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MODIFYING XENOGENEIC IMMUNE RECOGNITION AND ENGRAFTMENT BY GENETIC ENGINEERING

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To my wife Yu Shi
To my family
To the memory of my father

ABSTRACT

Transplantation using xenogenic organs, tissues and cells (*i.e.* xenotransplantation) is a potential solution to the shortage of those from human sources. Vascular endothelial cells (ECs) are the most immediate barrier between the xenogeneic donor organ and the host defense systems. In order to accomplish gene expression in ECs specifically, EC-specific promoters are preferable to be used. If human EC-specific promoters can be used in porcine ECs, time and efforts will be saved. In acute vascular rejection (AVR), the interaction between porcine endothelium and human NK cells/monocytes has been suggested to depend on the direct recognition of Galα1,3Gal (α-Gal) epitopes on porcine ECs. Genetic engineering of pancreatic islets prior to transplantation has the potential to improve islets' survival through the expression of genes encoding factors supporting islet acceptance by the host. The lentiviral vector system, *e.g.* an HIV-1 based vector system, has been shown to efficiently and stably transduce primary and post-mitotic cells.

The aims of this thesis were: (i) to investigate the activity and cell-specificity of the human EC specific promoters of *Flk-1*, *Flt-1*, *ICAM-2*, thrombomodulin and *vWf* in porcine cells; (ii) to evaluate the importance of lentiviral-mediated expression of α-Gal on ECs for its interaction with human NK cells and monocytes, and to evaluate the transduction efficiency in primary ECs; (iii) to investigate the ability of the lentiviral vector to transduce isolated rat pancreatic islets and its effect on islet function.

The promoters for human *Flk-1*, *Flt-1*, *ICAM-2*, thrombomodulin, and *vWf* supported similar levels of luciferase expression in human (HAECs) and porcine (HAECs) aortic ECs, with the Flk-1 promoter being the strongest. The human EC-specific promoters all showed less activity in porcine kidney microvascular ECs than in liver or brain microvascular ECs. The *thrombomodulin* and *Flk-1* promoters exhibited similar activity in porcine liver and kidney microvascular ECs, whereas the *Flk-1* promoter was stronger in aortic and brain microvascular ECs. No difference was detected between non-α-Gal and α-Gal expressing HAECs in terms of their susceptibility to NK cell-mediated lysis, ability to stimulate IFN-γ production by NK cells, or ability to support NK cell-adhesion under static and dynamic conditions. In addition, the α-Gal epitope did not appear to contribute to increased monocyte adhesion to, or migration across, endothelium. Human monocytes adhered less to PAECs than to HAECs under flow following human, but not porcine, TNF-α stimulation. Lentiviral transduction did not result in activation of HAECs, and transduced HAECs responded as expected to TNF-α and IFN-γ stimulation. Lentivirus transduction did not affect rat pancreatic islet's viability and insulin secretion *in vitro* and its ability to restore euglycemia in diabetic nude mouse *in vivo*. Furthermore, this vector sustained long-term expression of the transgene in islets.

Amongst the EC-specific promoters tested, the human *Flk-1* and thrombomodulin promoters are good candidate promoters for strong EC-specific gene expression in genetically modified pigs. Our work on the interaction between α-Gal epitopes and NK cells/monocytes suggest that efforts on preventing infiltration of these leukocytes in organ xenograft need to be focused on adhesion receptor-ligand interactions rather than on carbohydrate remodelling of donor pigs. In addition, the lentivirus vector can be used as a gene carrier to modify primary ECs as well as cells in pancreatic islets in order to improve engraftment and prevent rejection.

ORIGINAL PAPERS

This thesis is based on the following papers referred to in the text by their Roman numerals:

I. The *in vitro* activity and specificity of human endothelial cell-specific promoters in porcine cells. **He Z.**, She R., Sumitran-Holgersson S., Blomberg P., Islam KB., Holgersson J. Xenotransplantation. 2001, 8, 202-212

II. Aberrant expression of alpha-Gal on primary human endothelium does not confer susceptibility to NK cell cytotoxicity or increased NK cell adhesion. **He Z.**, Ehrnfelt C., Kumagai-Braesch M., Islam KB., Holgersson J. Eur J Immunol, 2004, 34, 1185-1195.

III. No role of α-Gal in human monocyte-endothelial cell interactions *in vitro*. Ehrnfelt C., **He Z.**, Holgersson J. Submitted.

IV. Long-term gene expression and metabolic control exerted by lentivirus-transduced pancreatic islets. **He Z.**, Wang F., Kumagai-Braesch M., Islam K., Permert J., Holgersson J. Submitted.

CONTENTS

LIST OF ABBREVIATIONS

α1,3GalT	α1,3-galactosyltransferase
α-Gal	Galα1,3Galβ1,4GlcNAc
γc	gamma chain
ψ	packaging signal
ALL	acute lymphoblastic leukemia
AVR	acute vascular rejection
BIV	bovine immunodeficiency virus
CA	capsid
CAR	Coxsackie-adenovirus receptor
CMV	cytomegalovirus
DAF	decay-accelerating factor
DNA	deoxyribonucleic acid
EC	endothelial cell
EF1α	elongation factor 1 α
EIAV	equine infectious anemia virus
Env	envelope glycoprotein
FGF	fibroblast growth factor
FIV	feline immunodeficiency virus
Flk	fetal liver kinase
Flt	fms-like tyrosine kinase
GFP	green fluorescent protein
HAEC	human aortic endothelial cell
HAR	hyperacute rejection
HIV-1	human immunodeficiency virus type 1
HLA	human leukocyte antigen
HSV	herpes simplex virus
IBMIR	immediate blood-mediated inflammatory reaction
ICAM-1	intercellular adhesion molecule-1
Ig	immunoglobulin
IL	interleukin
IN	integrase
ITR	inverted terminal repeat
JAM	junctional adhesion molecules
kb	kilobases
KIR	killer cell Ig-like receptor
LFA-1	lymphocyte function associated antigen-1
LTR	long terminal repeat
MA	matrix
MCP-1	monocyte chemottractant protein-1
MHC	major histocompatibility complex
MLV	murine leukemia virus
NC	nucleocapsid
NES	nuclear export signal
NK	natural killer
PAEC	porcine aortic endothelial cell
PBMEC	porcine brain microvascular endothelial cell

PCR	polymerase chain reaction
PD-L1	programmed death-1 ligand 1
PECAM-1	platelet endothelial cell adhesion molecule-1
PEG	polyethylene glycol
PEI	poly-L-ornithine, polyethyleneimine
PERV	porcine endogenous retroviruses
PKMEC	porcine kidney microvascular endothelial cell
PLMEC	porcine liver microvascular endothelial cell
PR	protease
R	Repeat
RCV	replication-competent virus
RRE	Rev-responsive element
RT	reverse transcriptase
SCID-X1	X-linked severe combined immune deficiency
SIN	self-inactivating
SIV	simian immunodeficiency virus
SLA	swine leukocyte antigen
SU	surface subunit
TAR	transactivation response element
TM	transmembrane
TNF	tumor necrosis factor
TU	transduction units
U5	unique 5’ region
VCAM-1	vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor
VLA-4	very late antigen-4
VSV-G	vesicular stomatitis virus G glycoprotein
vWf	von Willebrand factor
XNA	xenoreactive natural antibodies

1 INTRODUCTION

1.1 XENOTRANSPLANTATION

Organ or cell transplantation has been used to treat many human diseases (*1*). However, due to the immune rejection, the failure of implanted grafts veils the merit of this treatment. During past decades, following the improvements of the transplantation procedures in respect of organ procurement, immunosuppressive protocols, and pre-transplantation diagnostic techniques, there are more patients benefiting from successful long-term survival of the grafts. However, the critical shortage of human donor organs available for transplantation dramatically hampers the application of allotransplantation (*i.e.* transplantations between genetically different individuals of the same species). In the US, only about one third of patients on the transplantation waiting list were transplanted with donor organs in year 2002 (*2*). To meet the challenge, one attractive choice is to use xenogeneic cells, tissues and organs (from a non-human species) as sources for transplantation (*i.e*. xenotransplantation).

In human xenotransplantation, grafts from a concordant species such as nonhuman primates from the Old World (*e.g.* apes and baboons) will be more compatible than those from a discordant species such as pig. However, in clinical practice, using nonhuman primates as donors is hampered by a variety of hurdles, including long gestation periods, costs and difficulties of breeding, the risk of transmitting infection and ethical considerations. Pigs are considered as suitable xenograft donors because of the anatomical and physiological similarities between pig and human organs and easy breeding in pathogen-free conditions for clinical xenotransplantation. In addition, as pigs are used for food production, the use of pigs as tissue donors is deemed ethically acceptable. Even though pigs can be bred in pathogen-free conditions to minimize the risks of transmission of animal pathogens, the risk of transmitting porcine endogenous retroviruses (PERV) needs particular consideration. Either primary porcine cells or transformed porcine cell lines have been shown to contain in their genomes multiple copies of endogenous retroviruses (*3-6*). Because these viruses are inherited, breeding of PERV-free pigs is considered to be technically difficult (*7*). *In vitro* studies have shown that PERVs released from porcine primary cells or cell lines have the capability to establish a productive infection in human cell lines and primary cells (*7-10*). Thus, evaluating the risk of virus transmission is critical before xenotransplantation is practiced. However, there is no direct evidence suggesting that humans can be infected by PERV via exposure to porcine tissue (*11-15*).

1.1.1 Xenograft rejection

Following transplantation, rejection is the major hurdle preventing long-term function of xenografts. Depending on the kinetics and histopathological picture of vascularized organ xenograft rejection, it can be divided into hyperacute rejection (HAR), acute vascular rejection (AVR), cellular rejection and chronic rejection. HAR is mediated by xenoreactive natural antibodies (XNA) against α-Gal on porcine vascular endothelial cells (ECs), whereas AVR is believed, at least in part, to be triggered by xenoreactive induced Abs that can be α-Gal specific. The rejection of non-vascularized xenografts is usually cell-mediated, and dependent on T-lymphocytes. Cellular grafts like pancreatic islets transplanted by injection in the blood stream are usually damaged by an immediate blood-mediated inflammatory reaction (IBMIR) triggered by complement activation, coagulation and leukocyte (neutrophils and monocytes, mainly) infiltration.

1.1.1.1 Hyperacute rejection

The very early barrier of discordant xenotransplantion is a vigorous, rapid and irreversible rejection, which is defined as hyperacute rejection. The pathology of hyperacute rejection is characterized by intravascular thrombosis, interstitial hemorrhage, and severe injury to endothelial cells (*16*). HAR is initiated by XNA binding and complement activation along vascular ECs of the transplanted organ. XNA bind carbohydrate residues, Galα1,3Galβ1,4GlcNAc (α-Gal), on porcine ECs. α-Gal is produced by the α1,3-galactosyltransferase (α1,3GalT) (Fig. 1), the gene of which is mutated in humans, apes, and Old World monkeys such that these species don't express α-Gal. In man, the production of anti-α-Gal antibodies (Abs) may be caused by constant antigenic stimulation from α-Gal like epitopes located on the surface of bacteria in the gut flora. The majority of these antibodies are IgM, whereas IgG and IgA isotypes also exist, though at lower levels. Amongst these isotypes of XNAs, IgM has high binding avidity and was believed to be the only isotype having the capability to initiate HAR (*17, 18*). But recently, using an *in vivo* model, Yin D et al demonstrated that IgG (IgG3 and IgG1) also triggered HAR via complement activation (*19*).

$$\textbf{Gal}\beta\textbf{1,4GlcNAc-R + UDP-Gal} \xrightarrow{\alpha\textbf{1,3GalT}} \textbf{Gal}\alpha\textbf{1,3Gal}\beta\textbf{1,4GlcNAc-R + UDP}$$

Fig. 1. The biosynthesis of α-Gal epitopes. UDP, uridine diphospate.

Activation of the complement system is the major outcome of XNA-binding to α-Gal epitopes on porcine EC. The classical pathway of complement activation is initiated by Ig and is the major activation pathway in pig-to-primate transplants (*20*). Across other species barriers, *e.g.* guinea pig–to–rat, complement activation is via the alternative pathway (*21*). The final product of complement activation is the formation of the C5-9 membrane attack complex (MAC), which forms a pore in the EC membrane leading to lysis of the cell. Endothelial cell activation is important for the pathogenesis of HAR. This activation induces EC retraction, von Willebrand factor translocation to the EC surface, and loss of the antithrombotic phenotype (*22*). The changes of EC phenotype lead to intravascular thrombosis, extravascular hemorrhage and edema (*23*).

To prevent HAR, efforts have been taken to remove XNAs prior to transplantation, to prevent activation of the human complement system, and to reduce the expression of α-Gal epitopes on porcine ECs. Depletion of XNAs from the recipient's circulation using affinity columns bearing α-Gal could reliably prevent hyperacute rejection in pig-to-baboon xenograft models (*24*). In order to minimize complement activation, transgenic pigs have been engineered in which complement regulatory proteins, such as decay-accelerating factor (DAF/CD55), membrane cofactor protein (CD46) and CD59, are expressed (*25-27*). These pigs are less susceptible to XNA-mediated HAR (*25-27*). Recently, α1,3GalT knockout pigs have been made, and kidneys and hearts from such pigs transplanted into baboons did not suffer from HAR (*28, 29*).

