Epigenetic regulation of lentiviral and retroviral vectors in murine embryonic stem (ES) cells and genetic modification of ES cells with lentiviral vectors for application in regenerative medicine

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Abbrevations

5’-Aza	5-Azacytidine
Ad	adjust to exact volume
ATP	adenosine triphosphate
APS	Ammonium persulfate
BES	*N*,*N*-bis (2-Hydroxyethyl)-2-aminoethanesulfonic acid
BBS	Borate Buffered saline
bp	base pair
BS	bisulfate solution
BSA	bovine serum albumin
CA	virus capsid
CCR5	chemokine receptor type 5
CD4	glycoprotein, expressed on the surface of T lymphocytes
cDNA	complementary DNA
CMV	cytomegalo virus
CpG	cytosine and guanine dinucleotide separated by one phosphate
cPPT	central polypurine tract
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
Dnmt3a	*De-novo* Methyltransferase 3a
Dnmt3b	*De-novo* Methyltransferase 3b
dNTPs	desoxynucleotide mix
dTTP	Deoxythymidine triphosphate
E.coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
eGFP	enhanced green fluorescent protein
env	envelope gene of wildtype HIV
ES cell	embryonic stem cell
FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
gag	retroviral *gag* gene encoding group specific antigens
gp120	envelope glycoprotein gp120 of wildtype HIV
gp41	envelope glycoprotein gp41 of wildtype HIV
gDNA	genomic DNA
H&E	hematoxylin and eosin staining
HBSS	Hanks' Balanced Salt Solution
HEK293T	human embryonic kidney 293 cells expressing large T antigen from simian virus 40
HIV	human immunodeficiency virus

HRP	horse radish peroxidase
HSCs	haematopietic stem cells
ICM	inner cell mass
IMDM	Iscove's Modified Dulbecco's Medium
iPS	induced pluripotent stem cell
kb	kilo base
kDa	kilo Dalton
KH_2PO_4	Monopotassium phosphate
LB^+	Luria-Bertani medium with glucose
LTR	long terminal repeat
LVs	lentiviral vectors
MA	matrix proteins
MeCP2	methyl-CpG-binding protein 2
mRNA	messenger ribonucleic acid
MLV	murine leukemia viruses
PAGE	polyacrylamide gel electrophoresis
PB	primer binding site
PBS	phosphate buffered Saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
PGK	phosphoglycerate kinase
PIC	preintegration complex
pol	retroviral *pol* gene encoding polymerases
ψ (Psi)	packaging signal sequences of retroviruses
RVs	simple retroviral vectors
rev	*rev* gene of wildtype HIV, regulator of expression
rpm	revolutions per minute
RRE	*rev* response element
RSV	Rous sarcoma virus
RT	room temperature
SA	splice acceptor
SD	splice donor
SDS	Sodium dodecyl sulfate
SFCA	surfactant-free cellulose acetate
SI	subzonal injection
SIN	self inactivating
Taq	*Thermophilus aquaticus*
TAR	trans-activation response element, binding site for the *Tat* protein
tat	*tat* gene of wildtype HIV, *trans*-activator of transcription
TBE	Tris-borate-EDTA buffer
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline supplemented with Tween-20
TEMED	N, N, N', N'-tetramethylethylenediamine
Tris	Tris-hydroxyethyl aminomethan
TSA	Trichostatin A

UV	ultraviolet
vif	accessory genes of wildtype HIV
vpr	accessory genes of wildtype HIV
vpu	accessory genes of wildtype HIV
VSV.G	glycoprotein of vesicular stomatitis virus
WPRE	post-transcriptional regulator element of Woodchuck hepatitis virus
E.coli XL-1blue	*Escherichia coli* strain, used for transformation of plasmids (prepared as competent cells)

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1. Introduction

1.1. Retroviruses

Retroviruses belong to the *Retroviridae* family (Coffin, 1990; Coffin, 1992; Russell & Miller, 1996), which comprises various enveloped RNA viruses. The viral particles have a diameter of 80-100nm (Coffin, 1992). One characteristic of retroviruses is their capability to integrate into the genome of the host cells after reverse transcription of their viral RNA (Vogt, 1997). Figure 1 shows a schematic illustration of a lentivirus particle of *Retroviridae* family, i.e. the human immunodeficiency virus (HIV). The outer envelope membrane consists of a lipid bilayer with two transmembrane (TM) proteins (glycoprotein 120 (gp120) and 41 (gp41)) that are anchored on the surface of the envelope membrane (Figure 1) (Coffin, 1992; Vogt, 1997). Within the viral particle, matrix proteins (MA) are connected to the inside of the envelope membrane (Figure 1). Within the MA, a conical viral capsid (CA) contains two identical linear single-stranded RNAs with 7-12 kb in size (Vogt, 1997) and several viral enzymes including reverse transcriptase, integrase and proteinase (Figure 1) (Coffin, 1992).

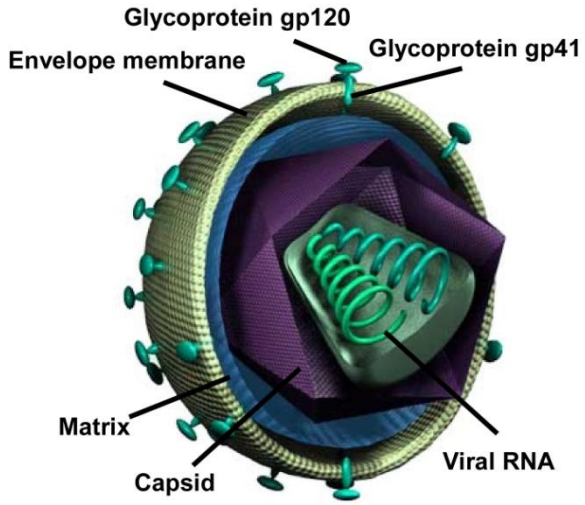

Figure 1: Schematic illustration of a lentivirus particle, human immunodeficiency virus (HIV-1). HIV-1 belongs to retroviruses. Graphic is modified from the cover of *Journal of the American Biological Safety Association* (Volume 12, Number 2, 2007, http://www.absa.org/abj/abj/ABJ2007v12n2.pdf)

All retroviruses carry three essential genes *gag*, *pol* and *env* (Figure 2, see also Figure 6). The *gag* genes encode domains for synthesis of MA, CA and structural proteins; the *pol* genes encode for the essential enzymes including reverse transcriptase, integrase and proteinase; and the *env* genes contain domains for surface and transmembrane components of viral surface proteins, i.e. glycoproteins (Figure 2).

The viral genome is flanked by regulatory *cis*-active sequences (Figure 2) that contain essential elements responsible for reverse transcription and also for the integration (Kao et al, 1987; Starcich et al, 1985). The R-region (R = redundant) on 5' and 3' ends of the viral genome contains identical sequences and is flanked with a cap structure at the 5'end (Modrow et al, 2010). Upstream of the U5 (U = unique) element a sequence is located that is mainly responsible for the integration of the provirus into the host cell chromosome. Downstream of U5 element exits the primer binding site (PB) to which a complementary cellular tRNA can bind. A splice donor (SD) downstream of PB is responsible for the production of spliced mRNA (Starcich et al, 1985). Following to SD, the packaging signal sequences (abbreviated as ψ) are required for encapsidation of viral RNA (Clever et al, 2000). A polypurine tract (abbreviated as PP) on 3' end of *gag*/*pol*/*env* genes is important for the initiation of synthesis of double-stranded DNA by reverse transcription. During the viral reverse transcription, U3-region will be duplicated and copied on to 5' ends. This results in the formation of long terminal repeat (LTRs) consisting of U3-R-U5 on both ends of provirus double-stranded DNA. Additionally, the U3-region contains promoter and *cis*-acting control elements where cellular proteins bind to regulate the gene transcription (Modrow et al, 2010).

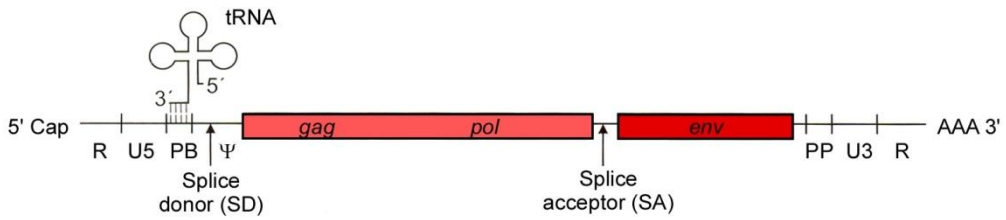

Figure 2: Viral RNA of retroviruses. Image is modified from Modrow et al, 2010 (Modrow et al, 2010)

1.2. Life cycle of retroviruses

To enter the host cell, the retroviral surface glycoprotein (i.e. gp120 in case of HIV) binds in the first step to specific plasma membrane receptors (i.e. CD4 receptor) (Figure 3). After attachment to the cells, TM glycoproteins mediate the fusion with the cell membrane and the uncoating of viral particles. Afterwards, the viral RNA and essential enzymes including reverse transcriptase are released into the cytoplasm of host cells. The reverse transcription, which takes place in the cytoplasm by the viral reverse transcriptase, leads to the

generation of double-stranded DNA from viral RNA. This double-stranded provirus DNA is further transported into the nucleus and inserted into the host cell's chromosome by the viral integrase. This results in an integrated provirus. The provirus is further transcribed by cellular RNA polymerase II and single-, multiple-spliced mRNAs and also the full-length virion RNA genome are generated. Viral mRNAs are translated by the cellular ribosome in the cytoplasm resulting in formation of ENV, Gag and Gag-Pol polyproteins. These virion proteins and the virion RNA genome are assembled at the cell periphery and the plasma membrane. Finally, all components of the virus including envelope, capsid and matrix form a complex virus (virion), bud through the plasma membrane and are released from the host cells.

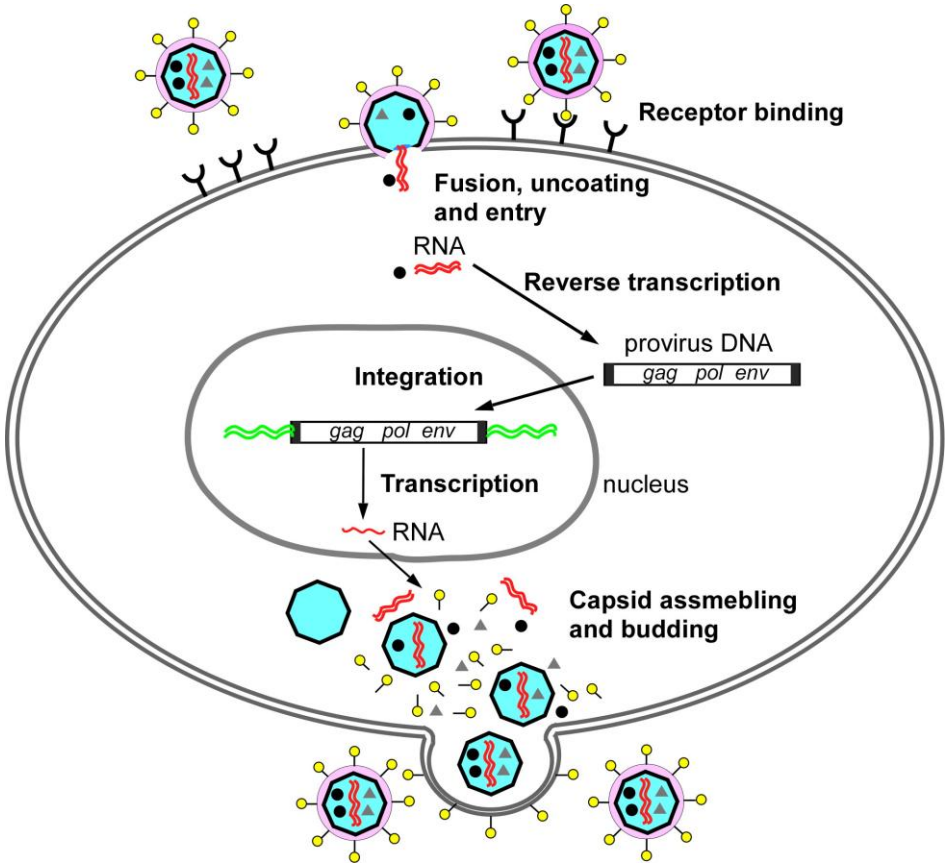

Figure 3: Life cycle of retroviruses. Modified after Pfeifer and Verma (2001) (Pfeifer & Verma, 2001a).

1.3. Simple and complex retroviruses

Retroviruses can be divided into two subgroups according to their genome: simple retroviruses and complex retroviruses (Coffin, 1992; Vogt, 1997). The simple retroviruses carry three major coding regions for viral proteins as already abbreviated as *gag*, *pol* and *env* (see also Figure 2). In addition to the three major coding regions, complex retroviruses

contain regulatory and accessory genes derived from multiple spliced mRNA (Vogt, 1997). For example, HIV that belongs to genus *Lentivirus* (complex retrovirus) has additional regulatory genes including *tat* (*trans*-activator of transcription) and *rev* (regulator of expression) and also accessory genes including *vif*, *vpr*, *vpu*, *nef* (see Figure 5). The Tat protein mediates the *trans*-active response (TAR) (Jones, 1997; Modrow et al, 2010) by binding to the TAR element that is essential for trans-activation of the viral promoter and virus replication (Kulinski et al, 2003). The Rev protein of HIV is responsible for post-transcriptional gene regulation during the replication cycle (Modrow et al, 2010; Pollard & Malim, 1998) by binding of Rev proteins to the RRE (*rev* response element). This enables an export of full-length viral mRNAs or multiple-spliced viral mRNA from the nucleus into the cytoplasm (Fischer et al, 1994). The Vif protein is a component of nuclear protein complexes of infectious viral particles that are necessary for HIV-1 replication *in vivo* (Marin et al, 2003; Modrow et al, 2010). The Vpr protein contributes to the transport of the pre-integration complex (PIC), which contains all necessary components for viral integration, into the nucleus. The Vpu protein enhances virus particle secretion (Geraghty et al, 1994; Schubert et al, 1996b) and enables more efficient shedding of viral particles (Schubert et al, 1996a). The Nef protein also increases the viral infectivity (Hirsch & Curran, 1996).

Besides these structural differences, the main difference between simple and complex viruses is their infection mode: The complex retroviruses (e.g. lentivirus, HIV-1) are able to infect dividing as well as nondividing cells (Figure 4 left) (Gallay et al, 1997; Lewis & Emerman, 1994) whereas the simple retroviruses (e.g. gammaretroviruses, MLV) are unable to infect the nondividing cells (Figure 4 right) (Lewis & Emerman, 1994; Yamashita & Emerman, 2006). In an early study it was suggested that the capsid protein plays an important role for the difference between HIV and MLV in their ability to transduce nondividing cells (Yamashita & Emerman, 2004). During fusion with the cellular membrane, HIV-1 capsid degrades quite early (Fassati, 2006), which leads to low abundance of CA. The minimal residual of capsid shell favors the cellular transport of PIC (Yamashita & Emerman, 2004). In contrast, MLV PIC contains a capsid shell until the nuclear entry (Fassati, 2006) that blocks the cellular transport of PIC (Yamashita & Emerman, 2004). Therefore, MLV provirus can not enter the nuclear membrane through nuclear pores. The provirus of MLV can only access the host cell chromosome during the cell mitosis when there is brief dissolve of nuclear membrane (Lewis & Emerman, 1994).

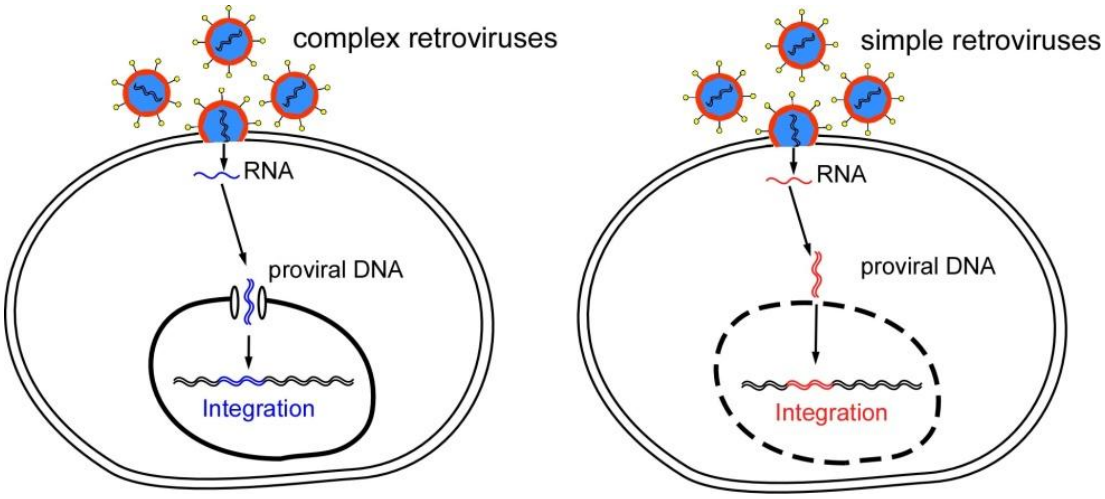

Figure 4: Schematic representation of integration progress of complex retroviruses (e.g. HIV-1)(left) and simple retroviruses (e.g. MLV)(right).

