>HMG-CoA reductase inhibitors (Statins) block HMG-CoA enzyme and thereby inhibit intracellular cholesterol synthesis, and upregulate normal LDLR allele</td>
<td>+++ 20-58% - / + 5%</td>
</tr>
<tr>
<td></td>
<td>Combination therapy: statins + intestinal cholesterol absorption inhibitors (Ezetimibe, plant stanols); or bile acid sequestrants (Colestevelm) that prevent the absorption of bile acids from the ileum and increase cholesterol catabolism in liver</td>
<td>++++ up to 70% + 20%</td>
</tr>
<tr>
<td>LDL apheresis combined with healthy lifestyle + lipid-lowering drugs</td>
<td>LDL particles are selectively removed from circulation through extracorporeal binding to affinity columns containing anti-apo B antibodies or dextran sulphate; heparin precipitation; or hemoperfusion from whole blood using polyacrylate-coated polyacrylamide beads</td>
<td>NR +++ 50-68%</td>
</tr>
<tr>
<td>Liver transplantation</td>
<td>Liver transplant that express normal levels of LDLR</td>
<td>NR ++++</td>
</tr>
<tr>
<td>Gene therapy</td>
<td>Liver expressing normal LDLR from gene transfer vector</td>
<td>NR to be determined</td>
</tr>
</tbody>
</table>

NR: not recommended

2.2.7. Animal models of FH

WHHL rabbit is an authentic animal model for human FH (Watanabe, 1980) characterized by the lack of functional hepatic LDLR (Schneider et al., 1983). These rabbits exhibit symptoms similar to human FH including severe unprovoked hypercholesterolemia, increased levels of plasma LDL, diffuse atherosclerosis and skin xanthoma (Goldstein et al., 1983). The inborn mutation of the LDLR gene is a 12 nucleotide in-frame deletion eliminating four amino-acids from the ligand binding domain of the LDLR (Yamamoto et al., 1986). This leads to abnormal folding of the protein, which impairs the intracellular transport to the plasma membrane. The mutation also renders the receptor unable to bind LDL (Class 2/3 phenotype, see Table 2); however, the few receptors that reach the cell surface retain the ability to bind VLDL remnants. While LDL clearance is decreased,
synthesis of LDL is increased leading to hypercholesterolemia in WHHL rabbits (Aliev and Burnstock, 1998).

The initial mouse model for hypercholesterolemia was the LDLR knockout (LDLR\textsuperscript{-/-}) mouse created by gene-targeting techniques (Ishibashi \textit{et al.}, 1993). These mice develop only a modest hypercholesterolemia when fed a normal diet, but are very susceptible to dietary modifications. On a high-fat diet the LDLR\textsuperscript{-/-} mice develop atherosclerotic lesions throughout the aorta (Ishibashi \textit{et al.}, 1994; Tangirala \textit{et al.}, 1995). Although used as a phenotype model for FH, LDLR\textsuperscript{-/-} mouse conveys fundamental differences in the lipoprotein metabolism when compared to humans. In the LDLR\textsuperscript{-/-} mouse, as well as in wild-type mice, HDLs predominate in the plasma, whereas the main cholesterol carriers in humans are LDLs. Additionally, in the mouse liver apo B mRNA is extensively edited by apo B mRNA editing polypeptide 1 (apobec-1) generating apo B-48-containing lipoproteins (Greeve \textit{et al.}, 1993), whereas in rabbits, as well as in humans, the liver normally produces only apo B-100 mRNA and fails to edit endogenous hepatic apo B mRNA. Lipoproteins containing the truncated apo B-48 have high levels of apo E and can be cleared efficiently from the plasma with mechanisms other than the LDLR, such as LRP (Martins \textit{et al.}, 2000). The presence of extensive apo B editing in liver is associated with a less atherogenic lipoprotein profile in comparison with animals that have no liver editing activity.

A double knock-out mouse, deficient in both LDLR and the apobec-1 gene (LDLR\textsuperscript{-/-}, apobec\textsuperscript{-/-}), has lipoprotein profiles that mimic human FH with high apo B-100 containing LDL cholesterol levels, and exhibit atherosclerosis by 8-months of age without high-fat diet mirroring the pathophysiology of human FH (Powell-Braxton \textit{et al.}, 1998).

2.3. Gene therapy

2.3.1. Definition

Gene therapy is defined as the delivery of nucleic acids into somatic cells of an individual for the treatment of an inherited or an acquired disease. The primary goals of gene therapy are to correct a patient’s genetic defect via gene repair or gene replacement, or to overexpress proteins that are therapeutically useful, for example, for gene expression regulation, signal transduction, immune system manipulation, or destruction of malignant and other cells (Boulikas, 1998). Gene therapy represents a new, innovative drug delivery system with roots as far back as 1944, when Avery, MacLeod, and McCarthy discovered, that a gene could be transferred within nucleic acids (Avery \textit{et al.}, 2000); and to the prediction of Edward Tatum in 1966 that viruses could be used to transduce genes (Wolff and Lederberg, 1994). Gene therapy technology today makes use of current technical and scientific advances including microbiology, virology, organic chemistry, molecular biology, biochemistry, cell biology, genetics, and genetic engineering. It is a complex process, involving multiple steps from delivery of the gene transfer vector to organs, tissue targeting, cellular trafficking, the regulation of gene expression level and duration, the biological activity of therapeutic protein, and the safety of the vector and gene product.

2.3.2. Gene transfer vectors

Gene transfer vectors are the vehicles used to transfer the gene or genes of interest (transgene) to the target cells, which will then will go on to express the protein(s) encoded
by the transgene(s). The success of gene therapy is fundamentally dependent on the development of a gene transfer vector that can deliver a gene to target cells selectively and efficiently with minimal toxicity. Because gene therapy is implemented in a wide variety of inherited as well as acquired diseases, no one ideal universal vector is available for all gene therapy applications. Numerous viral and nonviral vectors have been designed. Vector designs depend on the size of the transgene, whether long-term or transient expression is required, whether constitutive or inducible activity of vector promoter is needed, whether particular tropism to the target cell population is important, whether repeated administrations of the vector are necessary, and whether vector-mediated target cell killing is of interest. Each gene delivery system has distinct characteristics and preferential applications in therapy.

2.3.2.1. Viral gene transfer vectors

Viruses are intra-cellular parasites, designed through the course of evolution to infect cells, often with great specificity for a particular host and cell type. The experimental technique of viral gene delivery was built on the natural ability of viruses to efficiently introduce and express their nucleic acid in all types of recipient cells, including plant, animal and human. Viruses offer many fundamental advantages for use as gene therapy vehicles, such as specific cell-binding and entry properties, efficient targeting of the transgene to the nucleus of the cell, and the ability to avoid intracellular degradation. Viral-mediated gene transfer involves the engineering of an intact wild-type virus to a recombinant viral particle that will deliver the gene of interest into cells by the process of infection. For example, modifying or deleting genes involved in viral replication from the vector backbone renders the recombinant virus replication defective, a feature most often required from a gene transfer vector. In general, it is considered that the more severely stripped the viral vector is from its wild-type state, the less detrimental the virus for use in gene therapy protocols. Several different viruses have been considered potential vehicles for gene therapy. The viral vectors in laboratory and clinical use are based on murine or human RNA- and DNA-viruses. The most commonly used viral vectors in clinical gene therapy are retroviruses and adenoviruses (Ad) (Thomas et al., 2003; http://www.wiley.co.uk/genmed/clinical/). Other viruses used as gene transfer vectors include adeno-associated virus (AAV); lentiviruses (HIV, FIV, Simian immunodeficiency virus [SIV], Equine infectious anemia virus [EIAV]); foamy virus; baculovirus; Simian virus 40 (SV40); Hepatitis B virus (HBV); herpes viruses (Herpes Simplex virus [HSV]-1, Epstein-Barr virus [EBV]); vaccinia virus; polyomavirus (BK virus); papillomavirus (Bovine papilloma virus-1); and alphaviruses (Semliki Forest virus, Sindbis virus). In addition, a variety of hybrid vectors composed of different viral elements has been developed. Characteristics of some viral gene transfer vectors are summarized in Table 4.

2.3.2.1.1. MLV retroviral vectors

Retroviruses are classified into two categories: simple retroviruses that encode only the Gag, Pol and Env gene products; and complex retroviruses that also encode an array of small regulatory proteins with a range of functions (Goff, 2001). Retroviruses are lipid-enveloped RNA viruses composed of a homodimer of linear, positive-sense, single-stranded RNA genomes of 7-13kb. The viruses reverse-transcribe their RNA genome into
linear double stranded proviral DNA that enters the nucleus, and stably and irreversibly integrates into the chromosomes of the infected cell via the function of viral integrase. This property, the permanent alteration of the characteristics and function of the cell, makes retroviruses suitable vectors for permanent genetic modification of cells. Both simple and complex retroviruses have been utilized for gene transfer vector development. Retroviral vectors currently in use for gene therapy are mostly derived from murine leukemia viruses (MLVs). They were also the first viral vectors to be used in a clinical gene therapy trial (Blaese et al., 1995).

MLVs are classified as simple retroviruses. They assemble at the plasma membrane and contain a central, symmetrically placed, spherical inner core. MLV consists of three structural genes: gag, pol and env, flanked by the viral long terminal repeats (LTR), which are responsible for the regulation and expression of the viral genome (Goff, 2001). Reverse transcription of viral RNA into DNA makes it possible to manipulate the viral genome, since the proviral DNA can be used as the basis for vector construction. The removal of structural genes from the viral vector and the packaging signal from the packaging vector are the basis for the safe and efficient production of the recombinant virus (Pages and Bru, 2004).

In the replication defective retroviral vector construct, the structural genes of the retrovirus are replaced with the therapeutic transgene(s). The transgene is either placed directly under the viral LTR promoter, or under an internal promoter where the gene of interest is expressed from an internal heterologous promoter, or, alternatively, a second transgene is placed under a putative internal ribosomal entry site (IRES) sequence that allows the ribosome to translate the second cistron independently. The advantages of IRES vectors are the possibility of including larger genes and the efficient gene expression of two or more genes (Li and Zhang, 2004). Also the packaging signal (Ψ), essential for viral RNA packaging, is incorporated into the vector construct. The whole vector is then flanked by the viral LTRs that are necessary for the integration of the transgene into the target cell genome.

An important goal for manufacturing retroviral vectors is the production of recombinant viral particles at very high titer. For the viral vector production, the viral structural genes are provided by helper cells or by transient transfection of a packaging construct to packaging cells. After replication defective retroviral vector DNA is introduced into the packaging cells, integrated stably into the host cell DNA and transcribed into genomic retroviral RNA, which is packaged as infectious virus because of to the constitutive expression of the Gag-Pol and Env proteins in these helper cells.

In the early retrovirus packaging cells, only the viral Ψ packaging signal was removed from the integrated helper virus sequence (Mann et al., 1983). In the classical PA317 packaging cell line further modifications were made in order to reduce the risk of recombination between the integrated helper virus genome and the viral vector (Miller and Buttimore, 1986). Since then, numerous packaging cell lines have been designed to minimize the risk of generating replication competent virus (RCV) and to improve retroviral infection (Yu et al., 2003b; Merten, 2004). Furthermore, it has been shown that by producing MLV vector particles in a human cell line, the destruction of viral particles by human serum complement can be avoided (Takeuchi et al., 1994; Cosset et al., 1995). Rendering the retroviral vector self inactivating by introducing a deletion into the 3' LTR's U3 promoter/enhancer sequences of the vector has provided an additional biosafety
feature to retroviral vector technology (Yu et al., 1986). The inactivation of the 3′LTR is copied to the 5′ LTR during reverse transcription leading to improved efficiency of the internal promoter due to the removal of the LTR interference. Furthermore, the integrated proviral genome of the SIN vector is incapable of insertional activation of genes in the host genome because of the lack of a 3′ promoter.

FIG. 5 Production of VSV-G pseudotyped retroviral vector particles.

Infectivity of the virus is mediated by the envelope protein and the host range is determined by its receptor usage (Haynes et al., 2003). Amphotropic retroviruses are capable of infecting cells of murine and other species, including human, origin. In order to broaden the retroviral vector host range and to improve viral titers, envelope proteins of other viruses have been used to participate in the encapsidation of the vector genome and core components in a process referred to as pseudotyping. Specifically, the G-protein of Vesicular Stomatitis virus (VSV-G) has been shown to efficiently form pseudotyped virions with the genome and core components derived from MLV (Emi et al., 1991; Burns et al., 1993). These VSV-G pseudotyped retroviral vectors have a broad host range, increased infection efficiency and improved viral titers owing to protection of the virus particles from shedding during virus preparation and concentration. Because the VSV-G protein as such is cytotoxic to the cells, the VSV-G env gene is provided transiently via transfection of the helper cell (Fig. 5) or by controlling the expression of the gene using inducible expression strategies (Ory et al., 1996). Infectious VSV-G pseudotyped retroviral particles may also be produced by cell-free conversion of immature, noninfectious virus particles assembled in packaging cells by the addition of the VSV-G protein (Abe et al., 1998).
The advantages of standard MMLV retroviral vectors for use in gene therapy, in addition to the stable integration, are their nonimmunogenicity, a broad infectivity range of several target cell types, and the ability to carry reasonably large foreign genes (up to 8kb) (Miller, 1997; Boulikas, 1998). Among the disadvantages of standard MMLV retroviruses as vehicles for human gene therapy are the possibility of insertional mutagenesis in the target cell by random viral integration, the potential for generating RCV during virion packaging, the possibility of recombination between viral-based vectors and human endogenous retroviruses (HERVs), the instability of some retroviral vectors because of the inactivation of viral particles by human serum complement or by the phenomenon of transgene silencing, the lack of integration site specificity, low viral titers, and the inability of the simple retroviruses, derived from MLV, to productively infect non-dividing cells (Hansen and Pedersen, 2002).

2.3.2.1.2. Lentiviral vectors

Lentiviruses are complex retroviruses characterized by unique virion morphology with cylindrical or conical cores (Goff, 2001). They possess a complex genome composed of the structural genes common to all retroviruses. Matrix, capsid and nucleocapsid structural proteins are encoded by gag. pol encodes protease, reverse transcriptase, and integrase that are required for viral replication. env encodes for surface and transmembrane envelope glycoproteins. In addition, lentiviral genome encodes a plethora of regulatory and accessory genes involved in modulation of viral gene expression (tat, rev), assembly of viral particles (vif) and structural and functional alterations in the infected cell ( nef, vpr, vpu) (Fig. 6). (Freed and Martin, 2001)

**FIG. 6** HIV-1 and FIV proviral genome organization.

Lentiviruses are unique among retroviruses because of their ability to infect target cells independently of their proliferation status. They rely on active transport of the preintegration complex through the nucleopore by the nuclear import machinery of the target cell, which enables them to infect both dividing and non-dividing cells (Bukrinsky et al., 1992; Lewis et al., 1992; Gallay et al., 1996). Furthermore, specific virus-encoded
proteins are responsible for lentivirus integration in the absence of cellular mitosis (Greene and Peterlin, 2002). This feature makes them a valuable tool for gene therapy applications in which the target cells are non-dividing, such as hepatocytes in liver gene therapy, or terminally differentiated cell populations, such as neuronal tissue, hematopoietic cells, myofibres etc (Trono, 2000). Thus, lentiviral vectors offer potential for treatment of a wide variety of syndromes including genetic metabolic deficiencies (adenosine deaminase [ADA] deficiency, cystic fibrosis, hematopoietic disorders, Gaucher’s disease, FH) and acquired diseases (HIV infection, Parkinson’s disease, cardiovascular disease, cancer).

HIV-1, the causative agent of acquired immunodeficiency syndrome (AIDS), has been the most characterized lentivirus and the first lentiviral vector systems were based on this virus. As an alternative to the use of HIV-1 –derived vectors, other members of the lentivirus family have been used to generate vectors. These include HIV-2 (D’Costa et al., 2001), SIV (Kim et al., 2001), EIAV (O’Rourke et al., 2002), FIV (Sauter and Gasmi, 2001), and bovine immunodeficiency virus (Matukonis et al., 2002). These viral vectors may provide an added level of safety for human gene therapy. Furthermore, the non-human lentiviruses provide a tool for studying vector biosafety in the natural host.

HIV vectors

Lentiviral vectors are engineered to be defective for replication, so that only the early steps of the lentivirus life cycle (i.e. attachment, entry, reverse transcription, nuclear transport and integration) encoded by a fraction of parental lentiviral genome need be maintained in a lentivirus vector. In the case of HIV-1 –based vectors, new minimal packaging constructs, with viral packaging genes located on two or three separate plasmids and only the transgene located on the lentiviral vector, have been generated to increase viral biosafety (Fig. 7) (Kafri, 2001). In the third generation HIV-1 –based lentiviral vector, sequences coding for viral accessory proteins (vif, vpr, vpu, nef), critical only for the pathogenesis of the virus, have been deleted (Dull et al., 1998; Kim et al., 1998). The third generation packaging constructs express the HIV-1 gag and pol genes in the presence of the Rev protein, which is provided in trans by a separate non-overlapping construct. A strong constitutive promoter upstream of the vector transcriptional start site can replace the function of the regulatory protein Tat without reducing transduction efficiency of the vector.

Fig. 7 The third-generation SIN lentiviral vector system. A) Lentivirus vector construct; B-D) packaging constructs expressing gag, pol and RRE, rev, and VSV-G envelope protein.
These features result in an important biosafety gain because any RCV that could be generated during vector manufacturing would lack all factors essential for HIV-1 replication and virulence in vivo. Recently, a novel packaging system, the trans-lentiviral vector, has split the gag-pol component of the packaging construct into two separate parts thus further curtailting the possibility of RCV formation and retrovirus DNA mobilization for which functional gag-pol is absolutely required (Wu et al., 2000).

In the third generation lentiviral packaging system, the viral envelope, like all lentiviral proteins necessary for virus production, is supplied in trans by a separate plasmid (Fig. 7). This is necessary in order to evade the restricted host range of the HIV envelope. As with the retroviral vectors, VSV-G is the most often used envelope protein in lentiviral vectors. In addition, lentiviral vectors can be pseudotyped with numerous other viral surface glycoproteins, some of which may modulate the physicochemical properties of the vectors, their interaction with the host immune system as well as their host range (Verhoeven and Cosset, 2004).

For the assembly and production of recombinant virus particles most of the lentiviral vector studies have used transient transfection protocol in human derived 293T cells. A number of different transfection protocols have been utilized since they provide flexibility in the type of lentivirus produced, including the choice of the envelope pseudotype, accessory proteins and also expression cassettes (Karolewski et al., 2003). However, the transient production protocol is not easily standardized, and the product may be contaminated from cellular debris. In an attempt to overcome the drawbacks of the transient system, stable producer cell lines have been generated. However, many of the proteins required to assemble lentiviral vectors are toxic to cells when overexpressed, including VSV-G (Burns et al., 1993), protease (Konvalinka et al., 1995), Rev (Miyazaki et al., 1995), Tat (Li et al., 1995a), and Vpr (Bartz et al., 1996). Consequently cell lines expressing the toxic components under an inducible promoter have been generated (Yu et al., 1996; Kafri et al., 1999; Klages et al., 2000; Pacchia et al., 2001). The most recent, the second generation packaging cell line (Xu et al., 2001) is constructed with a minimal set of HIV-derived genetic information and a tetracycline (Tc) -dependent system to control expression of packaging and envelope functions. The viral titers from this packaging cell line were the equivalent of the vector produced by transient transfection, and their transducing activity was indistinguishable from, or superior to, that of this vector.