1.1.1.2 Acute vascular rejection

Acute vascular rejection, also known as delayed xenograft rejection, begins within 24 hours and leads to graft malfunction over a period of days to weeks. AVR is histologically characterized by EC swelling, focal ischemia, fibrinoid necrosis, diffuse

intravascular thrombosis with thrombi consisting mainly of fibrin, and infiltration of host inflammatory cells, *e.g.* monocytes, natural killer (NK) cells and neutrophils (*30, 31*).

When transplanted organs were derived from transgenic pigs expressing human DAF and/or CD59, AVR was delayed following depletion of circulating XNAs by immunadsorption and administration of the immunosuppressive agent cyclophosphamide (*32, 33*). In a similar pig-to-baboon model, XNAs were seen deposited in the transplanted porcine kidney (*34*). Thus in AVR, XNA including anti-Gal and anti-non-Gal Abs are important triggers of EC activation. This activation is associated with an up-regulated transcription of genes encoding i) cell adhesion molecules, *e.g.* E-selection (CD62E), vascular cell adhesion molecule-1 (VCAM-1/CD106), intercellular adhesion molecule-1 (ICAM-1/CD54), ii) cytokines/chemokines, *e.g.* interleukin (IL)-1, IL-6, IL-8, tumor necrosis factor (TNF)-α and monocyte chemottractant protein (MCP)-1 and iii) prothromboic molecules (*e.g.* tissue factor) (*35, 36*). In addition to XNAs, complement plays a role in triggering AVR. In a pig-to-primate model, classic complement activation was observed during the binding of XNAs to ECs of human DAF transgenic kidneys (*37*). In a similar model, AVR was reversed when the recipient was treated with a complement fragment C1-inhibitor (*38*). Furthermore, the incompatibility of porcine proteins with human counter-receptors may be a factor in the initiation of AVR. One example is that the porcine tissue factor pathway inhibitor poorly inhibits the human coagulation system (*39*).

As a result of endothelial cell activation, cell adhesion molecules involved in the recruitment of leukocytes into vascularized xenografts are up-regulated. Leukocyte infiltration is accomplished by a leukocyte adhesion cascade that includes the steps capture, rolling, firm adhesion, and transmigration. In the capture and rolling stages, E- and P-selectins on EC and L-selectin on leukocytes are engaged in the tethering and rolling of leukocytes by binding to carbohydrate counter-ligands on ECs and leukocytes, respectively. Activation of the rolling leukocyte by EC-bound chemokines leads to a rapid increase in the binding affinity of leukocyte integrins, such as CD11a/CD18 [lymphocyte function associated antigen-1 (LFA-1)], CD11b/CD18 (Mac-1) and CD11c/CD18 [very late antigen-4 (VLA-4)] which in turn bind to EC Ig superfamily receptors, *i.e*. ICAM-1, ICAM-2, ICAM-3, and VCAM-1 (*40*). In the last stage, transmigration is mediated by junctional molecules, *i.e.* platelet endothelial cell adhesion molecule-1 (PECAM-1, CD31) (*41, 42*), junctional adhesion molecules (JAM-A, -B and -C) (*43*) and CD99 (*44*). These juctional molecules may bind in a homophilic or heterophilic fashion (*41, 42, 45, 46*). JAM is also a ligand for LFA-1 (*43*). In pig-to-human xenotransplantation, the compatibility of human adhesion molecules with their specific porcine counter-receptors is likely to contribute to the leukocyte infiltrate. Most of the receptor-ligand pairs studied so far appear to be compatible(*47-50*), but it is still far from complete (*51, 52*).

Several types of cells, such as human neutrophils, NK cells, and monocytes, are involved in AVR. The interaction between human neutrophils and porcine EC has been described, and in one set of experiments this interaction required preactivation of EC with complement (iC3b) and/or XNAs (*53, 54*), while other experiments have showed that this interaction is independent of XNAs (*55, 56*). Recently, Cardozo *et al.* showed that neutrophil-EC interactions may lead to biologically relevant phenomena such as respiratory burst, diapedesis, and chemotaxis (*56*).

NK cells are not only important innate immune cells, but also essential regulators of adaptive immunity. They are usually defined as CD56+, CD16+ (FcγR III) and CD3-. According to the "missing-self" hypothesis, cells with missing or incomplete expression of host major histocompatibility complex (MHC) class I molecules are susceptible to NK cell killing (*57*). This recognition is mediated by killer cell Ig-like receptors (KIRs) and CD94/NKG2 C-type lectin receptors which recognize human leukocyte antigen (HLA)-A, -B and -C (*58-61*), and non-classical class I molecules (HLA-E) (*62*), respectively. The counterpart of HLA in pig is called swine leukocyte antigen (SLA). Examination of the amino acid sequence of SLA has indicated that the critical binding sites for human KIRs are missing, thus the incompatible SLA loses its ability to protect porcine cells from the lysis of human NK cells (*63*). This may in part explain the susceptibility of porcine cells to cytolysis by human NK cells. Seebach *et al.* also demonstrated that SLA class I molecules did not appear to protect porcine cells from human NK cell killing (*64*). By expression of human MHC class I molecules (*e.g.* HLA-Cw, -E and -G) in porcine EC, the rolling adhesion, migration and lytic activities of human NK cells were inhibited and porcine ECs were thus protected from lysis (*65-71*). In addition, it has been shown that HLA-E and HLA-G on porcine EC use both CD94/NKG2-dependent and –independent pathways to inhibit NK cells (*71*). That α-Gal could promote NK cell-binding was first suggested by Inverardi et al. who demonstrated that human NK cells bound to COS-7 cells transfected with the porcine α1,3GalT cDNA, but not to non-transfected COS-7 cells (*72*). In addition, NK cell-mediated cytolysis of porcine EC was reduced when porcine EC expressed an α1,2fucosyltransferase as a competitor of α1,3GalT to down-regulate the expression of α-Gal epitopes (*73*). In contrast, Sheikh et al. reported that human NK cell recognized porcine EC independently of α-Gal (*74*) and Baumann et al showed that NK cell adhesion and antibody-independent cytotoxicity were not reduced by the removal of α-Gal from porcine cells (*75*). Moreover, in Paper II our data show that expression of α-Gal on human EC does not induce human NK cell cytotoxicity nor does it increase NK cell adhesion or IFN-γ secretion in NK cells. Adhesion molecules, such as VLA-4 on human NK cells and VCAM-1 on porcine EC, have been shown to be important for both rolling and firm adhesion of human NK cells to porcine EC (*76, 77*). Human NK cells may also kill porcine EC in the presence of antibodies through the so called ADCC mechanism (*78*). In vitro experiments has revealed that cytolysis is mediated via the perforin/granzyme B pathway (*79*).

In vivo models has also revealed T cell-independent monocyte/macrophage infiltration in organ xenografts (*80-84*). The importance of graft-infiltrating macrophages for AVR was further suggested by prolonged survival of guinea pig hearts transplanted into C6-deficient rats in which the macrophages had been depleted prior to transplantation (*85*). However, results presented by van Overdam et al indicated that NK cells and macrophages infiltrating xenografts might not be important for rejection (*86*). The adhesion of human monocytes to porcine EC has proven to be dependent on human VLA-4 and porcine VCAM-1 interactions (*77, 87*). The α-Gal epitope on porcine EC has been shown to participate in the direct binding of human monocytes (*88-90*). However, using the static and dynamic adhesion models in Paper III, we demonstrated that aberrant expression of α-Gal in human EC did not increase the binding of human monocytes.

The protection of an organ from AVR in the presence of anti-graft antibodies and complement is known as accommodation (*91*). Several observations of this phenomenon have been reported in animal models of xenotransplantation (*91, 92*). In

accommodation, graft endothelium has been shown to increase the expression of anti-apoptotic genes bcl-x_L, bcl-2, A20 and an oxidative stress-responsive gene heme oxygenase-1 (*91*). In addition to anti-apoptotic effects, these genes also have the ability to inhibit the activity of NF-κB, which in turn prevents the expression of pro-inflammatory cytokine genes (*91, 93*).

To prevent AVR, depletion of induced XNAs from the host or elimination of α-Gal on the xenograft may be a strategy to facilitate graft survival (*78, 94*). Other strategies, such as anti-leukocyte adhesion (*77*) and transgenic expression of anti-inflammatory genes (*e.g.* hemoxygenase-1) in porcine EC (*95*) have also been considered.

1.1.1.3 Acute cellular rejection and chronic rejection

These stages of rejection follow HAR and AVR. Because organ xenografts are usually lost in HAR and/or AVR, acute T cell-mediated xenograft rejection has been difficult to study *in vivo*. However, there are evidence to suggest that T cells are involved in xenograft rejection (*96, 97*). Dorling *et al* demonstrated that T cell recognition of xenogeneic cells is mediated by both direct and indirect antigen presentation pathways (*98*). Compared to allogeneic combinations, indirect presentation of porcine antigens to human T cells led to a stronger primary response in cell cultures (*98*). Inhibition of T cell activity by immunosuppressive drugs or by inducing T cell tolerance may protect the graft from acute cellular rejection. There are to my knowledge no *in vivo* studies in which chronic rejection has been studied across a xenogeneic barrier.

1.1.2 Islet xenograft rejection

As a non-vascularized tissue, the rejection of islet xenografts is different from that of a rejection of vascularized organ xenografts. Without a vascular bed of donor origin islets do not suffer from HAR and AVR, but are subjected to a cell-mediated rejection following transplantation. Revascularization with vessels of host origin will occur during the first 1-2 weeks after islet transplantation (*99-101*). If islet grafts are exposed to host blood following intra-portal injection, they will trigger an instant blood mediated inflammatory reaction (IBMIR) that might prevent engraftment. If engrafted, islet xenografts are usually lost in a cell-mediated rejection executed by T-lymphocytes and macrophages.

1.1.2.1 IBMIR

For clinical islet transplantation, intra-portal injection is the optimal route of administration. Because islets is then directly exposed to blood, they trigger an IBMIR characterized by activation of platelets, coagulation and complement systems, and subsequent islet damage accompanied by infiltration of CD11+ leukocytes (*102, 103*). The expression of tissue factor on human islets is believed to trigger coagulation, and inhibiting tissue factor expression has been shown to protect islets from IBMIR (*104, 105*). However, whether porcine islets express tissue factor and if porcine tissue factor can trigger IBMIR remains to be determined. Both *in vitro* and *in vivo* studies have shown that complement is deposited on the porcine islet surface upon exposure to human and cynomologus monkey blood, respectively. Porcine islet damage could be avoided by pre-treatment of the recipient with either soluble complement receptor-1 and heparin or low molecular weight dextran sulfate (*103, 106*).

1.1.2.2 Cellular rejection

In pig-to-rodent islet transplantation models, infiltration of macrophages and T cells is seen in the islet graft. In mice depleted of CD4+ T-cells, prolonged survival of transplanted porcine islet grafts was reported (*107, 108*). In an immunodeficient mouse model, adoptively transferred human CD4+ T-cells could mediate rejection of fetal porcine islet cell clusters suggesting an important role for this cell type in rejection (*109*). Macrophages activated by CD4+ T-cells have been shown in mouse models to be important for recognition and rejection of islet grafts, and the deletion of macrophages will delay islet xenograft rejection (*110-112*). The MCP-1/CCR2 chemokine pathway was reported to be important regulators of CD4+ T-cell and macrophage infiltration (*113, 114*). In a porcine to cynomologus monkey model, CD8+ T-cells were the major graft-infiltrating cells (*115*). This may imply that in clinical islet transplantation, CD8+ T-cells can not be neglected. In addition, NK cells and eosinophils have been reported to be of minor importance in the rejection of islet grafts in mouse models (*116, 117*).

1.2 GENE THERAPY

Genes serve as functional units of heredity and control protein production. Many disorders in man, including inherited and acquired diseases, are related to altered gene function. The idea, to restore the defective gene, provides an attractive therapeutic method for these diseases.

Gene therapy involves transferring genetic material into human cells for therapeutic purposes. To date, many approaches for gene therapy have been evaluated in animal models of human diseases and in clinical trials. In spite of disappointing results from several clinical trials, including the occurrence of leukemia after gene therapy for X-linked severe combined immune deficiency (SCID-X1) (*118, 119*), the effort to improve the efficacy and safety of gene therapy has never been relented.

The success of gene therapy largely depends on the development of the gene transfer vector which is a carrier for therapeutic genes to be introduced into target cells. An ideal gene transfer vector is expected to induce efficient and stable expression of the transgene without disturbing normal host genes. In addition, production and manipulation of the vector should be easy. Further, the vector should not be toxic or immunogenic. Generally, vectors used in gene therapy are either non-viral or viral. Both vector classes have their advantages and disadvantages regarding their usage in gene therapy (Table 1).

Table 1. Characteristics of non-viral and viral vectors.