1.4. Retroviral and Lentiviral vectors

For using retro- or lentiviral vectors as a gene vehicle for transfer of transgenes into host cells leading to stable transgene expression, different systems were already established that are all based on the same principle. For the establishment of the viral vector system, the first step is to identify which viral genes are responsible for pathogenesis, replication and production of infectious particles. For safety propose, all pathogenesis-related genes were deleted from the viral genome. The essential viral genes for virus production were separated into two systems: the viral plasmids and the packaging constructs (Pfeifer & Verma, 2001a).

The viral plasmids contain the transgene cassette and *cis*-active sequences that are necessary for encapsidation of the viral vector genome and for viral transduction of the target cells. The packaging constructs contain viral sequences encoding genes for viral proteins essential for packaging of the vector genome and replication. For virus production, viral plasmids and packaging constructs are co-transfected into packaging or helper cells (e.g. HEK 293T cells). Afterwards, the recombinant viral particles are released from the packaging cells. These viruses are infectious but replication defective. Mostly, lentiviral vectors (LVs) generated in this way are based on HIV-1's sequence (Figure 5) and gammaretroviral vectors (RVs) on MLV (Figure 6).

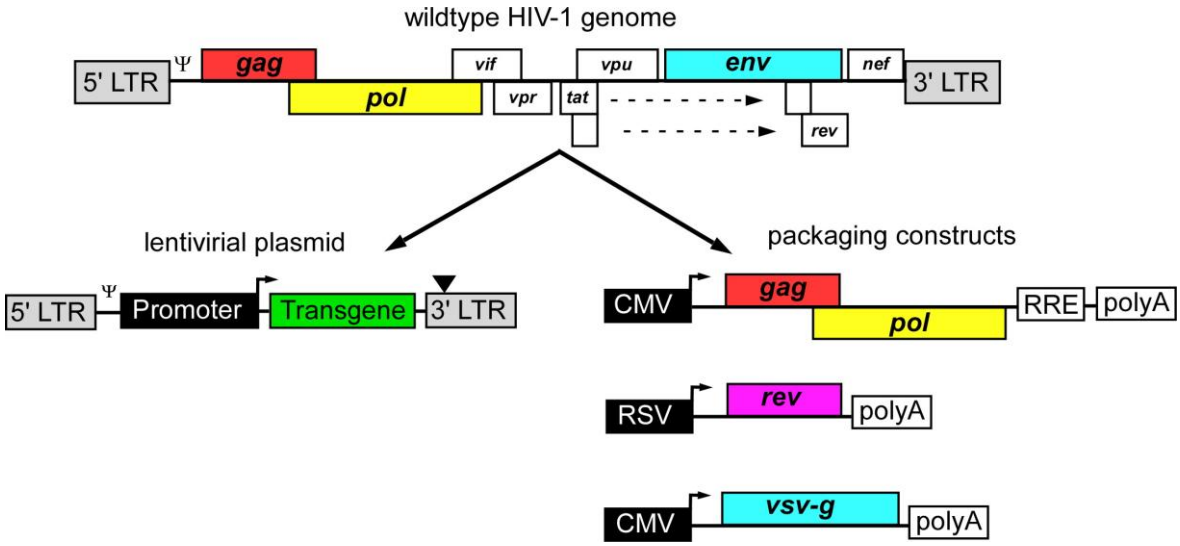

Figure 5: Schematic representation of lentiviral vector (LV) and packaging constructs derived from wildtype HIV-1 genome. Black inverse triangle indicates the deletion of LTR's promoter/enhancer at 3' LTR.

In detail, the wildtype HIV-1 accessory genes *vif, vpr, vpu* and *nef* (Figure 5) are related to viral pathogenesis. They are important for the viral life cycles *in vivo* but they are not essential for viral replication *in vitro* (Delenda, 2004; Kim et al, 1998; Zufferey et al, 1998). Therefore, to reduce the infectious risk of LVs removal of these viral elements from HIV genome led to the generation of lentiviral plasmid just containing the *cis*-active sequences and packaging signal sequences ψ of HIV-1 (Dull et al, 1998). Furthermore, it was shown that deletion of these elements does not compromise gene transduction (Zufferey et al, 1997). Furthermore, components of the 3' LTR were deleted (marked with black triangle in Figure 5). As parts of the 3' LTR are transferred to the 5' LTR during the process of reverse transcription (please refer to 1.1. or see below for details) this deletion leads to loss of promoter activity in the 5' LTR. Thus, an internal promoter was inserted in the lentiviral plasmid that can be ubiquitous and also tissue specific.

Besides the lentiviral plasmid several packaging plasmids encoding necessary genes have to be co-transfected in the producer cell line. The plasmid containing viral *gag-pol* cDNA provides the production of the essential enzymes and virion proteins. The regulatory protein Rev, encoded on a separate construct, interacts with RRE and enables production of high-titer viruses (Dull et al, 1998) as Rev enhance the nuclear export of unspliced *gag-pol* mRNA and viral vector RNA (Delenda, 2004). The native envelope gene *env* of HIV-1 restricts the infection of only cells that express CD4 protein and coreceptors (Berger et al, 1999; Wyatt & Sodroski, 1998). In order to increase the host cell spectrum, the *env* has been replaced with heterologous viral envelope proteins (pseudotyping), e. g. with the

glycoprotein of vesicular stomatitis virus (VSV.G) that is encoded on a third packaging plasmid (Naldini et al, 1996; Reiser et al, 1996). Besides the broader host tropism of VSV.G enveloped LVs, this envelope enables virus purification by ultracentrifugation. Furthermore, it facilitates production of viral vectors with higher titers and with increased resistance against freezing and thawing (Lever et al, 2004).

The retroviral genome of MLV is also separated into two groups: the retroviral plasmid and the packaging constructs (Figure 6). In detail, the viral plasmid contains the transgene cassettes that are under the control of the prototypic LTR promoter (Figure 6A) or internal promoter (Figure 6B). The essential virion and envelope proteins for packaging are divided into two expressing plasmids including *gag-pol* gene encoding virion proteins and the gene encoding for the wildtype ecotropic envelope proteins (Figure 6, right). In addition, RVs can also be pseudotyped with the VSV.G envelope.

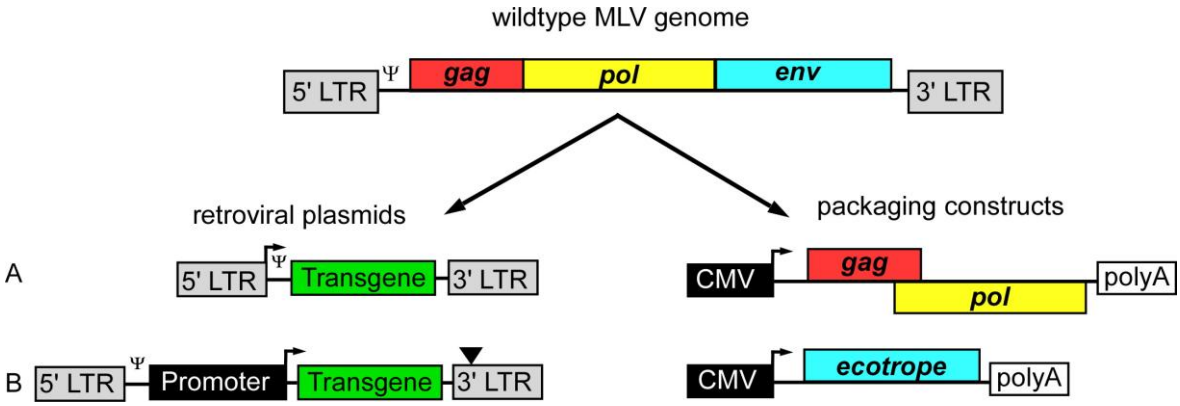

Figure 6: Schematic representation of retroviral vectors (RV) and packaging constructs derived from wildtype MLV genome with wildtype LTR-promoter (A) or with internal promoter (B). Black inverse triangle indicates the deletion of LTR's promoter/enhancer at 3' LTR.

During the viral reverse transcription in host cells, the 3' U3 and 5' U5 region of viral RNA are duplicated, which results in formation of LTR consisting of U3-R-U5 with the U3 region containing viral promoter and enhancer elements. Thus, the 5' LTR has promoter activity and regulates the transcriptional initiation of cellular RNA polymerase II (Figure 7 right). As retroviral or lentiviral vectors integrate randomly into host genome, therefore they may cause an insertional activation of cellular oncogenes (Miyoshi et al, 1998). This reveals a biosafety problem when prototypic LTR-promoters are included in viral vectors. Therefore, the U3 promoter/enhancer elements of 3' LTR are deleted in the lenti- or retroviral plasmids (black triangle in viral vectors in Figure 5, 6B and 7). During reverse transcription, these deleted elements are no longer transferred into 5' LTR, which prevents the transcriptional activity of 5' LTR in the integrated provirus. The viral vectors

carrying deleted U3 promoter are termed self-inactivating (SIN) vectors including SIN-RVs and SIN-LVs as they inactivate promoter activity during their own reverse transcription of RNA (Kim et al, 1998; Miyoshi et al, 1998; Yu et al, 1986; Zufferey et al, 1998). SIN vectors carry an internal promoter to drive transgene expression. Moreover, in SIN vectors the transcriptional interference between LTR promoter and the internal promoter is prevented (Zufferey et al, 1998).

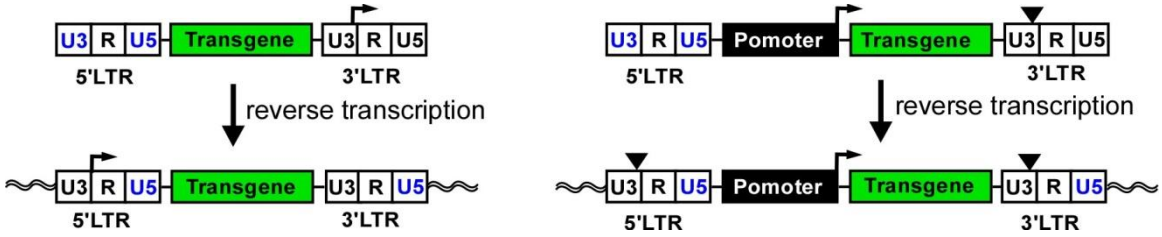

Figure 7: Schematic illustration of viral vectors with wildtype LTR promoter (left) and SIN vectors with internal promoter (right). . Black inverse triangle indicates the deletion of LTR's promoter/enhancer at 3' LTR.

In addition, the efficiency of gene transfer can be increased by inserting a regulatory sequence into the viral plasmid. For example, a central polypurine tract (cPPT) has been suggested to promote nuclear translocation of the PIC (Van Maele et al, 2003; Zennou et al, 2000). The insertion of a post-transcriptional regulatory element of Woodchuck hepatitis virus (WPRE) led to a substantial raise of expression and stability of the transgene (Zufferey et al, 1999).

1.5. Pluripotent stem cells: embryonic stem cells and induced pluripotent stem cells

Embryonic stem (ES) cells are derived from the primitive ectoderm of the inner cell mass of blastocysts (Figure 8) (Evans & Kaufman, 1981; Nagy et al, 1993). They are characterized by their self-renewal capability and their pluripotency, i.e. they can develop into the primary germ layers (ectoderm, endoderm, and mesoderm) (Tanaka et al, 2009; Wobus & Boheler, 2005). Because of their capacity to differentiate into all cell types of the adult body (Figure 8), ES cell-based therapies have been proposed for regenerative medicine and tissue replacement (Wobus & Boheler, 2005). Moreover, the isolation of ES cells from human blastocysts was a breakthrough for a potential therapeutic use of ES cells. In previous studies, various cell types derived from murine or human ES cells have been identified including neurons (Carpenter et al, 2001; Li et al, 1998), pancreatic β-cells (Assady et al, 2001; Soria et al, 2000), cardiomyocytes (Kaufman et al, 2001; Klug et al,

1996) and endothelial cells (Levenberg et al, 2002; Quinn et al, 2000). Thus, in the last decade, these ES cells have been provided a potential tool for future regenerative medicine (Keller, 2005; Wobus & Boheler, 2005). So far, retinal pigment epithelia derived from human ES cells has been already applied in first clinical trial for Stargardt's disease and macular degeneration (Schwartz et al, 2012).

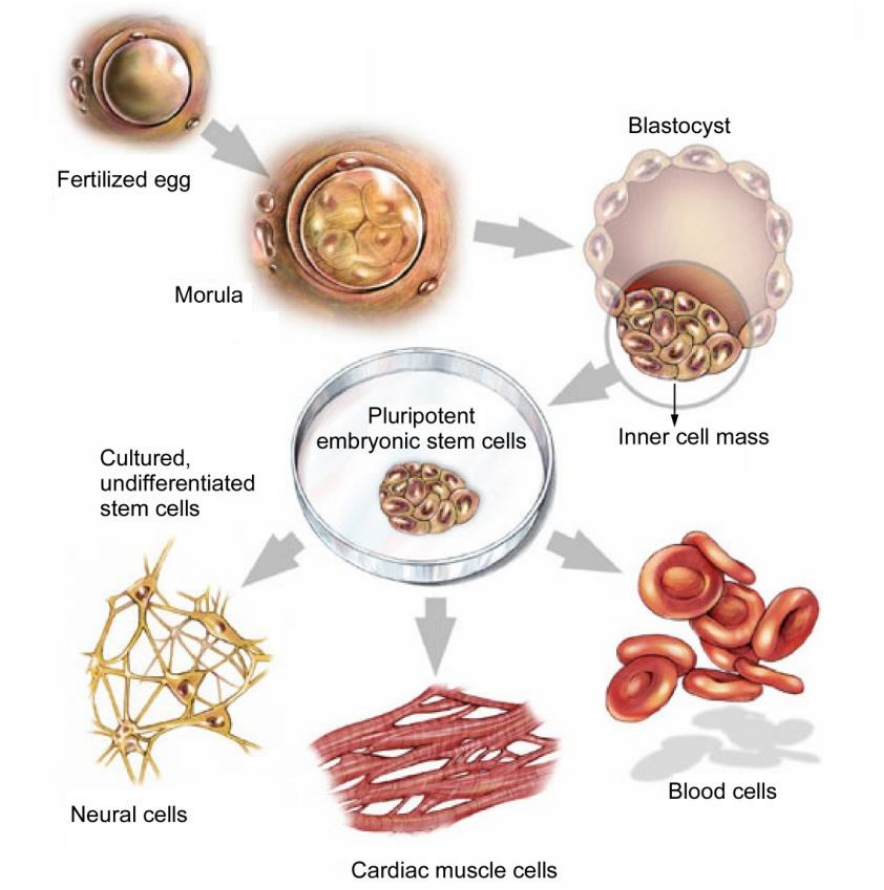

Figure 8: Schematic illustration for development pluripotent embryonic stem cells. (Modified from http://www.stemcellresearchfoundation.org/WhatsNew/Pluripotent.htm)

However, there are still ethic disputes about the usage of human ES cells. To avert from ethic controversies, Yamanaka and coworkers have successfully developed a method for conversion of somatic cells into embryonic stem like-cells named induced pluripotent stem (iPS) cells by using four transcriptional factors (Figure 9) (Takahashi et al, 2007a; Takahashi & Yamanaka, 2006). Since then, a number of further reports demonstrated the successful generation of iPS cells using different approaches (Li et al, 2009; Warren et al, 2010; Zhou et al, 2009). A recent report also shows the possibility to generate iPS by using only one transcription factor, i. e. Oct4 (Kim et al, 2009). Importantly, iPS cells are pluripotent like ES cells (Takahashi et al, 2007b) as they develop into the three germ layers involving all cell types of the adult body (Takahashi et al, 2007b). Furthermore, the

generation of patient-derived iPS cells has been proposed a possible solution to avoid immune responses after transplantation of ES cell-derived tissues (Maehr et al, 2009; Soldner et al, 2009). Therefore, iPS cells have been proposed as alternative cell sources for future cell replacement therapies or application in regenerative medicine instead of using ES cells (Robinton & Daley, 2012; Yamanaka & Blau, 2010).