A further improvement in the lentiviral vector system is an SIN vector. The SIN vectors contain a deletion in the 3' LTR that results in transcriptional inactivation of the upstream 5' LTR, thus diminishing substantially the risk of vector mobilization and recombination (Miyoshi et al., 1998; Zufferey et al., 1998; Bukovsky et al., 1999). In addition, the inclusion of the upstream central polypurine tract (cpp) element and the Woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) has improved the lentiviral vector system. The cpp allows internal initiation of second strand DNA synthesis and enhances the transport of the pre-integration complex to the nucleus (Follenzi et al., 2000; Zennou et al., 2000). The WPRE acts at the post-transcriptional level by promoting the nuclear export of transcripts and/or by increasing the efficiency of polyadenylation of the nascent transcript, thus increasing the total amount of mRNA in cells (Zufferey et al., 1999).

The most prominent limitation in the clinical use of HIV-based vectors is the biosafety consideration of introducing pathogen-derived sequences into humans (Debyser, 2003).
There is a possibility that a replication-competent virus might be generated through recombination with the packaging construct during vector production, or with HERVs in the target cell. Rescue of the integrated vector by a wild type helper virus resulting in vector spread is also a possibility. Indeed, development of highly specific and sensitive assays to detect RCV HIV in vector preparations is important. Furthermore, the possibility that vector preparations might be contaminated with HIV or a new virus which has incorporated an exogenous envelope, such as VSVG \textit{in vivo} is of concern. In addition, the lack of an indisputable animal model for the human pathogen derived vector brings a challenge into the HIV-1 based vector development.

**FIV Vectors**

FIV, a distant relative of primate lentiviruses (Olmsted \textit{et al.}, 1989; Talbott \textit{et al.}, 1989; Bachmann \textit{et al.}, 1997), is an appealing candidate for gene therapy as an alternative to the human pathogen, HIV-1, since neither seroconversion nor other detectable evidence of human infection has occurred (Lutz, 1990; Nowotny \textit{et al.}, 1995). FIV infects diverse Felidae, including the domestic cat and free-ranging populations of great cats worldwide (Olmsted \textit{et al.}, 1992). The infection and disease resembles HIV infection and AIDS; however, the speed of disease progression and overall mortality after FIV infection are not as rigorous as with HIV (Pedersen, 1993). The simple genome organization, which in addition to \textit{gag}, \textit{pol} and \textit{env} contains only three rather than six non-structural genes (\textit{vif, rev, orf-2}) (see Fig. 6), facilitates development of gene transfer vectors that can accommodate up to 8kb transgenes (Sauter and Gasmi, 2001).

After the development and \textit{in vitro} analysis of the first FIV –based VSV-G pseudotyped vectors (Poeschla \textit{et al.}, 1998) further improvements, following the general state-of-the-art design of HIV lentiviral vectors have been described with FIV vector systems (Johnston \textit{et al.}, 1999; Curran \textit{et al.}, 2000; Loewen \textit{et al.}, 2002). Furthermore, these FIV vector systems have been utilized for successful \textit{in vivo} applications targeting cells in the brain, liver, muscle, pancreas, eye, airway and hematopoietic system (Saenz and Poeschla, 2004). The current FIV vector systems consisting of the gene transfer vector with minimal gag region, and one or two packaging components with an envelope glycoprotein of VSV (Song \textit{et al.}, 2003) or Ross river virus (RRV) (Kang \textit{et al.}, 2002) are replication-incompetent and limited to a single round of infection process in the target cell without spreading (Sauter \textit{et al.}, 2003).

**2.3.2.1.3. AAV vectors**

AAV is a small, non-enveloped, single-stranded DNA dependovirus which belongs to the family of paroviridae. It requires a helper virus, preferably Ad or herpesvirus, or, alternatively, human papilloma virus or vaccinia virus to facilitate efficient, fully permissive AAV infection and replication (Bloom and Young, 2001). In the absence of helper virus, AAV establishes a latent infection and maintains itself as a latent provirus integrated into a unique site of human chromosome 19. AAV is able to efficiently infect a wide variety of cell types and also to infect both mitotic and post-mitotic, quiescent cells, through heparin sulphate proteoglycans and integrins (Summerford \textit{et al.}, 1999). AAV has not been associated with any human disease which substantially overcomes the safety concerns encountered with other vector systems.
Recombinant AAV vectors exhibit a broad host range and infect a variety of tissues and cells including liver (Snyder et al., 1997; Grimm et al., 2003; Harding et al., 2004), and vasculature (White et al., 2004; Gruchala et al., 2004a). The relatively simple genome of AAV can be easily manipulated to generate recombinant AAV vectors that lack all wild type coding sequences (Pfeifer and Verma, 2001) and therefore offer a reduced risk of undesirable host immune responses. The only cis-acting elements necessary for efficient encapsidation and integration are the inverted terminal repeats.

Up to 5kb (ideally between 4.1 – 4.9kb) expression cassettes can be accommodated by recombinant AAV particles (Dong et al., 1996), an obvious disadvantage of AAV vectors for wide spectrum of gene therapy applications. However, strategies have been developed to expand the transgene size limitation of AAV vectors. In a trans-splicing AAV vector system the transgene expression is driven from two independent AAV vectors (Duan et al., 2000; Nakai et al., 2000; Sun et al., 2000). Another approach to increase the packaging capacity of AAV vectors has been the generation of AAV-Ad (Goncalves et al., 2004) and AAV-HSV (Johnston et al., 1997) hybrid vectors which take advantage of the large cloning capacity of Ad or HSV.

Of the several serotypes of primate AAV (AAV-1 – AAV-8), AAV-2 is the best characterized and was both the first to be used (McLaughlin et al., 1988; Samulski et al., 1989), and today also the most commonly used in gene transfer vector technology. However, antibodies to AAV-2 are highly prevalent in humans. Cross-packaging AAV-2 particles with the capsids of other AAV serotypes, structurally and functionally different from AAV-2, has resulted in the escape of the anti-AAV-2 immune response (Grimm and Kay, 2003). The use of mutant AAV capsids has been shown to overcome major neutralizing effects of human AAV antisera (Buning et al., 2003). Recombinant AAV particles can also be derived from other AAV serotypes which may display some unique targeting capacities. For example vectors derived from the AAV serotype 8 can transduce the murine liver 100-fold more efficiently than AAV-2 viral vectors (Gao et al., 2002; Sarkar et al., 2004).

After infection the wild type AAV integrates into the human chromosome 19 (Huser et al., 2002), whereas the recombinant AAV has lost the site-specificity of integration because of the deletion of the rep gene in AAV vectors (Rutledge and Russell, 1997; Ponnazhagan et al., 1997). Random integration of AAV vector sequences is considered a risk involved with AAV-mediated gene therapy. However, episomal concatamers have been shown to predominate in vivo (Duan et al., 1998; Duan et al., 1999; Nakai et al., 1999) thus reducing the risk of insertional mutagenesis and activation of oncogenes (Tenenbaum et al., 2003). Some attempts have been made to restore the site-specific integration pattern of the AAV vector system by featuring the rep gene (Satoh et al., 2000; Huttner et al., 2003). Also, AAV-Ad, AAV-baculovirus and AAV-HSV hybrid vectors have been engineered to mimic the site-specific integration wild type AAV (Palombo et al., 1998; Recchia et al., 1999; Heister et al., 2002; Bakowska et al., 2003).

2.3.2.1.4. Adenoviral vectors

Human Ads belong to the family of adenoviridae and are associated with mild infections including upper and lower respiratory tract infections, epidemic conjunctivitis and infantile gastroenteritis in humans. They are non-enveloped, icosahedral double-stranded non-integrating DNA-viruses that remain as an extrachromosomal entity in the nucleus of the
host cell and are lost during cell division. To date, over 50 human Ad serotypes have been identified and classified into six subgroups, A to F, based on the percentage of guanine and cytosine in the DNA molecules and the ability to agglutinate red blood cells (Horwitz, 2001). Ad serotype 2 (Ad2) and Ad5, members of the C subgroup, are the most common serotypes to which most adults have been exposed. They are also the major Ad vectors currently used in clinical applications. However, other serotypes of human Ad as well as non-human Ads have been exploited for vector development in an attempt to avoid potential problems related to pre-existing immunity (Morral et al., 1999; Parks et al., 1999; Moffatt et al., 2000).

Replication-defective recombinant Ad-based vectors possess a very efficient nuclear entry mechanism which makes them attractive and widely used gene deliver vehicles for transduction of different cell types including quiescent, differentiated cells. The transduction efficiency of Ads is very high in almost all cell types compared with that of other viral vectors used for ex vivo or in vivo gene therapy. Ads are the second most commonly used viral vectors in gene therapy clinical trials today (Thomas et al., 2003; http://www.wiley.co.uk/genmed/clinical/). They exhibit low pathogenicity for humans, can be produced to high titers (10^{11} particles / ml), have a packaging capacity of over 30kb, and do not integrate into the host cell genome (Pfeifer and Verma, 2001).

A major disadvantage of using Ad as a gene transfer vector in vivo is the immune response, either an early innate inflammatory response or a later acquired immune response, induced against the viral vector, vector encoded proteins and infected cells resulting in the elimination of vector-transduced cells and inflammation (Tripathy et al., 1996; Kafri et al., 1998). Also, the length of the transgene expression is transient, a feature desirable for some applications such as cancer gene therapy; however, treatment of hereditary genetic disorders requiring long-term transgene expression is not efficiently achievable with the current Ad vectors. Furthermore, the high-titer recombinant Ads are potentially toxic, as exemplified in the death of an 18-year old patient with partial deficiency of ornithine transcarbamylase (OTC), after systemic inflammatory response in vascular endothelium and lung tissue leading to intravascular coagulation, acute pulmonary complications and multiorgan failure directly associated with a very high dose of second generation Ad vector administration (3.8 × 10^{13} particles) (NIH Report, 2002; Raper et al., 2003).

Recent advances in adenoviral vector development, including the high capacity or “gutless” Ad vector devoid of all coding viral genes (Parks et al., 1996; Kochanek et al., 2001), have substantially enhanced the properties of Ad vectors. Progress has been made on issues of immunogenicity and toxicity in vivo, long-term expression of the transgene, target cell specificity and tropism modification (Schagen et al., 2004). Several different generations of non-replicative and replicative Ad vectors are available today each of which will be most appropriate for certain applications (Volpers and Kochanek, 2004).

2.3.2.1.5. Other viral vectors

In addition to the most commonly used viral vectors described above, other viruses with specific features have been exploited for a variety of gene therapy purposes. SV40 based vectors can accommodate up to 4.7kb of exogenous DNA and are capable of integrating into both dividing and non-dividing cells thus exhibiting long-term transgene expression
(Strayer et al., 2002). They have been utilized in applications including gene therapy for inherited hepatic metabolic defect (Sauter et al., 2000) and gene therapy for HIV replication (Cordelier and Strayer, 2003). HBV-based vectors have been used for hepatocyte specific gene transfer because of their natural targeting to hepatocytes, efficiency of infection of quiescent hepatocytes, and the hepatocyte-specific promoter and enhancer elements they encompass (Klocker et al., 2003). HBV vectors lacking all viral protein expressing genes have been produced to high titer; however, the packaging capacity of the HBV vector is rather low, possibly only up to 1.6 kilobases (Untergasser and Protzer, 2004).

Recombinant baculoviral vectors have a substantial cloning capacity, and when controlled by a promoter active in mammalian cells, efficiently deliver and express transgenes in numerous cell types of human, primate, and rodent origin in vitro and in vivo (Ghosh et al., 2002), with a strong preference for hepatocytes of different origin, specifically human hepatocytes (Sandig et al., 1996a). However, baculovirus-mediated gene transfer is strongly reduced in the presence of serum complement, resulting in poor in vivo gene transfer potential (Hofmann et al., 1998). By employing baculoviral vectors in tissues with minimal complement expression, such as periventricular and central nervous system, efficient transduction can be achieved (Airenne et al., 2000; Lehtolainen et al., 2002). The recently developed complement-resistant baculovirus has been shown to enhance gene transfer in the livers of complement sufficient neonatal rats (Huser et al., 2001).

Foamy viruses constitute a subfamily of complex retroviruses, Spumaretrovirinae, (Goff, 2001) with a distinct replication pathway in which the virus reverse transcribes its genome into double stranded DNA before budding into the virus-producing cell (Delelis et al., 2003). Viral vectors derived from foamy viruses transduce efficiently non-dividing cells and so, are considered to be an alternative to lentiviral vectors for gene therapy applications. Foamy viral vectors can be concentrated by ultracentrifugation (Vassilopoulos et al., 2001), have broad tropism (Hill et al., 1999) and have been shown to transduce non-dividing cells more efficiently than oncoretroviral vectors (Russell and Miller, 1996), and at least as efficiently as VSV-G pseudotyped HIV-1 based lentiviral vectors (Leurs et al., 2003). Furthermore, resistance to human serum complement inactivation, and a high packaging capacity make them appealing alternatives for use in human gene therapy. Several versions of human foamy virus and simian foamy virus type 1-based vectors have been designed (Mergia and Heinkelein, 2003). They feature a three plasmid packaging system that split gag, pol, and env onto separate expression cassettes for minimizing the potential for replication-competent virus production by recombination.

The large capacity HSV-1 vectors have been employed in several applications, including gene transfer protocols that target neuronal tissue, such as the treatment of Parkinson’s disease, malignant gliomas and cerebral ischaemia (Goins et al., 2004). HSV-1 vectors efficiently transduce a variety of cell types and maintain as an extra-chromosomal DNA element in the nucleus of host cells establishing a long-lived asymtomatic infection in the sensory neurons of the peripheral nervous system and in some central nervous tissue. Another member of the herpesviridae, the EBV with a double stranded DNA genome has been engineered to express large DNA fragments. Since EBV establishes a latent state as an extra-chromosomal circular plasmid in the host nucleus it is suitable for long-term retention in the target cell. Like EBV, other episomal virus-derived
vectors such as polyomaviruses and papillomaviruses do not integrate into the host genome and thus avoid the possibility of transformation features that may result in secondary cancers (Van Craenenbroeck et al., 2000).

Alphaviruses, such as Semliki forest virus and Sindbis virus with their single-stranded RNA genome, can mediate efficient cytoplasmic gene expression in a variety of mammalian cells. Alphavirus vectors have shown promise for the safe tumor-killing and tumor-specific immune responses in gene therapy studies of cancer (Yamanaka, 2004). Also the non-integrating Vaccinia virus vectors have been developed for cancer gene therapy (Zeh and Bartlett, 2002).

**TABLE 4.** Characteristics of some viral gene transfer vectors

<table>
<thead>
<tr>
<th>Vector</th>
<th>Advantages</th>
<th>Limitations, SAE</th>
</tr>
</thead>
<tbody>
<tr>
<td>MLV</td>
<td>Stable integration, stable expression Low immunogenicity Broad tropism ≤8kb transgene capacity</td>
<td>Unable to transduce quiescent cells Low viral titers, low in vivo efficiency Inactivation by serum complement, transgene silencing Possibility of insertional mutagenesis Potential for pathogenic vector mutation</td>
</tr>
<tr>
<td>Lentivirus</td>
<td>Stable integration into quiescent cells Stable expression Low immunogenicity ≤8kb transgene capacity Wild-type virus (HIV-1) thoroughly characterized</td>
<td>Transient production protocol Lack of an indisputable animal model Possibility of insertional mutagenesis Potential for pathogenic vector mutation Potential for rescue of integrated vector</td>
</tr>
<tr>
<td>AAV</td>
<td>Transduce quiescent and dividing cells Stable / prolonged transgene expression Non pathogenic, low immunogenicity Broad tropism Simple genome easily manipulated</td>
<td>Small transgene capacity (&lt;4.7kb) Possibility of insertional mutagenesis</td>
</tr>
<tr>
<td>Ad</td>
<td>Transduce quiescent and dividing cells High efficiency Broad host range &gt;30kb transgene capacity Easy to produce in high titers</td>
<td>Transient expression Unable to integrate into host cell genome Repeated administration impossible Immunologic and inflammatory responses</td>
</tr>
<tr>
<td>Foamy virus</td>
<td>Transduce efficiently quiescent cells Resistant to inactivation by serum complement, broad tropism High transgene capacity ≥9kb</td>
<td>Low viral titer Not thoroughly characterized</td>
</tr>
<tr>
<td>SV40</td>
<td>Stable integration into dividing and non-dividing cells Long-term transgene expression</td>
<td>Small transgene capacity ≤4.7kb</td>
</tr>
<tr>
<td>Baculovirus</td>
<td>Strong preference for hepatocytes High transgene capacity Easy production Non-pathogenic</td>
<td>Transient expression Inactivation by serum complement</td>
</tr>
</tbody>
</table>
Since none of the currently available viral vectors can mediate targeted and efficient long-term gene transfer with no detrimental immune reactions, chimeric viral vectors featuring different viral and cellular elements are being developed (Lam and Breakefield, 2000; Mitani and Kubo, 2002). These hybrid vectors have potential to improve transduction efficiencies, alter the host range and entry specificity of virions, control the fate of transgenes in the host cells (integrating vs. non-integrating), and also regulate transgene expression.

2.3.2.2. Non-viral gene transfer vectors

Non-viral vectors have been generated to surmount some of the drawbacks associated with viral gene transfer vectors. Non-viral vectors are easy and less costly to manufacture in substantial quantities, more flexible in respect of transgene size and less immunogenic than their viral counterparts. Non-viral episomal vectors also avoid the potential risk of germline transmission or of random integration causing a deleterious or mutagenic effect. However, low transfection efficiency and transient transgene expression, the major disadvantages of using non-viral carriers, have hampered their clinical use (Schmidt-Wolf and Schmidt-Wolf, 2003).

Naked DNA

The simplest approach to non-viral gene transfer is the delivery of naked plasmid DNA into the target organ or the systemic circulation. However, in systemic circulation naked DNA is rapidly cleared by the liver, or degraded by restriction nucleases. Interestingly, liver parenchymal cells have been suggested to internalize naked DNA via receptor-mediated endocytosis (Budker et al., 2000). A number of tissues, including muscle, skin and tumors, are susceptible to naked DNA-mediated gene transfer. Gene transfer efficiency of naked DNA can be enhanced by several physical approaches, including physical pressure, electroporation, gene gun and ultrasound. These methods allow direct penetration of DNA through the cell membrane, bypassing the endosome/lysosome compartment to avoid enzymatic degradation, and in some cases delivery of DNA directly to the nucleus. While fairly inefficient as an in vivo gene transfer vehicle for therapeutic transgene expression, naked DNA has been exploited as an immunotherapeutic vaccine to establish antiviral or antitumor immunity (Liu, 2003).