Vector	Manipulation	Transfer efficiency *in vivo*	Chromosomal integration	Duration of expression	Transduction of postmitotic cells	Immuno-genicity
Non-virus	easy	+	no	transient	yes	low
Adenovirus	complicated	++++	no	transient	yes	high
AAV	complicated	+++	yes	stable	yes	moderate
Retrovirus	complicated	++	yes	stable	no	low
Lentivirus	complicated	+++	yes	stable	yes	low
HSV	complicated	+++	no	transient	yes	moderate

AAV, adeno-associated virus; HSV, herpes simplex virus

1.2.1 Non-virus vectors

Historically, non-viral vectors were developed earlier than viral vectors, although more viral based-vectors have been used in clinical trials. As shown in Table 1, non-viral vectors are easy to use and unlikely to be immunogenic. In addition, non-viral vectors can be produced on a large scale. Non-viral, naked plasmid DNA (deoxyribonucleic acid) can either be delivered by a physical method or combined with a chemical carrier such as a cationic lipid or polymer.

1.2.1.1 Naked DNA delivery

The simplest method for naked plasmid DNA delivery is direct injection of DNA into the tissue or via a vascular route. Wolff et al first reported that the skeletal muscle cell expressed transgenes following direct intramuscular injection of naked DNA (*120*). Since then, expression of transgenes delivered by naked DNA injection has been accomplished in various tissues and organs, including the liver (*121, 122*), skin (*123*), urological organs (*124*), and the thyroid (*125*). Systemic administration of DNA through intravenous injection has also been reported (*126*). However, low transfer efficiency limits the use of this method. Thus, different physical manipulations, ***e.g.*** the gene gun, electroporation, and ultrasound, have been used in combination with direct injection to improve gene transfer efficacy. It has been reported that the gene transfer can be carried out by shooting (using gene gun) gold particles coated with DNA into cells of different surgically exposed organs (*127*). Other *in vivo* applications involving gene gun also showed successful expressions of transgenes (*128, 129*). Electroporation, in which a controlled electric pulse is applied, can facilitate cell permeabilization and uptake of injected naked DNA ***(130).*** That electroporation can facilitate uptake of DNA has been reported *in vivo* in such organs as liver (*131*), muscle (*132, 133*), skin (*134*) and melanoma (*135*). However, electroporation has been reported to damage the tissue (*136*). Hartikka et al demonstrated that muscle lesions occurred after injection of plasmid DNA followed by electroporation, and interestingly this damage was plasmid-dependent (*137*). Recently, it was reported that the use of a syringe electrode minimized the damage of the tissue without losing transfection efficiency (*138*). Micro bubble-enhanced ultrasound has been used to facilitate naked DNA transfection and exhibited little tissue damage (*139-141*). Phase I and/or II clinical trials conducted by several groups have shown that naked DNA administration is a safe procedure (*142-145*).

1.2.1.2 Cationic lipids

Cationic lipids have the capacity to interact with and compact the negatively charged nucleic acids, therefore forming the self-assembled lipid/DNA complexes. These complexes are slightly positively charged, so they are capable to interact efficiently with the negatively charged cell membrane (*146, 147*). There are several kinds of cationic lipids, quaternary ammonium detergents, cationic derivatives of cholesterol and diacylglycerol, and lipid derivative of polyamines. The transfection by cationic lipids can be optimized by using a neutral helper lipid such as the naturally available dioleoyl phosphatidylethanolamine (DOPE), which facilitates the release of plasmid DNA from the endosome after endocytic uptake of the complex (*148*). With different administration pathways (*e.g.* airway, intravenous and intraperitoneal) , *in vivo* experiments have shown gene expression in lung, heart, liver, spleen, kidney and tumor following cationic lipid transfections (*149-156*).

1.2.1.3 Cationic polymers

Different from the hydrophobic cationic lipids, cationic polymers are a group of water soluble molecules including poly-L-lysine (PLL), poly-L-ornithine, polyethyleneimine (PEI), chitosan, and starburst dendrimer. For gene transfer, three structural types, *i.e.* linear, branched, and spherical, of cationic polymers have been used. Both branched (25kDa) and linear (22kDa) types of PEI have been used for efficient transfection both *in vitro* and *in vivo*. Boussif et al. suggested that the enriched nitrogen atoms in PEI provided high buffering capacity which protected DNA from nuclease degradation in the lysosome (*157*). PEI transfections have been achieved in major organs such as brain, kidney, liver and lung (*158-161*). Wightman *et al* demonstrated that due to the sensitivity to salt, linear PEI showed higher transfection capacity than branched PEI when systemically administered *in vivo* where saltier conditions prevailed (*162*). In Paper I, transfections with branched PEI (25kDa) was used in both primary cell cultures and established cell lines. In paper II-IV, we used the PEI transfection method to produce replication defective lentiviral infectious particles.

1.2.2 Virus vectors

As shown in Table 1, virus-derived vectors can transduce genes into target cells with high efficiency and some types of viral vectors can stably integrate into the host cell genome resulting in stable expression of the transgene. Some have the ability to infect postmitotic cells. Generally, the development of a viral vector is a process in which biosafety and virus biology has to be balanced. This is because it is necessary for safety reasons to delete genes important for virus pathogenicity; genes that are usually also essential for viral infectivity and thus gene transfer efficacy. A clinically useable vector should meet both safety and efficiency criteria. The main viral vectors that have been used for gene therapy purposes are adenovirus (Ad), adeno-associated virus (AAV), herpes simplex virus (HSV), and retro- and lentivirus vectors. Viral vectors based on Pox and Vaccinia viruses are also used in clinical trails.

1.2.2.1 Adenovirus vectors

The human Ad genome consists of a double stranded linear DNA molecule of approximate 36 kilobases (kb) in length. Ads can infect a broad range of cell types, including postmitotic cells. Based on neutralization assays, the viruses are classified into more than 50 serotypes. The most commonly used recombinant human Ad vectors are derived from serotype 2 (Ad2) and 5 (Ad5). The Ad genome is encapsidated in a nonenveloped icosahedral capsid which consists of hexons and pentons (penton bases and fiber monomers). The inverted terminal repeat sequences (ITRs), which are the *cis*-acting origins of replication of the viral DNA, are located at each end of Ad genome. By the gene expression time course in the viral replication, Ad genes are divided into early (E1A, E1B, E2, E3, and E4), delayed (IX and IVa2), and late transcription units (*163*). The infection of Ad is initiated by the binding of capsid proteins to the cell surface receptors, *i.e*. Coxsackie-adenovirus receptor (CAR) or $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins. After this receptor-mediated endocytosis, the virus DNA is released from the endosomal compartment by the lysis of the endosomal membrane, and subsequently enters into the nucleus where the transcription and replication begin. However, the virus genome does not integrate into the host cell genome. Next, viral DNA is replicated and packaged into virions by self-assembly of the capsid proteins, and then the infectious particle is released from the cell (*164*).

The first generation of Ad vector was constructed by deleting the E1 region that is necessary for *trans*-activating other genes (*165*). This deletion results in the prevention of expression of early transcribed genes, thus further limiting the viral replication and synthesis of late structural proteins. The production of the replication defective Ad is conducted in the complementing human cell line which is transformed by the E1 gene to provide E1 function *in trans*. Further, the deletion of the E3 gene in the first generation Ad vector improved the capacity of gene insertion and yield of vector. These vectors have been broadly used both for *in vitro* and *in vivo* gene transfers. However, infected cell cytotoxicity and host immune responses to the virus vector limit the use of Ad mediated gene transfer (*166, 167*). To overcome these drawbacks, more efforts have been invested in order to generate so called second generation Ad vectors. Ad vectors in which the E2 and E4 genes are further deleted are more efficient gene transfers with less cytotocity and immunogenicity (*168-170*). In addition, the "gutless" vectors that only contain ITRs and packaging signal have been developed (*171-173*). These constructions facilitate increased gene insertions and significantly reduced host immune responses. The vector propagation requires helper viruses to provide the functions for replication and assembly.

Ad vectors have been used extensively in research and clinical applications. In animal models, local and systemic administrations of Ad vectors have showed efficient gene transfers in various organs, such as lung, liver, kidney and heart (*166, 169, 174-176*). Large numbers of clinical trials world wide have also used Ad vectors (*177*).

1.2.2.2 Adeno-associated virus vectors

AAV is a single strand DNA virus belonging to the family of Parvoviridae. These viruses are considered as nonpathogenic to human and their replication requires the help of other viruses, usually Ad or herpes virus. Recombinant AAV derived from AAV serotype 2 (AAV2) is the most frequently used AAV vector. The AAV2 genome is composed of two open reading frames, rep which is required for virus genome replication and cap which encodes a structural protein, flanked by ITRs.

The recombinant AAV2 is constructed by swapping target gene in the position of rep and cap genes, thus only ITRs are derived from the original virus. The AAV2 vector can deliver transgenes of about 5kb size, and this limits the usage of AAV for large gene transfers. To supersede the initial protocol of production of AAV2 using Ad or HSV as helper virus, AAV2 vectors are co-transfected with two helper plasmids containing rep and cap genes, and a minimal number of adenoviral proteins required for AAV production (E2A, E4ORF6, and VA RNA), respectively (*178*). Another alternative approach is to generate packaging cell lines that contain the elements required for AAV production (*179, 180*). After recombinant AAV enters the host cell, it can either integrate randomly into the host genome or reside episomally. Both events lead to stable gene expression (*181-185*). *In vivo* administration of recombinant AAV has been shown to be associated with host immune responses to the virus, even though the response is not as strong as that caused by Ad (*186-188*).

In animal models, AAV vector-mediated gene transfers have been achieved in liver (*189, 190*), skeletal muscle (*191, 192*), heart (*193*), brain (*194*), and lung cells (*195*). Clinical trials with AAV vectors to introduce therapeutic genes have been performed on patients suffering from cystic fibrosis and hemophilia B (*196, 197*).

1.2.2.3 Herpes simplex virus vectors

Human HSV is an enveloped large DNA virus with double-stranded genome which is 152kb in size and encodes at least 80 gene products. The virus infection results in a latent infectious status in neurons where HSV persist episomally for the rest of the host's life. Two types of vectors, both derived from wild type HSV-1 and named replication defective and amplicon vectors, respectively, have been developed for gene delivery. The generation of replication-defective vectors is accomplished by depleting predecessor genes, inducing replication incompetence and minimizing virus cytotoxicity (*198-200*). Helping *in trans* with proper immediate-early genes in complementing cells, replication-defective viruses can be produced. The HSV-1 amplicon vector system includes a plasmid DNA and a standard HSV-1virion. The plasmid carries the trangene, a HSV-1 packaging/cleavage signal and the origin of HSV-1 DNA replication. The production of these vectors is based on the finding that HSV-1 has the capability to package the defective genome containing the origin of replication and packaging signal (*201*). A drawback of using the amplicon vectors is the contamination of helper virus. Recently, a helper-free amplicon vector was developed. When this vector was used, the contaminant helper virus was reduced (*202, 203*).

Both replication defective- and amplicon HSV-1 vectors are used for gene transfer not only to neurons, but also to a wide variety of other cell types. *In vivo* experiments have shown that HSV-1 vectors can be used for the treatment of nervous system related disorders, such as Parkinson's disease (*204, 205*) and pain disorders (*206-208*).

1.2.2.4 Retrovirus vector

Retroviruses are single-stranded RNA viruses that replicate through a DNA-dependent intermediate. These viruses can be classified into three subfamilies, *i.e.* oncoretroviruses, lentiviruses and spumaviruses. These viruses have been found to be associated with various diseases in human and many other mammals, such as malignancies, immunodeficiencies, and autoimmune disease. In general, the vectors derived from oncoretroviruses are known as oncoretrovectors, and those from lentiviruses known as lentivirus vectors.

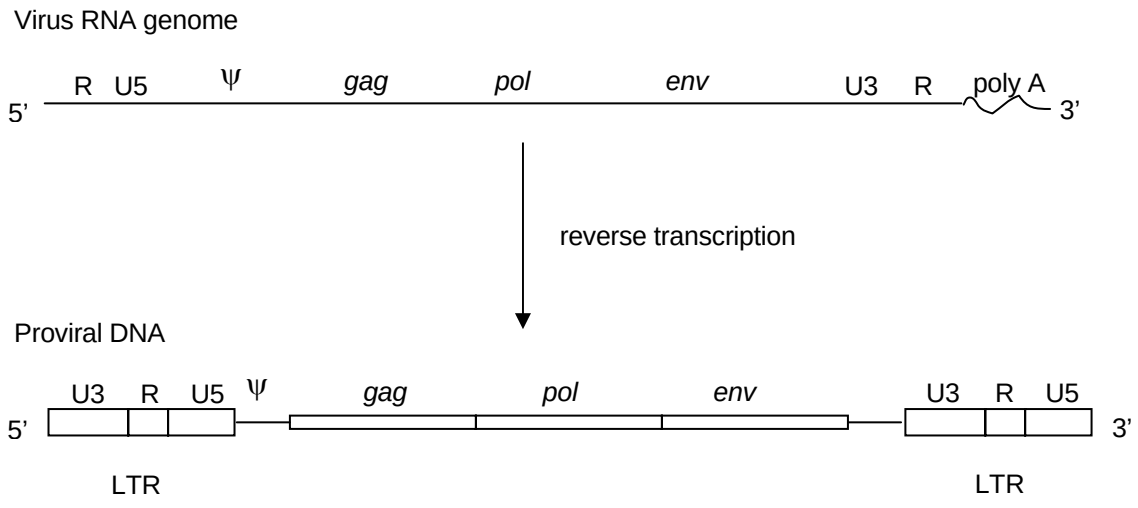

Fig. 2. Structure of retrovirus RNA genome and proviral DNA. Ψ, packaging signal.

