Aus der Klinik für Gastroenterologie, Hepatologie und Endokrinologie
der Medizinischen Hochschule Hannover

Cell-specific Infection of Human Cells Mediated by Lentiviral Vectors Pseudotyped with Measles Virus Hemagglutinin Fused to Single Chain Antibodies

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Teilergebnisse dieser Dissertation wurden im Juli 2013 im Artikel „*Specific gene delivery to liver sinusoidal and artery endothelial cells*" des Fachmagazins *Blood* veröffentlicht. Dabei dienten die Abbildungen 30 und 38 dieser Dissertation als Vorlagen für Teile der im Fachmagazin „*Blood*" veröffentlichten Abbildungen. Die Experimente wurden im Labor für Zell- und Gentherapie am Twincore unter der Leitung von Prof. Dr. med. M. Ott durchgeführt. Zusätzliche Experimente wurden im Labor für experimentelle Herz- Thorax- und Gefäßchirurgie unter der Leitung von PD. Dr. med. G. Warnecke und dem Labor für „Molekulare Biotechnologie und Gentherapie" unter der Leitung von Prof. Dr. rer. nat. C. Buchholz durchgeführt.

Parts of this dissertation were published in July 2013 in the article „*Specific gene delivery to liver sinusoidal and artery endothelial cells*" in *Blood* science magazine. Modified versions of figures 30 and 38 were implemented in this article. Experiments were conducted in the research group for "Cell- and Gene Therapy" at the Twincore under the supervision of Prof. Dr. med. M. Ott. Additional experiments were performed at the laboratory for experimental heart, thoracic and vessel surgery under the supervision of PD Dr. med. G. Warnecke, and the laboratory for "Molecular Biotechnology and Gene Therapy" under the supervision of Prof. Dr. rer. nat. C. Buchholz.

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Abbreviations

7-Aad	7-actinomycin D
AAV	adeno-associated vector
ASGPR	Whole ASGPR with 2 subunits H1 and H2
ASGR1	gene sequence coding for ASGPR H1
ASGR2	gene sequence coding for ASGPR H2
APC	allophycocyanin
APCs	antigen representing cells
BALB/c Rag2$^{-/-}$ IL-2R$\gamma_c^{-/-}$ mice	BALB/c mice with deleted Rag2 and IL-2 receptor gene
CD105	cluster of determination 105, endoglin
CMV	cytomegalovirus
CRD	carbohydrate recognition domain
CRDH1	carbohydrate recognition domain of subunit H1
dH_2O	distilled water
dNTPs	desoxynucleotides
ECBM	Endothelial Cell Basal Medium
ECGM	Endothelial Cell Growth Medium
ENG	Endoglin
eNOS	Endothelial nitric oxide synthase
FACS	fluorescence-activated cell sorting
Fig.	figure
eGFP	enhanced green fluorescent protein
HAR	hyperacute rejection
HBM	Hepatocyte Basal Medium
HCM	Hepatocyte Culture Medium
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
huASGPR	human ASGPR
HUVEC	human umbilical vein endothelial cell
IF	Immuno(histo)fluorescence
IL-2	interleukin 2
LDL	low density lipoprotein
LV-ASGPR	lentiviral vectors LV-Cao, LV-benhar and LV-benharP
LV-Cao/benhar/benharP	lentiviral vector with respective scFv against ASGPR
LV-huCD105	lentiviral vector with scFv against endoglin
LV-eGFP	lentiviral vector encoding for eGFP, VSV-G pseudotyped
MHC	major histocompatibility complex
MOPS	3-(N-morpholino)propanesulfonic acid
MSV	measles virus
MSV-HA	measles virus hemagglutinin
muASGPR	murine ASGPR
PAEs	primary arterial cells
PCR	polymerase chain reaction
PE	phycoerythrin
PVCs	primary venous cells
qPCR	quantitative PCR
rcf	rotational centrifugal force
rpm	rotations per minute
RRE	Rev responsible element
RT	room temperature
SFFV	spleen focus-forming virus
SLAM	signalling lymphocyte-activation molecule
TG	Tris-glycine
TGS	Tris-glycine-SDS buffer
UDP	Uridine diphosphate
VEGF	vascular endothelial growth factor
WB	Western blot

1. Introduction and Aims

1.1 The Liver

The liver plays an essential role to maintain the equilibrium of metabolic pathways. One of its main functions is the synthesis and storage of a wide variety of enzymes that are needed for conversion and degradation of substrates. For example, blood clotting factors II, VI, IX, X, and XI are synthesized in the liver, placing the organ on top of haemostasis regulation hierarchy together with the endothelium. Liver diseases may reduce levels of coagulation factors and bare the risk of massive haemorrhages. Careful treatment of patients with end-stage liver disease is therefore required. Other examples of molecules produced by the liver are hormonal and growth factors, and components of bile such as bile acids, cholesterol, phospholipids, and cholesterol. The liver is also the key regulator of glucose, glycogen, amino acid, lipid and phospholipid metabolism, emphasizing even more the metabolic key role of the liver (Ghany and Hoofnagle, 2008; Pratt and Kaplan, 2008).

In processes referred to as biotransformation, potentially hazardous metabolites and drugs are transformed by enzymes in phase I (70 % of substrates) and phase II reactions (30 %) (Yoshizato and Tateno, 2009). The chemical reactions render the metabolites more hydrophilic and available for renal elimination or bile acid excretion. Thus, patients with failing livers are threatened by the inability to dispose of waste products leading to liver coma and death. Pharmaceutical treatment of these patients is a difficult task for the responsible physician, as drug elimination is slowed down, endangering the patients' health even more.

In recent years, it has become clear that the liver has an important role in the modulation of the immune system. First of all, the liver produces most of the so called acute-phase proteins, a set of proteins that are secreted to the bloodstream upon inflammation. Amongst others is the protein alpha-1 antitrypsin (A1AT) that neutralizes proteinases from dead cells and C-reactive protein (CRP), which functions as an opsonizing agent (Fey et al., 1994; Parker and Picut, 2005). Secondly, the liver is intersected by a complex network of liver sinusoids, harbouring various cells of the immune system and sinusoidal endothelial cells, thereby building a link between the liver and the immune defence. Potentially hazardous materials such as cell debris, xenobiotics, antibody- and complement coated compounds are cleared by sinusoidal Kupffer cells while smaller amounts of various materials can be removed from the bloodstream by liver endothelial cell endocytosis (Parker and Picut, 2005). Both cell types also express members of the Fcγ receptor family, enabling them to ingest immunoglobulin complexes (Ravetch, 1994; Kuiper et al., 1994). Waste molecules resulting from general body turn-over and inflammatory

processes are often dispatched of by sinusoidal endothelial cells by the means of collagen, mannose and scavenger receptors (Lee et al., 2002; Smedsrød et al., 1986; Smedsrød et al., 1990). Special subsets of sinusoidal natural killer cells may trigger apoptosis in or actively attack circulating tumor cells (Vermijlen et al., 2002; Parker and Picut, 2012). The liver is involved in the regulation of immune responses by actively sequestering activated CD8+ T-cells in order to downgrade inflammatory response (Mehal et al., 1999; Kuniyashi et al., 2004). A review by Ian Crispe recapitulating antigen presenting cells (APCs) in the liver highlights the ability of sinusoidal endothelial cells to induce immune tolerance against presented antigens while subsets of dendritic cells can be either tolerogenic or activating APCs (Crispe, 2011).
The complexity of functions and its involvement in the basic metabolism of the body often pushes the liver to its limits, making the liver vulnerable to many diseases. Due to the wide variety of diseases that strike the liver, there is enormous potential in developing targeted therapies on the molecular level involving gene therapy. Recent findings have boosted research within the fields of targeted delivery of gene therapy vehicles to the liver, and further assessment using different techniques to efficiently target the liver is needed. This study investigates lentiviral vectors pseudotyped with measles virus hemagglutinin fused to single chain antibodies aiming towards specific transduction of hepatocytes.

1.2 The Endothelium

The endothelium is defined as the single cell layer of endothelial cells pointing towards the luminal side of the vessel, thereby physically separating the bloodstream from the rest of the vessel wall and the surrounding tissue. The endothelium acts as a barrier between the blood and the surrounding tissues and regulates the molecule exchange between the two body compartments. In addition, the endothelium is also involved in mediating immune responses. In normal tissue, endothelial cells are semi-permissive to circulating blood cells. In case of disease, however, certain mechanisms, e.g. the secretion of cytokines, are initiated to allow cell adhesion and translocation of circulating immune cells into the diseased tissue, processes that may – in a similar way – be partly activated when transplanted hepatocytes translocate through the liver sinusoidal endothelium to enter the parenchyma (Gauthier et al., 1996; Gupta et al., 1999). In a process referred to as rolling, endothelial cells of inflamed tissues are induced to express selectins that interact with receptors on circulating neutrophils and platelets in order to slow them down. Circulating cells are activated, adhere to the endothelial cell layer and finally migrate through the endothelial barrier to the point of inflammation. These processes are

mediated by multiple cytokines, integrins and molecules like intercellular adhesion molecule-1 (ICAM-1) amongst others (Cybulsky and Gimbrone, 1995).

One of the key functions of the endothelium is the regulation of the vascular tone and the flow resistance of the blood. The most crucial mediator of vascular tone is nitric oxide (NO), which is synthesized by endothelial nitric oxide synthase (eNOS) (Fleming and Busse, 1995). Triggered by the autonomous nerve system or by mechanical stress NO is released and activates guanylate cyclase, leading to an increase of cyclic 3', 5' guanosine monophosphate (cGMP) in smooth muscle cells and their subsequent relaxation. The constant release of basal levels of NO under physiological circumstances is the main factor keeping endothelial cells in a quiescent state (Arnold et al., 1977; Kharbanda and Deanfield, 2007).

Prostacyclin (PGI_2) and thromboxane A_2 (TXA_2) are products of endothelial cells that are also involved in smooth vessel relaxation as well as platelet aggregation. While PGI_2, which predominates under physiological circumstances, decreases the vascular tone and inhibits platelet aggregation, TXA_2 has opposite effects (Duffy et al., 1998). Further, anticoagulatory pathways of the endothelium are maintained by the expression of antithrombin and the ability of endothelial cell attached protein C to digest coagulation factors (Sagripanti and Carpi, 2000). In addition, there is some basal expression of plasminogen activator, rendering the endothelium in a fibrinolytic state in healthy conditions (Brown et al., 2000).

Endothelial cells are able to synthesize von Willebrand factor, fibronectin and thrombospondin if the endothelial cells become activated and the equilibrium is changed towards an inflammatory and pro-coagulatory state. Short periods of endothelium activation are not considered as a threat to health. Activation may actually be triggered by eNOS itself, as it is also capable of producing reactive oxygen species (ROS), which are needed to respond appropriately to limit inflammation or stress factors of various kinds (Deanfield et al., 2007). ROS result in alteration of effector proteins and activation of transcription factors, leading to the expression of cytokines, chemokines, and adhesion molecules (Hansson, 2005). However, the persistence of stress factors may lead to the lack of anti-oxidants (Deanfield et al., 2007; Li et al., 1995).

Atherosclerosis is the disease most commonly associated with endothelial cells and the key contributor to cardiovascular disease, the leading cause of death in the developed countries. It is initiated by the retention of low-density lipoprotein (LDL) into the artery wall (Skålén et al., 2002). Endothelial cells are activated by platelets and start producing adhesion molecules, subsequently sequestering immune cells like macrophages and $CD4^+$ T cells that secrete inflammatory cytokines like IFN-γ, TNFα and interleukin-1 (Cybulsky and Gimbrone, 1995;

Peiser et al., 2002; Janeway and Medzhitov, 2002). These induce systemic responses of the body, involving the production acute phase proteins in the liver in unstable angina or myocardial infarction (Liuzzo et al., 1994). Immune cells also produce proteolytic enzymes like metolloproteinases and cysteine proteases that may contribute to plaque rupture, the main cause of myocardial infarction and an important contributor to ischemic stroke (Jones et al., 2003; Liu et al., 2004).

As the endothelium is easily accessible through the bloodstream and involved in the development of atherosclerosis, the endothelium has been prioritized as a target for directed gene therapy. In this study, we investigate the possibilities of lentiviral vectors pseudotyped with measles virus hemagglutinin fused to antibodies specific for the endothelial marker Endoglin/CD105, aiming for a specific transduction of endothelial cells.

1.3 Gene therapy of the liver and the endothelium

1.3.1 Liver-directed gene therapy

The enzymatic processes taking place in the liver tissue are fragile and, therefore, predestined to give rise to numerous liver diseases. Some of the diseases are induced by external toxins such as alcohol, fungi, pharmaceuticals, or viral infections. Progression of the illness is determined by the initiation of medical treatment, discontinuation of toxin ingestion, environmental and individual characteristics. In end-stage liver disease, the only routine procedure today is to perform allogenic liver transplantation. In monogenetic liver disease, the development and propagation of the disease are caused by the failure of one single gene and its translational product. Diseases caused by a single defect can theoretically be overcome by replacing the defective gene by the means of gene and cell therapy, or a combination of both. In cell therapy, cells are transplanted to support or to replace malfunctioning endogenous cells in patients. Hepatocytes may be allogenic cells isolated from healthy individuals or autologous hepatocytes that have been treated with corrective gene therapy vectors *ex vivo* (Nguyen and Ferry, 2004, Nguyen et al., 2006). *Ex vivo* gene therapy in culture dishes delivers vectors to the hepatocytes and vectors can be washed away before re-transplantation, thus eliminating immune responses against the vector antigens (Nguyen and Ferry, 2004). Transgenes are expressed solely in transplanted hepatocytes without the need of hepatocyte-specific promoters.

Gene therapy trials in live humans have been almost exclusively performed on patients with haematological disorders, with promising results but with devastating side effects like the development of retroviral-induced leukaemia (Hacein-Bey-Abina et al., 2008). With regard to

the liver, *ex vivo* gene therapy clinical trials have been conducted. The group of Grossman et al. was able to transduce freshly isolated hepatocytes from patients suffering from familial hypercholesterolemia with murine lentiviral vectors carrying the information for expression of the low-density lipoprotein (LDL)-receptor to replace the defective endogenous receptors. Corrected cells were re-transplanted and translocated to the liver parenchyma via the portal vein. The experiments were partially successful as LDL plasma levels decreased by 6-20 %. Unfortunately, the magnitude of the decrease in serum LDL levels was insufficient to achieve a therapeutical benefit for patients and they were still at high risk of suffering atherosclerotic damage to their arteries (Grossman et al., 1994). Other studies using autologous fibroblasts or immortalised hepatocytes have shown to correct hyperbilirubinemia in Gunn Rat, but experiments have not been followed up because of the obvious tumorigenic risk (Seppen et al., 1997; Tada et al., 1998; Kawashita et al., 2008). In addition, preclinical studies using lentiviral vectors have already proven efficient in correcting the hyperbilirubinemia in Gunn Rat caused by the lack of bilirubin urine diphosphate (UDP)-glucosyltransferase (Nguyen et al., 2006). Also, handling of frozen hepatocytes and the process of transduction has been optimised, ensuring sufficient transduction efficiencies within a short time (Waern et al., 2012).

As an alternative to *in vitro* gene therapy, scientists have made huge efforts to develop tools that allow *in vivo* delivery of therapeutic genes. In this approach, therapy is directed towards endogenous cells that may not be able to repopulate. Mostly, gene therapy vectors are administered through intravenous injection, making the endothelium of large vessels an easily approachable target to gene therapy. However, if the vectors are thought to be delivered in specific organs and cell types, one has to consider modifications of the vector and the method of application to ensure targeted delivery. If approaching the liver in mice, injections are preferably given into the tail vein or intrasplenically, from which the vehicles circulate to the liver and penetrate the sinusoidal endothelium through 100 nm wide fenestrations to reach the hepatocytes. These fenestrations disappear in liver tumors, making the hepatic tumor tissue less accessible to such vectors (Nguyen and Ferry, 2004). Liver tumors can also be targeted by directing gene therapy vehicles to the site of angiogenesis, i.e. the endothelium of vessels feeding the tumor - this possibility has been addressed in this study using modified vector envelopes targeting the endothelium surface antigen human endoglin/CD105 (Anliker et al., 2010; Abel et al., 2013).

Several other methods to ensure efficient delivery of gene therapy vectors to the liver have been developed: In mice, gene therapy vehicles such as specially modified viral vectors or DNA-aggregates can be administered *in vivo* by tail vein injection in order to infect hepatocytes

(Herweijer and Wolff, 2003). Scientists have mostly focused on the development of viral delivery methods including adenoviral vectors, adenovirus-associated vectors, and lentiviral vectors. Adenoviral vectors are able to infect non-dividing hepatocytes, however, transduced hepatocytes tend to lose transgene expression over time due to the elimination by the immune system (Joos et al., 1998). It is today unknown whether large-scale production of less immunogenic adenoviral vectors can be conducted in a clinical setting (Ilan et al., 1997; Ehrhardt et al., 2003). Published results using adeno-associated vectors (AAV) as vehicles are promising and encouraging as they present a tool which allows production in large volumes and are in principle non-immunogenic as they lack common viral determinants. However, experiments have suggested that transgenes expressed by AAV may be presented by major histocompatibility complex (MHC) class I molecules, thereby inducing immune responses (Shakuran et al., 2001). There are also reports that $CD8^+$ T-lymphocytes may recognise AAV-capsids, resulting in cytotoxic responses against transduced hepatocytes (Mingozzi et al., 2007). Recently, liver-directed clinical trials inducing reconstitution of factor IX in haemophilia B patients are underway. Single dose intravenous infusion of serotype-8-pseudotyped AAV expressing codon optimised factor IX transgene resulted in AAV-mediated expression at 2-11% of normal factor IX levels in four out of six patients, while in two patients the injection intervals of recombinant factor IX could be extended (Nathwani et al., 2011). Investigators trying to develop gene therapy vehicles for the treatment of haemophilia A have been unsuccessful because of inefficient transgene expression and factor VIII immunity (High et al., 2014).

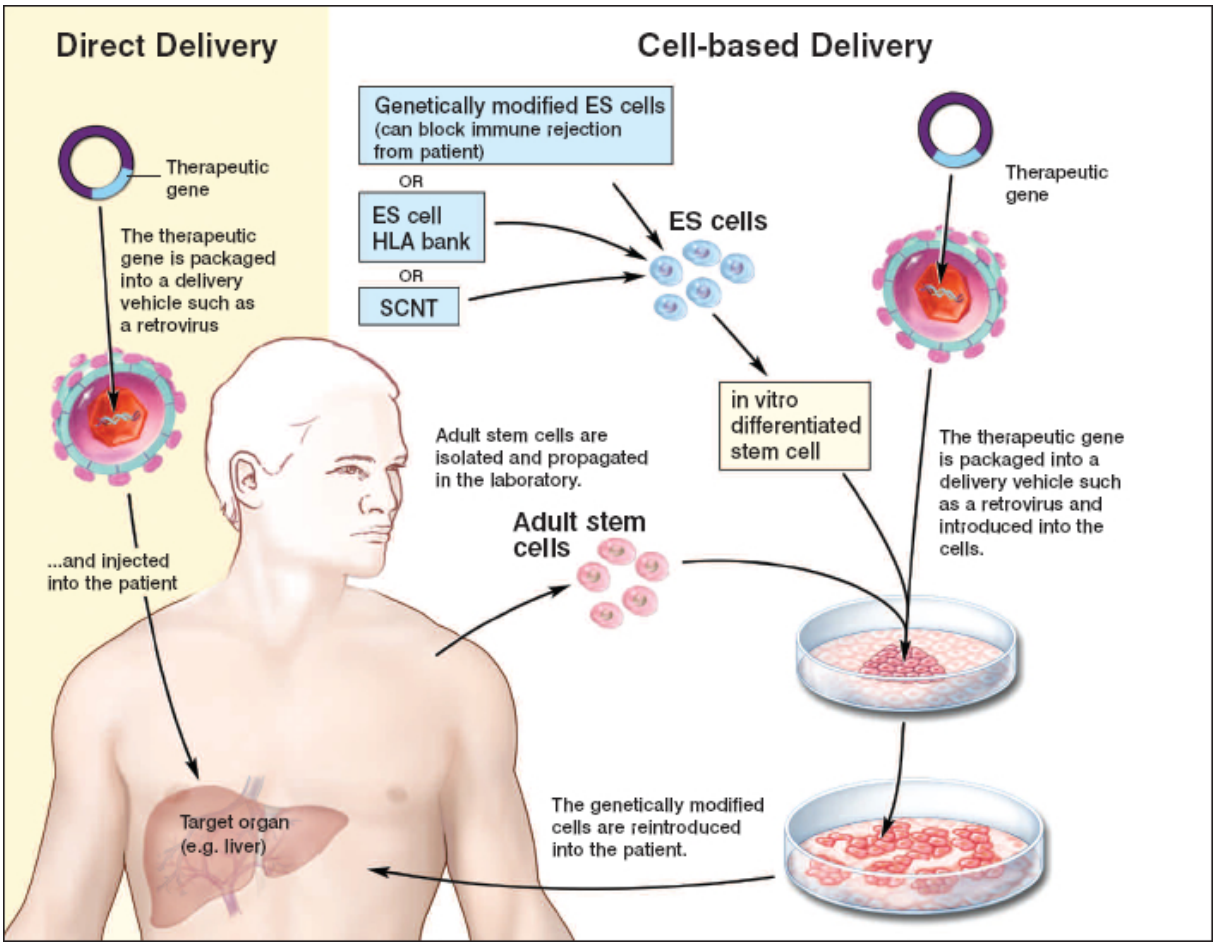

Figure 1: In vivo and ex vivo gene therapy. There are two possibilities to conduct gene therapy in humans. First, gene therapy vehicles such as lentiviral vectors can be directly injected into the human body. However, this method needs accurate targeting of the vector in order to prevent immune responses or other adverse effects of transgene expression in unwanted tissues (Folenzi et al., 2004). This can be achieved by restricting the viral particles to target cell types or by vector modification allowing transgene expression only in desired cells. The second option is the *ex vivo/in vitro* treatment of purified (autologous) cells with vectors, thereby resulting in transgene expression. The cells need to be transplanted into the patient after successful transduction. © 2006 Terese Winslow, http://stemcells.nih.gov

1.3.2 Gene therapy directed against the endothelium

Quite in contrast to the liver, the endothelium is more accessible by gene therapy vectors because of its direct contact with the bloodstream. In most cases, gene therapy vectors could easily be manoeuvred to the desired location by catheter guided systems used for angiographic interventions. The scope for gene therapeutical approaches of the endothelium is huge, reaching from treatment of blood coagulation disorders to the treatment of cardiovascular disease, the leading cause of death in Western civilisation.

Under certain conditions, diseased parts of large atherosclerotic vessels can be replaced by grafts. While hepatocytes can be accessed by *in vitro* and *in vivo* gene therapy and may functionally integrate into a structurally damaged liver parenchyma upon transplantation, undisrupted anatomy is conditional for the endothelium to fulfil its function flawlessly.

Most clinical trials using gene therapeutical approaches to correct vascular disease have concentrated on the effect on peripheral arterial occlusive disease (PAD). They collectively try to induce angiogenesis in the neighbourhood of occluded vessels by overexpression of angiogenetic factors like vascular endothelial growth factor (VEGF) and fibroblast growth factors (FGF). A review written by Sedighiani and Nikol in 2011 describes that expression of the transgenes has been accomplished by intramuscular or catheter mediated injection of plasmids or adenoviral vectors to the site of the occlusion, with varying results. One clinical phase III trial, however, did not present any benefit for patients with ischemic ulcers undergoing gene therapy treatment with NV1FGF (TAMARIS; Belch et al., 2011). Gene therapeutical approaches to challenging atherosclerosis involving delivery of anti-oxidant enzymes are underway but are struggling with problems regarding the choice of vector and the constitutive expression of the transgene (Van-Asche et al., 2011). Other possible targets of endothelium directed gene therapy include the endothelial cells located in the vessels of tumors that are dependent on angiogenesis to nourish tumorous tissues and tumor growth. Sinusoidal endothelial cells of the liver also represent an important target for endothelium-directed gene therapy. Endothelial cells in sinusoids and tumor vessels both express CD105/Endoglin, an endothelial cell surface marker, which can be targeted by lentiviral vectors with high affinity for $CD105^{+}$ (Anliker et al., 2010; Abel et al., 2013).

1.4 The lentiviral vector system

1.4.1 Design of the lentiviral vector system

In the search for an ideal viral vector system scientists have evaluated many possibilities. However, scientists have learned from drawbacks and disappointments that this task is not an easy one. With lentiviral vectors, concerns have been raised mainly with regards to the risk of insertional mutagenesis inducing leukaemia (Hacein-Bey-Abina et al., 2003; Hacein-Bey-Abina et al., 2008). There have even been cases were injected adenoviral vectors have caused death in a patient treated for ornithine transcarbamylase deficiency due to systemic inflammatory response (Raper et al., 2003). Despite the deadly events of some gene therapy trials, scientists have undertaken enormous efforts to develop new or improved methods of viral delivery of genes into target cells. Today, human immune deficiency virus (HIV)-derived, vastly modified lentiviral vectors are amongst the most promising tools in viral gene therapy. Several changes to the endogenous molecular machinery of HIV have been made to ensure

production of lentiviral vectors that are non-replicating, non-infectious but stably expressing the genes of interest (Sakuma et al., 2012).

The native HIV genome expresses three structural proteins (Gag, Pol and Env). While the *gag* gene harbours information for various structural core proteins, *pol* is responsible for the expression of proteins that mediate reverse transcription and insertion of the viral genome into the host cell. In comparison to Rev, Tat and Nef, the two proteins Gag and Pol are expressed with some delay from the same mRNA transcript by ribosomal frameshift and mediated by altered splicing mechanisms (fig 2B): Before expression of the structural proteins, fully spliced viral proteins Rev, Tat and Nef are translocated to the cytoplasm. Rev is relocated to the nucleus where it in a defined sequence of events initiates alternate splicing events that result in the expression of accessory proteins Vpr, Vfu, Vpu and structural proteins Gag, Pol, and Env (fig 2C).

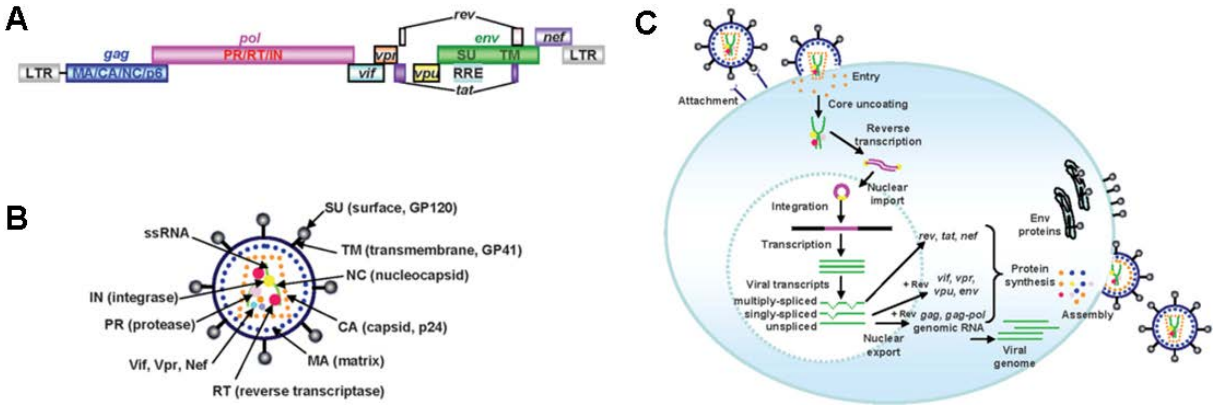

Figure 2: Lentivirus assembly and life cycle. A The lentiviral genome consists of nine genes. Three structural genes named Gag, Pol and Env, two regulatory genes names *tat* and *rev*, and four accessory genes *vif*, *vpu*, *vpr*, and *nef*. **B** While the structure genes encode for proteins that are needed for the assembly of the structural components and integration of the viral genome into the host genome, regulatory genes are necessary to mediate effective expression of the viral genes. Accessory genes are needed to let the virus that has infected a host cell evade from host immune mechanisms and to boost virus replication. **C** The encapsulated viral genome is released into the host cytoplasm through membrane fusion. Viral RNA is reverse transcribed and imported to the nucleus, where it integrates into the genome of the host cell. Upon integration, viral DNA is initially transcribed into multiply-spliced mRNA. Initial transcription of Tat, Rev, and Nef in the inserted viral genome is initiated by enhancer/promoter activity of the U3 region within the 5' long terminal repeat (LTR). The U5 region of the 3' LTR holds the polyadenylation signal. Therefore, both the U3 region of the 5'LTR and the U5 region of the 3' LTR are not transcribed into the mRNA of the provirus. Completion of the LTR occurs by reverse transcriptase before integration into the genome of the next target cell by copying the U3 region of the 3'LTR, thereby transferring the promoter activity to the integrating virus. Rev is imported back into the nucleus, where it binds to the rev responsible element (RRE) of singly-spliced and unspliced viral mRNA, facilitating to nuclear export. Expression of residual genes of the viral genome is initiated in the cytosol. Whole length viral genome and structural proteins give rise to a new generation of viral particles, which are released through the membrane to enter the bloodstream. In lentiviral vectors, only *gag*, *pol* and *rev* remain from the original viral genome. (Sakuma et al., 2012; © 2012 Biochemical Society)

In a series of modifications, the HIV genome has been genetically modified in order to ensure high biosafety standard and high expression at the same time. Nowadays, third generation

lentiviral vectors are used in laboratories to transduce target cells. In this approach, to minimize the possibility of generating replication-competent virions by recombination, the genes involved in the viral production are expressed separately from four different plasmids. Furthermore, all accessory proteins have been deleted. In the physiological HIV life cycle the accessory genes are needed to propagate efficiently *in vivo* as they can neutralize host antiviral factors (Vif, Vpu) or degrade cellular components like the MHC molecules needed for antigen presentation (Nef) (Harris et al., 2003; Neil et al., 2003; Collins et al., 1998).

The *env* gene has generally been replaced to the vesicular stomatitis virus glycoprotein (VSV-G), which is favoured as a glycoprotein because of its broad tropism (Burns et al., 1993). Although VSV-G increases the stability of the lentiviral vectors, it has been reported to have a cytotoxic effect. Furthermore, its capability to transduce most human cells is regarded as risky as many unspecific cells may be infected with VSV-G pseudotyped lentiviral vectors (Sakuma et al., 2012).

The viral genome is flanked by long terminal repeats (LTRs), which hold a U3, R and U5 region. Initial transcription of Tat, Rev, and Nef in the inserted viral genome is initiated by enhancer/promoter activity of the U3 region within the 5' LTR. In a series of events, the U3 region of 3'LTR is copied to the 5'LTR by reverse transcriptase to ensure continued transcriptional activity of newly integrated viral replicons (Sakuma et al, 2012). Today, scientists use lentiviral plasmids where the U3 region of the 3'LTR is deleted in order to prevent duplication of the U3 region when lentiviral vectors are produced. This eliminates the basal enhancer/promoter activity in lentiviral vectors.

Lentiviral vectors which are characterised by the elimination of the autonomous transcriptional activity are called self-inactivating (SIN) vectors. In these vectors, the expression of the transgene is driven by a strong mammalian promoter, e.g. cytomegalovirus-(CMV) or spleen focus-forming virus (SFFV)-promoters. Since the transactivation molecule Tat is not present in the third generation packaging, a promoter independent of Tat/TAR is used in the vector. Other modifications of this generation of lentiviral vectors include the central polypurine tract (cPPT), which improves mostly the reverse transcription of the vector. In addition, the woodchuck hepatitis virus post-transcriptional element (WPRE) has been added in front of the 3'LTR to enhance mRNA stability (Zuffrey et al., 1999). Other modifications to improve lentiviral vectors biosafety are under investigation. However, it will take some time until these modifications are implemented into standard lentiviral vectors production.

1.4.2 Measles virus pseudotyped lentiviral vector system

The tropism of lentiviral vectors is determined by its envelope glycoprotein. It can be expanded or limited to specific cells by exchanging the envelope glycoproteins with glycoproteins from other enveloped viruses, a process referred to as pseudotyping (Cronin et al., 2005). Normally, lentiviral vectors are pseudotyped with VSV-G. This has the benefit that all human cells and numerous animal cells can be transduced *in vitro* without difficulty. Higher transduction rates at one specific body part can be maintained by injecting the lentiviral gene vehicle in the bloodstream nearby leading to the desired tissue. However, unspecific spreading and transduction of unwanted tissues will be abundant. In *ex vivo* experiments, isolated cells are properly washed to remove lentiviral vectors before re-transplantation to prevent systemic infection (Waern et al., 2012; Nguyen et al., 2006). However, scientists now try to modify the vector envelope to target vectors to specific cell-types expressing characteristic receptors by the means of envelope-receptor interaction. This can be done by e.g. exchanging the pseudotyping glycoproteins to novel ones that are more specific to target cells (Cronin et al., 2005).

