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JONNA KOPONEN

Lentiviral Vector for Gene Transfer

A Versatile Tool for Regulated Gene Expression, Gene Silencing and Progenitor Cell Therapies

Doctoral dissertation

To be presented by permission of the Faculty of Natural and Environmental Sciences of the University of Kuopio for public examination in Auditorium, Tietoteknia building, University of Kuopio, on Saturday 19th April 2008, at 1 p.m.

Department of Biotechnology and Molecular Medicine
A.I. Virtanen Institute for Molecular Sciences
University of Kuopio

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ABSTRACT

Gene therapy holds promise to improve the treatment options of both inherited and acquired diseases like cardiovascular diseases. There is still a need for optimal gene delivery vectors for enhanced efficacy and safety. The aim of this research was to apply the human immunodeficiency virus-1 (HIV-1) derived lentiviral vector (LV) for different approaches of gene therapy. LVs have the ability to integrate into the host cell genome and are thus suitable for applications requiring long-term expression of the therapeutic gene. However, in such applications, there is a need to regulate the level of therapeutic protein expression. During this research, a LV system was developed and its efficacy tested for the capacity to adjust the amount of protein expressed or to switch the expression on and off by the addition of the antibiotic, doxycycline. This study demonstrates the ability to fine-tune the expression of a LV delivered therapeutic gene by adjusting the concentration of doxycycline within a range which can be achieved by oral administration. It also shows the functionality of the system *in vivo* in rat brain. Another approach for therapeutic gene regulation is to utilize an endogenous, pathophysiological stimulus of the target tissue. We designed a series of vectors exploiting a novel approach, oxidative stress induced gene regulation. This is an alluring concept for cardiovascular gene therapy applications, since oxidative stress plays a role in a number of cardiovascular diseases. Our results showed that antioxidant response elements introduced into LVs can be used for oxidative stress induced gene expression. Also, we studied whether LVs can be applied in a gene knock-down approach exploiting a small hairpin RNA (shRNA) – based method. Our results demonstrate efficient, long-term gene silencing by LV-shRNA both in cell culture and mouse brain. As a potential therapeutic application for LVs, we studied their ability to transduce cord blood (CB) derived progenitor cells and found that these cells could be efficiently transduced by LVs. CB is a unique source for hematopoietic stem cells and other progenitor cells, which can be exploited for novel cell therapy approaches. We also assessed the therapeutic potential of progenitor cells in a nude mouse model of hindlimb ischemia. We did not detect engraftment of progenitor cells into the target tissue. However, our results show enhanced regeneration of the ischemic muscle by progenitor cell injections. Based on these results, we suggest that progenitor cells may be beneficial in the recovery of injured tissue by indirect mechanisms. Taken together, this study demonstrates the applicability of HIV-1 based vectors as a basic research tool and a potential gene therapy vector, particularly for *ex vivo* approaches such as progenitor cell therapies.

National Library of Medicine Classification: QU 470, QU 475, QZ 52, QW 168.5.H6, QU 325
Medical Subject Headings: Gene Therapy; Gene Transfer Techniques; Genetic Vectors; Lentivirus; HIV-1; Gene Expression Regulation; Gene Silencing; Doxycycline; Brain; Rats; Oxidative Stress; Umbilical Cord; Blood; Stem Cells; Transduction, Genetic; Cardiovascular Diseases/therapy; Ischemia; Muscle, Skeletal; Mice

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ABBREVIATIONS

AAV	adeno-associated virus
AIDS	acquired immunodeficiency syndrome
ALS	amyotrophic lateral sclerosis
ARE	antioxidant responsive element
BMC	bone marrow mononuclear cell
CB	cord blood
CMV	cytomegalovirus
CNS	central nervous system
DEM	diethyl maleate
Dox	doxycycline
dsRNA	double stranded RNA
EIAV	equine infectious anemia virus
ELISA	enzyme linked immunosorbent assay
EPC	endothelial progenitor cell
ESC	embryonic stem cell
FGF	fibroblast growth factor
FKBP12	FK506-binding protein of 12 kDa
FRAP	FKBP rapamycin-associated protein
FRB	FKBP rapamycin-binding
FH	familial hypercholesterolemia
FIV	feline immunodeficiency virus
GAPDH	glyceraldehyde-3-phosphate
HGF	hepatocyte growth factor
HIF	hypoxia inducible factor
HIV-1	human immunodeficiency virus 1
HO-1	heme oxygenase 1
HSC	hematopoietic stem cell
HSV-tk	herpes simplex virus thymidine kinase
IHC	immunohistochemistry
KRAB	Kruppel-Associated Box
LDL	low density lipoprotein
LTR	long terminal repeat
LV	lentiviral vector
LVEF	left ventricular ejection fraction
MACS	magnet activated cell sorting
MCP-1	monocyte chemoattractant protein-1 gene
MCS	mesenchymal stem cell
MOI	multiplicity of infection
miRNA	microRNA
MLV	murine leukemia virus
Oas-1a	2',5'-oligoadenylate synthetase 1a gene
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
Pol III	RNA polymerase III
piRNA	Piwi interacting RNAs
RISC	RNA-induced silencing complex
RNAi	RNA interference
ROS	reactive oxygen species
RT-PCR	reverse transcription polymerase chain reaction
rtTA	reverse tetracycline transactivator
shRNA	small hairpin RNA
siRNA	small interfering RNA
SIN	self-inactivating
SIV	simian immunodeficiency virus
ssRNA	single stranded RNA
Tet	tetracycline
tetO	tetracycline operator
TetR	tetracycline repressor protein
TNF-α	tumor necrosis factor alfa
TRE	tetracycline-response element
tTA	tetracycline transcriptional activator
tTS	tetracycline trans-silencer
TU	transducing units
VCAM-1	vascular cell adhesion molecule 1
VEGF	vascular endothelial growth factor
VSV-G	vesicular stomatitis virus G-protein
qPCR	quantitative polymerase chain reaction
qRT-PCR	quantitative reverse transcription polymerase chain reaction
ZFHD1	zinc-finger homeodomain fusion 1

LIST OF ORIGINAL PUBLICATIONS

I Jonna K. Koponen, Hanna Kankkonen, Jani Kannasto, Thomas Wirth, Wolfgang Hillen, Hermann Bujard and Seppo Ylä-Herttuala.
Doxycycline-regulated lentiviral vector system with a novel reverse transactivator rtTA2^{S}-M2 shows a tight control of gene expression in vitro and in vivo.
Gene Therapy 2003 Mar;10(6):459-66.

II Hanna Hurttila, Jonna K. Koponen, Emilia Kansanen, Henna-Kaisa Jyrkkänen, Annukka M. Kivelä, Riina Kylätie, Seppo Ylä-Herttuala and Anna-Liisa Levonen.
Oxidative stress inducible lentiviral vectors for gene therapy.
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Umbilical cord blood-derived progenitor cells enhance muscle regeneration in mouse hindlimb ischemia model.
Molecular Therapy 2007 Dec;15(12):2172-7.

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Escherichia coli DNA contamination in AmpliTaq Gold polymerase interferes with TaqMan analysis of lacZ.
Molecular Therapy 2002 Mar;5(3):220-2.

* Authors with equal contribution. Also some unpublished data is presented.

CONTENTS

INTRODUCTION

The concept of gene therapy, an approach to treat disease by either modifying the gene expression or correction of abnormal genes, has been around since the first gene therapy applications were introduced in the early 1980s. By administration of DNA rather than a drug, many different diseases are currently being investigated as candidates for gene therapy. This has been influenced by the rapidly increasing knowledge of the human genome and its regulatory mechanisms. However, the success of clinical therapies is still limited due to the lack of optimal gene transfer vectors. Rather than aiming at a single vector that is suitable for all genetic therapies, different vectors with qualities tailored for each application is the objective. The most important features and requirements should be taken into account. These include the vector tissue tropism, the duration of gene expression, the possible genomic integration ability, the feasibility to switch off gene expression or to regulate its expression, the expected immune responses elicited by the vector, the possible need to repeated vector administrations, and safety and ethical considerations. Adenoviral vectors have been extensively and successfully used both in experimental and clinical settings and may be considered as the standard vector of choice for many applications that need short-term therapeutic gene expression. However, for therapies requiring long-term therapeutic gene expression, there is not such a standard vector. Also, when long-term expression of the therapeutic gene is desired, distinct safety and efficacy concerns need to be considered, such as the ability to regulate therapeutic gene expression within the therapeutic window, to switch off expression when required and the possibility of insertional mutagenesis in the case of integrating vectors. The increased data on HIV-1 molecular biology has been applied to gene therapy research to enable HIV-1 to be used as a gene therapy vector with a feature of stable integration into the target cell genome. With the latest generation HIV-1 vectors, only a minute proportion of the viral genome is exploited, both in the vector and the production system, resulting in a vector which does not transfer any viral genes, thus attenuating safety concerns. This thesis has focused on the development and appliance of HIV-1 derived lentiviral gene transfer vectors for regulated expression, gene silencing and progenitor cell therapies. Also, the efficacy of LV in animal models of cardiovascular diseases is evaluated.

REVIEW OF THE LITERATURE

GENE THERAPY

General concept

The basic concept of gene therapy is to insert genes into the somatic cells of an individual in order to treat a disease, either inherited or acquired. Hereditary diseases targeted by gene therapy usually aim at the correction of the function of one abnormal gene. However, in acquired diseases the activity of several genes is disturbed and the disease caused by these combined effects makes the gene therapy approaches of such diseases less straightforward.

Cardiovascular diseases

Despite major advances in therapies, cardiovascular diseases are still the leading cause of death in the Western world and are therefore attractive targets for gene therapy. Gene therapy approaches have been directed to hyperlipidemias, promotion of therapeutic angiogenesis in myocardium and skeletal muscle, post-angioplasty restenosis, hypertension, heart failure, the prevention of thrombosis and the protection of vascular by-pass grafts (reviewed by Ylä-Herttuala *et al.*, 2000, Rissanen *et al.*, 2007, Vincent *et al.*, 2007).

To date, the promotion of blood vessel growth, that is, therapeutic angiogenesis, has been the most studied aspect of cardiovascular gene therapy. Gene transfer for therapeutic angiogenesis has been targeted to both myocardial and lower limb ischemia, which are induced by atherosclerosis. Genes for vascular endothelial growth factors (VEGFs) (Mack *et al.*, 1998, Gowdak *et al.*, 2000, Arsic *et al.*, 2003, Rutanen *et al.*, 2004, Stewart *et al.*, 2006), fibroblast growth factors (FGFs) (Giordano *et al.*, 1996, Ueno *et al.*, 1997, Rissanen *et al.*, 2003a), platelet-derived growth factors (PDGFs) (Richardson *et al.*, 2001, Cao *et al.*, 2003, Li *et al.*, 2005b), and angiopoietins (Arsic *et al.*, 2003, Cho *et al.*, 2005) have been the mostly used therapeutic genes. Several clinical trials for therapeutic angiogenesis have been carried out (Rissanen *et al.*, 2007).

For genetic cardiovascular diseases, gene therapy is a conceivable treatment option especially for familial hypercholesterolemia (FH), which is caused by the lack of functional LDL-receptor. This results in serious hyperlipidemia, especially in individuals whose both alleles are defective. Promising results have been attained with LDL-receptor gene transfer targeted to the liver in animal models (Pakkanen *et al.*, 1999, Kankkonen *et al.*, 2004, Lebherz *et al.*, 2004).

Other targets

Other genetic disorders are also potential candidates for gene therapy. Probably the most known gene therapy studies are those directed to the primary immunodeficiency disorder SCID/ADA (Blaese *et al.*, 1995, Muul *et al.*, 2003). Other candidates with published gene therapy research include cystic fibrosis (Flotte *et al.*, 2007), inherited metabolic disorders like phenylketonuria (Ding *et al.*, 2006), lysosomal storage disorders like Gaucher's disease (Sands *et al.*, 2006), hematological disorders like hemophilias, hemoglobinopathies, anemias and thalassemias (Nathwani *et al.*, 2005) and muscular dystrophies (Foster *et al.*, 2006).

Cancer gene therapy covers a number of alluring treatment options for different types of cancer. These applications can be divided into three subgroups: immunotherapy, oncolytic therapy and gene transfer therapy. Immunotherapy covers the studies in which a cancer vaccine is produced by engineering

cancer cells to be more recognizable by the immune system. This can occur by the *in vitro* transfer of gene producing molecules which are pro-inflammatory (Simons *et al.*, 2006). Oncolytic gene therapy vectors are viruses which are modified to infect cancer cells and induce cell death through the propagation of the virus, expression of cytotoxic proteins and cell lysis (Rein *et al.*, 2005). The gene transfer concept involves the transfer of suicide genes (genes that cause cellular death when expressed) (Rasmussen *et al.*, 2002), antiangiogenesis genes (Ohlfest *et al.*, 2005) and cellular stasis genes (Eastham *et al.*, 2000). Suicide gene therapy, utilizing herpes simplex virus thymidine kinase (HSV-tk) gene transfer to a tumor followed by ganciclovir treatment, has shown potential in the treatment of the malignant brain tumor, glioblastoma (Immonen *et al.*, 2004).

In addition to genetic disorders and glioblastoma, there are a number of other pathologies which make the brain an important gene therapy target tissue. Gene therapy treatments have been engineered for neurodegenerative disorders like Alzheimer's disease, amyotrophic lateral sclerosis, Parkinson's disease (Cardone, 2007) and for multiple sclerosis (Martino, 2003), CNS injuries (Murray *et al.*, 2001), epilepsy (Noe' *et al.*, 2007) and cerebrovascular diseases like stroke (Jacobs *et al.*, 2005).

Gene therapy has also been studied for the treatment of viral infections, mostly for the HIV-1 infection (Dropulic *et al.*, 2006). In terms of endocrine and metabolic disorders, diabetes is probably the most abundantly studied (D'Anneo *et al.*, 2006).

GENE TRANSFER VECTORS

Overview of vectors

Principles of gene transfer

Gene transfer aims at the delivery of nucleic acids across the cell membrane and into the nucleus of target cells. These genes are introduced into the cells in vectors. The efficiency of therapeutic gene transfer is dependent on the ability of the vector to deliver the gene into target cells and on the transgene expression level. Different target tissues and cells require vectors with distinct properties. Also, the vector choice is dependent on the application, for example, on the desired duration of expression of the therapeutic gene. The development of optimal gene transfer vectors is one of the key issues in determining the applicability of gene therapy in clinical settings.

Nonviral gene transfer vectors

The concept of nonviral gene transfer covers plasmid vectors or oligonucleotides which are introduced into the cells either as naked DNA or by chemical or physical approaches. Early experiments suggested that a simple injection of naked DNA produced remarkable gene transfer efficiency in the muscle (Wolff *et al.*, 1990), liver (Hickman *et al.*,1994) and skin (Choate *et al.*,1997). However, gene transfer by naked plasmids has not proven efficient enough for *in vivo* applications. In terms of chemical approaches, DNA is formulated into condensed particles by using, for example; cationic lipids (Liu *et al.*, 2003a) or polymers (Neu *et al.*, 2005) as carriers. These compounds are useful for enhanced gene transfer efficiency *in vitro*. Physical approaches for gene transfer utilizing mechanical (particle bombardment or gene gun), electric (electroporation), ultrasonic, hydrodynamic or laser-based energy to penetrate the cell membrane have been explored (Gao *et al.*, 2007). Although

these methods may be efficient *in vitro*, they have not shown remarkable potency *in vivo*. In conclusion, nonviral vectors have not been able to improve upon the performance of viral vectors to date.

Viral gene transfer vectors

In viral vectors, parts of the native viral genome have been deleted and replaced by genetic elements needed for the expression of the therapeutic gene. Genetic engineering has meant that viral vectors do not carry the genetic elements needed for the formation of all the essential components of a virus particle such as viral structural proteins and enzymes. Therefore, they are not able to replicate and are not infectious. Elements for viral vectors are provided *in trans* by virus producing systems such as helper constructs or packaging cell lines, increasing the safety of the vectors. The tropism of viral vectors, that is the ability to transduce cells of different tissue types or animal species, may be modified by coating the viral particle with envelope proteins from another virus with known specificity.

Vectors based on DNA viruses

Adenoviral vectors are non-enveloped, double stranded DNA vectors, which deliver genes efficiently into a wide variety of cells both *in vitro* and *in vivo*, and are the most widely used viral vectors so far. Wild-type human adenoviruses are a general cause of benign respiratory and other infections in humans. Approximately 50 serotypes of adenovirus have been identified and gene therapy vectors derived from serotypes 2 and 5 are most commonly used. Adenoviral vectors are able to transduce both dividing and non-dividing cells. Their genome remains extrachromosomal in the host cell resulting in a transient expression of the therapeutic gene. Conditionally replicating adenoviral vectors have shown promise in cancer gene therapy (Carette *et al.*, 2007, Ranki *et al.*, 2007). However, a major problem of adenoviral vectors is their immunogenicity and toxicity (Liu *et al.*, 2003b). In fact, on one occasion, gene therapy using adenoviral vector delivery caused the death of a patient involved in a clinical trial for the treatment of ornithine transcarbamylase deficiency due to a huge immune response triggered by the vector (Marshall, 1999).