The retrovirus infectious particle consists of the outer envelope and the inner core which encapsids two identical single-stranded RNA genomes. The viral RNA genome contains the following structures from the 5' end: R (Repeat), U5 (unique 5' region), primer binding site, packaging signal (ψ), three essential structure genes (*gal*, *pol* and *env*), U3, R, and poly (A) tail (Fig. 2). Following the reverse transcription of viral RNA and the integration of viral DNA copies into host genome, the provirus is generated. Due to the rearrangement in this process, the regions containing the proviral U3, R, and U5 are formed on each end of the provirus (Fig. 2). These regions are nominated as long terminal repeats (LTRs). The viral replication initiation elements, the promoter and enhancer are located in the U3 region of 5' LTR. The *gag* gene mainly encodes three viral structural proteins: matrix (MA), capsid (CA), and nucleocapsid (NC). The *pol* gene encodes the viral replication enzymes: protease (PR), reverse transcriptase (RT), and integrase (IN). The *env* gene encodes the envelope glycoprotein (Env) which is subprocessed into transmembrane (TM) and surface (SU) subunits (*209*).

The life cycle of retrovirus involves the sequential multiple steps, e.g. virus entry, reverse transcription, integration, viral gene expression, and production of viral particles (Fig. 3). By the interaction of viral envelope proteins with their receptors on the host cell, the retrovirus adheres to and enters into the cytosol of host cell. Then, the viral RNA genome is uncapsidated and the viral reverse transcriptase initiates the reverse transcription to convert viral RNA into a double-stranded proviral DNA. Thereafter, the proviral DNA translocates to the nucleus, where the integration of the provirus into the cell genome occurs with the help of viral integrase. The translocation of oncoretroviruses such as murine leukemia virus (MLV) needs the nuclear membrane to loose its integrity during the mitogenic process of the cell. Thus, retroviruses infect only dividing cells. The integration sites of proviral DNA are random. After the integration, the promoter and enhancer in the 5' LTR are triggered by host cell transcription factors. As a result, the proviral DNA is transcribed. The transcription generates two distinct viral RNAs, one full-length unspliced form (from 5' R region to 3' R region) which can be packaged as viral genome into the viral particles and also serves as the template for *gag* and *pol* translation, and the other spliced form which acts as template for *env* translation. The translation of *gag* and *pol* leads to generation of two polyproteins, Gag and Gag-Pol precursors. Controlled by the packaging signal, two copies of viral RNA together with Gag and Gag-Pol precursors are packaged into viral particles. Further maturation of virions occur during or after the budding from host cell membrane by processing of the Gag and Gag-Pol precursors by viral protease. In this process, structural proteins (*e.g.* MA, CA and NC) and replication enzymes (e.g. RT and IN) are generated by cleavage of Gag and Pol, respectively (*209*).

The oncoretrovirus vectors have been developed from different oncoretroviruses, such as MLV, Rous sarcoma virus, spleen necrosis virus and avian leukosis virus (*210-213*). Replication defective vectors derived from the MLV are the most commonly used oncoretrovirus vectors. MLV vectors are generated from the proviral DNA form by replacing the *trans*-acting genes, i.e. *gag*, *pol* and *env*, with the transgene of interest, therefore only *cis*-acting genes of proviral DNA, i.e. LTRs, primer binding site, and ψ are left. The inserted transgene can be promoted by the viral promoter located in U3 region of LTR or by an exogenous promoter, such as of the immediate-early cytomegalovirus (CMV) or human elongation factor 1 α (EF1α) promoters, inserted upstream of the transgene. The *trans*-acting elements are necessary for virus replication, thus in the retrovirus vector system they are provided by the packaging cells which either transiently or stably express these genes. The early versions of

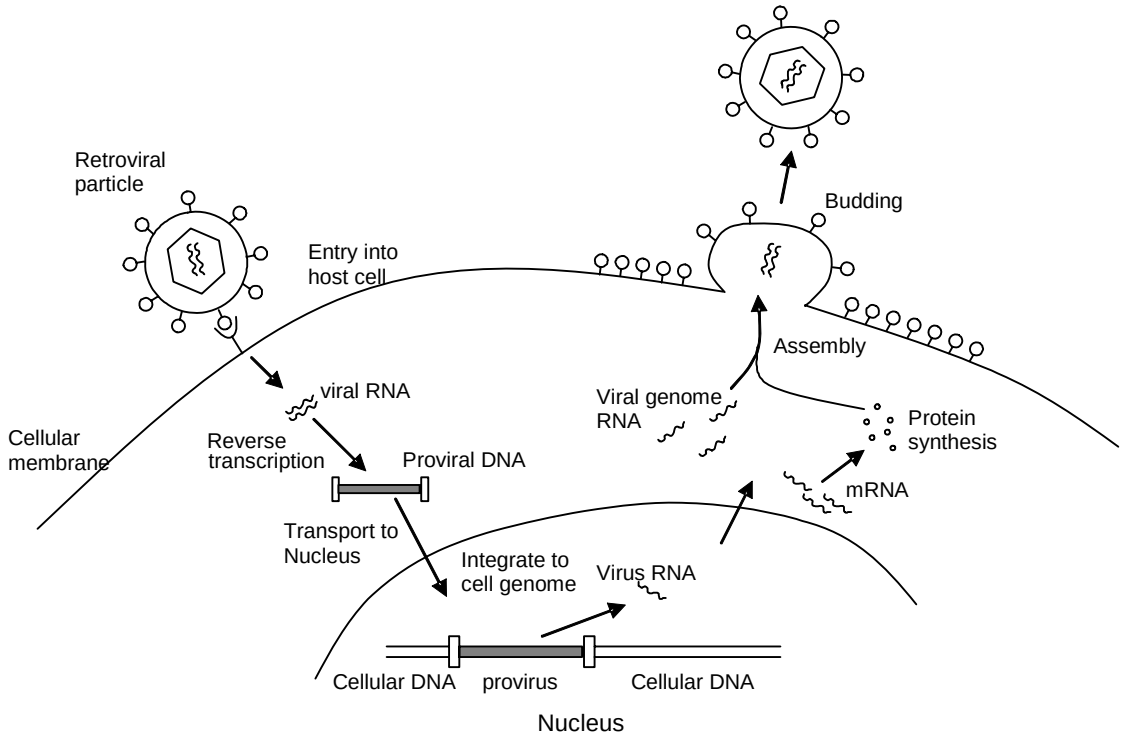

Fig. 3. The Retrovirus lifecycle. Retrovirus infection involves (i) Entry into host cells; (ii) Reverse transcription of the viral RNA genome and generation of the doublestrand proviral DNA; (iii) Integration of the proviral DNA into the host genome; (iv) Transcription of the viral genes and synthesis of viral proteins; (v) Production of viral particles.

packaging cell lines were generated by transfecting cells with the proviral DNA without the packaging signal sequence, and selecting the cells with stable expression of the transgene (*214, 215*). The retroviral particles can be efficiently produced in these helper cells. However, the problem of producing replication-competent viruses limits the use of these helper systems (*216*). This is due to homologous sequences between the vector and packaging plasmid facilitating recombination. To circumvent this problem, the packaging cells have been developed by stable transfection of two separate plasmids, one encoding *gag* and *pol*, and the other *env* (*217, 218*). In this system, the *cis*-acting elements are only located on the viral vector, whereas there are only *trans*-acting elements on the two plasmids. These modifications dramatically reduce the chance of recombination during virus propagation. Alternatively, high titer viruses can be produced by transiently co-transfect the virus vector, the plasmid containing *gag* and *pol*, and the plasmid containing *env* into a cell line (*219, 220*).

To improve the host range of the retrovirus vector, pseudotyped viruses have been developed by using an envelope from another virus to substitute that of the vector virus. A widely used envelope for pseudotyping is the G glycoprotein from vesicular stomatitis virus (VSV-G) (*221-224*). The VSV-G envelope can bind to ubiquitous phospholipid components of the cell membrane, therefore extending the host range of the pseudotyped virus. The VSV-G pseudotyped virus can withstand the shearing force of ultracentrifugation, making it appropriate for large scale production. Another improvement related to the safety of using the virus vector is to generate self-inactivating (SIN) vectors (*225, 226*). In the SIN vectors, the deletion of

promoter/enhancer sequences of the U3 region in the 3' LTR results in the inhibition of LTR-driven transcription. As a result, the expression of the transgene is only promoted by the internal promoter.

The retrovirus vectors used in most clinical trials are derived from the Moloney MLV (MoMLV). In addition to a tremendous number of applications in animal models of human disease, the retrovirus vectors are used in a number of clinical trials targeting for example adenosine deaminase (ADA) deficiency (*227-230*) and SCID-X1 (*231*). The outcome from these clinical trials has been encouraging. However, in the SCID-X1 treatments, 2 of 11 patients developed T-cell lymphomas due to the insertion of a single vector copy into the regulatory region of the LMO2 gene (*118, 119*). Thus, the biosafety of retrovirus vectors needs to be further assessed.

1.2.2.5 Lentivirus vectors

Lentiviruses belong to the retrovirus family and infect a number of species, including humans and other animals. Vectors derived from human immunodeficiency virus type 1 (HIV-1) are the most commonly used lentivirus vectors. Vectors have also been developed from other lentiviruses, such as human immunodeficiency virus type 2 (HIV-2), simian immunodeficiency virus (SIV), feline immunodeficiency virus (FIV), equine infectious anemia virus (EIAV), and bovine immunodeficiency virus (BIV). The HIV-1 replication cycle is similar to that of oncoretroviruses, but involves more regulatory genes and elements. In addition to *gag*, *pol* and *env*, HIV-1 carries two regulatory genes, *tat* and *rev*, and four accessory genes, *vif*, *vpu*, *vpr* and *nef* (Fig. 4). These additional elements facilitate virus replication and pathogenesis. Tat and Rev are essential for virus replication. Tat binds to the specific transactivation response element (TAR) in the R region of 5' LTR to up-regulate transcription. Rev contains a nuclear export signal (NES) and can interact with the Rev-responsive element (RRE) which is located within the *tat*/*rev* intron and facilitate export of unspliced and singly-spliced mRNAs that encode viral structural proteins. In the absence of either Tat or Rev, viral replication is inefficient (*232-234*). The accessory elements, *vif*, *vpu*, *vpr* and *nef*, are important for viral pathogenesis, but are not essential for viral replication in cell culture. As for oncoretroviruses, the lentivirus genome can integrate into the host cell genome and thus support stable expression of virus proteins.

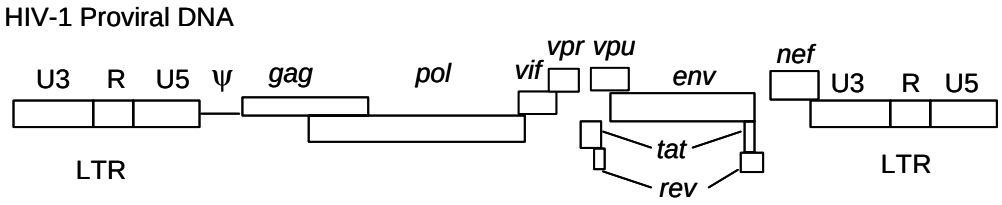

Fig. 4. HIV-1 proviral DNA. In addition to *cis* elements and structure genes, HIV-1 carries two regulatory genes, *tat* and *rev*, and four accessory genes, *vif*, *vpu*, *vpr* and *nef*.

The principle for making lentivirus vectors for gene therapy is similar to that of oncoretrovirus vectors. The lentivirus transfer vectors contain *cis*-acting sequences, including the LTRs and ψ, and an internal promoter upstream of the gene of interest (*235-237*). To improve the biosafety, SIN lentivirus vectors have been developed that has a mutated U3 region of 3' LTR (*238, 239*). The first generation of packaging plasmids contain the structural genes, *gag* and *pol*, and all HIV regulatory and

accessory genes (*235*). Thus, the resulting recombinant virus could potentially be pathogenic and replication-competent (RCV). Therefore, efforts have been made to delete accessory elements from the packaging plasmid without affecting the yield ofrecombinant viruses and their gene transfer ability (*240-242*). These so called second generation packaging plasmids carry structural elements*l*, and the regulatory elements, *tat* and *rev*. Further deletion of the *tat* gene and separation of the *gag*/*pol* and *rev* genes on two separate expression plasmids generated the third generation packaging system (*243*). These modifications improve biosafety by decreasing the pathogenesis of recombinant viruses and the risk of generating RCV. The envelope plasmid carries a gene encoding a heterogenous envelope, such as the VSV-G or lymphocytic choriomeningitis virus envelope (*241, 244, 245*). In addition, packaging cell lines which are stably transfected with the structural, regulatory and envelope genes have been generated (*246-249*). To produce recombinant viruses, the common method is to transiently co-transfect the three or four plasmids into packaging cells, such as 293T, and then to collect the virus-containing supernatant (Fig. 5). Further, virion particles can be concentrated by ultracentrifugation. The virus titer can be assessed by serial dilutions on target cells (293T or HeLa cells). Other assays, such as polymerase chain reaction (PCR) and real time PCR, have also been used for determining virus titers (*250, 251*).