In more sophisticated approaches, glycoproteins are genetically modified in order to limit and specify the lentivirus tropism. Some glycoproteins, e.g. from filoviruses and measles virus, need truncation of its wild-type cytoplasmic tails in order to achieve effective transduction if used for pseudotyping of HIV-based vectors (Cronin et al., 2005; Funke et al., 2008). In the case of measles virus, hemagglutinin and fusion protein cytoplasmic tails are truncated with 18 and 30 amino acids, respectively, to achieve high titer vector production. The hemagglutinin from the NSe variant of the measles virus vaccine strain Edmonston B binds to CD46, a complement regulatory protein, which is expressed on all types of cells except erythrocytes. NSe-derived hemagglutinin also binds to signalling lymphocyte-activation molecule (SLAM/CDw150), a receptor that can be found on the surface of activated T and B lymphocytes. The measles virus wild-type strain uses CD150 instead of CD46 for cell entry. Hemagglutinin functions as a complex with fusion protein to facilitate cell entry (Yanagi et al., 2006; Funke et al., 2008). However, proper functioning of hemagglutinin can be disturbed by the introduction of four amino acid changes which are vital for receptor binding (Vongpunsawad et al., 2004). Binding may be mediated by fusion of the mutated hemagglutinin to the variable fragment of a single chain (scFv) antibody instead, thereby directing binding of the hemagglutinin pseudotyped vector to cells specifically expressing the surface antigen targeted by the scFv antibody (Nakamura, et al., 2005; Yang et al., 2006). Suicide gene transfer to CD20 positive cells using lentiviral vectors pseudotyped with measles virus hemagglutinin fused to a scFv-CD20 antibody resulted in the elimination of these cells, indicating the potential specificity of this

method (Funke et al., 2008). The specificity of scFv bearing hemagglutinin glycoproteins was confirmed in experiments using scFv against CD133, a haematopoietic marker, and CD105, an endothelial cell marker (Anliker et al., 2010). Experimental modifications in comparison to VSV-G pseudotyped lentiviral production are minor, involving the exchange of the VSV-G plasmid to one fusion protein (F) plasmid and one hemagglutinin-scFv (H*mut*-scFv) plasmid (figure 3).

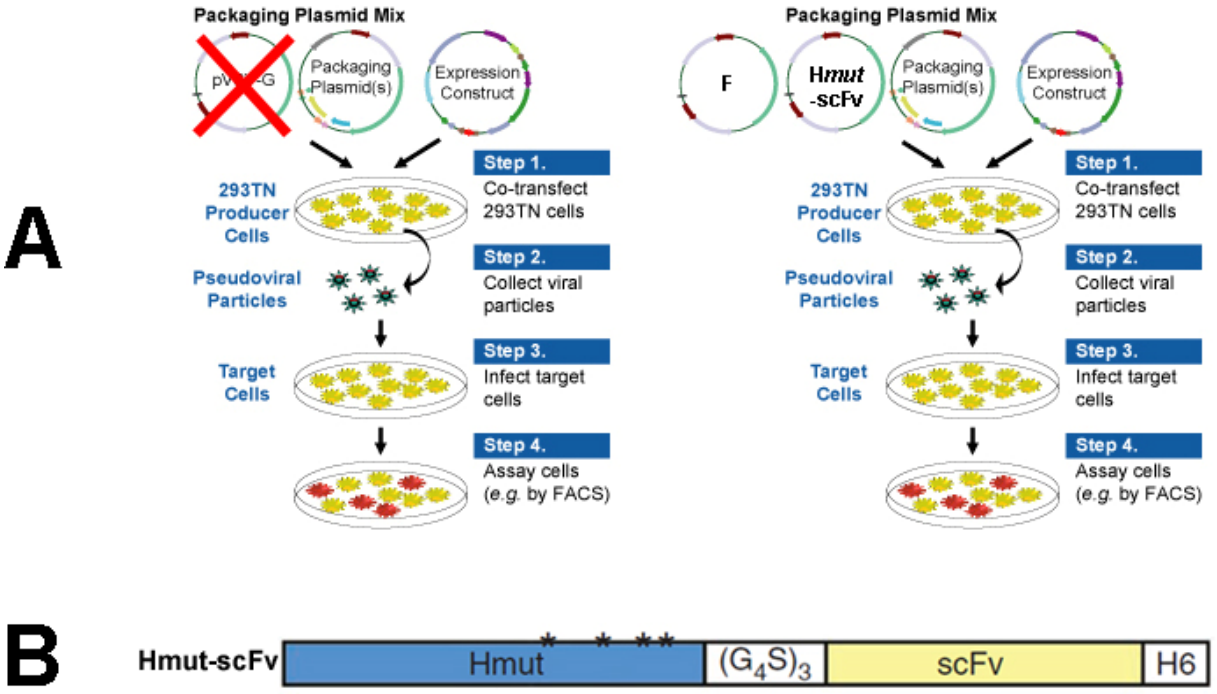

Figure 3. Measles virus pseudotyped HIV-based vectors. pVSV-G encoding for the VSV-G envelope is normally used in the production of lentiviral vectors. In measles virus (MSV) pseudotyped vectors, VSV-G is exchanged with pCG-Fnse-d30 (F, fusion protein) and pHL3-scFv-CD105. The modified hemagglutinin carries four point mutations (Y481A, R533A, S548L, F549S) making it impossible to properly interact with the receptor CD46 and SLAM that are natural receptors for the NSE measles virus vaccine strain Edmonston B. Instead, binding to target cells is now mediated by the single chain variable fragment (scFv), that is fused to the defective hemagglutinin by a glycine-serine polylinker ($(G_4S)_3$). In this study, the author uses scFv-antibodies against human asialoglycoprotein receptor (ASGPR) and human CD105. (Anliker et al., 2010 © Nature Methods)

Further development of the scFv-hemagglutinin measles virus HIV-based system and discoveries of suitable scFv-antibodies will allow more specific and efficient targeting *in vivo* of cells with time. The aim is to achieve *in vivo* targeting of specific cell types without the fear of contamination of unwanted organs or reactions of the immune system against the gene vehicle. Some organs will be easier to target than others because of their anatomical suitability, e.g. muscles and skin. However, also hepatic microvessels and liver are readily accessible by portal vein delivery and define promising targets for direct *in vivo* therapy using cell type-specific gene therapy vectors. In this study, the author is trying to direct lentiviral vectors to the

liver and the hepatic microvessels, two structures that are closely related to each other because of their anatomical proximity. For this purpose, the author used scFv against human asialoglycoprotein receptor (ASGPR) and human endoglin to target human hepatocytes and human endothelial cells.

1.5 Possible targets

1.5.1 Asialoglycoprotein receptor

Asialoglycoprotein receptor is a C-type lectin primarily expressed on hepatocytes. Its main function is to clear desialylated, galactose-terminal glycoproteins from the bloodstream by an endocytotic pathway (Morell et al., 1968 and 1971; Spiess, 1990). Endocytosis of proteins is Ca^{2+}-dependent and conducted by the clathrin-coated pathway, and ASGPR is recycled and transported back to the membrane surface to complete the degradation cycle, a process called receptor-mediated endocytosis (Bareford and Swan, 2007; Schwartz et al., 1984). Asialoglycoproteins are retained in lysosomes and degraded into its subunits for recycling in other metabolic pathways (figure 4) (Strous et al., 1985). Mouse deficient of ASGPR, however, demonstrate no impaired phenotype and proteins are cleared by an unknown alternate pathway, indicating that ASGPR-mediated clearance may not be vital (Rigopoulou et al., 2012).

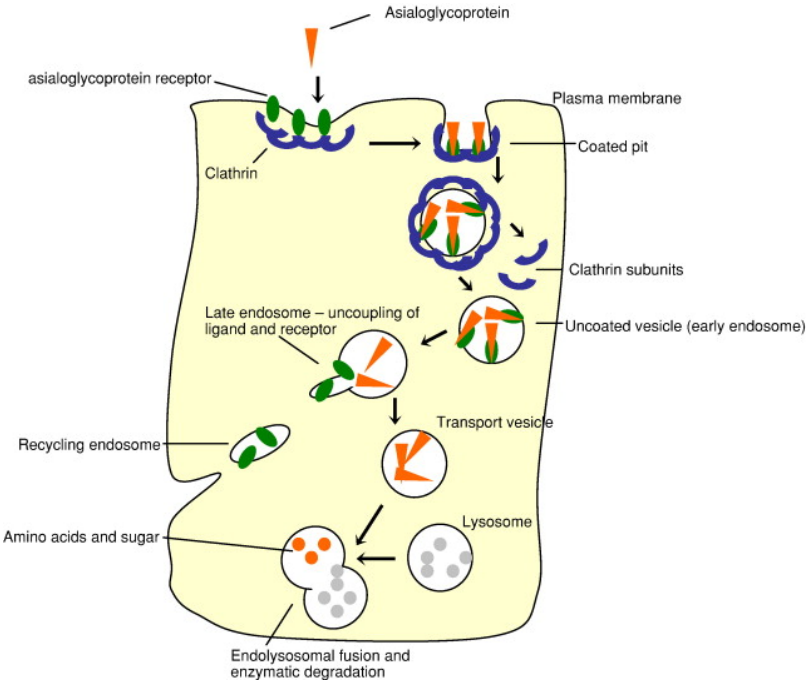

Figure 4: Receptor-mediated endocytosis. ASGPR binds proteins that have N-acetylgalactosamine residues. Clathrin subunits help the plasma membrane containing the receptor and its ligand to invaginate, forming a clathrin-coated pit. The clathrin subunits deattach and ligands are released from the endosomes. While ASPGR is returned to the plasma membrane, ligands are transported to lysosomes and degraded to smaller molecules. (© 2007, Bareford & Swaan)

ASGPR is a heterodimeric protein consisting of a major 48 kDa subunit ASGPR 1 (H1 encoded by the *ASGR1* gene) and a minor 40 kDa subunit ASGPR 2 (H2, encoded by *ASGR2* gene) (Stockert et al., 1995). Apparently, the two genes are expressed in a 3:1 ratio, making the H1

subunit the more abundant one (Spiess et al., 1990). Recently, some sub-isoforms have been identified without identifying their exact function (Liu et al., 2010). The ASGPR 1 major subunit consists of a 40 amino acid cytoplasmic domain followed by an approximately 20 amino acid single transmembrane domain. The extracytoplasmic part consists of 230 amino acid, and its 150 C-terminal amino acids form the carbohydrate recognition domain (CRDH1) (Meier et al., 2000). The carbohydrate recognition domain (CRD) is responsible for the interaction with specific protein-bound carbohydrates (Bider et al., 1996). The remaining 80 amino acids of the extracellular part mediate the oligomerisation between ASGPR 1 and ASGPR 2.

In addition, ASGPR has also been suggested to clear IgA from the bloodstream, however, experiments are contradictory. In this context, some scientists have proposed a mechanism where IgA are bound to known hepatotropic viruses and then are ingested by ASGPR into hepatocytes, thereby infecting the liver (Inamoto and Brown, 1991). This has specifically been shown for the hepatitis A virus (Dotzauer et al., 2000). Other hepatotropic viruses like Marburg virus and Hepatitis B virus are proposed to infect hepatocytes in an ASGPR-dependent manner (Treichel et al., 1994; Treichel et al., 1997). Recent investigations have also supported the idea that ASGPR may be involved in trapping of T cells and triggering their apoptosis within the liver, but convincing data is still missing (Guy et al., 2011).

Another function has been described quite recently, placing ASGPR as a main contributor in the regulation of lethal thrombocytopenia seen in infections with *Streptococcus pneumonia*. Lethal thrombocytopenia is caused by disseminated intravascular coagulation (DIC), in which platelets are consumed and fibrin is deposited systematically in organs, leading to multi-organ failure. *Streptococcus pneumonia* expresses sialidase, which removes the sialic acid-residues from the surface of platelets, which are in turn removed after recognition by ASGPR. In parallel, similar surface alterations on host cells are seen in advance of DIC, and may be regarded as triggering factors for hemostasis deregulation and initiation of DIC. Interestingly, in ASGPR$^{-/-}$-mice platelets cannot be removed by ASGPR, thereby contributing to an eventual DIC, underlining the possible importance of ASGPR in regulating the upcoming of DIC. In addition, ASGPR also sequesters von Willebrand factor in hepatocytes, making ASGPR an important regulator of coagulation factor levels in the bloodstream (Grewal et al., 2008).

Under physiological circumstances, ASGPR is expressed on the sinusoidal and the basolateral membrane of the hepatocytes. ASGPR distribution changes to the canalicular membrane during liver inflammation (Burgess, 1992). Also, in liver cirrhosis patients, ASGPR is overexpressed. Nevertheless, during increased impact by cytokines on hepatocytes, ASGPR functionality is

decreased, and serum levels of asialoglycoproteins are increased in cirrhotic patients (Nakaya et al., 1994).

In the pathophysiological mechanisms involved in autoimmune hepatitis (AIH), auto-antibodies against auto-antigens play an important role. The most important representative among liver specific proteins (LSP) is ASGPR, which can give rise to auto-antibodies by molecular mimicry (Oliviera et al., 2011; MacFarlane et al., 1984). T-cells from diseased AIH patients were able to induce autologous B cells to produce ASGPR auto-antibodies, indicating that ASGPR-induced AIH may be the results of dysregulation of the cellular immunity (MacFarlane et al., 1990; Lohr et al., 1990). Autoantibodies against ASGPR have also been described in some cases with hepatitis B and C infections, although auto-antibodies are far less redundant than in AIH.

1.5.2 Endoglin/CD105

Endoglin was initially discovered as an antigen of an antibody directed against pre-B leukemic cell lines and is expressed as a cell membrane 180 kD homodimer linked with a disulphide bond (Gougous et Letarte, 1988). It is divided into a large extracellular domain with a zona pellucida (ZP) and arginine-glycine-aspartic acid (RGD) integrin binding motif, a short transmembrane domain, and a cytoplasmic domain (Gougos et al., 1990). Two isoforms of endoglin are known, a longer L-endoglin form and a shorter variant, S-endoglin, which lacks a significant part of the cytoplasmic tail (Cheifetz et al., 1992). The cytoplasmic tail consists of several serine and tyrosine residues that are believed to be targets of intensive phosphorylation (Koleva et al., 2006). Mutations of endoglin have been associated with hereditary haemorrhagic telangiectasia type I (HHT1), also known as the Osler-Rendu-Weber syndrome arteriovenous malformations and bleeding disorders (McAllister et al., 1994). CD105 is mainly expressed on vascular endothelial cells, but expression is also found on hematopoietic progenitor cells, fibroblasts, stromal and vascular smooth muscle cells, macrophages and mesangial cells (Cho et al., 2001; Pierelli et al., 2001; Robledo et al., 1996; Rohklin, 1995; Lastres et al., 1992; Diez-Marques et al., 2002; Gougos and Letarte, 1988; Fonsatti et al., 2003). High levels of CD105 are found on the endothelial cells of human microvasculature and in tumor vasculature of solid malignancies, making CD105 an ideal target for tumor-directed therapeutics (Wang et al., 1993; Burrows et al., 1995; Fonsatti et al., 2001). Expression and promoter activity of CD105 can be upregulated by hypoxia inducible factor (HIF)-1 complex in the case of lack of oxygen, driving vessel formation in nutrient-deficient tumors (Sanchez-Elsner et al. 2002). In this context, CD105 has been found to be inversely correlated with apoptotic markers, suggesting an anti-apoptotic role

of CD105 (Tanaka et al., 2003). Interestingly, CD105 has been suggested to regulate the vascular tone by involvement in the eNOS pathway, which has been seen to be impaired in the HHT1 $CD105^{+/-}$ mouse model (Jerkic et al., 2006). Besides tumor angiogenesis, endoglin is also involved in regular vessel formation e.g. in embryogenesis. Mice with no endoglin expression die at gestational day 10.5 as they fail to form functional blood vessels in the yolk sack. In addition, CD105-deficient mouse embryos have morphogenic defects to the heart, highlighting the organogenesis function of CD105 (Arthur et al. 2000).

Endoglin mainly functions as a TGF-β type III auxiliary receptor (TβRIII) that modulates signalling by members of the TGF-β family like activin-A and bone morphogenic protein (BMP)-2 and -7 (Cheifetz et al., 1992, Lastres et al., 1996). Association of endoglin with TGF-β type II receptor (TβRII) is conditional for binding ligands like TGF-β1 or TGF–β3. Upon binding, the phosphorylation state of TβRII is changed, which in turn either triggers activation of the TβRI (ALK-5) pathway or the ALK-1 pathway. The two pathways trigger Smad 2/3 phosphorylation or Smad 1/5 phosphorylation, respectively. The latter one is extensively investigated and is known to trigger endothelial cell activation, adhesion, migration and expression of matrix components. Angiogenesis may be driven by the synthesis of vascular endothelial growth factor (VEGF), a pro-angiogenic factor that is often upregulated in tumor formation (Lastres et al., 1996; Ma et al., 2000; Guerrero-Esteo et al., 1999; Diez-Marques, 2002; Botella et al., 2004). It seems that activation of either pathway depends on the CD105 isoform expressed in one specific location. L-endoglin, which is preferably expressed in endothelial cells, rather activates the ALK-1 pathway, thereby activating endothelial cells. The truncated S-endoglin prefers activation of the inhibiting ALK5 pathway and results in plaque stabilisation by downregulation of inflammatory cytokines and upregulation of protective factors (fig. 5). The data collected from several experiments suggest that the two pathways control each other in order to keep a well-regulated equilibrium (ten Dijke et al., 2008).

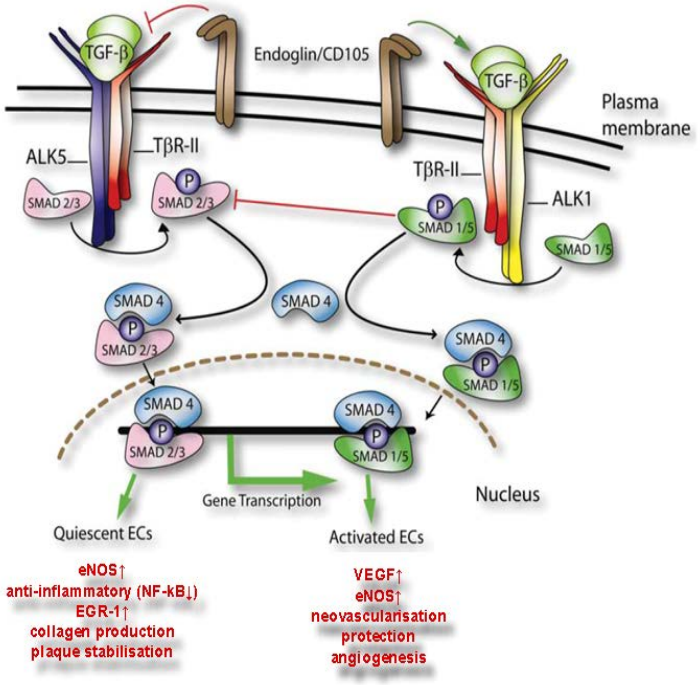

Figure. 5: Endoglin regulation. CD105 activation of TβR-II leads to activation of two alternate pathways: The TβR-II/ALK1 triggers cytosolic phosphorylation of Smad1/5 and leads to activation of endothelial cells by upregulation of VEGF and eNOS, which have angiogenic and protective qualities, respectively. In contrast, downstream activation of Smad2/3 leads to quiescence of endothelial cells, most apparently by downregulation of inflammatory cytokines and upregulation of protective factors, leading to plaque stabilisation. (adapted from Nachtigal et al., 2012, and Fonsatti et al., 2010)

High levels of a truncated, soluble form of endoglin (sCD105) is found in patients with solid and haematological neoplasms as a sign of ongoing angiogenesis (Takahashi et al., 2001a+2001b). Truncation is thought to be achieved by matrix metalloproteinase 14 (MMP-14) (Hawinkels et al., 2010). Rising levels of sCD105 have also been reported 2-3 months before the onset of severe pre-eclampsia and in patients with hypertension and hypercholesterinaemia. Though, the use of sCD105 as a prognostic marker for the disease is highly speculative at this time (Levine et al., 2006: Nachtigal et al., 2012).

The involvement of endoglin in atherosclerotic plaque formation has been described by several studies. While human aortic endothelium does not express endoglin under healthy circumstances, endothelial cells of microvessels do. In contrast, smooth muscle cells, endothelial cells and also macrophages strongly produce endoglin when located nearby atherosclerotic plaques (Conley et al., 2000; Piao and Tokunaga, 2006). Experiments in mice suggest that endoglin may only be expressed in endothelial cells and is engaged in repair of the endothelium, especially in mice receiving in apoE/LDLr-deficient mice with increased cholesterol levels (Nachtigal et al., 2009; Strasky et al., 2011).

1.6 Purposes and Aims

1.6.1 Purpose

Lentiviral gene therapy allows the expression of target genes and interfering micro-RNA molecules in transduced cells *in vitro* and *in vivo*. Cells from specific organs can be freshly isolated, transduced with lentiviral vectors and retransferred into animals or humans to correct specific metabolic errors. In some cases, however, cells are not easily isolated and re-integration into the target organ can be inefficient. Therefore, scientists now try to modify the envelope of lentiviral vectors to achieve cell type specificity when injected systematically into the bloodstream.

In this study, the lentiviral vectors' envelopes have been modified with the measles virus hemagglutinin that has been fused to single chain variable fragment antibodies. The scFv-antibodies are specific for human ASGPR and human CD105/Endoglin, which are considered to be characteristic antigens on hepatocytes and endothelial cells, respectively. Lentiviral vectors coated with hemagglutinin fused to single chain antibodies against human CD105/Endoglin have already successfully been tested *in vitro* on endothelial and fibroblastic cells lines.

This thesis focuses on testing the *in vitro* feasibility and specificity of the newly developed lentiviral vectors that are coated with measles virus hemagglutinin fused to scFv-antibodies specific against human ASGPR. Furthermore, the author sets out to conduct *in vitro* and *ex vivo* transduction experiments on endothelial cells and tissues using the previously developed lentiviral vectors pseudotyped with the measles virus hemagglutinin fused to scFv-antibodies against CD105/Endoglin. Finally, *in vivo* injections of the endoglin-specific lentiviral vectors are administered in mice that have been transplanted with a human artery to test whether the lentiviral vectors are capable of specifically targeting human endothelial cell layers.

1.6.2 Aims

Aim I: To transduce human hepatic cell lines with lentiviral vectors pseudotyped with measles virus hemagglutinin fused to single chain antibodies specific for asialoglycoprotein receptor *in vitro.*

Aim II: To transduce endothelial cell lines with lentiviral vectors pseudotyped with measles virus hemagglutinin fused to single chain antibodies specific for endoglin/CD105 *in vitro.*

Aim III: To test the susceptibility of human arteries and veins to lentiviral vectors pseudotyped with measles virus hemagglutinin fused to single chain antibodies specific for endoglin/CD105 *ex vivo*.

Aim IV: To transduce xenograft human arteries *in vivo* in immunodeficient mice with lentiviral vectors pseudotyped with measles virus hemagglutinin fused to single chain antibodies specific for endoglin/CD105.

2. Material and Methods

2.1 Material

2.1.1 Equipment

2-chamber slides	LabTek
+ 4°C freezer	Liebherr
- 20°C freezer	Liebherr
- 80°C freezer	Liebherr
Bacterial plate incubator	Thermo Corporation
Beaker	Duran
Biophotometer	Eppendorf
Cell counting chamber, improved (Neubauer)	Roth
Cell spreader	Sarstedt
Centricon Plus-70 Filter Centrifugation Device	Millipore
Centrifugation conical tube (15 ml, 50 ml)	Sarstedt
Centrifuge 5415D	Eppendorf
Cryotubes	Sarstedt
Cuvettes	Eppendord
Cuvettes (UV)	BioRad
Desktop PC	CS PC
Erlenmeyer flask	Duran
FACSCalibur	BDBiosciences
Filter paper	Whatman
Fluorescence microscope Olympus IX81 (IF-staining)	Olympus
Fluorescence microscope Olympus FV1000	Olympus
ICycler (PCR)	Biorad
Gel doc 2000 (DNA)	Biorad
Gloves, latex	Hartmann
Gloves, nitril	Kimberley-Clark
Heat incubator microcentrifuge tubes	Thermo Corporation
Heraeus Pico 17 Centrifuge	Thermo Corporation
Herasafe S1/S2 hood	Thermo Corporation
Incubator Heracell 240	Thermo Corporation
Inoculation loops	Sarstedt
Lightcycler 480	Roche
Measuring cylinder	Duran
Microcentrifuge tube centrifuge	Eppendorf
Microwave	Siemens
Needles	BD
Pipettes	Eppendorf
Parafilm	Roth
Petri dishes	Sarstedt
Pipette tips	Sarstedt
Pipette tips (cell saver)	Biozym
Pipette tips (for PCR)	Biozym
PCR 96-well plates	Sarstedt
PCR reaction tubes	Biozym
Polyvinylidendifluoride membrane	Millipore
Printer	HP/Canon

Rack	Roth
Reaction tubes (0.5, 1.5, 2 ml)	Biozym
SDS-Page tank	Biorad
Serological pipettes	Sarstedt
Sorvall Centrifuge	Sorvall
Sterile filters (0,45 µm, 0.22 µm)	Sarstedt
Syringe	Braun
Task wipes	Kimtech
Tissue culture dishes (10 cm, 6 cm, 6-, 12-, 24-, 96-well)	Sarstedt
Tissue culture flasks (25, 75, 175 cm^2)	Sarstedt
Thermomixer comfort/compact	Eppendorf
Water bath, small	GFL
Water incubator	Huber
Wet blotting system	Biorad
Western blot detection (digital)	INTAS
X-Ray film	GE Healthcare

2.1.2 Chemicals

Chemical	**Provider**
Acetone	Applichem
Acetic acid (96%)	Merck
Acrylamide solution 30 %	Roth
Agar	Fluka
Agarose, universal Gold	Peqlab
Agarose (low melting 100-1000 bp)	Biozym
Agarose (low melting > 1 kb)	Biozym
7-Amino-actinomycin D	Sigma-Aldrich
Ammonium acetate	Sigma-Aldrich
Ammonium persulfate	Roth
Ampicillin	Ratiopharm
Ampicillin	Sigma-Aldrich
Bacto yeast extract	BD Bioscience
Bacto tryptone	BD Bioscience
Bovine serum albumin (BSA)	Sigma-Aldrich
Bradford 1x Quickstart	BioRad
Bromophenol blue	Merck
Calcium chloride	Merck
5-Carboxytetramethylrhodamine (5-TAMRA)	Invitrogen
Chloroquine	Sigma-Aldrich
Coomassie Brilliant Blue R 250	Serva
Disodium hydrogen phosphate	Roth
Dimethylsulfoxide	Roth
dNTPs	Abgene
Dithiothreitol	Roth
DMEM, Gibco® 41966-029	Invitrogen
Donkey serum	Abcam

Ethanol, spoilt (96 %)	Th. Geyer
Ethanol, 100 %, molecular biology grade	Appli Chem
Ethidium bromide	Sigma-Aldrich
Ethylenediaminetetraacetic acid	AppliChem
Formaldehyde LSG 37 %	AppliChem
Fetal calf serum (FCS) Standard	PAA
Gelstar nucleic acid gel stain	Cambrex
L-Glutamine	PAA
Generuler 1 kb #SM1333	Fermentas
Generuler 100 bp #SM0243	Fermentas
Glycerine	Roth
Glycine	Roth
ß-Glycerolphosphat disodium salt pentahydrate	Fluka
Hepatocyte culture medium (HCM)	Lonza
HEPES pH 7.5	PAA
Hoechst 33342	Invitrogen
Hydrochloride acid	T.J. Baker
Isopropanol	Sigma-Aldrich
Loading dye (6x)	Fermentas
Magnesium chloride hexahydrate	Appli Chem
Magnesium hydroxide	Merck
β-Mercaptoethanol	Sigma-Aldrich
Methanol	T.J. Baker
3-(N-morpholino)propane sulfonic acid	Sigma-Aldrich
Oligonucleotides	MWG Operon
Ortho-phosphoric acid	Roth
Paraformaldehyde	Fluka
Penicillin/Streptomycin	PAA
pH-Meter calibration stock solutions (pH 4, pH 7, pH 10)	Roth
Ponceau S	Serva
Potassium chloride	Roth
Potassium dihydrogen phosphate	Roth
Prestained Protein Marker #P7708	NEB
Prestained Protein Marker #SM0671	Fermentas
Protein A	Sigma-Aldrich
RNAse ZAP	Sigma-Aldrich
RPMI1640, Gibco® 12633-012	Invitrogen
Rubidium chloride	Roth
Sodium dodecyl sulfate 10%	Gibco
Sodium acetate	Sigma-Aldrich
Sodium azide	Merck
Sodium carbonate	Appli Chem
Sodium chloride	Roth
Sodium citrate	Sigma-Aldrich
Sodium formamide	Sigma-Aldrich

Sodium hydroxide	Flucka
Sodium orthovanadate	AppiChem
Sodium phosphate	Sigma-Aldrich
Sodium sulfate	Merck
Skim milk powder	Appli Chem
TEMED	Roth
Tris	Roth
Tris base, acetic acid and EDTA buffer (TAE) (10x)	Roth
Triton X-100	Appli Chem
Trypan blue	Fluka
Trypsin-EDTA	PromoCell
Tryptone-peptone	Roth
Tween® 20	Roth
Tween® 80	Roth
Whatman filter paper	Roche
Yeast extract	Roth

2.1.3 Solutions

5 % blocking solution	5	g powdered carnation milk
	100	ml western washing buffer
Blocking solution (Immunohistofluorescence)	1	% (v/v) donkey serum
	0.3	% (v/v) Triton X-100
	in PBS	
$CaCl_2$ (2.5 M)	36.75	g $CaCl_2$
	100	ml dH_2O
	sterile filter	
DMEM (+ suppl.)	10	% (v/v) FCS
	1	mM penicillin/streptomycin
	1	mM L-glutamine
DMEM (hepatocytes transduction)	5	% (v/v) FCS
ECGM	500	ml ECBM
	2	% (v/v) FCS
	0.4	% (v/v) endothelial growth supplement
	0.1	ng/ml epidermial growth factor
	1	ng/ml basic fibroblast growth factor
	90	µg/ml heparin
	1	µg/ml hydrocortisone
EDTA (0.5 M, pH 8.0)	186.1	g EDTA*$2H_20$
	800	ml dH_2O
	adjust to pH 8.0 (NaOH)	
FACS buffer	5	% bovine serum albumin

	2 mM EDTA in PBS
HBS (HEPES buffered saline)	140 mM NaCl 1.5 mM $Na_2HPO_4 \cdot 2H_2O$ 50 mM HEPES pH 7.0 with NaOH
HCM (concentrations not provided)	500 ml HBM xx Ascorbic acid xx BSA-FAF xx Hydrocortisone xx Transferrin xx Insulin xx rhEGF xx GA-1000
Laemmli buffer (2x)	4 % (w/v) SDS 0.2 % (w/v) bromophenol blue 126 mM Tris, pH 6.8 20 % (v/v) glycerol
LB agar plates	10 g tryptone-peptone 5 g yeast extract 10 g sodium chloride 15 g agar autoclave
LB medium (5x)	50 g tryptone-peptone 25 g yeast extract 50 g sodium chloride pH 7.5 autoclave
Paraformaldehyde (4 %)	4 % (w/v) paraformaldehyde pH 7.5 with sodium chloride (6 M)
PBS (20x, pH 7.4)	160 g NaCl 4 g KCl 28.8 g Na_2HPO_4 4.8 g KH_2PO_4 ad 1 l dH_2O
Ponceau S stain	0.1 % (w/v) Ponceau S solution 5 % (v/v) acetic acid in PBS
Primary antibody diluent (WB)	1 % (w/v) BSA in PBS

SDS-PAGE gel buffer (accumulative)	0.5 M Tris 0.4 % SDS pH 6.8 (KOH)
SDS-PAGE gel buffer (seperative)	1.5 M Tris 0.4 % SDS pH 8.8 (KOH)
SOC medium	0.5 % (w/v) yeast extract 2 % (w/v) tryptone 10 mM sodium chloride 2.5 mM potassium chloride autoclave 20 mM magnesium chloride (sterile filtered) 20 mM glucose (sterile filtered)
Tris-glycine-SDS-PAGE buffer (5 x)	125 mM Tris 960 mM glycine 0.1 % (w/v) SDS
Transfer buffer	192 mM glycine 25 mM Tris 10 % (v/v) methanol
Transfection medium (TFM)	DMEM + suppl. 10 mM HEPES 25 µM chloroquine
Transformation buffer 1	100 mM $RbCl_2$ 50 mM $MnCl_2$ 30 mM potassium acetate 10 mM $CaCl_2$ 15 % glycerol pH 5.8 (acetic acid) sterile filter
Transformation buffer 2	10 mM MOPS 10 mM RbCl2 75 mM CaCl2 15 % glycerol pH 6.8 (KOH) sterile filter
Tris buffer	1 M Tris base Desired pH (sodium chloride)
Western blocking buffer	5 % (v/v) skim milk powder in PBS

Western wash buffer	0.05 % (v/v) Tween 20 in PBS

2.1.4 Kits

Name	**Provider**
Calcium Phosphate Transfection Kit	Sigma
Click It® Edu Flow Cytometry Assay Kit	Invitrogen
Complete lysis-M	Roche
ECL Plus Western Blotting detection reagent	GE Healthcare
iScript™ cDNA Synthesis Kit	BioRad
NuceloBond® Xtra Midi	Macherey & Nagel
NuceloBond® PC2000	Macherey & Nagel
Human Albumin ELISA Quantitation Set	Bethyl
One Shot® Mach1™-T1^R chemically competent *E. coli*	Invitrogen
One Shot® TOP10™ Chemically Competent *E. coli*	Invitrogen
One Shot® STBL3™ Chemically Competent *E. coli*	Invitrogen
PeqGOLD Plasmid Miniprep Kit	PeqLab
Plasmid Midi Kit	Machery & Nagel
Plasmid Mega Kit	Machery & Nagel
Phusion® High-Fidelity PCR Master Mix	Finnzymes
QIAquick Gel Extraction Kit	Qiagen
Quick Start Bradford Protein Assay	Biorad
Taq Polymerase Kit	Qiagen
pCR™ 4Blunt-TOPO® Cloning Kit for sequencing	Invitrogen
RNeasy Mini Kit	Qiagen
SuperScript™ III First-Strand Synthesis SuperMix	Invitrogen
Weighing scale BP 612/BL610	Sartorius
XL1 Blue chemically competent cells *E. coli*	Stratagene