Adeno-associated virus (AAV) derived vectors are single-stranded DNA vectors. The prototype of AAV gene therapy vectors is based on serotype 2. However, recent data from mouse experiments has shown that vectors derived from AAV serotype 8 show superior tropism for the liver (Nakai *et al.*, 2005) and those from serotype 6 for cardiac and skeletal muscles (Gregorevic *et al.*, 2004). Also, serotype 9 vectors have been shown to transduce the myocardium more efficiently than serotype 8 vectors (Inagaki *et al.*, 2006). AAV vectors are able to transduce both dividing and quiescent cells and although they remain extrachromosomal, long-term gene expression is achieved. In one clinical trial, the duration of therapeutic gene expression for up to several years has been reported (Jiang *et al.*, 2006). Native AAV is a parvovirus that is non-pathogenic in humans. In addition, AAV vectors are considered to be rather low in immunogenicity. However, a major drawback is the cumbersome virus production procedure, which is extremely difficult to upscale (Xiao *et al.*, 1998).

Other less frequently used gene transfer vectors derived from DNA viruses are those from baculovirus (Lehtolainen *et al.*, 2002), herpex simplex virus (Gao *et al.*, 2006) and Epstein-Barr -virus (Hellebrand *et al.*, 2006).

Vectors based on RNA viruses

Retroviral vectors are based on RNA viruses. The most extensively used retroviral

vectors are those derived from oncoviruses, such as murine leukemia virus (MLV) or lentiviruses (LV), such as human immunodeficiency virus-1 (HIV-1), simian (SIV), equine (EIAV) or feline (FIV) immunodeficiency viruses (reviewed by (Sinn *et al*. 2005)). Retroviral vectors carry their genetic information in the form of single stranded RNA (ssRNA). In the target cell, viral RNA is reverse-transcribed into double stranded DNA, which is then integrated into the host cell genome resulting in long-term transgene expression. The prototype of retroviral gene transfer vectors is derived from MLV (Mann *et al*., 1983). MLV vectors are only able to transduce dividing cells and they have been used for both *ex vivo* and *in vivo* applications. In clinical trials, MLV vectors have been used for the treatment of cancer, inherited and acquired monogenic disorders and AIDS. However, in a trial for the treatment of X-linked SCID patients with MLV vector gene transfer to hematopoietic stem cells *ex vivo*, vector induced leukemias were reported raising safety concerns (Hacein-Bey-Abina *et al*., 2003). In contrast, there have been no reports of insertional mutagenesis in ADA/SCID patients treated with MLV vector gene transfer to hematopoietic stem cells (Aiuti *et al*., 2007). Thus, the risks of insertional mutagenesis may depend on the vector system, the targeted cell types, the site of integration, the transgene and the underlying immunodeficiency, as suggested by the molecular analysis of the three affected patients' cells from the X-SCID trial (Hacein-Bey-Abina *et al*., 2003).

In contrast to MLV vectors, lentiviral vectors (LVs) are able to transduce both quiescent and dividing cells, which is an advantage for many experimental and clinical settings. Of the lentiviruses used for gene transfer, HIV-1 derived vectors are the most advanced and owing to species-specific restrictions, it is likely that they are more efficient than animal LVs for the transduction of many types of human cells. HIV-1 derived vectors are described in detail in the next chapter.

Lentiviral HIV-1 derived vectors

HIV-1 biology and genome

In the late 1970s and early 1980s, a new syndrome, with symptoms of immunologic dysfunction, was discovered in United States and Europe. A connective laboratory finding was the depletion of CD4+ T-lymphocytes in affected individuals. The disease was termed acquired immunodeficiency syndrome (AIDS). Later, a new retrovirus was isolated from both AIDS patients and infected, asymptomatic individuals from various risk groups. The new retrovirus causing a slow, progressive disease affecting the immune system and exhibiting morphologic and genetic characteristics typical of the lentivirus genus (*Lentivirinae*), was named human immunodeficiency virus (HIV) (Coffin *et al*., 1986) and subsequently HIV-1. Other lentiviruses include HIV-2 and nonhuman lentiviruses such as the feline immunodeficiency virus (FIV) of cats, simian immunodeficiency virus (SIV) of monkeys, bovine immunodeficiency virus (BIV) of cattle, equine infectious anemia virus (EIAV) of horses, Maedi/Visna virus and caprine arthritis encephalitis virus of sheep and goats.

Retroviral virion particles are spherical in shape and surrounded by a lipid membrane bilayer envelope with projections of glycoproteins. There is a spherical layer of protein under the membrane and an internal nucleocapsid whose shape varies from virus to virus. The members of the lentivirus genus are complex retroviruses with the morphology of cylindrical or conical cores.

Typically, all retroviruses carry three major genes that are critical for retroviral replication and assembly, *gag*, *pol* and *env*. The more complex retroviruses contain accessory

genes that are essential or contribute to efficient virus replication and persistence. HIV-1 encodes six additional genes: *tat*, *rev*, *vif*, *vpu*, *vpr*, and *nef*. (**Figure 1** and **Table 1**). The HIV-1 virion has a diameter of ~110 nm. The viral SU and TM glycoproteins are inserted into the lipid membrane surrounding the nucleocapsid. Proteins within the inner shell of a mature virion are cleavage products of the $Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$ polyproteins. The condensed inner core is formed by the capsid protein (CA), p24. Between inner core and the lipid membrane is the matrix protein (MA), p17, which remains associated with the lipid membrane. The virion core contains two copies of the single-stranded genomic RNA to which the NC protein is bound. Also packaged into the virion are the host transfer RNA; $tRNA_3^{Lys}$, and the viral proteins RT, PR, IN, Vif and Vpr. (Haseltine, 1991)

The HIV-1 life cycle

The HIV-1 replication cycle, started with the viral genome integrated into a host chromosome, leads to expression of viral gene products, production of new virus particles, infection of a new cell and reintegration of the viral genome. The HIV-1 life cycle may be split into 15 steps (Frankel *et al*., 1998). These are illustrated in **Figure 2** and are described below.

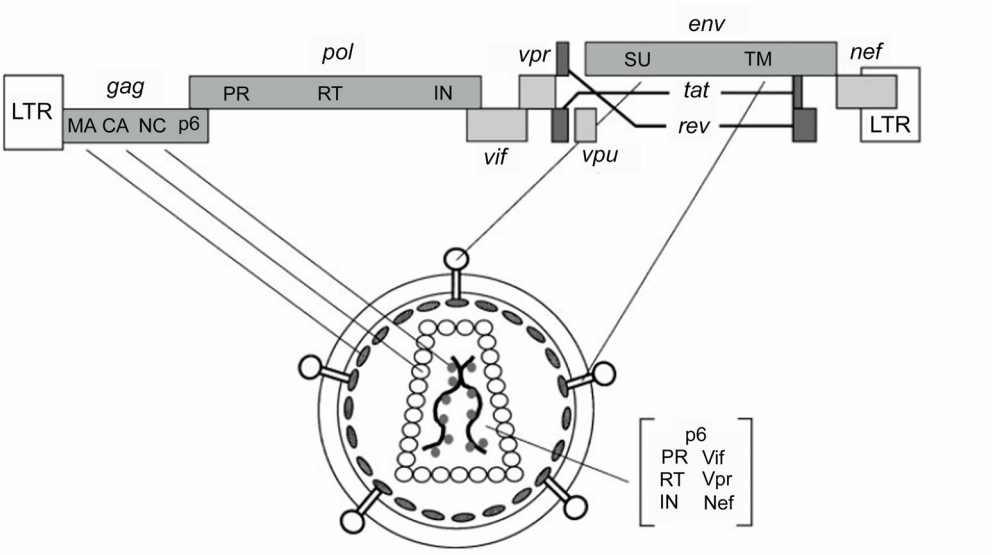

Figure 1. *Diagram of the HIV-1 genome and virion structure.* The genome is flanked by long terminal repeat (LTR). Nine genes (*gag*, *pol*, *env*, *tat*, *rev*, *vif*, *vpr*, *vpu* and *nef*) encode 15 proteins, see Table 1 for descriptions. Modified from Frankel *et al*., 1998.

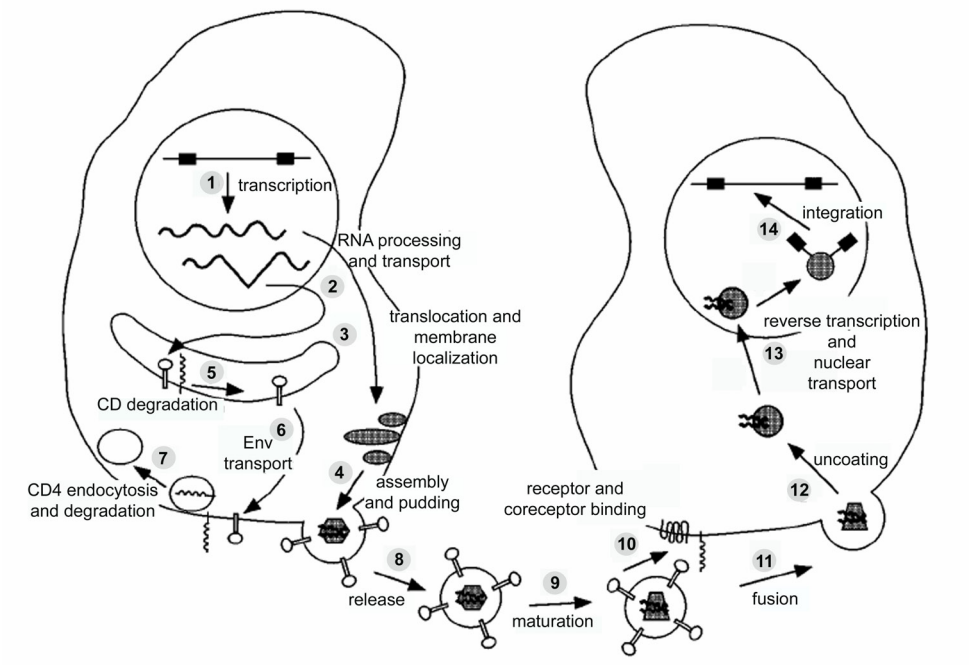

Figure 2. *The HIV-1 life cycle*. Details for the numbered steps can be found in the text. Picture modified from Frankel *et al.*, 1998.

Viral transcripts are expressed from the promoter located in the 5' long terminal repeat (LTR) (**1**), with Tat greatly enhancing the rate of transcription. Viral RNAs are then transported from the nucleus into the cytoplasm where they can be translated or packaged (**2**). This step is regulated by Rev. Some viral RNAs are translated by ribosomes in the cytoplasm to form Gag and Gag-Pol polyproteins, which localize to the cell membrane (**3**). The Env mRNA is translated at the endoplasmic reticulum and forms complexes with the co-expressed HIV-1 cell-surface receptor CD4. The virion core particle is constructed from the Gag and Gag-Pol polyproteins which are later processed into subunits (see **Table1**), accessory proteins Vif, Vpr and Nef, and the genomic RNA (**4**). The immature virion begins to bud from the cell surface. To provide surface (SU) and transmembrane (TM) proteins for the virion outer membrane, the Env polyprotein must be released from the complexes it has formed with CD4. Vpu assists this process by promoting CD4 degradation (**5**). Env is transported to the cell surface, where it must be protected from binding to CD4 (**6**). Nef promotes endocytosis and degradation of surface CD4 (**7**). As the virion particle buds and is released from the host cell surface (**8**), it undergoes maturation involving proteolytic processing of the Gag and Gag-Pol polyproteins by protease (PR) and Vif (**9**). After budding, the mature virion is ready to infect another cell. This is induced by interactions between surface protein SU and CD4 receptor and CC or CXC chemokine coreceptors of the target cell (**10**). After binding, the TM undergoes a conformational change that promotes virus-cell membrane fusion thereby allowing entry of the core into the cell (**11**). The virion core is then uncoated to expose a viral nucleoprotein complex containing

the viral proteins matrix (MA), reverse transcriptase (RT), integrase (IN), Vpr and viral RNA (**12**). During the microtubule based nuclear transport of this pre-integration complex, the viral single-stranded RNA genome is reverse transcribed into double-stranded RNA (**13**). The viral replication cycle is completed by IN catalyzing the integration of the viral DNA into a host chromosome (**14**).

Table 1. HIV-1 genes, gene products and their function. Modified from (Ramezani *et al*., 2002)

Gene	Encoded protein(s)	Function
Regulatory genes		
tat	Tat	Trans-activation of gene expression
rev	Rev	Nuclear export of late mRNAs Promotion of polysomal binding to RRE-containing RNAs
Accessory genes		
vif	Vif	Enhancement of virus transmission
vpr	Vpr	Nuclear transport of viral nucleoprotein complex Induction of G_2 arrest in dividing cells
vpu	Vpu	CD4 degradation Virus maturation and release
nef	Nef	CD4 and MHC-1 down-regulation Enhancement of virus replication
Structural genes	**polyproteins** cleaved into subunits	
gag	**Pr55^{gag}**: matrix MA (p17), capsid CA (p24), nucleocapsid NC (p9), p6	Formation of viral particles Packaging of viral genomic RNA
pol	**Pr160$^{gag\text{-}pol}$**: protease PR (p10), reverse transcriptase RT (p61/p52), integrase IN (p31)	Reverse transcription Integration Virus maturation
env	**gp160**: surface SU (gp120), transmembrane TM (gp41)	Binding and entry into the host cell

The development of HIV-1 derived gene transfer vectors

Like in any other viral gene transfer vectors, the generation of replication-defective LVs requires splitting the *cis*-acting sequences (vector sequences) needed for the transfer and expression of a transgene in target cells and the *trans*-acting sequences (packaging sequences) encoding the essential viral structural and enzyme proteins, onto separate genetic units. The tropism of viral vectors is broadened by pseudotyping; via encapsidation of the viral particle with the envelope of another virus. LVs are mostly pseudotyped with vesicular stomatitis virus G-protein (VSV-G), which is pantropic and highly stable. The transfer vector plasmid is cotransfected with the packaging and envelope plasmids into a cell line where virions are produced. Virions are assembled of viral proteins encapsidating the replication-defective transfer vector RNA.

The HIV-1 derived transfer vector *cis*-acting sequences include viral LTRs, the primer binding site, the packaging signal, the Rev responsive element, and an internal promoter linked to a transgene of interest constituting a transcriptional unit (Naldini *et al.*, 1996). The genetic elements derived from HIV-1 are required for viral encapsidation, reverse transcription and integration. Like MLV retroviral vectors, HIV-1 vectors do not transfer viral coding sequences into target cells, meaning that cells transduced with the HIV-1 vector do not express any viral proteins.

HIV-1 transfer vectors have been modified by introducing various internal promoters driving transgene expression, and by the inclusion of genetic elements such as the central DNA flap and the post-transcriptional element. The central DNA flap is a 99 nucleotide-long overlap formed after native HIV-1 reverse transcription and it is involved in the import of the HIV-1 preintegration complex into the nucleus. The sequence for this element, the central polypurine tract (cPPT), was omitted from early generation HIV-1 vectors but has been routinely included in current vector designs because of its beneficial effect on gene transfer efficiency (Follenzi *et al.*, 2000). The woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) sequence is also commonly included in current HIV-1 vectors. This element has been shown to enhance transgene expression from several types of promoters (Deglon *et al.*, 2000) by augmenting mRNA 3'-end processing and polyadenylation.

The HIV-1 vector packaging systems have been extensively developed. The first-generation packaging systems comprised three expression plasmids: the transfer vector, the plasmid for VSV-G envelope protein production and the packaging construct (Naldini *et al.*, 1996). In this system, only two of the nine native HIV-1 genes, *vpu* and *env*, were deleted. Subsequently, it was shown that none of the four HIV-1 accessory genes *vif*, *vpr*, *vpu* or *nef* were required for efficient production of VSV-G pseudotyped vector particles (Zufferey *et al.*, 1997). Therefore, the second-generation packaging system only utilized HIV-1 *gag*, *pol*, *rev* and *tat* genes, which further attenuated the potential for the generation of replication-competent viruses.

For the currently used third-generation HIV-1 vector system, several additional modifications have been made to ensure the safety of these vectors. Firstly, they have been modified to self-inactivate (SIN) by deleting the promoter sequences of the U3 region of the 3'LTR (Miyoshi *et al.*, 1998). Since the U3 region of the 3'LTR serves as a template for the U3 regions of both LTRs, the provirus carries the deletion in both LTRs after reverse transcription. As a result, the

LTRs of the integrated vector are almost completely inactivated. The inability to transcribe full-length vector RNA minimizes the chance of replication-competent virus generation and reduces the potential of oncogene activation by promoter insertional mutagenesis. To avoid the reconstitution of deleted U3 sequences by homologous recombination with intact 5' LTR during viral vector production, the U3 region of the 5' LTR is replaced with a heterologous promoter, usually a cytomegalovirus (CMV) promoter. Since the LTR promoter is dependent on Tat interaction, the use of the CMV promoter allows *tat* gene independent production of viral vectors. Therefore, from the third-generation HIV-1 vector packaging system, *tat* is deleted. This has enabled further refinement of the packaging system for increased safety, such as the expression of *gag-pol* and *rev* genes from two separate nonoverlapping plasmids. With 40% of the wild-type virus genome (three out of nine genes) left, the parental virus can not be reconstituted from such an extensively deleted packaging system. Also, in the absence of overlapping viral sequences the risk of recombination events between components of the viral production system is abolished, further limiting the possibility to yield replication-competent vectors. To date, replication-competent vector production has not been associated with the production of HIV-1 lentiviral vectors.