To date, many *in vitro* and *in vivo* applications with the lentivirus vector have been reported. The capability of lentivirus vectors to infect postmitotic cells allows this vector to support gene transfer in such cells as rat neurons (*235*), intact human islets (*252*), and murine hepatocytes (*253*). Recently, it was reported on the first ongoing clinical trial in which the lentivirus vector system is used for the treatment of HIV (*254*).

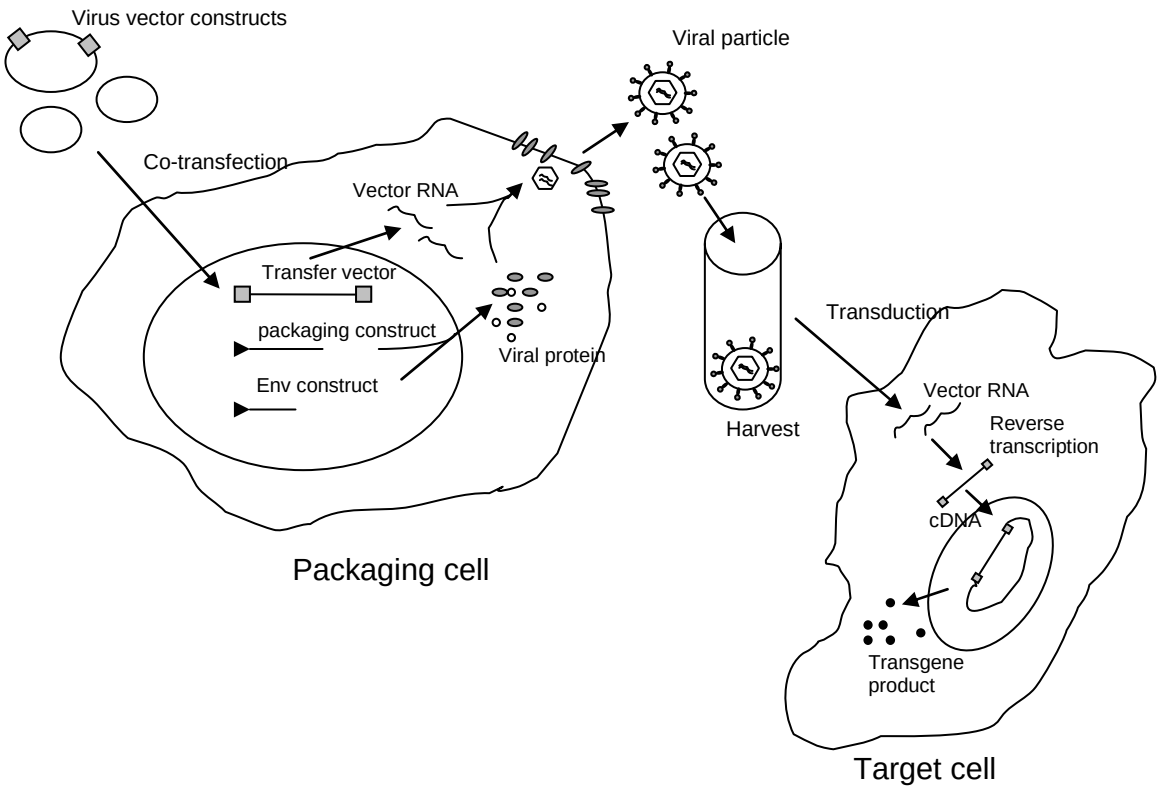

Fig. 5. Schematic of the generation of lentiviral vector infectious particle and the transduction of transgene into target cell.

1.2.3 Clinical trials of gene therapy

Since the first gene therapy clinical trial in 1989 (*255*), thousand human gene therapy trials have been completed or are ongoing in the world (*177*). The major human diseases involved in clinical trials of gene therapy include cancer, inherited monogenic diseases and vascular disease. Most of these clinical trials are in Phase I or II stages for assessment of safety and therapeutic efficacy.

ADA-SCID patients were the first to be included in a clinical trial of gene therapy for inherited human disease. These patients suffer from a deficiency of adenosine deaminase which is an enzyme catalyzing the deamination of adenosine and deoxyadenosine to inosine and deoxyinosine, respectively. The deficiency of this enzyme results in an accumulation of metabolites which are toxic for developing T cells, and trigger apoptosis of these cells, hence the clinical syndrome of SCID. If available, transplantation of bone marrow or stem cells from a haploidentical donor is the best treatment for ADA-SCID (*256*). Alternatively, exogenous enzyme replacement is conducted by direct injection of human or bovine ADA covalently attached to polyethylene glycol (PEG-ADA) (*257*). The first gene therapy trial in ADA deficiency patients who were under PEG-ADA treatment was performed using retroviral vectors carrying an ADA cDNA to transduce T lymphocytes (*228*). This study suggested that retroviral vectors were safe, but only low levels of reconstituted T cells were identified and no long term beneficial effect from the treatment was seen. Recently, successful treatment of two ADA-SCID patients was accomplished by retrovirus vector-mediated gene transfer to autologous CD34+ stem cells (*230*). In this clinical trail, the patients received non-myeloablative conditioning prior to the infusion of gene-modified stem cells. After the treatment, both patients showed reconstitution of multi-lineage lymphocytes, the restoration of immune responses and reduction of toxic metabolites.

Another congenital disorder addressed successfully by gene therapy is X-linked SCID (SCID-Xl). This X-linked hereditary disorder is caused by mutations in the gene encoding the common cytokine receptor gamma chain (γc) which is a subunit of the cytokine receptors for interleukins (IL) 2, 4, 7, 9, 15 and 21. Mutations in this receptor block the development of T and NK cells. The disease can be cured by transplantation of sibling bone marrow (*256*). For those who can not be treated with bone marrow transplantation, gene therapy is a way to restore the immune activities. Recently, Fischer's group reported a gene therapy approach in which a MoMLV based retrovirus vector encoding the γc cDNA was used to transduce autologous hematopoietic stem cells that were reinfused into SCID-Xl patients (*231, 258, 259*). Of the 10 patients treated with this protocol, 9 patients showed promising immunological reconstitution, including restoration of functional T and NK cells. Apart from the success, undesired incidents were reported in two patients after two and half years follow-up. Both patients developed an uncontrolled clonal T cell lymphoproliferative syndrome, similar to acute lymphoblastic leukemia (ALL) (*119*). By analyzing the insertion site of the retrovirus vector, patients' clonal T cells were shown to contain a single copy of the retroviral provirus inserted into the promoter or the first intron of the LMO2 gene, which is a T cell oncogene associated with the control of gene transcription (*260*). Thus, activation of LMO2 is likely to contribute to T-cell leukemia of the SCID-Xl patients treated with gene therapy. Recently, another SCID-Xl patient from the same treatment group was reported to have cancer (*261*). Although the investigation of the cause is still underway, the safety concerns of gene therapy are reinforced.

Clinical trials in which gene therapy has been used to treat hemophilia, including Factor VIII (F.VIII) deficiency (hemophilia A) and Factor IV (F.IV) deficiency (hemophilia), have been reported (*197, 262-264*). Using different gene transfer methods, including both virus vector-mediated and plasmid-mediated, genes encoding F.VIII and F.IV were administrated to patients. The expression of these factors was modest, resulting in limited efficacy in some of the patients. All of these clinical trials indicated that using the vectors was safe for the patients.

Gene therapy of cancer has been extensively investigated *in vitro* and in animal models, and the many encouraging preclinical studies have lead to the initiation of clinical gene therapy trials on human cancer. A number of different treatment strategies, including tumor growth suppression, boosting of host immunity, and killing cancer cells by oncolytic vectors, have been investigated. Administration of recombinant Ad encoding p53, a tumor suppressor, to tumor cells can suppress its growth and trigger apoptosis. This strategy has been used in lung (*265*) and ovarian (*266, 267*) cancer treatment clinical trials. Both of the trials showed that most of the patients expressed p53 *in vivo*. In the former trial, complete and partial responses have been observed in 6 of 19 patients after three months of p53 gene administration. In addition, the strategy of using conditionally replicating oncolytic viruses has been developed for cancer gene therapy. This is based on the finding that Ad deleted of E1A would not replicate in normal cells, but could grow in cells, *e.g*. tumor cells, lacking p53 leading the lysis of these cells. Using E1A deleted Ad, a clinical trial has been reported in patients with head and neck cancers and showed tumor regression in 21% of participating patients (*268*). Alternative gene therapy attempts have focused on enhancing tumor immunogenicity by introducing cytokine genes in the tumor. When plasmids carrying the IL-2 gene were injected locally in head and neck tumors, good safety and tolerance were seen. In terms of efficacy, however, only 1 of 11 patients benefited from the therapeutic gene (*269*). In another trial, tumor cells collected from a patient were transduced by Ad carrying the granulocyte/macrophage colony-stimulating factor (GM-CSF) gene and then injected back into the same patient. As a result, anti-tumor immune responses were seen (*270*).

Clinical trials of gene therapy for cardiovascular diseases have also been conducted. Plasmids or Ads carrying the vascular endothelial growth factor (VEGF) or fibroblast growth factor (FGF) genes were used in patients suffering from peripheral arterial disease or myocardial ischemic disease, and all vectors appeared safe and clinical treatment efficacy was accomplished (*142, 271-276*).

1.3 GENE THERAPY AND XENOTRANSPLANTATION

Genetic modification of xenograft donors or xenografts prior to transplantation has been considered an efficient way to avoid rejection by the host and improve graft function. Even though, currently there is no genetically modified xenograft that is used in clinical transplantation, encouraging results from *in vitro* and *in vivo* experiments foresee a promising future for this approach. To overcome xenorejection, different gene therapy strategies have been developed for different stages of xenograft rejection. Due to the strength of HAR and AVR, T cell-mediated rejection has been difficult to study *in vivo*. Thus to date, attempts to prevent xenograft rejection by gene therapy have been mainly directed against HAR and AVR.

Because of the importance of anti-Gal and its binding to α-Gal on porcine endothelium for the development of HAR and AVR, a major goal has been to eliminate or down-

regulate the expression of α-Gal on donor cells by genetic engineering. Two predominant approaches have been used to accomplish this. Before it was possible to use the nuclear transfer technology on pigs and to actually delete genes from the genome by homologous recombination (*277*), various strategies by which injected genetic material would prevent α-Gal biosynthesis was considered (Fig. 1). One such strategy is based on the competition for the same lactobiose substrate (N-acetyllactosamine) between an α1,2fucosyltransferase and the α1,3galactosyltransferase. Transgenic mice or pigs expressing the α1,2fucosyltransferase showed a reduction of α-Gal epitopes (*278*). *In vitro* and *in vivo* studies have shown that this α-Gal epitope "knock-down" process increases the resistance of xenogeneic cells to human serum-mediated lysis and prolongs the survival of organ xenografts, respectively (*279, 280*). However, once the nuclear transfer technology was adapted to pigs, the α1,3GalT encoding gene could be deleted by homologous recombination in primary pig fibroblasts in culture. Nuclei from such cells could then be transferred into fertilized, enucleated eggs from which a progeny was derived that lacked theexpression of α-Gal in its tissues (*281, 282*). Kidneys and hearts from α1,3GalT knock-out pigs transplanted into baboons pretreated by T-cell deletion and the addition of anti-CD154 mAbs exhibited substantially prolonged survival times suggesting that HAR was circumvented and AVR was weakened (*28, 29*). In addition, RNA interference has been used to down-regulate α1,3GalT mRNA and thus α-Gal expression in porcine endothelial cells (*283*). Whether this approach can be used also *in vivo* remains to be shown.

Although the disruption of the interaction between α-Gal and its antibody can prevent HAR and reduce AVR markedly, there may be additional donorantigens that can bind to XNAs and trigger complement activation and subsequent xenorejection. Alternative pathways of complement activation can also be involved. Therefore, inhibition and prevention of the complement reaction may serve as a way to protect xenografts from being destroyed by complement. In order to accomplish complement inhibition, the complement regulatory proteins DAF, MCP and CD59 have been transgenically expressed in pigs. In a pig-to-primate model, hearts from transgenic pigs co-expressing human DAF and CD59 survived complement mediated HAR (*26*).

Donor organ EC activation plays pivotal role in both HAR and AVR, therefore prevention of EC activation can weaken the rejection. It was reported that Ad mediated gene transfer of IκBα to human and porcine EC blocked the NFκB signaling pathway, and thereby inhibited the expression of VCAM-1, IL-1, -6, -8, and tissue factor. This resulted in an inhibition of the adhesion of the human promyelocytic HL-60 cells to ECs (*284*). When an EC stress response gene called A20 was transferred to PAEC via Ad, the over expression of A20 protected EC from apoptosis induced by TNF-α, Fas/CD95 and NK cells. This protection was due to the inhibition of caspase 8 by A20 (*285*).