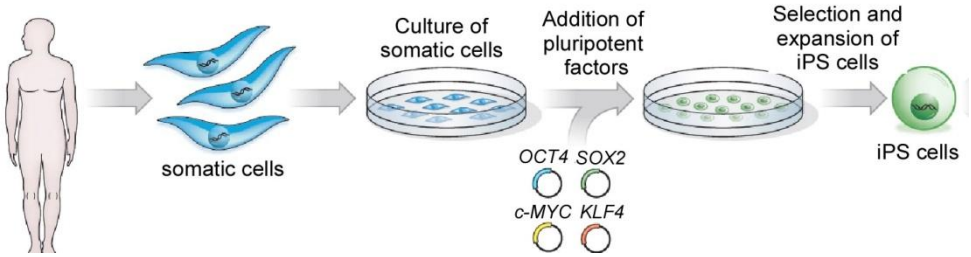

Figure 9: Schematic drawing of the generation of induced pluripotent stem (iPS) cells. Adult somatic cells are transduced with retroviruses encoding for four pluripotency factors (SOX2, KLF4, c-MYC and OCT4), leading to formation of iPS cells. Fully reprogrammed iPS cells have similar properties as ES cells. Schematic presentation is modified from Yamanaka and Blau, 2010 (Yamanaka & Blau, 2010).

1.6. Epigenetic regulation of LVs and RVs in ES cells

An early study showed that the injection of MLVs in mouse embryos led to transgene expression in newborn mice after transferring MLV-injected embryos in foster animals (Jaenisch et al, 1975). However, other studies revealed a transcriptional repression (gene silencing) of the transgene during the early mouse embryo development of MLV derived RVs (Jaenisch et al, 1982; Jahner et al, 1982; Speers et al, 1980). A *de nova* methylation of CpG nucleotides within proviruses has been proposed as a major cause of the transcriptional silencing (Challita & Kohn, 1994; Cherry et al, 2000; Jahner et al, 1982; Laker et al, 1998). DNA methyltransferases 3a and 3b (Dnmt3a and Dnmt3b) are probably responsible for this *de novo* methylation (Lei et al, 1996; Okano et al, 1999). In detail, methylated DNA can be targeted by the methyl-CpG-binding protein 2 (MeCP2) which is associated with transcriptional repressor complexes including histone methyltransferase as well as histone deacetylase (HDACs) which facilitate the remodeling of chromatin and repression of transcription (Bird & Wolffe, 1999; Fuks et al, 2003; Jones & Baylin, 2002; Lorincz et al, 2000). The HDACs in turn can remove acetyl groups from histones leading to silent chromatin structures by histone modifications (Jones et al, 1998; Nan et al, 1998).

It has also been reported that high methylation levels of wildtype HIV-1 led to transcriptional inactivation of viral DNA in mammals (Bednarik et al, 1990). Silencing of

LVs-mediated transgene has been first observed in hematopoietic stem cells (May et al, 2000). Silencing occurred mostly when using wildtype HIV-1 LTR promoter whereas only half of the integration sites were silenced when SIN-LVs were applied lacking the wildtype LTR promoter activity (Pannell et al, 2000). Previous studies demonstrated that SIN-LVs can be used for efficient gene transfer in ES cells and lead to expression of the transgene after differentiation (Hamaguchi et al, 2000; Pannell et al, 2000; Pfeifer et al, 2002). However, not all integration sites of lentiviruses lead to proper expression in transgenic animals indicating that transgene expression is still regulated by the cellular silencing machinery (Hofmann et al, 2006; Lois et al, 2002; Pfeifer et al, 2002). Furthermore, *de nova* methylase independent silencing of retroviral and lentiviral vectors has also been reported in ES cells, which suggests an important role of the modification of the chromatin structure and histones on retrovirus silencing (Pannell et al, 2000; Yao et al, 2004).

1.7. Risks of pluripotent stem cells

As mentioned before, several studies showed that murine or human ES cells can develop into different cell types like neurons, pancreatic β-cells and cardiomyocytes. In addition, iPS cell-derived neurons (Wernig et al, 2008) and cardiomyocytes (Mauritz et al, 2008) were also successfully generated. Thus, in the last decade, these pluripotent stem cells including ES and iPS cells have been provided a potential tool for future regenerative medicine (Keller, 2005; Wobus & Boheler, 2005)

However, the usages of pluripotent stem cells for gene therapies carry a potential risk of tumor (teratoma) formation *in vivo* if the transplanted cells are contaminated by residual undifferentiated cells (Bjorklund et al, 2002; Blum & Benvenisty, 2008; Nussbaum et al, 2007; Wakitani et al, 2003). Hence, removing the undifferentiated cells from differentiated cell populations has been considered as an essential procedure.

Up to now, the transfer of a suicide gene to potential risk cells has been established as a promising concept for tumor elimination in clinical trials (Portsmouth et al, 2007). One approach is based on the gene/prodrug combination herpes simplex virus thymidine kinase (HSV-TK) and ganciclovir (GCV) treatment. HSV-TK converts the prodrug GCV to a toxic metabolite leading to cell death. Importantly, the HSV-TK/GCV system has been widely used in several clinical trials e.g. for cancer therapy (Kubo et al, 2003; Sterman et al, 1998). Thus, one possibility to eliminate undifferentiated cells would be the implantation of a suicide gene, e.g. the mentioned TK cDNA and treatment of these cells

with GCV (Jung et al, 2007; Schuldiner et al, 2003). Indeed, several research groups already modified ES cells genetically in order to express HSV-TK (Goodwin et al, 2001; Jung et al, 2007; Naujok et al, 2010; Rong et al, 2012; Schuldiner et al, 2003). It has recently been shown that LV-mediated TK expression driven by the promoter of pluripotency gene in pluripotent stem cells might selectively ablate pluripotent stem cells. The use of LVs carrying TK cDNA did not alter the pluripotency and self-renewal capacity of the cells but made them sensitive to GCV (Cheng et al, 2012).

1.8. Aim of the work

Previous studies reported silencing of transgenes in murine embryos (Jahner et al, 1982) and embryonic stem (ES) cells when using wildtype retroviral vectors (RVs) as well as wildtype lentiviral vectors (LVs) (Pannell et al, 2000). In the next generation of these viral vectors a self inactivating (SIN) mutation was inserted in the LTR leading to loss of the wildtype promoter activity. In these vectors of retro- (SIN-RV) or lentiviral (SIN-LV) origin an additional internal promoter was inserted. Comparing the viral vectors showed that SIN-LVs resulted in more stable transgene expression as compared to wildtype RVs (Ikawa et al, 2003; Lois et al, 2002; Pfeifer et al, 2002). For SIN-RVs and SIN-LVs an equal potency has been reported in hematopoietic cells (Schambach et al, 2006a). However, no extensive studies have been done so far comparing SIN-LVs and SIN-RVs in murine ES cells or in transgenic mice. Within the first part of the present study it was therefore investigated how SIN-LVs and SIN-RVs are epigenetically regulated in murine ES cells. Besides the *in vitro* measurements transgenic mice were generated by applying subzonal injection of SIN-LVs and SIN-RVs to analyze the epigenetic regulation *in vivo* and to compare the efficiency when using the two different SIN viral vectors.

The second part of the present work focuses on the specific elimination of pluripotent stem cells for possible applications in regenerative medicine. Undifferentiated stem cells pose a great risk for the development of tumors after transplantation. Thus, this work aimed to remove such potential teratoma-forming cells by using a combination of the suicide gene thymidine-kinase that originally derives from Herpes simplex virus (HSV-TK) and treatment with the prodrug ganciclovir (GCV). HSV-TK converts GCV into a toxic metabolite leading to cell death. To genetically modify ES cells SIN-LVs were chosen carrying different promoters of pluripotency genes to drive TK expression. This in turn results in a specific elimination of only undifferentiated pluripotent stem cells upon GCV treatment. Besides the goal of maximal reduction of undifferentiated cells the study also focused on minimizing viral vector concentrations that had to be applied. Several *in vitro* analyses were performed using ES as well as iPS cells. Finally, genetically modified ES cells were applied *in vivo* in a mouse teratoma model and tumor formation was analyzed. The results are of interest for the potential use of pluripotent stem cells in regenerative medicine.

2. Materials and Methods

All chemicals and solutions used in this study were purchased from companies Roth, Merck, Sigma and VWR with the highest purity and molecular biology grade. Cell culture dishes, pipettes and other disposable consumables for cell culture or molecular biology have been supplied by VWR or Sarstedt. For the preparation of solutions purified water was used (Water Purification System, arium® 611VF, Sartorius AG).

Reagents

Acrylamide, Roti-phorese® Gel 30 (37.5:1) (Roth, Cat. No. 3029.1)
Ampicillin sodium salt (Roth, Cat. No. HP62.2)
CHIR 99021 (Axon Medchem, Cat. No. 1368)
Collagenase B (Sigma-Aldrich, Cat. No. 11088807001)
Coomassie dye, Coomassie brilliant blue (Merck, Cat. No. 1.15444.0025)
Cytotoxicity Detection Kit (LDH) (Roche, Cat. No.11644793001)
DNA *Taq* polymerase (Qbiogene, Cat. No. EPTQK109)
Donkey serum (Jackson ImmunoResearch, Cat. No. 017-000-001)
Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Cat. No. 61965, 31966 and 41966)
Ethidium bromide 10 mg/ml (Carl Roth GmbH, Cat. No. 2218.1)
FACS tube Polysterene 12x75mm (Sarstedt)
Fetal calf serum (FCS) (Biochrom AG, Cat. No. S0115)
Fox Chase SCID® Beige mice (Charles River).
Ganciclovir (Sigma-Aldrich, Cat. No. G2536-100MG)
H_2O (Sigma-Aldrich, Cat. No. W3500)
Hanks' Balanced Salt Solution (HBSS) (Gibco, Cat. No. 14175-046)
HEK 293T cells (ATCC, Cat. No. CRL-11268)
Hoechst 33258 (Sigma-Aldrich, Cat. No. 861405-100MG)
Hygromycin (Sigma-Aldrich, Cat. No. H3274-50MG)
iQ Multiplex Powermix (Bio-Rad, Cat. No. 1725849)
iQ SYBR Green Supermix (Bio-Rad, Cat. No. 170-8882)
Iscove's Modified Dulbecco's Media (IMDM) (Gibco, Cat. No. 12440)
Leukemia inhibitory factor (LIF) (Esgro, Cat. No. ESG1107)
MEM, Non-Essential Amino Acids Solution (Gibco, Cat. No. 11140-050)
mi-Pfu DNA polymerase (Metabion, Cat. No. mi-E6022)
N,N-Bis(hydroxyethyl)-2-aminoethanesulfonic acid (BES) (Roth,Cat. No. 9134.2)
NIH 3T3 cell (ATCC, Cat. No. CRL-1658)
NucleoBond® PC 500 EF maxi kit (MACHEREY-Nagel, Cat. No. 740550)
PD 0325901 (Axon Medchem, Cat. No. 1408)
Penicillin G/ Streptomycin (Biochrom AG, Cat. No. A2213)

PeqGOLD TriFast® (Peqlab, Cat. No. 30-2020)
Phenol/Chloroform/Isoamyl alcohol (Roth, Cat. No. A156.1)
Poly-L-lysine solution (Sigma-Aldrich, Cat. No. P4832)
Proteinase K (Roche, Cat. No .03115828001)
Restriction enzymes (New England Biolabs (NEB))
Reverse transcriptase ELISA (Roche, Cat. No. 11468120910)
ß-mercaptoethanol (Sigma-Aldrich, Cat. No. M7522)
Sucrose (Roth, Cat. No. 4621.1)
T4 DNA ligase (Invitrogen, Cat. No. 15224-041)
TA Cloning® Kit (pCR® 2.1 TOPO) (Invitrogen, Cat. No. 45-0046)
Transcriptor First Strand Synthesis Kit (Roche, Cat. No. 4896866)
Trypsin (Gibco, Cat. No. 35400-027)
E. coli XL-1 blue MRF (Stratagene, Cat. No. 200230)
α^{32}-P dCTP (Perkin Elmer ,Cat. No. NEG013Z001MC)

Antibodies

Primary antibodies:
Mouse monoclonal α-actinin (Sigma-Aldrich, Cat. No. A7811-.2ML)
Mouse monoclonal β-actin (Abcam, Cat. No. ab6276)
Mouse monoclonal GFP (CLONTECH, Cat. No. 8371-1)
Rabbit polyclonal Oct-3/4 (Santa Cruz, Cat. No. sc-9081)
Goat polyclonal Thymidine kinase (Santa Cruz, Cat. No. sc-28037)
Mouse monoclonal tubulin (Dianova, Cat. No. DLN-09992)

Secondary antibodies:
Goat polyclonal HRP (Jackson ImmunoResearch, Cat. No. 123-005-021)
Mouse polyclonal HRP (Jackson ImmunoResearch, Cat. No. 115-035-146)
Mouse polyclonal Dylight 649 (Jackson ImmunoResearch, Cat. No. 015-490-003)
Rabbit polyclonal DyLight 549 (Jackson ImmunoResearch, Cat. No. 011-510-003)

Equipment

1,5ml screw-cap reaction tube (Sarstedt, Cat. No. 72.693.005)
100 mm culture dishes (Sarstedt, Cat. No. 83.1802)
100 mm Petri dish (Greiner, Cat. No. 663102)
150 mm cell culture dishes (Falcon, Cat. No. 353025)
250 ml tensid-free cellulose acetate bottle-top filter (SFCA)(0.45µm) (Nalgene, Cat. No. 157-0045)
6-well/24-well cell culture plate (Sarstedt, Cat. No. 83.1839/83.1836,)
96-well plate reader SUNRISE RC/ST (Tecan)
96-well plates with optical caps (Peske, Cat. No. 353072)
Axio Observer.Z1 with Atopome system (Carl Zeiss)
AxioCam MRC5 (Carl Zeiss).

AxioStart (Carl Zeiss)
Cell scraper (Labomedic, Cat. No. 2015217)
Centrifugation tubes (Beckman Coulter, Cat. No. 358126 and 326819)
CL-1000 UV cross-linker (UVP)
Gene Screen Plus Hybridization Transfer Membranes (PerkinElmer, Cat. No. NEF988001PK)
Immobilon-P Transfer Membrane (PVDF)(Millipore, Cat. No. IPVH00010)
Incubator HERAcell 150 (Thermo Scientific)
Inverted cell culture microscope DMIL (Leica)
iQ5 Real Time PCR System (Bio-Rad)
P20, P200 and P1000 pipette (Gilson)
Photometer/ Biophotometer (Eppendorf)
Polysterene 12*75mm (Sarstedt)
Reciprocating shaker 3006 (GFL)
SW32Ti rotor (Beckman Coulter)
SW55 rotor (Beckman Coulter)
T1 Thermocycler (Biometra)
Thermomixer (Eppendorf)
Ultra-centrifuge Optima L-100 XP (Beckman Coulter)
UV light transilluminator, GelDoc® XR (Geldoc)

2.1. Lentiviral and retroviral vectors

The original virus plasmids are derived from the lab of Inder Verma (The Salk Institute for Biological Studies, Laboratory of Genetics, La Jolla, CA, USA). The viral vector system for production of recombinant viruses consist of two major parts: the retroviral or lentiviral plasmids and the packaging construct (see also Figure 5, 6). The plasmids and the procedures of production are described in details below.

2.1.1 Lentiviral plasmids and packaging constructs

pRRL.SIN-18-PGKGFP: The self-inactivating lentiviral vectors (SIN-LVs) (Figure 5) contains the *cis*-active sequences of HIV-1 that are required for encapsidation of viral vector genome and for viral transduction of target cells, as well as the eGFP expression under the control of human phosphoglycerate kinase (PGK) promoter. The 5' LTR contains enhancer elements and RSV promoter (Schambach et al, 2006a; Schambach et al, 2006b) as a substitute of the wildtype U3-region to drive expression of the vector RNA in the packaging cells. The U3 promoter/enhancer sequences in the region of the 3' LTR are

deleted leading to a SIN vectors (SIN as shown as black triangle in Figure 5) (Lois et al, 2002; Pfeifer et al, 2002).

pMDLg/pRRE: The construct pMDLg/pRRE (Figure 5) contains the *gag* and *pol* genes of HIV-1 driven by a Cytomegalovirus (CMV) promoter (Thomsen et al, 1984). Since the transcripts of the *gag* and *pol* genes contain *cis*-repressive sequences, they are only expressed by binding of *rev* to RRE (*rev* response element), which enables the export of the viral RNA from the nucleus (Dull et al, 1998).