2.1.5 Antibodies

Primary antibodies/Isotype controls

Antigen	raised in	clonality	dilution	Company	catalog #
Actin	rabbit	monoclonal	1/1000 (WB)	Sigma	A5060
ASGPR-PE	mouse	monoclonal	1/250	Santa Cruz	Sc-52623
CD31-PE (human)	mouse	polyclonal	1/11 (FACS) 1/3 (IF) 1/5 (IC)	Miltenyi	130-092-653
CD105-PE (human)	mouse	polyclonal	1/11 (FACS) 1/3 (IF) 1/5 (IC)	Miltenyi	130-094-941
CD105-APC (human)	mouse	polyclonal	1/11 (FACS) 1/3 (IF) 1/5 (IC)	Miltenyi	130-094-926
CD146 (human)	mouse	monoclonal	1/11 (FACS) 1/3 (IF) 1/5 (IC)	Miltenyi	130-092-849
CD309 (human)	mouse	monoclonal	1/11 (FACS) 1/3 (IF) 1/5 (IC)	Miltenyi	130-093-598
GFP-FITC	goat	polyclonal	1/200	Abcam	6662
GFP	rabbit	polyclonal	1/400 (IF)	Invitrogen	A11122
His-PE	mouse	monoclonal	1/11 (FACS)	Miltenyi	130-092-691

Secondary antibodies

Antigen	raised in	label	clonality	dilution	Company	catalog #
Goat IgG - H&L - F(ab)2	rabbit	HRP	polyclonal	1/1000 (WB)	Abcam	ab5755
Rabbit IgG - F(ab)2	goat	HRP	polyclonal	1/5000 (WB)	Abcam	ab6112
rat IgG whole	goat	HRP	monoclonal	1/1000 (WB)	Acris	R1614HRP
Goat IgG (H+L)	donkey	Dyelight488	polyclonal	1/500 (IF)	Abcam	ab98514
Goat IgG (H+L)	donkey	AF647	polyclonal	1/500 (IF)	Invitrogen	A-21447
Rabbit IgG (H+L)	donkey	AF488	polyclonal	1/500 (IF)	Invitrogen	A-21206
Rabbit IgG (H+L)	donkey	Dyelight594	polyclonal	1/500 (IF)	Abcam	Ab98500

2.1.6 Enzymes

Enzyme	**Provider**
*Bam*HI restriction enzyme (20 U/µl)	New England BioLabs
*Bgl*II restriction enzyme (10 U/µl)	New England BioLabs
Calf intestine alkaline phosphatase (CIAP)	Fermentas
DNAse	Fermentas
Endo H glycosidase	New England BioLabs
IScript reverse transcriptase	BioRad
*Nco*I restriction enzyme (10 U/µl)	New England BioLabs
Phusion polymerase	Finnzymes
Pwo polymerase	Peqlab
RNAsin	Promega
Ribolock RNAse inhibitor	Fermentas
Superscript reverse transcriptase	Invitrogen
T4 ligase (5U/µl)	Fermentas
Taq polymerase	Invitrogen

2.1.7 Software

Name	**Purpose**
Adobe Photoshop (Adobe) 7, CS5	Image processing
CellQuest Pro	FACS acquisition/analysis
Cell^M Acquisition (Olympus)	Immunohistofluorescence image processing
ChemStar (INTAS)	Western blot detection
FlowJo	FACS analysis
Fluoview 10 (Olympus)	Live image processing
LightCycler 480 Software (Roche)	Real-time PCR data acquisition and analysis
Image J	Image processing
MacOS 11.0 (Apple)	Operating system
Microsoft Windows 2000/XP/7	Operating system
Microsoft Office	Text processing, presentations, calculations
Microsoft Internet Explorer	Internet browsing
Mozilla Firefox	Internet browsing
SigmaPlot 8.0	Calculations, graph processing
Vector NTI 11.0 (Invitrogen)	Vector and cloning design
Quantity One (BioRad)	Agarose gel monitoring

2.1.8 Markers

2.1.8.1 DNA ladders

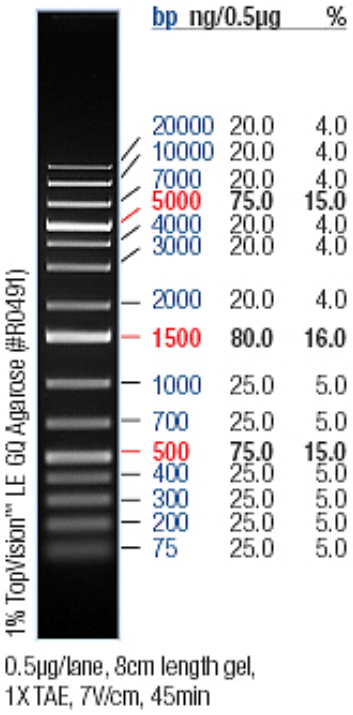

Figure 6: Generuler 1 kb Plus #SM1333

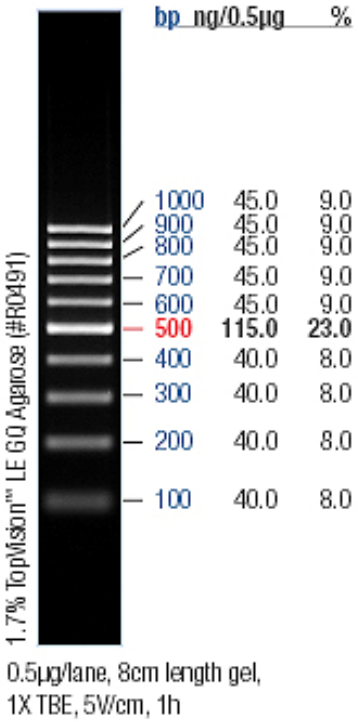

Figure 7: GeneRuler 100 bp #SM0243

2.1.8.2 Protein ladders

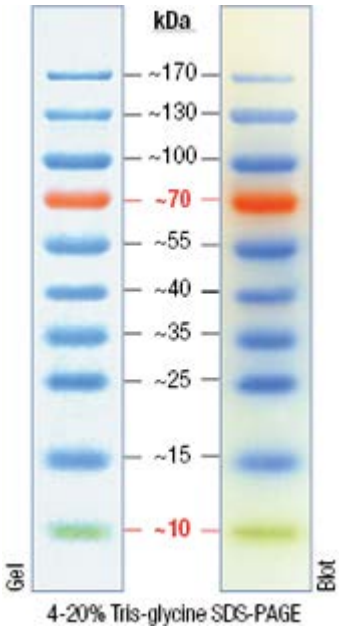

Figure 8: Prestained Protein Marker #SM0671

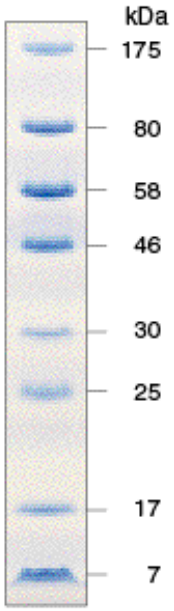

Figure 9: Prestained Protein Marker #P0771

2.1.9 Plasmids

pCR4®TOPO®-huASGPR

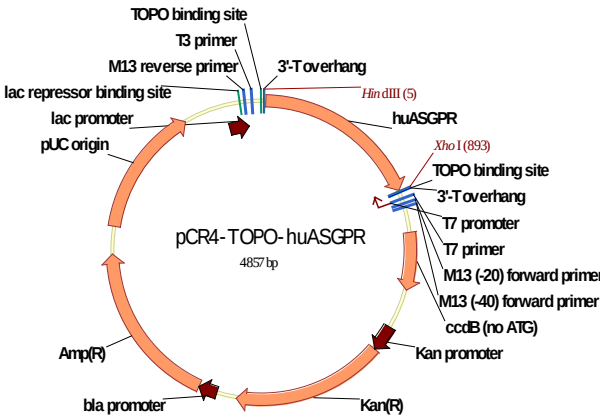

Figure 10: pCR4®TOPO®-huASGPR. 876 bp human ASGPR cDNA was subcloned into the pCR4®-TOPO® vector.

pcDNA3.1/Zeo(+)-huASGPR

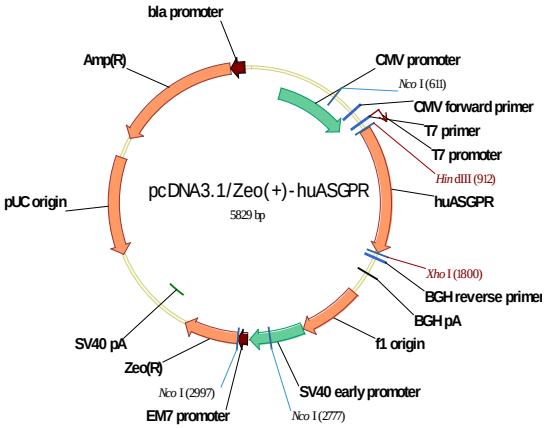

Figure 11: pcDNA3.1/Zeo(+)-huASGPR. The pcDNA3.1/Zeo(+) vector is a eukaryotic expression vector used for expression of proteins in mammalian cells. Expression of proteins is mediated by the strong CMV promoter and selection of mammalian cells is conducted with zeomycin. In this study, the 876 bp human ASGPR cDNA was inserted into the vector.

pSFFV-eGFP

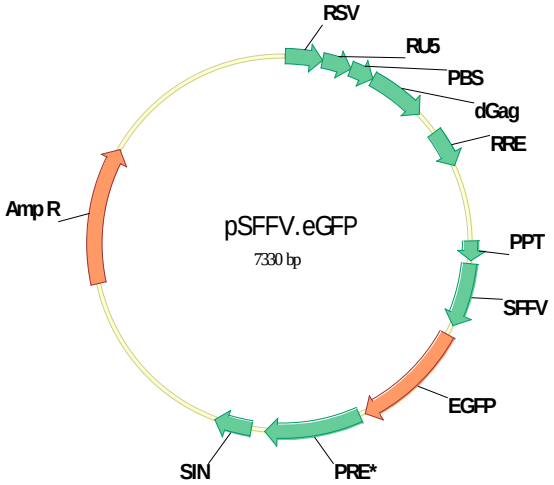

Figure 12: pSFFV-eGFP. pSFFV-eGFP transfer vector was used to generate LV-eGFP lentiviral vectors. The expression is mediated by a strong SFFV (spleen focus-forming virus) promoter. The orginal construct pRRL.PPT.PGK.GFPpre was kindly provided by L. Naldini (Milano, Italy).

Packaging plasmids:

pHLnse3-scFv-CRDH1 (Cao/benhar/benharP)

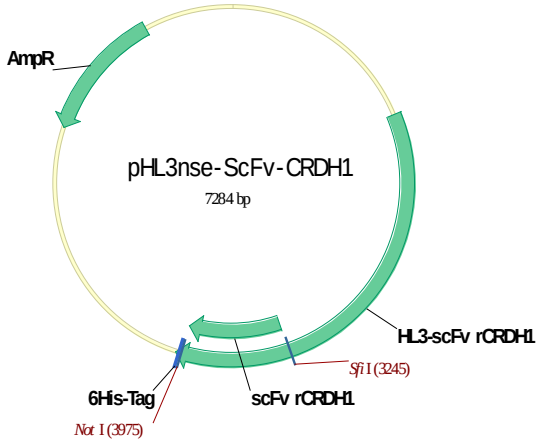

Figure 13: pHL3nse-scFv-CRDH1. The pHL expression plasmid contains a defective hemagglutinin gene which is fused by a linker (4x glycine-glycine-glycine-glycine-serine) to the nucleotide of a scFv directed against CRDH1 (rCRDH1). The fusion gene is ultimately followed by a 6x His-tag to detect expression of the fused gene. Binding and fusion to a target cell is mediated by the scFv in cooperation with the F-protein. The scFv part of the fusion gene was replaced by alternate scFv nucleotide sequences to test various scFv antibodies against ASGPR (pHL-Cao, pHL-benhar, pHL-benharP).

pHL3nse-scFv-A5/pHL3nse-scFv-CD105

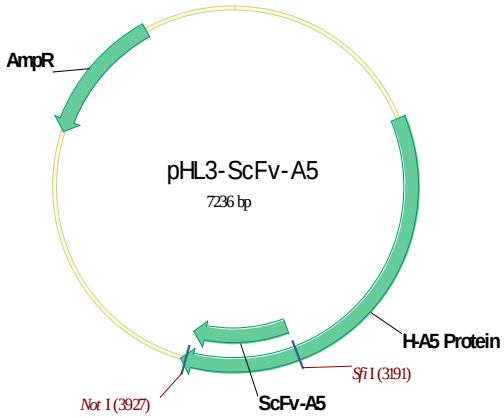

Figure 14: pHL3nse-scFv-A5/pHL3nse-scFv-CD105. The pHL expression plasmid contains a defective hemagglutinin gene which is fused by a polylinker (4x glycine-glycine-glycine-glycine-serine) to the nucleotide of a scFv directed against human endoglin (ENG, A5). The fusion gene is followed by a 6x His-tag to detect expression of the fused gene. Binding and fusion to a target cell is mediated by the scFv in cooperation with the F-protein.

pFcd3

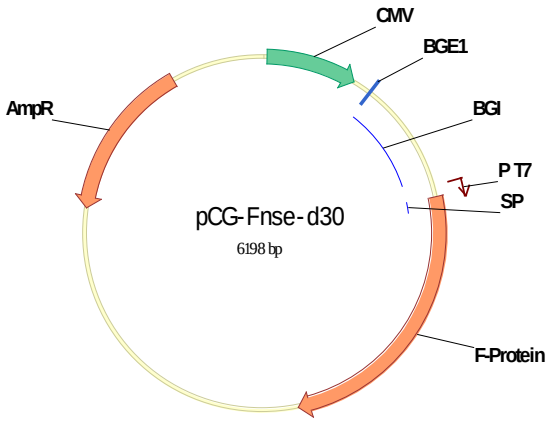

Figure 15: pCG-Fnse-d30: This plasmid contains the genetic information for the fusion protein (F-protein) that is necessary for the fusion of lentiviral vectors constructed with the measles virus envelope.

pMD2.G (VSV-G)

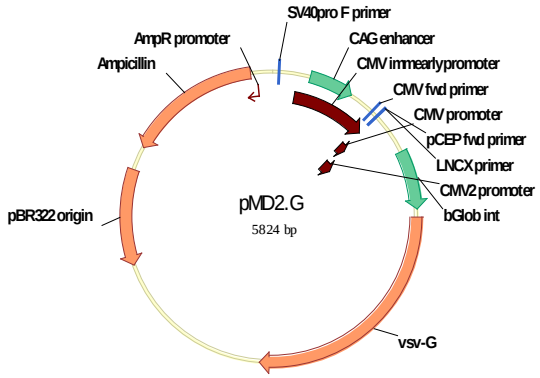

Figure 16: pMD2.G. The pMD2.G plasmid is a packaging plasmid encoding for the vesicular stomatitis virus (VSV-G) envelope glycoprotein, which enables lentiviral vectors to infect a broad variety of mammalian cells.

psPAX2 (Gag-pol-rev)

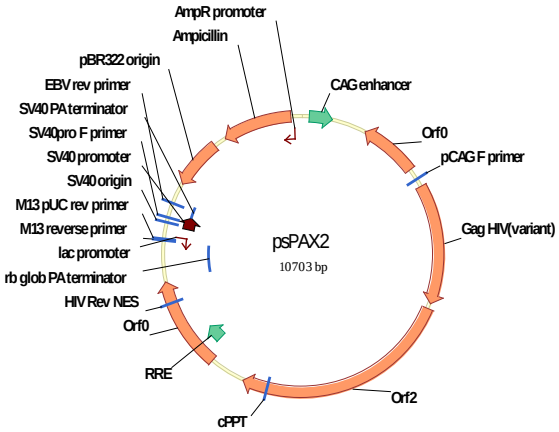

Figure 17: psPAX2. The psPAX2 packaging plasmid allows expression of HIV the gag and rev genes of HIV. The plasmid carries the information of regulatory elements of HIV to allow gene transcription in target cells.

2.1.10 Mouse model

BALB/c Rag2$^{-/-}$ IL-2R$\gamma_c^{-/-}$ mice

Humanized mice have previously been defined as immunodeficient mice that either transgenically express human genes or have engrafted human cells or tissues. In this study, we used mice with BALB/c background that were deficient of recombination activating gene 2 (Rag2$^{-/-}$) (Mombaerts et al., 1992). This mutation leads to impaired maturation of B and T cells, and immune cell development is further inactivated by the IL-2R$\gamma_c^{-/-}$ mutation by abolishing proper signalling between interleukins and its affinity receptors. In addition, deletion of the IL-2 receptor γ chain leads to complete absence of lymph nodes and NK cells (DiSanto et al., 1995; Cao et al., 1995).

2.1.11 Bacterial strains

One Shot® TOP10 *E. coli* (Invitrogen)

F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *deo*R *rec*A1 *end*A1 *ara*Δ139 Δ(*ara, leu*)7697 *gal*U *gal*K λ- *rps*L(StrR) *nup*G

E. coli TOP10 cells were used for initial cloning and maintaining plasmids. Their key features are high transformation efficiency and high yields of plasmid DNA due to its mutations in the *rec*A and the *end*A genes, which destroy mechanisms for recombination and replication events (Yanisch-Perron et al., 1985). This strain only expresses the β-fragment of β-galactosidase, which makes it useful for blue-white screening when transformed with plasmids expressing the α-fragment (Sambrook et al., 1989). The *mcr*A gene restricts DNA with methylcytosine in certain sequences (Promega guide for *E. coli* markers). The *deo*R gene encodes for a protein the DeoR repressor which regulates expression of the *nup*G nucleoside transport genes and *deo* operon transcription initiating from both the cyclic AMP (cAMP)/cAMP receptor protein-independent and the cAMP/cAMP receptor-dependent promoter-operator sites PO1 and PO2 (Singer *at al*., 1985). The mutation enhances the constitutive expression of genes for nucleotide synthesis and propagation of large plasmids. The TOP10 cells are affected in their ability to produce LPS and to metabolize galactose due to the mutation in the *gal*U and the *gal*K genes (Priebe et al., 2004; Choi et al., 2002). The mutation in the *ara* gene blocks arabinose catabolism (Kessler and Englesberg, 1969).

One Shot® Mach1™-T1^R *E. coli* (Invitrogen)

F$^-$ φ80(*lac*Z)ΔM15 Δ*lac*X74 *hsd*R($r_K^-m_K^+$) Δ*rec*A1398 *end*A1 *ton*A

One Shot® Mach1™-T1^R have the *hsd*R-mutation which prevents transformed DNA to be restricted by endogenous endonucleases. The *ton*A mutation changes an outer membrane protein so this bacterial strain can provide resistance to phage T1 Manual for OneShot® Mach1™-T1^R Chemically Competent *E. coli*).

STBL3 *E. coli*

F- *mcr*B *mrr hs*dS20 (r_B^-, m_B^-) *rec*A13 *sup*E44 *ar*a-14 *gal*K2 *lac*Y1 *pro*A2 *rps*L20 *(Str) xyl-5* λ^- *leu mtl-1*r

The Stbl3™ *E. coli* strain is derived from the HB101 *E. coli* strain and is recommended for use when cloning unstable inserts such as lentiviral DNA containing direct repeats. Unlike TOP10 *E. coli*, these cells reduce the frequency of unwanted homologous recombinations of long terminal repeats (LTRs) found lentiviral and other retroviral vectors (Manual for OneShot® Sbtl3™ Chemically Competent *E. coli*).

2.1.12 Eukaryotic cell strains

All cell cultures were grown in DMEM containing 10 % FCS, 1 mM L-glutamine, 100 I.U. penicillin and streptomycin and in 5 % CO_2, if not stated otherwise.

HEK293T

HEK293T cells were originally generated by transformation of human embryonic kidney cells with sheared adenovirus 5 DNA in the 70'ies by Graham et al. (1977). Later genomic analysis showed that 4.5 kb of viral DNA had become incorporated into chromosome 19. Recent results suggest that HEK293T derive from neuronal lineage cells, as they show a filamentous pattern when stained for major neurofilament subunit (Shaw et al., 2002). The cell line has proven to be very reliable in transfection experiments and is easily handled. The HEK293T cell lineage contains the SV40 large T antigen that allows episomal replication of transfected plasmids containing the SV40 origin of replication and temporary expression of genes of interest. As HEK293T cells express various adenoviral transcription units like E1 and E3 they are used for propagation of adenoviral vectors, in which these genes are deleted (Lieber et al., 1997). They

are also used for the production of lentiviral and retroviral vectors. HEK293T cell line is therefore called packaging cell line.

HepG2

The HepG2 cell line was isolated from tumor tissue of a 15-year-old boy with hepatocellular carcinoma in 1975. This cell line has been shown to produce a wide variety of liver-specific proteins and is deficient of the hepatitis B virus.

Hepa 1-6

Hepa 1-6 cells are derived from mouse hepatoma cells from the BW7756 tumor that arose in a C57L mouse. The cell line is adherent and grows as a monolayer. Secretion of several liver-specific products has been described (Darlington, 1987)

HT1080

The HT1080 cell line was isolated from a massive tumor located at the acetabulum of a 35 year-old Caucasian male in 1972 (Rashed et al., 1972). The HT1080 cell line is characterised by high proliferation rates and has especially proved useful in experiments with the expression of recombinant proteins.

Huh7/7.5

The Huh7 cell line was originally isolated from a hepatocellular tumor that was surgically removed from a 57-year-old Japanese male in 1982 (Nakabayashi et al., 1982). The immortalised cell line shows characteristics of epithelial-like tumorigenic cells. Huh7.5 is derivate from the Huh7 cell line which has been cured of hepatitis C by alpha-interferon therapy. The cell line offers the advantage of increased permissiveness to hepatitis C viral replication (Blight et al., 2002).

RAW264.7

The RAW cell line was originally acquired from ascites of a tumor within a male mouse by intraperitoneal injection of Abselon Leukaemia virus (A-MuLV). The cell line expresses numerous inflammatory mediators of mature murine macrophages, making the cell line a useful tool in research to investigate immune responses *in vitro*. The cells are able to lysis of cells by the means of antibody-opsonization (Raschke et al., 1978).

Primary Human Hepatocytes

BD Gentest™ Human CryoHepatocytes were acquired from BD Biosciences and cultivated in Hepatocyte Culture Medium (HCM, Clonetics).

Human umbilical vein endothelial cells (HUVEC)

Human umbilical vein endothelial cells (HUVECs) are cells isolated from an ordinary human umbilical cord of newborns (Davis et al., 2007; Baudin et al., 2007). The endothelial cells are removed from the cord by collagenase digestion and can be cultivated for about 16 passages. However, they lose functionality after passage five and experiments should be performed during early passages. HUVECs are CD31 and CD105-positive and offer scientists the possibility to study pathways involving angiogenesis and hemostasis (Gasowska et al., 2009). HUVECs were cultured in Endothelial Cell Growth Medium (Promocell).

Murine endothelial cell line bEnd.3

These murine endothelial cells were originally isolated from a brain endothelioma growing in the cerebral cortex of a BALB/c mouse. For immortalization, the cells were transformed by infection with the NTKmT retrovirus vector that expresses polyomavirus middle T antigen (Montesano et al., 1990). They represent a valuable source of cells to study hemostasis and low density lipoprotein (LDL)-mediated signalling pathways. bEnd.3 were cultivated in RPMI1640.

2.2 Methods

2.2.1 Cloning of human ASGR and scFv-hASGPR

2.2.1.1 Isolation of total RNA from human eukaryotic cells

$5*10^5$ HepG2 cells were plated on 6-well plates and grown in DMEM (+ suppl.) for 3 days to allow gene expression. Total RNA was isolated using the RNeasy Kit provided by Qiagen following the manufacturer's instructions. In short, cells were washed with PBS and 350 µl of RLT buffer containing 10 % β-mercaptoethanol was added. Cells were incubated for 3 minutes at room temperature and transferred to a microcentrifuge tube. 350 µl of 70 % ethanol was added and cells were lysed by aspirating several times with a syringe. The suspension was transferred into the RNeasy mini column and centrifuged at 10000 rcf for 1 minute. RNA bound to the column was washed once with 700 µl buffer RW1, twice with 500 µl of buffer RPE and remaining buffer was removed by centrifugation at maximum speed for 1 minute. RNA was eluted with 30 µl RNAse-free water. RNA concentration was determined with the Biophotometer (Eppendorf).

2.2.1.2 Reverse transcription into cDNA

RNA was transcribed into cDNA using the SuperScript™ III First-Strand Synthesis SuperMix kit according to manufacturer's instructions (Invitrogen). Normally, 0.5 µg of RNA was used during the transcription process and cDNA could be stored at – 20 °C for a month.

2.2.1.3 Polymerase chain reaction of human ASGPR

Forward (JW25) and reverse (JW26) primers with integrated restriction sites for *Hind*III and *Xho*I flanking the open reading frame of subunit H1 of human ASGPR (ASGR1) were designed based on the nucleotide sequence of *Homo sapiens* ASGR1 transcript variant 1 (accession number NM_001671.4).

Table 1: Primers for human ASGR1

Primer	Sequence	Restriction site
JW25	5' – GCGAAGCTTATCATGACCAAGGAGTATCAA – 3'	*Hind*III
JW26	5' – GCGCTCGAGAAATTAAAGGAGAGGTGGCTC – 3'	*Xho*I

The Phusion® High-Fidelity DNA Polymerase (Finnzymes) was used to amplify the huASGPR gene. Template cDNA was isolated from HepG2 cells as described above and reagents were mixed following the manufacturer's instructions. Details of reaction volumes and PCR cycling intervals are found in the following table.

Table 2: Reaction volumes of PCR of huASGPR

reagent	volume [µl]
Phusion Flash PCR Master Mix (2x)	25
JW25 (10 µM)	5
JW26 (10 µM)	5
Water	22.5
DMSO	1.5
cDNA HepG2	1
Total volume	50

Table 3: PCR cycles

step	Temp [°C]	Time	cycles	comments
1	98	30 sec	1	Denaturation
2	98	15 sec	35	Denaturation
	55	30 sec		Annealing
	72	45 sec		Elongation
3	72	3 min	1	Elongation
4	4	hold	1	Final step

The PCR product was analysed on a 1 % agarose gel prepared in Tris-acetate buffer along with 6x loading dye. DNA was stained with ethidium bromide for visualisation in ultraviolet light and photographed using the GelDoc 2000 (GFL).

2.2.1.4 Cloning into pCR™4Blunt-TOPO® cloning vector

The PCR product was used in the TOPO cloning reaction without previous purification. Reagents for the TOPO cloning reaction were added as suggested by the Invitrogen manual.

Table 4: Setup of TOPO cloning reaction

reagent	volume [µl]
huASGPR PCR product	2
Salt solution	1
water	2
pCR4® TOPO®	1
Total volume	6

The reaction mixture was incubated for 5 min at room temperature and immediately put on ice afterwards. The PCR product was hereby incorporated into the pCR®4-TOPO® to yield pCR®4-TOPO®-huASGPR.

2.2.1.5 Generation of competent *E. coli*

The following protocol is a slightly modified version of the protocol described by Hanahan (Hanahan, 1991). 25 µl of acquired One Shot® TOP10/ One Shot® Mach1™-T1R/ One Shot® STBL3 chemically competent *E. coli* were plated on LB-agar plates (no antibiotics) and grown overnight at 37 °C. Colonies were picked and transferred into 100 prewarmed SOC medium (no antibiotics) and grown at 37 °C. When optical density at 600 nm measured 0.55, bacterial cultures were cooled for 5 minutes on ice and cells were pelleted by centrifugation (2500 rcf, 10 min, 4 °C). The cells were resuspended in 30 ml transformation buffer 1 and incubated on ice for 90 minutes. The cells were collected by centrifugation (2500 rcf, 10 min, 4 °C). The cells were resuspended in 4 ml ice-cold transformation buffer 2, aliquoted into pre-chilled 1.5 microcentrifugation tubes and snap-freezed in liquid nitrogen. The competent cells were stored at –80 °C.

2.2.1.6 Transformation of TOPO cloning reaction

The TOPO cloning reaction was used for transformation of competent One Shot® TOP10 *E. coli*. 2 µl of TOPO cloning reaction were transferred into 25 µl of competent cells. After incubation on ice for 10 minutes, the cells were heat-shocked for 30 seconds at 42 °C. 250 µl of SOC medium (no antibiotics) were added and the cells were grown at 37 °C for 1 hour. 100 µl were plated on LB-Agar dishes (+80 µg/ml ampicillin) and plates were incubated overnight at 37 °C. Positive colonies were inoculated in LB-medium (+ 80 µg/ml ampicillin) at 37 °C overnight.

2.2.1.7 Purification of plasmids

Plasmid DNA was isolated from transformed *E. coli* by using the peqGOLD Plasmid MiniPrep Kit according to manufacturer's instructions (PeqLab manual). DNA concentration was determined by measuring UV extinction at 260 nm and 280 nm with the Biophotometer (Eppendorf). The isolated plasmids were prepared for sequencing by GATC (Konstanz) and SeqLab (Göttingen) according to the company's instructions along with M13 sequencing primers provided with the TOPO®-Cloning Kit for Sequencing.

Table 5: Sequencing primers for pCR4®-TOPO®-huASGPR

Primer	Sequence
M13 forward primer	5' – GTAAAACGACGGCCAG – 3'
M13 reverse primer	5' – CAGGAAACAGCTATGA – 3'

Plasmid DNA was transformed into One Shot® TOP10 competent cells and purified in large scale following the manufacturer's instructions (Macherey & Nagel NucleoBond® Xtra Midi manual; Macherey & Nagel NucleoBond® PC 2000).

2.2.1.8 Restriction analysis of pCR®4-TOPO-huASGPR

2.2.1.8.1 Qualitative restriction digest

Restriction analysis of pCR®4-TOPO®-huASGPR was performed to investigate the presence of the huASGPR-insert. *Hind*III and *Xho*I were selected for qualitative restriction analysis as its restriction sites had been introduced in the previous PCR flanking the huASGPR insert. However, restriction enzymes (RE) were interchangeable with each other according to the specific needs and desired restriction patterns, taking into account specific activity levels of the respective restriction enzymes in buffer NEB 3.

Table 6: Restriction digest of pCR®4-TOPO®-huASGPR

reagent	Volume [µl]	Volume [µl]
pCR®4-TOPO® -*Cd47*	5	5
NEB 3 (10 x)	2	2
RE 1	0.5	--
RE 2	0.5	0.5
nuclease-free water	12.5	12
Total volume	20	20

The mixtures were incubated at 37 °C for 2 hours and heat-inactivated at 65 °C for 15 minutes. Restriction patterns were analysed on a 1 % agarose gel and visualised as described before.

2.2.1.8.2 Preparative restriction digest

In preparation for the cloning of huASPGR into the eukaryotic expression vector pcDNA3.1/Zeo(+), pCR®4-TOPO®-huASGPR and the target vector pcDNA3.1/Zeo(+) were digested with *Hind*III and *Xho*I restriction enzyme according to the reaction stated below.

Table 7: Preparative restriction digest

reagent	pcDNA3.1 [µl]	pCR®4-TOPO®-huASGPR
DNA	6 µg	6 µg
NEB 3 (10x)	2	2
*Hind*III	3	3
*Xho*I	3	3
nuclease-free water	Ad 20	Ad 20
Total volume	20	20

The mixtures were incubated at 37 °C for 2 hours and heat-inactivated at 65 °C for 15 minutes. The DNA was analysed on a 1.5 % agarose gel as described above and appropriate bands were purified from the gel following the manufacturer's instructions (QIAquick Gel Extraction Kit).

2.2.1.9 Ligation

Digested and purified huASGPR and pcDNA3.1/Zeo(+) were ligated using T4 ligase. The insert was ligated with 200 ng vector backbone in 5:1, 3:1 and 1:1 ratios. The amount of digested huASGPR insert (875 bp) was calculated according to the following formula:

$$ng(insert) = \frac{ng(vector) \cdot size(kb, insert)}{size(kb, vector)} \cdot ratio\left(\frac{insert}{vector}\right)$$

1 µl of T4 ligase (5 U/µl) was added along with 2 µl 10x T4 ligase reaction buffer and samples were filled up with dH_2O to a total volume of 20 µl. Ligation reaction was performed at 25 °C for 16 hours. Ligase was inactivated by heating to 65 °C for 15 minutes and stored at 4 °C until transformation into TOP10 chemically competent cells. The ligated product was named pcDNA3.1/Zeo(+)-huASGPR. The product was transformed into chemically competent TOP 10 cells, positive clones were analysed by growth selection on ampicillin plates and plasmids were purified as stated above. Sequencing and restriction analysis were performed to verify the insertion of the huASGR1 gene into pcDNA3.1/Zeo(+). Sequencing was performed with specifically designed primers.