Following injection of xenogenic and avascular pancreatic islets into the portal vein, IBMIR may destroy the islet xenograft. IBMIR is characterized by platelet consumption, complement activation and initiation of coagulation. Schmidt et al reported that adult porcine islets expressing human DAF or CD59 following adenovirus gene-transfer were partially protected from being lysed by human serum (*286*). The same group also reported that the pancreatic islets isolated from a human DAF transgenic pig was partially protected from lysis by human serum (*287*). Transduction of the anti-apoptotic Bcl-2 gene into xenogenic islets has been shown to prolong the

survival of transplanted islets by increased resistance to complement-mediated cytotoxicity (*288*). Another strategy to protect xenogeneic islets from damage could be to introduce into islets the gene encoding catalase, an enzyme protecting cells from oxidative damage by converting hydrogen peroxide into water and oxygen. *In vitro* experiments showed that the catalase gene when expressed in porcine pancreatic islets partially reduced the islets' susceptibility to oxidant stress (*289*). Other genes, such as those encoding CTLA-4Ig and heme oxygenase-1, have been suggested to improve the survival of transplanted xenogeneic islets. However, this has yet to be established.

2 AIMS OF THE PRESENT STUDY

- To investigate the activity and cell-specificity of the human endothelial cell specific promoters of *Flk-1*, *Flt-1*, *ICAM-2*, thrombomodulin and *vWf* in porcine cells

- To assess the lentivirus transduction efficiency in, and its effects on, primary endothelial cells

- To evaluate the importance of lentiviral-mediated expression of α-Gal on endothelium for its interaction with human NK cells and monocytes

- To investigate the ability of lentiviral vector to transduce isolated rat pancreatic islets and its effect on islet function *in vivo*

3 METHODOLOGICAL CONSIDERATIONS

Materials and methods utilized in this thesis have been described in detail in each paper or manuscript. Here, I will give a brief description of some of the methods used.

3.1 PRIMARY ENDOTHELIAL CELLS

At 37°C in 5% CO_2 and 95% humidified air, HAECs (Clonetics, Walkersville, MD; Cascade Biologics, Portland, OR) were cultured in gelatin-coated tissue culture flasks using EBM-2 (Clonetics) or Medium-200 (Cascade Biologics) medium. These media were supplemented with extra components (Which components? If you don't know, remove this sentence) as recommended by the suppliers. HAECs used in this series of experiments were propagated less than 13 passages.

Using established methods (What are the established methods, describe briefly), porcine vascular and microvascular ECs were isolated from different sources, i.e. aorta (PAEC), kidney (PKMEC), liver (PLMEC) and embryonic brain (PBMEC) *(77, 290, 291)*. PAECs were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) or supplemented EBM-2 medium. PKMECs and PLMECs were maintained in DMEM containing 10% FBS, whereas PBMECs were cultured in MCDB 131 medium supplemented with 10% FBS and endothelial growth supplements (EGM-2 single quots). All porcine ECs were cultured in gelatin (0.2%) coated tissue culture flasks at 37°C in 5% CO_2.

Porcine ECs were characterized by their cobble-stone morphology and the expression of EC surface markers, *i.e.* CD31 and VCAM-1. It has been demonstrated that ECs from different vascular beds exhibit phenotypic and functional differences (*292*). The genetic inherence of different EC and the local microenvironment may cause these differences. Indeed, in Paper I, the expression of CD31 and VCAM-1 was increased in PLMECs and PAECs and decreased in PKMECs and PBMECs following a TNF-α stimulation. For *in vitro* experiments, primary EC cultures are better than established EC lines, as the latter may lose some EC features due to transformation and long-term culture.

3.2 LUCIFERASE ASSAYS

A fragment was selected from each human EC specific promoter including the genes encoding Flk-1 (fetal liver kinase-1), Flt-1 (fms-like tyrosine kinase), ICAM-2, thrombomodulin, and vWf (von Willebrand factor), and was then cloned into the promoter site of the pGL3-control plasmid encoding a luciferase gene. Using a commercial kit (Promega, Madison, WI), dual-luciferase reporter assays were performed to assess the activity of the promoter in question. Cells were co-transfected with the pGL3 construct and an internal control plasmid, pRL-TK in a ratio of 50:1. One day after transfection, cells were collected and lysed. After adding a substrate to the cell lysates, the luciferase activity, which correlated to the promoter activity, was measured using a luminometer. The luciferase reporter assay is sensitive and the internal control included reduces intra- and inter-experimental variations.

3.3 LENTIVIRAL VECTOR SYSTEM

As described in preceding sections, in Paper II-IV, a three-plasmid lentivirus vector system was used to introduce target genes into HAECs and rat pancreatic islets,

respectively. The lentivirus transfer vector used included pHR'EF1αGFPSIN or pHR'EF1αGalTSIN carrying the gene of interest controlled by the EF1α promoter. In addition, a second generation packaging plasmid pCMVΔ8.91 containing virus structural and regulatory genes, and an envelope plasmid pMD.G encoding a VSV-G envelope were used (Fig. 6). In the transfer vector, the multiple cloning site is located between restriction enzyme sites *EcoR*I and a 3' *Kpn*I flanked by the EF1α promoter and a 3'SIN-LTR. To produce replication defective viral particles, the three plasmids were co-transfected in 293T cells using the PEI transfection method. The cells were then cultured in fresh culture media (2 % FCS) into which virion particles were released. The medium was collected, filtered through a 0.45-μm filter, and concentrated by ultracentrifugation. To determine the virus titer, 293T and HeLa cells were infected by a virus suspension and infected cells were examined by FACS analysis. As the transduction permissibility differs between cells, the titers determined in 293T cells were higher than those in HeLa cells. Therefore, viral titers were determined mainly in 293T cells.

Target cells like HAECs and isolated pancreatic islets can be transduced by incubating them with concentrated virions. However, in order to have efficient gene transfer in different target cells, preliminary tests are needed to determine the viral transduction efficacy. When HAECs were infected with viruses encoding green fluorescent protein (GFP) at an MOI of 0.5, around 70% of the cells expressed GFP. Upon infection of rat islets with viruses carrying the GFP gene at $5x10^3$ transduction units (TU)/islet, 33% ± 8 islet cell expressed GFP.

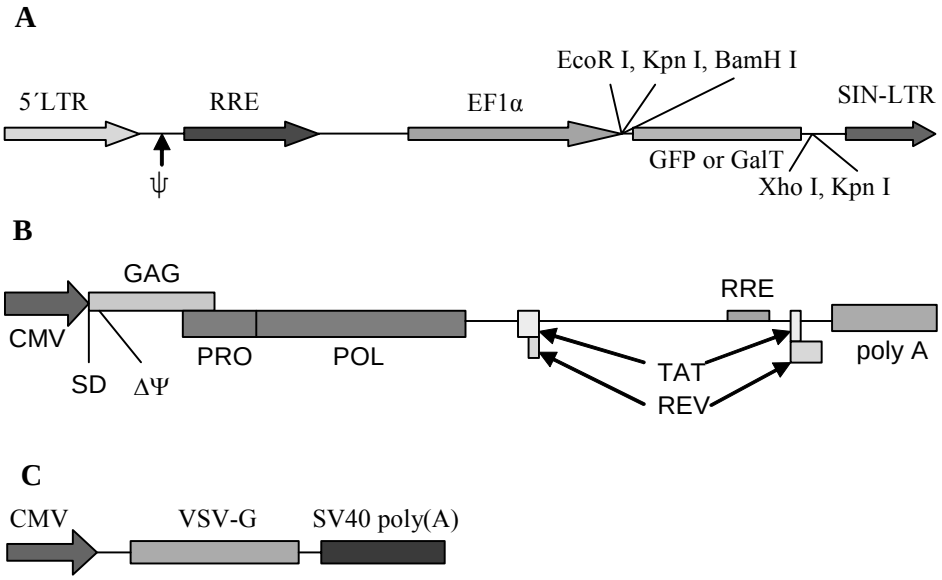

Fig. 6. The lentivirus vector system. (A) The HIV-1 transfer vector containing the gene or cDNA of interest and the minimal cis-acting elements of HIV-1. (B) The packaging plasmid containing the structural and regulatory genes. (C) The envelope plasmid encoding the envelope glycoprotein of vesicular stomatitis virus (VSV-G).

3.4 PURIFICATION OF HUMAN ANTI- α -GAL ANTIBODIES

Human anti-α-Gal Ab was purified form pooled AB serum using agarose beads with covalently linked Gal1,3Gal disaccharides (Calbiochem, San Diego, CA). In brief, 200 ml of human serum was applied at 0.5 ml/min to a column (10 mm in diameter) containing 4 ml of slurry (2 ml of packed beads). Thereafter, the column was extensively washed with PBS, and bound Abs were eluted with 0.1 M glycine/HCl (pH2.5) at 1 ml/min. Serial fractions (4 ml in each) were collected in tubes containing 0.4 ml of neutralizing buffer (1.5 M Tris/HCl, pH8.8). The absorption at 280 nm was read spectrophotometrically, and tubes containing Abs were pooled, dialyzed against 1% PBS, lyophilized, and resuspended in distilled water. The protein concentration was determined using the BCA Protein Assay kit (Pierce, Rockford, IL).

In this study, we used purified human anti-α-Gal Ab to assess α-Gal expression both on cell surfaces (FACS analysis) and in whole-cell extracts (Western blotting). Purified human anti-α-Gal Ab is more specific for α-Gal epitopes relevant to xenograft rejection than the lectin, *Griffonia simplicifolia* I-B_4, which has a broader specificity and can also bind α-Gal epitopes that are not α-1,3-linked (*293*).

3.5 ADHESION ASSAYS

Static adhesion assays were performed by incubating one million human NK cells or monocytes with transduced and non-transduced HAECs as well as PAECs for 15 min at 37°C. Non-adhered cells were carefully removed by rinsing three times with medium. The number of adhered cells was counted in ten visual fields at a magnification of 100x.

Flow adhesion assay was carried out in a flow chamber (Glycotech, Rockville, MD) mounted and held in place by vacuum on plastic culture dishes with confluent monolayers of ECs. Human NK cells or monocytes in a density of $5x10^5$ cells/ml were perfused through the chamber at 1 Dyne/cm^2 corresponding to the shear stress found in post-capillary venules where the interactions studied are believed to occur. Following 15 min of perfusion, adhered cells were counted in ten visual fields at a magnification of 100x.

3.6 RAT ISLET ISOLATION

Rat pancreatic islets were isolated by collagenase digestion from rats weighing 300-350 g. Briefly, under anesthesia, the abdomen was opened and the common bile duct was clamped at both ends. The pancreas was distended by injection of 10 ml Hanks' buffer containing 10 mg of collagenase XI (Sigma) and 0.1 mg of DNase-1 (Sigma). The pancreas was resected, incubated at 37°C for 15 min, and then vigorously shaken for 30 seconds. The dissociated islets were separated from exocrine debris either by hand-picking or gradient centrifugation.

3.7 *IN VIVO* ISLET TRANSPLANTATION MODEL

In Paper IV, a rat islet-to-nude mouse xenotransplantation model was used to assess the engraftment and function of transplanted, non-transduced and transduced rat islets. Diabetes was induced in athymic nude mice by a single injection of streptozotocin (STZ, 250 mg/kg body weight) via the penile vein. Rat islets, either non-transduced or transduced with viruses encoding GFP, were cultured *in vitro* for 5 days, and then transplanted under the kidney capsule of diabetic nude mice. Mice were kept for at least

8 weeks. During this period, the mice were checked for changes in body weight, blood glucose and glucose tolerance.

Nude mice are characterized by a low number of functional mature T-lymphocytes because they lack a normal thymus. In this small mammal model, the innate immunity components alone, such as complement, myeloid lineage leukocytes and NK cells, are not sufficient to trigger the rejection of islet xenografts (*294*). As a result, xenografts are accepted by nude mice. Thus, these rodents have been extensively used in various models of xenografting aiming at investigating factors of importance of engraftment and physiological function of islet xenografts *in vivo*.

4 RESULTS AND DISCUSSION

4.1 HUMAN EC-SPECIFIC PROMOTERS OPERATE EQUALLY WELL IN PORCINE EC AND EXHIBIT VASCULAR BED SPECIFIC EXPRESSION LEVELS

In pig-to-human organ transplantation, the porcine vascular endothelium is one of the premier targets of xenorejection. The activation of porcine EC plays a pivotal role for subsequent interaction between the graft and the host immune system. Thus, genetic modification of porcine EC, such as addition of genes encoding immunoregulatory molecules, is believed to be a promising approach to reduce organ xenograft rejection. To have the transgene selectively expressed in EC, it has to be controlled by EC-specific promoters. Because human EC-specific promoters are better characterized than their porcine counterparts, using human EC specific promoters in pigs can save efforts in isolating and characterizing the porcine counterparts of human sequences. In Paper I, we investigated the activity and cell specificity of human EC specific promoters, *i.e*. the *Flk-1, Flt-1, ICAM-2, thrombomodulin and vWf* promoters, in porcine ECs from different vascular beds.