RSV-rev: The construct RSV-rev (Figure 5) expresses the *rev* gene driven by Rous sarcoma virus (RSV) promoter (Lois et al, 2002).

pMD.G: The construct pMD.G (Figure 5) encodes a heterologous envelope protein from vesicular stomatitis virus (VSV.G) to pseudotype the viral vector. VSV.G is under the control of the CMV promoter (Lois et al, 2002).

2.1.2 Retroviral plasmids and packaging constructs

pSRS11-PGKGFP: SIN gammaretroviral vector (SIN-RV) contains the sequences of murine leukemia viruses (MLV) required for packaging, reverse transcription and integration, as well as the eGFP expression cassette under the control of human PGK promoter. The 5' LTR of SIN-RV also contains enhancer elements and RSV promoter (Schambach et al, 2006a; Schambach et al, 2006b) as a substitute of the wildtype U3-region to drive expression of the vector RNA in the packaging cells. The promoter/enhancer sequences in the U3 region of 3' LTR were deleted to generate self-inactivating vectors (Figure 6B) (Schambach et al, 2006a; Schambach et al, 2006b; Yu et al, 1986).

pCLMFGGFP: gammaretroviral vector contains the *cis*-active sequences of MLV required for packaging, reverse transcription and integration, as well as the eGFP expression cassette under the control of wildtype LTR promoter (Naviaux et al, 1996) (Figure 6A).

pCMV-gagpol: The construct pCMV-gagpol expresses the *gag* and *pol* genes of MLV driven by a CMV promoter (Figure 6).

pMD.G: The construct pMD.G (Figure 5) encodes a heterologous envelope protein from vesicular stomatitis virus (VSV.G) to pseudotype the viral vector. VSV.G is under the control of the CMV promoter (Lois et al, 2002).

2.1.3. Virus-plasmid maxi preparation

In order to obtain enough viral and packaging plasmid DNA for viral vector productions, plasmids were transformed into chemical (or heatshock) competent cells of strain *E. coli* XL-1 blue MRF (Stratagene). Cells were cultivated and plasmids were isolated by using an endotoxin-free Maxi kit (NucleoBond PC 500 EF).

First, 50 ng plasmids were each incubated with competent cells on ice for 30 minutes. Afterwards, the cell suspension with plasmids was heat shocked at 42 °C in a water bath for 45 seconds. Subsequently, 1 ml Luria-Bertani (LB^+) medium without antibiotics was added and cells were incubated at 225 rpm shaking and 37°C for 1 hour. After spinning down the cells at 3600 rpm for 5 minutes the supernatant was decanted. The cell pellet was resuspended in remaining medium and plated on LB plates containing ampicillin (100 µg/ml). Plates were incubated at 37°C for 16 hours or overnight.

On the next day, single bacteria colonies were picked and inoculated in 5 ml LB^+ medium supplemented with ampicillin (100 µg/ml). After 6 hours incubation at 37°C with 225 rpm shaking, the medium with growing bacteria was transferred into 100 ml LB^+ medium supplemented with ampicillin with 225 rpm shaking overnight. On the next day, bacteria were harvested by centrifugation (6K15 with 11150 rotor, Sigma) with 3600 rpm for 15 minutes at 4 °C. Subsequently, plasmids were isolated with an endotoxin-free Maxi kit NucleoBond PC 500 EF kit according to the manufacturer's instructions (MACHEREY-Nagel).

LB^+ plate

LB-Medium (Lennox)	20 g
Glucose	1 g
H_2O	Ad 1 l
Agar-agar	15 g
pH (with HCl)	7.2-7.5

Autoclaved, poured into 100-mm petri dishes and stored at 4°C

LB^+ Medium

LB-Medium (Lennox)	20 g
Glucose	1 g
H_2O	Ad 1 l
pH (with HCl)	7.2-7.5

Autoclaved and stored at 4°C

2.1.4. Production of recombinant lentiviral and retroviral vectors

For the production of recombinant lentiviral and retroviral vectors, HEK 293T (ATCC) cells were used as virus packaging cell. Twelve 150-mm cell culture dishes were coated with 8 ml poly-L-lysine solution per dish at room temperature (RT) for 15 minutes. After removing poly-L-lysine solution, 18 ml DMEM medium (DMEM supplemented with 10% fetal calf serum (FCS)) was applied in each dish. For plating virus packaging cells 150-mm cell culture dishes were used with HEK 293T cells of 100% confluency. Cells were washed with PBS, trypsinized with 2 ml trypsin and resuspended in 18 ml virus medium (20 ml at final volume). 2 ml of cell suspension was each transferred on poly-L-lysine coated dishes containing 18 ml medium. HEK 293T cells were then cultured at 37 ℃, 10% CO_2 overnight.

Poly-L-lysine solution		**DMEM medium**
0.01% (w/v) poly-L-lysine	50 ml	DMEM (Cat. No. 61965)
1xPBS	450 ml	10% FCS
		100 U/ml Penicillin G
		100 µg/ml Streptomycin.
stored at 4℃		stored at 4℃

18 hours after cell split HEK 239T cells had a confluency of 40-60%. For transfection of the plasmids, transfection mixture was prepared by mixing the lentiviral or retroviral plasmids as well as packaging plasmids (Table 1). Afterwards, $CaCl_2$ was added to the mixture and mixed by inverting. Finally, 2x BBS was added and mixed gently by inverting. After 15 minutes incubation at RT, the transfection mixture was applied dropwise to each cell culture dish with HEK cells. The dishes were swirled to distribute the mixture and cells were incubated at 37 ℃, 3% CO_2 overnight.

Table 1: Transfection mixture for co-transfection of HEK 293T cells (Pfeifer & Hofmann, 2009)**.**

	Transfection mixture		
	SIN Lentivirus	SIN Retrovirus	Retrovirus
viral plasmid	SIN-LVs	SIN-RVs	pCLMFGGFP
packaging plasmids	pMDLg/pRRE	pCMV-gagpol	pCMV-gagpol
	RSV-Rev	pMD.G	pMD.G
	pMD.G		
	$CaCl_2$	$CaCl_2$	$CaCl_2$
reagent	2x BBS	2x BBS	2x BBS

*Different LVs and RVs were produced. They are detailed described in the Result part.

1x PBS	
NaCl	40 g
KCl	1 g
Na_2HPO_4	7.2 g
KH_2PO_4	1.2 g
H_2O	Ad 5 l
pH (with HCl)	7.4
Autoclaved and stored at 4℃	

2x BBS	
N,N-bis(2-Hygroxyethyl)-2-Amionethansulfon acids (BES)	4.26 g
NaCl	6.45 g
Na_2HPO_4	0.085 g
H_2O	Ad 400 ml
pH (with HCl)	6.95
Autoclaved and stored at 4℃	

Next day, the medium was replaced with fresh DMEM medium and incubated at 37 ℃, 10% CO_2 overnight. If a viral vector carries a fluorescence-reporter gene, e.g. eGFP, more than 80% of the cells expressing fluorescence were observed showing high transfection efficiency.

One day after medium change, the supernatants containing released recombinant viral particles in cell dishes were collected and 16 ml fresh virus medium per dish was added. The cells were again cultured at 37 ℃, 10% CO_2 overnight. Thereafter, the cell debris in the supernatants was removed through SFCA bottle-top filters. The filtered supernatants were further centrifuged by an ultracentrifuge with SW32Ti rotor at 61,700g at 17 ℃ for 2 hours. Subsequently, the upper mediums were discarded and each virus pellet was resuspended in 50 µl HBSS. The virus suspensions were stored in sterile screw-cap reaction tubes at 4 ℃ until second harvest.

24 hours after first harvest, the supernatants in cell dishes were collected and centrifuged as mentioned above. The virus suspensions from first and second harvest were mixed and transferred into a centrifuge tube on top of a 2 ml 20% (w/v) sucrose cushion. The viruses were concentrated by an ultracentrifuge with a SW55Ti rotor at 53,500g at 17 ℃ for 2 hours. After discarding the medium, the virus pellet was resuspended in 120 µl HBSS and the virus suspension was transferred to sterile screw-cap reaction tubes. The tubes were shacked at 1,400 rpm at 16℃ for 45 minutes and centrifuged at 16,000g for 60 seconds to spin down debris. The opaque supernatants were aliquoted in sterile screw-caps reaction tubes and stored at -80 ℃.

2.1.5. Measurement of virus titer

Biological titer

If LVs/RVs carry a fluorescence reporter gene, the biological virus titers were measured by infecting cells with serial dilutions of the virus-preparation combined with flow cyctometry analysis. Therefore, $1x10^5$ NIH 3T3 (murine embryonic fibroblasts) cells per well were seeded on 24-well cell culture plate. On the next day, 1 µl virus was dissolved in 330µl DMEM and mixed completely (Dilution No.1). Then, 30µl of Dilution No. 1 was further transferred in new reaction tube with 300µl DMEM and mixed completely (Dilution No. 2). The Dilution No. 3 and No. 4 were prepared according to the same procedure as for No. 2. Finally, all serial dilution of No. 1 to 4 including control (medium without virus) were applied to 5 individual wells of 24-well plate growing NIH 3T3 cells. Another well was used for cell counting. Medium was changed 18 hours later and cells were always incubated at 37 ℃. After 72 hours, NIH 3T3 cells were washed with PBS, trypsinized and fixed with 4% paraformaldehyde (PFA) for 15 minutes on ice. Subsequently, cells were spun down by centrifugation with 1100 rpm at 4℃ and resuspended in 1 ml PBS for flow cyctometry analysis (Pfeifer & Hofmann, 2009). The multiplicity of infection (MOI) and the biological titer of the virus in infectious units (IU) per ml (IU/ml) were calculated as follows

MOI = -ln (percentage of eGFP-negative cells/100)

Virus titer (IU/ml) = (No. of infected cells) x (MOI) x (dilution factor) x 1000

4% PFA	
paraformaldehyde	4 g
1X PBS	Ad 100 ml
pH	7.4
stored at -20℃	

Physical titer

To determine the physical titer of lentiviral vector preparations Enzyme Linked Immunosorbent Assays (ELISA) against reverse transcriptase or the lentiviral p24 capsid protein were used. In the present study, a commercially available reverse transcriptase ELISA was applied (see Reagents) giving the ng reverse transcriptase/µl virus suspension. Besides, the physical titer can be referred to as the integrant numbers of provirus per genome in transduced host cells (titer in integrants/ml) by applying quantitative real time PCR (2.6.3).

2.2. Cultivation of pluripotent stem cells

2.2.1. Cultivation of ES and iPS cells

ES cells including R1 (3.1) and D3 (3.2) ES cell line and iPS cells including iPS and iPS-Oct-GFP cell lines (3.2.2) were stored in liquid nitrogen. One day before thawing cells, cell culture dishes or plates were coated with a layer of mitotically inactived murine fibroblast cells. They were used a feeder cells that preserve the self-renewal and maintain the pluripotency of pluripotent stem cells (Pfeifer & Hofmann, 2009; Wobus & Boheler, 2005). Cells were briefly thawed in a 37℃ water bath and transferred to 15-ml conical tube with 5 ml medium. After centrifugation at 200 g for 5 minutes, the supernatants were aspirated and cells were resuspended in fresh medium. Then, ES or iPS cells were cultured at 37 ℃, 5% CO_2. In order to maintain the pluripotency of cells, cell medium was changed every day. Moreover, cells were split on new dishes before cell grew to high confluency or they were split if cell colonies became visibly large or if they get in contact to each other.
iPS cell lines used in present work were kindly provided by Jun.-Prof. Dr. Philipp Sasse.

2.2.2. Transduction with LVs/RVs

In order to transduce pluripotent cells efficiently, viral transduction was performed after single cells have attached to the feeder cells layer. Viral transduction of ES or iPS cells was performed by removing the medium and incubating the cells with fresh medium containing dissolved virus overnight. The required amount of virus was calculated as follows

amount of virus (µl) = cell numbers per well × MOI / physical titer (Integrants/ml)

Since LVs can also transduce feeder cells highly efficient, the number of feeder cells had to be considered for the calculations.
The medium containing virus was replaced with fresh medium 18 hours after transduction. The viral transduced cells were either further cultured until next regular passaging of cells or frozen in cell freezing medium (ES or iPS cell medium supplemented with 10% DMSO) at -80 °C. 2 days later the frozen cells were stored in liquid nitrogen.

R1 ES cell medium (3.1)

DMEM (Cat. No. 31966)
20% FCS
0.1mM ß-mercaptoethanol
100 U/ml Penicillin G
100 µg/ml Streptomycin.
1,000 U/ml LIF

D3 ES cell Medium (3.2)

DMEM (Cat. No. 41966)
15% FCS
1x non-essential amino acids (MEM)
0.1mM ß-mercaptoethanol
100 U/ml Penicillin G
100 µg/ml Streptomycin.
1,000 U/ml LIF

iPS cell medium

DMEM (Cat. No. 41966)
15% FCS
1x non-essential amino acid (MEM)
0.1mM ß-mercaptoethanol
100 U/ml Penicillin G
100 µg/ml Streptomycin
1,000 U/ml LIF
3 µM CHIR 99021
4 µM PD 0325901

2.2.3. Generation of ES cell clones

After transduction of ES cells with LVs/RVs, 100 to 500 ES cells were seeded on 100-mm tissue dishes coated with feeder cells ($4x10^6$ feeder cells per dish). 7 days later the feeder cells around single colonies were removed by circling with a filtered pipette tip connected to pipette P20 (Gibson). The ES cell colonies were scraped slightly to dislodge the colonies from the feeder cell layer. Subsequently, ES cell colonies were sucked into the pipette tips and transferred into a well of a 24-well plate containing 120 µl 0.05% trypsin/EDTA. After incubation at 37°C for 5 minutes, the ES cell colonies were resuspended gently to dispense to single cells. Three times 40 µl of the cell suspension was each transferred into a separated freshly prepared well of a 24-well plate with adherent feeder cells ($1x10^5$ feeder cells per well) and 300 µl of ES cell medium, respectively. Then, ES cells were further cultivated for 3-4 days until 70% confluency. The three wells per ES cell clone were either used for Southern blot analysis, flow cyctometry analysis or for freezing of cells.
The SIN-LV- and SIN-RV-transduced ES cell clones for epigenetic regulation analysis were kindly provided by Dr. Andreas Hofmann.

2.2.4. Differentiation of ES cells

Differentiation of ES cells *in vitro* was performed by culturing ES cells as embryoid bodies (EBs) in the absence of mouse embryonic fibroblast feeders and leukemia inhibitory factor (LIF) (Wobus & Boheler, 2005) in liquid mass culture (Boheler et al, 2002; Doetschman et al, 1985). ES cells were trypsinized and then transferred in differentiation medium. The cell suspensions were incubated in sterile petri dishes at 37°C with 80 rpm shaking for 14 days. The differentiation medium was replaced every 3 days. At the end of the procedure the EB aggregates were formed.

Differentiation medium

IMDM (Cat. No. 12240)
20% (v/v) FCS
0.1 mM ß-mercaptoethanol
100 U/ml Penicillin G / 100 µg/ml Streptomycin

2.3. Extraction of genomic DNA

Genomic DNA (gDNA) of ES cells was extracted by the classical phenol-chloroform method: After washing of cells in a 6-well or 24-well plate with PBS, PBS was removed and cells were treated with 500 µl extraction buffer per well for 5 minutes at 37℃. The dissolved cells were resuspended by gently pipeting. Subsequently, the samples were transferred into 1.5ml reaction tubes and incubated in a thermomixer at 800 rpm shaking at 55℃ overnight. On the next day, 500µl Phenol/chloroform/isoamyl alcohol (50:48:2) was each added to the samples and the tubes were inversed vigorously. After centrifugation at 13000 rpm for 15 minutes, the upper aqueous phase containing gDNA was each transferred into a new tube without residual of cells. For a second removal of the residues, 500 µl chloroform was added to the gDNA solution and mixed well. After centrifugation with 13000 rpm for 15 minutes, the upper aqueous phase containing gDNA was transferred into a new reaction tube. For gDNA precipitation, 800 µl isopropanol was added to the aqueous phase and gDNA was spun down by centrifuging at 13000 rpm for 15 minutes. The gDNA pellets were dried at 37℃ for 30 minutes and finally dissolved in 100 µl H_2O at 55℃ overnight. gDNAs were stored at 4℃.

DNA extraction buffer

5 mM EDTA
0.2% (w/v) SDS
200 mM NaCl
0.1 mg/ml Proteinase K

For isolation of gDNA from mouse tail, 0.3 to 0.5 cm of the mouse tail was minced and treated with 500 µl extraction buffer at 55 ℃ overnight. The further procedure was performed as mentioned above.