Lipoplexes

For intravenous injection of plasmid DNA a carrier vector is necessary. A number of synthetic vectors primarily based on cationic lipids and –polymers have been developed. Cationic lipid-DNA complexes, lipoplexes, are easily manipulated and have the potential to deliver large polynucleotides into somatic cells (Tranchant et al., 2004). They form electrostatic complexes with the negatively charged DNA and can be taken up by cells. Lipoplexes have a limited capacity for systemic application because of their rapid clearance from plasma, accumulation in lung tissue and toxic side effects. Incorporation of PEG lipids into the lipoplex increases their circulation time. Direct in vivo application of lipoplex particles to the site of pathology has resulted in clinical response in human gene therapy trials for the treatment of cancer (Hortobagyi et al., 2001; Stopeck et al., 2001), cystic fibrosis (Hyde et al., 2000) and restenosis and myocardial and lower limb ischemia (Laitinen et al., 2000; Mäkinen et al., 2002).
**Polyplex and lipopolyplex systems**

Polyplex systems consist of complexes formed by nucleic acid and a cationic polymer such as polyethylenimine, poly-L-lysine and cationic peptides (Brown et al., 2001). Compared to cationic lipids, cationic polymers are more efficient in condensing DNA and consequently in protecting DNA from nuclease degradation. Small size has also been associated with increased nuclear localization and transgene expression. The accessible free amino groups on these agents facilitate conjugation of a variety of targeting ligands such as galactose, asialorosomucoid, transferrin and monoclonal antibodies to enhance the transfection efficiency of the complex in specific cell types. To aid the escape of the DNA-cationic carrier complex from the endosome the polycation gene delivery systems are usually coupled with synthetic peptides mimicking viral fusogenic peptides, pH-responsive histidine-based systems, polycations with intrinsic endosomolytic activity based on a buffering effect, and polyanions mimicking the anionic amphiphilic peptides (Cho et al., 2003).

Lipopolyplex systems are hybrid complexes prepared by condensing DNA with a polycation and entrapping this polyplex within anionic or neutral liposomes (Brown et al., 2001). The method provides better protection from endonucleases and results in less toxic gene transfer when compared with cationic liposomes alone. Hybrid vectors that combine the merits of viral and non-viral gene transfer vectors have been generated to augment nuclear delivery and enhance transgene expression (Niidome and Huang, 2002; Schmidt-Wolf and Schmidt-Wolf, 2003).

2.3.3. Development of targeted and regulated gene transfer vectors

Viruses have adapted to overcome the barriers of strong containment of the genome, the natural barrier which all life has evolved to maintain the integrity of genetic information. For this reason, viruses appear to be the ideal natural gene delivery vehicles. Indeed, numerous viral features can be utilized for gene therapy purposes; however, in order to comply with the requirement of safe and efficient gene therapy, a number of factors must be taken into account while developing optimal viral vectors.

First, the choice of parental virus must be considered taking into account potential viral pathogenicity, toxicity and other factors hazardous to humans. The possibility of replication-competent virus formation should be eliminated, and also the possibility of recombination between viral-based vectors and human endogenous viruses should be minimized. The inclusion of selectable markers into the vector backbone should be evaluated because of their potential immunogenicity (Riddell et al., 1996). The vector infectivity and duration of transgene expression in the target tissue in each specific application should be considered. Where pertinent, features enabling controlled evasion of host defense systems such as the serum complement system and immune responses (against viral vector, proteins encoded by the vector, and the transduced cells) would be of interest for incorporation into the vector system. Targeting and specificity of the vector to desired tissues or organs should be contemplated in order to avoid unpredictable side effects due to ectopic expression of the transgene in normal tissues. The regulation and control of the transgene expression post-cell transduction should be considered in order to enable transgene activation when necessary for the maintenance of transgene expression within a therapeutic window, and for possibility of transgene silencing if that becomes
necessary. Many of these features have been meticulously studied in a number of viral vector systems.

### 2.3.3.1. Targeted vectors

The general applicability and safety of gene therapy depends on the efficiency of the gene transfer in directing a therapeutic gene specifically to a target tissue and/or cell. Furthermore, targeting the transgene to a specific locus in the host genome by homologous recombination would be of interest when correcting a genetic defect by gene therapy.

#### 2.3.3.1.1. Targeting cell surface receptors

In order to redirect the viral host range and achieve specific binding of viral particles to the target cells *in vivo*, chimeric viral envelopes or capsids have been engineered. Natural ligands of surface molecules, antibody derivatives, and single-chain antibodies for the extra cellular domain of cell surface receptors, have been inserted into the receptor binding envelope or capsid subunit of the vector (Girod et al., 1999; Nguyen et al., 2003; Ráty et al., 2004; White et al., 2004). However, the infection efficiency of such chimera has been poor, limiting their feasibility for clinical use (Zhao et al., 1999). Bridging the virus vector and the target cell by antibodies or ligands is another approach (Boerger et al., 1999; Korokhov et al., 2003). Further, the choice of the viral serotype has an effect on the tissue specificity of AAV vector (Buning et al., 2003), whereas, the choice of viral envelope has its impact on tissue specificity among retro- and lentiviral vectors (Kang et al., 2002; MacKenzie et al., 2002; Sung and Lai, 2002; Kahl et al., 2004). Indeed, viral envelope proteins derived from HBV (MLV vector) (Sung and Lai, 2002), and RRV (FIV vector) (Kang et al., 2002) have proven potential for liver-specific targeting.

**Liver-targeted receptor-mediated endocytosis**

The asialoglycoprotein (ASGP) receptor, a high affinity cell-surface receptor exclusively expressed in hepatocytes, and ASGP receptor-mediated endocytosis have been widely used as a liver-targeting system in combination with non-viral vectors (Fukuma et al., 2000; Arangoa et al., 2003). The ASGP receptor-mediated endocytosis pathway was further exploited when the MMLV vector was pseudotyped with Sendai virus glycoprotein F (SV-F) (Spiegel et al., 1998). A unique feature of SV-F is its ability to function as a ligand for the hepatocyte-specific ASGP receptor, which resulted in enhanced expression in HepG2 cells *in vitro* after MMLV / SV-F transduction. The same approach has also proven functional in combination with lentiviral vectors *in vitro* (Kowolik and Yee, 2002). However, the transduction efficiency of the SV-F pseudotypes was relatively low possibly owing to inefficient release of the internalized vector from the endosome compartment. In addition, the recovery after concentration of the SV-F pseudotypes was reduced when compared to the VSV-G pseudotypes, but still 50 times higher than with MMVL env. SV envelope (F-virosome) has also been utilized in a viral – non-viral hybrid system in which the transgene was packed and delivered to hepatocytes *in vivo* within the F virosome (Ramani et al., 1998).

Another receptor-mediated targeted gene transfer was successfully manifested when a ligand derived from apo E was incorporated in an AAV vector (Loiler et al., 2003). The
mutant AAV – apo E capsid was shown to direct the vector particle to the endocytosis pathway mediated by LDLR or other receptors of the LDLR family.

2.3.3.1.2. Transcriptional targeting

Transcriptional targeting using a combination of cell-specific promoter/enhancer elements that exhibit cell-type specific gene expression is another approach to cell-specific targeting. Transcriptional targeting can be used to compensate for the lack of cell-type specificity of the vector thereby allowing efficient and stable expression of the transgenes. These include genes coding for proteins whose accurate performance is dependent on their synthesis in the correct cell type because of, for instance, the requirement for distinct post-translational modifications. With this technique, gene transfer can result in widespread vector assimilation, but gene expression is limited to the target cell population. Cell-type specific promoters have been used for a variety of targeted gene expression studies in numerous cell populations and tissues (Walther and Stein, 1996).

Liver-specific promoter/enhancer elements

A number of natural liver-specific promoter/enhancer elements are known. These include phosphoenol-pyruvate carboxykinase (PEPCK), human α1-antitrypsin (hAAT), albumin, and transthyretin promoters (Guo et al., 1996; Walther and Stein, 1996; Papadakis et al., 2004). Several studies have been performed in which the liver-specificity of these and other liver-specific promoters has been demonstrated (Sandig et al., 1996b; Le et al., 1997; Koeberl et al., 1999). The major disadvantage of the use of the liver-cell specific promoters in gene therapy is their lower transcriptional activity by comparison with strong viral and cellular enhancers such as cytomegalovirus (CMV), LTR, SV40 viral enhancer, and human elongation factor 1α (Hafenrichter et al., 1994; Guo et al., 1996). However, their potency lies in the lower cellular and humoral immune responses in vivo, which is due to the lack of ectopic expression of the transgene in other tissues; and the resulting prolonged transgene expression (Ding et al., 2002; Follenzi et al., 2004).

Recently chimeric liver-specific promoters have been generated. With an AAV-2 vector expressing clotting factor IX (FIX) under the transcriptional control of a synthetic liver-specific promoter, liver-specific transgene expression was achieved in mice and dogs after portal vein or intravenous injection. The promoter contained the thyroid hormone-binding globulin (TBG) promoter sequences, two copies of α1-microglobulin/bikunin enhancer sequences (ABP), and a 71-base pair leader sequence (Ill et al., 1997) alone (Wang et al., 1999) or in conjunction with cis-acting regulatory elements (β-globin intron and WPRE) (Harding et al., 2004). Further, a conglomerate of minimal regulatory sequences from the strong SV40 viral enhancer together with the liver-type pyruvate kinase promoter harboring its TATA box and a hepatocyte nuclear factor 1α binding site, was shown to create a potent artificial tissue-specific promoter (Park et al., 2004).

In order to identify the most suitable combination of known liver-specific promoter and enhancer elements (i.e. alcohol dehydrogenase 6 (ADH6), hAAT, cholesterol 7alpha-hydroxylase, clotting FIX, apo E/C-I, and ABP) Gehrke et al. carried out a comparative investigation of a total of 25 synthetic hepatocyte-specific transcriptional control elements in vitro and in vivo. The conclusion was that a combination of the ADH6 basal promoter linked to two tandem copies of an apo EC-I enhancer element was found to be the strongest of those analyzed transcriptional control units for the liver-specific expression of
transgenes. This chimeric promoter led to 117-fold transcriptional activity in HepG2
human hepatoma cells in vitro, and 40- to 45-fold activity in mouse liver after intravenous
plasmid DNA injection by comparison with the transcriptional activity achieved with the
SV40 promoter (Gehrke et al., 2003).

A comparable study evaluated tissue specificity, activity, and length of gene expression
driven by chimeric constructs merging regulatory sequences of the albumin, hAAT, HBV
core protein, and hemopexin genes in vitro and in vivo (6 h – 50 days) (Kramer et al.,
2003). The results were compared to the transcriptional activity of CMV promoter, which
was shown to be 10 times higher in comparison with the SV40 promoter. The hAAT
promoter alone or when linked to the albumin or HBV enhancers was shown to be the
most potent in directing stable gene expression in liver cells. Almost 40% of CMV activity
(4-fold activity compared to SV40) was yielded when the hAAT promoter was used alone,
and when used in combination with the HBV enhancer up to 253-fold enhancement was
achieved yielding 180% of CMV activity in HepG2 cells. Furthermore, the in vivo
transgene expression levels from the hAAT-HBV enhancer chimera were comparable to the
expression levels from the CMV promoter for one week, after which the transgene
expression levels from the CMV promoter decreased more than 100-fold, whereas the
expression level from the hAAT-HBV was shown to be more stable. The results from these
comparative studies, however, may not directly correlate with viral gene transfer (Miao et
al., 2000) and further evaluation is needed to determine its feasibility with viral vector
applications.

2.3.3.1.3. Gene targeting

Gene targeting, the use of homologous recombination to produce defined alterations to
target cell genome, can lead to accurate, site-specific correction of genetic defects as well
as constituent endogenous regulation of the “corrected” gene. Unlike viral-mediated gene
insertion, gene repair causes no damage to improper regions of the genome nor does it
produce deleterious mutations. Numerous gene targeting approaches have been
attempted in vitro as well as in vivo employing chimeric RNA-DNA oligonucleotides or
chimeraplasts, single-stranded oligonucleotides, triplex-forming oligonucleotides, small-
fragment homologous replacement, and viral vectors with varying levels of success
(Andersen et al., 2002; Hendrie et al., 2003; Liu et al., 2004b). However, the current
gene targeting technology is too inefficient for in vivo use and further understanding of the
cellular mechanisms involved in the activation of the endogenous repair pathways and the
underlying protein factors, as well as further improvements and optimization in the gene
targeting methodology, will be needed in order to fulfil the therapeutic potential of this
strategy.

Liver-specific gene targeting

Gene targeting can be utilized for gene repair of a number of single-nucleotide genetic
disorders originating in the liver. Gene targeting utilizing chimeric RNA-DNA
oligonucleotides and single-stranded oligonucleotides has been implemented in a few in
vivo liver-directed gene therapy studies with varying success (Richardson et al., 2002; Lu et
al., 2003). However, the methodology of gene targeting in vivo is still inefficient, and
further developments are needed for it to become clinically applicable.
2.3.3.2. Transcriptionally regulated vectors

The ability to regulate the timing, duration and level of transgene expression in the target cell via exogenous stimuli facilitates the maintenance of a gene product within a therapeutic range, makes it possible to adapt to disease evolution via adjustments in the transgene expression, and broadens clinical efficacy and safety as well as the application range of gene therapy. The development of ligand-dependent transcriptional regulatory systems for use in gene therapy is therefore of great importance. For a pharmacologically regulated gene therapy vector, several criteria should be met: 1) the ligand should activate rather than silence the transgene expression in order to avoid prolonged exposure to the silencer drug, and avoid the delay in induction due to the rate of drug clearance from the body; 2) no interference should occur with endogenous metabolic pathways; 3) gene expression should be tightly regulated in a dose-dependent way by an orally bioavailable, well-tolerated inducer drug that penetrates all tissues; and 4) induction and repression of the gene expression should be fully and rapidly reversible. (Toniatti et al., 2004)

The first attempts to develop inducible transcriptional regulation of the transgene utilized inducible internal promoters responding to heat shock, heavy metal ions, steroid hormones (Yarranton, 1992) or radiation (Manome et al., 1998). These applications suffer from severe limitations such as pleiotropic activity of the inducing agent and high basal activity. Since then several inducible systems combining functional domains from prokaryotic, eukaryotic and viral proteins to create chimeric transactivators have been developed (Toniatti et al., 2004). However, the number of clinically applicable regulatory systems available is limited, and all such systems require significant improvements. Common to all current regulatory systems are the use of a chimeric ligand-dependent transcription factor, and an artificial inducible promoter, comprising multiple binding sites for the DNA-binding domain of the transcription factor followed by a minimal promoter. Upon interaction with the exogenously added drug or hormone such as Tc, mifepristone (a synthetic progesterone antagonist), tamoxifen, ecdysone (insect hormone), rapamycin, pristinamycin, and erythromycin the transcription factor is recruited to (or released from) the target sequences of the promoter resulting in activation or silencing of transgene transcription.

2.3.3.2.1. TetON, TetOFF

The Tc dependent transcriptional switch has major advantages over the other gene regulation systems. Its inducer drug, Dox, has a well-known safety profile, is well tolerated, is orally bioavailable, has germane half-life of 14 – 22 h, and demonstrates good tissue penetration of the inducer drug (Klein and Cunha, 2001). The most widely studied eukaryotic gene regulation systems are the Tet-OFF2 (Gossen and Bujard, 1992) and Tet-ON3 (Gossen et al., 1995) systems based on the Escherichia coli (E.coli) Tn10 Tc resistance repressor protein (tetR) that binds to specific operator sequences (tetO) of the Tc resistance operon with high specificity (Orth et al., 2000). The interaction between tetR and tetO is prevented by Tc or its more potent analog, Dox. Dox binds to the repressor protein and induces an allosteric change resulting in the release of the repressor from its target DNA sequence.
In the TetOFF system, transcription activation from a Tc-responsive promoter results from binding of the Tc-regulated transactivator (tTA), a fusion protein of tetR and the VP16 activation domain of HSV, in the absence of the ligand (Dox) (Baron and Bujard, 2000). The TetOFF system has been widely used in animal gene therapy studies; however, due to its unfavorable kinetic properties (the drug is used to silence the transgene), it is unlikely to be implemented in clinical gene therapy applications.

In the reverse Tc-regulated TetON system, addition of the ligand (Dox) induces transcription activation via binding of a reverse Tc-transactivator (rtTA) on its target promoter (Fig. 8). The major drawbacks of the original TetON system, i.e. the requirement for high Dox doses for activity, the low intrinsic background activity due to residual affinity of the rtTA to tetO in the absence of Dox, and the instability of the rtTA (Baron and Bujard, 2000), were dramatically improved when two novel rtTA variants, rTA-S2 and rTA-M2, were isolated. Following the generation of these variants by random mutagenesis of the Tc repressor gene and screening for alleles with reverse binding properties, the variants were fused with three minimal activation domains, instead of the VP16, and engineered into the synthetic transactivators, rTA2S-S2 and rTA2S-M2, by gene optimization. These variants have highly improved properties with respect to basal activity, inducibility, Dox responsiveness and stability thus allowing stringent regulation of target genes in stably transfected eukaryotic cells. While functioning at 10-fold lower Dox concentrations than the original rtTA, rTA2S-M2, the most promising new transactivator, exhibits no basal activity in the OFF state, and is more stable in eukaryotic cells (Urlinger et al., 2000).

![FIG. 8](image)

**FIG. 8** The TetON transcriptional regulation system.

Both novel transactivators have been shown to be capable of tightly controlling transcription of various transgenes after delivery to a number of tissues in vivo with diverse vectors (Aurisicchio et al., 2001; Lamartina et al., 2002). However, utilization of high vector doses resulted in partial slackening of the stringency of transcription control, which demonstrated an upper limit to the amount of vector that can be delivered.

Another strategy to circumvent the high basal activity of the rtTA system is to incorporate the Tc-responsive transcriptional silencer (tTS) in the TetON system (Freundlieb et al., 1999). In the absence of Dox tTS binds the promoter responsive for rtTA and inhibits basal transcription, whereas on addition of Dox, tTS dissociates from the tetO and
activation of rtTA initiates transcription. Using an optimized vector (Ad; AAV) the combination of rtTA/tTS was shown to significantly reduce the basal activity in vivo (Mizuguchi and Hayakawa, 2001; Rendahl et al., 2002). Furthermore, combining the rtTA2S2-S2 or rtTA2S2-M2 with the tTS has resulted in more stringent control than when either is used alone (Lamartina et al., 2003; Mizuguchi et al., 2003).