Applications of HIV-1 derived gene transfer vectors

HIV-1 derived gene transfer vectors show efficient delivery, integration and long-term expression of transgenes in both dividing and nondividing cells, thus making them excellent vehicles for basic studies of gene overexpression and knockdown. As such, they represent an attractive tool for most potential targets of gene therapy, whether the targets are early precursors or terminally differentiated cells. While third generation HIV-1 vectors are able to transduce virtually all types of cells *in vitro*, it seems that the accessory protein, Vpr, is important for the transduction of macrophages and hepatocytes (Naldini *et al*., 1996, Kafri *et al*., 1997). Also, although HIV-1 vectors do not require cell division, like the native HIV-1 virus, they are unable to successfully transduce T lymphocytes during the G_0 stage of the cell cycle. This is due to blocks at the levels of reverse transcription and nuclear import. However, HIV-1 vectors mediate efficient stable transduction of many cell types which are poorly transduced by other vectors. For example, gene transfer to progenitor and stem cells is one of the most important applications of HIV-1 derived vectors.

Embryonic stem cells (ESCs) are cells derived from the inner cell mass of an early embryo. They can be maintained in an undifferentiated state indefinitely and can be genetically manipulated *in vitro* without losing their differentiation potential. This unique property of ESCs suggests that they may provide a useful tool to analyze developmental pathways and are a promising cell source for transplantation therapies. Efficient genetic manipulation of ESCs is critical for both development, biology research and for maximizing the therapeutic potential of ESCs. HIV-1 derived vectors have been shown to efficiently drive transgene expression in mouse (Kosaka *et al*., 2004) and human (Gropp *et al*., 2003) ESCs. ´

Of all blood cell types, only hematopoietic stem cells (HSC) can self-renew, persist throughout a lifetime and reconstitute the whole lympho-hematopoietic system of an individual. HIV-1 LVs can efficiently transduce *ex vivo* mouse (Moreau-Gaudry *et al*., 2001), non-human primate (Horn *et al*., 2002) and human (Miyoshi *et al*., 1999) HSCs in the absence of cytokine stimulation and cell cycle induction. This is important

because culture conditions which facilitate the proliferation of HSCs without the loss of their stem cell capacity have not been identified. HIV-1 derived LVs efficiently transduce human $CD34^+$ cells, a heterogenous population of HSCs and progenitor cells. The LV-transduced $CD34^+$ cells are capable of engraftment and multi-lineage differentiation in NOD/SCID (non-obese diabetic/severe combined immunodeficient) mice (Miyoshi *et al*., 1999). Such genetically modified cells can be passed to secondary transplants (Woods *et al*., 2000) which further confirms the transduction of true HSCs and not only the multipotent progenitor cells.

The stereotactic injection of HIV-1 LV was the model initially used to illustrate the ability of these vectors to transduce nondiving cells *in vivo* (Naldini *et al*., 1996). Numerous studies have reported successful long-lasting and efficient transgene expression in terminally differentiated neurons of rodent brain after a single injection of only a few microliters of high titer (magnitude of 10^9 TU/ml) vector stock. In addition to neurons, LVs are able to transduce most cell-types within the CNS *in vivo*, including astrocytes, oligodendrocytes, adult neuronal stem cells and glioma cells (Jakobsson *et al*., 2003, Consiglio *et al*., 2004, Miletic *et al*., 2004). LVs have a property of highly efficient retrograde transport providing access to a wide area of the brain after a single injection, thus enabling potential therapy for widely disseminating neurological disorders. Also, the delivery of *ex vivo* LV transduced HSCs trafficking to the CNS has been exploited. Promising therapeutic effects of HIV-1 LV mediated gene transfer has been documented in animal models of Alzheimer's disease (Dodart *et al*., 2005), Huntington's disease (de Almeida *et al*., 2001), Parkinson's disease (Kordower *et al*., 2000), amyotrophic lateral sclerosis (ALS, Raoul *et al*., 2005a) and lysosomal storage diseases (Biffi *et al*., 2004). Also, LV gene transfer has been utilized in the development of new animal models of Huntington's (Regulier *et al*., 2003) and Parkinson's disease (Lo Bianco *et al*., 2002). In these models LV gene transfer has been used to induce overexpression of the mutated form of protein present in these diseases.

The liver is an important target tissue for gene therapy because of the numerous genetic defects that cause defects in liver function resulting in severe disorders such as hemophilia A and B and FH. Also, the liver is a target of chronic virus infections such as hepatitis B and C. Despite the regeneration capacity of the liver, hepatocytes divide only occasionally in the adult. Several studies have reported that LVs can transduce nondividing rodent and human hepatocytes, both *ex vivo* and *in vivo* (Kafri *et al*., 1997, Nguyen *et al*., 2002, VandenDriessche *et al*., 2002, Follenzi *et al*., 2004). However, mouse studies have shown higher LV gene transfer efficiency in neonates and after partial hepactomy (Park *et al*., 2000, Ohashi *et al*., 2002, Park *et al*., 2003), suggesting that proliferating hepatocytes are more prone to LV transduction. Some properties of the architecture of the hepatic lobule or the tightness of the endothelial barrier in hepatic blood vessels may be influenced by liver growth or regeneration, thus favouring viral entry. Alternatively, it has been suggested that the HIV-1 accessory protein Vpr, absent from later generation LVs, can enhance hepatocyte transduction (Kafri *et al*., 1997). However, in a study of LV mediated LDL-receptor gene transfer in rabbit model of FH, a long term therapeutic effect without hepactomy was reported (Kankkonen *et al*., 2004). Although only a modest gene transfer efficiency of 0.01% of the liver cells was achieved, the results showed a significant (44%) decrease in the serum cholesterol level of the treatment group at a one year timepoint compared to controls. These

results support further research of LV mediated liver gene therapy.

When the early generations of HIV-1 LVs were developed, high expectations of their performance in *in vivo* gene therapy applications were raised. So far, these expectations have been fulfilled only in the targets of the central nervous system (CNS), lympho-hematopoietic system and to a lesser extent in the liver. More work is needed to evaluate the true utility of LVs in targeting tissues such as skeletal muscle and the myocardium. The first clinical trial utilizing HIV-1 LV for the treatment of HIV-1 infection is currently in process (Levine *et al*., 2006). In this study, an antisense approach against the HIV-1 envelope was utilized by *ex vivo* transduction of the patients' T-cells. A LV with wild-type LTRs was used and therefore, expression of the antisense sequence was up-regulated upon the wild-type HIV-1 infection of the vector bearing cell. The results demonstrate safe and efficient gene delivery and good persistence *in vivo* and also, an improvement of the immune function in four out of five patients. However, the use of LV in patients infected with wild-type HIV-1 presents a problem, the potential of the wild-type virus to infect a cell modified by the vector. As a result, the wild-type virus infection would mobilize the vector genome by packaging it and transferring it to new cell. For HIV-1 patients, such a spread of the vector might actually be beneficial. However, it poses complex biosafety and ethical problems and should be avoided. The patients from this trial were monitored for over one year (Levine *et al*., 2006). Only a long-term follow-up after at least three years will reveal the true safety of such treatment. Nonetheless, based on the results from the first clinical trial with LV, it possesses the potential to be used for the therapies involving prior *ex vivo* genetic modification of cells of the lympho-hematopoietic system.

A few years ago, lentiviral HIV-1 derived vectors made a breakthrough in the generation of transgenic animals (reviewed in Park, 2007). Previously, transgenesis has been achieved by pronuclear injection of naked DNA. This is a rather inefficient and tricky technique requiring a clearly visible oocyte pronucleus mostly inapplicable to species other than mouse. Also, mouse transgenesis utilizing MLV retroviral vectors failed as a result of transgene silencing during development (Cherry *et al*., 2000). HIV-1 LVs have been successfully used to generate transgenic mice and rats by the transduction of single-cell embryos, early blastocysts or embryonic stem cells (Lois *et al*., 2002, Pfeifer *et al*., 2002). In these experiments, LV mediated transgenesis resulted in very high embryo viability with 80% of mice carrying the provirus. Unlike MLV retroviral vectors, HIV-1 LVs appear to escape epigenetic silencing. The reason for this remains unknown but might be linked to different integration site preferences. MLV vectors have been found to integrate predominantly close to transcriptional start regions and CpG islands (Wu *et al*., 2003). In contrast, LVs, studied to date, integrate across the entire transcribed gene region with no preference to the proximity to the transcriptional start site. Also, LV genomes contain fewer CpG dinucleotides susceptible to cytosine methylation than the onco-retroviral vectors, which may partially explain the finding that they are less prone to silencing. Successful LV-mediated transgenesis has also been extended to larger animal species including cattle (Hofmann *et al*., 2004) and pig (Hofmann *et al*., 2003). In addition to offering models of human diseases, especially large transgenic animals may find applications in future bioindustry for example as producers of human proteins for drug use or as a potential source of humanized organs for transplantation.

CELL THERAPIES FOR CARDIOVASCULAR DISEASES

General concept

Among treatment options for cardiovascular diseases, there is a definite need for alternative therapies, particularly for advanced and severe disease. Experimental studies have indicated that progenitor or stem cells derived from different sources possess regenerative capacity in the heart and vasculature, which has raised expectations of clinically applicable cell therapy for tissue repair in cardiovascular diseases. The initial concept for this research was based on the cell plasticity-hypothesis, which suggests that progenitor cells can transdifferentiate *in vivo* across generally agreed tissue lineage boundaries. The concept of plasticity has, however, been challenged by data proposing that HSCs are committed to differentiate into cells of hematopoietic lineages and do not own the capacity to transdifferentiate (Wagers *et al.*, 2002). Cell fusion has since been proposed as an alternative explanation for observed transdifferentiation events. On the other hand, by secretion of paracrine factors, progenitor cells might affect vasculogenesis, tissue repair and remodelling without the need to undergo transdifferentiation or cell fusion. Also, stem cell niches have been identified from myocardium. The concept of stem cell niche covers the local tissue environments of surrounding cells which are important for the regulation of stem cells controlling and balancing self-renewal and differentiation (Moore *et al.*, 2006, Morrison *et al.*, 2008). There is evidence of nesting cardiac stem cells and progenitors that are connected structurally and functionally to myocytes and fibroblasts by junctional and adhesion proteins, such as connexins and cadherins (Urbanek *et al.*, 2006). A novel fascinating mechanism proposed to play a role in cell therapy is the putative stimulation of endogenous tissue repair pathways which might contribute to the regeneration of stem cell niches (Mazhari *et al.*, 2007).

With the evolving experimental data, the concept of cell therapy for cardiovascular diseases has shifted from the original idea of progenitor cells taking part in the regeneration of injured myocardium or skeletal muscle or playing a part in the induction of angiogenesis by the direct involvement of progenitor cells into newly forming vessels. Instead, a broader hypothesis suggests that cell therapy might in fact facilitate complementary aspects of tissue repair (**Figure 3**). These effects might include augmentation of cell survival (for example, in limiting apoptosis), tissue oxygenation by angiogenesis or improvement in positive tissue remodelling. The most potent target diseases for cell therapy include myocardial infarction, ischemic cardiomyopathy and peripheral vascular disease causing skeletal muscle ischemia in lower limbs.

Cell types and sources

Several sources of progenitor cells for cardiovascular cell therapy exist in adults, including unfractioned or fractioned hematopoietic and mesenchymal stem cells from bone marrow, circulating progenitor cells, skeletal myoblasts and resident progenitor cells for example, from adipose tissue. Also, cord blood HSCs and cardiomyocytes derived from embryonic stem cells have been used in animal models.

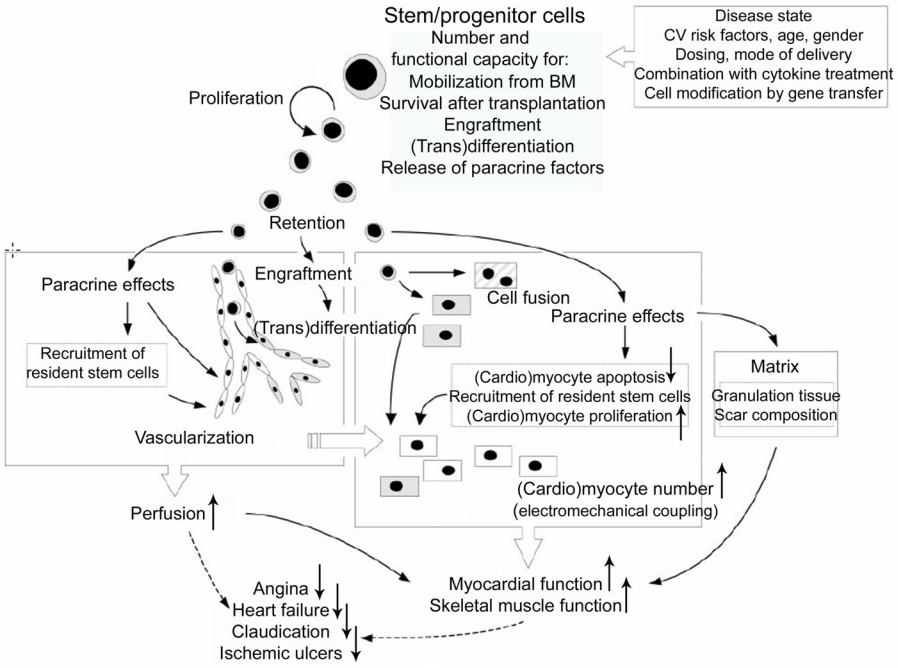

Figure 3. *A working hypothesis of cell therapy for myocardial and skeletal muscle regeneration.* Cell therapy can have a favourable impact on tissue healing by alternative mechanisms presented in the figure. Stem and progenitor cell numbers and functional capacity are influenced by a patient's age, gender, cardiovascular risk factors and underlying disease state which all contribute to the natural response in the injured tissue and also to preparation of autologous cell preparations for therapy. Figure modified from Wollert *et al.*, 2005.

For cardiovascular gene therapy, bone marrow has been proposed as a source of hematopoietic, vasculogenic and mesenchymal stem cells. Initial experimental evidence suggested a significant degree of myocardial regeneration by the administration of lineage negative c-kit$^+$ bone marrow mononuclear cells (BMCs) into a murine model of myocardial infarction (Orlic *et al.*, 2001). However, this has been questioned by subsequent studies showing little or no tissue integration of these BMCs in similar animal models (Balsam *et al.*, 2004, Murry *et al.*, 2004). These findings challenged the paradigm of BMC transdifferentiation, although did not exclude the possibility that such cells could potentiate myocardial repair by other mechanisms. Bone marrow mesenchymal stem cells (MSCs) are a component of marrow stroma. They are self-renewing, clonal precursors, which expand easily in culture, exhibit multipotency and have also been shown to differentiate to cardiomyocytes and vascular cells (Jiang *et al.*, 2002). Endothelial progenitor cells (EPCs) have been proposed to induce angiogenesis and re-endothelization in the models of ischemia and vascular injury (Madeddu *et al.*, 2004, Nowak *et al.*, 2004). Mechanisms suggested for EPC-mediated angiogenesis include integration of the EPC into newly formed micro- and macrovessels and the secretion of growth, survival and cell-modulatory factors. EPCs have been identified and enriched from bone marrow and peripheral blood by the expression of surface antigens such as CD31, CD133, VEGFR-2 and Tie-2.

Whether these cells, exhibiting endothelial plasticity, offer a significant therapeutic advantage remains unclear in the absence of convincing data.

Skeletal myoblasts are satellite progenitor cells in muscle. In response to muscle injury, they are able to proliferate and fuse to regenerate new multinucleated cells. The potential advantage of using these cells in cell therapy applications include their autologous origin, ease of isolation, high *in vitro* proliferative capacity, *in vivo* ischemic tolerance and myocyte restricted lineage commitment which limits the risk of oncogenetic transformation (Deasy *et al.*, 2004). In animal models of myocardial ischemia, autologous skeletal myoblasts augmented contractile function (Taylor *et al.*, 1998) and findings from clinical studies suggested that implanted myoblasts engrafted viably in scarred myocardium (Pagani *et al.*, 2003). The enthusiasm for myoblast therapy has faded by the lack of evidence for cardiomyocyte differentiation of myoblasts and further, due to arrhytmias observed in a clinical trial, presumably caused by the inability of myoblasts to integrate into the conduction system of the heart (Menasche *et al.*, 2003). Recently, a population of myoendothelial cells with multilineage capacity, including skeletal and cardiac muscle regenerative potential, has been identified within human skeletal muscle (Zheng *et al.*, 2007). Further research will show whether these cells, showing myogenic and endothelial properties, can be envisioned as a therapy for muscle diseases.

The ability of human embryonic stem cell (ESC) derived cardiomyocytes to survive and integrate structurally and functionally into healthy and post-infarct cardiac tissue has been demonstrated in animal models (Kehat *et al.*, 2004, Laflamme *et al.*, 2005, Xue *et al.*, 2005, Caspi *et al.*, 2007). The recent breakthrough findings show that mouse and human fibroblasts can be reprogrammed to pluripotent ESC-like cells by the transfer of three to four transcription factor genes (Takahashi *et al.*, 2006, Takahashi *et al.*, 2007, Wernig *et al.*, 2007, Yu *et al.*, 2007, Nakagawa *et al.*, 2008). The resulting induced pluripotent stem cells have the potential to be used in future treatments for cardiovascular diseases.