Table 8: Sequencing primers for pcDNA3.1/Zeo(+) inserts

Primer	Sequence
pcDNA3.1/Zeo(+) forward primer	5' – ATTAATACGACTCACTATAG – 3'
pcDNA3.1/Zeo(+)reverse primer	5' – AAGGCACAGTCGAGGCTGAT – 3'

Proper plasmids were transformed into One Shot® TOP10 competent cells and plasmid DNA were purified in large scale following the manufacturer's instructions (Macherey & Nagel NucleoBond® Xtra Midi manual; Macherey & Nagel NucleoBond® PC 2000).

2.2.1.10 Transient transfection of HT1080 cell with pcDNA3.1/Zeo(+)-huASGPR

To test expression of the ASGPR1 protein from pcDNA3.1/Zeo(+)-huASGPR, HT1080 cells were transiently transfected with the Lipofectamine 2000 kit following the manufacturer's instructions. Cells were harvested 3 days after transfection and expression of huASGPR was analysed by FACS. For this, 100000 cells were stained with primary antibody anti-ASGPR-PE (Santa Cruz, 1:250 dilution) for 10 minutes and washed 3 times with FACS buffer before analysing the ASGPR expression by FACS.

2.2.1.11 Cloning of the nucleotide sequences of single chain antibodies directed against human ASGPR (scFv-huASGPR) and human Endoglin (scFv-huCD105) into defective hemagglutinin pseudotyping vectors

The eukaryotic expression vector pHL3nse represents one of the pseudotyping vectors used to produce lentiviral vectors pseudotyped with measles virus hemagglutinin. Expression of defective hemagglutinin is driven by a strong eukaryotic promoter. Hemagglutinin is linked to a single chain antibody, which eventually directs binding to specific target cells and mediates integration of the viral complex. In this study, we used hemagglutinin fused to single-chain antibodies against human ASGPR (scFv-huASGPR, figure 13) and human CD105/Endoglin (scFv-huCD105; figure 14).

2.2.1.11.1 scFv-huASGPR

The available nucleotide sequences of the single chain variable fragment antibodies against ASGPR 1 (scFv-huASGPR, an aggregate name for scFv-Cao, scFv-benhar, and scFv-benharP, see supplementary section) were synthesized and cloned into the commercially available pCG vector with flanking *Sfi*I and *Not*I restriction sites using the services of MWG Operon. A special algorithm was applied to predict unfortunate secondary and tertiary structures that could prevent

proper expression and folding of the hemagglutinin-scFv fusion protein (Friedel et al., 2015). In the case of benhar-scFv, modifications needed to be implemented into the scFv nucleotide sequence: The modified scFv nucleotide (benharP) was taken along with the scFv-constructs that were previously mentioned. Upon receiving the plasmids, a control restriction digest was performed to ensure the presence of the scFv-insert. The scFv-inserts were cloned into the pHL pseudotyping vector to yield pHL3nse-scFv-CRDH1 (an aggregate name for pHL-Cao, pHL-benhar and pHL-benharP). In this vector the defective hemagglutinin gene is fused to a scFv-antibody specific against human ASGPR (figure 13). Lentiviral production using the pseudotyping pHL3nse-scFv-CRDH1 yields LV-ASGPR with desired affinity for ASGPR-expressing cells.

2.2.1.11.2 scFv-huCD105

The pHL3nse-scFv-A5/pHLnse3-scFv CD105 was kindly provided by S. Kneissl from the research group Buchholz (Funke et al., 2010). The nucleotide sequence of the single chain antibody against CD105/Endoglin had previously been synthesized and subcloned into the commercially available pCG vector with flanking *Sfi*I and *Not*I restriction sites using the services of MWG Operon. The scFv-huCD105-insert was cloned into the pHL pseudotyping vector to yield pHL3nse-scFv-A5/pHLnse3-scFv CD105. In this vector, the defective hemagglutinin gene is fused to a scFv-antibody specific against human CD105/Endoglin (figure 14). Lentiviral production using the pseudotyping pHL3nse-scFv-A5/pHL3nse-scFv-CD105 yields LV-huCD105 with an affinity for CD105/Endoglin expressing cells.

2.2.1.12 Expression analysis by fluorescence activated cell sorting (FACS)

To test expression of the scFv-constructs, HEK293T cells were transiently transfected with the Lipofectamine 2000 kit following the manufacturer's instructions. Cells were harvested three days after transfection and prepared for FACS as mentioned above using the anti-His primary antibody (Miltenyi Biotec, 1:11 dilution)

2.2.2 Production of lentiviral vectors

2.2.2.1 Cell propagation

Most eukaryotic cell lines were grown in DMEM (+ suppl.) if not stated otherwise. HUVECs were grown in ECGM. In general, cells were passaged in a 1:10 dilution every 4-5 days and medium was changed every 2-3 days. To passage cells, the cell monolayer was washed with prewarmed PBS and treated with 2x trypsin-EDTA for 5 minutes. Prewarmed DMEM (+

suppl.) was added to stop trypsin reaction. Cells were transferred to a conical tube and centrifuged at 700 rcf for 5 minutes. The supernatant was removed and cells were resuspended in an appropriate amount of DMEM (+ suppl.) or ECGM. The cells were transferred into a new cell culture flask in an appropriate dilution. To determine the exact number of cells, viable cells were counted in an improved Neubauer cell counting chamber using trypan blue dead cell exclusion.

2.2.2.2 Transient transfection of HEK293T cells for lentiviral vector production

Lentiviral vectors were prepared by using calcium phosphate-mediated transfection of subconfluent HEK293T cells (Duff et al., 1998). It was essential to use highly proliferating HEK293T in order to get high levels of virus production. This was accomplished by passaging cells 3 times a week in a 1:5 dilution prior to transfection.

24 hours prior to infection, $5*10^6$ HEK293T cells were seeded on a 10 cm dish. 7 ml of DMEM (+ suppl.) were added. 2 hours prior to transfection, the medium was replaced with transformation medium consisting of DMEM (+ suppl.) with 10 mM HEPES and 25 µM chloroquine.

For each 10 cm dish, 7 µg of F-plasmid was mixed along with 2.33 µg hemagglutinin-scFv-fusion plasmid (pHL3nse-scFv-Cao, pHL3nse-scFv-benhar, pHL3nse-scFv-benharP or pHL3nse-scFv-A5), 12 µg gag-pol-rev plasmid and 10 µg pSFFV-eGFP in a 15 ml conical tube. For the control vector LV-eGFP, 10 µg of pSFFV-eGFP was mixed along with 12 µg gag-pol-rev plasmid and 1.5 µg VSV-G plasmid in a 15 ml conical tube for a 10 cm dish. Plasmids were diluted with dH_2O to 450 µl. 50 µl of 2.5 M $CaCl_2$ were added. The $CaCl_2$-plasmid mixture was added slowly to 500 µl of 2x HBS while air-bubbling. The solution was incubated at room temperature for 20 minutes and added dropwise to the HEK293T cells. The plates were incubated at 37 °C. 12 hours later, the medium was replaced with DMEM (+ suppl.) containing 10 mM HEPES. The lentiviral vectors produced by transfected HEK293T cells are further referred to as LV-ASGPR (an aggregate name for LV-Cao, LV-benhar, and LV-benharP) and LV-huCD105.

2.2.2.3 Concentration

Medium containing the lentiviral vectors was collected after 24, 36 and 48 hours post transfection and sterile filtered through a 0.45 µm filter to retain HEK293T cells. The filtered medium was concentrated using Centricon Plus-70 filter centrifugation device by centrifugation at 3500 rcf for 15-25 minutes to a final volume of approximately 3 ml (20x concentration). The

non-filtered supernatant containing concentrated LV-ASGPR, LV-huCD105 or LV-eGFP was aliquoted to microcentrifuge tubes and stored at -80 °C.

2.2.3 *In vitro* transduction of cell lines

2.2.3.1 Transduction of cells

$1*10^5$ HepG2 cells were plated in 24-well plates and treated for 2 hours with DMEM (+ suppl.) containing 10 mM HEPES and 4 µg/ml protamine sulfate. HUVECs were incubated with ECGM. Concentrated LV-Cao, LV-benhar, LV-benharP were added to HepG2 cells, in amounts of 10, 50, 100, 200 and 500 µl. Concentrated LV-eGFP or LV-huCD105 was diluted 1:20 and 1:10 in DMEM (+ suppl.), respectively, and 1, 2, 5, 10, 20 µl were added to HUVECs. The medium was replaced after 24 hours and cells were grown for 3 days to allow expression of transgenes and eGFP. The medium was changed every second day. Fluorescence i.e. successful transduction was detected by fluorescence microscopy. To determine the transduction efficiency of the virus, fluorescence-activated cell sorting (FACS) analysis of transduced HepG2 cells was performed to quantify eGFP fluorescence (Schambach et al., 2006). In addition, various other cell lines, e.g. murine endothelial cell (bEND.3) and fibroblast cell line HT1080 were transduced with lentiviral vectors to examine specificity.

2.2.3.2 Fluorescence activated cell sorting and staining

To conduct fluorescence activated cell sorting (FACS), cells were detached from the dish by trypsin-EDTA and transferred into 96-well chambers. The 96-well plate was centrifuged at 1200 rcf for 2 minutes and the supernatant was removed. Cells were washed once in FACS buffer.

If staining for surface antigens was required, cells were resuspended in 100 µl FACS buffer. An appropriate amount of FACS-staining antibody was added along with 0.25 µg 7-amino-actinomycin D for dead cell exclusion. Antibodies were incubated with cells for 10 minutes at 4 °C in the dark and cells were washed twice in FACS buffer. Cells were resuspended in an appropriate amount of FACS buffer and sampled for FACS analysis. Desired cells were gated according to their size and forward scatter (FSC) and side scatter (SSC). Fluorescence was detected in appropriate fluorescence channels at the FACSCalibur.

2.2.3.3 Calculation of virus titers

Commonly, virus titers are determined by counting distinct colonies of infected cells - derived from one infection event followed by cell division - at sufficiently high virus dilutions and

subsequent multiplication of the numbers of colonies counted with the dilution used. When eGFP or its derivates (that are particularly suited for FACS-analyses) are used as marker genes encoded by a viral vector, the virus titer can also be determined via the percentage of transduced, fluorescent protein expressing cells measured by FACS-analysis.
The fraction of eGFP-positive, transduced cells (P; $0 \leq P \leq 1$, where P stands for the mathematical term 'probability') can be deduced from the Multiplicity Of Infection (ratio of the number of infectious agents applied vs. the number of cells targeted; MOI, m) according to the Poisson-distribution (Ellis and Delbrück, 1939):

Equation 1) $P(k) = e^{-m}m^k/k!$

The average percentage of infected cells after inoculation with virus at a known MOI is given as (Flint et al., 2003; O'Reilly et al., 1994):

Equation 2)

$$P(n > 0) = 1 - P(n = 0) = 1 - \frac{m^0 \times e^{-m}}{0!} = 1 - e^{-m}$$

or

Equation 3) $P = 1 - e^{-0,5m}$

Equation 2) is valid for virus vectors that do not require cell division to occur for successful infection (for example lentiviral vectors (HIV-1 based) in theory), whereas equation 3) applies to virus vectors that do need cell division for successful transduction/infection (retroviral vectors (MLV, murine leukemia virus). However, MOI vs. P titration series (determination of the virus titer in the classical way via counting infected cell colonies and subsequent FACS-analyses of the same cell lines infected at defined MOIs) revealed that lentiviral vector titration on standard cell lines is also closely matched by equation 3) (rather than equation 2)), indicating that under conditions of frequent cell divisions (HUVECs, HepG2), lentiviral vectors prefer integration into the DNA-genome after nuclear breakup during cell division instead of employing their possibilities to actively enter intact nuclei (Albanese et al., 2008; Michael Bock, National Institute of Medical Research, London,UK, now MHH; unpublished data). Thus, for calculating the MOI of lentiviral vectors used here, equation 3) is used, too.

Equation 4) $m = -2 \times \ln(1 - P)$ (derived from Equation 3; with ln: natural logarithm)

Knowing the absolute number of cells seeded for transduction (n_{cells}), the dilution of the virus preparation applied (dil_{virus}) and the resulting percentage of transduced cells (P , i.e. fraction of EGFP-positive cells ($0 \leq P \leq 1$), as measured by FACS-analysis), the virus titer (T) of the virus preparation [transducing virus particles / ml] is obtained thereafter as:

Equation 5) $T = m \times n_{cells} \times dil_{virus}$

Virus titers were determined for each specific virus batch. Only samples with measured fluorescent values below 20 % were considered for the calculation of titers to minimize the risk of multiple infection events.

Example:

100000 HUVEC cells in 500 µl medium were inoculated with 2 µl of 1:10 diluted LV-CD105 and the fraction P of eGFP expressing cells was determined after three days according to 2.2.3.2. In an example calculation, we assume that the measured transduction efficiency was 3.28%. The background (mock) fluorescence (autofluorescence) was measured to be 1.16 %. Thus, the effective transduction efficiency was determined to be P = 2.12 %.

According to equation 4, $m = -2 * \ln(1 - \frac{2.12}{100}) = 0.0429$.

The virus titer T is then calculated by equation 5: $T = m \times n_{cells} \times dil_{virus} = 0.0429 \times 100000 \times \frac{500 \times 10}{2} = 10713000 = 1.0713 \times 10^7$ transducing virus particles per millilitre.

Titration was performed with 1, 2, 5, 10 and 20 µl and the respective calculated titers were averaged to calculate an approximate titer of that particular virus batch.

2.2.4 *Ex vivo* transduction of mammalian vessels

2.2.4.1 Surgical removal of human vessels

The left pulmonary artery (A. pulmonalis sinistra), side branches of the mammarian artery (A. mammaria) and the great saphenous vein (V. saphena magna) were acquired from patients that underwent coronary bypass surgery at the Department for Thoracic Surgery at the Medizinische Hochschule Hannover. Patients had given their consent to use of biological tissue the day before the surgical procedure. The patients' full consensus was acquired according to the ethical guidelines of the Medizinische Hochschule Hannover.

Vessels were quickly transferred into ice-cold PBS and rinsed from blood. Within 1.5 hours after removal from the patients' bloodstream, vessels were transferred into 2 ml of pre-warmed ECGM in a 6-well plate. Vessels were either kept for subsequent transduction with LV-huCD105 and LV-eGFP or treated with collagenase to isolate endothelial cells.

2.2.4.2 Transduction of vessels in plastic plates

Vessels were treated with $7*10^6$ viral particles and incubated for 24 h with lentiviral vectors. To increase the concentration of the viral vectors on the luminal side of the vessels, the ends were clamped with metal clips and vectors were injected with a syringe into the clamped lumen. The medium was removed and transgene GFP expression was allowed for another 8-10 days. Fluorescence was monitored by fluorescence microscopy. The medium was changed every 2 days.

2.2.4.3 Immunohistofluorescence of whole vessels section

Transduced vessels were transferred into a 15 ml conical tube and incubated with 4 % paraformaldehyde for approximately 6 hours at room temperature. Vessels were transferred to 30 % sucrose and incubated overnight at 4 °C. Tissue samples were embedded in Tissue-Tek® and cross-sectioned at the cryotome. Slides were stored at –80 °C for subsequent immunohistofluorescence staining.

Tissue cross sections were air-dried for 20 minutes at room temperature and washed twice in PBS for 10 minutes. Sections were blocked with PBS containing 5 % donkey serum and 0.3 % Triton X-100 for 30 minutes. Sections were incubated with primary rabbit anti-GFP antibody (A11122, Invitrogen, diluted 1:200 in blocking solution) and primary mouse anti-human CD105-PE or CD105-APC (130-094-941, 130-094-926, 1:3 dilution) for 1 hour at room temperature or left untreated as a control. Slides were washed three times for 5 minutes with PBS. Positive and negative controls were incubated with secondary antibodies anti-rabbit AF488 (1:200) to gain GFP fluorescence along with 1:2000 dilution of Hoechst 33342 for 30 minutes covered from light at room temperature. Sections were washed three times in PBS for 5 minutes and fluorescence was detected with the Olympus IX81 fluorescence microscope. Overlay pictures were assembled by analysing and merging the eGFP, Texas Red, AF647 and Hoechst fluorescence channels.

2.2.4.4 Transduction and transplantation into the A. descendens of BALB/c Rag2$^{-/-}$ IL-2R$\gamma_c^{-/-}$ mice

Side branches of the mammarian artery were acquired from patients undergoing coronary bypass surgery as described above. The small vessels were transferred into PBS, washed, transferred into endothelial cell medium and dissected. Transduction of the vessel was performed for 4-12 hours with $7*10^6$ viral particles. Animal surgery was performed by animal technicians from the research group of Gregor Warnecke. BALB/c Rag2$^{-/-}$ IL-2R$\gamma_c^{-/-}$ were anesthetized using isoflurane. The mice underwent laparotomy and the aorta was clamped at two locations within the infrarenal part of the abdominal aorta. The clamped section of the aorta was cut and interposed with the human artery graft as described before (Koulack et al., 1995; Nadig et al., 2010). The abdominal cavity was closed with sutures and mice replaced to their cave to recover.

2.2.4.5 Preparation and transduction of vessels from *Callithrix jacchus* (Marmoset)

Sample vessels from the *Callithrix jacchus* species were acquired from the Institute of Reproductive Medicine of the University in Münster in collaboration with Thomas Müller from the Rebirth Cluster of Excellence at the Medizinische Hochschule Hannover. Vessels were transduced as described above.

2.2.5 Isolation and transduction of human endothelial cells

2.2.5.1 Collagenase digestion and mechanical isolation

Human vessels were acquired and transferred into cultures plates as described above. Collagenase 4 (Sigma-Aldrich) was added to the medium from a 1 mg/ml stock solution to a final concentration of approximately 1.5 pZ-U/ml. Vessels were incubated for 90 minutes at 37°C and the medium was replaced to remove the collagenase. Vessels were cut into slices and transferred into smaller 12-well plates. The luminal side (endothelium) of the vessels were scraped with a scalpel to remove endothelial cells that had dissolved from the basal lamina by collagenase digestion (from now on referred to as a mechanical disruption). This generated cells cultures of primary venous cells (PVCs) and primary arterial cells (PACs). The medium was exchanged every 4 days and cells were allowed to grow for 2-3 weeks before further propagation of dividing cell cultures. In addition, aliquots were transduced with LV-huCD105 or LV-eGFP as a control. Cell growth and cell culture architecture were monitored by native light microscopy.

2.2.5.2 FACS of isolated endothelial cells

Isolated endothelial cells were stained and analysed by FACS as described above using primary antibodies against CD105, CD31, and CD146.

2.2.5.3 Preparation of endothelial cells from *Callithrix jacchus* (Marmoset)

Endothelial cells from the *Callithrix jacchus* (Marmoset) species were isolated following the procedure described above. Cells were monitored with bright-field and direct fluorescence microscopy.

2.2.6 *In vivo* systemic transduction of human endothelial cells

2.2.6.1. Removal of mammarian artery from human patients

Human vessels were acquired from patients undergoing coronary bypass surgery and transferred into cultures plates as described above, quickly transferred into ice-cold PBS and rinsed from blood. Vessels were transplanted at once into the A. descendens of BALB/c $Rag2^{-/-}$ IL-$2R\gamma_c^{-/-}$ mice.

2.2.6.2 Transplantation into A. descendens of BALB/c Rag2$^{-/-}$ IL-2R$\gamma_c^{-/-}$

Side branches of the human mammarian were prepared and rinsed. They were transplanted into an interposing position between the ends of the intersected abdominal aorta of immunosuppressed BALB/c Rag2$^{-/-}$ IL-2R$\gamma_c^{-/-}$ mice by end-to-end anastomosis (2.2.4.4). Mice were left to recover for 48 hours.

2.2.6.3 Systemic administration of the LV-ENG

On day 3 after transplantation of the human vessel into the mice, $4*10^6$ viral particles of LV-ENG were injected into the tail vein and distributed through the body's blood flow. Injections were repeated on day 7 and day 10. Mice were sacrificed after 16 days to allow strong expression of the eGFP marker gene signalling infection by LV-ENG.

2.2.6.4 Immunohistofluorescence

Human grafts located in the A. descendens of transplanted mice were macroscopically identified and removed for preparation for immunohistofluorescence as described before (2.2.4.3). As controls, V. cava inferior, heart, lung, and liver were removed and processed along with the human graft for comparative analysis.

3. Results

3.1 Cloning of human ASGR1 and scFv-huASGPR

3.1.1 Cloning of human ASGR1

Human ASGR1 was successfully amplified from HepG2 cDNA by polymerase chain reaction (figure 18, left) and subcloned into the pCR4®TOPO® vector containing the *Xho*I and *Hind*III restriction sites flanking the ASGR1 gene. Restriction analysis using *Xho*I and *Hind*III verified the presence of the ASGR1 insert in the pCR4®TOPO® vector. Sequencing with M13 forward and reverse primers supplied by Invitrogen confirmed the absence of point mutations (data not shown). The ASGR1 cDNA was cloned into the pcDNA3.1/Zeo(+) eukaryotic expression vector to yield pcDNA3.1/Zeo(+)-huASGPR. Insertion of the gene of interest was verified by DNA-sequencing using specifically designed primers and restriction analysis. Digestion with *Xho*I and *Hind*III of pcDNA3.1/Zeo(+)-huASGPR revealed the presence of a 4.94 kb band and another one at 0.88, the first one representing the linearized vector and the latter one the insert, respectively (figure 18 right).

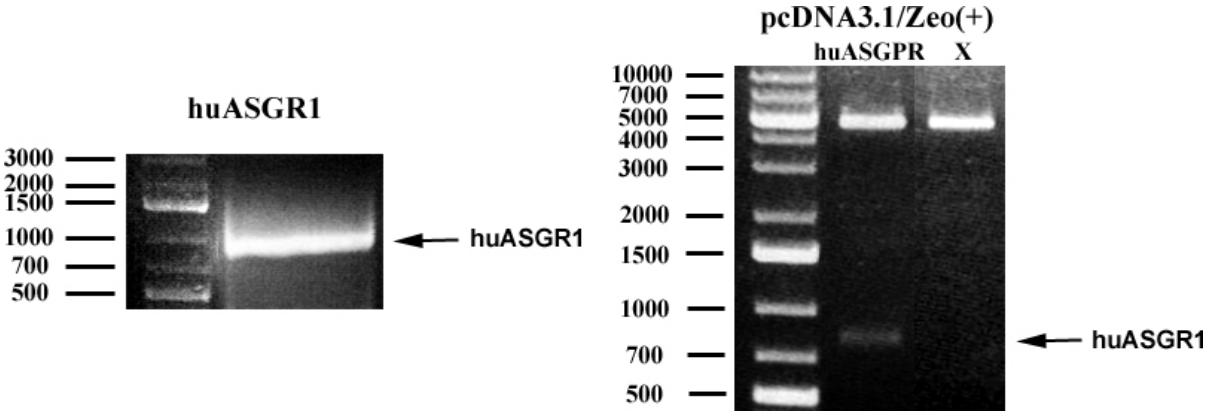

Figure 18: Cloning of the human ASGR1 gene. left The ASGR1 amplification product at 0.88 kb was electrophoresed and visualised by ethidium bromide. **right** ASGR1 was cloned into the pcDNA3.1/Zeo(+) vector and insertion of the cDNA was analysed by restriction digest with *Xho*I and *Hind*III (**huASGR1:** pcDNA3.1/Zeo-huASGPR, bands at 4.93 kb represents the linearised vector and 0.88 kb the gene insert; **X:** empty vector, linearised pcDNA3.1/Zeo(+) at 4.93 kb). The pictures are representatives from three independent experiments.

3.1.2 Cloning of scFv-huASGPR

3.1.2.1 Design of the scFv polypeptides

In preparation for this study, scFv-huASGPR nucleotide sequences were acquired by searching articles for the keyword “ASGPR scFv” or “ASGPR single chain antibody” in the PUBMED database in October 2009 and February 2010. The searches returned two publications with sequences for scFv antibodies directed against human ASGPR published by Cao and co-workers and Tratenherts and Benhar (Cao et al., 2006; Tratenherts and Benhar, 2009). The first

nucleotide sequence, referred to as scFv-Cao, contained 720 base pairs resulting in the expression of a 240 amino acid polypeptide. The second polypeptide, referred to as scFv-benhar, consisted of 243 amino acids. *In silico* modelling using the "XY"-algorithms, which is not explained in detail in this study because of its complexity, predicted unfortunate secondary structures within the scFv-benhar polypeptide (Friedel et al., 2015 and personal communication with research group Buchholz 2009-2015). Therefore, the algorithms suggested an amino acid exchange at position 193 (S193P) to optimise surface expression of the scFv-polypeptide. The modified scFv polypeptide was named scFv-benharP. The scFv nucleotide sequences were inserted into the previously described measles virus vector to generate a fusion protein with the mutated hemagglutinin glycoprotein (Funke et al., 2010). Expression analysis of the fusion protein was made possible by the addition of a C-terminal His-tag.

When comparing the two scFv polypeptides scFv-Cao and scFv-benhar with each other, one finds a higher variance of amino acids in the variable region of the light chain. Based on the scFv-benhar sequence, 17 out of 115 amino acids are replaced in scFv-Cao in the variable heavy chain, resulting in 85.2 % sequence homology. However, 56/110 amino acids are exchanged in the variable light chain, leading to a homology of just 49%. Thus, the main difference between the two scFv antibodies lies within the variable light chain rather than the whole molecule (figure 19).

```
scFv-benhar      (1) EVQLLESGGGLVQPGGSLRLSCAASGFTFSDYYMSWVRQAPGKGLEWVSA
scFv-benharP     (1) EVQLLESGGGLVQPGGSLRLSCAASGFTFSDYYMSWVRQAPGKGLEWVSA
scFv-Cao         (1) EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSG

scFv-benhar     (51) ITTGGGSPNYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCARDR
scFv-benharP    (51) ITTGGGSPNYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCARDR
scFv-Cao        (51) INGTGYTTSYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKS-
                                  VH←|       linker       |→VL
scFv-benhar    (101) TAGYFDYWGQGTTVTVSSGGGGSGGGGSGGGGSQSVLTQ--PPSASGTPG
scFv-benharP   (101) TAGYFDYWGQGTTVTVSSGGGGSGGGGSGGGGSQSVLTQ--PPSASGTPG
scFv-Cao       (100) -GGDFDYWGQGTLVTVSSGGGGSGGGGSGGGGSTDIQMTQSPSSLSASVG

scFv-benhar    (149) QRVTISCTGSSSGIGNAYVSWYQQLPGKAPKLLIYKNGQRPSGVSDRFSG
scFv-benharP   (149) QRVTISCTGSSSGIGNAYVSWYQQLPGKAPKLLIYKNGQRPSGVPDRFSG
scFv-Cao       (149) DRVTITCRASQS--ISSYLNWYQQKPGKAPKLLIYSASSLQSGVPSRFSG
                                                        VL←|
scFv-benhar    (199) SKSGTSASLAISGLRSEDEADYYCAAWDDSLNGWVFGGGTKLTVL-AAAR
scFv-benharP   (199) SKSGTSASLAISGLRSEDEADYYCAAWDDSLNGWVFGGGTKLTVL-AAAR
scFv-Cao       (197) SGSGTDFTLTISSLQPEDFATYYCQQSSSNPN--TFGQGTKVEIKRAAAR
                        His-tag
scFv-benhar    (248) GSHHHHHH
scFv-benharP   (248) GSHHHHHH
scFv-Cao       (245) GSHHHHHH
```

Figure 19: Amino acid sequences of scFv-huASGPR. Three different scFv-huASGPR antibodies were included in this study: scFv-Cao, scFv-benhar and scFv-benharP. A linker connects variable regions of the heavy region (**V_H**) and of the light region (**V_L**). Note the amino acid exchange at position 193 (scFv-benhar) from serine to proline in scFv-benharP. The variable light chain is followed by a His-tag to identify expression of the novel scFv polypeptides.

3.1.2.2 Cloning of scFv into hemagglutinin pseudotyping vectors

The scFv-Cao, -benhar, -benharP and -huCD105 nucleotide sequences were synthesised by MWG Operon and inserted into a pCG expression vector with flanking *Sfi*I and *Not*I restriction sites. Nucleotides were digested with appropriate restriction enzymes and ligated into the pHL3nse-vector, yielding pHL-Cao, pHL-benhar, pHL-benharP and pHL3nse-scFv-A5/pHL-CD105. pHL-Cao and pHL-CD105 were prepared by our collaboration partner at the Paul-Ehrlich Institut in Langen Germany. Insertion of the scFv nucleotides into the pHL3nse-vectors was confirmed by restriction analysis, showing the presence of a ~0.7 kb band when digested with *Sfi*I and *Not*I (figure 20).

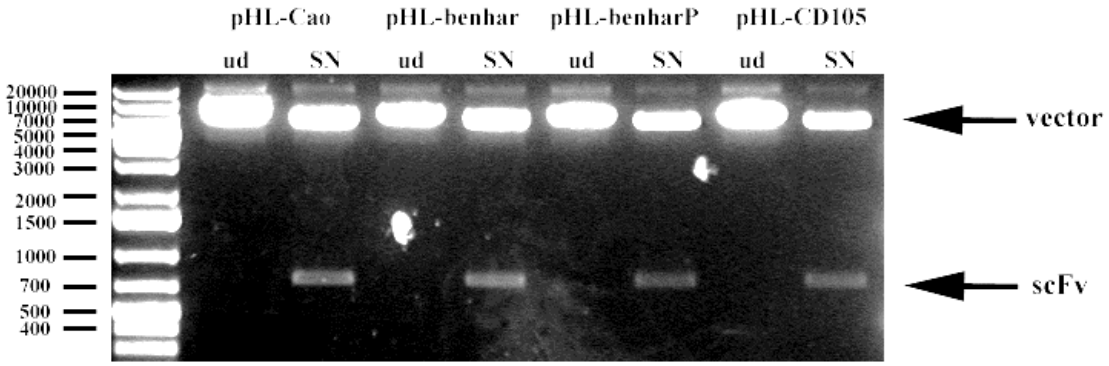

Figure 20: Restriction of pHL-scFv vectors. The pHL3nse-vectors containing the scFv nucleotides were digested with *Sfi*I and *Not*I (**SN**) or not (**ud**). Digestion resulted in the appearance of the linearised pHL-vectors (**vector**) and a band representing the excised scFv insert (**scFv**). The pictures are representatives from three independent experiments.

3.1.3 Recombinant expression of human ASGPR and His-tagged scFv

To test recombinant expression of human ASGPR and scFv polypeptides from its respective eukaryotic expression vector, HT1080 and HEK293T cell lines were transiently transfected with the respective vectors. Expression of the recombinant polypeptides was allowed for three days and cells were prepared for expression analysis by FACS using fluorescence-labeled antibodies.

FACS analysis of HT1080 cells transfected with pcDNA3.1/Zeo(+)-huASGPR revealed that recombinant human ASGPR was detected by the anti-ASGPR antibody. In repeating experiments, approximately 10-17.7% of transfected HT1080 cells showed ASGPR expression (with figure 21 showing the sample with the strongest expression of ASGPR). Stable transfection with linearised pcDNA3.1/Zeo(+) and selection of positive clones by Zeomycin

increased the proportion of ASGPR expressing cells (data not shown). However, this approach was very time-consuming and did not improve results in subsequent experiments.