2.3.4. Gene transfer techniques

Two main gene therapy approaches for somatic cells have been pursued. First, ex vivo gene therapy involves removal of a segment of a specific organ, tumor or blood cells of an individual and the isolation of syngeneic cells to be grown in primary cell culture and subjected to gene transfer in vitro. These genetically altered cells are then transplanted back into the body where they produce the therapeutic protein. The benefits of the ex vivo approach include targeted transduction of a specific cell type and the possibility of avoiding vector particle inactivation by the active complement present in the host serum. Another method for delivering genes to the body is the direct in vivo injection of gene transfer vectors, thus eliminating the cumbersome steps of explanting, cultivating and transplanting the cells. The in vivo approach is usually less invasive than any procedure that entails surgery. During in vivo delivery, however, the gene transfer vehicles, whether viral or non-viral, may be attacked by components of immune system present in the blood before they have a chance to reach the target cell membrane. Furthermore, the in vivo setting offers less control over which cells are exposed to the gene transfer vector. However, with targeted and tissue specific vectors transduction can be limited to target cells.

A newly evolved approach, prenatal in utero gene therapy, may provide an alternative for a variety of hereditary and acquired diseases. It is based on the concept that application of gene therapy vectors to the fetus in utero may prevent the development of severe clinical disease by early intervention, allow targeting of otherwise inaccessible organs, tissues and stem cell populations with relatively low vector doses, avoid immune reaction against gene transfer vector and transgene protein because of the prenatal tolerance of the immature immune system, and provide postnatal tolerance against the therapeutic transgenic protein (Coutelle and Rodeck, 2002). However, in utero somatic gene therapy is still an experimental concept and insufficient data is available to date to ensure the safety of this procedure or to proceed to human clinical trials. A preliminary stage to in utero gene therapy might be the pre-natal cell transplantation procedures for the treatment of severe combined immunodeficiency disease (SCID) fetuses (Westgren et al., 2002; Peranteau et al., 2004).

2.3.4.1. Vascular gene transfer

The principal methods of introducing genetic material into blood vessels include in vivo gene transfer into the vascular wall and ex vivo gene transfer to vessel segments. Gene transfer into the artery wall can be accomplished from the lumen and from adventitia (Ylä-Herttuala, 1997). After the initial double-balloon catheter-mediated retroviral gene transfer to porcine iliofemoral arteries (Nabel et al., 1990) a number of intra-luminal catheter-and stent-based delivery methods have been generated for local in vivo gene transfer into the vessel wall (Gruchala et al., 2004b). Intra-vascular gene transfer has potential during
angioplasty and other intravascular manipulations (Ylä-Herttuala and Martin, 2000). Recently, adventitial gene transfer has shown promise as an alternative route for delivery of therapeutic genes into the artery wall (Laitinen and Ylä-Herttuala, 1998).

**Adventitial gene transfer**

Adventitial gene transfer, in contrast to intra-arterial gene transfer, circumvents the physical barrier of the endothelium, internal elastic lamina and atherosclerotic lesions and avoids interrupting blood flow and plasma interference (Fig. 9). In addition, inflammatory reaction against the vector may be diminished (Schneider et al., 1999) and systemic distribution of the gene transfer vector is reduced when periadventitial vector delivery is employed (Hiltunen et al., 2000). Biodegradable polymers mixed with antisense oligonucleotides (Simons et al., 1992), plasmid DNA (Indolfi et al., 1995) and Ad vectors (Feldman et al., 1997) have been used for gene transfer to the outer surface of vessels. Vector solutions have also been injected directly into the adventitia (Rios et al., 1995; Schneider et al., 1999) or into isolated adventitial space within silastic (Laitinen et al., 1997; Turunen et al., 1999; Airenne et al., 2000) and biodegradable collars (Pakkanen et al., 2000) or within biodegradable gel (Siow et al., 2003). Vector producing encapsulated cells have also been used for adventitial implantation (Armeanu et al., 2001). Adventitial gene transfer can be used for the delivery of diffusible or secreted therapeutic products into the arterial wall during such surgical procedures as coronary artery bypass surgery, peripheral vascular graft surgery, prosthesis and anastomosis surgery and endarterectomy (Ylä-Herttuala and Martin, 2000). Indeed, gene expression appears stronger after plasmid-liposome-mediated adventitial gene transfer than after intra-luminal gene transfer to canine saphenous vein grafts (Kalra et al., 2000).

**Cell-based vascular gene transfer**

Another approach for vascular gene therapy is cell based gene transfer, which requires harvesting of vascular cells, ex vivo transduction and subsequent re-implantation. By this method a specific cell type can be isolated, gene transfer efficiency can be optimized and gene expression confirmed prior to re-implantation. Both of the major vascular wall cell types; SMCs and endothelial cells, have been utilized for cell-based vascular gene transfer (Clowes, 1997; Parikh and Edelman, 2000). SMCs have many properties that make them reliable for cell-based vascular gene transfer. In culture conditions these well-differentiated cells are robust and simple to handle with no stringent growth requirements, and also relatively easy to transduce. A number of different vectors have been used to transduce vascular SMC, including MMLV retroviral vectors (Clowes, 1997; Beltrao-Braga et al., 2002), Ad vectors (Ribourtout et al., 2003), AAV vectors (Lynch et al., 1997) and lentiviral vectors (Dishart et al., 2003). Moreover, including an SMC-specific promoter together with enhancer elements in the vector has been shown to increase transgene expression in
vascular SMCs (Appleby et al., 2003). Transduced SMCs do not appear to be transformed and are long-lived after implantation. After the initial demonstration of SMC-based intravascular gene transfer (Plautz et al., 1991) a number of SMC-based applications have been reported for endovascular remodeling after injury (Clowes et al., 1994; Ribourtout et al., 2003), for biological lining of vascular grafts (Geary et al., 1994; Eton et al., 2004) and for systemic delivery of gene products (Osborne et al., 1995). Systemic delivery has also been accomplished after adventitial delivery of transduced SMCs (Beltrao-Braga et al., 2002), and after subcutaneous and intraperitoneal transplantation of transduced vascular SMCs within a bioisolator device (Yanay et al., 2003).

2.3.4.2. Liver-directed gene transfer

The liver is one of the most attractive sites for gene transfer because of its major role in many metabolic processes and involvement in a large variety of diseases. The liver fulfills many vital processes in mammals. It is the central organ of energy metabolism, biotransformation of xenobiotics, and synthesis of plasma proteins under physiological and pathophysiological conditions. The liver is the organ that synthesizes most of the body’s circulating plasma proteins, including lipoproteins. In addition to other functions, such as degradation of drugs and toxins, uptake, processing and storage of several vitamins and iron, the liver is involved in many important metabolic pathways, including lipid metabolism. Thus, hepatic gene therapy may provide an approach to therapy for various diseases of hepatic function, inherited as well as acquired or multifactorial. By means of gene therapy, the liver hepatocytes may also be used as bioreactors to secrete therapeutic proteins into the blood.

2.3.4.2.1. Delivery route

Gene transfer into mammalian hepatocytes has been accomplished using both ex vivo and in vivo (Ghosh et al., 2000) and also in utero (Lipshutz et al., 1999; MacKenzie et al., 2002) approaches. Hepatocytes are easily accessible to vectors injected into the circulation through large pores in liver capillaries (Fraser et al., 1995). Liver-directed gene therapy has been studied for the treatment of several diseases like dyslipidemia (Oka and Chan, 2002), hemophilia A and B (Van den Driessche et al., 2003), lysosomal storage disorders (Cheng and Smith, 2003), and diabetes mellitus (Nett et al., 2003). In vivo targeting to the liver has been performed by injecting DNA or viral vectors into the liver parenchyma (Kuriyama et al., 2000), splenic capsule (Chen et al., 2000), isolated liver lobes via portal vein or bile duct (Zhang et al., 2001), hepatic artery (Raper et al., 2002), or portal vein (Kozarsky et al., 1994; Pakkanen et al., 1999b) or through bile duct or portal vein perfusion of the liver (De Godoy et al., 2000). The development of a safe and efficient gene transfer vector has been a major challenge in liver-directed gene therapy. None of the currently available means of gene transfer to the liver is optimal for all types of applications. Several viral and non-viral vectors have been generated for liver gene therapy for use in specific situations.
2.3.4.2.2. Vectors for liver-directed gene transfer

Long-term correction of the genetic diseases affecting the liver will require stable integration of the therapeutic gene into the host genome. This has been accomplished by a number of gene transfer vectors.

Injection of naked plasmid DNA under the transcriptional control of a liver-specific promoter into mouse tail vein has resulted in low level episomal transgene expression of up to 1.5 years from mouse livers (Zhang et al., 2000; Miao et al., 2001; Miao et al., 2003). This is not surprising, since episomal persistence of DNA has been used in nature by viruses to establish a latent state in host cells conferring life-long expression of some genes from the viral genome (Goins et al., 1994). Recent innovations including mechanically massaging the liver (Liu et al., 2004a), and plasmid DNA delivery into the rabbit portal vein via a balloon occlusion catheter (Eastman et al., 2002) have further enhanced the level of gene expression after intravenous injection of naked plasmid DNA. In addition, a hammering bullet gene gun instrument (Kuriyama et al., 2000), microencapsulation of recombinant cells (Sun et al., 1986; Hortelano et al., 1999), nanoparticles (Yamada et al., 2003), and utilization of transposon technology (Yant et al., 2000; Mikkelsen et al., 2003) have shown potential for liver gene therapy. Short term efficient gene transfer has been accomplished by these methods; however, further development is needed before implementation in human gene therapy.

First-generation Ads as well as the newly developed helper-dependent gutless Ad vectors have been shown to transduce a significant proportion of hepatocytes after intravenous delivery in animal models (Connelly, 1999; Ehrhardt et al., 2003). The longevity of the adenoviral transgene expression in the liver has been improved by incorporating liver-specific promoters in the vector (Pastore et al., 1999; Oka et al., 2001; Van Linthout et al., 2002).

AAV virus based vectors have yielded promising results in hepatic gene transfer (Koeberl et al., 1997; Snyder et al., 1999; Mount et al., 2002; Mochizuki et al., 2004). Successful results in animal models of hemophilia B have prompted the initiation of human clinical trials of direct AAV vector delivery to the liver via a catheter to the hepatic artery (High, 2001). However, there are indications that in some settings, the AAV vector may initiate a detectable cellular and humoral immune response to the transduced gene product in vivo (Brockstedt et al., 1999). By using alternatives to AAV-2 serotype based AAV vectors or pseudotype AAV vectors it may be possible to evade the immune response to the AAV-2 capsid protein as well as enhance liver directed gene transfer (Mingozzi et al., 2002; Grimm et al., 2003; Sarkar et al., 2004). Furthermore, incorporation of a liver-specific promoter into the vector backbone has been shown to improve the potency of the AAV vector to transduce hepatocytes in vivo after delivery into the mouse liver (Xiao et al., 1998; Nakai et al., 1998; Wang et al., 1999). In the study of Harding et al. tail vein injection of an AAV-2 vector into mice resulted in 20-30% lower transgene expression than portal vein injection; however, when an AAV-2 vector under a liver-specific promoter was used 25.6 ± 12.6% of hepatocytes stained positive for the transgene, whereas a vector under a ubiquitous promoter led to 5.2 ± 3.2% transduction efficiency (Harding et al., 2004).
Hepatocyte transduction with retroviral vectors has been performed on cultured primary hepatocytes in vitro (Wolff et al., 1987; Armentano et al., 1990; Grossman et al., 1991) and ex vivo (Grossman et al., 1992; Kay et al., 1992), and also in vivo after hepatocyte stimulation either by partial hepatectomy (up to 70%) alone (Ferry et al., 1991; Cai et al., 1998; Podevin et al., 2000) or in combination with thymidine-kinase – ganciclovir pretreatment (Pakkanen et al., 1999a; Pakkanen et al., 1999b), by toxic gene products (Lieber et al., 1995) and by hepatocellular mitogens (Bosch et al., 1996; Patijn et al., 1998; Xu et al., 2003). The use of the traditional MMLV-based vectors has resulted in modest gene transfer efficiency, rarely exceeding 1%. Pseudotyping the standard MMLV retrovirus with VSV-G envelope protein resulted in improved and prolonged transgene expression in rat and in WHHL rabbit liver (Shiraishi et al., 1999; Pakkanen et al., 1999a). Hepatocyte transduction has also been enhanced by complete vascular exclusion and in situ perfusion of the liver with retrovirus-containing solution after partial hepatectomy (Ferry et al., 1991; De Godoy et al., 2000). However, the need for major surgery or liver damage before integrative gene transfer with retroviruses is clinically undesirable.

HIV-1 based lentiviral vectors have been shown to transduce stably quiescent hepatocytes in vivo and ex vivo (Kafri et al., 1997; Nguyen et al., 2002); however, some studies have shown that lentivirus-mediated gene transfer to liver is greatly enhanced when provided during hepatocellular cycling (Park et al., 2000a) or regeneration induced by partial hepatectomy or direct hyperplasia (Park et al., 2000b; Ohashi et al., 2002). Further improvements to the vector backbone, however, reduced the need for cell cycle progression for lentiviral vectors to transduce quiescent hepatocytes in vivo (Park and Kay, 2001; Pleifer et al., 2001; Follenzi et al., 2002; Tsui et al., 2002; Van den Driessche et al., 2002; Giannini et al., 2003). Incorporation of a liver specific promoter to drive the lentiviral expression cassette has been shown to restrict the transgene expression to hepatocytes and to alleviate the activation of the host immune system (Follenzi et al., 2002; Oertel et al., 2003; Park et al., 2003). Pseudotyping of a lentiviral vector with SV-F (Kowolik and Yee, 2002) or RRV glycoproteins (RRV-G) (Kang et al., 2002) conferred hepatocyte specificity on the vectors. RRV-G also caused less cytotoxicity in comparison to the more common VSV-G. Furthermore, lymphocytic choriomeningitis virus glycoprotein pseudotyped lentiviral vectors have been shown to result in minimal hepatic injury compared to the VSV-G pseudotyped lentiviral vector in mice (Beyer et al., 2002).

2.3.5. Apolipoproteins in gene therapy for hyperlipidemia

2.3.5.1. Apo E

Apo E is considered to have therapeutic potential for improving lipoprotein metabolism based on its physiological role in mediating the interaction of lipoproteins with cell surface receptors, both LDLR and LRP, and thereby modulating the in vivo catabolism of various lipoproteins, including the atherogenic, cholesterol-rich VLDL and chylomicron remnants. Furthermore, its molecular properties, namely stability, water solubility, and simultaneous binding of lipids and other molecules, make it an attractive molecule for therapeutic purposes. In order to evaluate the potential of apo E in gene therapy for hyperlipidemia and atherosclerosis, several studies have been performed using a variety of animal models.
Among the first pieces of evidence of the potential of apo E in treating hyperlipidemia were the results from direct injections of apo E protein into the circulation of WHHL rabbits. Up to 60% reductions in plasma cholesterol levels, mostly owing to an accelerated plasma clearance of remnant lipoproteins, were detected after intravenous injection of recombinant apo E in WHHL and cholesterol fed rabbits (Mahley et al., 1989; Yamada et al., 1989). Sustained administration (3 times a week for 8.5 months) of purified rabbit apo E had no effect on plasma cholesterol levels but was shown to prevent the progression of atherosclerosis in WHHL rabbits (Yamada et al., 1992). Moreover, hepatic overexpression of rat apo E reduced plasma cholesterol levels and suppressed diet-induced hypercholesterolemia as well as atherosclerosis in the absence of functional LDLR in transgenic LDLR<sup>−/−</sup> mice (Osuga et al., 1998). More recently, hepatic overexpression of human apo E3 (20 – 43mg/dl) has been shown to induce highly significant regression of pre-existing advanced atherosclerosis without marked changes in total plasma cholesterol levels or lipoprotein composition after adenoviral gene transfer in LDLR<sup>−/−</sup> mice (Tangirala et al., 2001).

Generation of the apo E knockout mouse (apoE<sup>−/−</sup>) (Plump et al., 1992; Zhang et al., 1992) provided the basis for many studies regarding the anti-atherogenic potential of apo E. The severely hypercholesterolemic phenotype and spontaneous atherosclerosis of the apoE<sup>−/−</sup> mouse (Nakashima et al., 1994; Reddick et al., 1994) can be prevented by bone marrow transplantation (Linton et al., 1995; Hasty et al., 1999), and macrophage-specific expression of the human apo E transgene (Bellosta et al., 1995; Gough and Raines, 2003). Macrophage-derived apo E has been suggested to delay the development of atherosclerosis through a receptor-dependent pathway (Yoshida et al., 2001). Several studies have shown that the anti-atherogenic nature of apo E functions via the plasma compartment as well.

Therapeutic apo E expression has been achieved after intradermal engraftment of murine embryonic yolk sac endothelial cells (Cioffi et al., 1999), and naked DNA, adenovirus and AAV-mediated gene transfer to liver and muscle (Kashyap et al., 1995; Tsukamoto et al., 1999; Rinaldi et al., 2000; Tangirala et al., 2001; Harris et al., 2002b). Direct comparison of two non-macrophage-specific delivery routes, the intramuscular and systemic delivery (liver-targeting) showed that liver-specific expression of apo E (but not muscle-derived expression) resulted in normalization of plasma cholesterol levels and retarded progression of early atherosclerosis and regression of late atherosclerotic plaques after adenovirus-mediated gene transfer of apo E (7.5±0.98mg/dl) (Harris et al., 2002a). However, the low transduction efficiency of muscle, possibly due to the 100 times lower levels of coxsackie and adenovirus receptors in muscle tissue than in liver tissue, probably affected the outcome of the treatment.

Apo E3, the most common fully functional apo E isoform, has been shown to be more potent than apo E2 or apo E4 for gene therapy of hyperlipidemia and for regression of both early atherosclerotic lesions and advanced complex lesions. A direct comparison of the apo E isoforms by adenoviral gene transfer into the liver of apoE<sup>−/−</sup> mice demonstrated the impaired ability of apo E2 to clear VLDL, IDL and remnant lipoproteins from the circulation. Moderate levels of apo E3 and apo E4 expression lead to normalization of the hypercholesterolemia and a dose-dependent, but moderate increase in serum triglyceride levels (Tsukamoto et al., 1997; Tsukamoto et al., 1999). However, apo E2 and apo E4 exhibit residual anti-atherogenic activity and have been shown to prevent the
progression of early atherosclerosis, but not advanced complex lesion regression (Tsukamoto et al., 1999; Athanasopoulos et al., 2000).

An optimal level of apo E is required for lipoprotein clearance from the circulation. Normal cells maintain optimal apo E level via apo E recycling (Heeren et al., 1999b; Swift et al., 2001). Genetic deficiency or the presence of dysfunctional forms of apo E generally cause hypercholesterolemia or type III HLP with marked increase in VLDL and LDL levels and premature atherosclerosis both in humans (Mahley et al., 1999) and in mice (Plump et al., 1992; Zhang et al., 1992). At physiological concentration apo E promotes efficient clearance of apo E-containing lipoprotein remnants (van Dijk et al., 1999). However, marked overexpression of human apo E3 causes massive hypertriglyceridemia (HTG) in transgenic mice (Huang et al., 1998), and HTG together with hypercholesterolemia in transgenic rabbits, apoE\(^{-/-}\) knockout mice and apoE\(^{-/-}\)LDLR\(^{-/-}\) double knockout mice (Huang et al., 1999; van Dijk et al., 1999; Maugeais et al., 2000). Further, a subtle to moderate increase in apo E expression results in accumulation of LDL cholesterol in plasma of transgenic rabbits (Fan et al., 1998).