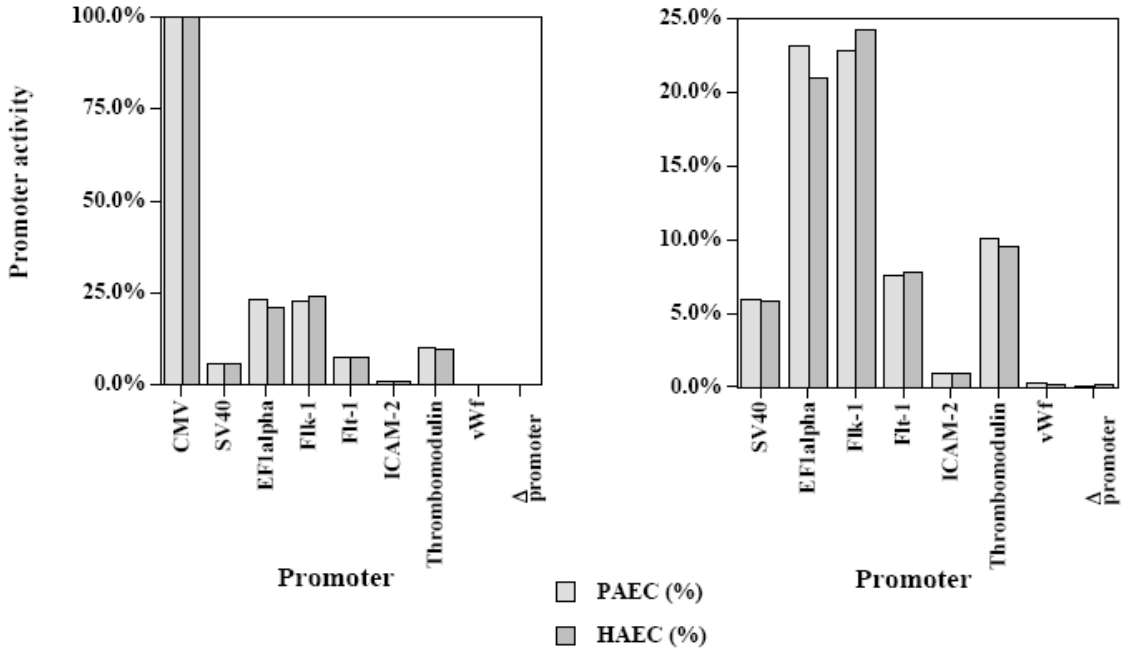

Fig.7. Functional analysis of the *CMV*, *SV40*, *EF1α*, *Flk-1*, *Flt-1*, *ICAM-2*, *thrombomodulin* and *vWf* promoters in porcine and human aortic endothelial cells using firefly luciferase reporter gene constructs. All constructs contained the pRL-TK vector carrying the Renilla luciferase under the control of the Herpes simplex virus thymidine kinase promoter to correct transfection efficiency. The luciferase activity was expressed as a percentage of the luciferase activity seen when the CMV promoter in the pGL3 control vector was used. Both panels are essentially identical except for their Y-axis scales.

According to the dual-luciferase reporter assay, the human *Flk-1, Flt-1, ICAM-2, thrombomodulin and vWf* promoter sequences mediated expression of the luciferase

reporter gene equally well in human and porcine primary aortic ECs. The *Flk-1* promoter showed the strongest activity amongst the EC-specific promoters, followed by the *thrombomodulin*, the *Flt-1*, the *ICAM-2* and the *vWf* promoters (Fig. 7). The activity of the EC-specific promoters investigated was lower in porcine non-endothelial cell lines, *e.g*. PK-15 and ST, than in PAECs, which suggests that the activity of these promoters is, to a certain degree, tissue- or cell-specific in pigs. The results indicate that these human promoters regulate gene transcription in a similar manner in pigs, and the interaction between transcription factors and these promoter sequences in pigs are comparable to that in men.

However, there are reports that the regulatory mechanism of the *Flk-1* sequence was different between humans and mice (*295, 296*). It has been shown that the activities of EC-specific promoters, such as those controlling transcription of *tie-1*, *vWf* and *ICAM-2*, are discordant between *in vitro* and *in vivo* situations (*297-300*). Thus, our *in vitro* data need to be verified *in vivo* in transgenic pigs to see if the promoters' activity and EC-specificity remain.

In Paper I, we also showed that the EC-specific promoters all had less activity in PKMECs than in PLMECs and PBMECs. The *Flk-1* and thrombomodulin promoters showed similar activity in PLMECs and PKMECs. The *Flk-1* promoter exhibited stronger activity in PAECs and PBMECs than in other porcine ECs (Fig. 8).

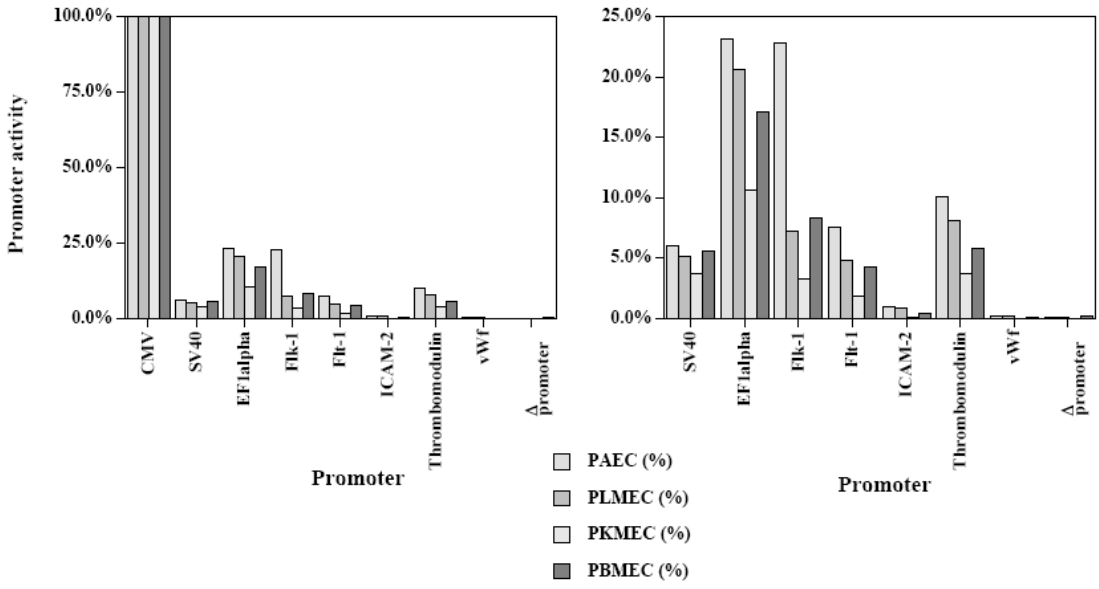

Fig. 8. Functional analysis of the *CMV*, *SV40*, *EF1α*, *Flk-1*, *Flt-1*, *ICAM-2*, thrombomodulin and vWf promoters by transfection of primary porcine aortic and porcine liver, kidney, and brain microvascular endothelial cells with firefly luciferase reporter gene constructs. All constructs contained the pRL-TK vector for correction of transfection efficiency. The luciferase activity was expressed as a percentage of the luciferase activity obtained with the CMV-pGL3 control vector. Both panels are essentially identical except for their Y-axis scales.

Although our *in vitro* data show different activities of these promoters in ECs from different vascular beds, this may not reflect the activity in different vascular beds *in*

vivo because the microenvironment surrounding the cells *in vivo* is likely to be different from that of *in vitro* cell culture and critical for EC transcriptional regulation (*292*). Thus the activity of these promoters in ECs from different sources needs further investigation.

4.2 LENTIVIRUS IS AN EFFICIENT GENE CARRIER FOR ISOLATED HUMAN ENDOTHELIAL CELLS AND RAT PANCREATIC ISLETS

The difficulty of efficiently and stably introducing genes into primary cells and postmitotic cells is well known. The, recently developed lentivirus vector for gene transfer provides a tool for these tasks. In Papers II-IV, we have used a lentivirus vector system to transduce primary HAECs and intact rat pancreatic islets.

In Papers II and III, primary HAECs were infected with virion particles carrying the GFP reporter gene. At an MOI of 2.5, the fraction of GFP expressing HAECs reached 98.5%. Analysis of GFP expression in these cells following lentiviral transduction showed that GFP expressing cells appeared one day after the infection. The proportion of GFP positive cells increased in the following days, peaking on Day 5, and staying high thereafter. The gradual increase in the number of transgene-expressing cells is likely to be due to accumulating events at all stages of virus infection including virus entry, reverse transcription, integration, viral gene expression, and production of target protein. Because of random integration of viral DNA in the genome, transgene expression levels will vary between cells within a transduced cell population. It has been reported that rat EC become activated following Ad transduction, which precludes the use of Ad for therapeutic purposes aiming at genetically modifying graft endothelium (*301*). In present study (Paper II), using lentiviruses carrying the GFP cDNA, we determined the effect of lentivirus transduction on EC by using flow cytometry to detect the EC activation markers, E-selectin, VCAM-1 and MHC class II, with or without TNF-α or IFN-γ stimulation. According to the data, lentivirus transduction did not activate ECs and transduced HAEC were as responsive to activation as non-transduced HAEC (Fig. 9). In Paper III, the data revealed that following lentivirus-mediated α1,3GalT gene expression, the expression levels of E-selectin, VCAM-1 and ICAM-1 in resting as well as TNF-α stimulated ECs were as expected with an increase in the expression of E-selectin and VCAM-1 following activation. Thus, neither lentivirus transduction *per se* nor expression of the α1,3GalT transgene influenced the expression levels of these adhesion molecules.

In order to draw a firm conclusion on the involvement of the α-Gal epitope in the adhesion of NK cells to endothelium in our model system, it is of significance that the α-Gal epitope density is similar between α1,3GalT-transduced HAECs and normal PAEC. Flow cytometric analysis using human anti-α-Gal Abs revealed that the fluorescence intensities of PAEC and α-Gal expressing HAEC were overlapping indicating similar α-Gal epitope densities. Two populations of α-Gal epitope expressing HAECs with distinct α-Gal epitope expression levels were identified by flow cytometry using anti-α-Gal Abs. This may result from a regulatory event that was not related to the expression of the transgene,

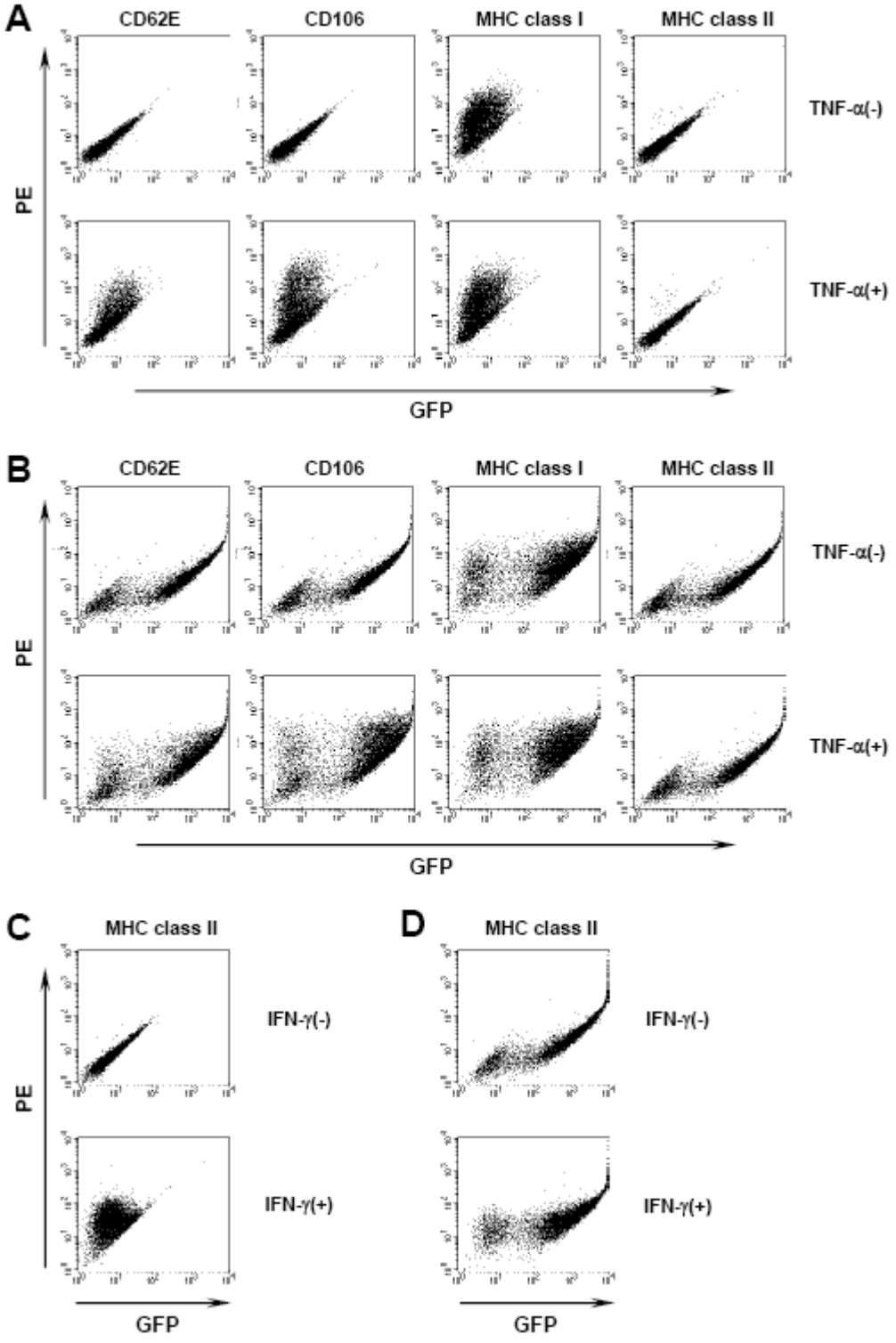

Fig.9. Phenotypic analyses of GFP-transduced and non-transduced primary HAECs. On Day 5, non-transduced (A, C) and transduced (B, D) primary HAECs were cultured with or without TNF-α or IFN-γ. Following staining with PE-conjugated mAbs, the expression of CD62E, CD106, MHC class I and II was assessed (Y axis) on GFP-expressing (B, D) and non-GFP-expressing (A, C) cells (X axis) by flow cytometry. One representative experiment out of three is presented.

but acted at an epigenetic level, for instance, carbohydrate precursor chain or activated sugar-donor availability. Western blotting analysis using human anti-α-Gal Abs under reducing conditions, PAEC and α1,3GalT transduced HAEC showed a very similar staining pattern of the stained major protein components of 150 kDa and 155 kDa, respectively. This finding indicates that proteins carrying precursor chains for the α1,3GalT may be phylogenetically conserved and that the glycosylation of a particular protein is tightly regulated.