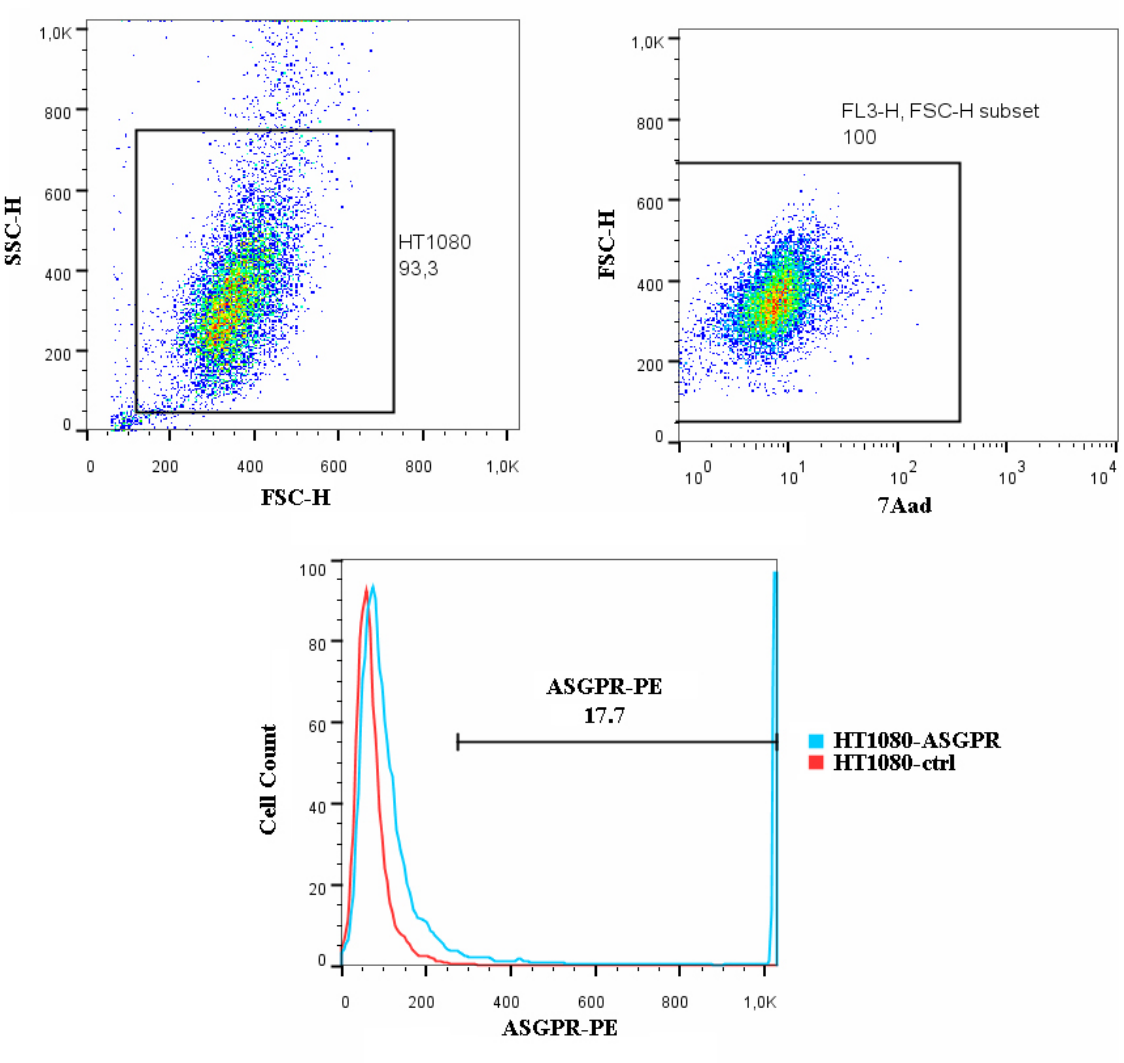

Figure 21: Expression of ASGPR. HT1080 cells were transiently transfected with pcDNA3.1/Zeo(+)-huASGPR (**HT1080-ASGPR**) or pcDNA3.1/Zeo(+) as control (**HT1080-ctrl**) and cells were harvested after three days. Cells were prepared for FACS analysis using an anti-ASGPR-PE antibody to detect surface expression of ASGPR. 17.7 % of HT1080-ASGPR show distinct expression of ASGPR. Each plot is representative for three independent experiments.

For expression of the scFv polypeptides, HEK293T cells were transfected with appropriate pHL3nse constructs (pHL3nse-scFv-CRDH1 and pHL3nse-scFv-CD105). Since no antibodies were commercially available to detect expression of the scFv polypeptides, they were fused to His-tags allowing detection with anti-His antibodies. FACS analysis using the anti-His antibody revealed surface expression of all four scFv-constructs (scFv-Cao, scFv-benhar, scFv-benharP, scFv-CD105). Surface expression of the scFv polypeptides proved variable reaching from 7.8

% (scFv-Cao) to 32.3 % (scFv-CD105) of transfected HEK293T cells being positive for His-tag expression (figure 22 C-F).

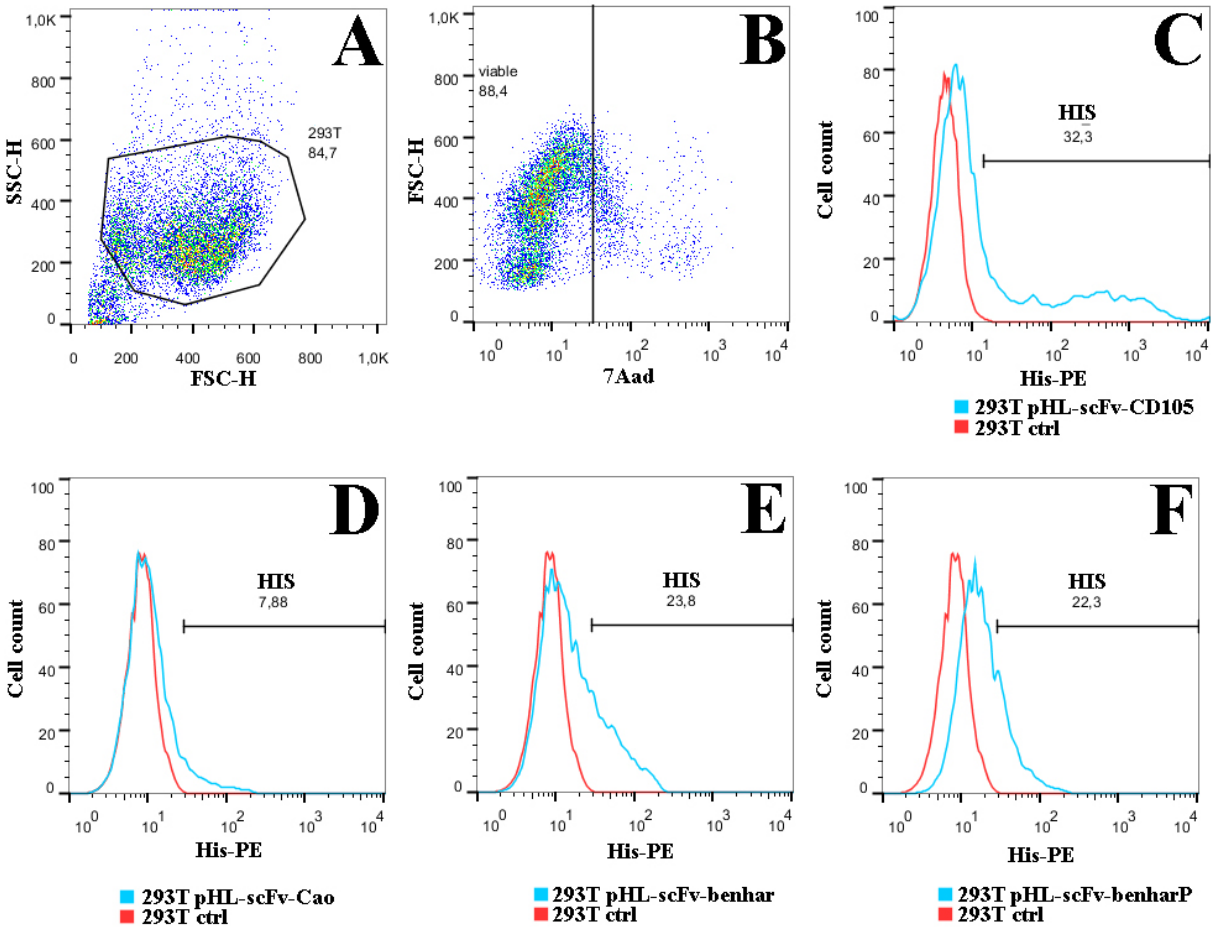

Figure 22: scFv surface expression. HEK293T cells were transiently transfected with pHL-scFv constructs, harvested after three days and prepared for FACS by staining with an anti-His-PE antibody. **A, B** Cells were gated and stained for viable cells. **C-F** His-PE fluorescence of viable cells was detected as a measurement for scFv-expression. scFv-CD105 demonstrated strongest surface expression (**C**, 32.3%) while scFv-Cao (**D**, 7.88%) showed weak expression. scFv-benhar (**E**, 23.8) and scFv-benharP (**F**, 22.3%) showed moderate-strong expression. Each plot is a representative for three independent experiments.

3.1.4 Endogenous expression of ASGPR

HepG2 cells were prepared for FACS analysis to detect endogenous ASGPR expression. Interestingly, HepG2 cells demonstrated inconsistencies with regard to ASGPR expression: In ASGPR$^+$ HepG2 cells, ASGPR expression was variable within the same batch of cells reaching from 40-92%, with strongest ASGPR expression detected in less often propagated cells (figure 23). Expression of ASGPR seemed highly variable between different batches: HepG2 cells acquired from another laboratories in the Twincore facility did not express ASGPR. Experiments were repeated with Huh 7 cells, where ASGPR expression could not be identified in any of the tested batches (data not shown).

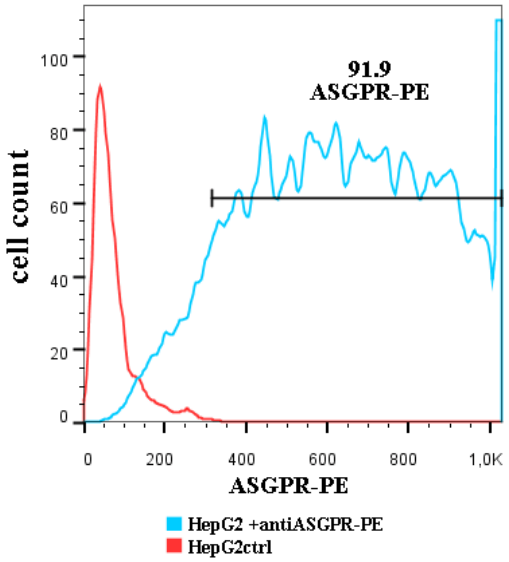

Figure 23: HepG2 ASGPR expression. HepG2 cells were prepared for FACS analysis of surface ASGPR expression. This plot is a representative of the HepG2 cell batch with the strongest expression. The HepG2 cells had been propagated 10 times when FACS analysis was performed. The plot is a representative from three independent experiments measuring the ASGPR expression of one batch of HepG2 cells.

3.2 Transduction with lentiviral vectors pseudotyped with measles virus hemagglutinin fused to scFv-ASGPR

3.2.1 Transduction of human hepatic cell lines with LV-ASGPR

After surface expression of the hemagglutinin-scFv fusion polypeptides had been successfully tested we initiated lentiviral vector production using HEK293T cells. Vector titers usually need to be determined with appropriate target cells that can be transduced with the lentiviral vector. For this reason, the human hepatic ASGPR$^-$ Huh7.5 and HEK293T cell lines (control cell lines) and HepG2 cells expressing ASGPR were incubated with 20x concentrated lentiviral vectors LV-ASGPR (LV-Cao, LV-benhar and LV-benharP). All cell lines were efficiently transduced with the control vector LV-eGFP. Unfortunately, both control cell lines and HepG2 remained non-transduced by LV-Cao and LV-benhar (data not shown), even at high vector volumes.

In addition, also primary human hepatocytes were subject to transduction by LV-Cao, LV-benhar and LV-benharP. Again, no transduction of target cells was detected (data not shown). As target cells were not transduced by LV-scFv-ASGPR it became impossible to calculate accurate lentiviral titers of LV-ASGPR.

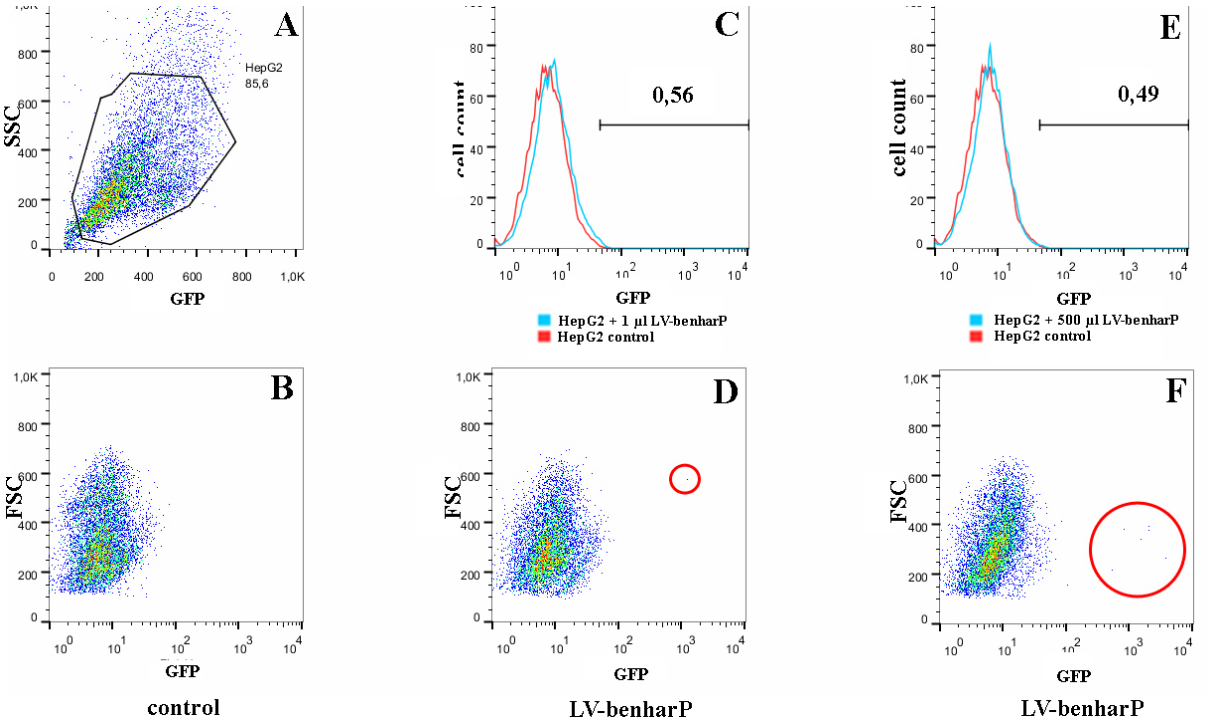

Figure 24: HepG2 cells transduced with LV-benharP. HepG2 cells were treated with either 1 µl (C, D) or 500 µl (E, F) of LV-benharP. **A** Cells were prepared for FACS and gated for detection of GFP expression. **B** In untreated cells, no GFP expression was detected (HepG2 control). HepG2 cells treated with LV-benhar did not demonstrate any eGFP-like fluorescence at all. **C-E** HepG2 cells treated with increasing amounts (1µl and 500 µl) of 20x concentrated LV-benharP showed distinct granulous GFP fluorescence spots, which appeared as outliers in the FACS analysis, (D, F, red circles). The unspecific staining did not lead to an increase of the GFP-positive fraction of transduced HepG2 cells (C, E). The plots are representatives from three independent experiments.

3.2.2 Transduction of HT1080 cells with LV-Cao and LV-benhar

HT1080 fibroblast and HEK293T cell lines transiently expressing human ASGPR were generated to test whether transduction by antigen-specific LV-ASGPR could be induced by recombinant expression of ASGPR. Cells were treated with 20x concentrated LV-Cao, LV-benhar and LV-benharP, and transduction was analysed by FACS and direct fluorescence microscopy. Unfortunately, no cells were transduced by the lentiviral vectors LV-Cao and LV-benhar (figure 25). However, if treated with 10 µl of LV-benharP, a small fraction of approximately 5.81 % of HT1080-ASGPR and 4.22 % in control HT1080 cells showed GFP fluorescence, demonstrating the ability of this specific vector to transduce HT1080 human fibroblast cells (figure 25). However, both ASGPR$^+$ and control HT1080 cell lines were equally transduced in repetitive experiments, indicating unspecific transduction with of LV-benharP independent of ASGPR expression on cells. Transduction efficiencies using LV-benharP remained similar between HT1080-ASGPR and HT1080 control cells even if transduced with high lentiviral vector volumes. Experiments were repeated unsuccessfully using HEK293T

transiently expressing ASGPR (data not shown). Also here, the inability to conduct stable transduction with LV-ASGPR made it impossible to calculate LV-ASGPR titers.

Thus, with two anti-ASGPR lentiviral vectors yielding failing to transduce ASGPR$^+$ target cells we stopped further attempts in this direction.

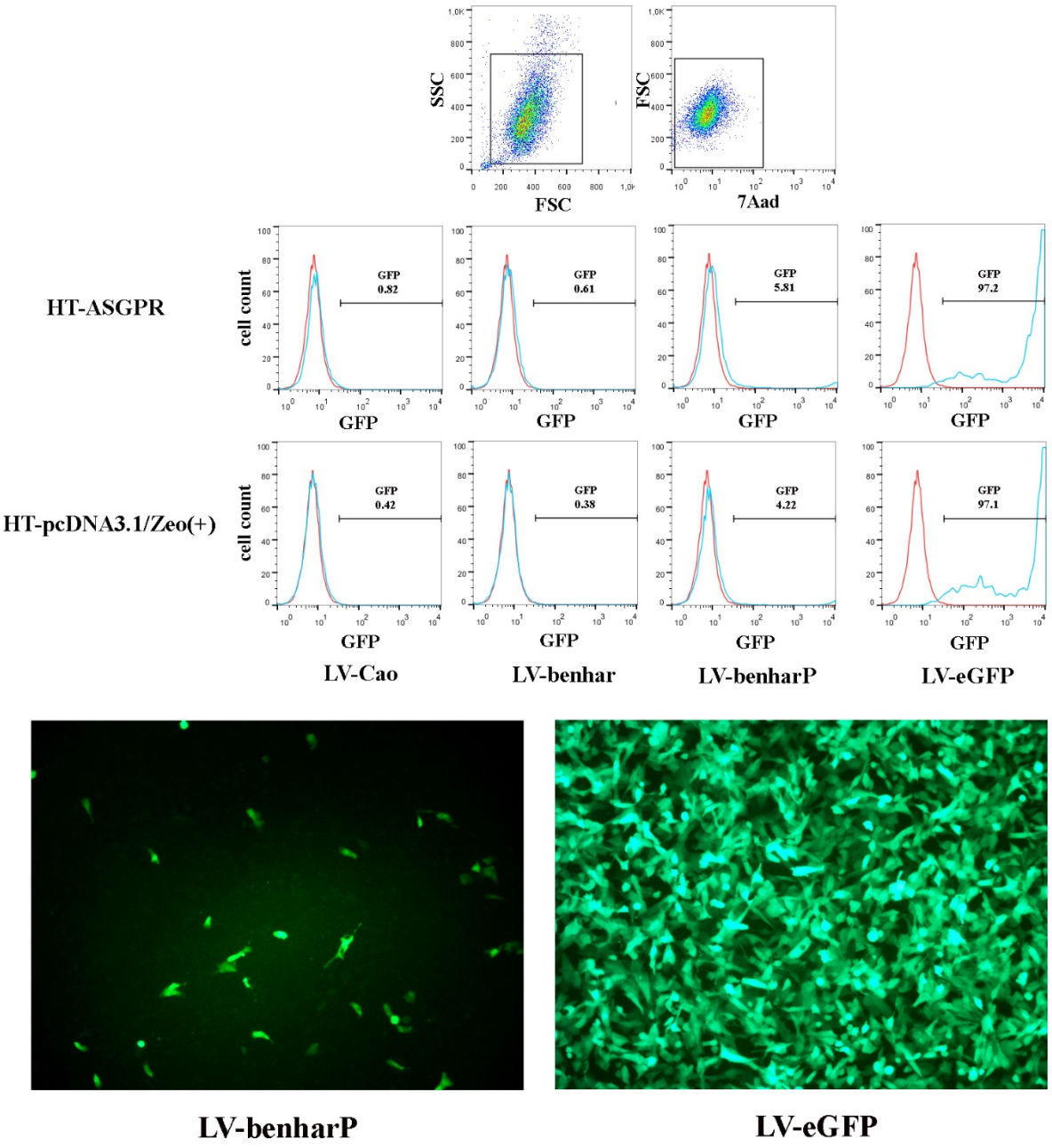

Figure 25: Transduction of HT-ASGPR cells. HT1080 cells were transiently transfected with pcDNA3.1/Zeo(+)-ASGPR (**HT-ASGPR**) or empty vector pcDNA3.1/Zeo(+) (**HT1080-pcDNA3.1/Zeo(+)**) and transduced with **LV-Cao**, **LV-benhar**, **LV-benharP** or **LV-eGFP** as a control. While both cell lines were efficiently transduced by control vector LV-eGFP (~97%), no cells were transduced by LV-Cao and LV-benhar. Approximately 4-6% of the cells were transduced by 10 µl of LV-benharP in both HT-ASGPR and the control group. The difference was not statistically significant, demonstrating the non-specificity of the LV-benharP towards ASGPR. Direct fluorescence microscopy pictures show HT1080 cell transduced with LV-benharP (**left**) and LV-eGFP (**right**) for comparison. The plots and pictures are representatives for three independent experiments.

3.2.3 Production efficiency of LV-huCD105

Production of lentiviral vectors specific to huCD105 (LV-huCD105) and transduction of HUVECs has been successful in previous reports (Funke et al., 2010). Lentiviral vectors were generated using HEK293T producer cells. LV-huCD105 was concentrated approximately 20-fold and titers were calculated by measuring transduction efficiency of HUVEC cells as previously described (Schambach et al., 2006). Vector titers proved to be highest 24-36 hours after transfection of HEK293T cells, reaching a mean titer of approximately $7.03*10^7$ (+/- $9,66*10^6$) infectious particles per millilitre 24 hours after transfection and $6.1*10^7$ (+/- $1.51*10^7$) 36 hours after transfection. Titers of LV-huCD105 were rapidly declining 48 hours after transfection and were not used for further experiments (figure 26, A).

Previous experiments had shown that LV-huCD105 was specific for cells expressing human CD105 (Funke et al., 2010). In preparation for this study, several transduction experiments involving human cell lines were carried out to examine the specificity of LV-hu-CD105. Just to mention a few, human glioblastoma multiforme cell line T98G, human thymoma cell line EL4, cervical carcinoma cell line HeLa, hepatic cell lines HepG2, Huh7, and Huh7.5 failed to be transduced by LV-huCD105, showing that LV-huCD105 is not able to transduce this specific subset of CD105 negative cells. On the other hand, the $CD105^+$-HT1080 fibroblast cell line described above was transduced by LV-huCD105 to some extent.

In addition, various non-human mammalian cell lines were included to test transduction susceptibility by LV-huCD105. These tests also included murine brain endothelial cell line b.End3, representing a cell line expressing murine CD105. Repeated experiments confirmed that these cell lines could not be transduced by LV-huCD105 (figure 26, C).

Later experiments also included testing of marmoset endothelial cells isolated from the abdominal aorta representing endothelial cells from another primate species. However, transduction of the cells was also unsuccessful (figure 38).

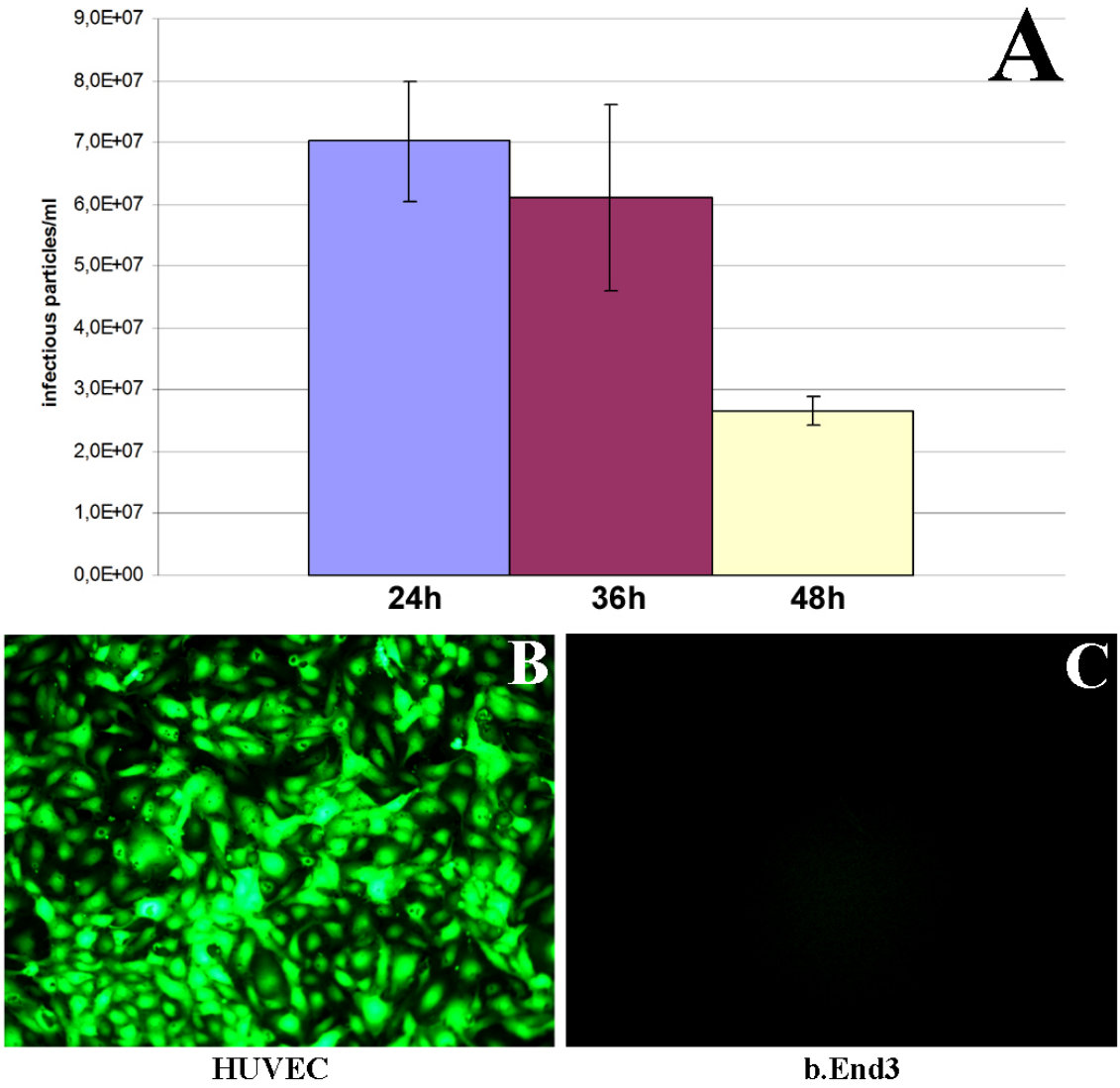

Figure 26: Production and transduction of LV-huCD105. A LV-huCD105 were produced in HEK293T cells and titers were determined by FACS analysis of transduced HUVECs. Mean titers of LV-huCD105 were between $2.0*10^7$ to $1.0*10^7$ infectious particles per millilitre. **B** HUVEC cells were efficiently transduced by LV-huCD105. **C** To examine the specificity against human endothelial cells, the murine endothelial cell line b.End3 expressing murine CD105 was exposed to LV-huCD105. LV-huCD105 was not capable of transducing b.End3 cell line. The pictures are representatives from at least three independent experiments.

3.2.4 Vein infection

As transduction experiments of human endothelial cell lines were promising, additional experiments involving life tissue were designed to test the lentiviral vectors in a more elaborate setting. For this reason, human saphenous veins and human mammary arteries were acquired from patients undergoing coronary bypass surgery at the Department of Thoracic Surgery at the Medizinische Hochschule Hannover, Germany. The vessels were quickly transferred into ice-cold PBS and rinsed from excessive blood. Cleaned vessels were initially cut into smaller pieces measuring approximately 5-30 mm, cultivated in human endothelial growth medium and

transduced with approximate titers of $5*10^7$ infectious particles of LV-huCD105. GFP expression of transduced tissue cells appeared late after approximately 7-10 days after transduction, quite in contrast to non-primary cells that show GFP expression within 2-3 days after transduction with lentiviral vectors. Initial experiments with both LV-huCD105 and LV-eGFP transduced vessels resulted in low transduction efficiencies, but results were improved as handling of the samples was optimised (figure 27, A and B). As transduction events were rare, it became impossible to evaluate whether LV-huCD105 did transduce fewer cells than unspecific LV-eGFP, as would be expected.

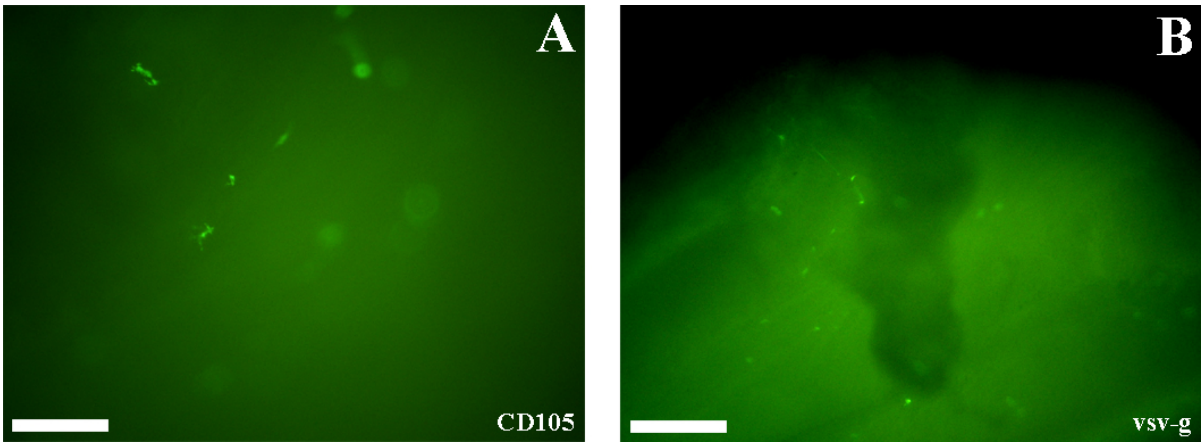

Figure 27: Infection of human veins. Human saphenous veins were acquired from patients undergoing coronary bypass, transferred into cell culture dishes containing endothelial growth medium and treated with LV-huCD105 (**A**) or VSV-G pseudotyped LV-eGFP (**B**) as a control. The pictures are representatives from one vein transduced approximately three hours after surgery. Bars represent 250 µm (**A**) and 600 µm (B).

In general, survival of tissue cells was experienced to be more dependent on the handling of the sample and the duration from surgery until transduction. Therefore, several experiments were carried out to optimise the procedures and to shorten the latency until actual transduction of the vessels occurred. In the end, sample processing involving clamping, removal from the patient, rinsing in ice-cold PBS, transfer into the appropriate medium and transduction was reduced to a total duration of approximately 50 minutes. Workflow optimisation markedly increased i) the transduction efficiencies in both venous and arterial tissue and ii) the efficiency to isolate primary cells from the vessels. Generally spoken, handling of venous tissues was easier compared to that of arterial tissue. As a consequence, venous vessels were more readily transduced and isolation of primary cells from the vein was more successful.

Saphenous veins cut into pieces not longer than 10 mm were transduced with LV-huCD105 and LV-eGFP as positive controls. The veins were covered with endothelial cell medium and monitored by immunohistofluorescence microscopy. The vessels transduced with LV-

huCD105 showed intense GFP expression after 7-10 days with the highest intensity located near the lumen of the vessel (figure 28 A, B). The specific lumen-near distribution of LV-huCD105 transduced cells was later confirmed by immunohistofluorescence. Vessels transduced with LV-eGFP demonstrated more intense expression of eGFP, both in regard to fluorescence intensity and the total number of transduced cells. However, transduced cells appeared more randomly distributed than in vessels transduced with LV-huCD105 (figure 28 C, D).

In a series of experiments, factors positively contributing to transduction efficiency were identified. Transduction efficiency positively correlated with the transduction length if cells were transduced for four to 24 hours. Transduction rates were also improved if the vessels were repeatedly infected with lentivirus three days in a row. Interestingly, transduction events were more abundant if vessels were intensively treated with both enzymatic digestion and mechanical disruption before adding lentiviral vectors if compared to enzymatic digestions only.