Major contributors to the apo E induced hyperlipidemia are apo E-dependent stimulation of hepatic VLDL production, enhanced VLDL clearance and interference with apoC-II-dependent VLDL lipolysis (van Dijk et al., 1999; Tsukamoto et al., 2000a). The carboxy-terminal segment of apo E has been associated with the increased production rate of VLDL (Kypreos et al., 2001b). Therefore, the hypertriglyceridemic effect of apo E overexpression can be surmounted by deletion of the C-terminal lipid binding domain of apo E (Kypreos et al., 2001a; Gerritsen et al., 2003).

Low plasma apo E levels independent of delivery route appear to possess therapeutic potential in limiting the progression of the early stages of atherogenesis without normalizing the plasma concentration of atherogenic remnant lipoprotein particles (Hasty et al., 1999; Athanasopoulos et al., 2000; Thorngate et al., 2000; Harris et al., 2002b). It has been suggested that the low level of apo E present in the circulation may prevent the accumulation of remnant lipoproteins on the vessel wall, a development of atherosclerotic lesions that precedes the attachment of monocytes, and thus protect against early lesion development. Other anti-atherogenic properties of apo E may also be involved in the apo E-mediated prevention of atherosclerosis.

2.3.5.2. ApoA-I, apoA-I\(_{\text{Milano}}\)

Another anti-atherogenic apolipoprotein is the apolipoprotein A-I (apo A-I), the major protein constituent of HDL. HDL is one of many factors that participate in the regulation of the atherosclerotic process and reduced levels of HDL are associated with higher incidence of CHD (Young et al., 2004). In addition to its antioxidant, anti-inflammatory, and anti-thrombotic properties HDL mediates reverse cholesterol transport from peripheral tissues, including the arterial wall, to the liver for excretion. This feature of HDL is considered responsible for the strong inverse correlation between plasma HDL levels and CHD. As demonstrated by numerous in vivo experiments apo A-I seems to carry the major responsibility in the anti-atherogenic activity of HDL. Overexpression of human apo A-I leads to an increase in the number of circulating HDL particles and a decrease in diet- or gene-induced atherosclerosis in transgenic mice and rabbits (Paszty et al., 1994; Duverger et al., 1996). In humans an infusion of apo A-I – phospholipid complexes has been shown to lead to an increased cholesterol efflux with production of small HDLs, and to enhanced
bile acid and neutral sterol excretion (Eriksson et al., 1999; Nanjee et al., 1999). An apo A-I–mediated protective effect on the arterial wall has also been reported after gene transfer to apoE<sup>−/−</sup> and LDLR<sup>−/−</sup> mice (Kawashiri et al., 2001; Pastore et al., 2004).

Apo A-I<sub>Milano</sub>, a natural variant of human apo A-I (Weisgraber et al., 1980) originally described in a population with low cardiovascular risk (Franceschini et al., 1980), has been shown to confer on its carriers significant protection against vascular disease despite their reduced HDL levels (Sirtori et al., 2001). The HDL fraction of individuals with the apo A-I<sub>Milano</sub> variant and another cardioprotective apo A-I isoform, the apo A-I<sub>Paris</sub>, has been shown to consist primarily of the small HDL-3 (Bruckert et al., 1997). Unlike the larger HDL-2 particles, HDL-3 have been associated with a reduced risk of progression of CHD (Yu et al., 2003a) and inhibition of LDL oxidation (Kontush et al., 2003). Also, both apo A-I variants, apo A-I<sub>Milano</sub> and apo A-I<sub>Paris</sub> have been reported to exhibit more potent antioxidant activity than the wild type apo A-I (Bielicki and Oda, 2002). Moreover, repeated systemic infusions or a single localized injection of recombinant apo A-I<sub>Milano</sub> (Calabresi et al., 1994) – phospholipid complexes have been shown to produce vasculoprotective effects via rapid mobilization of cholesterol from existing atherosclerotic plaques in rabbits and pigs after arterial injury (Chiesa et al., 2002; Kaul et al., 2003), and in cholesterol-fed apoE<sup>−/−</sup> mice (Shah et al., 2001). In humans short term weekly infusions of apo A-I<sub>Milano</sub> – phospholipid complexes have resulted in an average reduction of 4.2% in the volume of coronary atheroma (Nissen et al., 2003). Although the preliminary data appears promising, more data from larger clinical trials will be needed to confirm the effects of apo A-I<sub>Milano</sub> in reducing atherosclerotic plaque burden.

### 2.3.5.3. Apobec-1

Given that apo B-100–associated lipoproteins (VLDL and LDL) are much more atherogenic than apo B-48–associated lipoproteins (chylomicrons and their remnants and VLDL), apobec-I, the catalytic subunit of apo B editing enzyme, has been considered potentially useful for gene therapy of hyperlipidemia. It was shown to limit the generation of atherogenic apo B-100–containing lipoproteins and to significantly reduce plasma LDL-cholesterol levels in rabbits (Greeve et al., 1996; Kozarsky et al., 1996a). However, uncontrolled transgenic expression of apobec-I leads to nonspecific editing of mRNAs and hepatocellular dysplasia and carcinoma in transgenic mice and rabbits (Yamanaka et al., 1995). While no adverse effects were observed when apobec-I was expressed at low or moderate levels (Qian et al., 1998), the possibility of using apobec-I directly as a therapeutic agent through a protein transduction domain of HIV-1 Tat protein has been suggested, as in this way precisely controlled levels of the transgene induction were transiently maintained in primary rat hepatocytes in vitro (Yang et al., 2002b).

### 2.3.6. Human gene therapy

Human gene therapy is a complex, multiphase process involving the identification of genes causing or related to disease, development, in vitro testing and manufacturing of the gene transfer vectors, preclinical testing and toxicology studies in animal models, and clinical development of gene therapy products through the three phases of clinical trials. Many metabolic diseases are potential candidates for treatment with gene therapy. Candidate diseases include those caused by a single gene defect such as SCID and FH, more
complex multifactorial diseases, including diabetes mellitus and atherosclerosis, and acquired diseases like AIDS.

The first phase I gene therapy clinical trial, which aimed to provide detailed information on the safety and feasibility of the gene therapy procedure, began on September 14, 1990 when a 4-year-old girl with SCID was injected with autologous T cells transduced \textit{ex vivo} with a retroviral vector containing the human ADA cDNA (Blaese et al., 1995). A 12-year follow-up of that study has been reported, which has shown that, ten years after the last \textit{ex vivo} transduced cell infusion, approximately 20% of the patients lymphocytes still carry and express the retroviral gene; however, the expression was not considered sufficient to allow withdrawal of the supplementary PEG-ADA treatment (Muul et al., 2003). Two other patients, treated with the same transduction protocol (Blaese et al., 1995; Onodera et al., 1998), developed precipitating antibodies against fetal bovine serum, present in the infused cell suspension. Antibodies to MMLV p30 core protein were also detected from the patients following the cell infusions. One patient was positive for p30 antibodies as long as eight years after the final cell infusion (Muul et al., 2003).

Subsequently, successful gene therapy after retroviral \textit{ex vivo} gene transfer to the autologous hematopoietic stem cells of nine out of eleven infants with sustained (up to over four years) correction of SCID phenotype has been reported (Cavazzana-Calvo et al., 2000; Hacein-Bey-Abina et al., 2002; Hacein-Bey-Abina et al., 2003b). Unfortunately, almost three years after completing therapy, two children developed T cell leukemia, possibly owing to insertional mutagenesis associated with retrovirally mediated gene transfer (Hacein-Bey-Abina et al., 2003a; Hacein-Bey-Abina et al., 2003b). Another adverse event, systemic inflammatory response syndrome, leading to the death of an 18-year-old male who participated in a safety study of E1,E4 deleted Ad-mediated gene transfer of human OTC into the right hepatic artery at high dose, has been reported (Raper et al., 2003). Despite these drawbacks, human gene therapy clinical trials are ongoing and success has been achieved e.g. in the treatment of malignant glioma (Sandmair et al., 2000). More than 900 gene therapy phase I to III clinical trials have been approved worldwide during the last 15 years. The majority (66%) of these trials were directed towards the fight against cancer, about 10% to the treatment of monogenic diseases and 8% to the treatment of vascular diseases (http://www.wiley.co.uk/genmed/clinical/).

2.3.7. FH gene therapy

2.3.7.1. \textit{Ex vivo} approach

The first gene therapy technique developed for FH utilized the \textit{ex vivo} approach. In it recombinant amphotropic retroviruses carrying the LDLR gene were used to transduce hepatocytes in WHHL rabbits (Wilson et al., 1990; Chowdhury et al., 1991), in baboons (Grossman et al., 1992), and eventually in the first human FH gene therapy clinical trial (Grossman et al., 1994; Grossman et al., 1995). Prior to use in human clinical trials, the techniques of hepatocyte delivery were refined using dogs and baboons (Grossman et al., 1992; Grossman et al., 1993). In the procedure, the patients underwent hepatic resection and placement of a portal vein catheter. Primary hepatocyte cultures were prepared from the resected liver and transduced with the retrovirus. The autologous hepatocytes were subsequently transplanted back into the donor liver via the portal circulation. In human
clinical trials, the technique was feasible and relatively safe. Nevertheless, it was laborious, difficult and time consuming, and resulted in only a modest lipid-lowering effect (Raper et al., 1996) probably owing to the difficulty of stably reimplanting a sufficiently high number of engineered hepatocytes to produce LDLR. In addition to autologous hepatocytes, human amniotic epithelial cells (HAEC) have been utilized for ex vivo gene transfer of LDLR cDNA in an adenoviral vector to WHHL rabbits (Takahashi et al., 2001). The genetically modified HAEC were transplanted to the liver of the rabbits and substantial, but transient (< 3 weeks) decreases in serum cholesterol were observed.

2.3.7.2. **In vivo approach**

Even though further developments have been reported in the ex vivo gene transfer methods utilizing lentiviral vectors (Nguyen et al., 2002; Giannini et al., 2003; Oertel et al., 2003), the direct *in vivo* approach offers an attractive alternative for liver gene therapy. In addition to being less invasive, *in vivo* gene therapy benefits from recent advances in targeted vector development (Lundstrom, 2003). Moreover, the generation of a non-invasive method utilizing a scintillation camera for monitoring successful liver-directed gene transfer of LDLR (Tietge et al., 2004) provides for further advances in the gene therapy methodology available for clinical gene therapy trials of FH.

**Non-viral vectors**

An *in vivo* approach, utilizing a DNA–protein complex capable of targeting LDLR genes to hepatocytes, temporarily reconstituted hepatic LDLR activity after intravenous infusion into WHHL rabbits (Wilson et al., 1992), thus demonstrating the potential of *in vivo* gene transfer for developing non-invasive approaches to liver-directed gene therapies. Another non-viral approach for FH gene therapy was represented in the study of Parise et al. (Parise et al., 1999), in which a soluble chimera of LDLR and transferrin fusion protein was shown to bind LDL specifically and was internalized by WHHL rabbit fibroblasts *in vitro*. The chimera was also shown to produce a transient physiological effect in LDLR-/- mice after naked plasmid injection *in vivo* (Razzini et al., 2004). However, in order to obviate the potential undesired cholesterol accumulation in the extrahepatic tissues mediated by the ubiquitous representation of the transferrin receptor, LDLR should be bound to a ligand protein that is preferentially bound by a liver-specific receptor, such as the asialoglycoprotein (Wu et al., 2002). In another study transferrin served as an intravenous delivery vehicle for a cationic liposome rabbit LDLR cDNA complex to the leukocytes of WHHL rabbits (Shichiri et al., 2003). The method demonstrated a significant, but temporary (up to 3 weeks) decrease in plasma LDL cholesterol in rabbits.

**Adenoviral vectors expressing LDLR, VLDLR, apobec-1**

*In vivo* administration of the first generation, E-1 deleted Ad vectors resulted in efficient, yet transient (<3 weeks), LDLR gene transfer effect in LDLR-/- mice (Ishibashi et al., 1993) and in WHHL rabbits (Kozarsky et al., 1994; Li et al., 1995b; Cichon et al., 2004). Owing to the induction of humoral and cellular immune responses to the LDLR protein, a neoantigen in LDLR-deficient animals, overexpressing human VLDLR [94% homologous to the mouse protein (Gafvels et al., 1993)], instead of human LDLR [77% homology to the mouse counterpart (Polvino et al., 1992; Hoffer et al., 1993)], was considered as an alternative. It was hypothesized that ectopic VLDLR expression in liver would reduce serum cholesterol levels by catabolizing LDL precursors. Indeed, the use of VLDLR in an adenoviral vector led
to more prolonged metabolic correction in \( \text{LDLR}^{-/-} \) mice, partly because of the less immunogenic nature of the transgene protein in the \( \text{LDLR}^{-/-} \) mouse model (Kobayashi et al., 1996; Kozarsky et al., 1996b). Despite being more protracted, the transgene expression lasted only 3 – 9 weeks. This was not unexpected, because the first generation Ad vector, used in the studies, has been shown to induce inflammation and an immune response (Yang et al., 1994a; Yang et al., 1994b). Amelioration, although modest and transient (up to three weeks), of lipoprotein profile in the liver of WHHL rabbits was also obtained by recombinant Ad-mediated expression of apobec-1 (Kozarsky et al., 1996a).

The new gutless Ad vector, devoid of all viral coding regions, was shown to be less immunogenic than the first generation E-1 deleted Ad vector. Hence, gene transfer of the VLDLR under transcriptional control of a liver-specific PEPCK promoter (Short et al., 1992) within the new gutless Ad vector exhibited prolonged transgene expression of over six months in \( \text{LDLR}^{-/-} \) mice (Oka et al., 2001). Also blocking of a CD40 ligand has resulted in inhibition of immune-mediated loss of transgene expression enabling long-term (\( \geq 3 \) months) reduction in plasma cholesterol levels after Ad-LDLR gene transfer to \( \text{LDLR}^{-/-} \) mice (Stein et al., 2000). However, the chromosomal integration of Ad vectors is extremely rare (Harui et al., 1999) and therefore Ad vectors may not provide the best tool for stable amelioration of a genetic defect. Furthermore, Ad vectors have shown evidence of a potential to cause a fatal inflammatory immune response in the recipient (Lehrman, 1999; Marshall, 1999; Anonymous, 2002).

**AAV vectors**

AAV vectors have shown the potential for stable transgene expression after intrasplenic injection of VLDLR in \( \text{LDLR}^{-/-} \) mice where Chen and coworkers (Chen et al., 2000) demonstrated transgene stability over a period of seven months with a 40% reduction in plasma cholesterol levels. Moreover, serotypes AAV-7 and AAV-8 have been shown to be more potent in normalizing serum lipids and preventing atherosclerosis than the traditional AAV-2 serotype after LDLR gene transfer to the portal vein of \( \text{LDLR}^{-/-} \) mice (Lebherz et al., 2004).

The outcomes of the \( \text{LDLR}^{-/-} \) mouse studies, however, are unpredictable in humans since the \( \text{LDLR}^{-/-} \) mouse does not strictly reflect the human disease. The lipoprotein metabolism of mice is inherently different from humans (Rader and FitzGerald, 1998; Daugherty, 2002), and unlike humans, LDLR deficient mice have only a mild elevation of serum cholesterol and little atherosclerosis. The double knock-out (LDLR negative and apobec-1 negative) mice would represent a better model of the human disease because they exhibit elevated serum LDL cholesterol and develop severe atherosclerosis (Powell-Braxton et al., 1998). Moreover, the WHHL rabbit provides a unique animal model for FH gene therapy because of the human-like distribution of their cholesterol between lipoprotein subclasses (LDL:HDL ratio) and because of their susceptibility to spontaneous atherosclerosis without high-fat challenge (Watanabe, 1980).

**Retroviral and lentiviral vectors**

In order to improve the efficiency of the in vivo retrovirus-mediated gene transfer, stimulation of hepatocyte proliferation by partial liver resection and cytotoxic thymidine kinase – ganciclovir treatment were combined with repeated injections of retroviral vectors, carrying the LDLR cDNA, into the portal vein of WHHL rabbits (Pakkanen et al., 1999b). This led to a maximum of 35% decrease in total plasma cholesterol levels 2 – 3 months
after the gene transfer. However, when amphototropic retroviruses are used the need of stimuli for hepatocyte proliferation renders the method invasive and laborious. Subsequently lentiviruses have provided a new tool for liver-directed gene transfer. In contrast to retroviruses, lentiviruses are capable of integrating into chromosomes of non-dividing cells, such as hepatocytes, leading to long-term transgene expression thus making them a valuable candidate for liver gene therapy (Kafri et al., 1997).

The ex vivo and in vivo gene therapy studies for treating FH are summarized in Table 5.