For cell therapy research of cardiovascular diseases to date, bone marrow derived progenitor cells are the most commonly used. An important issue complicating the interpretation and comparison of both experimental and clinical data is the heterogeneity of cell preparations, since both unfractioned mononuclear cells and fractioned preparations selected for $CD34^+$ or $CD133^+$ have been used.

Cell therapy combined with gene therapy

Gene therapy has been widely applied for the therapy of cardiovascular diseases, most popularly in the concept of angiogenic growth factor therapy for ischemic myocardium or skeletal muscle. Although the biological effects of such growth factors are well understood, these therapies have not proven efficient in clinical trials presumably due to the limited efficacy of current gene transfer technology. One approach to improve the delivery of growth factors might be the combination of cell and gene therapy to utilize progenitor cells as carriers. After a transgene is introduced, these engineered progenitor cells would home into the target area and secrete therapeutic proteins. Also, by gene transfer, the chemokine expression profile of the progenitor cell might be altered to improve homing of endogenous progenitor cells into the injured area (Askari *et al.*, 2003). Another approach using cell based gene therapy is to engineer progenitor cells to express a protein which is not secreted but modifies the biology of the cell itself. Such a modification might aim at improving

cell survival by inhibiting apoptosis for example (Mangi *et al*., 2003), or by strengthening resistance to ischemia or scavenging free radicals.

Clinical trials and future prospects

Small clinical trials have focused on the safety and feasibility of progenitor cell therapy in cardiovascular diseases, including ischemic cardiomyopathy (Fuchs *et al*., 2003, Perin *et al*., 2003, Tse *et al*., 2003), peripheral vascular disease (Tateishi-Yuyama *et al*., 2002b) and myocardial infarction (Strauer *et al*., 2002, Britten *et al*., 2003, Fernandez-Aviles *et al*., 2004, Wollert *et al*., 2004). While the safety of cell therapy has been demonstrated it has been difficult to compare data because of variations in the methods used. These include variations in routes of cell delivery, preparation of cells and chosen endpoint parameters for efficacy evaluation. To date, several randomized, controlled trials of intracoronary application of bone marrow cells for patients with acute myocardial infarction have been reported (Chen *et al*., 2004, Bartunek *et al*., 2005, Erbs *et al*., 2005, Hendrikx *et al*., 2006, Janssens *et al*., 2006, Kang *et al*., 2006, Meyer *et al*., 2006, Cleland *et al*., 2007). The change in the left venctricular ejection fraction (LVEF) after cell therapy has been assessed by angiography, magnetic resonance imaging or ultrasound and compared to a control group. Statistically significant LVEF improvements have been obtained in some of the studies (Chen *et al*., 2004, Erbs *et al*., 2005, Hendrikx *et al*., 2006, Kang *et al*., 2006, Cleland *et al*., 2007). Nevertheless, it has been pointed out that these improvements in myocardial function are not likely to be significant in a clinical context. In fact, experts have agreed that before pursuing further clinical trials a better understanding of the mechanisms of progenitor cell mediated therapy, the methods used to produce the therapeutic cell preparations, the application route, the timing of treatment and patient selection needs to be attained (Bartunek *et al*., 2006). This will show the true potential of progenitor cell therapy as a clinical treatment for cardiovascular diseases.

GENE TRANSFER VECTORS WITH REGULATED GENE EXPRESSION

General concept

In terms of both experimental and clinical gene therapy applications, one of the key issues is the ability to regulate the expression of a therapeutic gene, in order to produce levels of protein within a therapeutic window, and to switch off the expression if desired. Regulated gene expression vectors are based on the insertion of sequences binding to transcriptional activators preceding the minimal promoter. These activators will bind, and thus, activate gene expression when a particular inducer compound is present. Binding is either achieved subsequent to a conformational change in the activator or by heterodimerization of two distinct factors, one that is responsible for specific DNA binding and the other for transcription activation. Regulated gene expression systems consist of at least two separate expression cassettes: one that contains the transcriptional activator under the control of either a constitutive or a tissue specific promoter, and the other contains a transgene under the control of the regulated, transcriptional activator responsive promoter. To deliver these expression cassettes into target cells, either two separate gene transfer vectors are used or, all the elements are combined into a single vector. To date, tetracycline-dependent gene regulation systems are the most utilized and advanced. These and some other commonly applied systems are introduced in the following sections. A direct comparison of different

regulation systems is difficult and therefore, each system should be selected according to the requirements of the particular application. When regulatable gene expression is used in clinical applications, the pharmacology of the inducer drug plays a key role in selection of the system.

Tetracycline-regulated gene expression

Tetracycline (Tet) –regulated transcriptional expression systems have been adapted from studies of the *E.coli* tetracycline resistance mechanism (Hillen *et al*., 1994). In bacteria, the TetR protein inhibits the transcription of genes in the tetracycline-resistance operon present in the Tn10 transposon by docking to the Tet operator (tetO) sequences, in the absence of tetracycline. Therefore, the transcription of the genes needed to metabolize Tet is inhibited by TetR in the absence of Tet. When Tet is present, it is bound by TetR, inducing a conformational change, which results in the release of TetR from tetO, allowing transcription. In the Tet-off system (**Figure 4**), TetR is modified to function as a transcriptional activator by fusion with a viral protein domain VP16, a eukaryotic transactivator derived from herpes simplex virus type 1. As a result, TetR is converted from a transcriptional repressor to an activator, and is termed the Tet transactivator (tTA). The tTA is expressed independently of the tetracycline response element (TRE, a framework of seven tandem sequences upstream of a minimal CMV promoter) -driven transgene encoding cassette. In the absence of Tet the tTA binds to the TRE, and activates transcription of the transgene from an otherwise silent minimal promoter. When Tet is present, it binds to tTA, which is then released from tetO and transcription is no longer continued. Thus, Tet-off system driven gene expression is switched on and off in the absence or presence of the inducer, respectively. As an inducing drug, the Tet analog doxycycline (Dox) is often used.

When mutations were randomly introduced into the TetR part of the tTA a separate protein was produced which exhibited opposite binding properties and led to the development of the Tet-on system (**Figure 4**). The mutant of tTA, termed rtTA, triggers transcription by activating the TRE in the presence of the inducer Tet. The Tet-on gene regulation system, that utilizes rtTA, has been widely applied, but the system is somewhat limited with respect to the precision of regulation. The rtTA has been found to also show some degree of affinity for tetO sequences in the absence of Tet, therefore inducing a low basal level of gene expression in the off state. To further improve the Tet-on system, the inventors of the system screened for mutant rtTA molecules in yeast to obtain a transactivator with more specific binding properties. One mutant with superior features was identified and this novel transcriptional activator was named rtTA2^{S}M2. The Tet-on system with rtTA2^{S}M2 showed negligible basal expression and was fully induced with a 10-fold lower Dox concentration than rtTA (Urlinger *et al*., 2000). To date, the rtTA2^{S}M2 has been successfully used in a wide variety of applications (Lamartina *et al*., 2002, Chenuaud *et al*., 2004, Vogel *et al*., 2004, Pluta *et al*., 2005).

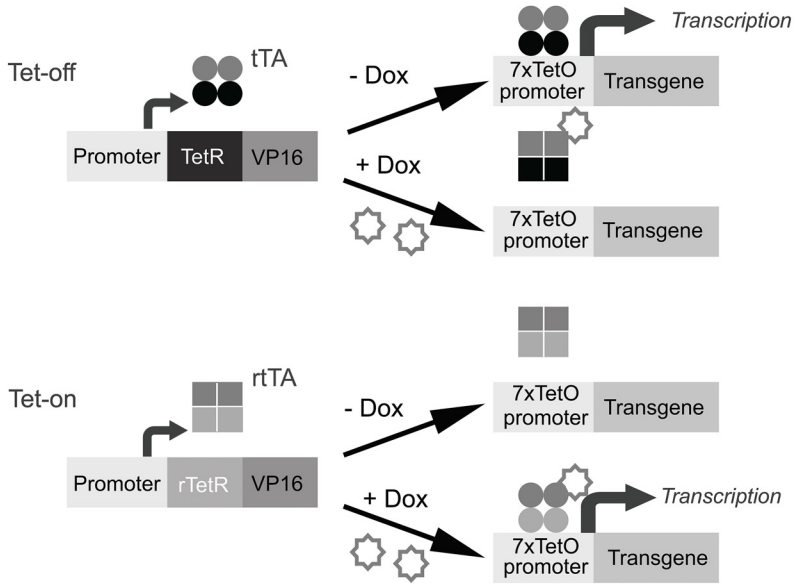

Figure 4. *Components of the Tet-off and Tet-on gene expression regulation system.* Both systems consist of two transcriptional units: one for the expression of transactivator (tTA or rtTA) and the other has the regulated transgene under the control of the transactivator binding promoter. The transactivator consists of the tetracycline operon (TetO) binding domain fused to the transactivation domain VP16 originating from HSV virus. In the Tet-off system the TetO binding domain, tetracycline repressor (TetR) is able to bind in the absence of tetracycline or its derivative, doxycycline (Dox) and thus, transcription is activated by the VP16 domain of the tTA in the absence of Dox. When Dox is present, it binds to the tTA and induces a conformational change, releasing tTA from TetO and deactivating transcription. In the Tet-on system the binding properties of TetR are mutated for reversed binding properties. Thus, the resulting transactivator, rtTA induces transcription in the presence of Dox.

In addition to its tight regulation and the possibility to strictly adjust the transgene expression level dose-dependently, the tetracycline-regulated gene expression system has several advantages over other systems. The inducer drug has been used as an antibiotic for decades and thus, has well characterized pharmacological properties in a clinical setting. Doxycycline is non-toxic at doses required for gene induction and the tissue concentrations needed are achieved by oral administration of the drug. Doxycycline is lipophilic and hence efficiently absorbed by cells. It is also rapidly metabolized and cleared from the body making it an ideal drug for rapid induction and repression of gene expression. The components of the Tet-on system recognize unique sequences of DNA, thus reducing the risk of side effects. However, because the transactivator protein has both bacterial and viral protein components, an immune response is possible. In fact, the immunogenicity of rtTA2^{S}M2 transactivator has been reduced by humanized amino acid codon optimization (Urlinger *et al.*, 2000).

Steroid hormone receptor -based regulated gene expression

Steroid hormones can easily cross epithelial barriers and plasma membranes. Endogenously, steroid hormones bind to their receptors in the cytoplasm and these complexes are then translocated to the nucleus where they bind to DNA to regulate gene expression. Thus, steroid hormone receptor ligands bear the potential to be utilized as soluble drugs for the regulation of transgenes in therapy applications.

A gene regulation system exploiting a truncated form of the ligand-binding domain of the human progesterone receptor has been developed (Wang *et al*., 1994). This system relies on the ability of the modified ligand-binding domain to bind to synthetic antiprogestins as agonists. An antiprogestin-dependent, site-specific chimeric transcription factor was generated by linking the modified ligand-binding domain to a heterologous DNA-binding domain, yeast GAL4, and to a transcription activation domain, NF-κβ p65 subunit. In the presence of an antiprogestin, such as mifepristone, the chimeric transactivator binds to its target sequence, the 17-mer GAL4 sequence positioned upstream of the minimal promoter, to activate transcription of the transgene. Mifepristone regulated gene expression has been used both in *in vitro* and *in vivo* applications (Wang *et al*., 1997, Oligino *et al*.,1998, Burcin *et al*., 1999). An advantage of the antiprogestin gene regulation system is that it mostly comprises of modified human proteins and thus, should be less immunogenic. However, inducers of the system are generally able to activate native steroid hormone receptors, which creates a risk of side effects. In fact, mifepristone is a drug which is used to induce abortion in women, although with a dose well above that is sufficient for transgene regulation. These concentrations of mifepristone are similar to those in estrogen replacement therapies used during menopause (Wang *et al*., 1994) and thus, known to have biological effect.

Another strategy to utilize steroid hormone regulation is the use of a non-mammalian steroid hormone receptor, such as the ecdysone receptor. The ecdysone receptor is of insect origin, and is involved in triggering metamorphosis in *Drosophila*. Ecdysone regulated gene expression systems consist of a chimeric transactivator protein composed of the VP16 activation domain fused to the ecdysone receptor with altered DNA-binding specificity and with the ability to hetorodimerize with the retinoid X receptor. The response element, which is combined to the transgene, is synthetic and not recognized by natural nuclear receptors. The presence of an ecdysone analog elicits heterodimerization of the transactivator leading to transgene expression. Ecdysone regulated gene expression has been successfully applied in animal models (Johns *et al*., 1999, Karns *et al*., 2001, Galimi *et al*., 2005). As gene expression inducers, ecdysteroids have certain advantages compared to human steroids. Ecdysteroids have short half-lives, which aids in precise gene induction. Also, they are considered relatively non-toxic and do not appear to affect mammalian physiology (No *et al*., 1996). However, a disadvantage of the system is that expression of insect proteins *in vivo* may induce an immune response in the host.

Rapamycin-regulated gene expression

Rapamycin-regulated gene expression systems are based on the ability of the immunosuppressant drug rapamycin to dimerize two cellular proteins, immunophilin FKBP12 (FK506-binding protein of 12 kDa) and FRAP (FKBP rapamycin-associated protein). The system utilizes two fusion proteins. The first is a fusion of the FKBP12

domain and a synthetic DNA-binding domain ZFHD1 (zinc-finger homeodomain fusion 1). The second protein is a fusion of a domain of FRAP, called FRB (FKBP rapamycin-binding) and a transcriptional activation domain of the p65 subunit from the NF-κβ. Rapamycin induced dimerization is able to constitute a functional transcriptional activator which binds to the ZFHD1 recognition site located upstream of the transgene cassette containing a minimal promoter and thus, induce transcription when rapamycin is present (Rivera *et al.*, 1996). Rapamycin-regulated gene expression has been applied in animal models for erythropoietin expression and cancer therapy (Crittenden *et al.*, 2003, Rivera *et al.*, 2005, Nguyen *et al.*, 2007). An advantage of the rapamycin-regulated gene expression system is that it is free of bacterial and viral protein components. However, the immunosuppressive activity of the inducer drug rapamycin limits its potential use in clinical applications.

Physiologically regulated gene expression

Instead of controlling transgene expression by dosing of a drug, gene expression may be regulated by an endogenous physiological stimulus. A prototype of such a "vigilant vector" is the hypoxia regulated vector, which has potential in gene therapy of myocardial and skeletal muscle ischemia. In its current form, hypoxia-induced gene regulation is achieved by an oxygen sensitive transcription activator, which is composed of the oxygen degradation domain of hypoxia-inducible factor 1α fused to the transcriptional activation domain p65 and to the DNA binding domain GAL4. This engineered transcription factor is degraded in normoxic conditions, but in hypoxia it is able to bind to its GAL4 target sequence, placed upstream of the transgene, and activate transcription (Tang *et al.*, 2005). Other pathophysiological stimuli which might be utilized in therapeutic transgene regulation are vascular shear stress and oxidative stress. Oxidative stress has been implicated to play a role in a number of cardiovascular pathologies including hypertension, atherosclerosis, myocardial infarction, reperfusion injury and restenosis (Levonen *et al.*, *in press*) and thus, oxidative stress induced gene regulation could be exploited in a variety of potential gene therapy applications. Therapeutic gene expression regulation by a physiological stimulus typical for the targeted pathology is an alluring concept and presumably, will be increasingly applied in future therapies.

Radiation induced gene expression

Genetic radiotherapy was introduced based on the finding that a specific sequence of the early growth factor response-1 gene promoter, called the CArG element, mediates ionizing radiation-induced gene transcription (Hallahan *et al.*, 1995). This application to cancer therapy is based on a vector where tumor necrosis factor-α (TNF-α) expression is under the control of the CArG elements and combines both the spatial and temporal control of therapeutic gene expression. By using an adenoviral vector, radiation induced TNF-α expression has resulted in high intratumoral TNF-α concentration and produced antitumor effects in animal models of human cancers and has been tested in phase I clinical trials for the treatment of advanced solid tumors (reviewed by Mezhir *et al.*, 2006).

GENE EXPRESSION KNOCK-DOWN BY RNA INTERFERENCE (RNAi)

Introduction to RNAi

The Nobel Prize awarded discovery in 1998, showed that long double-stranded RNA (dsRNA) could silence gene expression in the nematode *Caenorhabditis elegans* (Fire *et al.*,1998). This was followed by the finding in 2001, that showed that short synthetic RNAs could induce the same phenomenon in mammalian cells (Elbashir *et al.*, 2001). These findings have led to an increasing amount of information of the role of small RNAs in gene regulation and the subsequent use of RNAi based therapeutics in preliminary clinical trials. To date, several small regulatory RNAs have been discovered. These include short interfering RNAs (siRNAs), microRNAs (miRNAs) and Piwi-interacting RNAs (piRNAs). In addition to uncovering new mechanisms of gene silencing and revolutionizing the understanding of endogenous mechanisms of gene regulation, the discovery of RNAi has provided a powerful new tool for biological research and a potential therapeutic approach for the *in vivo* inactivation of proteins linked to human disease progression and pathology.