2.4. Isolation of total RNA and cDNA synthesis

Total RNA of cells was extracted by Guanidin-isothiocyanate/phenol (Chomczynski & Sacchi, 1987) using peqGOLD Tris-FAST (peqLAB). Firstly, cells from a well of a 6-well plate were washed with PBS. After removing the PBS, 1ml of peqGOLD Tris-Fast® was each added to cells. After incubation for 5 minutes at RT, dissolved cells were resuspended by gently pipeting. The following steps were progressed according to the manufacturer's

instructions. The measurement of total RNA's concentration was performed by using a photometer (Eppendorf).

For cDNA synthesis by reverse transcription of RNA, Transcriptor First Strand cDNA Synthesis Kit **(**Roche) and random hexamer primers were applied according to the manufacturer's instructions.

2.5. Isolation of protein

Cells of wells from a 6-well or 24-well plate were washed with PBS. After removing of PBS, cells were treated with 200 µl Radioimmunoprecipitation assay (RIPA) lysis buffer containing complete protease inhibitor (Roche) for 5 minutes at RT and then resuspended by gently pipeting. Thereafter, protein lysates were centrifuged at 13000 rpm at 4℃ for 15 minutes. The upper protein lysate solutions were each transferred in a new tube and stored at -80℃. Protein concentration was measured by using Coomassie blue Bradford method (Bradford, 1976). The Bradford method is based on the binding of amino acids to Coomassie blue G250 dye resulting in an absorbance shift. 2 µl of protein lysate was each added to 98 µl 0.15 M NaCl solution. After addition of 1 ml Coomassie solution, samples were incubated for exactly 2 minutes at RT and the absorbance was measured by a photometer at 595 nm (Eppendorf). A calibration curve for absorbance was performed by using BSA standards of known protein concentration.

RIPA lysis buffer

150 mM NaCl
50 mM Tris (pH 7.5)
0.1% (w/v) SDS
0.25% (w/v) Deoxycholic acid sodium salt
1% Nonidet P40
stored at 4℃

Coomassie blue Bradford regant

Coomassie G250	100 mg
98%(v/v) EtOH	50 ml
85% (v/v) H_3PO_4	100 ml
H_2O	Ad 1 l
stored in darkness at 4℃	

For isolation of protein from mouse tails, 0.3 to 0.5 cm of the mouse tail was minced and homogenized by using a hand mixer in a reaction tube with 500 µl RIPA buffer at 4 ℃. The further procedure was performed as mentioned above.

2.6. Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) (Saiki et al, 1988) is a widely used method for amplification of defined nucleic acid sequences. Within the first step (denaturation) double-stranded DNA is melted by heat denaturation. The second step (annealing) allows annealing of forward and reverse primers to the complementary sequence, respectively. In the third step (extension) the DNA polymerase synthesizes a new complementary DNA strand by adding deoxynucleoside triphosphates (dNTPs) in 5' to 3' direction. The repeated cycles of these three steps results in production of huge amounts of DNA.

2.6.1. Genotyping PCR of transgenic mice

The following GFP primers were used to detect the eGFP gene from integrated provirus in probably transgenic mice resulting from subzonal injections with SIN-LV/RV. AP18 (forward) and KISAX (reverse) primers were used as PCR loading controls targeting housekeeping gene Protein Kinase G 1 (PKG1). The TaqCORE kit (Qbiogen) was applied for genotyping PCR reactions by using the following PCR preparation and programs.

Primer name	**Sequence of genotyping PCR primers**
GFP forward	5'-ATGGCTTCGTACCCCTGCCA-3'
GFP reverse	5'-TCAGTTAGCCTCCCCCA-3'
AP18	5'-GCTCTACTCGTCCGAAACCT-3'
KISAX	5'-GCCGCTCGAGTAAGGGAAACTAATGAGAAACTGCT-3'

PCR preparation	
gDNA	3 µl
GFP forward (10 pmol/µl)	2.5 µl
GFP reverse (10 pmol/µl)	2.5 µl
dNTPs (1.25 mM/each)	8 µl
10 x reaction buffer	5 µl
DNA polymerase *Taq*	0.5 µl
H_2O	28 µl
Total	50 µl

PCR program

Step	Temperature	Time (seconds)
1.	95 °C	300
2.	95 °C	30
3.	57°C	30
4.	72 °C	30
5.	72 °C	300

Step 2 to 4 was repeated for 40 cycles

PCR preparation		**PCR program**		
gDNA	3 µl	Step	Temperature	Time (seconds)
Ap18 (10 pmol/µl)	2.5 µl	1.	95 °C	300
KISAX (10 pmol/µl)	2.5 µl	2.	95 °C	30
dNTPs (1.25 mM/each)	8 µl	3.	58°C	30
10 x reaction buffer	5 µl	4.	72 °C	30
DNA polymerase *Taq*	0.5 µl	5.	72 °C	180
H_2O	28 µl			
Total	50 µl	Step 2 to 4 was repeated for 30 cycles		

2.6.2. Cloning PCRs for generation of LVs expressing TK

For the generation of TK expressing LV constructs, different promoters of pluripotency genes as well as the cDNA of Herpes simplex TK were cloned after PCR amplification using *mi*-Pfu DNA polymerase (Metabion). The *mi*-Pfu DNA Polymerase catalyzes DNA polymerization and possesses also a proofreading activity to excise base misinsertions rapidly that may occur during the polymerization process.

The promoters of pluripotency genes Nanog and Oct-3/4 including enhancer elements (Boer et al, 2007) were amplified from gDNA of wildtype mice using "cloning" PCR. The promoters of pluripotency genes EOS-C3 and EOS-S4 were amplified from plasmids: PL-SIN-EOS-C(3+)-EiP and PL-SIN-EOS-S(4+)-EiP (Hotta et al, 2009). The four promoters were each cloned into either the *Sal*I and *BamH*I or *Cla*I and *BamH*I site of the 3rd generation LV plasmid pRRLSIN.cPPT.eGFP.WPRE (Follenzi et al, 2000; Pfeifer & Verma, 2001b).

Primer name	**sequence of Cloning PCR primers**
Nanog forward	5’-cgtgat*GTCGAC*AATTTCTTCTTCCATTGCTTAGACGG-3’ *Sal*I
Nanog reverse	5’-tgcgcc*GGATCC*AAGGGATTTCTGAAAAGGTTTTAGGC-3’ *BamH*I
Oct-3/4 forward	5’-ttcatt*ATCGAT*TCTAGGCACGCTTAGGGC-3’ *Cla*I
Oct-3/4 reverse	5’-ttcatt*AGATCT*CCGAGCCGGGGGCCTGGTGG-3’ *Bgl*I
EOS-C3 forward	5’-ggaa*ATCGAT*TTTATCCAGCCCTCACTCCT-3’ *Cla*I
EOS-C3 reverse	5’-aatt*GGATCC*TGGCTTTACCAACAGTACCG-3’ *BamH*I
EOS-S4 forward	5’-ggaa*ATCGAT*TTTATCCAGCCCTCACTCCT-3’ *Cla*I
EOS-S4 reverse	5’-aatt*GGATCC*TGGCTTTACCAACAGTACCG-3’ *BamH*I

After cloning of the promoter the eGFP transgene was each replaced with TK cDNA of herpes simplex virus between the restriction sites *BamH*I and *Xho*I. TK cDNA was amplified from a plasmid containing TK cDNA that was originally obtained from Promega. For the generation of the double cassette LV construct EOS-S4-TK-PGK-Hygromycin (STPH) (see also Figure 49), an expression cassette with hygromycin resistance gene under the control of the PGK promoter was cloned into the LV construct EOS-S4-TK (ST) between TK cDNA and the WPRE element. The cDNA of the hygromycin resistance gene was amplified from a plasmid that was kindly supplied by Prof. Fleischmann’s lab. The following PCR preparations and programs were used for cloning PCR reactions. The annealing temperature varied depending on the primer pair used.

Transgene	**Sequence Cloning PCR primers**
TK forward	5’-gatt*GGATCC*ATGGCTTCGTACCCCTGCCA-3’ *BamH*I
TK reverse	5’-aatt*CGCGAG*TCAGTTAGCCTCCCCCA-3’ *Xho*I
hygromycin forward	5’-ttgg*GGATCC*AGCCGCCACCATGAAAAAGC-3’ *BamH*I
hygromycin reverse	5’-ggtt*CTCGAGATCGAT*CTATTCCTTTGCCCTCGGACGAGTGC-3’ *Xho*I-*Cla*I

PCR preparation	
gDNA/plasmid DNA	5 µl
primer forward (10 pmol/µl)	2.5 µl
primer reverse (10 pmol/µl)	2.5 µl
dNTPs (2.5 mM/each)	4 µl
10 x reaction buffer	5 µl
mi-Pfu polymerase (2.5 units)	0.5 µl
H_2O	30.5 µl
Total	50 µl

PCR program

Step	Temperature	Time (seconds)
1.	95 ℃	300
2.	95 ℃	30
3.	55-60℃	30
4.	72 ℃	300
5.	72 ℃	600

Step 2 to 4 repeated for 40 cycles

2.6.3. Quantitative real-time PCR for analysis of provirus integration numbers

For an accurate detection of the provirus integration number using quantitative real-time PCR (qPCR), the supercoiled gDNAs were firstly digested using restriction enzymes to obtain smaller DNA fragments that are more suitable for subsequent qPCR reactions. The gDNAs were obtained as described in section 2.3. Around 5 µg gDNA was digested with 5 units of each enzyme *Bam*HI and *Eco*RI at 37 ℃ overnight. The digested DNA was precipitated with 500 µl 100% isopropanol by inverting and subsequent freezing at -80 ℃ for at least 1 hour. Afterwards, digested DNA was spun down at 13,000 rpm at 4 ℃ for 15 minutes. After discarding supernatants, 500 µl 70% ethanol was added and tubes were inverted several times. Subsequently, DNA was spun down again at 13,000 rpm at 4 ℃ for 15 minutes. After discarding supernatants, the digested DNAs were dried at 37 ℃ for 1 hour. Finally, the digested DNAs were dissolved in 30 µl H_2O at 55 ℃ overnight and stored at 4 ℃.

The qPCR analysis was performed using iQ5 Real-time PCR System and the iQ Multiplex Powermix (BioRad) in 25 µl final volumes according to the manufacturer's instructions. To measure exactly the LV integrants per genome of transduced cells or in transgenic mice, a duplex qPCR approach was used in which the copies of the LVs and the DNA loading (housekeeping gene, i.e. Burkitt lymphoma receptor 1 (BLR1) gene) are measured in one-and-the-same tube. Integrated provirus DNA was detected by using a FAM-labeled Late-RT probe and Late-RT primers and the house keeping gene BLR1 was detected by using Texas-Red–labeled BLR1 probe and BLR1 primers (Pfeifer et al, 2010). The following

PCR preparations and program were used for qPCR reactions. For each sample the reaction was performed (at least) in triplet.

Primers/Probes	**Sequence qPCR primers/probes**
Late-RT forward	5'-TGTGTGCCCGTCTGTTGTGT-3'
Late-RT reverse	5'- GAGTCCTGCGTCGAGAGAGC-3'
Late-RT probe	5'-FAM- CAGTGGCGCCCGAACAGGGA-BHQ1-3'
BLR1 forward	5´-CGGAGCTCAACCGAGACCT-3´
BLR1 reverse	5´-TGCAAAAGGCAGGATGAAGA-3
BLR1 probe	5´-TexasRed-CTGTTCCACCTCGCA GTAGCCGAC-BHQ1-3´

PCR preparation

digested gDNA	2 µl
Late-RT for (5 pmol/µl)	1.5 µl
Late-RT rev(5 pmol/µl)	1.5 µl
Late-RT probe (5 pmol/µl)	1 µl
BLR1 for (5 pmol/µl)	1.5 µl
BLR1 rev (5 pmol/µl)	1.5 µl
BLR1 probe (5 pmol/µl)	1 µl
iQ Powermix	12.5 µl
H_2O	2 µl
Total	25 µl

PCR program

Step	Temperature	Time (seconds)
1.	95 °C	180
2.	95 °C	15
3.	60°C	60

Step 2 to 3 was repeated for 40 cycles

The absolute copy number per genome can be determined by comparison of the cycle threshold values from virus specific probe versus the BLR1 probe. Copy numbers were evaluated as follows

Copy numbers = $2^{\Delta Ct}$ with ΔCt=Ct(BLR1)-Ct(Late-RT) (Pfeifer et al, 2010).

2.6.4 Quantitative real-time PCR for TK expression on mRNA level

Quantification of TK expression on mRNA level was performed by using the iQ SYBR Green Supermix (Bio-Rad) on iQ5 Real-time PCR System (Rio-Rad) with the following specific primer combinations. As loading control GAPDH was used. Preparation of cDNA is explained in detail in 2.4.

primers	Sequence qPCR primers
TK-RT forward	5'-GATGACTTACTGGGCAGGTG-3
TK-RT reverse	5'-GATGGCGGTCGAAGATGAG-3'
GAPDH-RT forward	5'-CCACTCACGGCAAATTCAAC-3'
GAPDH-RT reverse	5-'GTTCACACCCATCACAAACATG-3'

PCR preparation

cDNA (2ng/µl)	2 µl
RT forward (5 pmol/µl)	1.5 µl
RT reverse (5 pmol/µl)	1.5 µl
iQ SYBR Green Supermix	12.5 µl
H_2O	7.5 µl
Total	25 µl

PCR program

Step	Temperature	Time (seconds)
1.	95 ℃	180
2.	95 ℃	30
3.	57℃	30
4.	72 ℃	30

Step 2 to 4 were repeated for 40 cycles

Melting curve

Step	Temperature	Time (seconds)
5.	72 ℃	180
6.	55 to 95℃	30

Step 6 was repeated for 81 cycles. 0.5℃ was increased and acquisition per cycle.

Relative quantification of mRNA levels was performed based on the cycle threshold (Ct) values of the amplification curves as follows

relative mRNA level = $2^{\Delta Ct}$ with ΔCt = Ct(GAPDH)-Ct(TK)

2.7. Separation, isolation and cloning of DNA

2.7.1. Enzyme restrictions of DNA

For digestion of double stranded DNA on specific sequences, restriction enzymes are the most important tool. Restriction enzymes were purchased from NEB. The digestion of DNA was performed according to the manufacturer's instructions using the buffers supplied with the enzymes by NEB. The general reaction condition was used as follows

DNA digestion	
DNA	5 µg
Restriction enzyme	20 units
10 x buffer	3µl
10 x BSA	3µl
H_2O	Ad 30 µl

Reaction was incubated at 37 ℃ over night.

2.7.2. Separation and elution of digested DNA

Nucleic acids are negatively charged and DNA fragments of different molecular weights can be separated by gel electrophoresis. By staining the gels with the fluorescent dye ethidium bromide visualization of the separated DNA under UV light (302nm) can be obtained. A DNA marker (1kb Ladder, Invitrogen) is used to determine the size of DNA-fragments.. Agarose gel electrophoresis is a common method of separating and analyzing DNAs within the size of 0.5 to 12 kb. Therefore, agarose was melted in 1x TBE by using a microwave and ethidium bromide (800 ng/ml final) was added before pouring in a gel tank. The gel electrophoresis was performed in 1x TBE as running buffer and a voltage of 100V was applied.

10x TBE buffer	
Tris	540 g
boric acid	275 g
0.5M EDTA pH 8.0	200 ml
H_2O	Ad 5 l

For elution of DNA from agarose gels the target DNA fragment was cut out and eluted by using the GFX^{TM} System (Amersham) according to the manufactory's instruction.

2.7.3. Ligation of DNA

For reconstructing new virus plasmids, T4 DNA ligase (NEB) was used for catalyzing the formation of a phosphodiester bond between juxtaposed 5' phosphate and 3' hydroxyl termini in duplex DNA. The following ligation reaction was used in general.

DNA ligation	
DNA (vector)	50 ng
DNA (insert)	200 ng
T4 DNA ligase	2.5 units
10 x Ligation buffer	1,5µl
H_2O	Ad 15 µl

The reaction mixture was incubated at 16°C over night.

On the next day, 7 µl of the DNA ligation was each transformed into chemical competent *E. coli*. XL1 blue cells. The transformation was performed as already described for the retransformation of plasmids (2.1.3).