**TABLE 5.** Summary of ex vivo and in vivo FH gene therapy studies

<table>
<thead>
<tr>
<th>VECTOR</th>
<th>TRANS-GENE</th>
<th>ANIMAL</th>
<th>PHYSIO-LOGICAL EFFECTS</th>
<th>REF.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>EX VIVO</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>retrovirus</td>
<td>LDLR</td>
<td>baboon</td>
<td>modest</td>
<td>(Wilson et al., 1990; Chowdhury et al., 1991)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HUMAN</td>
<td></td>
<td>(Grossman et al., 1992)</td>
</tr>
<tr>
<td>adenovirus</td>
<td>LDLR</td>
<td>WHHL</td>
<td>transient</td>
<td>(Grossman et al., 1994; Grossman et al., 1995)</td>
</tr>
<tr>
<td></td>
<td>DNA-protein</td>
<td>LDLR</td>
<td>transient</td>
<td>(Wilson et al., 1992)</td>
</tr>
<tr>
<td>liposome-DNA</td>
<td>LDLR</td>
<td>WHHL</td>
<td>efficient, transient</td>
<td>(Shichiri et al., 2003)</td>
</tr>
<tr>
<td>transferrin</td>
<td>LDLR</td>
<td>WHHL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>fusion protein</td>
<td>LDLR</td>
<td>LDLR-/- mice</td>
<td>transient</td>
<td>(Razzini et al., 2004)</td>
</tr>
<tr>
<td><strong>IN VIVO</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenovirus</td>
<td>LDLR</td>
<td>LDLR-/- mice</td>
<td>efficient, &lt;3wk</td>
<td>(Ishibashi et al., 1993)</td>
</tr>
<tr>
<td></td>
<td>VLDLR</td>
<td>LDLR-/- mice</td>
<td>3-9wk</td>
<td>(Kozarsky et al., 1994; Li et al., 1995b)</td>
</tr>
<tr>
<td></td>
<td>apobec</td>
<td>WHHL</td>
<td>modest, &lt;3wk</td>
<td>(Kozarsky et al., 1996a)</td>
</tr>
<tr>
<td></td>
<td>LDLR</td>
<td>LDLR-/- mice</td>
<td>≥ 3 months</td>
<td>(Stein et al., 2000)</td>
</tr>
<tr>
<td></td>
<td>LDLR</td>
<td>LDLR-/- mice</td>
<td>efficient, chol crystals, &lt;4mo</td>
<td>(Cichon et al., 2004)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SCID mice</td>
<td>efficient, chol crystals, &lt;3wk</td>
<td></td>
</tr>
<tr>
<td>gutless Ad</td>
<td>VLDLR</td>
<td>LDLR-/- mice</td>
<td>significant, &gt; 6mo</td>
<td>(Oka et al., 2001)</td>
</tr>
<tr>
<td>gutless Ad (PEPCK)</td>
<td>VLDLR</td>
<td>LDLR-/- mice</td>
<td>- 40 % chol &gt; 7mo</td>
<td>(Chen et al., 2000)</td>
</tr>
<tr>
<td>AAV-2</td>
<td>VLDLR</td>
<td>LDLR-/- mice</td>
<td>significant, ≥ 20wk</td>
<td>(Lebherz et al., 2004)</td>
</tr>
<tr>
<td>AAV-7,AAV-8</td>
<td>LDLR</td>
<td>LDLR-/- mice</td>
<td>significant, ≥ 20wk</td>
<td></td>
</tr>
<tr>
<td>retrovirus</td>
<td>LDLR</td>
<td>WHHL</td>
<td>- 35 % chol &gt; 1y</td>
<td>(Pakkanen et al., 1999b)</td>
</tr>
</tbody>
</table>
**Gene transfer of LDLR locus**

The most recent development in FH gene therapy utilized a high-capacity HSV based amplicon vector for gene transfer of a complete 135kb LDLR genomic locus containing all 18 exons, introns and the native promoter (Wade-Martins et al., 2003). Efficient expression was detected in human and rodent cells *in vitro* for over three months; and the infectious LDLR locus retained the classical expression regulation by sterol levels in human cells. Regulation of LDLR expression at physiologically relevant levels may prove essential in FH gene therapy since high levels of unregulated LDLR expression may be toxic to cells. Indeed, it has been shown that high, unregulated levels of LDLR expression lead to a massive buildup of extracellular lipids and a formation of cytotoxic cholesterol-derived precipitates and crystals (Cichon et al., 2004).
3. AIMS OF THE STUDY

This study was implemented to analyze and improve the efficacy and safety of the currently available retroviral and lentiviral gene transfer vectors and technology for use in gene therapy of FH first in cell culture studies *in vitro*, and thereafter, in a rabbit and rat models *in vivo*. A further objective was to evaluate the potential of retroviral and lentiviral vectors for vascular *ex vivo* gene therapy in a rabbit model.

More specifically, the following issues were addressed:

→ Can VSV-G pseudotyped MMLV-retrovirus and FIV-lentivirus based vectors be used for *ex vivo* gene transfer of rabbit vascular SMC? Can autologous SMCs be re-implanted back to the adventitial surface of rabbit carotid artery using a collar model? Can apo E be used as a therapeutic agent in the adventitial *ex vivo* gene therapy approach? Can the adventitial *ex vivo* gene therapy approach be exploited for FH gene therapy?

→ Can HIV-1 based third-generation lentiviral vectors under the transcriptional control of a liver-specific promoter be used for *in vivo* gene therapy of FH without the need for liver resection?

→ Are traditional MMLV-retroviral and third generation HIV-1 based lentiviral vectors safe for use in liver gene therapy?

→ Is the novel improved version of the reverse Tc-regulated TetON system feasible (functional, tightly regulated, dose-dependent, effective for several switch-on/off cycles) in conjunction with the third-generation HIV-1 based lentiviral vectors for *in vitro* and *in vivo* gene transfers?
4. MATERIALS AND METHODS

The materials and methods used in articles I-IV are summarized below and described in detail in the indicated publications.

**TABLE 6. Methods used in this study**

<table>
<thead>
<tr>
<th>Method</th>
<th>Description</th>
<th>Used in</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA cloning</td>
<td>Lentiviral vector design and construction</td>
<td>II, IV</td>
</tr>
<tr>
<td>Production of viral vectors</td>
<td>Retroviral vector production and concentration</td>
<td>I, III</td>
</tr>
<tr>
<td></td>
<td>HIV vector production and concentration</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>FIV vector production and concentration</td>
<td>II, III, IV</td>
</tr>
<tr>
<td>Analysis of recombinant viral vectors</td>
<td>Retroviral vector titer determination: transduction</td>
<td>I, III</td>
</tr>
<tr>
<td></td>
<td>Lentiviral vector titer determination: transduction, ELISA (p24)</td>
<td>II, III, IV</td>
</tr>
<tr>
<td></td>
<td>Replication competent virus detection</td>
<td>II, III</td>
</tr>
<tr>
<td>Lipoprotein preparation</td>
<td>LDL isolation and labeling (DiI; Na(^{125})I)</td>
<td>I, II</td>
</tr>
<tr>
<td></td>
<td>Lipoprotein deficient serum (LPDS) preparation</td>
<td>I, II</td>
</tr>
<tr>
<td>Cell culture</td>
<td>Primary cell isolation and culture</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>Transduction</td>
<td>I, II, IV</td>
</tr>
<tr>
<td>In vitro analysis</td>
<td>Immunocytochemistry</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>ELISA (apo E)</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>Fluorescence microscopy</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td>Flow cytometry</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td>(^{125})I-LDL degradation assay</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td>DiI-LDL binding and uptake assay</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td>LacZ activity assay (x-gal staining, luminescence assay)</td>
<td>I, IV</td>
</tr>
<tr>
<td></td>
<td>DNA, RNA isolation; PCR, RT-PCR</td>
<td>I, II</td>
</tr>
<tr>
<td>In vivo procedures</td>
<td>Anesthesia, sacrificing, tissue sample collection</td>
<td>I, II, III, IV</td>
</tr>
<tr>
<td></td>
<td>Periadventitial collar implantation</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>Portal vein surgery</td>
<td>II, III</td>
</tr>
<tr>
<td></td>
<td>Stereotactic intracranial injection</td>
<td>IV</td>
</tr>
<tr>
<td></td>
<td>Blood sample collection</td>
<td>I, II, III</td>
</tr>
<tr>
<td>Analytical methods</td>
<td>Clinical chemistry assays of plasma samples</td>
<td>I, II, III</td>
</tr>
<tr>
<td></td>
<td>ELISA (apo E)</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>Immunohistochemistry</td>
<td>I, II, III, IV</td>
</tr>
<tr>
<td></td>
<td>DNA, RNA isolation (Trizol / QiaAmp)</td>
<td>I, II, III</td>
</tr>
<tr>
<td></td>
<td>PCR, RT-PCR</td>
<td>I, II, III, IV</td>
</tr>
<tr>
<td></td>
<td>Veterinarian pathologist examination</td>
<td>II, III</td>
</tr>
<tr>
<td>Statistical analysis</td>
<td>mean ± SEM or SD</td>
<td>I, II, III, IV</td>
</tr>
<tr>
<td></td>
<td>ANOVA</td>
<td>I, II, III</td>
</tr>
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</table>
**TABLE 7.** Plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Description</th>
<th>Source</th>
<th>Used in</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCMV-G</td>
<td>VSV-G envelope expression plasmid</td>
<td>T. Friedmann, UCSD, La Jolla, CA, USA</td>
<td>I, IV</td>
</tr>
<tr>
<td>pCF1Δenv</td>
<td>FIV vector packaging plasmid</td>
<td>E. Poeschla &amp; F. Wong-Staal, UCSD, La Jolla, CA, USA</td>
<td>I</td>
</tr>
<tr>
<td>pMDLg/pRRE</td>
<td>Packaging plasmid encoding HIV-1 gag-pol</td>
<td>I. Verma, Salk Institute, La Jolla, CA, USA</td>
<td>II, III, IV</td>
</tr>
<tr>
<td>pRSV-Rev</td>
<td>Packaging plasmid encoding HIV-1 rev</td>
<td>I. Verma, Salk Institute, La Jolla, CA, USA</td>
<td>II, III, IV</td>
</tr>
<tr>
<td>pMD.G</td>
<td>VSV-G envelope expression plasmid</td>
<td>I. Verma, Salk Institute, La Jolla, CA, USA</td>
<td>II, III</td>
</tr>
<tr>
<td>pHIV-CS</td>
<td>SIN HIV-1 vector (U3 region of the 5’LTR replaced with CMV promoter; 133bp deletion in U3 region of the 3’LTR) used as a backbone for HIV-rTA2²-M2 and HIV/TRE/LZ vectors</td>
<td>I. Verma, Salk Institute, La Jolla, CA, USA</td>
<td>IV</td>
</tr>
<tr>
<td>pUHrT61-1</td>
<td>hCMV – rTA2²-M2 cDNA containing expression plasmid</td>
<td>H. Bujard, ZMBH, Heidelberg, Germany</td>
<td>IV</td>
</tr>
<tr>
<td>pTRE2</td>
<td>TRE expression plasmid</td>
<td>Clontech, Palo Alto, CA, USA</td>
<td>IV</td>
</tr>
</tbody>
</table>

**TABLE 8.** Cell lines used in this study

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Description</th>
<th>Source</th>
<th>Used in</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMC</td>
<td>Primary rabbit vascular SMCs</td>
<td>central ear artery of WHHL, NZW rabbits</td>
<td>I</td>
</tr>
<tr>
<td>293GP</td>
<td>Human embryonal kidney epithelial cells expressing MMLV gag and pol genes</td>
<td>T. Friedmann, UCSD, La Jolla, CA, USA</td>
<td>I</td>
</tr>
<tr>
<td>293T</td>
<td>Human embryonic renal epithelial cells expressing SV40 large T antigen</td>
<td>ATCC</td>
<td>I, II, III, IV</td>
</tr>
<tr>
<td>NIH3T3</td>
<td>Mouse fibroblasts</td>
<td>ATCC</td>
<td>I</td>
</tr>
<tr>
<td>HT1080</td>
<td>Human fibrosarcoma</td>
<td>ATCC</td>
<td>I</td>
</tr>
<tr>
<td>WHHL</td>
<td>Primary WHHL rabbit skin fibroblasts</td>
<td>WHHL rabbit skin</td>
<td>II</td>
</tr>
<tr>
<td>PA317</td>
<td>Mouse fibroblasts</td>
<td>T. Friedmann, UCSD, La Jolla, CA, USA</td>
<td>III</td>
</tr>
<tr>
<td>209F</td>
<td>Rat fibroblasts</td>
<td>T. Friedmann, UCSD, La Jolla, CA, USA</td>
<td>III</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary cells</td>
<td>ATCC</td>
<td>IV</td>
</tr>
<tr>
<td>HepG2</td>
<td>Human hepatocellular carcinoma</td>
<td>ATCC</td>
<td>II</td>
</tr>
<tr>
<td>SupT1</td>
<td>Human T-lymphoblasts</td>
<td>ATCC</td>
<td>II</td>
</tr>
<tr>
<td>EAhy926</td>
<td>Human endothelial hybridoma cells</td>
<td>C-J. Edgell, UNC, Chapel Hill, NC, USA</td>
<td>IV</td>
</tr>
</tbody>
</table>
### TABLE 9. Viral vectors used in this study

<table>
<thead>
<tr>
<th>Vector backbone</th>
<th>(plasmid code)</th>
<th>Promoter</th>
<th>Transgene</th>
<th>Other elements</th>
<th>Source</th>
<th>Used in</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMLV</td>
<td>retro-lacZ (pBAG)</td>
<td>MMLV-LTR</td>
<td>lacZ</td>
<td>RSV / neo</td>
<td>C. Cepko, Harvard Medical School, Boston, MA, USA</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>LTR-LDLR (pLDRNL)</td>
<td></td>
<td>human LDLR</td>
<td></td>
<td>T. Friedmann, UCSD, La Jolla, CA, USA</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td>LTR-lacZ (pLZRNL)</td>
<td></td>
<td>lacZ</td>
<td></td>
<td>T. Friedmann, UCSD, La Jolla, CA, USA</td>
<td>III</td>
</tr>
<tr>
<td>FIV</td>
<td>FIV-lacZ (pCTRZLb)</td>
<td>CMV</td>
<td>lacZ</td>
<td></td>
<td>E. Poeschla &amp; F. Wong-Staal, UCSD, La Jolla, CA, USA</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>FIV-apoE3</td>
<td></td>
<td>human apoE3</td>
<td></td>
<td>A. Miyahara &amp; T. Friedmann UCSD, La Jolla, CA, USA</td>
<td>I</td>
</tr>
<tr>
<td>HIV-1 / SIN</td>
<td>CMV-LDLR</td>
<td>CMV</td>
<td>rabbit-LDLR</td>
<td>HIV-1 cppt; WPRE</td>
<td>Hanna Kankkonen, University of Kuopio, Finland and /or R. Marr &amp; I. Verma, Salk Institute, La Jolla, CA, USA</td>
<td>II, III</td>
</tr>
<tr>
<td></td>
<td>CMV-GFP</td>
<td></td>
<td>GFP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>LSP-LDLR</td>
<td>LSP</td>
<td>rabbit-LDLR</td>
<td>HIV-1 cppt; WPRE; LE6</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>LSP-GFP</td>
<td></td>
<td>GFP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIV-1 / SIN</td>
<td>HIV-TRE-LZ</td>
<td>TRE/CMV</td>
<td>lacZ</td>
<td></td>
<td>J. Koponen, University of Kuopio, Finland</td>
<td>IV</td>
</tr>
<tr>
<td></td>
<td>HIV-rtTA2S-M2</td>
<td>CMV</td>
<td>rtTA2S-M2</td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

### TABLE 10. Vector production protocols used in this study

<table>
<thead>
<tr>
<th>Vector</th>
<th>Envelope</th>
<th>Packaging plasmids</th>
<th>Packaging cells</th>
<th>Production protocol</th>
<th>Concentration method</th>
<th>Titered on / method</th>
<th>Titer Tu/ml</th>
<th>Used in</th>
</tr>
</thead>
<tbody>
<tr>
<td>retro-lacZ</td>
<td>VSV-G</td>
<td>pCMV-G</td>
<td>293GP</td>
<td>CaPO₄ transfection</td>
<td>50000g, 4°C, 2h</td>
<td>NIH3T3 / x-gal</td>
<td>1 × 10⁸</td>
<td>I</td>
</tr>
<tr>
<td>LTR-LDLR</td>
<td>MMLV-env</td>
<td>-</td>
<td>PA317</td>
<td>packaging cell</td>
<td>PEG precipitation</td>
<td>209F / neo</td>
<td>1 × 10⁵</td>
<td>III</td>
</tr>
<tr>
<td>LTR-lacZ</td>
<td>MMLV-LTR</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FIV-lacZ</td>
<td>VSV-G</td>
<td>pCMV-G</td>
<td>293T</td>
<td>CaPO₄ transfection</td>
<td>50000g, 4°C, 2h</td>
<td>HT1080 / x-gal</td>
<td>7.5 × 10⁸</td>
<td>I</td>
</tr>
<tr>
<td>FIV-apoE3</td>
<td>VSV-G</td>
<td>pCMV-G CF1Δenv</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CMV-LDLR</td>
<td>VSV-G</td>
<td>pMD.G (or pCMV-G)</td>
<td>293T</td>
<td>CaPO₄ transfection</td>
<td>68,000g, RT, 2h + 59000g, RT, 2h (or 50000g, 4°C, 2h)</td>
<td>HIV p24 ELISA and / or 293T / FCM</td>
<td>3.5 × 10⁹</td>
<td>II, III</td>
</tr>
<tr>
<td>CMV-GFP</td>
<td>VSV-G</td>
<td>pMDLg/PRRE</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LSP-LDLR</td>
<td>VSV-G</td>
<td>pRSV-Rev</td>
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<tr>
<td>LSP-GFP</td>
<td>VSV-G</td>
<td>pMD.G (or pCMV-G)</td>
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<td></td>
</tr>
<tr>
<td>HIV-rtTA2S-M2</td>
<td>VSV-G</td>
<td>-</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>HIV-TRE-LZ</td>
<td>VSV-G</td>
<td>-</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Vector – gene</td>
<td>PCR</td>
<td>Forward Primer</td>
<td>Reverse Primer</td>
<td>Fragm. Length bp</td>
<td>Anneal Temp. °C</td>
<td>Mg²⁺ Conc. mM</td>
<td>Cycles</td>
<td>Used in</td>
</tr>
<tr>
<td>--------------</td>
<td>-----</td>
<td>----------------</td>
<td>----------------</td>
<td>-----------------</td>
<td>----------------</td>
<td>--------------</td>
<td>--------</td>
<td>--------</td>
</tr>
<tr>
<td>Retro – lacZ</td>
<td>1</td>
<td>5’ – GGATTTGGGCGACGACT – 3’</td>
<td>5’ – GGGGCGGGACTATGGTG – 3’</td>
<td>317</td>
<td>60</td>
<td>1.5</td>
<td>35</td>
<td>I</td>
</tr>
<tr>
<td>FIV – ApoE3</td>
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<td>5’ – GCAAAATGCCCCACCACCCAC – 3’</td>
<td>5’ – GCGCAGGTAATCCCAAAAGC – 3’</td>
<td>491</td>
<td>68</td>
<td>1.5</td>
<td>38</td>
<td>I</td>
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<td></td>
<td>2</td>
<td>5’ – GGAAAGACTTAAGGCAGCGGC – 3’</td>
<td>5’ – CCCGGCTGCTCCACCTGGG – 3’</td>
<td>293</td>
<td>68</td>
<td>1.5</td>
<td>38</td>
<td>I</td>
</tr>
<tr>
<td>LSP – rabbit LDLR</td>
<td>1</td>
<td>5’ – CCAGTGGCCCAATGGCATCACC – 3’</td>
<td>5’ – GCAATACGTGATACAAAGGC – 3’</td>
<td>1069</td>
<td>64.5</td>
<td>1.5</td>
<td>36</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>5’ – GGACAAAGTGTCTGGAGGC – 3’</td>
<td>5’ – GCAGGCTATCCACATAG – 3’</td>
<td>877</td>
<td>64.5</td>
<td>1.5</td>
<td>38</td>
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<tr>
<td>rabbit GAPDH</td>
<td>1</td>
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<td>5’ – GCTAAGCATGGGTGTCAGGA – 3’</td>
<td>424</td>
<td>60</td>
<td>3</td>
<td>35</td>
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</tr>
<tr>
<td>LTR – human LDLR</td>
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<td>5’ – CCGTCTGTGGCTCCTATGAA – 3’</td>
<td>563</td>
<td>56</td>
<td>2.5</td>
<td>35</td>
<td>III</td>
</tr>
<tr>
<td>LTR – control</td>
<td>1</td>
<td>5’ – CCTGAAAATGACCTGTACC – 3’</td>
<td>5’ – GGCACGAGAATCACAACAC – 3’</td>
<td>937</td>
<td>56</td>
<td>1.5</td>
<td>40</td>
<td>III</td>
</tr>
<tr>
<td>LSP – GFP</td>
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<td>5’ – ACCCCGACCACATGAAGCAGC – 3’</td>
<td>5’ – CGTTGGGGCTTTGCTCAGGG – 3’</td>
<td>417</td>
<td>60</td>
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<td></td>
<td>2</td>
<td>5’ – ACGACGGCACTACAAGACC – 3’</td>
<td>5’ – ACCTTGATGCCCAGTTCTT – 3’</td>
<td>184</td>
<td>60</td>
<td>1.5</td>
<td>20</td>
<td>III</td>
</tr>
<tr>
<td>HIV – TRE/LZ</td>
<td>1</td>
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<td>5’ – TGAGGGAAGGACGAAGATAT – 3’</td>
<td>463</td>
<td>58</td>
<td>4</td>
<td>40</td>
<td>IV</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>5’ – GGGATCCTCTAGCAG – 3’</td>
<td>5’ – GGGATCCTCTAGCAG – 3’</td>
<td>329</td>
<td>58</td>
<td>4</td>
<td>40</td>
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<tr>
<td>HIV-rtTA2S-M2</td>
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<td>5’ – CGCGATGTGAGAGGAGAGA – 3’</td>
<td>267</td>
<td>58</td>
<td>4</td>
<td>40</td>
<td>IV</td>
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</table>
TABLE 12. Animals used in this study