The mechanism of RNAi

In the RNA-guided gene-silencing pathway discovered thus far, dsRNA is the trigger molecule. Long dsRNA can derive from various sources, such as simultaneous sense and antisense transcription of specific genomic loci or from viral replication intermediates. However, the predominant form of dsRNA in mammalian cells is derived from endogenously expressed miRNAs. miRNAs are derived from imperfectly paired non-coding hairpin RNA structures and are encoded within introns. They are transcribed by a RNA polymerase II enzyme, processed in the nucleus by the Drosha enzyme complex and exported to the cytoplasm as precursor molecules called pre-microRNAs. In the cytoplasm, the pre-microRNAs are further shortened and processed by an RNAse III endonuclease enzyme called Dicer to produce imperfectly matched, double-stranded miRNAs. Similarly, Dicer processes long, perfectly matched dsRNAs into shorter siRNAs. In the next step, a multi-enzyme complex, including the Argonaute 2 –protein and the RNA-induced silencing complex (RISC), binds to miRNA or siRNA duplex and discards one strand, to form an activated complex containing the guide or antisense strand. If complementarity of the strands is imperfect, as with a miRNA and mRNA, the enzyme complex blocks translation. When complementarity is perfect or nearly perfect, such as with a siRNA and mRNA, the complex degrades the RNA strand in a guided fashion. In both cases, translation of the target mRNA is inhibited, either by translational repression or mRNA cleavage. (Kim *et al.*, 2007). The principle of siRNA and miRNA function in the cytoplasm of mammalian cells is presented in the **Figure 5**.

The goal of RNAi-based research applications and therapies is to activate selective mRNA cleavage for efficient gene silencing. It is possible to harness the endogenous pathway in two ways: either by using a gene transfer vector to express a short hairpin RNA (shRNA) that resembles a micro-RNA precursor, or by introducing synthetic siRNAs into the cytoplasm that readily mimic the Dicer cleavage product. To date, only synthetic siRNAs have been utilized in clinical RNAi therapeutics, while vector mediated shRNA-based approaches are widely used in basic research and pre-clinical gene therapy applications.

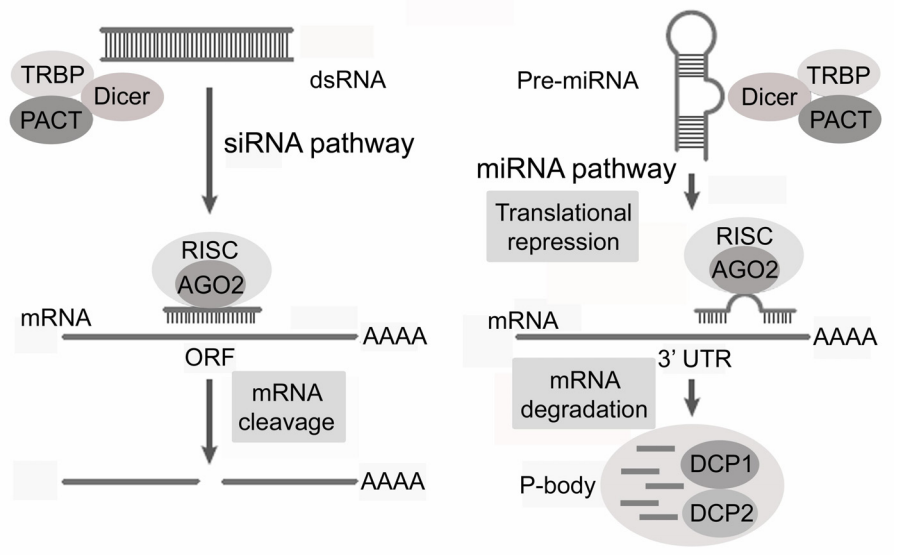

Figure 5. *Mechanisms of siRNA and miRNA pathways in the cytoplasm of mammalian cells.* Cytoplasmic dsRNAs or pre-miRNAs are processed by an enzyme complex consisting of Dicer, TAR RNA-binding protein (TRBP) and protein activator of protein kinase PKR (PACT) into siRNAs or miRNAs which are loaded into Argonaute 2 (AGO2) and the RNA-induced silencing complex (RISC). The siRNA guide strand recognizes the target sites to direct mRNA cleavage, which is carried out by the catalytic domain of AGO2. In the case of the miRNA-pathway, the mature miRNA recognizes target sites in the 3' untranslated region (3' UTR) of mRNAs to direct translational inhibition and mRNA degradation in processing P-bodies that contain the decapping enzymes DCP1 and DCP2. ORF: open reading frame. Figure modified from Kim *et al.*, 2007.

shRNA delivery by gene transfer vector

Vector mediated shRNA delivery is based on DNA-based cassettes expressing shRNAs from RNA polymerase III (Pol III) promoters (Brummelkamp *et al.*, 2002). These promoters include the human ribonuclease P RNA component H1 or the human or mouse U6 small nuclear RNA promoter. They have well defined transcriptional initiation and precise termination sites, and have therefore become a popular choice for vector based gene silencing. The minimal shRNA expression cassette includes a Pol III promoter, directly followed by at least 19 nucleotides of the sense target sequence, a loop of 4-10 nucleotides, the complementary antisense target sequence, and a stretch of four to six uridylates as a terminator (**Figure 6**). The duplex shRNAs are substrates for nuclear export by the exportin-5 pathway, and they are further processed by Dicer to yield functional siRNA duplexes. In contrast, the siRNAs enter the RISC directly. The most critical factor in determining the success of RNAi mediated gene silencing is the choice of target sequence. Despite the presence of different algorithms predicting the performance of a particular sequence, not all predicted sequences result in efficient silencing. Thus, each siRNA or shRNA must be tested independently. The expression cassettes for shRNAs may be introduced into cells within a plasmid vector or, for improved efficiency and stable expression, within a viral vector such as an adenoviral vector (Shen *et al.*, 2003), a retroviral vector

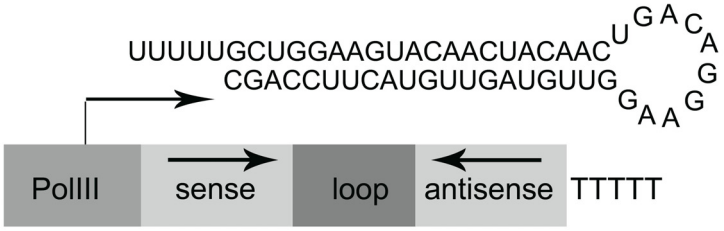

Figure 6. *A diagram of a polymerase III (Pol III) driven shRNA hairpin expression cassette used in gene transfer vectors*. Expression cassette consists of a Pol III promoter, a hairpin forming sequence and a termination sequence of thymidines (T) and the resulting shRNA molecule after transcription.

(Barton *et al*., 2002), a lentiviral vector (Rubinson *et al*., 2003) or an AAV-vector (Han *et al*., 2004). The LV-mediated approach has proven efficient in the delivery of shRNAs into stem cells and in the development of gene-knockdown in pre-implantation mouse embryos (Rubinson *et al*., 2003, Tiscornia *et al*., 2003).

RNAi applications

In addition to *in vitro* applications of RNAi it has also been exploited in transgenesis using lentiviral vectors (Singer *et al*., 2006). In comparison to the standard method of generating knock-down rodents, that is the induction of targeted deletions in the mouse genome via homologous recombination, LV-mediated RNAi has proven efficient in both loss-of-function analysis and the establishment of disease models. As a therapeutic approach, RNAi has enormous potential in a vast range of pathologies. Viral diseases are potent targets of RNAi, and the efficacy of this approach has been demonstrated against the HIV infection by successful targeting of most viral transcripts in cells infected with HIV (reviewed by Rossi, 2006). Clinical trials for the treatment of respiratory syncytial virus infection and of HIV infection were scheduled to begin in year 2007. Also, RNAi therapeutic approaches directed to ocular disease and age-related macular degeneration, are currently in progress. These trials are based on the intraocular delivery of siRNAs targeting VEGF and VEGF-receptor 1. For cancer therapy, various molecular pathways may be intervened by the employment of RNAi, and antiproliferative or proapoptotic effects have been reported in cell culture studies or animal models (reviewed by Pai *et al*., 2006). Suggested targets for cancer therapy include gene products involved in carcinogenesis, e.g. cell cycle regulators, molecules of oncogenesis pathways and cell senescence factors. Also, molecules crucial for tumor-host interactions, factors involved in tumor resistance to chemo- or radiotherapy and molecules related to DNA repair mechanisms represent potential targets for RNAi mediated cancer therapy. RNAi based gene therapy has also been studied in animal models of neurodegenerative diseases. For example, the delivery of a shRNA against mutant ataxin-1 by AAV vector into the brain of mice representing the pathology of spinocerebellar ataxia type 1, led to a beneficial effect on neuronal cells (Xia *et al*., 2004). Also, in a mouse model of ALS, LV-mediated delivery of shRNA against the mutant superoxide dismutase 1 led to long-term, stable gene silencing along with improved survival of motor neurons and a delayed onset of the disease phenotype in mice (Ralph *et al*., 2005, Raoul *et al*., 2005a).

Although RNAi holds a clear potential for therapeutic applications, the current technology has its limitations. Due to similarities in nucleic acid sequences, RNAi mediated therapy may mediate off-target effects (Jackson *et al*., 2006). Also, non-specific immune stimulation, such as interferon response and dendritic cell activation through toll-like receptors, has been observed (Judge *et al*., 2005). However, it is likely that a greater understanding of the selection criteria for optimal siRNA or shRNA sequence will at least partly overcome these issues. As in the case of gene therapy, the potential success of RNAi therapy is much dependent on the development of improved delivery methods, relating both to synthetic siRNA delivery and transfer vector based shRNA delivery.

AIMS OF THE STUDY

The aim of this study was to develop HIV-1 derived lentiviral gene transfer vectors for gene therapy, mainly for applications of gene therapy for cardiovascular diseases. Since LVs are able to integrate into the host cell genome, there is a requirement for regulated therapeutic gene expression. Therefore, approaches to regulate LV-mediated gene expression were explored in this study. During the recent years two novel technologies, RNA interference and progenitor cell therapy, have emerged. We aimed at combining these approaches to lentiviral gene transfer technology.

More specifically, the following objectives were addressed:

The design of an exogenously regulated lentiviral vector system (I)

For regulated gene expression by administration of an orally available drug, can the novel transactivator rtTA2^{S}M2 for the Tet-on system be successfully applied to the HIV-1 derived vector system?

The design of an oxidative stress induced lentiviral vector (II)

Can the antioxidant response element (ARE) sequence be utilized for an oxidative stress inducible gene transfer vector? Which of the studied ARE-elements are the most potent and what is the most effective combination? Is the HIV-1 derived lentiviral vector suitable for oxidative stress induced gene expression applications?

Long-term therapeutic gene expression in cardiovascular gene therapy applications (unpublished)

Is the HIV-1 based lentiviral vector effective for *in vivo* gene transfer to the rabbit carotid artery by intraluminal or periadventitial approach, rabbit skeletal muscle by intramuscular injection and to the porcine myocardium by intramyocardial injection?

Cord blood derived progenitor cell therapy in skeletal muscle ischemia (III)

Do HIV-1 derived lentiviral vectors efficiently transduce different subfractions of human CB derived progenitor cells? Do CB progenitor cells possess therapeutic potential in the mouse model of skeletal muscle ischemia? Does lentiviral transduction of the VEGF-D$^{\Delta N \Delta C}$ growth factor gene to progenitor cells enhance their therapeutic potential?

The design of a lentiviral vector for gene silencing by RNA-interference (IV)

May HIV-1 derived lentiviral vector be utilized for long-term gene silencing by RNAi? Which RNA polymerase III promoter is more powerful for efficient silencing; U6 or H1?

Real-time quantitative PCR - approach for the analysis of lacZ marker gene expression (V)

Can a real-time quantitative PCR method be developed for the accurate quantification of *lacZ* marker gene transcription?

MATERIALS AND METHODS

SUMMARY OF THE MATERIALS AND METHODS

The materials and methods used in this study are summarized in the following tables and described in detail in the original publications (I, III-V) and in the manuscript (II). The methods used for assessing lentiviral vector efficacy in animal models that are not intended for publishing are described below.

Table 2. A summary of the prime methods used for this study.

Principal method	Description	Used in
DNA cloning	Vector construction	I, II, IV
Production of lentiviral vectors	FuGENE^{R} transfection	I
	Calcium phosphate transfection	II, III, IV
	Ultracentifugation for virus concentration	I, II, III, IV, up.
Analysis of lentiviral vector quality	Titer determination by p24 ELISA	I, II, III, IV
	Titer determination by FACS	III
Cell biology methods	FACS	III, IV
	Fluorescence microscopy	III, IV
	Luminescent β-galactosidase assay	I
	Luciferase assay	II
	Chemical induction of oxidative stress by DEM or 15dPGJ$_2$	II
	hCB cell subfraction isolation	III
	Progenitor cell differentiation assays	III
DNA-based analysis methods	PCR	I, II, III, up.
	Southern blot	IV
RNA-based analysis methods	RT-PCR	I, III, up.
	Quantitative real-time PCR (qPCR)	IV, V
Immunological methods	Immunocytochemistry	III, IV
	Immunohistochemistry	III, IV, up.
	ELISA	I, II, III, IV
	Western blot	II
	MACS	III
Tissue sample processing	Fixation, embedding, sectioning, liquid nitrogen freezing	I, III, IV
Other analysis methods on tissue samples	X-gal staining for β-galactosidase activity	I, up.
	Hematoxylin-eosin -staining	III
	Morphometric analysis	III
	Miles assay	up.

Abbreviations used: up.: unpublished results, FACS: fluorescence activated cell sorting, ELISA: enzyme linked immunosorbent assay, DEM: diethyl maleate, 15 dPGJ$_2$: 15-deoxy-12-14-prostaglandin J2, PCR: polymerase chain reaction, MACS: magnet activated cell sorting

Table 3. Plasmids used in this study.

Plasmid	Description	Origin	Used in
pCMV-G	VSV-G envelope expression plasmid	T. Friedmann, USCD, La Jolla, CA, USA	I, II, III, IV
pMDLg/pRRE	HIV-1 Gag-Pol encoding packaging plasmid	I. Verma, Salk Institute, La Jolla, CA, USA	I, II, III, IV
pRSV-Rev	HIV-1 Rev encoding packaging plasmid	I. Verma	I, II, III, IV
pHIV-CS	Third generation HIV-1 vector plasmid, SIN-vector due to deletion in 3' LTR	I. Verma	I
pUHrT61-1	hCMV-rtTA2^{S}M2 encoding plasmid	H. Bujard, ZMBH, Heidelberg, Germany	I
pHIV-rtTA2^{S}M2	HIV-CS based vector encoding rtTA2^{S}M2	J. Koponen, University of Kuopio, Finland	I
pTRE2-LacZ	Plasmid with lacZ cDNA under the control of tetracycline responsive element containing promoter	Clontech	I
pHIV-TRE-LacZ	pHIV-CS based vector with TRE-LacZ	J. Koponen	I
pGL3-Promoter	Plasmid luciferase cDNA under the minimal SV40 promoter	Promega	II
pGL-hNQO1, pGL-hGCLM, pGL-mHO1	Luciferase reporter constructs with antioxidant response elements (AREs) of respective genes in combinations of of 1, 2 and 3 copies of each element	H. Hurttila, University of Kuopio, Finland	II
pLV-hNQO1, pLV-hGCLM, pLV-mHO1	Lentiviral plasmid vectors of ARE reporter constructs, vectors with 1 and 2 copies of each element	H. Hurttila	II
pLV-PGK-GFP	Third generation HIV-1 SIN-vector with cPPT and WPRE elements. Human PGK promoter driving the expression of the GFP marker gene.	L. Naldini, IRCC, Turin, Italy	III
pLV-PGK-VEGF-D$^{\Delta N\Delta C}$	As pLV-PGK-GFP, but GFP replaced with VEGF-D$^{\Delta N\Delta C}$ cDNA	P. Mäkinen, University of Kuopio, Finland	III
pBluescriptII	Cloning vector	Stratagene	IV
pLV-PGK-ΔNGFR	As pLV-PGK-GFP, but GFP replaced with ΔNGFR marker gene cDNA	L. Naldini	IV
pLV-U6shGFP, pLV-H1shGFP, pLV-U6shGFP-H1shGFP	shGFP vectors with U6 or H1 promoter or both, based on pLV-PGK- ΔNGFR	P. Mäkinen	IV
pLV-U6shGAPDH, pLV-H1shGAPDH, pLV-U6shGAPDH-H1shGAPDH	shGAPDH vectors with U6 or H1 promoter or both, based on pLV-PGK-ΔNGFR	P. Mäkinen	IV
pRRL	Third generation HIV-1 SIN-vector with cPPT and WPRE elements. CMV promoter for transgene expression.	W. Osborne, University of Washington, Seattle, WA, USA	up.
pRRL- VEGF-D$^{\Delta N\Delta C}$	pRRL-based vector encoding VEGF-D$^{\Delta N\Delta C}$	P. Mäkinen	up.
pRRL-LacZ	pRRL based vector encoding lacZ	P. Mäkinen	up.

Table 4. Cell lines and primary cell types used in this study.