2.8. Southern Blot

Southern Blot analysis (Southern, 1975) is a recommended method to identify specific genes in gDNA and the procedure is described in detail below. Briefly, gDNA was digested with a suitable restriction enzyme to obtain smaller fragments for the analysis. DNA fragments were then separated by agarose gel electrophoresis (2.7.2) and transferred from the agarose gel to a nylon membrane by blotting. Finally, the membrane with DNA was hybridized with a radioactive probe that has a specific complementary sequence of the target DNA. The radioactive labeled target DNA was detected by use of a PhosphorImager (Southern, 1975) or by exposing to an autoradiography film that can be developed by AGFA CP 1000 film processor.

2.8.1 gDNA digestion for Southern blot analysis

For analysis of integration numbers of provirus DNA in host cells or transgenic mice, gDNA was isolated (2.3) and digested with the restriction enzyme *BamH*I (NEB) at 37°C over night. Integrated provirus DNA in the host genome can only be digested once with *BamH*I. The size of digested DNA fragments depends on the integration site and thus, on further restriction sites in the genome (Figure 10). For the analysis of the methylation status of integrated provirus, an additional methylation-sensitive enzyme was used to digest gDNA (3.1.4).

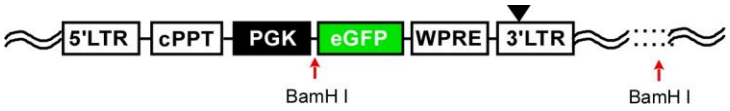

Figure 10: Schematic illustration of integrated provirus. The gDNA of SIN-LV-transduced cells was digested with *BamH*I for Southern blot analysis.

2.8.2. DNA denaturation

The digested DNA was loaded on a 0.7% (w/v) agarose gel and separated by electrophoresis. The agarose gel was then visualized under the UV light transilluminator, GelDoc® XR (Geldoc) together with a phosphorescent ruler and an image was taken. Thereafter, DNA in the agarose gel was depurinated by bathing in 0.2M NaCl for 10 minutes and neutralized by bathing in 0.5M NaOH/1.5M NaCl for 30 minutes. This step enables a denaturation of the DNA leading to single stranded DNA that can be hybridized with a probe. Denaturation was performed in 0.5M Tris/3.0M NaCl (pH 7.4) for 30 minutes.

2.8.3. Blotting

For transferring the single stranded DNA from the agarose gel onto a nylon membrane, the transfer agent 10x SSC buffer was used for blotting. The blot was set up as sandwich (from bottom to top): large plastic dish with 10x SSC buffer, sponge, Whatman filter paper, gel, nylon transfer membrane (Gene Screen Plus Transfer Membrane; Perkin Elemer Life Science), Whatman filter paper and blotting paper towel. During the transferring of buffer from dish to blotting paper by capillary action overnight, DNA was transferred onto the membrane. For fixation of DNA on the membrane a CL-1000 UV cross-linker (UVP) was used at 120,000 µJ/cm^2 for 1 minute.

20x SSC

NaCl	350 g
Na_3-citrate-2H_2O	176 g
H_2O	Ad 2 l
pH (with HCl)	7.0

2.8.4. Probe hybridization

An eGFP-probe was used for hybridization with provirus DNA containing eGFP cDNA. For preparing the eGFP-probes, eGFP cDNA (748 bp) was digested from a LV plasmid with restriction enzymes *BamH*I and *Sal*I. 200ng probe-cDNA in 14 µl H_2O was boiled at 97℃ for 10 minutes and then cooled down on ice for 10 minutes. Afterwards, eGFP cDNA was labeled with deoxynucleoside triphosphates (dATP, dGTP and dTTP) and phosphorus-32 labeled dCTP (α-P^{32}-dCTP) (PerkinElmer) by using the Random Primed Labeling Kit (Roche). After incubation at 37℃ for 30 minutes, radioactive labeled-eGFP probe was purified through NICK Columns (Amersham) according to the manufactory's instructions. The radioactivity of the probe was measured using a radioactivity counter. A proper amount of radioactive labeled-probe was transferred into 1ml TE buffer in a 15 ml tube and heated in water bath at 100℃ for 10 minutes.

The membrane with fixed DNA was transferred into a glass column and hybridized with Church buffer including probe by rotating at 60 ℃ over night. On the next day, the membrane was washed twice with 2x SSC/1% (w/v) SDS at 60 ℃ for 20 minutes and 0.4x SSC/1 % (w/v) SDS for 40 minutes. The autoradiography film was put on the membrane and exposed for 7 days. Finally, the film was developed by AGFA CP 1000 film processor.

eGFP probe	
eGFP cDNA	14 µl
mix of dATP, dGTP, dTTP	6 µl
Hexanucleotide	4 µl
αP^{32}-dCTP	14 µl
Klenow enzyme	2 µl

1x TE buffer	
1M Tris (pH 8.0)	5 ml
0.5M EDTA (pH 8.0)	1ml
H_2O	Ad 500 ml

Church buffer	
20% (w/v) SDS	175 ml
ssDNA	5 ml
BSA	5 g
1M Na_2HPO_4	193.5 ml
1M NaH_2PO_4	56.5 ml
0.5M EDTA pH 8.0	1 ml
H_2O	Ad 500 ml

ssDNA	
salmon sperm DNA	500 mg
H_2O	Ad 50 ml

2.9. Western Blot

Western blot is a widely used tool for detection of specific proteins by using specific antibodies. Briefly, proteins were separated using sodium dodecyl sulfate-polyacrylamide-polyacrylamid Gelelectrophorese (SDS-PAGE) (Burnette, 1981). Afterwards, proteins were electro-transferred onto polyvinylidene difluoride (PVDF) membranes. The transferred proteins on the membrane were then specifically targeted using specific antibodies and visualized was performed by a chemiluminescent detection method.

For protein separation, acrylamide gels were used, i.e. a stacking gel on top of a separating gel. The polymerization of gel was each initiated by adding ammonium peroxodisulfate (APS) as radical initiator and TEMED as catalysator. The percentage of acrylamide leads to a varying property of gels for stacking and separating of proteins of different molecular weights.

Protein lysates (2.5) were diluted to optimal concentrations of 2-5 µg/µl with RIPA buffer containing 6x Laemmli loading buffer (Laemmli, 1970) supplemented with 5% (v/v) ß-mercaptoethanol. The proteins were boiled on a thermomixer at 97 ℃ for 5 minutes and separated by using SDS-PAGE with 12% separating and 5% stacking gel. Subsequently, proteins were transferred to PVDF membranes at 220mA for 1 hour using a semi-dry method.

After transferring, the membrane was rinsed once in methanol and subsequently in water. The membrane was blocked in 5% (w/v) skim milk in Tris-buffered saline with 0.1% Tween-20 (TBS-T) for 30 minutes. After washing three times with TBS-T buffer, the membrane was incubated with the primary antibody against either GFP, β-actin or tubulin at RT for 1 hour. In addition, the membrane was incubated at 4 ℃ over night when using the primary antibody against TK. After washing three times with TBS-T buffer, the membrane was incubated with the suitable secondary antibody conjugated to Horse Radish Peroxidase (HRP) (Jackson ImmunoResearch) at 1:10,000 dilution. For detection of the HRP signal, the SuperSignal west pico chemiluminescent substrate (Thermo), ECL detection film (GE Healthcare) and film developer (AGFA) were used. The following antibodies were used.

Name of the antigen	Company	Dilution
GFP	Clontech	1:1000
β-actin	Abcam	1:1000
TK	Santa Cruz Biotechnology	1:500
tubulin	Dianova	1:1000
mouse-HRP	Jackson ImmunoResearch	1:10000
goat-HRP	Jackson ImmunoResearch	1:10000

12% Separating gel (20 ml)	
H_2O	6.9 ml
30% Acrylamide (Roti-phorese® Gel 30)	8 ml
4x 1.5M Tris-Cl/0.4% SDS pH 8.8	5 ml
20% (w/v) APS	100 µl
TEMED	8 µl

5% Stacking gel (6 ml)	
H_2O	3.5 ml
30% Acrylamide (Roti-phorese® Gel 30)	1 ml
4x 0.5M Tris-Cl/0.4% SDS pH 6.8	1.5 ml
20% (w/v) APS	30 µl
TEMED	6 µl

4x 1.5M Tris-Cl/0.4% SDS pH 8.8	
Tris	91 g
SDS	2 g
H_2O	Ad 500 ml
pH (with HCl)	8.8

4x 0.5M Tris-Cl/0.4% SDS pH 6.8	
Tris	9.06 g
SDS	0.6 g
H_2O	Ad 150 ml
pH (with HCl)	6.8

6x Laemmli buffer	
4x 0.5M Tris-Cl/ 0.4% SDS pH 6.8	6 ml
Bromphenol blue	0.12 g
Glycerol	9 ml
SDS	2.4 g
H_2O	Ad 20 ml

10x Electrophoresis buffer	
Tris	60.2 g
Glycine	288 g
H_2O	Ad 2 l

Transfer buffer	
10x Electrophoresis buffer	100 ml
Methanol	200 ml
H_2O	Ad 1 l

10x TBS	
Tris	24.2 g
NaCl	160.46 g
H_2O	Ad 2 l
pH (with HCl)	8.0

TBS-T buffer	
10x TBS	100 ml
Tween-20	1 ml
H_2O	Ad 1 l
pH (with HCl)	8.0

2.10. Bisulfite Sequencing

The bisulfite sequencing is a method to determine the 5-methyl-cytosine resulting from methylation of cytosine in CpG nucleotides (Clark et al, 1994). By treating DNA with sodium bisulfate, all cytosine residues are converted to uracil except cytosines carrying 5-methyl residues (Frommer et al, 1992). The target fragments are amplified with strand-specific primers (Frommer et al, 1992) and then cloned into plasmids for further sequencing. The numbers and positions of methylated CpG sites can be determined by comparing to unmethylated ones.

2.10.1. DNA digestion and purification

5 µg gDNA (2.3) was digested with restriction enzyme *EcoR*I. DNAs were then extracted with phenol and precipitated with 500µl 96% (v/v) ethanol as described before (2.6.3). DNA pellets were dried by vacuum in a speed vac and further dissolved in TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0). The DNA was fully denatured by twice shock freezing at -80℃ and thawing at RT. DNA was then boiled at 100 ℃ for 10 minutes and concentrations were detected using a photometer (Eppendorf).

2.10.2. Bisulfite-conversion of DNA

2µg digested and purified DNA was added to NaOH solution (0.3 M NaOH final) in total volume of 20µl and denatured at 37 ºC for 15 minutes. Subsequently, 120 µl BS solution was added and DNA was incubated in a themocycler (Biometra) using the following steps: 15 cycles with 30 seconds at 95 ℃ and 15 minutes at 50 ℃. Finally, the DNA was purified with Wizard DNA Clean-Up System (Promega) according to the manufactory's instructions. DNA was then eluted with 103.6 µl TE buffer. 6.4 µl NaOH (5M) was added to the eluted DNA solution and the solution was incubated at 37 ℃ for 15 minutes. After adding of 47 µl NH_4OAc (10M, pH 7.0) for neutralization, DNA was precipitated with 500 µl 96% (v/v) ethanol , dried in vacuum and dissolved in 50 µl TE buffer. DNA was stored at -20℃.

BS solution

3.6 M Sodium bisulfite
0.6 mM Hydroquinone
pH 5.0

10 µl converted DNA was then used for amplification of eGFP resulting from provirus integration. The following primers, PCR preparation and program were used.

Primer name	**Sequence of primers**
GFP-BS forward	5'- GGGTATAAGTTGGAGTATAA-3'
GFP-BS reverse	5'-CTCCAACAAAACCATATAAT-3'

PCR preparation

BS convert DNA	10 µl
GFP-BS forward (10 pmol/µl)	2.5 µl
GFP-BS reverse (10 pmol/µl)	2.5 µl
dNTPs (1.25 mM/each)	8 µl
10 x reaction buffer	5 µl
DNA polymerase *Taq*	0.5 µl
H_2O	21 µl
Total	50 µl

PCR program

Step	Temperature	Time (seconds)
1.	95 ℃	300
2.	95 ℃	30
3.	40 ℃	60
4.	72 ℃	60
5.	72 ℃	600
6.	4 ℃	∞

Step 2 to 4 were repeated for 50 cycles

2.10.3. Subcloning of PCR products

The PCR products obtained from 2.10.2 were separated by Agarose-gel electrophoresis (2.7.2.) and extracted by using the GFX^{TM} System (Amersham) (2.7.2.). The purified fragments were sub-cloned into pCR2.1 vector (Topo TA cloning kit, Invitrogen) according to the manufactory's instructions and transformed into chemical competent *E. coli* XL-1 blue cells (as mentioned for retransformation of plasmids in 2.1.3). On the next day, single bacteria colonies were picked and inoculated in 5 ml LB^{+} medium supplemented with ampicillin (100 µg/ml). After overnight incubation at 37℃ with 225 rpm shaking, bacteria were harvested by centrifugation with 3600 rpm for 15 minutes at 4 ℃. Thereafter, the plasmid-DNAs was isolated using the minipreparation of plasmid DNA by alkaline lysis with SDS (Sambrook & Russell, 2001). The following primer was used for sequencing.

Primer name	**Sequence of primers**
M13 uni (-21)	5'- TGTAAAACGACGGCCAGT-3'

2.11. LDH assay

The lactate dehydrogenase (LDH) assay is a colorimetric assay for the quantification of LDH that is released from the cytosol of damaged cells into the cell culture supernatant. Therefore, it can be used as cytotoxicity assay in the context of the effect of GCV on TK expressing ES cells. The LDH in cell culture supernatants was either directly analyzed using LDH assay or LDH was measured after complete lysis of cells of each set of samples: $3x10^3$ ES cells transduced with NT, OT, CT and ST or untransduced (2.2.2) were seeded on a 24-well plate coated with a layer of feeder cells. One day after ES cell seeding, the medium was replaced by 500 µl fresh medium with 20 µM GCV. 36 hours later, further 500 µl fresh medium with 20 µM GCV was added on the cells. Further 36 hours later (=72 hours with GCV treatment), 50 µl of the supernatants from ES cells with GCV treatment and ES cells with GCV treatment after adding of lysis buffer for 15 minutes were each analyzed using the LDH assay in accordance to the manufacturer's instructions. To obtain "background" signals feeder cells without ES cells were seeded as well and treated with GCV. 50 µl of the supernatants from feeder cells alone and feeder cells alone after adding of lysis buffer for 15 minutes were analyzed. These values with or without lysis were each

substracted from the extinction values obtained from ES cells. The differences between values with or without lysis represent the number of ES cells that survived GCV treatment. Extinction was each measured using ELISA reader (TECAN) with absorbance at 492 nm.

2.12. *in vivo* experiments

To investigate the fate of LV-TK-transduced ES cells *in vivo*, 100µl cell suspension with $1x10^6$ untransduced ES cells, STPH-transduced ES cells with 1.5 copies per genome in average with hygromycin pre-selection or STPH-transduced ES cells with 3.8 copies per genome in average without hygromycin pre-selection (2.2.2) were injected into the hind limbs of Fox Chase SCID® Beige mice (Charles River) (3.2.4). Three hours after ES cell injection, mice were administered with either saline solution (0.9% (w/v) NaCl) or GCV (20 mg/kg/day) for 12 days. Three weeks later, mice were sacrificed for the analysis of potential teratoma formation. The teratomas were analyzed by measuring their weight and size. For further analysis, teratomas were fixed in 4% (w/v) PFA in PBS, embedded in O.C.T. (Tissue-Tec) and stored at -80 °C. Sections were prepared in 10 µm thickness for H&E staining (see also 2.13).

2.13. Hematoxylin and Eosin (H&E) staining

H&E staining displays a common staining procedure to visualize structures of tissue. Hematoxylin solution is used for nuclei staining leading to visualization of nuclei in a blue color. Eosin stains the cytoplasm and connective tissue resulting in a variety of red or pink color.

Teratomas sections (2.12.) were twice incubated in PBS for each 5 minutes. Afterwards, slides were treated with hematoxylin (Mayers hemalaun, Merck) for 1 minute and then washed in fluent water for 5 minutes. Thereafter, slides were stained with eosin (Eosin G supplemented with 0.5 % acetic acid, Merck) for 30 seconds and washed in fluent water for 5 minutes. Finally, slides were mounted with Roti® -Histokitt (Carl Roth). Images were taken using a AxioStart (Carl Zeiss) and AxioCam (Carl Zeiss).

2.14. Immunochemistry

Immunochemistry is a widely used method to detect specific antigens in cells or tissues by applying specific antibodies. The specific antigens are visualized by immunofluorescence resulting from antibodies conjugated to fluorescent dyes.