<table>
<thead>
<tr>
<th>Animal model</th>
<th># of animals</th>
<th>Target tissue / delivery route</th>
<th>Transgene</th>
<th>Used in</th>
</tr>
</thead>
<tbody>
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<td>NZW rabbit</td>
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<td>carotid artery / <em>ex vivo</em></td>
<td>lacZ-retro</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td></td>
<td>FIV-lacZ</td>
<td></td>
</tr>
<tr>
<td>WHHL rabbit</td>
<td>6</td>
<td>carotid artery / <em>ex vivo</em></td>
<td>FIV-apo E3</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td></td>
<td>FIV-lacZ</td>
<td></td>
</tr>
<tr>
<td>WHHL rabbit</td>
<td>13</td>
<td>liver / portal vein</td>
<td>LSP-LDLR</td>
<td>II, III</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td></td>
<td>LSP-GFP</td>
<td>II, III</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td></td>
<td>CMV-GFP</td>
<td></td>
</tr>
<tr>
<td>WHHL rabbit</td>
<td>7</td>
<td>liver / portal vein</td>
<td>LTR-LDLR</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td></td>
<td>LTR-control</td>
<td></td>
</tr>
<tr>
<td>WHHL rabbit</td>
<td>9</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Wistar rat</td>
<td>20</td>
<td>brain / direct injection</td>
<td>HIV/rtTA2-M2 + HIV/TRE/LZ</td>
<td>IV</td>
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</table>

All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Kuopio, Kuopio, Finland.

TABLE 13. Antibodies and their ligands used in this study

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Target</th>
<th>Dilution</th>
<th>Application</th>
<th>Source</th>
<th>Used in</th>
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<tr>
<td>HHF-35</td>
<td>SMC</td>
<td>1:500</td>
<td>ICC, IHC</td>
<td>EnzoDiagnostics, Farmingdale, NY, USA</td>
<td>I</td>
</tr>
<tr>
<td>anti-β-gal</td>
<td>lacZ</td>
<td>1:100</td>
<td>IHC</td>
<td>Sigma, St. Louis, MO, USA</td>
<td>I</td>
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<tr>
<td>9-H8</td>
<td>Apo E</td>
<td>1:200</td>
<td>ELISA</td>
<td>Cappel/ICN Pharmaceuticals, Aurora, OH, USA</td>
<td>I</td>
</tr>
<tr>
<td>RAM-11</td>
<td>Macrophages</td>
<td>1:50 – 1:1000</td>
<td>IHC</td>
<td>Dako, Hamburg, Germany</td>
<td>I, II, III</td>
</tr>
<tr>
<td>MCA-805</td>
<td>T-cells</td>
<td>1:10</td>
<td>IHC</td>
<td>Serotec Inc., Raleigh, NC,USA</td>
<td>I, II, III</td>
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<tr>
<td>JL-8</td>
<td>GFP</td>
<td>1:100</td>
<td>ICC, IHC</td>
<td>BD Biosciences, Palo Alto, CA, USA</td>
<td>II</td>
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<tr>
<td>MCA-812</td>
<td>B-cells</td>
<td>1:10</td>
<td>IHC</td>
<td>Serotec Inc., Raleigh, NC,USA</td>
<td>III</td>
</tr>
<tr>
<td>2AB02B</td>
<td>rabbit IgG</td>
<td>1:100</td>
<td>IHC</td>
<td>Serotec Inc., Raleigh, NC,USA</td>
<td>III</td>
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</tbody>
</table>
5. RESULTS AND DISCUSSION

Gene therapy provides an attractive alternative for the treatment of FH when compared to the more traditional treatment methods, which are time consuming and expensive (LDL apheresis) or involve a significant morbidity and mortality risk (liver transplantation). The choice of an optimal gene transfer vector expressing an appropriate transgene and necessary regulatory elements is crucial for safe and successful somatic gene transfer. Furthermore, understanding the pathophysiology of the disease is essential for accurate tissue targeting and for selecting the pathway to introduce the gene.

5.1. Article I

5.1.1. Adventitial ex vivo gene transfer resulted in efficient gene expression

Gene transfer into the arterial wall provides an alternative for the treatment and prevention of vascular diseases. However, development of safe and efficient gene transfer vectors together with targeted delivery to the vascular wall has presented substantial challenges over the last decade (Ylä-Herttuala and Martin, 2000). We developed a novel local adventitial cell grafting system for use in vascular medicine. In our model, genetically engineered vascular SMCs were transplanted onto the adventitial surface of the rabbit carotid arteries using a silicone collar or biodegradable collagen sheet. In comparison to the intra-arterial vascular SMC transplantation based on endothelial scraping of the carotid artery (Clowes, 1997), or direct injection of transduced SMCs into the adventitia (Beltrao-Braga et al., 2002), our model provides a less aggressive method.

Vascular SMCs were chosen for the delivery target because they are well-differentiated cells which are easy to manipulate in vitro and relatively susceptible to transduction. In our study SMCs were also easily obtained from the rabbit ear artery. Vascular SMCs could also be derived from veins. In addition, adventitial fibroblasts could serve as target cells. However, they are usually less well defined than vascular SMCs. Other cell types prospective for ex vivo vascular gene therapy include vascular precursor or stem cells and leukocytes.

In our study, the vascular SMCs transduced with high efficiency with MMLV-retroviral and FIV-lentiviral vectors, and grafted around the rabbit carotid artery using a silicone collar or a biodegradable collagen sheet. The transduced cells were shown to reside in the adventitia within the encapsulated space of the collar during the whole follow-up period (up to 47 weeks). In this study, we used a non-degradable collar for cell grafting but collars can also be made of biodegradable materials (Pakkanen et al., 2000). On the other hand, if necessary a non-degradable collar containing the transduced cells can later be removed surgically. Even though positioning of a silicone collar around the carotid artery has been shown to induce vascular injury and hindrance of transmural flow, resulting in intimal thickening (De Meyer et al., 1997), no substantial intimal thickening at the site of cell implantation or SMC migration into the intima was observed in our study at any time point.
5.1.2. Gene expression was transient

Owing to their integration into the host cell genome, retroviral and lentiviral proviruses are highly influenced by the host-cell gene-regulation machinery. Thus, retroviral vector constructs with a variety of strong viral or cellular promoters are vulnerable to silencing effects that interfere with the promoter activity.

In vivo silencing of retroviral transcription is a well-documented phenomenon in a wide variety of cell types. Moreover, lentiviral vectors have been shown to suffer from promoter silencing even though lentiviral vectors have shown more resistance to in vivo silencing than classical MLV-based vectors (Blömer et al., 1997; Kafri et al., 1997; Gerolami et al., 2000; Indraccolo et al., 2002). The mechanism of provirus silencing is controversial and considerable diversity in variables affecting the transgene expression has been reported. The decrease of gene expression in retroviral systems has been suggested to be due to methylation of cytosine residues in retroviral LTR, and to occur through a two-step mechanism whereby methylation of transcriptional elements is followed by repressive alterations in chromatin structure (Pannell and Ellis, 2001). Moreover, the lack of transcription factors and presence of some cytokines have been shown to inhibit gene expression from the CMV promoter (Gribaudo et al., 1993; Svoboda et al., 2000). The mechanisms of lentiviral gene expression inefficiency have been suggested to be integration site-dependent shutdown (Jordan et al., 2001), species-restricted lower transcriptional levels (Price et al., 2002) and vector specific mRNA instability (O’Rourke et al., 2002). Species-specific, post entry barriers have also been shown to affect lentiviral gene transfer efficiency (Hofmann et al., 1999).

In our study, the transgene expression from the transplanted SMCs transduced ex vivo with retrovirus or FIV vectors declined after a few months or weeks (respectively). The apparent difference in the transgene silencing behavior between the retroviral and lentiviral vectors in our study may be due to the divergent promoter sequences of the vector constructs. The retroviral vector under the transcriptional control of retroviral LTR may not be as prone to silencing as the CMV-driven FIV vector. Indeed, a widely used CMV promoter is particularly prone to expression shutdown in the in vivo environment. It has been shown to be active only in cell types naturally infected by CMV (Baskar et al., 1996). Since the removal of all viral transcriptional elements from the viral LTR has been shown to circumvent retroviral vector shutdown (Prasad Alur et al., 2002), one approach to overcome the LTR or CMV-originated silencing of gene expression could be to produce vectors with an internal physiological tissue-specific promoter.

5.1.3. Extrahepatic apo E expression resulted in elevated plasma cholesterol levels

Treatment of FH by LDLR or VLDLR gene therapy requires the delivery of these genes to the liver. However, secreted therapeutic proteins, like LDLR-transferrin (Parise et al., 1999) may have potential in treating FH when expressed from a non-hepatic source. Apo E derived from hepatocytes has been shown to be more effective than non-hepatocyte derived apo E in plasma cholesterol clearance in the presence of LDLR (Raffai et al., 2003). However, the significance of hepatic vs. extrahepatic expression of apo E3 in
cholesterol clearance from plasma in the absence of functional LDLR is unclear, and controversial results have been reported (Linton et al., 1998; Yu et al., 2000). In our study human apo E3 expressed and secreted from an extrahepatic source, namely the genetically engineered SMCs implanted around the carotid artery of WHHL rabbits, resulted in impaired cholesterol homeostasis with elevated plasma cholesterol levels. This could be due to interference or blockade of LDL uptake, by large apo E rich remnant particles competing with LDL for binding to hepatic lipoprotein receptors, like LRP, and by non-lipoprotein bound apo E. Despite the increased plasma cholesterol levels, possible beneficial effects on atherosclerotic vascular disease cannot be excluded after vascular expression of apo E3. In fact, in an early study of Yamada et al. repeated intravenous injections of purified apo E to WHHL rabbits reduced atherosclerosis while plasma cholesterol levels remained unaffected (Yamada et al., 1992). Also physiological levels of macrophage apo E in the vessel wall have been shown to have anti-atherogenic properties in the absence of functional LDLR, and to affect later stages of plaque development (Fazio et al., 2002). However, a conflicting report demonstrated that elimination of macrophage-derived apo E from LDLR−/− mice reduced atherosclerosis by slowing hepatic VLDL production (Shi et al., 2004).

Functional LDLR has been demonstrated to be essential for efficient removal of extrahepatic (macrophage-derived) apo E-enriched lipoprotein remnants from the circulation, and consequently for normalization of plasma cholesterol levels and protection against atherosclerotic lesion development in apoE−/− mice (Van Eck et al., 2001). Hepatic apo E3 overexpression, on the other hand, has been shown to significantly inhibit the progression of atherosclerosis in LDLR−/− mice with little effect on plasma cholesterol levels (Thorngate et al., 2000; Tsukamoto et al., 2000b; Tangirala et al., 2001). Amelioration of hypercholesterolemia has been reported after moderate increase in liver-derived apo E3 levels in LDLR−/− mice (Osuga et al., 1998). Therefore, hepatic expression of exogenous apo E3 may be more likely to have therapeutic effects in the absence of functional LDLR.

Co-expression of LDLR and apo E has been shown to result in a substantial increase in large apo E-rich HDL particles; however, no additive effects of expression of the LDLR and apo E on cholesterol lowering or atherosclerosis reduction could be demonstrated (Kawashiri et al., 2001). Thus, apo E3 gene therapy may be beneficial only for FH patients with dysfunctional apo E.

5.2. Articles II and III

5.2.1. Liver-specific lentiviral vectors were functional in vitro

Third-generation SIN lentiviral vectors expressing rabbit LDLR or GFP under the transcriptional control of a synthetic liver-specific promoter (LSP) or a CMV promoter were produced to high titers and were shown to be free of replication-competent virus particles. Cell type specificity, transgene expression and LDLR functionality from the provirus were analyzed on HepG2 cells and WHHL rabbit skin fibroblasts transduced with LSP-LDLR, CMV-LDLR, LSP-GFP, and CMV-GFP. Vectors under the LSP promoter were shown to express transgenes only in the liver cells, whereas the CMV-driven lentiviral vectors transduced efficiently both liver cells and skin fibroblasts. The LDLR in the transduced cells was shown to be functional by 125I-LDL degradation and Dil-LDL uptake assays. RT-PCR
analysis confirmed the transgene expression in the CMV-LDLR and LSP-LDLR-transduced HepG2 cells and CMV-LDLR transduced WHHL fibroblasts 4 weeks after transduction.

5.2.2. Liver-directed gene transfer resulted in a long-term therapeutic effect and stable transgene expression in WHHL rabbits

Several research groups have demonstrated the therapeutic effects of LDLR gene transfer for hypercholesterolemia in animal models (see Chapter 2.3.7). Many of them showed short-term amelioration of hypercholesterolemia in WHHL rabbits and LDLR⁺/⁻ mice after in vivo LDLR gene transfer using non-viral vectors and first generation Ad vectors (see Table 5). We were the first to show the therapeutic effect of lentivirus-mediated gene transfer of LDLR in a rabbit model. In our studies marked long-term reductions in plasma cholesterol levels were detected in WHHL rabbits treated with in vivo intraportal injections of LSP-LDLR and LTR-LDLR. Seventy percent (9/13) of the LSP-LDLR treated rabbits demonstrated a positive treatment effect with lowered plasma cholesterol levels (25 ± 8%) during the first 4 weeks (Article II). An additional 23% of the LSP-LDLR treated rabbits (3/13) responded to the treatment only after 4 weeks, which may be related to late integration and expression of the provirus. One of the 13 LSP-LDLR-treated rabbits failed to respond to the LDLR treatment. In all the LSP-LDLR treated rabbits 44 ± 8% and 34 ± 10% decreases were detected in the plasma cholesterol levels at the 1-year and 2-year time points, respectively (Fig. 10). At the end of the 3-year follow-up period the plasma cholesterol levels of LSP-LDLR and LTR-LDLR treated rabbits were 26.5% and 20% (respectively) below the age-adjusted mean values of our WHHL rabbit colony (Article III). The CMV-GFP and LSP-GFP treated control animals followed the steady cholesterol-declining pattern typically observed in our WHHL colony (population control) (Fig. 10).

![Fig. 10](image-url)

**FIG. 10** Changes in total plasma cholesterol levels in WHHL rabbits after retrovirus (LTR-LDLR, n=7) and lentivirus (LSP-LDLR, n=13; LSP-GFP, n=10) –mediated gene transfer to the liver. Data are expressed as percentage (mean ± SD) changes in comparison with the pretreatment values in each treated animal. Dashed line (--) indicates the values (+SD / -SD) of age-matched control population (n=38).

In order to achieve long-term amelioration of FH, sustained production of therapeutic protein is fundamental. The ability of retroviral and lentiviral vectors to integrate into the host genome facilitate stable long-term expression of the transgene. In our study, the transgene expression was detected from liver for at least one year after the gene transfer.
WHHL rabbits that carry LDLR gene defect causing class 2/3 phenotype are a receptive animal model for FH gene therapy by LDLR gene transfer. However, patients with class 1 phenotype susceptible to immune reaction against transduced LDLR may benefit from overexpression of the other LDLR family receptors that bind apoB containing lipoproteins with high affinity. Also the anti-atherogenic apolipoproteins: apo A-I, apo A-I_Milano and apobec-1 may have therapeutic potential for gene therapy of hypercholesterolemia and subsequent atherosclerosis.

Prolonged correction of hyperlipidemia has also been demonstrated after gutless Ad vector and AAV-2–mediated gene transfer of VLDLR to LDLR<sup>-/-</sup> mice (Chen et al., 2000; Oka et al., 2001). Even though the gutless Ad vector is capable of producing highly efficient gene transfer to the mouse liver with >95% hepatocytes transduced after tail vein injection of 3·10<sup>11</sup> Ad particles, its extremely low rate of chromosomal integration eventually results in marked reduction in transgene expressing hepatocytes. The in vivo predominating episomal concatamer forms of AAV have been shown to convey the potential of long term transgene expression in quiescent and slowly dividing cells (Duan et al., 1998) thereby reducing the risks involved in chromosomal integration (Tenenbaum et al., 2003) and signifying the potential of AAV as gene delivery vector for FH gene therapy. Moreover, the novel AAV serotypes 7 and 8 have been shown to be potent vehicles for liver-targeted gene therapy (Gao et al., 2002). In the study of Lebherz and coworkers, vectors based on AAV-7 and AAV-8 were shown to be able to mediate up to 85% gene transfer efficiency and stable transduction of hepatocytes in vivo (Lebherz et al., 2004). Intraportal injection of 1·10<sup>12</sup> genome copies of AA-7 and AAV-8 vectors expressing LDLR under a liver-specific promoter resulted in significant and sustained (at least 20 weeks) reductions in plasma cholesterol levels in LDLR<sup>-/-</sup> mice.

In our study, the transduction efficiency in the rabbit liver after intraportal injection of 1·10<sup>9</sup> TU of LSP-GFP vector was modest with fewer than 0.01% of the liver cells expressing the GFP transgene. In comparison, the transduction efficiency achieved after LTR-lacZ gene transfer (7.5·10<sup>5</sup> TU) to hepatectomized and thymidine-kinase – ganciclovir treated WHHL rabbit liver was at least two times higher (Pakkanen et al., 1999b). Partial hepatectomy may have potential in increasing the transduction efficiency of the liver-directed lentiviral vectors; however, cellular damage induces the immune response to antigens associated with injured cells (Shi et al., 2003) and may therefore reduce overall transduction efficiency. By optimizing the vector dose corresponding to animal species and target tissue size, gene transfer efficiency may be markedly enhanced without the need for extensive surgery. Indeed, an equivalent dose (10<sup>5</sup> TU) of third-generation lentiviral vectors injected into the tail vein or portal vein of mice, an animal 100 times smaller than the rabbit, has been reported to result in markedly higher transduction efficiency (an average of 5%) in hepatocytes (Pfeifer et al., 2001; Follenzi et al., 2002; Tsui et al., 2002; Van den Driessche et al., 2002). The functionality and purity of the virus preparation may also affect the saturation and toxicity levels of transduction efficiency as well immune response against transduced cells. Therefore, the toxicity of vector preparations related to the presence of viral proteins, or of compounds derived from the production system, should be minimized, or ideally eliminated.