Cell line or type	Description	Origin	Used in
293T	Human embryonic kidney epithelial cell line expressing SV40 large T antigen	American type culture collection (ATCC), Manasass, VA, USA	I, II, III, IV
CHO	Chinese hamster ovary cell line	ATCC	I
EAhy926	Human endothelial hybridoma cell line	C.J. Edgell, UNC, Chapel Hill, NC, USA	I
HUVEC	Human umbilical vein endothelial cells	Primary cells isolated from umbilical cord	II
hCB $CD133^+$ and $CD34^+$ MNCs	Human $CD133^+$ and $CD34^+$ MACS-purified mononuclear cell subfractions	Primary cells isolated from cord blood	III
hCB MSCs	Human mesenchymal stem cells	Primary cells isolated from cord blood	III
c166-GFP	Mouse endothelial cell line with stable expression of GFP marker gene	ATCC	IV
HeLa	Human cervical adenocarcinoma cell line	ATCC	IV
PA317	Mouse fibroblast cell line with stable expression of LacZ marker gene	T. Friedmann, USCD, La Jolla, CA, USA	V

Table 5. Primary antibodies used in this study.

Antibody	Description	Application	Distributor	Used in
CD34 and CD133	Mouse anti-human antibodies conjugated with magnetic microbeads	MACS, FACS	Miltenyi Biotec	III
CD34, CD133, CD13, CD29, CD44, CD49e, CD73, CD90, CD105, HLA-ABC, CD14, CD45, HLA-DR	Mouse anti-human antibodies for the detection of cell surface antigens conjugated with fluorochromes	FACS	BD Biosciences	III
CD34	Rat anti-mouse antibody for the detection of blood vessel endothelium	IHC	HyCult Biotechnology	III
mMQ	Rabbit anti-mouse antibody for the detection of macrophages	IHC	Accurate Chemical and Scientific	III
VEGF-D	Mouse anti-human antibody	ELISA	R&D Systems	III
NGFR	Mouse anti-human PE-conjugated and non-conjugated antibody targeted to NGFR	FACS, IHC	BD Biosciences	IV
HO-1	Rabbit anti-human polyclonal antibody	WB	Stressgen	II
GCLM	Rabbit anti-human polyclonal antibody	WB	T. Kavanagh, University of Washington, WA, Seattle, USA	II
β-actin	Rabbit anti-human polyclonal antibody	WB	Cell Signaling	II

Abbreviations used: MACS: magnetic activated cell sorting, FACS: fluorescence activated cell sorting, PE: phycoerythrin, NGFR: nerve growth factor receptor, IHC: immunohistochemistry, ELISA: enzyme-linked immunosorbent assay, WB: western blotting, GCLM: glutamate-cysteine ligase modifier subunit

Table 6. HIV-1 lentiviral vectors and their properties used in this study.

Vector	Promoter	Transgene	Additional elements	Origin	Used in
HIV-rtTA2 ^{S}M2	CMV	Reverse tetracycline transactivator rtTA2 ^{S}M2		J. Koponen, University of Kuopio, Finland	I
HIV-TRE-LacZ	Minimal CMV with seven copies of TRE	LacZ marker gene encoding for β-galactoside		J. Koponen	I
LV-hNQO1-Luc, LV-hGCLM-Luc, LV-hGCLM-HO-1	ARE elements (1 or 2 copies) of respective genes combined with minimal SV40	Luciferase or heme oxygenase 1	cPPT, WPRE	H. Hurttila, University of Kuopio, Finland	II
LV-PGK-GFP	Human PGK	GFP marker gene	cPPT, WPRE	L.Naldini, IRCC, Turin, Italy	III
LV-PGK-VEGF-D$^{\Delta N \Delta C}$	Human PGK	VEGF-D$^{\Delta N \Delta C}$	cPPT, WPRE	P. Mäkinen, University of Kuopio, Finland	III
LV-U6shGFP, LV-H1shGFP, LV-U6shGFP-H1shGFP	Human U6 or/and H1 and PGK	small hairpin RNA targeting GFP; shGFP, ΔNGFR marker gene	cPPT, WPRE	P. Mäkinen	IV
LV-U6shGAPDH, LV-H1shGAPDH, LV-U6shGAPDH-H1shGAPDH	Human U6 or/and H1 and PGK	small hairpin RNA targeting mouse GAPDH; shGAPDH, ΔNGFR marker gene	cPPT, WPRE	P. Mäkinen	IV
RRL- VEGF-D$^{\Delta N \Delta C}$	CMV	VEGF-D$^{\Delta N \Delta C}$	cPPT, WPRE	P. Mäkinen	up.
RRL-LacZ	CMV	LacZ marker gene	cPPT, WPRE	P. Mäkinen	up.

Abbreviations used: CMV: cytomegalovirus, TRE: tetracycline responsive element, hNQO1: human NAD(P)H quinone oxidoreductase, hGCLM: human glutamate-cysteine ligase modifier subunit, HO-1: heme oxygenase 1, ARE: antioxidant response element, PGK: phosphoglycerate kinase, GFP: green fluorescent protein, cPPT: central polypurine tract, WPRE: woodchuck hepatitis virus post-transcriptional element, ΔNGFR: truncated form of nerve growth factor receptor, GAPDH: glyceraldehyde-3-phosphate dehydrogenase, up.: unpublished results

Table 7. PCR-methods used in this study.

Target gene or region	Application	Used in
Beta-galactosidase (lacZ)	gPCR, RT-PCR, qPCR	I, V, u.p.
Tetracycline reverse transactivator rtTA2 ^{S}M2	gPCR, RT-PCR	I
hNQO1, hGCLM AREs	PCR cloning	II
hHO1	qPCR	II
Human chromosome 17 α-satellite region	gPCR	III
Human U6 and H1 promoter	PCR cloning	IV
Green fluorescent protein (GFP)	qPCR	IV
Rodent GAPDH	qPCR	IV
Mouse Oas-1a, MCP-1, VEGF-A, PDGF-β	qPCR	IV
Eukaryotic ribosomal 18S RNA	qPCR	IV, V
Human VEGF-D$^{\Delta N \Delta C}$	RT-PCR	up.

Abbreviations used: gPCR: genomic PCR, RT-PCR: reverse-transcription PCR, qPCR: quantitative PCR, hNQO1: human NAD(P)H quinone oxidoreductase, hGCLM: human glutamate-cysteine ligase modifier subunit, ARE: antioxidant response element, hHO1: human heme oxygenase -1, Oas-1a: 2',5'-oligoadenylate synthetase -1a, MCP-1: monocyte chemoattractant protein-1, VEGF-A: vascular endothelial growth factor -A, PDGF- β: platelet derived growth factor – β, up: unpublished results

Table 8. Animal models used in this study.

Animal	Model	Used in	n
Rat	Intracerebral injection of lentiviral vector	I	20
Mouse	Hindlimb ischemia model, intramuscular injection of progenitor cells	III	40
	Intracerebral injection of lentiviral vector	IV	28
Rabbit	Intraluminal administration of lentiviral vector into carotid artery	up.	4
	Periadventitial administration of lentiviral vector into carotid artery		4
	Intramuscular injection of lentiviral vector	up.	9
Pig	Intramyocardial injection of lentiviral vector	up.	3

Abbreviations used: n: number of animals, up: unpublished

Methods of assessing HIV-1 lentiviral vector efficacy in animal models of cardiovascular gene therapy (unpublished)

General methodology

Pilot animal studies to assess the efficacy of third generation lentiviral gene transfer vector in previously established animal models are described below. For these studies RRL-LacZ, RRL-VEGF-$D^{\Delta N\Delta C}$ or LV-PGK-VEGF-$D^{\Delta N\Delta C}$ vector preparations with viral titers of 7×10^{8}-1×10^{9} TU/ml were used.

Intraluminal and periadventitial approach for lentiviral vector administration into the rabbit carotid artery

Intraluminal administration of the viral vector in to the rabbit carotid artery was performed as previously described (Gruchala *et al*., 2004). New Zealand White (NZW) rabbits (n=2) were used. Briefly, a segment of exposed carotid artery was isolated between atraumatic vascular clamps, and two cannulas were introduced into the vessel lumen through proximal and distal arteriotomies. The lumen of the vessel was flushed with saline, and a volume of 100 µl of RRL-LacZ vector was administered into the vessel lumen and allowed to dwell for 20 min. After the incubation, blood flow was restored. In a subgroup of rabbits (n=2), the carotid artery was mechanically injured 4 days before gene transfer. Injury was done by introducing an angioplasty balloon catheter into the artery and passing it three times to denude the endothelial layer and disrupt the internal elastic lamina. Animals were sacrificed 7 days after viral vector administration and artery samples collected for *lacZ* gene expression determination by X-gal, RT-PCR and PCR.

Periadventitial administration of the viral vector was done with the extravascular model taking advantage of a silastic collar positioned around the rabbit carotid artery (n=4) (Laitinen *et al*., 1997). Briefly, a collar (2 cm in length, Ark Therapeutics, Kuopio, Finland) was placed around the exposed carotid artery. The vector solution (300 µl) was administered into the collar and tissue glue was used to seal the collar. The animals were sacrificed 10 (n=2) or 28 (n=2) days after viral vector administration and samples collected for lacZ gene expression determination by X-gal, RT-PCR and PCR.

Intramuscular injection of lentiviral vector in rabbit skeletal muscle

Intramuscular injection of the RRL-LacZ LV in non-ischemic NZW rabbit semimembranosus thigh muscle (n=3) was performed as previously described (Rissanen *et al*., 2003a). A total volume of 1 ml of vector stock was divided into 10 separate injections. Seven days after the injections, the animals were sacrificed and muscle samples collected for lacZ gene expression analysis by X-gal staining, PCR and RT-PCR.

The angiogenic potency of RRL-VEGF-$D^{\Delta N\Delta C}$ was studied by skeletal muscle injections. Also, the potential gene transfer efficiency enhancing effect of bupivacaine, a myotoxic local anaesthetic, was studied by bupivacaine pre-treatment of muscle (5 mg/ml, 10 injections 0.1 ml each) 3 days before vector injections. Lentiviral vectors RRL-LacZ (n=2, of which one rabbit was pre-treated with bupivacaine) and RRL-VEGF-$D^{\Delta N\Delta C}$ (n=4, of which 2 rabbits were pre-treated with bupivacaine) were injected. Contrast enhanced ultrasound imaging was used to asses muscle perfusion 7 and 21 days after vector administration. Animals were sacrificed after 21 days and samples collected for X-

gal staining, PCR and RT-PCR. To analyse the angiogenic effect, samples were subjected to immunohistochemical staining of capillary vessels and vascular permeability by the Miles assay as previously described (Rissanen *et al.*, 2003a).

Intramyocardial injection of lentiviral vector in porcine myocardium

The efficacy of RRL-VEGF-D$^{\Delta N \Delta C}$ in porcine myocardium (n=3) was studied by utilizing a NOGA electromechanical mapping and injection system offering a percutaneous route of intramyocardial injection as previously described (Rutanen *et al.*, 2004). Briefly, electromechanical mapping of the left ventricle was performed with the NOGA system and injection catheter. After mapping, ten myocardial injections of the RRL-VEGF-D$^{\Delta N \Delta C}$ vector (0.2 ml per injection, total volume 2 ml) were performed to the anterolateral wall. Six days after the injections, myocardial perfusion was studied with contrast echocardiography. The animals were sacrificed 14 days after vector administration and samples collected for a Miles permeability assay and histological analyses.

RESULTS AND DISCUSSION

Doxycycline regulated lentiviral vector system (I)

A lentiviral vector system utilizing the revised tetracycline transactivator, rtTA2^{S}M2, showed a tight regulation of lacZ gene expression with negligible basal level in the non-induced stage. The gene expression level could be adjusted by the dosing of doxycycline, and expression could be repeatedly switched on and off. These results were obtained by *in vitro* cotransduction of two LVs, one encoding the rtTA2^{S}M2 and the other the lacZ marker gene under the control of the tetracycline responsive promoter. By cotransducing CHO and EAhy926 cell lines at a low viral multiplicity of infection (MOI 1 for HIV/TRE/LacZ and MOI 2 for HIV/ rtTA2^{S}M2), a 132 and 17 –fold induction in β-galactosidase activity, respectively, was detected. During long-term culture of co-transduced CHO cells, the lacZ gene could be repeatedly switched on and off by the addition and withdrawal of doxycycline. Importantly, the induction level was dose-dependent and full induction was achieved at a doxycycline concentration of 500 ng/ml, which can be obtained by oral administration. LVs efficiently transduce several cell types of brain. Also, doxycycline is known to penetrate the blood-brain barrier. Therefore, as proof of the principle of the *in vivo* functionality of the vector system, vectors were injected into the rat brain. Following cotransduction of viral vectors into the rat striatum (n=20), RT-PCR revealed that doxycycline induced lacZ transcription in 5 out of 7 rats and not in the 3 rats where doxycycline was not administred. Ten rats were used for X-gal staining to study β-galactosidase activity in frozen brain sections. In all rats which received doxycycline (n=5) X-gal positive cells were detected. No positive cells were seen in samples from rats without doxycycline treatment (n=5), further confirming the tightness of gene induction with our vector system. DNA PCR confirmed the presence of both vectors in the brain of every animal. However, this does not confirm that cotransduction of both vectors targeted the same cells.

The use of rtTA2^{S}M2 for tight doxycycline dependent regulation has also been reported by others, for example, in the generation of conditionally transgenic animals (Michalon *et al*., 2005, Katsantoni *et al*., 2007) and in *in vivo* gene transfer approaches with AAV-vectors in mouse and nonhuman primate muscle and retina (Chenuaud *et al*., 2004, Stieger *et al*., 2006). However, dose-response experiments have demonstrated that tightness of control may be partially lost at higher vector doses (Lamartina *et al*., 2002). These findings stress the importance of specifying an optimal vector dose for each application.

There are specific advantages achieved by introducing the elements for doxycycline regulated gene expression into two separate vectors. The first, is the ability to adjust the proportion of each transcriptional unit by simply changing the ratio and amount of vector particles applied for optimal gene regulation. Secondly, in the two vector system the transcriptional units are not in close proximity and therefore, the regulated promoter is not disturbed by promoter interference from an adjacent promoter constitutively expressing the transactivator, an apparent disadvantage of the single vector system (Bornkamm *et al*., 2005). The two vector -system enables the generation of transgenic animals with induced transgene expression accomplished by first generating separate mouse lines that contain the transactivator and target constructs, and then producing double transgenic animals by breeding. Since lentiviral vectors are proven to be exceptionally powerful tools in transgenesis, our vector system shows the

potential to be exploited in the generation of conditionally transgenic animals.

Despite the advantages of a two-vector system, there are numerous applications where repeatable transfer of the transcriptional units required for regulated gene expression require the use of a single vector. These include all *in vivo* gene transfer procedures. Vogel and coworkers have described a HIV-1 based single-vector system, in which the transcriptional unit for rtTA2^{S}M2 has been inserted in the antisense orientation and separated from the regulatable unit by the chicken β-globin insulator (Vogel *et al*., 2004). The authors report transgene expression regulation in the rat striatum over two orders of magnitude by the addition or withdrawal of doxycycline. The latest and most advanced versions of the Tet-on system where all components are on a single vector combine the rtTA2^{S}M2 with the use of a transrepressing factor, doxycycline-dependent trans-silencer called tTS. tTS is a fusion protein consisting of the KRAB (Kruppel-Associated Box) transrepressing domain of the human Kid-1 protein fused to the wild-type TetR (Salucci *et al*., 2002, Lamartina *et al*., 2003, Epanchintsev *et al*., 2006). The KRAB-domain can induce transcriptional silencing to within 3 kb of its binding site. In the absence of doxycycline, tTS binds to the tetO target sequence and inhibits basal transcription. As doxycycline is added, the tTS dissociates allowing rtTA2^{S}M2 to bind and trigger activation. This design is preferred in single-vector applications since the transrepressing factor, that ensures the inactivity of the regulated promoter in the off-state, abolishes any potential promoter interference. It may also reduce interference from genomic elements which come into proximity after integration when integrating vectors are used to deliver the regulatory system. However, a disadvantage of using the tTS means the inclusion of an additional transcriptional unit, which complicates the vector system and increases the size of genomic material to be inserted into the transfer vectors.

Overall, particularly from a clinical point of view, the doxycycline system has a clear pharmacological advantage over other gene regulation systems like mifepristone or rapamycin. Namely, that doxycycline has a half-life of 14-22 h, has excellent tissue penetration due to its lipophilic nature and is well tolerated. The most serious side effect is a dose-dependent photosensitivity that may occur in 1-3% of patients after prolonged administration (Klein *et al*., 2001). The latest versions of the Tet-on system can be fully induced in rodents and monkeys by doxycycline at doses comparable to those commonly used for antibiotic therapy (Lamartina *et al*., 2003). The major concern with respect to the use of an antibiotic-induced regulatory system is the possibility of raising resistance to the antibiotic itself. Although tetracycline derivatives are less used than in the past, they may still represent the treatment of choice for certain infectious diseases such as Lyme disease (Klein *et al*., 2001). Thus, the generation of Tet-system compatible, non-antibiotic doxycycline analogs would be desirable.