EBs (2.2.4.) were collected and washed twice with PBS. After that, EBs were incubated in Collagenase B (0.1 mg/ml, Roche) for 20 minutes in a shaker with 600 rpm at 37°C. $3x10^5$ single cells from dissociated EBs were seeded per well of a 24-well plate with cover slips that were coated with gelatin (0.1% (w/v) in PBS). After reattaching of the cells on the cover slips (24 hours later), cells were fixed with 500 µl 4% (w/v) PFA at RT for 15 minutes. The cover slips with fixed cells were removed from the 24-well plate and used for immunostaining: Therefore, they were incubated with primary antibodies in PBS solution containing 0.05% donkey serum (Jackson ImmunoResearch), 0.1% (v/v) triton, anti Oct-3/4 (1:100, Santa Cruz Biotechnology) and anti α-actinin (1:400, Sigma-Aldrich) for 2 hours. After removing the solution with the primary antibodies and washing three times with PBS, the cover slips were further incubated with secondary antibodies containing anti Dylight 549 and anti Dylight 649 (both 1:400, Jackson ImmunoResearch) in Hoechst stain solution (Sigma-Aldrich) for 1 hour. Images were taken using Axio Observer.Z1 with Apotome system (Clar Zeiss) and AxioCam MRC5 (Clar Zeiss).

3. Results

3.1. Epigenetic regulation of LVs and RVs

3.1.1. Viral vectors transduction of mouse embryonic stem cells (mixed populations)

3.1.1.1. Viral vectors-transduced cells under undifferentiation conditions

In order to compare the epigenetic regulation of LVs and RVs in murine ES cell populations, murine ES cells (R1 ES cell line) were transduced with VSV.G pseudotyped SIN-LV, SIN-RV and PCLMFG (MOI = 50) carrying each the eGFP reporter gene from the jellyfish *Aequorea victoria* driven either by an internal PGK promoter or by the wildtype 5' LTR promoter (Figure 11) (see also 2.2.2). These ES cell populations were neither further selected after transduction nor further subcloned, but the whole populations including transduced and untransduced cells were used for analysis. Cell populations that were generated in this way are referred to as mixed populations in the following text.

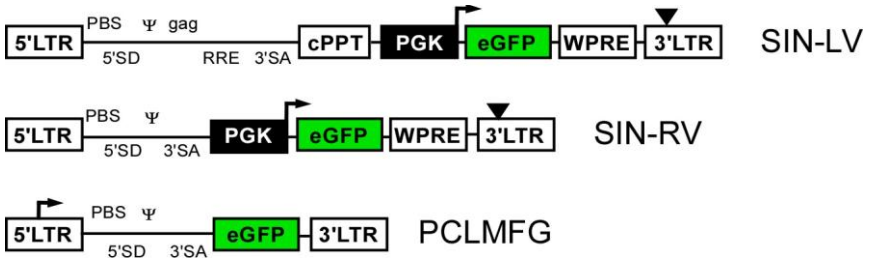

Figure 11: Constructs of SIN-LV, SIN-RV and PCLMFG carrying eGFP expressing cassette driven by internal PGK promoter or LTR promoter. PB: primer binding site; ψ: packaging signal ; SD: splice donor; SA: splice acceptor; RRE: *rev* response element.

After viral transduction, the ES cells were further cultured under undifferentiation conditions for 17 days and fluorescence images were taken. For quantification of eGFP expressing cells, flow cyctometry was applied. As the process of provirus integration, transcription and translation of the integrated transgene need approximately 48 hours, transgene expression was analyzed two days after virus transduction and additionally on day 17 post transduction. Images were each taken from undifferentiated ES cells that were transduced with the three vectors mentioned above (Figure 12). On day 17 post transduction, eGFP-positive cells were only observed in SIN-LV- and SIN-RV-transduced cells (Figure 12A). For quantification of eGFP-positive cells using flow cyctometry the "cut off" of the applied flow cytometer has to be set to 10% for untransduced (GFP negative) cells (Pfeifer & Hofmann, 2009). This means that 10% are equal to no eGFP

expression. Flow cyctometry analysis revealed more eGFP-positive cells (65.33±3.44%) on day 2 and on day 17 (59.28±4.88%) (Mean±SEM) for SIN-RV-transduced cells as compared to SIN-LV and PCLMFG, respectively (Figure 12B): SIN-LV-transduced cells resulted in 41.25±1.89% and 44.77±0.79% eGFP-positive cells on day 2 and 17 post transduction, whereas PCLMFG-transduced cells resulted in 38.36±10.51% eGFP-positive cells on day 2 post transduction and no eGFP expressing cells were detected on day 17 post transduction. A high reduction of eGFP-positive cells of PCLMFG-transduced cells to nearly the same level as untransduced cells (WT) was already observed on day 5 post transduction as analyzed by fluorescence microscopy (data not shown) as well as by flow cytometry. This indicates a strong silencing of integrated provirus DNAs in PCLMFG-transduced ES cells under undifferentiation conditions. In contrast, SIN-LV and SIN-RV transduction resulted in a stable transgene expression in undifferentiated ES cells with overall a higher percentage of eGFP-positive cells for SIN-RV transduction.

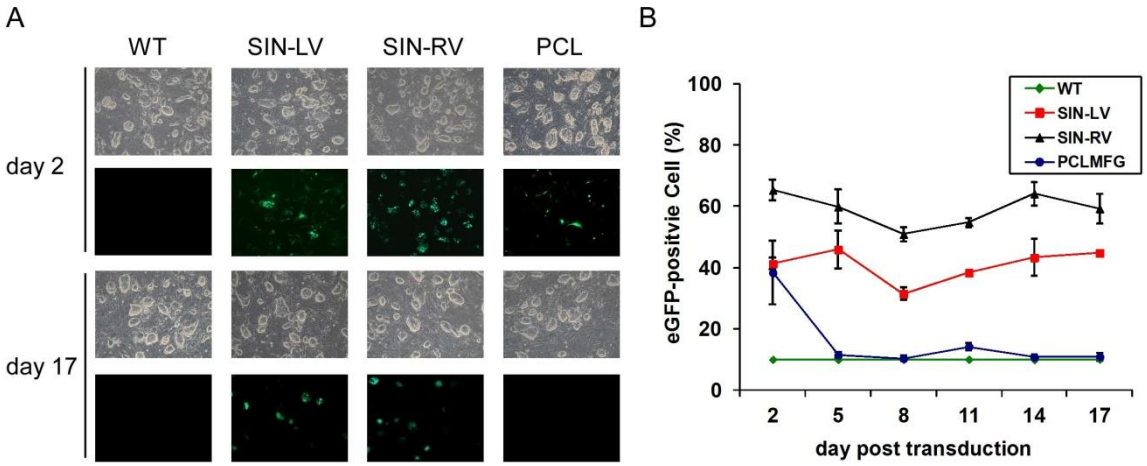

Figure 12: **eGFP expression analysis of untransduced (WT) and SIN-LV-, SIN-RV- and PCLMFG-transduced ES cells (mixed population, MOI=50) under undifferentiation conditions**. (**A**) Brightfield and fluorescence images of untransduced (WT) ES cells or ES cells transduced by SIN-LV, SIN-RV and PCLMFG on day 2 and 17 post transduction. (**B**) Percentage of eGFP-positive ES cells as analyzed by flow cyctometry after viral transduction under undifferentiation conditions. Green rhombus: untransduced ES cells (WT). Red square: SIN-LV-transduced ES cells (SIN-LV). Black triangle: SIN-RV-transduced ES cells (SIN-RV). Blue circle: PCLMFG-transduced ES cells (PCLMFG) (n=3, Mean±SEM).

3.1.1.2. Viral vectors-transduced cells under differentiation conditions

In the next step, ES cells were transduced with the three viral vectors (Figure 11) and eGFP expression was analyzed under differentiation conditions. Therefore, untransduced

as well as transduced ES cells were cultured in suspension as aggregates (embryoid bodies (EBs) (Wobus & Boheler, 2005) (2.2.4). The EBs were either harvested on day 5 or 14 post differentiation and eGFP-positive cells were detected by fluorescence microscopy and flow cyctometry.

For PCLMFG-transduced cells, the eGFP-positive cells disappeared already on day 5 post differentiation (Figure 13A, B). In SIN-LV- and SIN-RV-transduced cells, very few eGFP-positive cells were observed on day 5 post differentiation (Figure 13A upper) and nearly no eGFP-positive cell was detectable on day 14 post differentiation (Figure 13A lower). Flow cytometry analysis was consistent with the fluorescence images: The eGFP-positive cells of SIN-LV- and SIN-RV-transduced cells decreased dramatically from 41.25±1.89% and 65.33±3.44% to 19.61±1.15% and 32.75±8.9% (Mean±SEM) on day 5 post differentiation, respectively (Figure 13B). Moreover, only 12.12±1.58% (SIN-LV) and 19.55±2.93% (SIN-RV) eGFP-positive cells were detected on day 14 post differentiation.

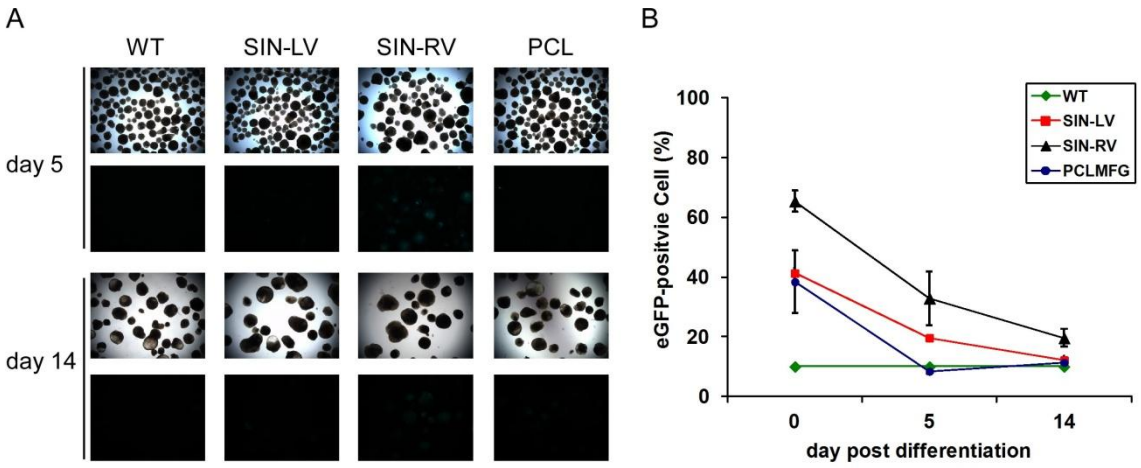

Figure 13: eGFP expression of untransduced (WT) or SIN-LV-, SIN-RV- and PCLMFG-transduced ES cells (mixed population, MOI=50) under differentiation conditions. (**A**) Brightfield and fluorescence images of untransduced (WT) or ES cells transduced with SIN-LV, SIN-RV and PCLMFG on day 5 and 14 post differentiation. (**B**). Percentage of eGFP-positive cells during 2-week differentiation after viral transduction. Green rhombus: untransduced ES cells (WT). Red square: SIN-LV-transduced ES cells (SIN-LV). Black triangle: SIN-RV-transduced ES cells (SIN-RV). Blue circle: PCLMFG-transduced ES cells (PCLMFG) (n=3, Mean±SEM).

Taken together, PCLMFG-mediated gene transfer led to complete silencing of the transgene in a mixed ES cell population already on day 2 post transduction under both undifferentiation or differentiation conditions. The SIN-LV- and SIN-RV-transduced mixed ES cell populations resulted in stable transgene expression for 17 days under undifferentiation conditions whereas the transgene expression in differentiated cells already declined on day 5 post differentiation. Epigenetic silencing of the transgene for

both SIN-LV and SIN-RV was obviously observed on day 14 post differentiation although a few eGFP-positive cells still remained in the SIN-RV-transduced cell population (Figure 13).

3.1.1.3. qPCR analysis of proviruses integration numbers

In order to study whether the transgene expression is declining due to disappearing integrations of proviruses in undifferentiated or differentiated cell cultures, copy numbers of proviruses have been analyzed by using quantitative real-time PCR (qPCR). The loss in integration numbers can be due to overgrowing of infected cells by untransduced cells or due to the death of transduced cells. Thus, the gDNA of SIN-LV-, SIN-RV- and PCLMFG-transduced ES cells was isolated and used as templates for qPCR analysis, respectively. The quantifications of provirus integrations were each performed for undifferentiated as well as for differentiated cells (Figure 14).

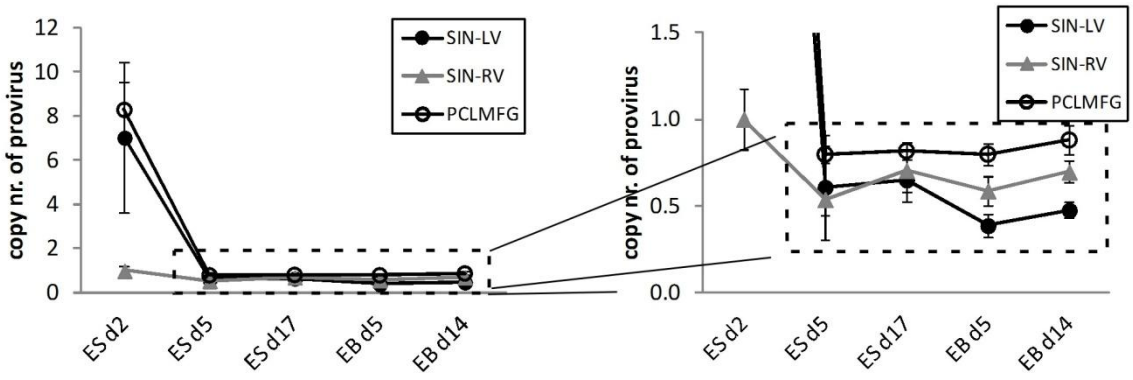

Figure 14: Copy number of provirus in SIN-LV-, SIN-RV- and PCLMFG-transduced ES cell s (mixed population, MOI=50) under undifferentiation (ES) and differentiation (EB) conditions. The copy numbers of the three viral vectors were analyzed by qPCR. Viral transduced ES cells were analyzed on day 2, 5, and 17 post transduction and EBs were analyzed on day 5 and 14 post differentiation. As compared to ES d5 of each construct, copy numbers of ES d17, EB d5 and EB d14 show no significant difference, respectively (n=3, Mean±SEM, ANOVA, analysis of variance).

In contrast to SIN-RV-transduced ES cells, SIN-LV- and PCLMFG-transduced ES cells contained on day 2 post transduction higher copy numbers of proviruses (7.04±3.42 and 8.30±1.26 (Mean±SEM)) (Figure 14 left). The copy numbers immediately declined to less than 1 for both SIN-LV and PCLMFG vectors on day 5 post transduction (Figure 14 right). The already low copy number of the SIN-RV-transduced ES cell population was further reduced by around 30% to 0.64±0.19 from day 2 to 5 post transduction under

undifferentiation conditions (Figure 14). For all vector types these copy numbers then remain on approximately the same level until day 17 post transduction under undifferentiation conditions. ES cells were then differentiated into EBs and qPCR analysis was performed on day 5 and day 14 post differentiation. This also revealed no significant differences as compared to day 5 or day 17 post transduction under undifferentiation conditions.

Different reasons are possible to explain the declining of provirus integration in the beginning. As reported by Butler et al (Butler et al, 2001), released proviruses in the cytoplasm after infection of host cells that were not integrated can form 1 or 2-LTR circle constructs within 24 hours post transduction. These LTR circles are also detected by qPCR but do not reveal from provirus integrations. Due to degradation of the LTR circles they can not be detected at later time points. As the SIN-LVs, SIN-RVs and PCLMFG vectors used are VSV.G pseudotyped they can transduce feeder cells as well, on which ES cells are seeded. Thus, transduction of these feeder cells also contributes to the copy numbers as determined by qPCR, although eGFP expression is only analyzed in ES cells. On day 5 post transduction the feeder cells are replaced by fresh feeder cells when the ES cells are passaged. Therefore, the contribution of transduced feeder cells to the analyzed copy numbers has only be considered for provirus determination on day 2. Therefore, a comparison of the three vector types can only be performed on day 5 post transduction as well as on later time points.

In conclusion, integration of provirus DNA is similar among the three vector types and therefore, this is not the reason for different eGFP expression patterns.