Viral titer has been considered the main obstacle in efficient retroviral gene transfer (Bowles et al., 1996; Kitten et al., 1997; De Godoy et al., 1999). A model of an in vivo, in situ perfusion of regenerating rat livers after partial hepatectomy has demonstrated
limited extrahepatic diffusion and improved retroviral transduction efficiency with up to 34.4% hepatocytes transduced (De Godoy et al., 2000). With this model the limits of a single passage through the liver via portal vein injection, namely the limited viral volume and thus the limited number of infectious viral particles delivered into the liver, and the limited duration (20 min) of portal vein clamping, could be diminished. In addition, the unsolicited leakage to the systemic circulation and possible biodistribution of the viral vector to other organs could be reduced.

5.2.3. Transgene expression was liver-specific

In addition to expressing the transgene on a long-term basis, an optimal gene transfer vector for the treatment of FH should be able to transfer genes specifically to the liver. Even though ex vivo gene therapy provides a means of specifically targeting isolated hepatocytes, direct in vivo gene therapy provides a less invasive method when compared to the in vitro transduction and subsequent transplantation of isolated and cultured hepatocytes. However, systemic delivery of the third generation lentiviruses containing ubiquitous promoters has been shown to result in transduction of and transgene expression from liver, spleen, and bone marrow in mice (Pfeifer et al., 2001; Tsui et al., 2002; Van den Driessche et al., 2002; Kootstra et al., 2003; Follenzi et al., 2004).

We demonstrated liver targeting by physical targeting of vector preparations through direct in vivo injection into the portal vein, and by transcriptional targeting. In vivo injection of the HIV-lentiviral vectors (LSP-LDLR, LSP-GFP) under the transcriptional control of a synthetic liver-specific ABP/TBG enhancer/promoter resulted in long-term liver-specific expression of the transgenes with no transgene expression in other tissues. However, in some animals, spleen, lung, heart, and muscle samples were positive for provirus DNA (1 – 15 months time points). In comparison, Follenzi and coworkers demonstrated in mice that gene transfer of a third-generation lentiviral vector containing a liver specific albumin promoter restricted the transgene expression to hepatocytes while spleen and bone marrow were also transduced (Follenzi et al., 2002).

The major advantage of the liver-specific promoters over the ubiquitous promoters is the lack of ectopic expression of the transgene in other than the target tissues, and the resulting prolonged transgene expression due to lower cellular and humoral immune responses in vivo (Follenzi et al., 2004). However, most of the liver-specific promoters lack the high transcriptional activity of the strong viral and cellular enhancers (Kramer et al., 2003). Constitutive highly active transgene expression, on the other hand, may not be imperative for gene therapy of FH. It has been suggested that non-physiological overexpression of LDLR from adenoviral vectors driven by strong viral promoters (RSV, CMV) induces an imbalance between the LDL uptake and metabolism rates leading to pathological accumulation of lipids and cholesterol in transduced cells in vitro and in vivo (Inoue et al., 1991; Heeren et al., 1999a; Cichon et al., 2004). Moreover, overexpression of LDLR in transgenic mice with human apo E4 has caused severe atherosclerosis with marked elevation of plasma cholesterol on a Western-type diet (Malloy et al., 2004). Therefore, a physiologically controlled expression element driving the transgene expression from the gene transfer vector may be vital for FH gene therapy.
5.2.4. Liver-directed in vivo gene therapy proved safe

5.2.4.1. Animal well-being and survival

WHHL rabbits treated with liver-directed gene transfer of MMLV retroviral vectors (6.5 – 7.5x10^5 TU) or HIV-1 based liver-specific lentiviral vectors (1x10^9 TU) tolerated the gene transfer procedure well and recovered from the operations without any problems, however three animals were lost due to surgery-related technical complications within 4 weeks of the gene transfer. All of the retrovirus-treated rabbits that were not sacrificed according to the study protocol (n=10) (Pakkanen et al., 1999b), and all the LSP-LDLR lentivirus-treated rabbits that were not sacrificed according to the study protocol, survived over two years (n=5) after the gene transfer. Meanwhile, 42% of the non-treated control rabbits died of natural causes (atherosclerotic stroke, n=1; intestinal fur ball blockade, n=2) before the age of 2 years. Two LSP-GFP treated rabbits died of atherosclerotic stroke before the 2 year time point (age 20.2 and 26.7 months), and one CMV-GFP treated rabbit died of acute necrotic colitis at the 30 months time point (age 35.8 months). One LSP-LDLR treated rabbit died of a parasitic fever (caused by Encephalitozoon cuniculi) at 26 months (age 30.4 months). The number of sacrificed animals by age, their cause of death and pathological findings are listed in Table 14.

5.2.4.2. Liver function and histology after gene transfer

In the follow-up of clinical chemistry parameters (CRP, ASAT, ALAT, AFOS), no permanent changes were detected during the three-year follow up of WHHL rabbits treated with MMLV-retroviral or lentiviral gene transfers, while normal liver function was observed with no sign of infection or inflammatory changes. Liver biopsy operation was shown to elevate the plasma ASAT and ALAT levels which returned to baseline levels within a week. No signs of inflammatory responses were detected in liver biopsy samples taken 2–4 weeks after the gene transfer as judged by general histology and immunostainings for macrophages and T cells. Veterinarian pathological examination revealed modest to frequent periportal lymphocyte infiltration and centrilobular vacuolization of the liver tissue in most of the rabbits in all the groups in this study, including the non-treated control group. No signs of inflammatory responses were detected from postmortem liver samples as judged by immunostainings for macrophages and T-cells.

5.2.4.3. Pathology, SAE

Veterinarian pathologist examination of the euthanized rabbits revealed symptoms related mainly to atherosclerosis. In all the groups in this study the heart contained scarred areas, some rabbits had atheroma plaques in coronary and pulmonary arteries and in most rabbits in all the groups the aorta was severely atherosclerotic with mineralization. Few rabbits had cholesterol accumulation or cholesterol clefts in heart valves, kidney medulla, and choroids plexus. In one rabbit cholesterol accumulation was found also in eyes (Fig. 11). One LTR-LDLR treated rabbit (age 33 months) showed decreased activity of testis (Fig. 11) and a few MMLV-retrovirus or lentivirus treated rabbits (age 30 – 41 months) showed uterine cystic hyperplasia or endometritis. Four rabbits were diagnosed with a neoplasm (Table 14). Of the other sacrificed, non-symptomatic animals, veterinarian pathologist examination revealed no obvious gene-transfer-related pathological findings.
**TABLE 14.** Number and age of WHHL rabbits sacrificed in the liver-directed gene transfer studies, cause of death and SAE of individual animals

<table>
<thead>
<tr>
<th>Virus</th>
<th>Vector</th>
<th>Rabbits</th>
<th>Animals sacrificed / died at age (months)</th>
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</thead>
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<td></td>
<td></td>
<td></td>
<td>0-11</td>
</tr>
<tr>
<td>MMLV retro</td>
<td>LTR-LDLR</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>LTR-control</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>HIV-1 lenti</td>
<td>LSP-LDLR</td>
<td>13</td>
<td>2C</td>
</tr>
<tr>
<td></td>
<td>LSP-GFP</td>
<td>10</td>
<td>1C</td>
</tr>
<tr>
<td></td>
<td>CMV-GFP</td>
<td>3</td>
<td>1S</td>
</tr>
<tr>
<td>none</td>
<td>none</td>
<td>9</td>
<td>2S</td>
</tr>
</tbody>
</table>

**Cause of death:**
- A\(^\text{1}\): Atherosclerosis;
- C\(^\text{7}\): Surgery related complication;
- S\(^\text{8}\): Sacrificed according to study protocol;
- ND: Not determined;
- Encephalitis;
- Necrotic colitis;
- Intestinal blockade.

**List of pathological findings:**
- Lowered activity of testis;
- Uterine adenocarcinoma;
- Lymphoma;
- Nephroblastoma;
- Trichoepithelioma;
- Eyes filled with cholesterol.

**FIG. 11**
Pathological findings of WHHL rabbits 14 – 27 months after retrovirus and lentivirus – mediated gene transfer to the liver. (A) Lowered activity of testis. Note atrophic seminiferous tubules. Lowered activity [L], normal activity [N] (LTR-LDLR rabbit, age 33 months); (B) Ocular accumulation of cholesterol crystals and lipophages accompanied by chronic inflammatory reaction and multinucleated cells. Lens [L], vitreous body [VB] (LSP-GFP rabbit, age 26.7 months).
5.2.4.4. Biodistribution

5.2.4.5. Provirus integration and possibility of insertional mutagenesis

Although retroviral and lentiviral vectors facilitate long-term transgene expression in vitro and in vivo, the chromatin milieu at the semi-random proviral integration sites (Bushman, 2002; Bushman, 2003) modulates all aspects of transgene expression, including the length of expression and expression level (Jordan et al., 2001). While MLV-retroviral vectors have been shown to prefer integration near the start of transcriptional units (Wu et al., 2003), HIV-1 targets active genes (Schröder et al., 2002). These distinct integration schemes of MLV-retroviruses and HIV-1-lentiviruses may result in different biosafety risk profiles of these vectors.

Insertion of a vector into a gene required for cell viability, especially if the cell contains only one functional copy of the gene, could lead to cell death. Viral-mediated gene integration can also result in significant changes in genome-wide methylation (Muller et al., 2001), which could result in epigenetic gene silencing and tumorigenesis (Baylin et al., 2001). Furthermore, the potential for abnormal regulation of cell growth resulting from retroviral, or lentiviral vector insertion into the host genome and the subsequent development of malignancy poses a major concern.

Replication-defective retrovirus–induced insertional mutagenesis has generally been assumed to be extremely rare. However, it has been reported in mice after ex vivo gene transfer of a marker gene to bone marrow (Li et al., 2002) and in two of eleven children treated with retroviral ex vivo gene transfer of yc gene to autologous hematopoietic stem cells (Hacein-Bey-Abina et al., 2003b). In the two children, retroviral integration in proximity to the LMO2 proto-oncogene promoter led to high-level of LMO2 expression and development of T-cell lymphoproliferation and leukemia. The LMO2 targeting was hypothesized to be due to a physical hotspot at the LMO2 locus or to growth advantage of the activated LMO2 mutants. Also the transgene, yc gene, has been suggested to act as a synergistic factor in driving the proliferation of a rare cell with the LMO2 targeted integration, and to induce leukemia (Baum et al., 2004; Dave et al., 2004).

In our long-term study of MMLV-retroviral and HIV-1 lentiviral gene transfer to the liver of WHHL rabbits four of 33 rabbits were diagnosed with neoplasms, of which three were malignant (Article III). Even though the overall frequency (12%) is rather high compared to the average frequency (2.6%) of neoplasms found in rabbits (Weisbroth, 1994) all the neoplasm forms found from in the rabbits in our study groups have been previously described in rabbits (Greene, 1959; Ingals et al., 1964; Allman et al., 1978; Weisbroth, 1994; Shibuya et al., 1999; Gomez et al., 2002). Especially uterine adenocarcinoma, found in a 4.5 year old doe in the LTR-LDLR study group, has been observed in over 60% of female rabbits over four years of age. The only neoplasm found positive for provirus DNA was the nephroblastoma isolated from an 11.5 month old male rabbit 6.5 months after the LSP-GFP mediated gene transfer. In addition to the nephroblastoma, liver, spleen and lung samples of the same rabbit were positive for provirus DNA. The relationship of the provirus to the development of the nephroblastoma currently remains unknown. However, a number of chromosomal loci involved in the development of human nephroblastoma have been identified (Dome and Coppes, 2002). Thus, the possibility that insertional mutagenesis may have been involved in the initiation of the nephroblastoma malignancy cannot be excluded. Therefore, it will be of interest to determine the flanking
DNA regions and the integration site of the provirus within the clonal composition of the nephroblastoma sample.

5.3. Article IV

5.3.1. Dox-regulated HIV-1 vector system showed tight control of gene expression

We generated a novel tightly regulated Dox–dependent lentiviral vector system that was shown to transduce both dividing and non-dividing cells in vitro and in vivo. Co-transduction of HIV/TRE/LZ and HIV/rtTA2²-M2 to CHO and EAhy926 cell lines resulted in Dox–dependent induction in both cell lines as measured by β-galactosidase activity. Transgene expression was detected by RT-PCR from co-transduced CHO cells after Dox-induction but not from uninduced cells. Provirus DNA was present in both induced and uninduced cells as determined by genomic PCR. In vivo gene transfer of the Dox-regulated lentiviral vectors to rat brain resulted in efficient gene transfer with all animals positive for both HIV/rtTA2S-M2 and HIV/TRE/LZ proviruses ten days after the transduction as detected by PCR. RT-PCR analysis showed the expression of the reverse transactivator in every animal. No lacZ transgene expression was detected in animals that were not treated with Dox (n=3) whereas rats receiving Dox (1 mg for 4 days) were found positive for lacZ expression by RT-PCR and X-gal staining.

The regulation was dose-dependent with below detection levels of β-galactosidase activity when no Dox was present and with full induction with Dox concentration of 500ng/ml. This suggests that the level of the therapeutic protein could be finely adjusted with Dox concentrations administered orally. Exposing of co-transduced CHO cells to cycles of Dox on/off inductions demonstrated the potential of rapid on/off switching of the transgene in long-term cultures. All three Dox-inductions during 13 cell passages after the co-transduction resulted in increases in the β-galactosidase activity.

In this study, we used a two-vector system in which the transgene under the inducible promoter and the construct driving the transactivator expression were supplied in separate vectors. However, for clinical trials, a single vector encoding both regulator and inducible genes would be preferable and of great interest with respect to safety and dosage control issues. Indeed, a number of expression cassettes coexpressing the transgene, the transactivator (rtTA2S-M2) and/or the tTS in a single vector have been constructed and their functionality has been demonstrated in vitro and in vivo (Lamartina et al., 2002; Salucci et al., 2002; Lamartina et al., 2003; Mizuguchi et al., 2003; Chenuaud et al., 2004; Vogel et al., 2004). In these vectors, optimal inducibility and minimal background activity in the absence of the inducer were achieved when the promoters driving the expression of the transgene and the rtTA2²-M2 transactivator were positioned apart from each other, but oriented in the same direction, or alternatively, when they were separated by a non-coding spacer independent of the orientation of the transcriptional unit. Combining the rtTA2²-M2 with the tTS was shown to result in more stringent control than with either alone; however, the maximum activity of rtTA2²-M2 was slightly reduced by co-expressed, IRES-dependent tTS (Lamartina et al., 2003). The lower maximum activity of IRES-based constructs was suggested to be due to a higher level of expression of the activator from monocistronic than from bicistronic transcripts.
Incorporating a liver-specific promoter into the regulated lentiviral vector system may further improve the vector safety for FH gene therapy. A combination of tight regulation and liver-specificity has been reported in conjunction with gutless adenoviral vectors containing a mifepristone-inducible system (Wang et al., 2004) and in a Dox-dependent (tetON) plasmid system containing the trans-activator and transgene under liver specific promoters in a single plasmid vector (Zabala et al., 2004). The Dox-dependent basal and final protein levels were shown to depend on the strength of the promoter that directs the transcriptional activator as well as on the relative orientation of the two genes in the same plasmid vector.

5.4. Aspects of the future development of gene delivery strategies

In this study, we have demonstrated the value of the current gene transfer vectors for FH gene therapy; however, the construction of vector systems directed towards targeted and regulated expression of a carefully chosen therapeutic gene from a distinct target organ, with accurate choice of gene delivery approach, will improve the safety and efficacy of gene therapy applications. In addition, the potential risks of insertional mutagenesis and germ-line alteration as well as issues concerning the possibility of mosaic virus generation, the possibility of the rescue of integrated vectors by wild type helper viruses, the possibility of replication-competent virus formation, and the potential for reactivation of dormant endogenous viruses should be taken into account in the development of new gene delivery strategies. Ultimately the applicability of gene therapy depends on the balance of risk over against alternative treatments.
6. SUMMARY AND CONCLUSIONS

1. We established a new *ex vivo* gene transfer technique for potential use in long-term local or systemic gene therapy applications during vascular surgery. VSV-G pseudotyped MMLV-retroviruses were potent vehicles for stable gene transfer to autologous rabbit SMCs transplanted around the carotid artery of a rabbit model. Transgene expression from FIV vectors under transcriptional control of CMV promoter were silenced more rapidly *in vivo* than transgenes expressed from MMLV vectors driven by viral LTR. FIV-mediated adventitial *ex vivo* gene transfer of human apo E3 resulted in efficient, albeit transient, apo E secretion into rabbit plasma with subsequent elevation in plasma cholesterol levels demonstrating the importance of careful evaluation of transgene properties and of the delivery route when the aim is to treat hypercholesterolemia.

2. We demonstrated the potential value of the third-generation self-inactivating liver-specific lentiviral vectors in the treatment of FH using direct intraportal liver gene therapy without the need for liver resection. Liver-specific HIV-1 based lentiviral vector–mediated gene transfer of LDLR to the liver of WHHL rabbits resulted in stable transgene expression and decrease in plasma cholesterol levels up to three years after the gene transfer (end of study). Normal liver function and morphology were reported and no signs of major infection or inflammatory changes were detected.

3. We demonstrated the long-term safety of MMLV-retroviral and HIV-1 lentiviral vectors as vehicles for liver-directed *in vivo* gene transfer. We demonstrated prolonged lifespan of rabbits treated with LDLR encoding vectors. No obvious gene transfer related pathological findings were detected in the treated WHHL rabbits up to four years after the gene transfer. However, four of the 33 treated and control rabbits were found to show neoplasms, one of which was positive for provirus.

4. We constructed a novel Dox–regulated self-inactivating HIV-1 lentiviral vector system and showed its functionality and applicability *in vitro* and *in vivo*. We demonstrated that the dose-dependent and repeatedly inducible vector system was capable of efficient expression and explicit regulation of the transgene in rat brain *in vivo*.

In conclusion, we developed a novel adventitial *ex vivo* gene transfer technique for potential use in gene therapy applications during vascular surgery. We demonstrated the value of HIV-1 lentiviral vectors, and also the standard MMLV-retroviral vectors, in the treatment of FH in WHHL rabbits. We constructed a novel regulated lentiviral vector system that has potential in improving the vector safety in FH gene therapy.
7. REFERENCES


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81


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