4.3 NK CELLS AND MONOCYTES DO NOT DIRECTLY RECOGNIZE THE α-GAL EPITOPE

Infiltration of NK cells and monocytes/microphages in xenografts is a common phenomenon in AVR and has been suggested to involve the α-Gal epitope on the endothelium of xenografts. However, the importance of the α-Gal epitope for leukocyte binding and extravasation is controversial. In Papers II and III, we established an *in vitro* model for studying the importance of this epitope for investigations on the interaction between host NK cells or monocytes and endothelium.

In Papers II and III, HAECs were transduced with the α1,3GalT gene by lentivirus-mediated gene transfer. The α-Gal expressing cells accounted for 70-95% of the whole population. Using these α-Gal expressing HAECs, the effect of α-Gal epitope on human NK cell and monocyte adhesion, and on NK cell cytotoxicity, was investigated. No differences were seen between non-α-Gal- and α-Gal-expressing HAEC in terms of their susceptibility to NK cell mediated lysis, capability to stimulate IFN-γ production by NK cells, or ability to support NK cell or monocyte adhesion under static and dynamic conditions, with or without TNF-α stimulation. Our observations are discordant with some reports (*72, 73*) and concordant with others (*74*). There are several potential explanations for the diversified results. First, there are many phenotypic differences between human and porcine EC. One example is that the porcine MHC class I molecules have been claimed to be incompatible with the NK cell killer inhibitory receptors (*63*). Second, using COS cells aberrantly expressing α-Gal to study the interaction between porcine EC and human leukocytes may not be relevant because of the differences in the repertoire of α-Gal structures between the two different cell types, and the lack of EC-specific cell adhesion molecules on COS cells. Third, α-Gal binding lectins or IgG $F(ab)'_2$ may sterically block α-Gal substituted molecules which are involved in the conjugation of the NK cell to the target even though the α-Gal epitope *per se* is not involved (*302*). The *Griffonia simplicifolia* I IB_4 isolectin, has been shown to activate porcine ECs, which may affect their susceptibility to NK cell-mediated lysis (*303, 304*). Finally, reducing the α-Gal epitope density on porcine endothelium by over-expression of an α1,2 fucosyltransferase competing for the same precursor as the α1,3GalT, or by treating ECs with an α-galactosidase cleaving terminal α-Gal, may have profound effects on the glycosylation phenotype of these cells. In addition, over-expression of an α1,2 fucosyltransferase may decrease the level of sialylation thereby making the cell surface less negatively charged, which in itself will decrease the repulsive forces between two opposing cells and thereby increase adhesion (*305, 306*).

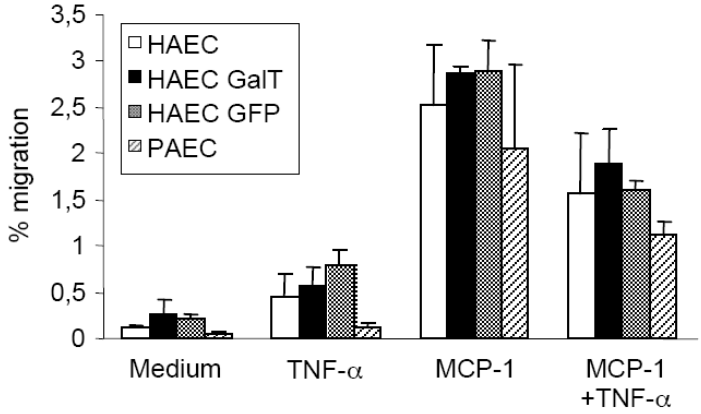

Fig. 10. Transendothelial migration of human monocytes across PAECs, GalT-, GFP- and nontransduced HAECs. The migration experiment was performed in Transwell® inserts (pore size 8 μm, 6.5 mm in Ø) and the ECs were cultured for at least 5 days to reach confluence. The ECs were treated with medium or human TNF-α (20 ng/ml) for 5 hrs before the assay. MCP-1 (50 ng/ml) was used as a chemotactic factor and was added to the lower compartment. One million human monocytes were added to the upper compartment and allowed to migrate for 2 hrs at 37°C. The migrated monocytes were collected by centrifugation, fixed in 1% paraformaldehyde and counted in a Bürker chamber. Results are expressed as mean ± SEM from three experiments.

In Paper III, porcine EC and non-α-Gal- and α-Gal-expressing HAEC showed similar activity with regard to their ability to support human monocyte transendothelial migration (Fig. 10), indicating that the α-Gal epitope expressed on porcine EC is not directly involved in monocyte migration during xenograft rejection. In our previous studies, porcine EC supported the migration of different human leukocytes including monocytes, as well as did human endothelium (77). Even though porcine and human endothelium supports monocyte migration equally well, the process may be different on a molecular level. The migration across TNF-α-activated endothelium in the presence of MCP-1 was decreased in all types of EC as compared with the addition of MCP-1 alone. A possible explanation may be that TNF-α-activated endothelium supports monocyte EC adherence to such a degree at which the cells' migration is prevented.

4.4 THE IN VIVO FUNCTIONAL CAPACITY OF LENTIVIRUS-TRANSDUCED RAT ISLETS EQUALS THAT OF NON-TRANSDUCED ISLETS.

Using GFP as a reporter, in Paper IV, we evaluated the transduction efficacy of the lentivirus vector on isolated rat pancreatic islet and its effect on islet function *in vitro* and *in vivo*. Following islet transduction with lentiviruses encoding GFP at a ratio of $5x10^3$ TU/islet, all islets contained fluorescent cells at the end of the five-day incubation period. Flow cytometric analysis of single cell suspensions prepared from transduced islets showed that 33±8 % of dispersed islet cells expressed GFP. The possible explanation for the relatively low transduction efficacy on a cellular level may be attributed to the difficulty for the viruses to reach the islet core. However, if the

application is to express a secreted protein or a cell membrane bound protein that excerts its function at the islet surface, expression limited to the peripheral cells of an islet may suffice. Raising the number of infectious particles may facilitate the infection of the islet core. However this may also increase the chance of multiple infections of the same islet cells. Thus, finding the optimal ratio of virion particles to isolated pancreatic islets is crucial to reach maximal transduction efficiency with minimal multiple infection. Similar insulin secretion and similar ADP/ATP radios between transduced and non-transduced rat pancreatic islets suggest that, the transduction per se did not affect the viability and insulin secretion of the islet. In addition, we transplanted intact and transduced rat pancreatic islets into nude mice with SZT-induced diabetes. Transduced islets and control islets restored the animals' euglycemia in a similar manner. The euglycemia was maintained for at least 8 weeks until the islet-bearing kidney was resected (Fig. 11). Under the fluorescent microscope, GFP-expressing cells were seen in the removed kidney and in the cryosections prepared from the kidney. Similar long term expression of transgene mediated by lentivirus in rodent pancreatic islets has been reported by others (*307*).

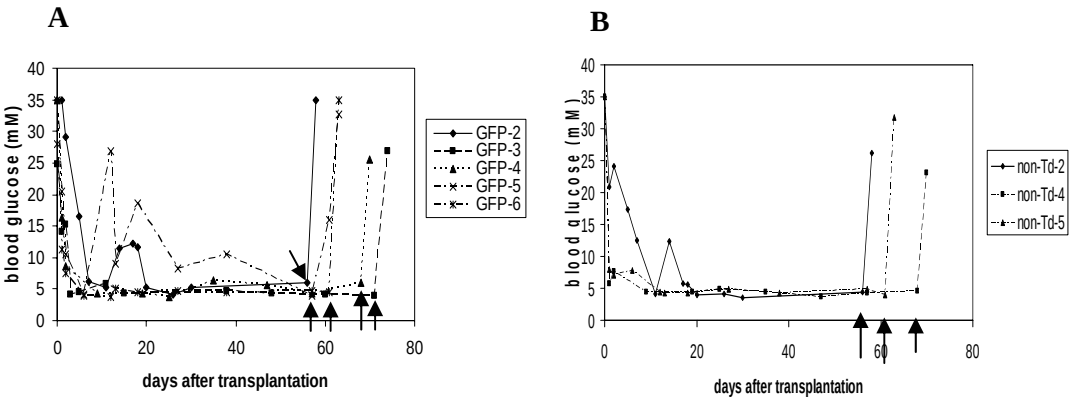

Fig. 11. Blood glucose levels of nude mice carrying transduced (A) or control islets (B). Blood glucose levels were followed regularly for more than 8 weeks. Arrows indicate the time of graftectomy.

One concern recently raised is the toxicity of the VSV-G pseudotypic viruses to islet cells (*245*). However, in our *in vivo* transplantation studies transduced islets did not perform worse than non-transduced islets with regard to islet engraftment and glucose control. In hematoxylin and eosin stained paraffin sections of the islet-bearing kidney, there was no sign of inflammatory cell infiltration into the transduced islet graft. Thus, the lentivirus vector system used can carry out safe, stable and efficient gene transfer to intact rat pancreatic islet.

5 CONCLUSIONS

- The human *Flk-1*, *Flt-1*, *ICAM-2*, *thrombomodulin* and *vWf* promoters supported the same degree of luciferase activity in porcine and human ECs, whereas the strength of these promoters is different in microvascular ECs from porcine kidney, liver and brain.

- *In vitro*, the *Flk-1* and thrombomodulin promoters were the most active of the promoters tested.

- Lentiviruses constitute very efficient gene transfer vectors for primary EC and lentivirus transduction does not influence the endothelial state of activation.

- Neither under static nor flow conditions, does the α-Gal epitope contribute to the interaction of ECs with human NK cells and monocytes. Upon human, but not porcine, TNF-α stimulation, monocytes adhered less to porcine than to human endothelium under the condition of flow.

- Lentivirus transduction does not affect islet morphology or function *in vitro* or *in vivo*, and can thus be used as a gene carrier to modify the cells in pancreatic islets in order to improve engraftment and prevent rejection.

6 FUTURE PROJECTS

- The *in vitro* data shown in Paper I suggest the possible use of the human endothelial-specific promoters in porcine ECs. Further *in vivo* investigations need to be performed to confirm these *in vitro* conclusions. As shown in Papers II and III, the lentivirus vector is an efficacious gene transfer vector for primary endothelium. Thus, it would be interesting to subclone the human endothelial-specific promoters into the lentivirus vector and subsequently to introduce systemically these promoter constructs followed by a reporter gene into pigs. The EC specificity and activity will be assessed *in vivo* following infection. Also producing transgenic pigs by using human endothelial-specific promoters would be an attractive project to evaluate the specificity of these promoters *in vivo*.

- In Paper III, we have demonstrated that TNF-α from human and porcine have different effects on the stimulation of porcine EC with regard to their ability to support human monocyte adhesion. Even thought there is no difference in the expression of adhesion molecules, *i.e*. E-selectin, VCAM-1 and ICAM-1, on porcine EC upon stimulation with both human and porcine TNF-α, there may be other differences in surface molecule expression following human and porcine TNF-α stimulation. Therefore, further investigations of these differences may indicate how monocyte migration and infiltration in AVR is regulated *in vivo*.

- We will further use the lentivirus vector to transduce rat islets with functional genes such as programmed death-1 ligand 1 (PD-L1), which is the ligand of programmed death-1, an inhibitory receptor expressed on activated T, B cells and monocytes. The ability of PD-L1 expressed on the surface of islets to perturb immune stimulation and cytotoxicity *in vitro* as well as to accomplish prolonged graft survival *in vivo* will be investigated.

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