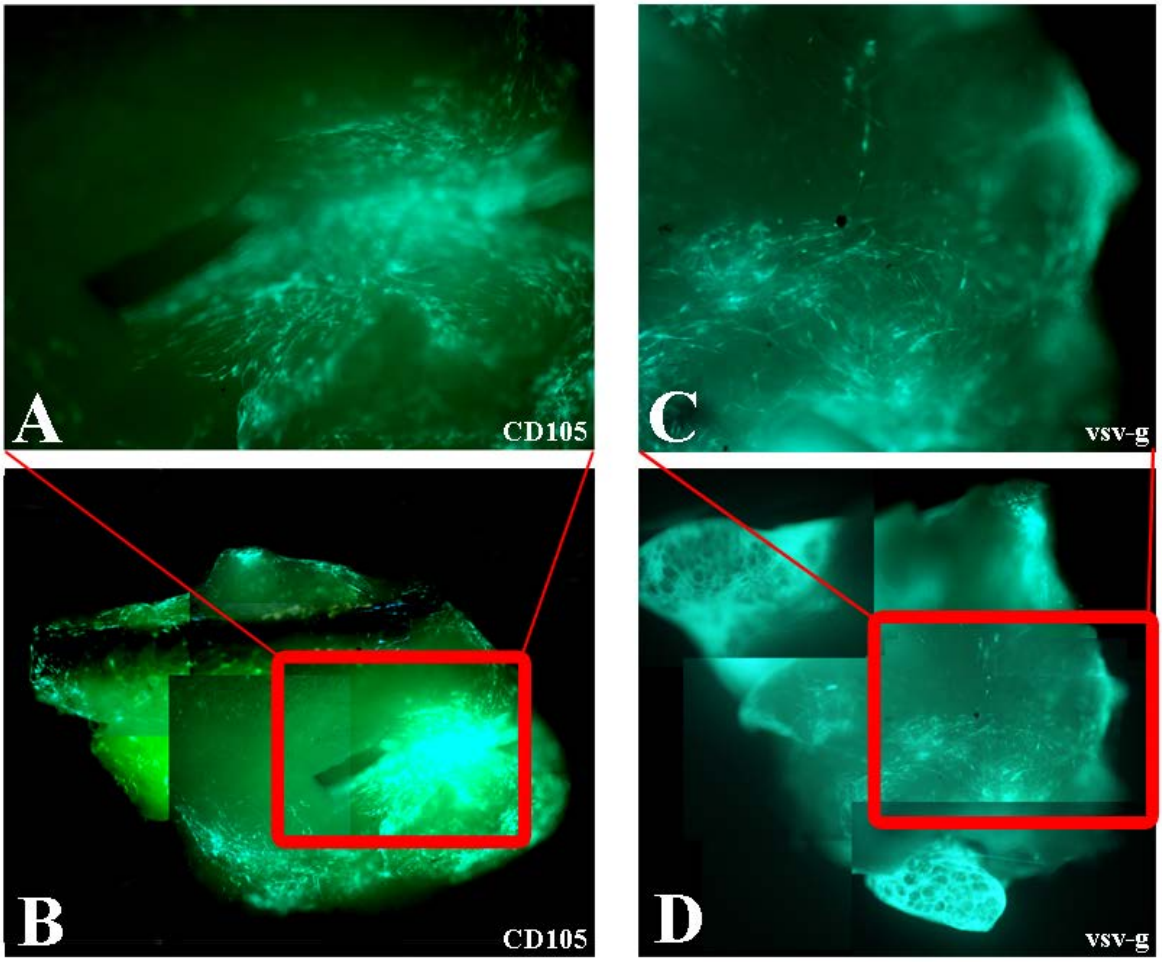

Figure 28: Infection of human saphenous veins. Human saphenous veins were acquired from patients undergoing coronary bypass, transferred into cell culture dishes containing appropriate medium and treated with LV-huCD105 (A, B) or VSV-G pseudotyped LV-eGFP (C, D) as a control. **A** Veins transduced with LV-huCD105 show intense lumen-near expression of GFP. **B** Overview of a human vein transduced with LV-huCD105. **C** Veins transduced with control LV-eGFP demonstrated transduction events that appeared more randomly distributed. **D** Overview of the vein transduced with LV-eGFP. GFP expression is more evenly scattered and more intense in veins transduced with VSV-G pseudotyped LV-eGFP. The pictures are representatives from one vein transduced approximately 50 minutes after surgery and show more intense staining than in previous experiments with increased latency between surgery and transduction. The same samples were prepared for immunohistofluorescence staining as demonstrated below. Experiments were successfully repeated with three veins, although with varying transduction rates.
Assembly of the pictures: Raw picture data were obtained by detecting GFP fluorescence at 15 (VSV-G: seven) adjacent locations of the human vein explant using the fluorescence microscope Olympus FV1000. To obtain an overview of the whole vein, the 15 (VSV-G: seven) pictures were stitched together using Adobe Photoshop software. The noise was reduced by balancing colour levels. Due to the assembly of pictures that had been pre-processed by Cell^M Olympus Imaging software during detection it was not possible to create an image where edges smoothly fitted to each other. This resulted in vertical and horizontal lines that can be observed in B and D. Hangover areas located in the corners of the assembled picture were filled with black colour to generate a high-contrast background.

To further identify the characteristics of the transduced luminal cells immunohistofluorescence was performed. Serial sections of transduced veins were prepared for immunohistofluorescence and stained for huCD105 and GFP. Co-localisation of $CD105^+$ cells (figure 29 A, E) and GFP^+ (figure 29 B, F) transduced cells was detected in cells lined up reminding of an endothelial boundary to the lumen of a vessel (figure 29 D, H).

Unfortunately, initial serial sections of transduced veins were not conserved as a whole piece but ripped apart in the course of processing for immunohistofluorescence. However, as handling was improved, some veins were maintained in one piece also after the strenuous treatment with paraformaldehyde, freezing and sectioning in preparation for immunohistofluorescence. As endoglin and GFP-expression were analysed in the well-preserved cross-sections of a transduced vein one could clearly locate co-localisation of GFP^+/$huCD105^+$ cells to the inner endothelial layer facing the lumen (figure 30).

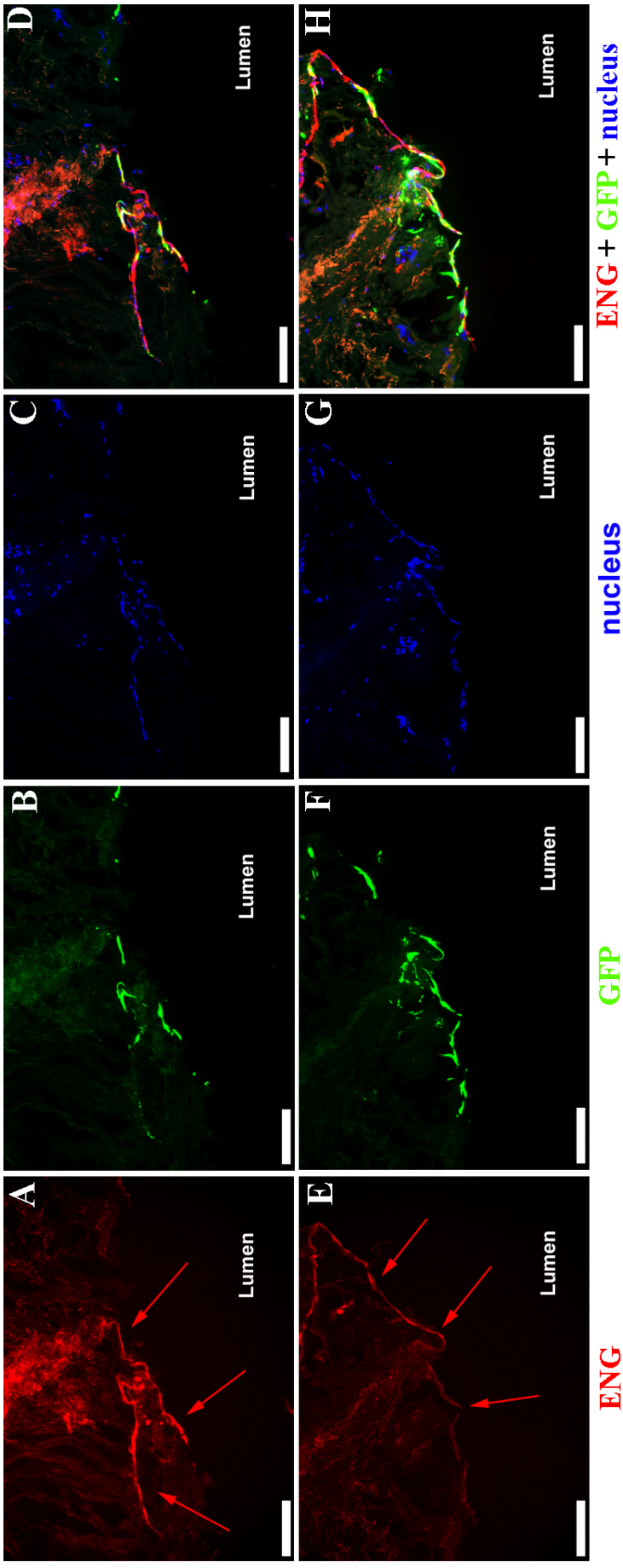

Figure 29: Immunohistofluorescence of cross-sectioned, transduced human saphenous veins. Human saphenous veins were acquired from patients undergoing coronary bypass, transferred into cell culture dishes containing appropriate medium and treated with LV-huCD105. Veins transduced with LV-huCD105 were prepared for immunohistofluorescence staining with anti-huCD105 (**A, E, red**), anti-GFP (**B, F, green**) and Hoechst nucleus staining (**C, G, blue**). **D, H** Co-localisation of $CD105^+$ and transduced GFP^+ cells (yellow). Transduced GFP^+-cells are lined up along with $huCD105^+$ cells reminding of an endothelial cell layer facing the lumen in a vessel. These pictures are representatives from three independent experiments using human saphenous veins. Bars represent 250 µm.

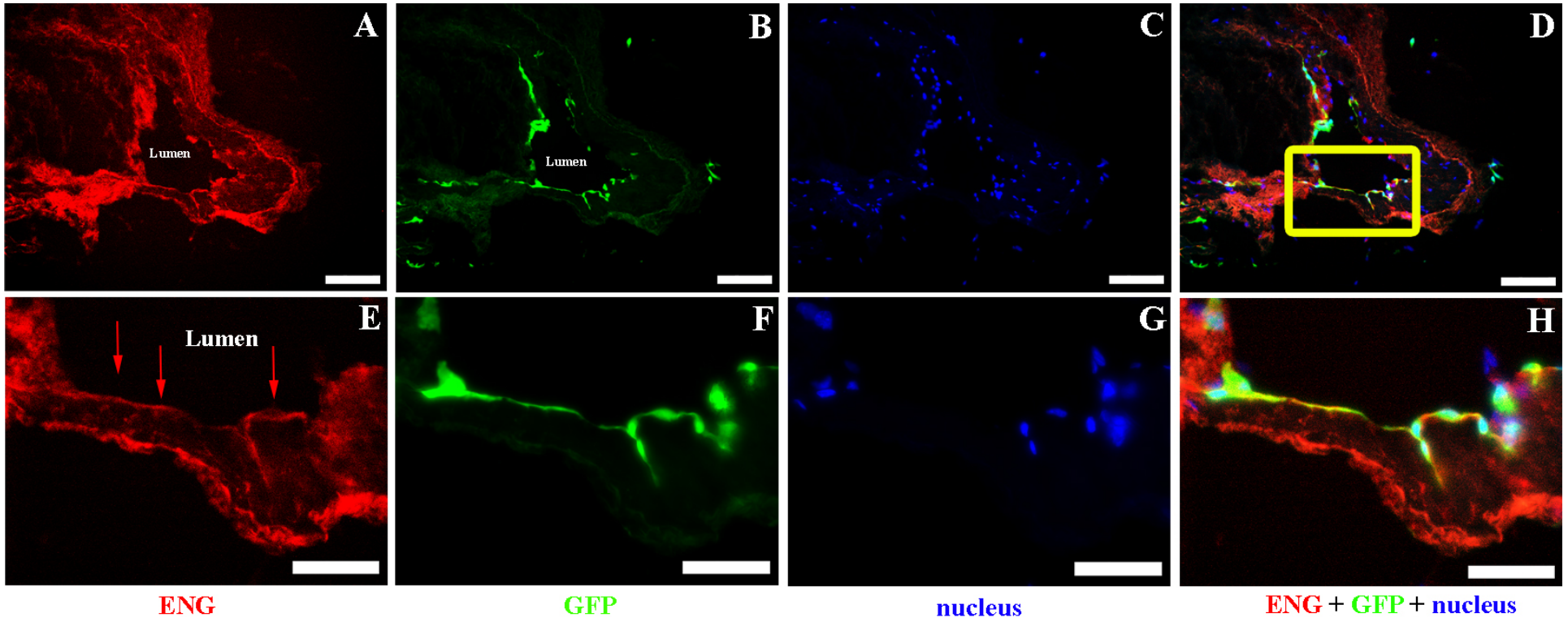

Figure 30: Immunohistofluorescence of transduced veins, cross-section. Human saphenous veins were acquired from patients undergoing coronary bypass, transferred into cell culture dishes containing appropriate medium and treated with LV-huCD105. Human saphenous veins transduced with LV-huCD105 were prepared for immunohistofluorescence staining with anti-huCD105 (**A and E, red**), anti-GFP (**B, and F, green**) and Hoechst nucleus staining (**C and G, blue)**. **D** Overlay picture of CD105, GFP and cell nucleus staining (A-C). The yellow box marks the area which is presented in E-H. **E-H** Magnified sections of corresponding pictures A-D. **H** Co-localisation of CD105+ and GFP+ cells facing the lumen of the vessel used GFP^+-cells (green-yellow). These pictures are representatives from three independent experiments using one well-preserved saphenous vein. Bars represent 250 µm in A-D, and 50 µm in E-H.

3.2.5 Artery infection

Complementary to human veins, human mammary arteries were subject to *ex vivo* transduction with LV-huCD105. Just like their venous counterparts, flow processes had to be optimised to achieve high transduction efficiency and to acquire primary cells from arteries (figure 31). In general, limitation of the time until the artery was transferred into medium and fast transduction after surgery were found to be the most crucial factors influencing transduction success. Arteries transduced with VSV-G pseudotyped LV-eGFP demonstrated higher GFP^+-fluorescence intensity of transduced cells (figure 31 D, F) than arteries transduced with equal amounts of LV-huCD105. However, arteries transduced with LV-huCD105 were also readily transduced (figure 31 A, B, C, E). In general, arteries were more fragile and transduction efficiency was lower compared with transduction experiments involving human saphenous veins.

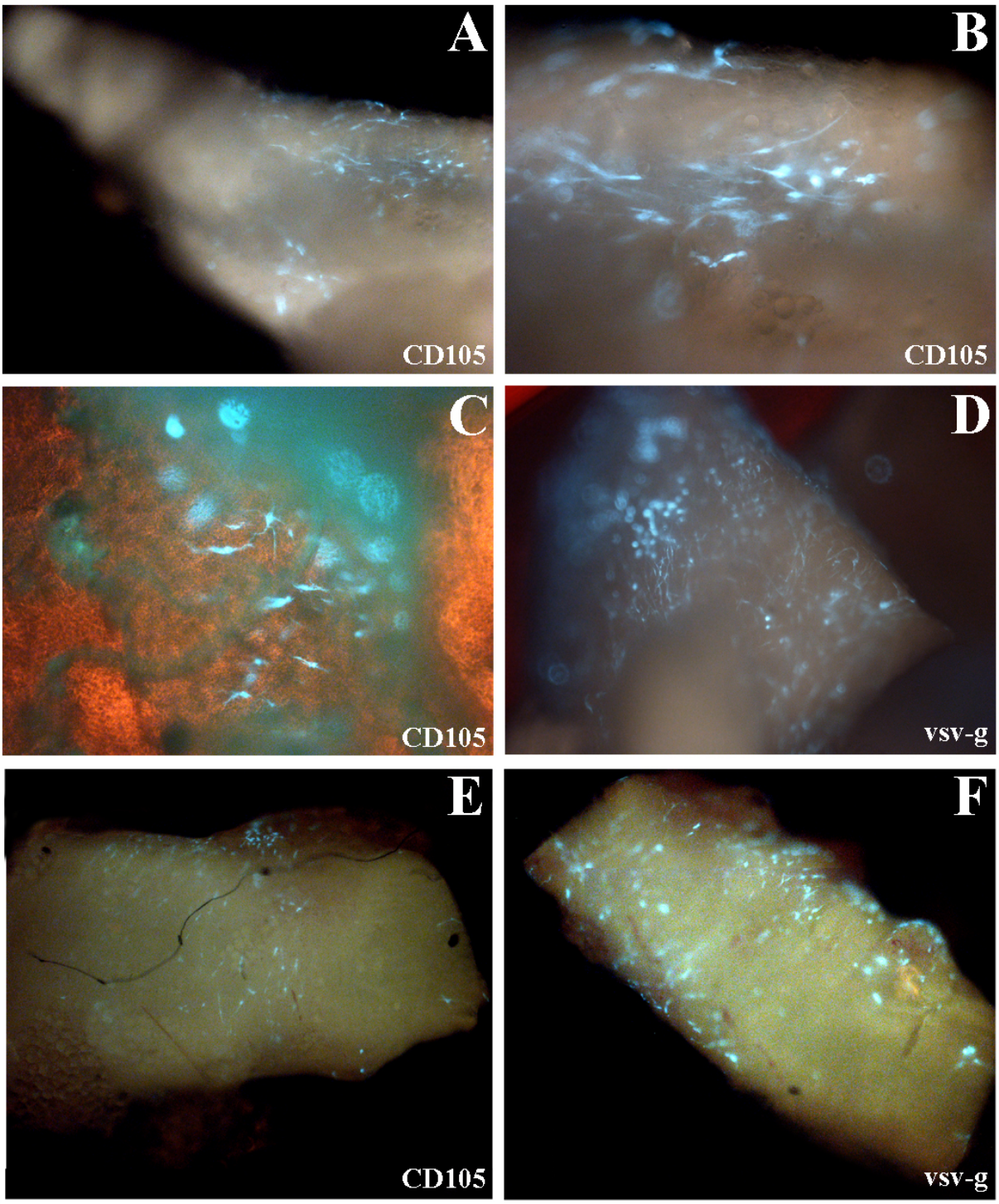

Figure 31: Transduction of a human artery. Human mammary arteries were acquired from patients undergoing coronary bypass, transferred into cell culture dishes containing appropriate medium and treated with LV-huCD105 (A, B, C, E) or VSV-G pseudotyped LV-eGFP (D, F) as a control. In general, arteries were more efficiently transduced with LV-eGFP than with LV-huCD105. The pictures are representatives from arteries transduced approximately 1.5 hours (A-D) and 50 minutes (E, F) after surgery. The artery depicted in E and F shows more intense staining than the other one due to the shorter duration between surgery and transduction. The pictures are representatives from two independent experiments, respectively.

3.2.6 Primary cell isolation

3.2.6.1 HUVECs

The isolation of HUVECs was performed by collaborating laboratories at the surgical and the paediatrics' department due to their access to human umbilical cords. To characterise the acquired HUVECs, an analysis of the expression profile was conducted. As HUVECs change expression characteristics and dedifferentiate into fibroblasts in the course of time, testing had to be repeated after cells had been propagated for more than five times. HUVECs were prepared for immunocytochemistry or FACS and stained with appropriate antibodies against huCD105 (figure 32 A), huCD31 and huCD146. HUVECs demonstrated strong expression of huCD105 and huCD31 and moderate, diffuse surface expression of huCD146 as demonstrated in immunocytochemistry (figure 33) and FACS (figure 37, upper row). HUVECs were readily transduced by LV-huCD105 resulting in intense GFP fluorescence (figure 32 C).

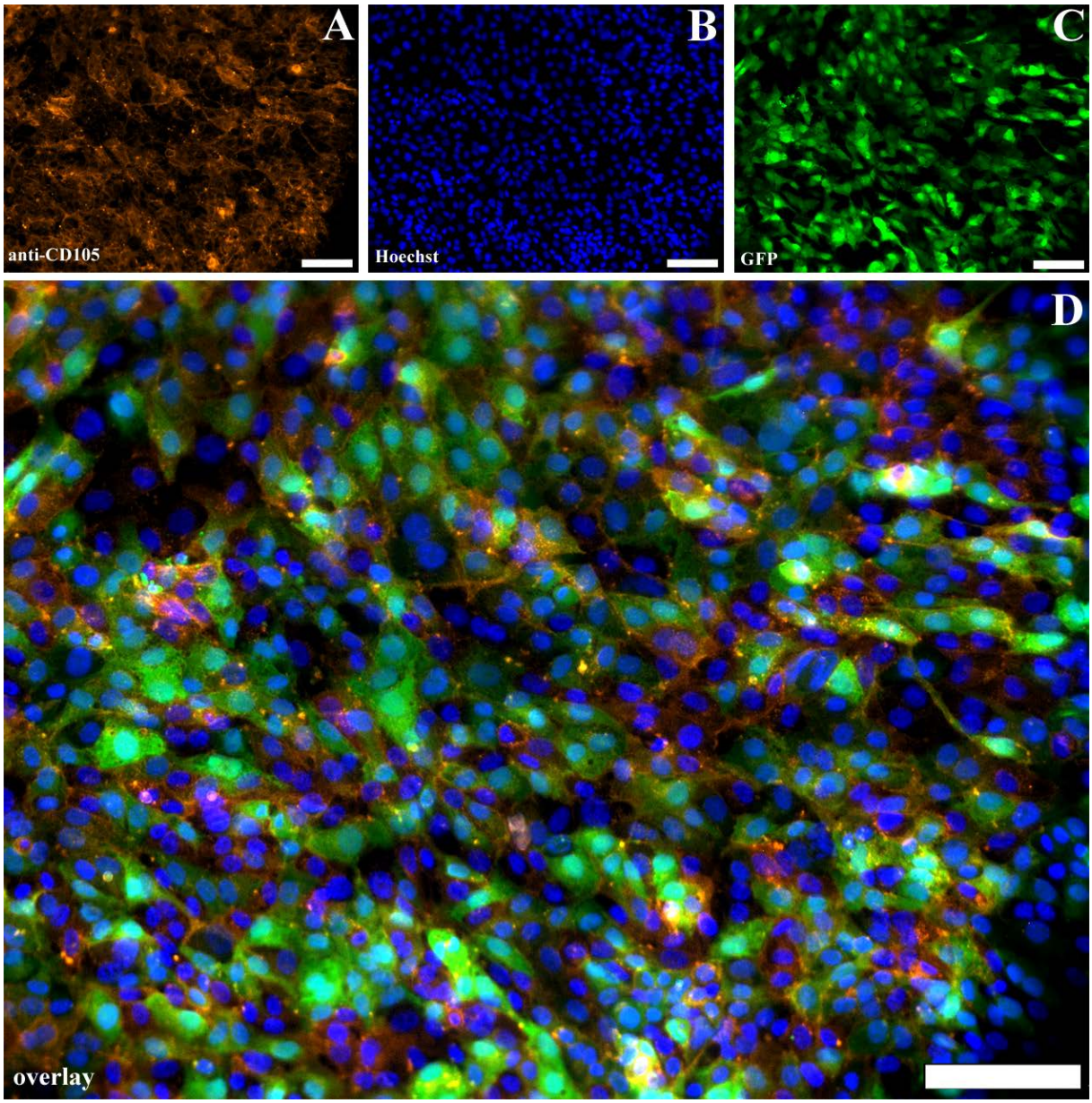

Fig. 32: Transduction of HUVEC cells with LV-huCD105. HUVEC cells were transduced with LV-huCD105 and prepared for immunocytochemistry. **A** HUVEC cells were stained with antibodies against huCD105. **B** Cell nuclei were stained with Hoechst. **C** Transduced HUVEC cells show GFP transgene expression. **D** Overlay of A-C. Bars represent 250 µm. The pictures are representative for 35 transduction experiments.

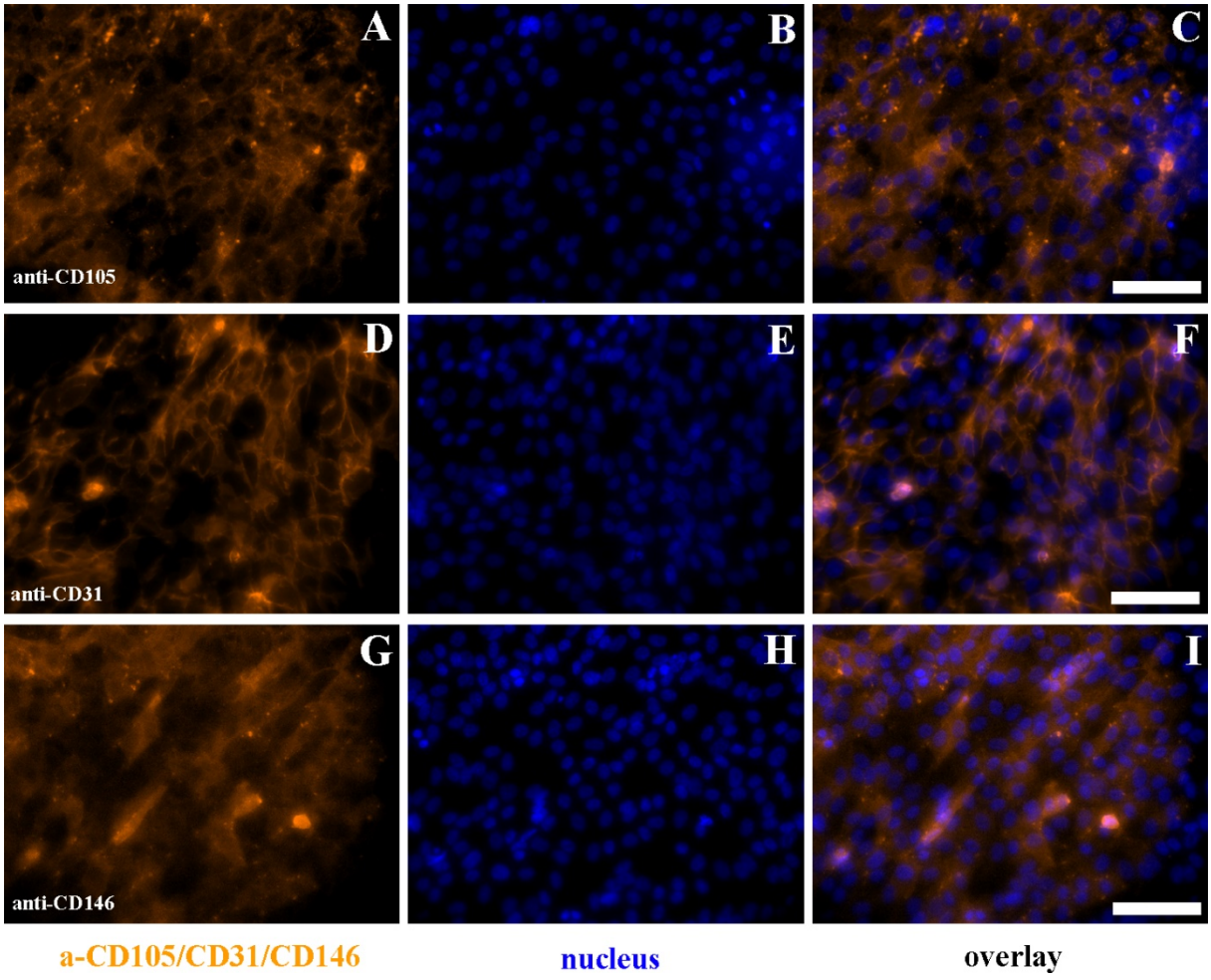

Figure 33: Surface expression of HUVECs. HUVECs were isolated and were prepared for immunocytochemistry to analyse the expression of endothelial cell markers. **A, D, G** Cells were stained with antibodies against huCD105 (**A**), CD31 (**D**) and CD146 (**G**) labelled with APC-fluorochrome. **B, E, H** Cell nuclei were stained with Hoechst 33342. **C, F, I** Overlay pictures were assembled showing endothelial cell markers and cell nuclei. HUVECs stain positive for endothelial markers CD31, CD105 and CD146. Bars in C, F, I represent 50 µm. The pictures are representative for two independent experiments and for all HUVEC cell lines used in this study.

3.2.6.2 Primary venous cells (PVCs)

Handling of surgical tissues was optimised over time, and quality and survival of explanted tissues was improved. These circumstances did not only improve transduction efficiencies, they also helped to generate primary cell cultures from tissues. In addition to collagenase digestion, the tissues had also been scraped on their luminal side using a scalpel to ensure both enzymatic and mechanical disruption. Vessel were transduced with LV-eGFP (figure 34 A-B) or LV-huCD105 (figure 34 C-L). Approximately 10 days post-surgery, dividing GFP^+ cells had sprouted from the vessel (figure 34 B) and attached in colonies to the cell culture dish and on a titan clamp which had been used during surgery (figure 34 A). Further investigation of cell cultures revealed about seven colonies in total. Two of them are depicted in figure 34 C-L. High contrast direct fluorescence microscopy of GFP (turquoise colouring of the GFP) made it easier to identify the colonies, as colonies were almost invisible to the human eye if visualised with bright-field microscopy. The morphology of some of the isolated cells that were single spotted reminded of the morphology HUVECs present at young passages (figure 34, H).

Other cells appeared to be stretched, resembling the geometry of fibroblasts (figure 34 D, F). The cell cultures were generally slow-growing and were propagated for about ten rounds until they were discarded after 2-3 months. In this period, cells underwent transformation into fibroblast-like cells, a phenomenon that is well-known from HUVEC cell lines. Remarkably, the PVCs formed three-dimensional cell clusters (figure 34 F), which has been observed frequently with HUVECs as well (figure 34 B). PVCs could be isolated from three different saphenous veins acquired from three different patients.

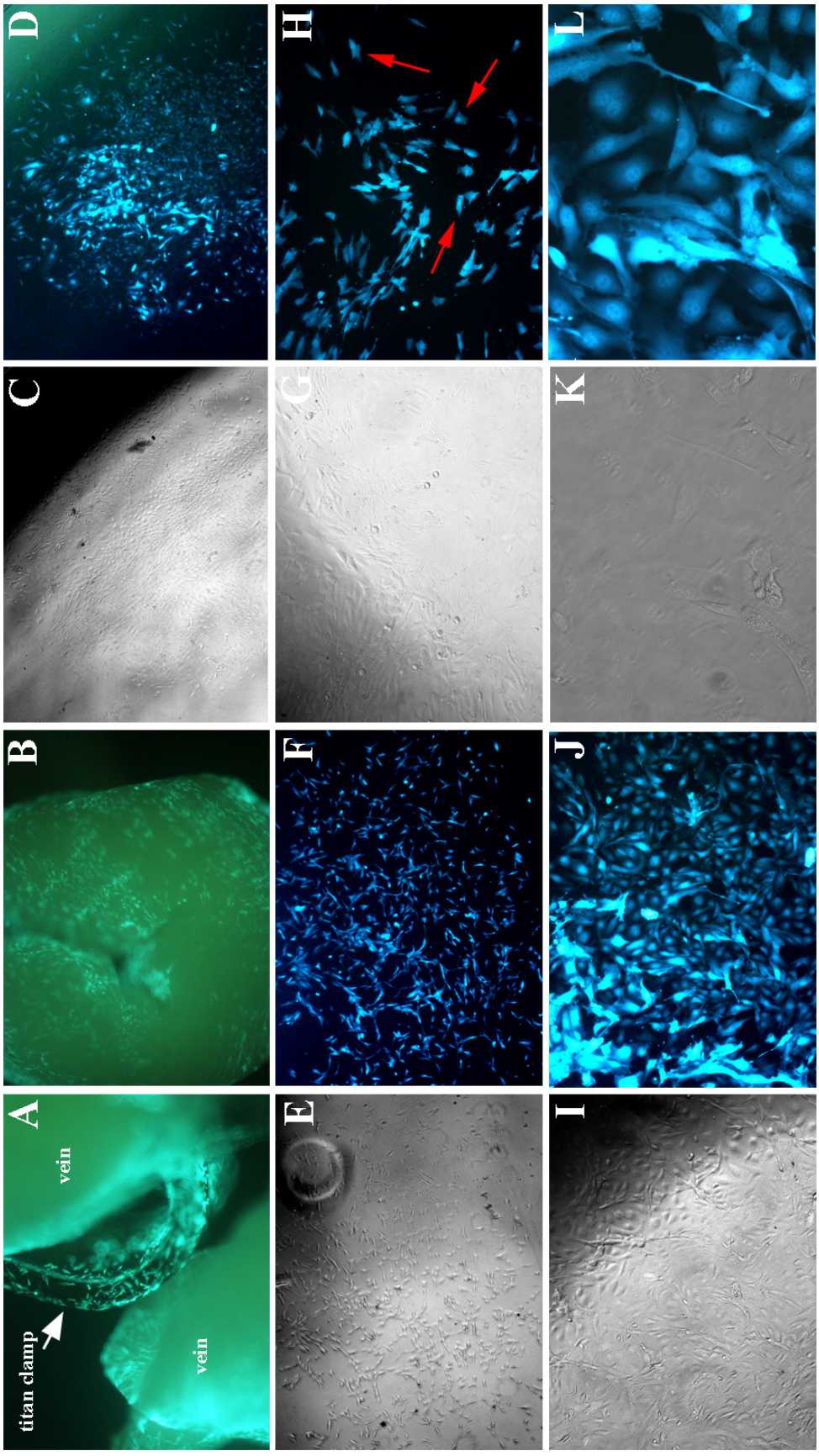

Figure 34: Isolation of primary cells from human saphenous vein. Human saphenous veins were acquired from patients undergoing coronary bypass, transferred into cell culture dishes containing endothelial cell medium and treated with VSV-G pseudotyped LV-eGFP (A, B) as control or LV-huCD105 (C-L). Veins were digested with collagenase and mechanically scraped on the luminal side using a scalpel to de-attach possible endothelial cells. Cultures were monitored for 2-4 weeks by direct fluorescence microscopy. After 10 days, primary cells could be identified. **A** Some primary cells had attached to a titanium clamp used during surgery of the saphenous vein (40x magnification). **B** Saphenous vein transduced with LV-eGFP (40x magnification). **C-F** Cells aggregated into several colonies, two of which are depicted in C-F (40x magnification). Cells demonstrated GFP fluorescence as a sign of transduction by LV-huCD105. Some cells appeared stretched resembling the geometry of fibroblasts. **G-H** Other cells showed polygonal cell geometry like early HUVECs, indicating their possible endothelial nature (arrows, 100x magnification). **I-L** Cells that were arranged in clusters and showed a three-dimensional arrangement (G-J: 100x and K, L: 400x magnification). These pictures are representative for three independent experiments.

During propagation of PVCs, expression of huCD105 and the ability of LV-huCD105 to transduce PVCs was repeatedly tested. Several experiments with cell cultures from all three patients were carried out and cells remained readily transducable by LV-huCD105 three months and 10 propagations after isolation. The cells remained positive for huCD105 as determined by FACS and by immunocytochemistry (figure 35). Unfortunately, despite some success in cultivating the PVCs, cells became quiescent after approximately two-three months. This prohibited the author to further analyse endoglin expression and expression of other antigens.