Oxidative stress inducible lentiviral vector (II)

We studied if the antioxidant response element (ARE), a cis-acting sequence found in the promoters of the genes induced by oxidative stress, can be utilized for gene induction in a transfer vector. The ARE sequences of three genes, human NADPH quinine oxidoreductase 1 (NQO1), human glutamate-cysteine ligase modifier subunit (GCLM) and mouse heme oxygenase 1 (HO-1), were subcloned into a reporter plasmid vector in combination with one, two or three copies of the ARE sequence, upstream of the minimal promoter. After plasmid transfections, oxidative stress was mimicked

by the addition of a chemical, either diethyl maleate (DEM) or 15-deoxy^{12,14}-prostaglandin J_2 (15-dPGJ_2), and luciferase activity of the cell cultures was analysed. In all constructs, luciferase activity was induced upon chemical induction of oxidative stress. However, there was some basal expression of luciferase without induction and the expression increased in relation to the number of ARE elements in the constructs. The basal expression level could be due to reactive oxygen species and oxidative products of macromolecules produced by normal cell respiration and thus cannot be avoided. Also, it should be noted that the oxygen levels in cell culture conditions (21%) are much higher than the naturally occurring levels in most tissues (Roy *et al*., 2003), and it is likely that basal ARE-activity would be lower in an *in vivo* situation. In our study, for more efficient and long-lasting expression, the reporter constructs with one and two copies of AREs from hNQO1 or hGCLM genes were cloned into LVs and their efficacy for oxidative stress induced gene expression studied in human umbilical vein endothelial cells (HUVECs). Endothelial cells represent a potential target for antioxidant gene therapies (Levonen *et al*., *in press*) and were therefore selected for these studies. Upon chemical induction of oxidative stress, a clear induction of luciferase activity was seen with all lentiviral constructs. When HUVECs were transduced at the MOI of 5, the highest induction in luciferase activity (nine fold activity compared to the uninduced state) was seen with a construct carrying two copies of hGCLM ARE. Thus, this vector was used for the following studies. When the luciferase reporter gene was replaced with the potential therapeutic gene, heme oxygenase 1 (HO-1), a 10-fold induction of gene expression was seen. This is a significant result, as the most advanced hypoxia-induced vector system yield seven fold induction in gene expression upon hypoxia in cell culture (Tang *et al*., 2005). In addition, we used TNF-α to mimic vascular inflammation in HUVECs and studied the effect of ARE-induced HO-1 therapeutic gene expression on the expression of the proinflammatory factor, VCAM-1. Our results showed that ARE induced HO-1 gene expression was able to decrease the expression of VCAM-1 and thus, the system holds potential for the therapy of oxidative stress.

Based on our results the genetic ARE elements, induced cytoprotective genes by oxidative stress, and can be exploited in gene transfer vectors. Since oxidative stress can not be completely mimicked in cell culture conditions, the true performance of our ARE vectors need to be defined in animal models. Considering the lentiviral vector tropism for brain tissue, an experimental model of stroke might be optimal for these studies. Also, for the promotion of cell survival in oxidative conditions in progenitor cell therapy approaches, our lentiviral ARE constructs may be readily applicable. As oxidative stress plays a role in atherosclerosis, restenosis and ischemia-reperfusion injury of myocardium and skeletal muscle, the ARE induced expression of therapeutic genes using AAV vector, which has proved to be more effective at the transduction of these tissues, is an appealing concept.

The performance of lentiviral HIV-1 derived vector in cardiovascular gene therapy applications (unpublished data)

Most of the previous gene therapy applications for cardiovascular diseases have used adenoviruses. Despite their efficiency, only short-term transgene expression is achieved; maximal protein expression is achieved after four days and this declines to below detection level after two weeks in most applications. Several cardiovascular applications would benefit from long-term therapeutic gene expression.

Therefore, we tested the efficiency of the HIV-1 derived, third generation self-inactivating LV to transduce skeletal muscle, blood vessel and myocardium and mediate a therapeutic effect. The results of these pilot studies are summarized in **Table 9**.

The intraluminal approach may be performed simultaneously with angioplasty and is a potential choice for the administration of vectors for gene therapy of restenosis. We studied the efficacy of LV in the blood vessel by intraluminal administration in a rabbit model (Gruchala *et al*., 2004). In a subgroup of rabbits, mechanical injury by balloon catheter was performed simultaneously to disrupt the endothelial cell layer and the internal elastic lamina of the blood vessel in order to model balloon angioplasty revascularization procedure. As a result, no LacZ gene expression was detected by X-gal staining or RT-PCR in arterial samples of rabbits sacrificed seven days after vector administration into the lumen of intact arteries, whereas in the samples of denuded arteries LacZ gene expression could be detected with RT-PCR. However, β-galactosidase positive cells were not detectable by X-gal staining, suggesting a low level of transgene expression only detectable with the sensitive RT-PCR method. LacZ expression present only in arteries preconditioned with balloon denudation may be due to the abolishment of the endothelial cell layer or, induced proliferation of endothelial and smooth muscle cells due to injury, thus facilitating the viral transduction. In intraluminal gene transfer of the rabbit carotid artery, the use of AAV and adenoviral vectors have resulted in effective transduction (Gruchala *et al*., 2004). AAV vectors showed efficient transduction of smooth muscle cells lasting for at least 100 days and therefore, can be regarded as more potent arterial gene transfer vehicles than LVs for prolonged therapeutic gene expression.

To study whether adventitial exposure of LV would result in a significant gene transfer efficiency into the blood vessel, we assessed a model of periadventitial administration of vector making use of a silastic collar placed around the rabbit carotid artery (Laitinen *et al*., 1997). Ten days after LV administration, transgene expression could be detected with RT-PCR, while X-gal staining did not show the presence of positive cells suggesting a very low efficacy of gene transfer. 28 days after LV administration, LacZ expression was no longer detectable by RT-PCR. Based on these results, we concluded LV is not an ideal vector for periadventitial arterial gene transfer requiring long-term transgene expression.

Table 9. Pilot studies for the evaluation of LV gene transfer efficacy for cardiovascular gene therapy

Animal	Model	Vector	n	Follow-up (days)	Analysis methods and results for LV gene transfer
Rabbit	Carotid artery, intraluminal administration (Gruchala *et al* 2004)	RRL-LacZ	2	7	X-gal staining (-), RT-PCR (-)
	Carotid artery, intraluminal administration, preconditioned with balloon angioplasty (Gruchala *et al* 2004)	RRL-LacZ	2	7	X-gal staining (-), RT-PCR (+)
	Carotid artery, periadventital administration with silastic collar (Laitinen *et al* 1997)	RRL-LacZ	2	10	X-gal staining (-), RT-PCR (+)
			2	28	X-gal staining (-), RT-PCR (-)
	Skeletal muscle, intramuscular injection (Rissanen *et al* 2003)	RRL-LacZ	3	7	X-gal staining (-), RT-PCR (+), PCR (+)
			4	21	X-gal staining (-), RT-PCR (-), PCR (-), perfusion (-)
		RRL-VEGF-D	2	21	RT-PCR (-), PCR (-), Miles assay (-), perfusion (-), IHC (-)
	Skeletal muscle, intramuscular injection, preconditioned with bupivacaine (Rissanen *et al* 2003)	RRL-LacZ	1	21	X-gal staining (-), RT-PCR (-), PCR (-), perfusion (-)
		RRL-VEGF-D	2	21	RT-PCR (-), PCR (-), Miles assay (-), perfusion (-), IHC (-)
Pig	Intramyocardial injection (Rutanen *et al* 2004)	RRL-VEGF-D	3	14	perfusion (-), Miles assay (-), IHC (-)

Abbreviations used in the table: LacZ: β-galactosidase, VEGF-D: vascular endothelial growth factor-D, RT-PCR: reverse transcription polymerase chain reaction, IHC: immunohistochemistry

The efficacy of LV gene transfer was studied in rabbit skeletal muscle to assess its potential to be utilized in long-term gene therapy applications in a rabbit model of hind limb ischemia. Seven days after intramuscular injections, LacZ gene expression and LV vector presence could be detected by RT-PCR or genomic PCR. However, in tissue sections, transgene expression was not detectable by X-gal staining. Three weeks after LV injections, transgene expression or vector presence could not be detected with RT-PCR or genomic PCR. Also, in animals injected with LV-VEGF-$D^{\Delta N \Delta C}$, the effects typical for this growth factor could not be detected, including enhanced blood perfusion assessed with ultrasound, increased extravasated plasma proteins from newly formed vessels within the tissue measured by Miles assay or, promoted capillary vessel formation detected by immunohistochemistry (Rissanen *et al*., 2003b). In a subgroup of animals, we studied if LV transfer efficiency could be increased by muscle preconditioning with injections of local anaesthetic, bupivacaine, which is considered myotoxic and therefore might enhance proliferative activity of muscle cells (Wells, 1993). As studied 21 days after LV injections, bupivacaine pretreatment of muscle was not seen to promote LV transduction.

In our hands, LV mediated gene transfer in rabbit models of cardiovascular gene therapy did not show potency. Supporting our finding, there are reports suggesting that rabbit cells are to some extent resistant to transduction by HIV-1 derived, VSV-G pseudotyped LVs *in vitro* (Hofmann *et al*., 1999, Ikeda *et al*., 2002). According to the results of Hofmann *et al*, human, hamster and pig cell lines are most prone to HIV-1 LV transduction, while mouse, rat and rabbit cells lines are less efficiently transduced, listed in order of reducing transduction efficiency. This is in line with our own observations that human primary cells and cell lines are very efficiently transduced *in vitro* even with low multiplicities of infection (MOI) of the viral vector, while mouse, rat and rabbit cell lines require higher MOIs for efficient transduction (Koponen JK and Mäkinen P, unpublished data). These observations may be explained by species-specific host cellular factors that limit the post-entry events of the viral vector, such as the nuclear translocation (Hofmann *et al*., 1999). Based on the *in vitro* results showing HIV-1 being more prone to the transduction of human cell lines, it is impossible to predict if LVs could mediate efficient gene transfer in human tissues.

The myocardium is an important target tissue for cardiovascular gene therapy including many applications where sustained expression of the therapeutic gene would be essential. We studied the efficacy of LV in pig myocardium by the intramyocardial approach. We assessed a gene transfer vector injection technique readily applicable for clinical use, the NOGA-catheter -assisted injections with electromechanical mapping of the myocardium (Rutanen *et al*., 2004). After injections of LV- VEGF-$D^{\Delta N \Delta C}$ we searched for the effects of the growth factor previously seen in both skeletal muscle (Rissanen *et al*., 2003b) and myocardium (Rutanen *et al*., 2004). We were unable to see VEGF-$D^{\Delta N \Delta C}$ mediated effect by ultrasound measurement of blood perfusion (6 days after injections), or by Miles assay for the detection of extravasated proteins from newly formed capillary vessel or induced capillary angiogenesis in immunohistochemical stainings (14 days after the injections). Therefore, we concluded LV gene transfer insufficient to trigger therapeutic effect in pig myocardium.

As the pilot animal studies with LVs, performed in our previously established animal models of cardiovascular gene therapy, and described in this thesis,

resulted in no positive LV transduced cells (as detected by histological methods) and was inefficient in terms of the effect of transgene expression, we concluded that the gene transfer efficiency was below what is required for a therapeutic effect. In fact, at least in their current form, LVs seem comparatively poor at delivering genes into the liver and muscle. Despite some encouraging results originally obtained with HIV-1 LVs (Kafri *et al*., 1997), further experiments have generally revealed low efficacies, at least in adult animals. In a previous study from our research group, a modest LV gene transfer efficiency of less than 0.01% of the liver cells has been reported in a rabbit model of FH (Kankkonen *et al*., 2004). However, in this model even a low level of LDL receptor gene transfer was sufficient to produce a long-term serum cholesterol reducing effect probably due to the fact that even a low number of cells with a functional LDL receptor are adequate for such an effect. In terms of gene transfer efficiency, it appears that adenoviral and AAV vectors are better suited for therapies targeted to the liver or muscle, which seems to apply to blood vessel as well (Gruchala *et al*., 2004). Nevertheless, LVs still show excellent performance in gene delivery into the central nervous system, retinal cells and into the cells of lympho-hematopoietic system. In relation to cardiovascular gene therapy, the ability of LVs to transduce HSCs may hold therapeutic potential. For example, *ex vivo* genetic manipulation of progenitor cells of the monocyte lineage could be utilized to modify the effect of macrophages in the development of atherosclerotic lesions or restenosis or, to take advantage of the recruitment of macrophages into areas of pathology where they could express the therapeutic protein. Also, genetic modification of stem and progenitor cells for combined gene and cell therapy for cardiovascular diseases like myocardial ischemia is a fascinating prospect for which HIV-1 LV would be a highly potent vector of choice.

Cord blood-derived progenitor cells in a mouse model for skeletal muscle ischemia (III)

Progenitor cell therapy is a novel approach which may be utilized for the treatment of ischemia in the myocardium and skeletal muscle. Combining gene therapy with cell therapy applications allows for the modification of progenitor cell properties, such as cytokine release, surface receptor expression and factors potentially promoting cell survival in conditions like ischemia or oxidative stress. LVs have enabled the genetic modification of HSCs and embryonic stem cells that were nonpermissive to earlier vectors, especially in the quiescent state. Cytokines, for example, stem cell factor and interleukins, may be used to stimulate HSCs to proliferate and facilitate transduction. However, as it is important to preclude unwanted differentiation of the cells by cytokine induction, an efficient transduction method for unstimulated cells is essential. It has been reported that cytokine pre-treatment of human $CD34^+$ cells reduces their long-term engraftment ability in mice indicating a defect in cell survival or homing to bone marrow (Ahmed *et al*., 2004, Wulf-Goldenberg *et al*., 2007). In our study, we assessed whether cord blood derived HSC subfractions of $CD34^+$ and $CD133^+$ cells can be efficiently transduced by LV without the need for cytokine induced proliferation. At a LV MOI of 100, we achieved comparable transduction efficiencies with and without cytokine induction of both $CD34^+$ (57.0% and 52.5%, respectively) and $CD133^+$ (53.0% and 52.5%) cell subfractions as judged from FACS detecting GFP marker gene fluorescence. Also, we used cell cycle analysis to confirm that cytokines induced cell cycling in HSCs (data not shown). In the majority of published reports utilizing LV gene transfer into HSCs, cytokine stimulation

has been used. However, to exclude cytokine induced effects, optimal transduction conditions requiring minimal modifications of the HSCs should be determined for each application. By a colony forming assay, we were able to show that the ability of $CD34^+$ and $CD133^+$ cells to differentiate into cells of the hematopoietic lineage was not altered by LV transduction, suggesting that their progenitor capacity was not affected. Also, we were able to efficiently transduce MSCs and as detected by adipogenic and osteogenic differentiation assays, the transduced MSCs did retain their differentiation ability.

Cord blood (CB) is an excellent source of progenitor cells in terms of a high percentage of stem cells and the presence of immature HSCs derived from a young individual. We assessed the therapeutic potential of two sub-populations of progenitor cells, $CD133^+$ and MSCs, in a nude mouse model of hind limb ischemia. Cells were transduced with a LV vector encoding the GFP marker gene or the VEGF-$D^{\Delta N\Delta C}$ growth factor gene. Due to the more primitive nature of $CD133^+$ compared to $CD34^+$ cells (Hemmoranta *et al.*, 2006), the $CD133^+$ subpopulation was selected to be tested in the animal model. Also, since $CD133^+$ antigen expression has been connected to endothelial progenitor cells (Gehling *et al.*, 2000), and CB derived $CD133^+$ cells have been reported to be capable of differentiation into endothelial and cardiomyocyte-like cells *in vitro* (Bonanno *et al.*, 2007), we wanted to see if these cells could incorporate into newly forming vessels or regenerating muscle. MSCs have been shown to be capable of multilineage differentiation, for example into skeletal muscle cells (Pittenger *et al.*, 1999) and therefore, might have therapeutical potential in the regeneration process of ischemic muscle.

Because of the contradictory results published earlier, mostly concerning cell therapy of myocardial ischemia, we also analysed whether progenitor cell therapy might mediate a therapeutic effect without cells directly participating in the regeneration process. As a model of progenitor cell mediated gene therapy, we also assessed whether CB cell subfractions expressing VEGF-$D^{\Delta N\Delta C}$ could mediate an angiogenic effect, previously shown to be induced by this particular growth factor (Rissanen *et al.*, 2003b, Rutanen *et al.*, 2004, Kholova *et al.*, 2007). In our model, we injected progenitor cell suspensions (total cell number ~1×10^5) intramuscularly into the left hindlimb muscles of nude mice, for which unilateral hind limb ischemia was induced by femoral artery ligation immediately before injections. Animals were sacrificed 3, 7 or 21 days after the operation. We were unable to show the presence of progenitor cells by immunohistochemistry at any of the time points, as studied by GFP- or human cell mitochondrial or cytoskeleton -specific antibodies in immunohistochemistry (data not shown). By human chromosome 17 α-satellite region specific PCR, we were able to show the presence of human genetic material in the injected muscles three days after injection. However, seven days post-injection human DNA was no longer present. Also, PCR-based detection does not indicate if the cells are alive. Our result is in line with the previous observation by Tateno et al., who have shown that mouse bone marrow -derived mononuclear cells disappeared from mouse ischemic muscle three days after implantation (Tateno *et al.*, 2006). It is unknown, whether these observations are due to the general inability of progenitor cells to engraft or are attributed to ischemic conditions of the muscle, presumably limiting cell survival. By counting capillary vessels, we did not observe enhanced capillary angiogenesis in mice groups injected with CD133+ cells or MSCs transduced with LV-VEGF-$D^{\Delta N\Delta C}$ or LV-GFP, when compared to the control group. Since the typical effect of VEGF-$D^{\Delta N\Delta C}$ was not seen, we concluded

that the injected cells did not secrete enough growth factor to induce therapeutic angiogenesis. This might be due to a too short survival time of the cells in the ischemic muscle. The direct incorporation of bone marrow derived progenitor cells into new or remodelling blood vessels in the ischemic myocardium and skeletal muscle has been previously reported. The magnitude of cellular incorporation highly varies between studies. Although a high occurrence of capillaries containing transplanted cells has been depicted in some studies (Asahara *et al*., 1997, Kocher *et al*., 2001, Kawamoto *et al*., 2003), only a single transplanted cell in the circumference of the capillary vessel is required for the vessel to be counted as positive. This may limit the interpretation of the true effect of cell incorporation into structures of a vascular bead. Furthermore, some studies have reported only small numbers of positive vessels, despite impressive improvements in blood perfusion (Tomita *et al*., 1999, Wang *et al*., 2001, Iba *et al*., 2002), suggesting the existence of alternative mechanisms to the direct cellular incorporation mediating the effects.