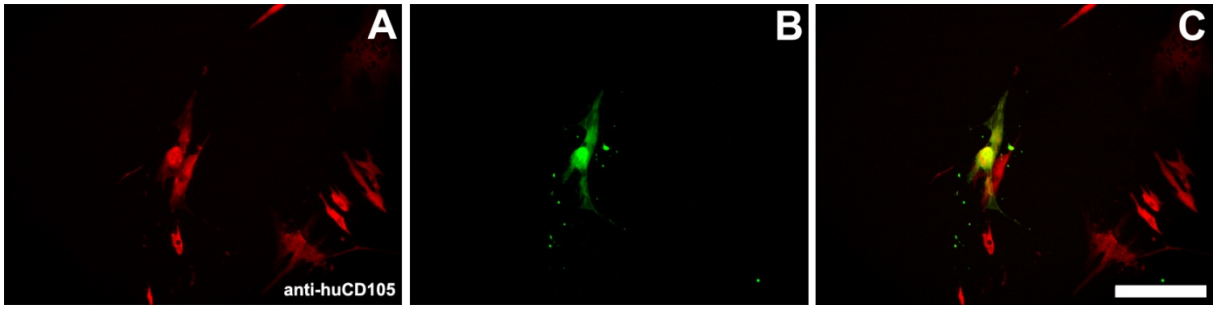

Figure 35: Immunocytochemistry of primary cells from a human saphenous vein. Human saphenous veins were acquired from patients undergoing coronary bypass, incubated with LV-huCD105, transferred into cell culture dishes containing the appropriate medium and treated with LV-huCD105. Primary cells were isolated from human saphenous veins by collagenase digestion and mechanical disruption. Cells were cultivated in endothelial cell medium and prepared for immunocytochemistry. **A** Cells stained with anti-huCD105-PE show strong expression of human CD105. **B** Some isolated cells expressed GFP as a sign of transduction by LV-huCD105. **C** Cells transduced by LV-hu-CD105 demonstrate co-localisation of GFP and huCD105. These pictures are representatives from three independent experiments from one batch of isolated cells. Bars in C represent 50 µm.

3.2.6.3 Primary arterial cells (PACs)

Human arteries were more difficult to handle and some concern was raised whether primary cells could be isolated from the fragile tissues. Nevertheless, primary cells from human mammary arteries (PACs) were isolated using the same combined enzymatic and mechanical approach as described before. However, colonies were more sparsely distributed. From two human arteries, only four colonies were acquired for subsequent testing. Cells remained transducable by LV-huCD105 for at least four weeks after isolation (figure 36 A, B). Once the PAC cell culture turned into a confluent cell monolayer they had adapted a fibroblast-like morphology (figure 36 C).

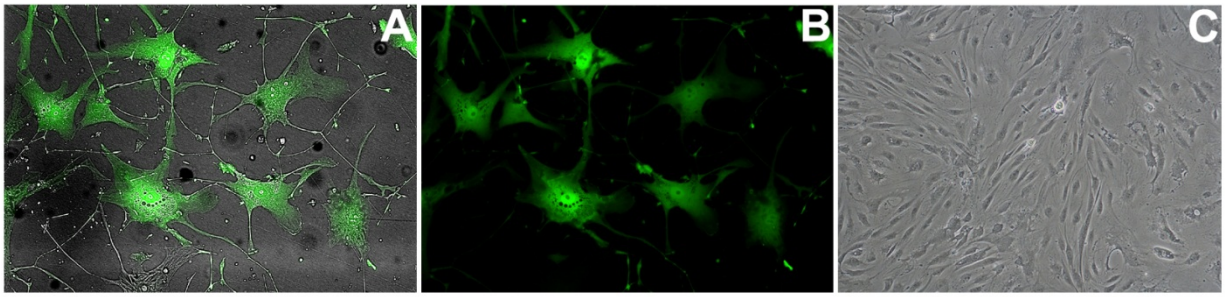

Figure 36: Primary cells from human mammary artery. Human mammary arteries were acquired from patients undergoing coronary bypass, transferred into cell culture dishes containing endothelial cell medium and transduced with LV-huCD105. The vessels were digested with collagenase and scraped on the luminal side to de-attach possible endothelial cells. Primary cells isolated from a human mammary artery (PACs) settled in cell culture dishes, were treated with LV-huCD105 and imaged by direct fluorescence microscopy. **A, B** Cultures were monitored for 2-4 weeks and remained susceptible to transduction. Cell clusters were detected after approximately 10 days and demonstrated GFP-fluorescence as a sign of transduction. **C** After 4 weeks, cell layers grew to dense cell layers. These pictures are representative for two independent experiments.

To characterise isolated PACs, surface expression of huCD31, huCD105, and huCD146 was analysed by FACS using specific fluorochrome labelled antibodies. HUVEC cells were analysed as positive controls. Interestingly, PACs strongly expressed huCD105 but lacked expression of huCD31 and huCD146. One has to point that PACs were repeatedly negative for huCD31, a characteristic surface marker of endothelial cells (figure 37). Similar results were acquired with PVCs: PVCs were strongly positive for huCD105 but lacked expression huCD31 and huCD146 (data not shown). Unfortunately, cells could only be propagated two-three times and became quiescent thereafter. Therefore, expression analysis experiments had to be limited to endothelial cell markers and expression analysis of other cell markers was left out.

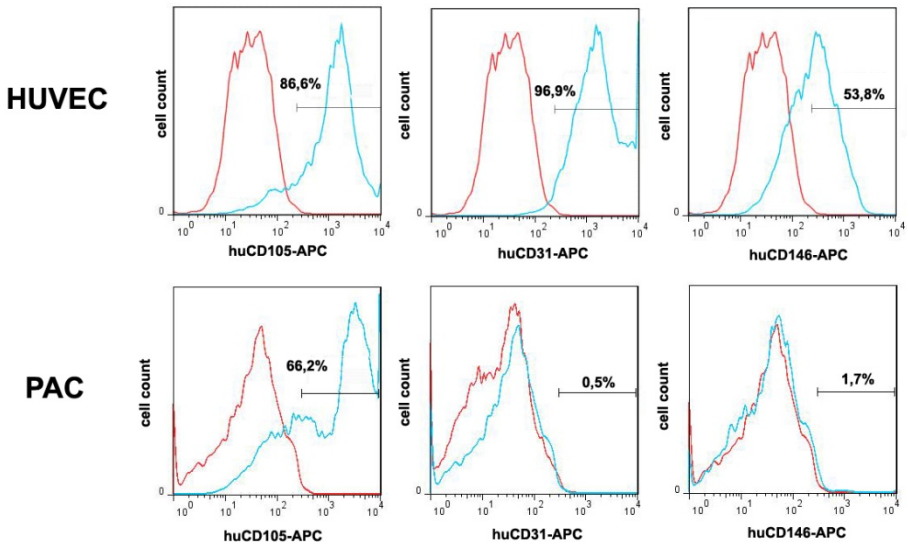

Figure 37: FACS of HUVEC and PACs. Human mammary arteries were acquired from patients undergoing coronary bypass and transferred into cell culture dishes containing endothelial cell medium. The vessels were digested with collagenase and scraped on the luminal side to de-attach possible endothelial cells. HUVEC cells and primary cells isolated from human mammary artery (PACs) were prepared for FACS to analyse expression of endothelial cell surface markers. While HUVECs are positive for CD105, CD31 and CD146, PACs only show expression of CD105, which is the target of the LV-huCD105. These plots are representatives from three independent experiments with one batch of PACs.

3.2.7 Marmoset

In an experiment to test the LV-huCD105 in a primate, non-human organism, arteries from the marmoset species *Callithrix jacchus* were acquired from the Institute of Reproductive Medicine of the University Münster, Germany. The tissues were acquired with the help of Dr. rer. nat. T. Müller, head of the Service Unit Embryonic Stem Cell at the Medizinische Hochschule Hannover. Arteries were prepared and transduced *ex vivo* with LV-huCD105 following the same procedures as with human arteries. Repeatedly, the author was not able to show successful transduction of marmoset vessels by LV-huCD105 (data not shown).

In parallel, primary cells from Marmoset arteries were isolated using collagenase digestion and cultivated for a couple of days before transducing them with LV-huCD105. Direct fluorescence microscopy identified some dividing GFP positive cells as a sign of transduction (figure 38). These cells, however, were sparse and transduction was not constantly seen in repeating experiments.

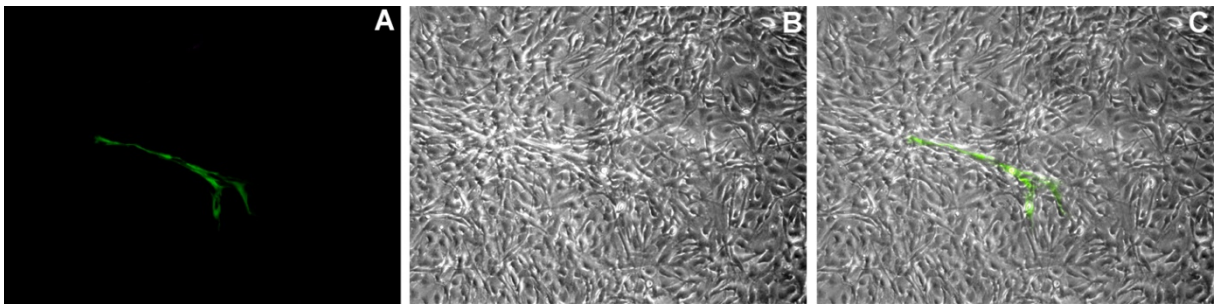

Figure 38: Transduction of marmoset primary cells. Primary cells from marmoset were isolated using collagenase digestion and cultivated. **A** Cells were transduced with LV-huCD105. Unfortunately, only a few cells were transduced by LV-huCD105 (green cells) in one single experiment. **B** Bright field microscope picture. **C** Overlay of transduced cells. Transduction could not be repeated in subsequent experiments. The picture is a representative of three spots with transduced cells from one single experiment.

3.2.8 Transplantation experiments

3.2.8.1 In vivo transduction of human arterial xenografts

Thus, LV-huCD105 was specific against human $CD105^{+}$ cells and able to transduce live solid tissue *ex vivo*. Encouraged by these results we set out to investigate the specific targeting capabilities of the lentiviral vehicle in living animals. For this reason, side branches of the human mammary artery were transplanted into an interposing position between the ends of the intersected abdominal aorta of immunosuppressed BALB/c $Rag2^{-/-}$ IL-$2R\gamma_c^{-/-}$ mice by end-to-end anastomosis. LV-huCD105 was repeatedly injected into the tail vein. The injected LV-huCD105 circulated through the bloodstream, passing the V. cava inferior, the heart, lung arteries, the lung, lung veins, the heart to finally reach the human endothelium of the xenograft.

Eventually, LV-huCD105 bound to the huCD105$^+$ cells and managed to transduce the cells. Initially, titers below $1*10^7$ infectious particles of LV-huCD105 were used in trying to get successful transduction of the xenograft endothelial layer. Although single cells of the human endothelial layers demonstrated GFP fluorescence as a sign of transduction in initial experiments, titers of LV-huCD105 were increased to $3*10^7$ infectious particles per 800 µl injection sample to ensure maximum load of lentiviral vector particles in the bloodstream. In this setting, three transplanted mice were treated with three lentivirus injections at day 3, 5 and 8 and sacrificed after 16 days to analyse transduction of the xenograft endothelium. The xenografts showed several locations with intense GFP$^+$ fluorescence as a sign of transduction (figure 39). In some parts of the xenograft endothelial layer, transduced endothelial cells were lined up beside one another and formed a distinct GFP$^+$ boundary between the connective extracellular matrix of the vessel wall and the lumen of the xenograft. Other murine tissues, e.g. the heart and the most proximal part of the V. cava inferior were tested for the presence of GFP$^+$ cells. The V. cava inferior is one of the first body compartments that is passed by highly concentrated LV-huCD105. The high number of lentiviral particles passing the murine endothelial barrier in the V. cava inferior increased chances of transduction if LV-huCD105 were non-specific to human CD105$^+$ cells. Also, the heart may harbour high lentiviral concentrations for a period of time. However, both tissues did not reveal any signs of transduction by the human specific LV-huCD105 (figure 40).

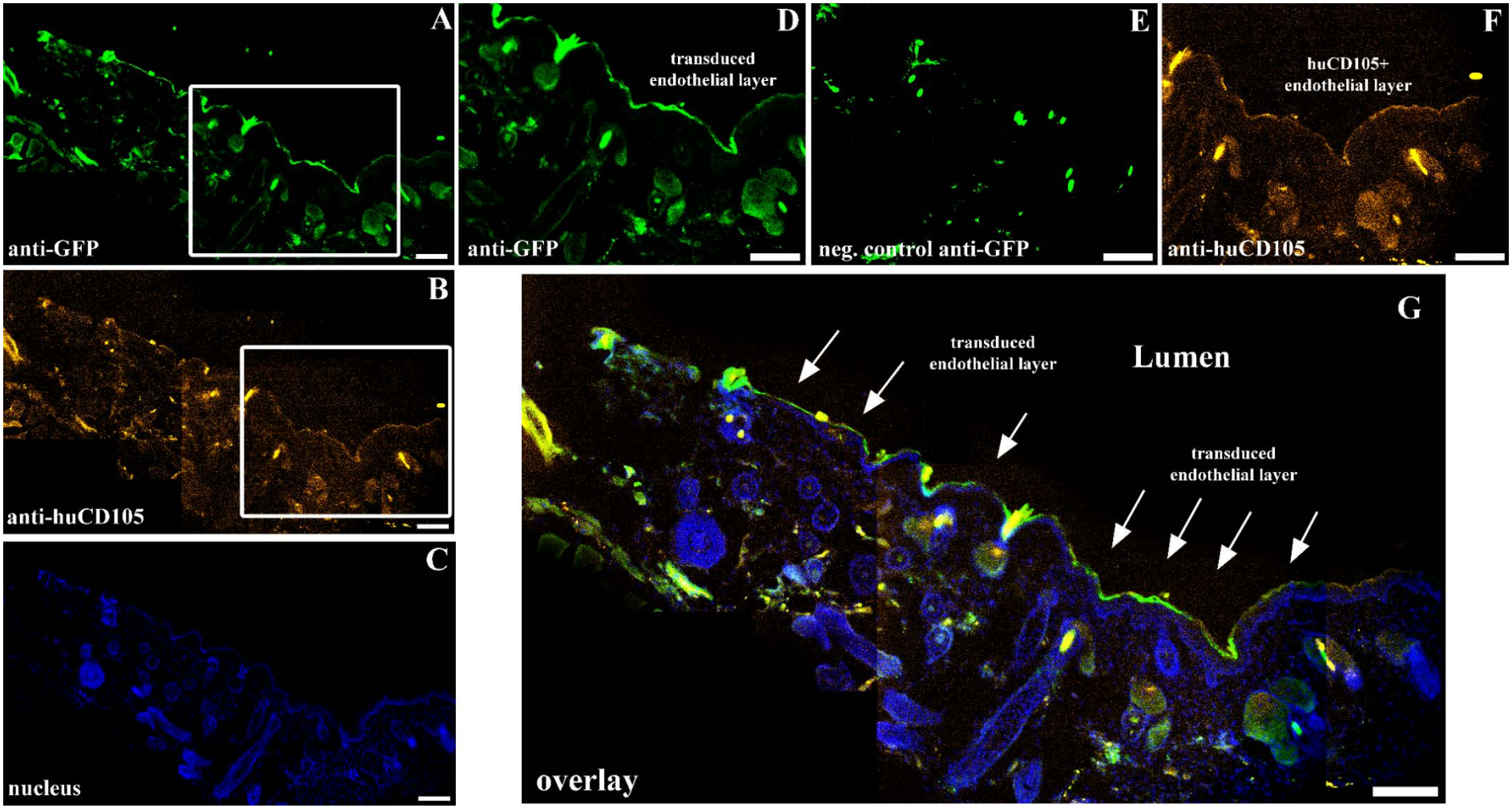

Figure 39: Transplantation of human mammary artery into mice: Human mammarian arteries were acquired from patients undergoing coronary bypass operation. The arteries were inserted in an interposing position between the two ends of the dissected abdominal aorta by end-to-end anastomosis. Transplanted mice recovered for 48 hours before initial *in vivo* injection of 800 µl LV-huCD105 into the tail vein. Injections were repeated at day 5 and 8. Mice were sacrificed after 16 days and organs including the transplanted human artery were prepared for immunohistofluorescence microscopy. **A-C** Human artery tissue was analysed for expression of GFP (**A**), huCD105 (**B**) and cell nucleus (**C**). Circular aggregations of cell nuclei in C may result from minor arteries originating from the larger human mammarian artery. **D** A magnified section of figure 39A clearly shows that transduced cells are aligned towards the lumen of the transplanted artery. **E** A negative control for the GFP staining demonstrated the absence of GFP staining, clearly showing that staining of GFP in A is specific. **F** A magnified section from figure 39 B demonstrates the alignment of huCD105^{+} endothelial cells towards the lumen of the transplanted artery. **G** An overlay of figures A-C depicts yellowish co-localisation of GFP and huCD105, indicating transduced cells are endothelial cells. This picture is a representative from artery sections from three transplanted mice. The pictures were acquired by detecting GFP, Hoechst and APC fluorescence at four adjactant locations along the luminal endothelial layer of the transduced graft. The channels were merged using the Olympus Cell^M image processing software. The pictures were assembled using Adobe Photoshop software. Noise was reduced by balancing colour levels equally for assembled pictures. The assembly of the pictures was not able to create an image where edges smoothly fitted to each other, and hangover areas were filled with black colour to generate a high-contrast background. All bars represent 250 µm.

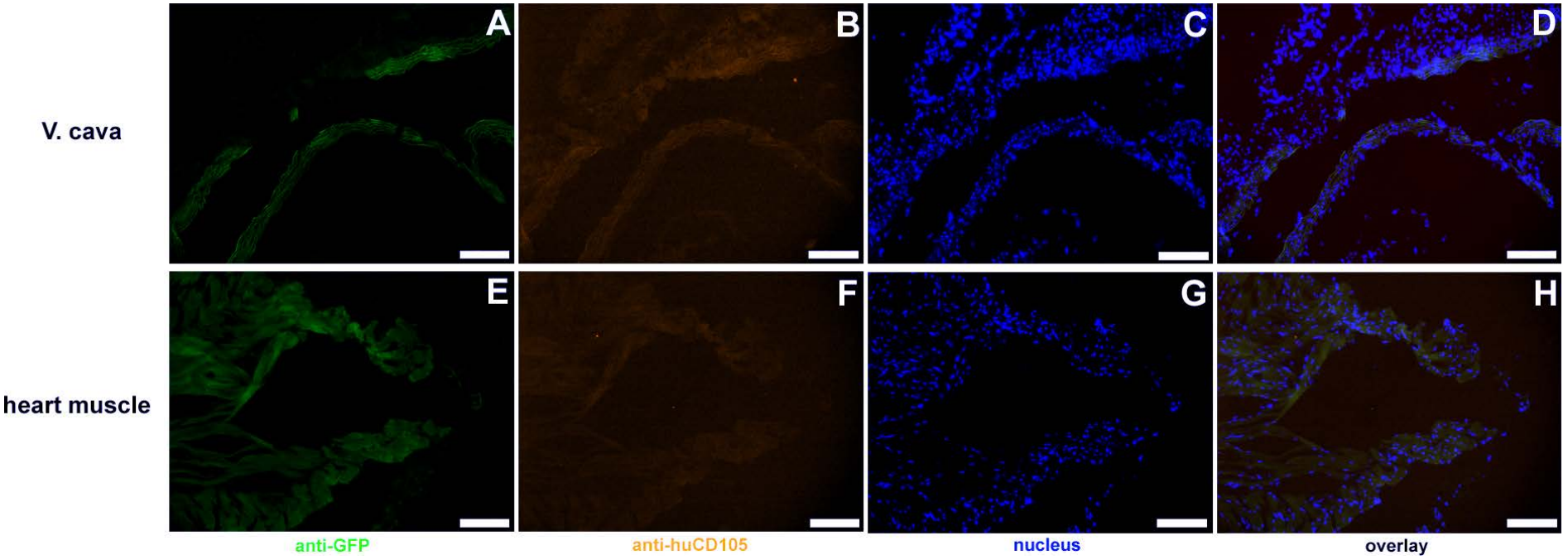

Figure 40: Transplantation of human mammary artery into mice, control: Human mammarian arteries were acquired from patients undergoing coronary bypass operation and transplanted into the intersected abdominal aorta of immunodeficient mice by end-to-end anastomosis. Transplanted mice recovered for 48 hours before initial *in vivo* injection of 800 µl LV-huCD105 into the tail vein. Injections were repeated at day 5 and 8. Mice were sacrificed after 16 days and organs were prepared for immunohistofluorescence microscopy. The **upper row (A-D)** demonstrates a representative immunohistofluorescence picture of the proximal part of the Vena cava inferior, which is circulated early by LV-huCD105. The **lower row (E-H)** shows a representative picture of the heart muscles, which can be identified by their characteristic histology. **A, E** No GFP^+ transduced cells can be identified in either one of the tissues indicating that murine tissues are not transduced by LV-huCD105. **B, F** As expected, murine tissues are devoid of human CD105. **C, G** Nucleus staining of the V. cava (C) and the heart muscle (G). **D, H** Overlay pictures of the V. cava (D) and the heart muscle (H). Pictures have been colour-balanced to increase the intensity of the blue colour (cell nucleus). Bars represent 250 µm.

3.2.8.2 *Ex vivo* transduction and transplantation

The previous experiment described how human mammarian artery was transplanted into BALB/c Rag2$^{-/-}$ IL-2R$\gamma_c^{-/-}$ mice within 2 hours after removal from the human patient. *In vivo* transduction by tail vein injection was conducted 48 hours after transplantation and repeated several times to achieve positively transduced cells in the xenograft artery. The method is dependent on survival, vector stability in the bloodstream of the mouse and the survival of the graft. Although transplantation experiments were conducted successfully in three mice, the method remains highly experimental and fragile.

In two independent experiments, the author tried to transplant already transduced arteries into the intersected abdominal aorta to overcome the difficulty of *in vivo* instability of the lentiviral vector. However, these experiments were not successful as mice died during the night after surgery. Autopsies were carried out and revealed that the xenograft arteries had undergone necrosis and burst, leading to massive haemorrhage into the abdominal cavity of the mice. Further experiments were abandoned because of the discouraging results of the initial experiments and from an ethical point of view.

4. Discussion

The body homeostasis is regulated by numerous enzymatic pathways and transport mechanisms that deliver desired substrates and remove waste molecules. The metabolic network of the liver is unmatched by other organs as it does not only dispose of toxic metabolites but also is the main producer of biomolecules (Ghany and Hoofnagle, 2008). Both metabolites and biomolecules have to pass through a barrier of endothelial cells to reach the liver parenchyma and other tissues while microbiological agents and other contaminants are held back (Parker and Picut, 2005). Therefore, hepatocytes and endothelial cells live in close functional proximity that requires more attention in terms of drug development than it has received by now.
Both hepatocytes and endothelial cells can be targeted by gene therapy vectors. In some cases, hepatocytes can be isolated from the diseased patient, undergo *ex vivo* gene therapy and be re-transplanted by injection into the portal vein (Grossman et al., 1994). Another possibility is to transplant allogenic hepatocytes from healthy individuals without previous treatment with lentiviral gene therapy vectors. However, recipients have to undergo immunosuppressive treatment to prevent rejection (Schneider et al., 2006). Transplanted hepatocytes translocate into the native liver parenchyma and show independent metabolic activity before complete integration (Gupta et al., 1999). Quite in contrast, the integrity of the endothelial cell layer is a pre-requisite for full functionality of endothelial cells in regard to haemostasis, biomolecule transport and inhibition of atherosclerotic plaque formation. Cell transplantation of endothelial cells to reconstitute functions of the endothelium appears to be less suitable. Scientists therefore now focus on developing gene therapy vehicles specifically directed against surface molecules on endothelial cells to allow proper targeting (Anliker et al., 2010).

There have also been advances to target gene therapy vectors to specific antigens on hepatocytes in order to allow *in vivo* administration of therapeutics. In recent years, specially designed lentiviral vectors covered with single chain variable fragment (scFv) antibodies have been investigated to make cell-specific delivery more efficient. The scFv antibodies are characterised by a high affinity to specific surface antigens, and lentiviral vectors displaying the scFv antibodies on their envelope eventually attach and subsequently transduce target cells expressing the specific antigen (Nakamura et al., 2005). In this study, we chose to modify the lentiviral vectors' envelope with scFv antibodies against ASGPR, a hepatocyte-specific surface marker, and CD105, an antigen that is generally expressed on endothelial cells of microvessels. While the lentiviral vector against human CD105 has already been successfully tested against

$CD105^+$ human cell cultures, tests with living human tissues have not been conducted at all (Anliker et al., 2010).

To begin with, we successfully detected expression of all hemagglutinin-scFv fusion constructs against CD105 and ASGPR. Proper folding and externalisation of the scFv polypeptide is a pre-requisite for the functionality of lentiviral vectors pseudotyped with measles virus glycoprotein fused to single chain antibodies. Positive detection of surface scFv expression was needed as previous data suggested that some hemagglutinin-scFv fusion constructs may not fold properly and, therefore, cannot be assembled into the envelope of lentiviral vectors (Friedel et al., 2015). In general, scFv constructs against ASGPR were less efficiently expressed compared to scFv antibodies against CD105, ranging from 7.88 % to 23.8 % for ASGPR-scFv constructs and 32.3 % for CD105-scFv constructs. Previous publications have discussed that the fused part of a chimeric envelope, here the scFv-antibody, may dimerize and aggregate in the endoplasmatic reticulum. Upon aggregation in the endoplasmatic reticulum, membrane transport of the scFv antibodies would be disrupted (Fielding et al., 1998). It is unclear, to which extent the scFv-ASGPR and scFv-CD105 dimerize under physiological conditions and whether scFv-ASGPR dimerizes to a higher degree than scFv-CD105 as suggested by the lower expression levels. In addition, transgene expression levels may be impaired by low levels of substrates such as specific amino acids or tRNA-molecules (Kim et al., 1997).

Lower production levels of scFv-ASGPR may have resulted in lower amounts of externalised LV-huASGPR viral particles, thus resulting in less frequent transduction events in $ASGPR^+$ cells. In our experiments, externalisation of scFv-ASGPR (7.8%-23.8%) was slightly lower than the externalisation of scFv-CD105 (32.3%). If we consider that HepG2 cells and HUVEC cells generally had similar susceptibility to lentiviral vectors pseudotyped with measles virus hemagglutinin fused to scFv-antibodies, we would expect – on the basis of lower externalisation of LV-ASGPR – a slight decrease of transduction events in HepG2 cells treated with LV-ASGPR compared to HUVEC cells treated with LV-huCD105. In this case, none of the HepG2 cells were transduced by LV-ASGPR, indicating insufficient affinity of LV-ASGPR against its target antigen or other structural issues that may be responsible for the inability to transduce target cells.

Unfortunately, it proved difficult to test LV-ASGPR on $ASGPR^+$ cell lines. We tested HepG2 and Huh7 cell lines that supposedly are characterised by abundant ASGPR expression (Schwartz et al. 1982; Treichel et al., 1994; Tratenherts 2009). In fact, the majority of cells did not express ASGPR. Several batches of HepG2 cells from the Twincore facility were tested for

ASGPR expression. Only one batch of HepG2 cells proved to be a suitable ASGPR expressing positive control for transduction experiments with LV-ASGPR as suggested by previous publications (Tratenherts 2009). To rule out the possibility that the loss of ASGPR expression in HepG2 cells was a facility-related phenomenon, our collaboration partners from the Paul-Ehrlich-Institute (research group Buchholz) tested ASGPR expression in the HepG2 cells available at their facility. Tests revealed that HepG2 cells located at the Paul-Ehrlich Institute did not express ASGPR, confirming our suspicions that ASGPR may not be abundantly expressed in all HepG2 cells (personal communication with research group Buchholz). This suggests that ASGPR expression in HepG2 cells can be deregulated and vanish. To analyse this phenomenon, we would have to test whether freshly acquired $ASGPR^+$ HepG2 cells lose ASGPR expression in cell cultures over time.

Several Huh7 cell lines were also tested negative for ASGPR expression, despite contrary information in previous publications (Schwartz et al. 1982; Treichel et al., 1994). One report suggests that ASGPR expression is lost after plating and expansion of cells, as primary hepatocytes have been reported to undergo changes in their expression profile in cell culture after plating (Richert et al., 2006). Furthermore, Zijderhand-Bleekemolen and co-workers have shown that plasma membrane situated ASGPR may translocate to the trans-Golgi reticulum and the compartment of uncoupling receptor and ligand upon stimulation with ASGPR ligands and weak bases. The proportion of total ASGPR presented on the cell surface was diminished from 34 % to 4 % of the total ASGPR content in HepG2 cells if incubated with weak bases for 30-40 minutes (Zijderhand-Bleekemolen et al., 1987). In our *in vitro* experiments, HepG2 cells were cultured in DMEM supplemented with foetal calf serum, L-glutamine and penicillin/streptomycin. We can only speculate about whether the medium itself or its supplements may trigger complete relocation of ASGPR in long-term cultures. Conclusively, transduction of HepG2 cells will fail once ASGPR has translocated to intracellular compartments as surface ASGPR will not be available for binding by ASGPR-scFv antibodies. We have found that ASGPR expression fluctuates between cell lines and different batches, and testing of an appropriate cell line for ASGPR expression is crucial before experiments start. In addition, the literature on cell lines endogenously expressing ASGPR needs to be revised (Tratenherts 2009; Schwartz et al. 1982; Treichel et al., 1994). To overcome fluctuating ASGPR expression levels in specific cell lines, the author generated HT1080 cell lines transiently expressing ASGPR to ensure antigen presentation to LV-ASGPR at the time of transduction. Fortunately, the HUVEC cells used for this study readily expressed CD105, which allowed straightforward testing of LV-huCD105.

Despite extensive testing of LV-Cao, LV-benhar and LV-benharP lentiviral constructs, no specific transduction of ASGPR-expressing cells was detected. Single ASGPR$^+$ HepG2 cells treated with LV-Cao, LV-benhar and LV-benharP demonstrated eGFP fluorescence as a sign of transduction, however, this was interpreted as non-specific transduction by lentiviral vectors. Interestingly, LV-benharP was able to transduce HT1080-ASGPR cells, but no significant difference was detected in comparison to control ASGPR$^-$ HT1080 cells. Thus, efficient specific transduction of ASGPR$^+$ cells failed although high vector volumes were used and different scFv-antibodies against ASGPR were tested. The most probable reason for failing to transduce ASGPR$^+$ target cells may be the limited affinity of the scFv antibodies against its antigen ASGPR. In fact, the same research group that had originally identified the first scFv-antibody against ASGPR concluded that its affinity towards ASGPR was low (Wang et al., 2006).

Secondly, we are not able to present numbers on how many infectious lentiviral particles are used in transduction experiments. Normally, vector titers are determined by the means of qPCR or fluorescence that detect expression of the transgene in transduced cells. In case of LV-ASGPR, no target cells were transduced. Therefore, an accurate determination of transduction events and subsequently viral titers was impossible. Thus, we do not know if lentiviral titers were sufficient to transduce target cells. Instead, we used vector volumes comparable with those from transduction experiments using LV-huCD105. The issue will be solved once appropriate scFv-antibodies against ASGPR are found.

Third, there is also the theoretical chance that lentiviral production itself has failed in the case of LV-ASGPR. This possibility is nevertheless considered as hypothetical out of two reasons: First, surface expression and externalisation of the His-tagged hemagglutinin-scFv fusion protein scFv-Cao, scFv-benhar and scFv-benharP was confirmed by FACS. Second, the same structural and lentiviral transfer plasmids were used in parallel during all lentivirus production of LV-ASGPR and LV-huCD105 with the exception for the hemagglutinin-scFv fusion protein plasmids (pHL3nse-scFv-ASGPR or pHL3nse-scFv-A5). Production of LV-Cao, LV-benhar, and LV-benharP were conducted in parallel with LV-eGFP and LV-huCD105 to acquire lentivirus production controls. LV-eGFP and LV-huCD105 were efficiently produced and worked well in subsequent transduction experiments. Combined with the fact that scFv-ASGPR was efficiently externalised it seems highly unlikely that formation of the lentiviral particles itself had become disrupted. Thus, any failure in the functionality of LV-huASGPR would be related to the specific characteristics of pHL3nse-scFv-ASGPR plasmids or their products, i.e. the scFv-ASGPR antibodies.

Fielding and co-workers suggested that polypeptides presented on the envelope of retroviral particles may be prone for a sequestration pathway by specific receptors other than the regular lentiviral entry pathway, a phenomenon known as inverse targeting. In their experiments, they displayed stem cell factor (SCF) on the surface of retroviral vectors with the aim to target cells that express Kit, a known SCF-receptor. Unexpectedly, transduction rates declined, and the scientists suggested that SCF on viral envelopes may, in fact, be sequestered away from the retroviral entry pathway. Transduction rates were increased again by adding ligands that competed with SCF for binding places on SCF-receptors (Fielding et al., 1998). In parallel, the single chain variable fragments against ASGPR presented in this study may undergo sequestration away from the lentiviral entry pathway. As Fielding and co-workers suggested, this could be prevented by adding soluble ligands to the cell transduction medium as competitors of scFv-ASGPR for the $ASGPR^+$ receptor on target cells. However, in the experiments described by Fielding and co-workers, transduction efficiency of target cells remained between 3.6-4.3% if no precautionary measures were taken to circumvent sequestration of SCF. In our study, transduction of $ASGPR^+$ was non-existent and it is rather unlikely that transduction would be improved by the addition of soluble ASGPR ligands.
Due to the frustrating attempts to transduce cells with LV-ASGPR, experiments were put on hold. Experiments will be continued once new scFv antibodies have been developed. Forgoing cell-specific gene delivery with surface manipulated lentiviral vectors future projects should concentrate on screening various phage antibody libraries and *in vitro* testing with the aim to identify high-affinity antibodies against ASGPR. Moreover, alternative hepatocyte surface markers other than ASGPR may be found, and scFv-antibodies could be directed against novel hepatocyte-specific markers.