In our study, we observed increased regeneration of ischemic muscle by progenitor cell injections (regenerating muscle area 86.7% - 93.8% in progenitor cell injected groups versus 73.2% in the control group) as measured from the histology sections 21 days after the operation. Our results suggest that both CB $CD133^+$ and MSC –progenitor cells increase the regeneration capacity of ischemic muscle by a mechanism unrelated to progenitor cell engraftment. As an alternative mechanism for progenitor cell -mediated therapeutic response in ischemic skeletal muscle, a paracrine effect has been suggested (Kinnaird *et al*., 2004, Ziegelhoeffer *et al*., 2004, Tateno *et al*., 2006). This might be mediated either by the factors released by the progenitor cells themselves or by tissue resident cells stimulated by the progenitor cells. Immunohistochemical staining for macrophages showed a higher count of infiltrated macrophages in the progenitor cell injected muscles. In fact, impaired macrophage recruitment has been reported to cause delayed recovery of hind limb function, increased muscle atrophy and fibrosis in a hind limb ischemia model in CD4-knock-out mice (Stabile *et al*., 2003). Therefore, the regenerative effect of progenitor cell injection might be explained by their attraction of monocytes resulting in a higher number of infiltrating macrophages, which might further stimulate regeneration for example by the release of cytokines. However, as we studied the effects of human CB cells in mouse skeletal muscle ischemia, an immune deficient nude mouse model was required, limiting the interpretation of issues concerning inflammatory mechanisms.

Overall, the mechanisms of progenitor cell mediated response in ischemic myocardium and skeletal muscle require further evaluation. Therefore, new experimental studies are warranted. Clinical trials have been focused mostly on myocardial ischemia (Wollert *et al*., 2005) rather than skeletal muscle ischemia (Tateishi-Yuyama *et al*., 2002a, Ishida *et al*., 2005). Nevertheless, skeletal muscle ischemia might be a more potent candidate for progenitor cell therapies, since there is no need for electrical coupling of engrafting cells and because of the presumed easier administration of the cell preparation in the skeletal muscle compared to the myocardium. Also, the most potent subtypes and sources of progenitor cells need to be identified. CB is enriched with HSCs as compared to other sources like bone marrow or peripheral blood. A world-wide CB banking system provides rapid supply to HLA-typed CB tested for infectious agents as the material can be shipped easily anywhere (Brunstein *et al*., 2006). This makes cord blood an optimal source for progenitor cell

preparations for clinical use. Thus, the potential of CB derived cells in different therapies is worth studying.

A lentiviral vector for gene silencing by RNAi (IV)

A wide range of experimental and therapeutic applications would benefit from sustained RNAi-mediated gene silencing, which is not achieved by introducing synthetic siRNA oligonucleotides. In these situations shRNA-mediated silencing delivered into a target cell within a transfer vector is applicable. However, specific constructs are required for each application. Pol III promoters have well-defined transcriptional start sites and they produce RNA transcripts lacking a polyadenylation tail and are therefore ideally suited for the production of shRNAs. We compared the efficiency of two widely used Pol III promoters, the human U6 small nuclear promoter and the human H1 promoter for their effects on gene silencing after LV mediated transfer of a shRNA against GFP. We chose the marker gene GFP as a target gene because it is easily detected by FACS and fluorescence microscopy. Also, cell lines with stable GFP expression, such as the mouse endothelial cell line c166-GFP, and GFP transgenic mice are commercially available. Our results showed that by LV mediated transduction, the U6 promoter is more powerful than the H1 promoter for GFP marker gene expression silencing *in vitro* in a GFP expressing endothelial cell line as judged at the protein level by FACS (94% and 86% silencing for U6 and H1, respectively) and at the RNA level by quantitative RT-PCR (88% and 71%). Similar results were obtained *in vivo,* in mouse brain as revealed by immunohistochemistry. Also, the U6 promoter was found to be more effective than the H1 promoter in the silencing of the GAPDH housekeeping gene as studied by RT-qPCR (35% and 22%). Compared to the silencing effect of the U6 promoter driven vectors, we did not find enhanced silencing of GFP or GAPDH by a vector with two shRNA cassettes driven by both promoters. This might be due to maximal loading of the small RNA-guided gene-silencing pathway in the transduced cells achieved by strong transcription from the U6 promoter. The fact that we reached far more efficient maximal silencing of GFP than GAPDH gene transcription (88% versus 35%) might be due to tight regulation and higher expression levels of the GAPDH housekeeping gene and more likely, a less efficient RNAi target sequence. By assessing viral vector copy number in the genome of transduced cells by southern blot we were able to confirm the more powerful effect of the U6 LVs compared to the H1 LVs. Namely, in comparison to a single copy of a U6 LV, a higher copy number of H1 LV (5-10 copies) was required for an equal 80% silencing effect. The powerful knock-down effect obtained with a single integrated copy of U6 LV suggests that our vector design has the potential for *in vivo* applications and RNAi applications in cells which are not easily transduced *in vitro*, including stem and progenitor cells and thus, has the applicability in the generation of transgenic mice.

We also studied long-term gene silencing mediated by LVs *in vitro* and *in vivo*. Since the silencing of the GFP marker gene does not lead to a loss of function -effect, it is a potential target for studying long-term silencing. Our *in vitro* results showed that with all constructs, silencing of GFP lasted up to 28 weeks and remained at constant level, as assessed by FACS. Further, stereotactic injection of LV-U6shGFP or LV-H1shGFP vectors into the brain of GFP transgenic mice showed GFP gene silencing for up to 9 months as studied by fluorescence microscopy of frozen brain sections. The merged images of GFP fluorescence and immunohistochemical stainings with a fluorescent signal to detect

marker gene ΔNGFR expression showed the co-localization of diminished GFP fluorescence and the expression of ΔNGFR, further confirming vector-mediated GFP silencing. This suggests the usefulness of our vector in the field of brain research, for example, in developing either models or therapeutic tools for neurodegenerative diseases.

Originally, siRNA induced gene silencing was introduced as a mechanism which can be utilized without inducing a type 1 interferon response when dsRNA sequences shorter than 30 bp are used (Elbashir *et al.*, 2001). However, it has been shown that dsRNA molecules mediating RNAi may trigger an interferon response by alternative pathways, like via Toll-like receptors (Seth *et al.*, 2006). Therefore, the possibility of evoking an interferon response needs to be evaluated for each application. In our study, we used RT-qPCR to study the potential induction of the mouse Oas-1a gene, whose expression is upregulated by an interferon response. We found that LV-mediated RNAi did not induce Oas-1a gene expression and thus did not evoke an interferon response. RNAi induced off-target effects also need to be assessed for each application. For this, we analyzed the expression levels of MCP-1, VEGF-A and PDGFR-β genes, which are all expressed in endothelial cells. We found them unaffected by shRNA expressing LV transductions. Despite the limited gene expression array analysis, the unchanged expression of these representative genes suggests a lack of widespread off-target effects by our vector design.

LV-mediated shRNA delivery has been successfully applied by others in various applications *in vitro* (Li *et al.*, 2005a), *in vivo* (Raoul *et al.*, 2005b, Pfeifer *et al.*, 2006) and *ex vivo* (Anderson *et al.*, 2007, Ohmori *et al.*, 2007) and also, in the generation of knock-out mice (Tiscornia *et al.*, 2003). Combining the efficient LV-mediated generation of transgenic mice with the shRNA approach results in a feasible tool. In fact, we have also used our U6 driven shRNA LVs in the generation of knock-down mice, and the analysis of the generated mouse lines is currently in progress (Hämäläinen *et al*, unpublished data).

To date, the advantages of utilizing Pol III promoters in inducing RNAi have been clearly demonstrated. However, one major limitation of Pol III promoters is their lack of tissue specificity. Therefore, Pol II promoters have also been utilized in RNAi vectors. The Pol II based systems mimic miRNA-based silencing (Zeng *et al.*, 2005), meaning that the transcript needs additional processing by the cellular enzyme Drosha to produce a shRNA before Dicer-mediated processing to yield siRNAs. Because this system mimics the natural miRNA pathway, it might prove advantageous. However, it adds a level of complexity which may be detrimental in some settings. In addition, Pol II based RNAi systems may directly utilize regulatory systems, such as the tetracycline systems. In fact, several systems have been developed for both Pol II and Pol III based RNAi regulation, either reversibly by doxycycline or irreversibly by Cre-Lox based systems (reviewed in Wiznerowicz *et al.*, 2006). Conditional knock-down approaches are useful for therapeutic applications that require long-term silencing, for developmental research, when the effect of knockdown of lethal genes is desired or, for modelling human pathologies in animal models, that is to induce and revert the shutoff of a disease affecting gene, such as an oncogene or tumor suppressor gene. Although using Pol II promoters for RNAi has certain advantages, a more systematic comparison of the levels of RNAi obtained with Pol III-driven shRNAs versus Pol II-driven miRNA-based shRNA is needed. Finally, it is likely that for RNAi applications ranging from screening of shRNA libraries to

gene-based therapies, both systems may be useful.

A real-time quantitative PCR - approach for the analysis of lacZ marker gene expression (V)

Quantitative reverse transcription PCR (qRT-PCR) is a method for quantitative determination of gene expression. The real-time -method, a method monitoring the PCR reaction in the thermal cycler as it progresses, has been extensively utilized since first discovered (Gibson *et al*., 1996). The real-time method utilizes 5' exonuclease activity of Taq-DNA polymerase enzyme through the use of sequence-specific fluorogenic hydrolysis (TaqMan®-chemistry). During the amplification stage of the PCR cycle, a probe that is specific to the amplified region will bind, and as the DNA-polymerase synthesizes the complementary strand, its 5'-exonuclease activity degrades the bound probe. As a result, once the fluorescent molecules, that are tagged to the 5'-end of the probe are released from the close proximity of the 3'-end fluorescent quenching molecules, a fluorescence signal is generated. This signal fluorescence can be measured and it is proportional to the amplified product amount, and thus, relative to the amount of starting material. qPCR or qRT-PCR methods taking advantage of the sequence specific probe are sensitive, specific and enable quantitative determination of traces of sample DNA or very low levels of target gene expression.

We assessed whether a real-time qRT-PCR method utilizing TaqMan®-chemistry would be suitable for the quantification of LacZ marker gene expression. This method was intended for determining the LacZ gene expression level in our doxycycline regulated LV-constructs and for assaying gene transfer efficiency and biodistribution of various viral vectors in animal models. However, despite the appropriate design of primers and probe, qRT-PCR analysis constantly resulted in a strong amplification signal from the controls which did not include any sample. Further, we did not observe a signal in the negative controls for 18S RNA amplification, an endogenous control used to normalize the results. Since the possibility of external contamination of reagents was also ruled out by testing several aliquots, we reasoned that the amplified genetic material was originating from the commercial reagents used. The LacZ gene, encoding for the bacterial β-galactosidase enzyme, origins from *E.coli*. Unfortunately, there is contaminating bacterial DNA present in the Taq polymerase (Bottger, 1990, Rand *et al*., 1990), which gave rise to the strong amplification signal in our qRT-PCR method. The enzyme (AmpliTaq Gold DNA polymerase®) is commercially synthesized in *E.coli* as a recombinant protein, and the incomplete purification of the enzyme results in traces of bacterial DNA in the preparation. In our hands, the amplification of the control sample without sample resulted in a strong amplification signal with a relatively low threshold cycle (C_t=32), indicating a remarkable amount of contaminating sequence. The resultant amplification of the negative control could not be ruled out by mathematical elimination, since the amplification of low concentrations of target DNA or RNA would presumably be masked by the contamination at this level. Therefore, we concluded that our method was not applicable for the intended use. Also, if the detection of other bacterial genes is performed with a comparable method, adequate controls should be included in each run to rule out amplification originating from the DNA polymerase. Today, commercial DNA polymerase enzymes of greater purity are available. In fact, Tondeur *et al* reported that the qRT-PCR method for LacZ gene expression designed by us can be successfully utilized by using a polymerase of higher purity, AmpliTaq Gold DNA polymerase low DNA®, or alternatively,

by treating the standard AmpliTaq Gold DNA polymerase® with DNAse enzyme prior to use (Tondeur *et al*., 2004). This observation enables the use of our qRT-PCR design for the quantification of LacZ gene expression and detection of the LacZ encoding vector DNA.

CONCLUSIONS AND FUTURE PERSPECTIVES

Studies included in this thesis indicate the versatile applicability of HIV-1 derived LVs. In our studies, third generation LVs were successfully used as a platform for regulated gene expression induced by the orally available drug, doxycycline or an endogenous pathophysiological stimulus, oxidative stress. The SIN-design of third generation LVs, whereby after vector integration into host cell genome no promoter activities are left in the LTR regions flanking the integrated sequence, means that possible promoter interference with the regulated promoter is abolished and, may be one key factor in successful transgene regulation. In fact, inactive LTRs flanking the transgene cassette might also operate as genomic insulators and hinder the interference of genomic promoters near the integration site with transgene promoter regulation. Also, LVs exploited for RNAi resulted in powerful, long-term gene silencing. The *in vivo* performance of LV in cardiovascular disease gene therapy models, whereby the rabbit skeletal muscle and blood vessel and pig myocardium were used as target tissues, did not prove to be efficient. However, as shown by us and others, LVs are valued because of their high transduction efficiency into the stem and progenitor cells such as HCSs and ESCs and are thus, excellent candidates for gene transfer vectors in progenitor cell therapies and the generation of transgenic animals. In our studies, we also took advantage of the high transduction efficiency of LVs into the brain tissue. In their current form, LVs represent excellent tools for long-term genetic modification of the cells of the lympho-hematopoietic and central nervous system. From the viewpoint of experimental research, third generation HIV-1 LVs are easy to produce by a simple co-transduction protocol of four separate plasmids. However, for the clinical use of LVs, a stable production of vectors would be desirable. Safety issues regarding the use of HIV-1 LVs in clinical treatments are of concern. Herein, it should be noticed that third generation LVs do not transfer any viral genes into the host cell genome and, the packaging system is deleted of genes that are not absolutely necessary for vector production. Given these modifications HIV-1 LVs can be considered as highly safe for experimental use. However, the use of integrating vectors in clinical gene therapy trials is under the spotlight because of the lymphomas that occurred in the trial for the treatment of X-linked SCID. It can not be predicted if these serious adverse effects could have been ruled out with the use of SIN-vector possessing inactive LTRs. It may be proposed that the risk of insertional mutagenesis might have been reduced by the use of SIN-vectors. The results of the first clinical trial with the HIV-1 LV for the treatment of the HIV-1 infection suggest that *ex vivo* modification of autologous T-cells by LV is safe and efficient and transduced cells persist *in vivo*. Moreover, based on this study, LVs hold promise for efficient gene transfer into cells of the lympho-hematopoietic system, and thus, may be utilized in a variety of therapies, for example in combating viral infections, in the treatment of blood-cell disorders such as hemoglobinopathies, storage and metabolic diseases, immunodeficiencies and cancer. In the design of these therapies the possibility to use the LV as a platform for regulated transgene expression is of an advantage.

The concept of progenitor and stem cell therapies has shifted from the original idea of

cells taking part in the regeneration process through mechanisms such as transdifferentiation or cell fusion, into a broader hypothesis that cell therapy might facilitate complementary aspects of tissue repair. The beneficial effects, although arguable, that have been observed in the clinical cell therapy trials for myocardial infarction, may be explained by the involvement of indirect mechanisms such as the secretion of paracrine factors by the therapy cells, or by their effects mediating endogenous regenerative pathways of the tissue. In our study, we demonstrated a beneficial effect on the regenerating ischemic skeletal muscle by the injection of progenitor cells. As we could not detect engraftment of the cells, we concluded that the effect was indirect and might be associated with enhanced recruitment of macrophages into the ischemic muscle area. Thus, progenitor cell induced immunostimulatory processes may also play a role in cell therapies. In conclusion, a better understanding of cell therapy mechanisms and a critical evaluation of the results from both experimental and clinical studies are crucial for the development of new therapies.

Finally, genetic therapies, either alone or combined with cell therapies, hold promise for the treatment of cardiovascular diseases. The development of suitable gene transfer vectors for each specific application plays a critical role in this progress. Along with the evolving knowledge of the human genome and its regulatory mechanisms and the molecular details of different viruses, improved gene transfer vectors will be developed. It may be speculated that the future gene transfer vectors will not be directly based on any natural viruses but instead, will be based on engineered, synthetic virus-like vectors mimicking combined features of several different viruses.

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