In parallel, this study tested gene therapy vehicles directed against endothelial cells using scFv-antibodies directed against the endothelial cell marker CD105. While the liver can be approached by targeted liver therapy to cure monogenetic liver diseases, endothelial cells may be even more suitable for targeted gene therapy of the liver because of its anatomic accessibility. Endothelial sinusoidal cells create the only barrier between the bloodstream and the liver parenchyma, and the endothelium may be a useful instrument in directing therapeutics to the liver. Endothelial cells may also play a role in the angiogenesis involved in liver tumor formation and defines a molecular target in anti-tumor therapies (Wang et al., 1993; Burrows et al., 1995; Fonsatti et al., 2001). Furthermore, endothelial cells are also involved in plaque formation in atherosclerosis, the leading cause of death in the civilised world, and haemostasis

(Conley et al., 2000; Piao and Tokunaga, 2006). Therefore, in collaboration with the research group around Professor C. Buchholz in Langen, Germany, we decided to aim for *ex vivo* and *in vivo* experiments proving the effectiveness of lentiviral delivery of transgenes to endothelial cells.

First, lentiviral vector production was tested on freshly isolated and cryo-conserved HUVECs. Vector production quality was sufficient with mean titers around $7.0*10^7$ infectious particles per milliliter, resembling titers described in previous publications (Anliker et al., 2010). High output titers would later prove to be essential for subsequent *ex vivo* and *in vivo* experiments, as these required high viral loads to mark distinct transgene fluorescence as a sign of positive transduction.

Initial experiments transducing human veins with LV-huCD105 were strenuous, as experiments of this kind have not been carried out before. Some details specific for the *ex vivo* transduction of vessels were initially unknown but turned out to be vital for successfully accomplishing our ambition to specifically transduce endothelial cells in living tissue with LV-huCD105. Specific details had to be continuously identified and improved during the handling of new samples and lead the way to finally demonstrate the specific transduction of $CD105^+$ cells *ex vivo* and *in vivo*.

In normal cell cultures, fluorescence in transduced cells can be detected about 24-48 hours after transduction. In this specific study, we transduced blood vessels surgically removed from patients undergoing bypass operation. Transduced cells located within the vessel tissue were detected approximately 8-10 days after transduction in all assessed samples. This delay could not be improved by higher vector titers, gentle handling, or transduction time, and, therefore, has to be accepted as a specialty of the system used. One explanation may be that in *ex vivo* explants cell numbers tend to stagnate due to paused cell division. Therefore, fewer cells can be transduced by lentiviral vectors and transduction events are less frequent. As previously described, lentiviral transduction may become less efficient in non-dividing cells because of the nuclear membrane barrier, contributing to the relative scarcity of infected cells (Ravot et al., 2002; Albanese et al., 2008). In addition, soft tissues experience stress when explanted from the body, shifting cell metabolism to more vital pathways, thereby inhibiting synthesis and folding of transgenic eGFP, possibly explaining the delayed transgenic expression of transduced cells. Secondly, transduction efficiency was increased if vessels were enzymatically digested and mechanically disrupted. At first glance, this may increase the risk of damaging vital tissue and cells. On the other hand, mechanical separation of cells increases the accessibility by lentiviral vectors, thereby boosting transduction events. Third, fluorescence

was dependent on transduction length and whether it was repeated. In detail, transduction was essentially maximized if transduction occurred for 24 hours and repeated once. Extension of the transduction times did not result in improved transduction efficiency, probably because of the limited viability of lentiviral vectors (Sakuma et al., 2010). All these considerations were taken into account before advancing to animal experiments.

Fluorescence microscopy of the vessels transduced with LV-huCD105 had suggested that the transduced cells were located luminal rather than in the vessel wall. In comparison, vessels treated with unspecific LV-eGFP demonstrated eGFP fluorescence more randomly scattered through the entire vessel wall. This suggested that LV-huCD105 favoured transduction of luminal cells which would be consistent with the localisation of endothelial cells. Immunohistofluorescence staining against CD105 was performed to identify whether specifically $CD105^+$ cells were transduced or not. In a series of experiments, transduced arterial and venous tissues were analysed for overlapping GFP and $CD105^+$ expression with the aim to validate CD105-specific transduction of cells. As hypothesized, transduced GFP^+ cells lined up along the luminal side of the vessel. This was concurrent with the expression of CD105 in endothelial cells on the luminal side of the vessel, hinting at successfully targeted transduction of endothelial cells. In fact, no $CD105^-$ cells were transduced during these experiments, indicating the high specificity of LV-huCD105. Occasionally, cells located in the subendothelial matrix, i.e. tunica intima, demonstrated GFP fluorescence. Without identifying the character of these cells any further, these may have been fibroblast cells that are known to express CD105 (Rokhlin et al., 1995). If LV-huCD105 was further developed, additional staining experiments using antibodies against fibroblast markers such as proline-hydroxylase would be needed to confirm the nature of these cells and to evaluate the specificity of the LV-huCD105 (Janin et al., 1990).

When evaluating the effectiveness of the CD105-specific vectors in this experimental setting some certain considerations have to be taken into account: First, all transduction experiments were conducted with vessels that had been removed from patients undergoing coronary bypass. These patients tend to suffer from extensive atherosclerosis, and CD105 expression is induced in vessels with atherosclerosis. Vessels from diseased patients may actually have elevated basal expression levels of CD105 that is unmatched with those in healthy individuals, leading to overrepresented transduction events in the vessels used in this study (Conley et al., 2000). Secondly, the endoglin expression may not only be induced by atherosclerotic damage, but by

the surgical procedure itself that imposes mechanical stress on the tissue. Colazzo and co-workers have described that human adipose derived stem cells may have elevated levels of endothelial cells markers such as CD31 when exposed to VEGF and shear stress (Colazzo et al. 2014). In parallel, endothelial markers such as CD105 may be induced in removed artery tissue by the surgical procedure itself. Again, higher levels of CD105 due to mechanical damage may trigger more frequent transduction events *in vivo* than in healthy patients. Inadequately high expression levels of CD105 in surgically removed tissue may be limited by swift handling of vessels to anticipate events of gene induction.

In fact, one has to critically evaluate the specificity of LV-huCD105 as other cells have been described to express CD105 (Cho et al., 2001; Pierelli et al., 2001; Robledo et al., 1996; Rokhlin, 1995; Lastres et al., 1992; Diez-Marques et al., 2002; Gougos and Letarte, 1988; Fonsatti et al., 2003). In our *in vitro* experiments, we were able to transduce the human $CD105^+$ HT1080 fibroblast cell line, consistent with previous findings (Funke et al., 2010). In addition, our *ex vivo* transduction experiments suggested some - although minor – transduction of fibroblast cells in the subendothelial matrix. These findings redefine LV-huCD105 as a vector that is not necessarily specific for human endothelial cells, but for $CD105^+$ cells including fibroblasts. In practical terms, this means that LV-huCD105 will transduce fibroblast tissues offside of the targeted endothelial tissue. Also, CD105 is expressed on e.g. monocytes, macrophages, erythroid precursor cells, and many more in humans, and these cells mark potential targets for transduction by LV-huCD105. However, LV-huCD105 will be primarily confronted with endothelial $CD105^+$ cells pointing towards the luminal side of the vessel as it is administered by intravenous injection. Other cell types will only be secondary targets upon transduction by LV-huCD105. Summing up, one might conclude that LV-huCD105 is a lentiviral vector specific to CD105 and semi-specific to endothelial cells. Once pre-clinical testing has ended, *in vivo* application of LV-huCD105 in humans will finally answer the question regarding the specificity and whether solely endothelial cells can be preferably targeted. Some background transduction of non-endothelial cells may be tolerated in critically ill patients if side effects in the non-endothelial cells are limited.

An interesting finding of this study is that maybe stem cell-like cells were isolated from human arterial and venous tissues using a combination of enzymatic digestion and mechanical disruption. This method may be further improved by decreasing transfer time from surgical removal to cell medium. Furthermore, to evaluate the efficiency of the cell isolation procedure, one has to define a measure to evaluate the percentage and purity of isolated cells. At this point,

it is unclear which measure could be used to determine the percentage of isolated cells from total cells. Rigorous enzymatic digestion and mechanical separation from each other will still leave numerous cells firmly attached to the connective tissues of the vessel. However, purified cells could be qualitatively characterised by analysing the expression of endothelial and fibroblast cell markers.

In this study, isolated primary cells demonstrated high expression levels of CD105, indicating either endothelial or fibroblast origin. Quite in contrast to HUVECs, they did not express typical endothelial cell markers like CD31 or CD146. This indicates that the isolated cells, in fact, were fibroblast-like rather than endothelial cells at the time of expression analysis. One has to consider that cells by the time of analysis had been expanded several times and cultivated for at least four weeks because of their slow propagation. This may have altered the expression profile, a phenomenon that is commonly known from HUVECs (Eman et al., 2006; Hamel et al., 2006). Isolated cells may have initially expressed CD31 and CD146, but the expression of these distinct endothelial markers may have been lost during propagation rendering endothelial cells fibroblastic. This is also consistent with the morphology of isolated cells: Isolated cells that were analysed one week after separation from the vessels showed a polygonal cell geometry if not surrounded by other daughter cells, while cells that had undergone cell division appeared more longitudinal in shape as a sign of fibroblastoid transformation. Furthermore, the isolated primary cells could have been fibroblastoid from the beginning, therefore lacking typical endothelial cell markers. Finally, primary cell isolation from vessels may have created a mixture of fibroblasts and endothelial cells. Fibroblasts within the mixture may have propagated faster and overgrown the endothelial cells, explaining the transformation of the majority of cells from polygonal to longitudinal cells and the absence of endothelial cell markers once enough cells were available for FACS analysis. In future experiments, scientists should focus on analysing the nature of isolated cells as early as possible, e.g. by flow cytometry analysis directly after separation from the tissue or some days later to allow the cells to recover. However, cell numbers are low directly after isolation, reducing statistical validity if cells are processed and analysed by FACS. One way to solve this problem would be to run cells collected from multiple tissues as one sample, making the results more representative.

Isolated cells demonstrated high CD105 expression levels and were readily transduced with LV-huCD105, indicating that isolated cells do not lose their susceptibility to LV-huCD105 *in vitro*. Cultivated cells can be tested for lentiviral transgene expression and its impact on cell metabolism. In addition, appropriate tests can be performed *in vitro* to evaluate transgene functionality. If desired, the generation of immortalised cell lines from primary cells could

further expand the possibilities to test the consequences of transgene expression in long-term cell cultures.

One of the major efforts of the lentiviral vectors described in this work is their specificity against certain antigens depending on the nature of the scFv antibodies attached to the lentiviral vector envelope. Here, we use scFv antibodies against human CD105, a common marker of endothelial cells. LV-huCD105 has already been tested *in vitro* against cells from other non-primate species. While control human endothelial cells were positively transduced by LV-huCD105, endothelial cells from e.g. rodents were not. These findings were confirmed in preparative experiments at the beginning of this study (Anliker et al., 2010). To test whether LV-huCD105 could infect cells isolated from primates other than humans we continued testing LV-huCD105 *ex vivo* on vessels and isolated cells from Marmoset monkeys (*Callithrix jacchus*). Apparently, LV-huCD105 did not transduce cells from Marmoset monkeys, indicating that LV-huCD105 specificity may, in fact, be limited to humans or just a very limited number of primates. Further testing on other primate species more closely related to humans, e.g. chimpanzees, have to be performed to determine whether LV-huCD105 is absolutely specific to humans. If LV-huCD105 was found to transduce cells from any other primate, this specific species could be used for pre-clinical animal testing when ethical concerns have been addressed. Long-term sustainability of transgene expression would have to be measured in the living animal, as only a very limited number of highly differentiated cells are transduced. High turn-over rates of targeted endothelial layers would pose a considerable threat regarding continuous transgene expression, and repetitive application of lentiviral vectors would become necessary (Foteinos et al., 2008).

Encouraged by the successful *ex vivo* transduction of explanted human vessels we set out to transplant *ex vivo*-transduced vessels into immunodeficient mice. However, grafts failed soon after transplantation. Autopsy revealed that necrosis may have been the cause of graft failure. In an attempt to minimize graft failure and increase survival of the recipient mice, the *ex vivo* transduction time was shortened to just a few hours. However, this did not improve survival of the recipient mice, and experiments were cancelled after the second attempt due to ethical concerns. One can assume that a combination of hypoxia, mechanical stress, lack of nutrients and temperature changes caused failure of the graft.

Instead, grafts were directly transplanted into the abdominal cavity of mice to replace the abdominal part of the aorta. Ischemia time was minimized to 45 minutes. Mice recovered for

48 hours and repetitive tail vein injection with LV-huCD105 was performed. Using our experience from previous injections of the lentivirus into mice we assumed that 800 µl of the concentrated virus was insufficient to transduce a suitable amount of endothelial cells and to acquire detectable levels of transgene expression. The majority of the injected volume may be lost at the site of application, in connective tissues or distributed to other organs by the bloodstream, explaining the high injection volumes (Ott and Yuan, personal communication). In total, three injections were performed to maximize transduction of human endothelial cells of the graft. In three out of three mice, eGFP fluorescence was detected in CD105 expressing graft cells as a sign of transduction by the injected vector. As previously mentioned, CD105 expression may be upregulated in stress situations, making CD105 overrepresented in grafts compared to physiological conditions (Conley et al., 2000). In this specific case, CD105 may have risen over a certain threshold that is required to transduce human endothelial cells by systemic injection of LV-huCD105.

However, successful transduction of the graft endothelium was found to be our major achievement. The graft had been subject to severe mechanical and physiological stress situations and LV-huCD105 was still able to specifically transduce human endothelial cells. The results indicate that lentiviral vectors pseudotyped with measles virus glycoproteins and scFv antibodies against human CD105 are specific for human endothelial grafts in mice. In the future, functional transgenes other than eGFP could be transduced by means of LV-huCD105 to investigate whether sustained transgene expression in specifically targeted endothelial cells is possible (Abel et al., 2013). Xenotransplants are mainly rejected by cell-mediated and humoral responses against xenograft vessels, with subsequent clotting and hemorrhage (Yang and Sykes, 2007). One way to overcome xenograft rejection would be to transduce endothelial cells with anti-coagulatory factors or factors that render them invisible to the host immune system (Waern et al., 2012). Transduction of the xenograft endothelial cells would be performed with LV-huCD105 if anti-viral immune reactions against components of the lentivirus have been excluded.

One has to emphasize that the *in vivo* experiments suggest that gene therapy vectors may be directed against specific cell types without administering the vector at a nearby site. Here, lentiviral vectors were administered through the tail vein, and they consecutively circulated through the bloodstream passing the inferior vena cava, the right heart, the lungs, and the left heart before reaching the aorta. Still, lentiviral vectors arrived at the xenograft to transduce human endothelial cells, without unspecifically transducing non-human cells on its way. This is unprecedented before and marks a substantial advancement in organ directed gene therapy.

However, one has to critically mention that due to technical reasons mice with the aortic xenograft were not transplanted with other human tissues in order to investigate the distribution and specificity of LV-huCD105 amongst human xenografts.
Humans may become subject to lentiviral gene therapy one day, and scientists have to investigate by which means lentiviral vectors can be introduced into the human body most effectively. Perioperative local infusion of lentiviral vectors nearby the target tissue would bear the risk of surgical complications such as bleeding and infections. If cell-specific lentiviral vectors were prepared in sufficient amounts and with an adequate potency to transduce distant target tissues, surgical procedures could become obsolete and replaced by a common peripheral intravenous application into the extremities. To ensure patient safety, scientists have to guarantee that side effects of potent lentiviral vectors are negligible.

While transduction of endothelial cells, epidermal skin cells, conjunctivae of the eye and cells close to the nasopharyngeal tract are easily accessible by the means of targeted gene therapy, parenchymal cells of solid organs are separated by physical borders. If administered through peripheral veins, gene therapy vehicles would have to penetrate the endothelial cell layer in order to enter the parenchyma. This is quite similar to the events that have been identified when hepatocytes are transplanted into the portal vein. Transplanted cells squeeze through the fenestrations between endothelial cells to enter the parenchyma. However, engraftment can be improved by disrupting the endothelial cell layer with cyclophosphamide (Malhi et al., 2002). In parallel, if lentiviral access to the organs was limited, endothelial cell layers or other mediators controlling the exchange of molecules between the body fluids and certain parenchymal organs could be disrupted by chemicals to allow lentiviral vectors to enter. Similar attempts to increase viral transduction of specific organs have shown effective before: Application of mannitol to rats effectively disturbed the blood-brain barrier and allowed delivery of inactivated 35S herpesvirus to brain astrocytes (Neuwelt et al., 1991). Furthermore, hepatocyte transduction by lentiviruses injected by the portal vein was increased using macrophage-blocking agent gadolinium chloride (van Til et al., 2004). In addition, endothelial cells could be initially targeted by LV-huCD105 holding suicidal genes. Similar approaches using various viral vectors as vehicles of suicide genes have been previously described (Funke et al., 2008; Mathis et al., 2006; Nestler et al., 1997; Culver et al., 1992). The endothelium would become apoptotic and allow access to parenchymal organs by lentiviral vectors. Of course, this bears the risk of uncontrolled apoptosis, possibly even disruption of the vessel wall and subsequent hemorrhage (Dimmeler and Zeiher, 2000; Benjamin et al., 1997; Carmeliet et

al., 1996). Moreover, $CD105^+$ tumor vessels undergoing angiogenesis represent an ideal target for LV-huCD105 if equipped with pro-apoptotic transgenes (Dimmeler and Zeiher, 2000). Theoretically, endothelial cells of tumor cells could also be genetically modified by LV-huCD105 to synthesize anti-tumorous drugs to complement cytostatic therapy (Fonsatti et al., 2003).

Several questions regarding the feasibility of lentiviral vectors pseudotyped with measles virus hemagglutinin for targeted gene therapy remain unanswered. However, advances are made continuously in the field of targeted gene therapy. In this study, LV-huCD105 is tested both *ex vivo* and *in vivo* regarding its feasibility to specifically transduce human endothelial cells. The results indicate that LV-huCD105 may be targeted specifically to $CD105^+$ cells *in vivo*, underlining the future possibilities of targeting lentiviral vectors pseudotyped with measles virus glycoproteins fused to scFv antibodies against specific antigens. Following these findings, a wide array of new scFv antibodies targeting novel surface antigens will soon come forth, enhancing efforts to evolve targeted gene therapy to the clinical stage.

5. Summary/Abstract

Gene therapy is a promising tool to cure various genetic disorders that arise from mutations in the germline. After several setbacks, new approaches are being developed to meet the high standards in accordance to the strict guidelines of good clinical practise and especially in regards to patient safety. The use of self-inactivating, non-replicating lentiviral vehicles has emerged to become a valuable technique to deliver genes into diseased cells to replace malfunctioning substrates of biochemical pathways.

The tropism of lentiviral vectors can be altered by modifying the viral envelope, i.e. pseudotyping. Recently, mutated forms of the measles virus hemagglutinin (MSV-HA) have been used fused to single chain variable fragment (scFv) antibodies to restrict transduction by lentiviruses to a specific subset of cells.

In this study, scFv-antibodies against asialoglycoprotein receptor (ASGPR) and endoglin (ENG/CD105) were integrated into the viral envelope to achieve cell-specific transduction of hepatocytes and endothelial cells, respectively. However, only endoglin-directed lentiviral vectors (LV-huCD105) proved efficient in various *in vitro* transduction experiments. *Ex vivo* protocols were established to transduce human arteries and veins as well as primary endothelial cells isolated from human vessels. Immunohistofluorescence staining showed that transduced cells all expressed endoglin, thereby demonstrating the specificity of the lentiviral vector. In concluding experiments, BALB/c $Rag2^{-/-}$ IL-$2R\gamma_c^{-/-}$ mice were transplanted with human arteries into the abdominal aorta and LV-huCD105 was injected into the tail vein. Remarkably, only the xenograft, but not murine vessels, showed successful transduction by LV-huCD105, proving not only the species-specificity of LV-huCD105, but also its ability to specifically transduce human endothelial cells in mice upon circulation in the bloodstream.

Summing up, this study demonstrates that the integration of scFv-antibodies directed against endoglin into the viral envelope allows the generation of high-titer lentiviral vectors capable of targeted infection of human endothelial cells. The findings are a major breakthrough in gene therapy, as they implicate that targeted *in vitro* gene transfer into endothelial cells is possible after systemic application of the lentiviral vehicles. Targeting transgenes to endothelial cells will help to deliver inhibiting drugs to sites of atherosclerotic inflammation or anti-tumor pharmaceuticals to the site of angiogenesis.

6. Literature

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Last but not least I want to thank my family, my mother and Rebecca, for their support behind the scenes during the time within and out of the laboratory.

8. Supplementary

Sequence of human ASGR1 transcript variant 1 (876 bp)

```
1     ATGACCAAGG AGTATCAAGA CCTTCAGCAT CTGGACAATG AGGAGAGTGA
51    CCACCATCAG CTCAGAAAAG GGCCACCTCC TCCCCAGCCC CTCCTGCAGC
101   GTCTCTGCTC CGGACCTCGC CTCCTCCTGC TCTCCCTGGG CCTCAGCCTC
151   CTGCTGCTTG TGGTTGTCTG TGTGATCGGA TCCCAAAACT CCCAGCTGCA
201   GGAGGAGCTG CGGGGCCTGA GAGAGACGTT CAGCAACTTC ACAGCGAGCA
251   CGGAGGCCCA GGTCAAGGGC TTGAGCACCC AGGGAGGCAA TGTGGGAAGA
301   AAGATGAAGT CGCTAGAGTC CCAGCTGGAG AAACAGCAGA AGGACCTGAG
351   TGAAGATCAC TCCAGCCTGC TGCTCCACGT GAAGCAGTTC GTGTCTGACC
401   TGCGGAGCCT GAGCTGTCAG ATGGCGGCGC TCCAGGGCAA TGGCTCAGAA
451   AGGACCTGCT GCCCGGTCAA CTGGGTGGAG CACGAGCGCA GCTGCTACTG
501   GTTCTCTCGC TCCGGGAAGG CCTGGGCTGA CGCCGACAAC TACTGCCGGC
551   TGGAGGACGC GCACCTGGTG GTGGTCACGT CCTGGGAGGA GCAGAAATTT
601   GTCCAGCACC ACATAGGCCC TGTGAACACC TGGATGGGCC TCCACGACCA
651   AAACGGGCCC TGGAAGTGGG TGGACGGGAC GGACTACGAG ACGGGCTTCA
701   AGAACTGGAG GCCGGAGCAG CCGGACGACT GGTACGGCCA CGGGCTCGGA
751   GGAGGCGAGG ACTGTGCCCA CTTCACCGAC GACGGCCGCT GGAACGACGA
801   CGTCTGCCAG AGGCCCTACC GCTGGGTCTG CGAGACAGAG CTGGACAAGG
851   CCAGCCAGGA GCCACCTCTC CTTTAA
```

Protein sequence of human ASGPR isoform 1a (291 aa)

```
1     MTKEYQDLQH LDNEESDHHQ LRKGPPPPQP LLQRLCSGPR LLLLSLGLSL
51    LLLVVVCVIG SQNSQLQEEL RGLRETFSNF TASTEAQVKG LSTQGGNVGR
101   KMKSLESQLE KQQKDLSEDH SSLLLHVKQF VSDLRSLSCQ MAALQGNGSE
151   RTCCPVNWVE HERSCYWFSR SGKAWADADN YCRLEDAHLV VVTSWEEQKF
201   VQHHIGPVNT WMGLHDQNGP WKWVDGTDYE TGFKNWRPEQ PDDWYGHGLG
251   GGEDCAHFTD DGRWNDDVCQ RPYRWVCETE LDKASQEPPLL
```

Sequence alignment of ASGPR-scFv

```
                      1                                                  50
scFv-benhar     (1)   GAGGTGCAGCTGTTGGAGTCTGGGGGAGGCTTGGTACAGCCTGGGGGGTC
scFv-benharP    (1)   GAGGTGCAGCTGTTGGAGTCTGGGGGAGGCTTGGTACAGCCTGGGGGGTC
scFv-Cao        (1)   GAGGTGCAGCTGTTGGAGTCTGGGGGAGGCTTGGTACAGCCTGGGGGGTC
                      51                                                 100
scFv-benhar    (51)   CCTGAGACTCTCCTGTGCAGCCTCTGGATTCACCTTCAGTGACTACTACA
scFv-benharP   (51)   CCTGAGACTCTCCTGTGCAGCCTCTGGATTCACCTTCAGTGACTACTACA
scFv-Cao       (51)   CCTGAGACTCTCCTGTGCAGCCTCTGGATTCACCTTTAGCAGCTATGCCA
                      101                                                150
scFv-benhar   (101)   TGAGCTGGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGGGTTTCAGCT
scFv-benharP  (101)   TGAGCTGGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGGGTTTCAGCT
scFv-Cao      (101)   TGAGCTGGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGGGTCTCAGGT
                      151                                                200
scFv-benhar   (151)   ATTACTACTGGTGGTGGTAGCCCGAACTATGCAGACTCCGTGAAGGGCCG
scFv-benharP  (151)   ATTACTACTGGTGGTGGTAGCCCGAACTATGCAGACTCCGTGAAGGGCCG
scFv-Cao      (151)   ATTAATGGTACTGGTTATACTACATCTTACGCAGACTCCGTGAAGGGCAG
                      201                                                250
scFv-benhar   (201)   GTTCACCATCTCCAGAGACAATTCCAAGAACACGCTGTATCTGCAAATGA
scFv-benharP  (201)   GTTCACCATCTCCAGAGACAATTCCAAGAACACGCTGTATCTGCAAATGA
scFv-Cao      (201)   GTTCACCATCTCCAGAGACAATTCCAAGAACACGCTGTATCTGCAAATGA
                      251                                                300
scFv-benhar   (251)   ACAGCCTGAGAGCCGAGGACACGGCCGTGTATTACTGTGCGAGAGACAGG
scFv-benharP  (251)   ACAGCCTGAGAGCCGAGGACACGGCCGTGTATTACTGTGCGAGAGACAGG
scFv-Cao      (251)   ACAGCCTGAGAGCCGAGGACACGGCCGTATATTACTGTGCGAAAT-----
                      301                                                350
```

```
scFv-benhar   (301) ACCGCCGGGTACTTTGACTACTGGGGCCAGGGCACCACCGTCACGGTCTC
scFv-benharP  (301) ACCGCCGGGTACTTTGACTACTGGGGCCAGGGCACCACCGTCACGGTCTC
scFv-Cao      (296) -CTGGTGGTGATTTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTCTC
                    351                                             400
scFv-benhar   (351) CTCAGGCGGAGGAGGGTCCGGTGGTGGTGGTTCTGGCGGCGGCGGCTCCC
scFv-benharP  (351) CTCAGGCGGAGGAGGGTCCGGTGGTGGTGGTTCTGGCGGCGGCGGCTCCC
scFv-Cao      (345) GAGCGGTGGAGGCGGTTCAGGCGGAGGTGGCAGCGGCGGTGGCGGGTCGA
                    401                                             450
scFv-benhar   (401) ---AAAGCGTGCTGACTCAG---CCACCCTCAGCGTCTGGGACCCCCGGG
scFv-benharP  (401) ---AAAGCGTGCTGACTCAG---CCACCCTCAGCGTCTGGGACCCCCGGG
scFv-Cao      (395) CGGACATCCAGATGACCCAGTCTCCATCCTCCCTGTCTGCATCTGTAGGA
                    451                                             500
scFv-benhar   (445) CAGAGGGTCACCATCTCTTGTACTGGCAGCAGCTCCGGCATTGGGAATGC
scFv-benharP  (445) CAGAGGGTCACCATCTCTTGTACTGGCAGCAGCTCCGGCATTGGGAATGC
scFv-Cao      (445) GACAGAGTCACCATCACTTGC-CGGGCA--AGTCAGAGCATTAGCA--GC
                    501                                             550
scFv-benhar   (495) TTATGTGTCCTGGTACCAGCAGCTCCAGGAAAGGCTCCCAAACTCCTCA
scFv-benharP  (495) TTATGTGTCCTGGTACCAGCAGCTCCAGGAAAGGCTCCCAAACTCCTCA
scFv-Cao      (490) T-ATTTAAATTGGTATCAGCAGAAACCAGGGAAAGCCCTAAGCTCCTGA
                    551                                             600
scFv-benhar   (545) TTTATAA-GAATGGTCAGCG-GCCCTCAGGGGTCTCTGACCGGTTCTCTG
scFv-benharP  (545) TTTATAA-GAATGGTCAGCG-GCCCTCAGGGGTCCCTGACCGGTTCTCTG
scFv-Cao      (539) TCTATTCTGCAT--CCAGCTTGCAAAGTGGGGTCCCATCAAGGTTCAGTG
                    601                                             650
scFv-benhar   (593) GCTCCAAGTCTGGCACCTCAGCCTCCCTGGCCATCAGTGGGCTGCGGTCC
scFv-benharP  (593) GCTCCAAGTCTGGCACCTCAGCCTCCCTGGCCATCAGTGGGCTGCGGTCC
scFv-Cao      (587) GCAGTGGATCTGGGACAGATTTCACTCTCACCATCAGCAGTCTGCAACCT
                    651                                             700
scFv-benhar   (643) GAGGATGAGGCTGATTATTACTGCGCAGCATGGGATGACAGCCTG-AATG
scFv-benharP  (643) GAGGATGAGGCTGATTATTACTGCGCAGCATGGGATGACAGCCTG-AATG
scFv-Cao      (637) GAAGATTTTGCAACTTACTACTGT-CAACA------GTCTTCTTCTAATC
                    701                                       741
scFv-benhar   (692) GTTGGGTGTTCGGCGGAGGCACCAAGCTCACCGTCCTC---
scFv-benharP  (692) GTTGGGTGTTCGGCGGAGGCACCAAGCTCACCGTCCTC---
scFv-Cao      (680) CTAATACGTTCGGCCAAGGGACTAAGGTGGAAATCAAACGG
```

Sequence of the ENG-scFv

```
1       ATGGCCGAAG TGCAGCTGCT GGAAAGCGGC GGTGGCCTGG TGCAGCCGGG
51      TGGCTCTCTG CGTCTGTCTT GCGCGGCTAG CGGCTTCACC TTTAGCAGCT
101     ATGCTATGAG CTGGGTTCGC CAGGCGCCCG GGAAAGGTCT GGAATGGGTT
151     TCTGCTATTT ATGGTAGCGA TGGTGATACC ACGTACGCGG ATTCCGTGAA
201     AGGCCGCTTC ACCATCAGCC GTGATAACTC TAAAAACACC CTGTATCTGC
251     AGATGAACAG CCTGCGCGCC GAAGACACGG CGGTGTATTA CTGCGCGCGC
301     GTGTTTTATA CGGCGGGCTT CGATTATTGG GGCCAGGGTA CGCTGGTCAC
351     CGTCTCGAGC GGTAGCGATT CCAACGCGGG GCACGCCAGC GCCGGTAACA
401     CCTCTGATAT TGAGCTCACC CAGTCTCCGT CCTCCCTGTC TGCATCTGTT
451     GGCGATCGTG TGACCATCAC CTGCCGCGCA TCCCAGAGCA TTAGCTCTTC
501     TCTGAACTGG TACCAGCAGA AACCGGGCAA AGCCCCGAAA CTGCTGATCT
551     ATGCTGCGTC CAGCTTGCAG AGCGGCGTGC CGTCTCGCTT CAGCGGATCC
601     GGTTCTGGCA CCGATTTCAC CCTGACCATC AGCAGCCTGC AGCCGGAAGA
651     TTTTGCAACT TACTATTGTC AACAGGCGCC GGCGAAGCCG CCGACGTTCG
701     GCCAGGGCAC CAAACTGGAA ATTAAACGT
```

Amino acid sequence of scFv-ENG

```
1       MAEVQLLESG GGLVQPGGSL RLSCAASGFT FSSYAMSWVR QAPGKGLEWV
51      SAIYGSDGDT TYADSVKGRF TISRDNSKNT LYLQMNSLRA EDTAVYYCAR
101     VFYTAGFDYW GQGTLVTVSS GSDSNAGHAS AGNTSDIELT QSPSSLSASV
151     GDRVTITCRA SQSISSSLNW YQQKPGKAPK LLIYAASSLQ SGVPSRFSGS
201     GSGTDFTLTI SSLQPEDFAT YYCQQAPAKP PTFGQGTKLE IKR
```

Curriculum vitae

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Abel, T., El Filali, E., Waern, J., et al., 2013. Specific gene delivery to liver sinusoidal and artery endothelial cells. Blood **122,** 2030-2038

Ort, Datum Johan Waern

Declaration/Eidestattliche Erklärung **(§ 2 Abs. 2 Nr. 6 +7 PromO, MHH)**

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Cell-specific Infection of Human Cells Mediated by Lentiviral Vectors Pseudotyped with Measles Virus Hemagglutinin Fused to Single Chain Antibodies

in der Klinik für Gastroenterologie, Hepatologie und Endokrinologie, MHH
unter Betreuung von

Prof. Dr. med. M. Ott

und in Zusammenarbeit mit

PD Dr. med. G. Warnecke
Prof. Dr. C. Buchholz (PEI, Langen)

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