3.1.1.4. Methylation analysis by using methylation-sensitive Southern blot method

Early reports have proposed *de novo* methylation of the LTR promoter of MLV leading to transgene silencing during mouse embryogenesis (Challita & Kohn, 1994; Jahner et al, 1982). DNA Methylation of provirus DNA has been also considered as a major reason of gene silencing in ES cells, originally in order to silence foreign DNA (Cherry et al, 2000). Methylation-sensitive Southern blot is a common method to detect the methylation state of integrated provirus DNA. Therefore, gDNAs of transduced ES cells and EBs were harvested on day 2, 5 and 17 post transduction and day 5 and 14 post differentiation. The gDNA was digested by two restriction enzymes involving a “methylation-insensitive” as

well as a "methylation-sensitive" enzyme (Figure 15). The methylation-sensitive enzyme can not digest cytosine-methylated fragments. By applying both enzyme types at the same time and analysis of the restriction pattern conclusions can be drawn concerning the methylation status of digested DNA.

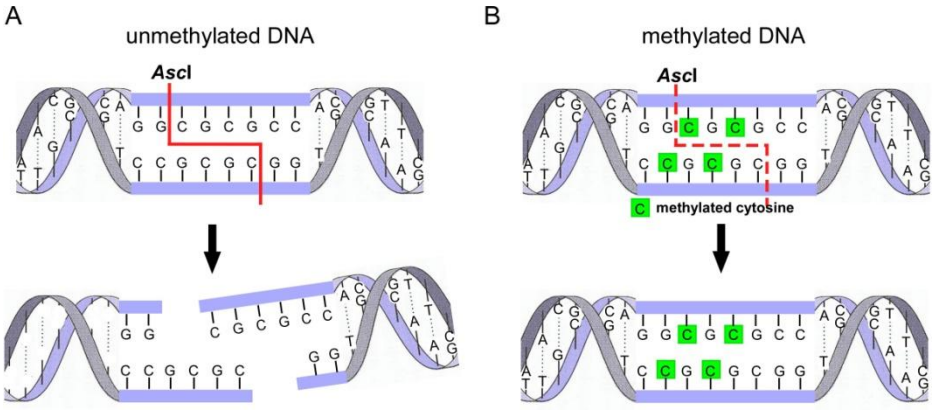

Figure 15: Schematic illustration of methylation-sensitive restriction.(**A**) methylation sensitive enzyme *Asc*I is able to digest the unmethylated CpG DNA sequence. (**B**) Methylated CpG island sequence blocks the activity of *Asc*I.

Digesting the gDNA with the enzyme *EcoR*I and the methylation-sensitive enzyme *Asc*I would result in a 1.8 kb fragment if SIN-LV provirus is methylated (Figure 16A). In case of unmethylated provirus SIN-LVs a 1.5 kb band would be found (Figure 16A). The restricted DNA is separated on an agarose gel and after blotting the bands can be targeted by a radioactive eGFP probe for visualization on a Southern blot (Figure 16B). On day 2, 5 and 17 post transduction obviously only unmethylated bands of SIN-LV in undifferentiated ES cells were observed indicating no evidence of methylation (Figure16B, C). However, on day 5 and 14 post differentiation methylated bands were detected suggesting that the methylation of SIN-LV occurred in differentiated cells (Figure16B, C).

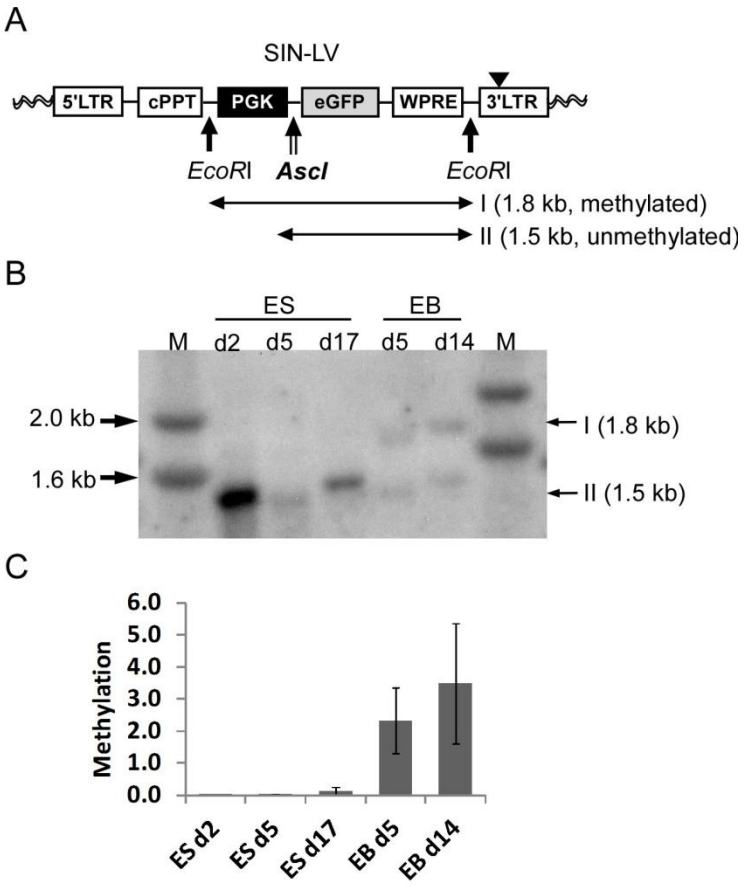

Figure 16: Analysis of the methylation status of SIN-LV proviruses using the methylation-sensitive restriction enzyme *Asc*I. (**A**) DNA methylation of eGFP region was analyzed by digesting genomic DNA with *Eco*RI and methylation-sensitive enzyme *Asc*I. Methylation of the CpG dinucleotide present in the *Asc*I site results in a 1.8-kb band (I) in the Southern blot. Unmethylated DNA yields a 1.5-kb band (II) after *Eco*RI/*Asc*I digestion. (**B**) Southern blot analysis of ES cells transduced with SIN-LVs on day 2, 5 and 14 post transduction and EBs on day 5 and 14 post differentiation. Radioactive labeled eGFP probe was used to detect digested fragments. (**C**) The methylation status of integrated proviruses were quantified by calculating the ratio of methylated band (I) to unmethylated band (II) using Quantity One 1-D Analysis Software (Bio-Rad) (n=3, Mean±SEM).

For the analysis of the methylation status inSIN-RV-transduced ES cells the methylation-sensitive enzyme *Sal*I was used as well as the methylation-insensitive enzyme *Acc65*I. This results in a 2.6 kb methylated band (I) in methylated SIN-RV proviruses and a 1.8 kb unmethylated band (II) in unmethylated SIN-RV proviruses (Figure 17A). In comparison to SIN-LVs, weak methylated bands were already observed in undifferentiatedSIN-RV-transduced ES cells (Figure 17B, C). Moreover, methylated bands obviously appeared on day 5 and 14 in EBs indicating apparently a methylation of SIN-RV proviruses under differentiation condition (Figure 17B, C).

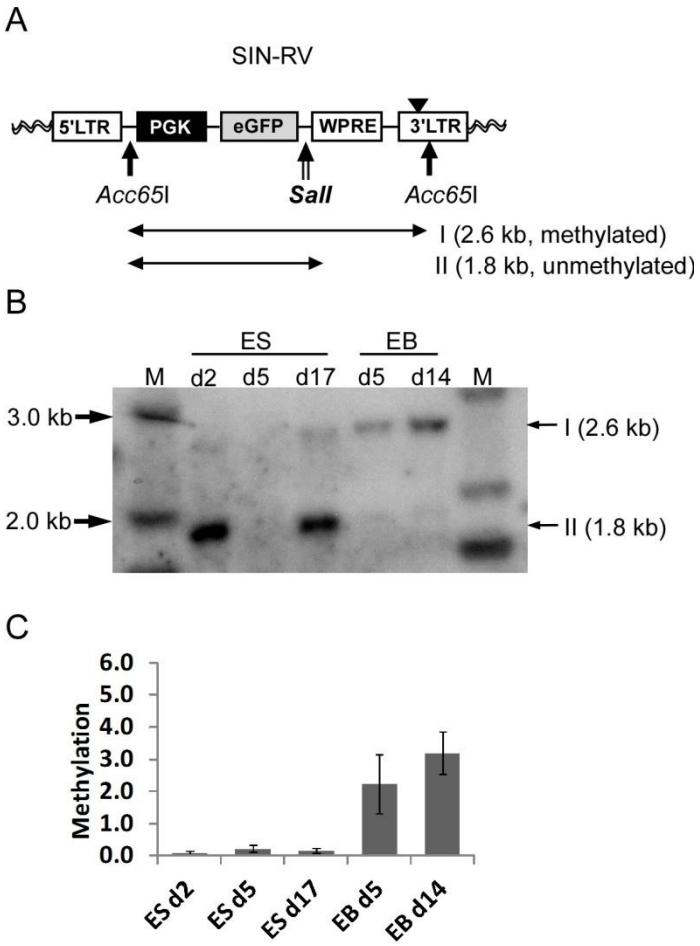

Figure 17: Analysis of the methylation status of SIN-RV proviruses using the methylation-sensitive restriction enzyme *Sal*I. (**A**) DNA methylation of eGFP region was analyzed by digesting gDNA with *Acc65*I and methylation-sensitive enzyme *Sal*I. Methylation of the CpG dinucleotide present in the *Asc*I site results in a 2.6-kb band (I) in the Southern blot. Unmethylated DNA yields a 1.8-kb band (II) after *Acc65*I/*Sal*I digestion. (**B**) Southern blot analysis of ES cells transduced with SIN-RVs on day 2, 5 and 14 post transduction and EBs on day 5 and 14 post differentiation. Radioactive labeled eGFP probe was used to detect digested fragments. (**C**) The methylation status of integrated proviruses were quantified by calculating the ratio of methylated band (I) to unmethylated band (II) using Quantity One 1-D Analysis Software (Bio-Rad) (n=3, Mean±SEM).

In summary, methylation levels are extremely low at undifferentiation stage of both SIN-LV- and SIN-RV-transduced ES cells (Figure 16C and 17C). The methylation occurred firstly on day 5 post differentiation, and is even stronger on day 14 post differentiation.

The correlations between eGFP-positive cells and methylation level are displayed in a diagram (Figure 18). This reveals strong inverse correlations between the methylation coefficient and the percentage of eGFP-positive cells for both SIN-LV and SIN-RV (Figure 18) and indicates that lower eGFP expression rates indeed are the consequence of provirus methylation. Interestingly, this is true for both vector types analyzed under undifferentiation as well as under differentiation conditions.

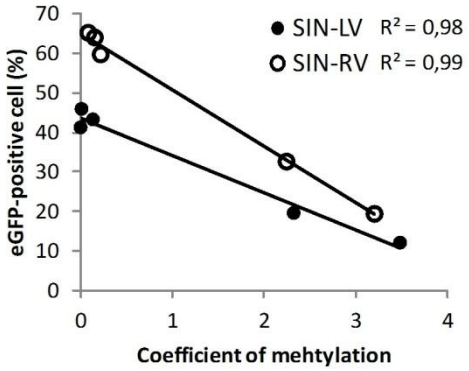

Figure 18: Correlation between percentage of eGFP-positive cells and methylation coefficient. The methylation level of SIN-LV (hollow circles) and SIN-RV (solid circles) proviruses in transduced ES cells were compared to percentage of eGFP-positive cells under undifferentiation and differentiation conditions (Data are obtained from Figures 12B, 13B, 16C and 17C)

3.1.2. Single copy clones of SIN-LV- and SIN-RV-transduced ES cells

3.1.2.1. Generation of single copy SIN-LV and SIN-RV ES cell clones

As figured out, methylation plays an important role in epigenetic regulation of SIN-LV and SIN-RV in mixed ES cell populations (see also Figure 18). For a more accurate analysis of SIN-LV and SIN-RV mediated gene expression single copy ES cell clones were generated instead of the use of mixed cell populations analyzed so far. Therefore, ES cell were transduced with SIN-LV and SIN-RV and single clones were picked. The gDNAs from picked cell clones were subsequently isolated, digested by *BamH*I enzymes and finally screened by using Southern blot analysis to obtain ES cell clones with single integrant (Figure 19).

For the following analysis of eGFP expression, SIN-LV and SIN-RV ES cell clones with only one integrant were chosen, that were identified by Southern blot analysis (SIN-LV: #40 and #52; SIN-RV: #10 and #14) (Figure 19).

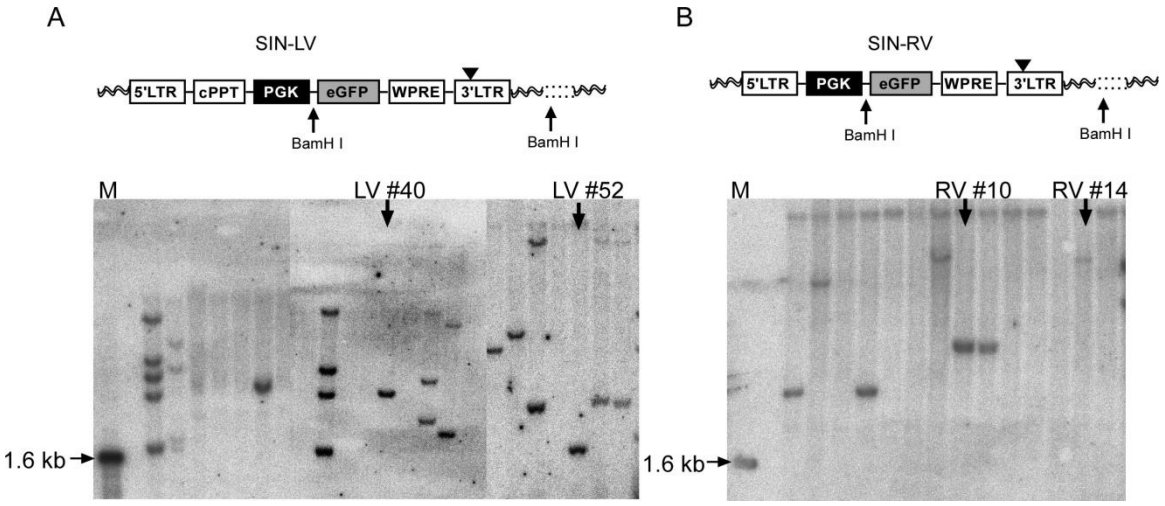

Figure 19: gDNA digestion of picked cell clones from SIN-LV- and SIN-RV-transduced ES cells and Southern blot analysis. ES cells were transduced with SIN-LVs (**A**) or SIN-RVs (**B**), clones were picked, expanded and gDNA was isolated and digested with *BamH*I. Southern blot analysis was used to detect the number of provirus integrations. Marked by an arrow are single copy SIN-LV ES cell clones #40 (3 kb) and #52 (1.8 kb)(**A**) and single copy SIN-RV ES cell clones #10 (7 kb) and #14 (12 kb) (**B**).

Quantification using flow cyctometry revealed that for both constructs the ES cell clones showed either low or high eGFP expression levels, respectively (Figure 20). Theses single copy ES cell clones were chosen for further investigations.

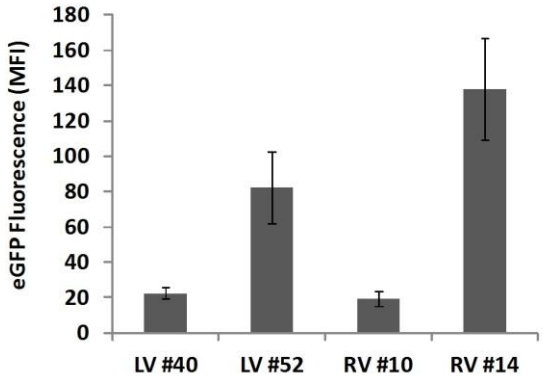

Figure 20: Mean fluorescence intensity (MFI) of single copy ES cell clones. ES cell clones with single integrant were identified by Southern blot analysis. eGFP expression levels were analyzed by flow cyctometry. SIN-LV ES cell clones #40 (LV #40) and #52 (LV #52) express low and high levels of eGFP, respectively; SIN-RV ES cell clones #10 (RV #10) and #14 (RV #14) are express low and high levels of eGFP, respectively (n=3, Mean±SEM).

3.1.2.2. Single copy ES cell clones under undifferentiation conditions

The four ES cell clones obtained from picked clones (3.1.2.1) were then cultured under undifferentiation conditions for 15 days and eGFP expression was analyzed every three

days. As shown on Figure 20, clones LV #40 and RV #10 expressed low eGFP levels. This could also be seen under undifferentiation conditions as analyzed by fluorescence microscopy (Figure 21A upper). High eGFP level expressing clones LV #52 and RV#14 (see also Figure 20) showed also high levels of eGFP-positive cells on day 3 post plating under undifferentiation conditions (Figure 21A upper). On 15 day post plating, high eGFP level expressing clones LV #52 and RV#14 still showed the high eGFP expressions and for LV #40 and RV #10 the same slight eGFP expressions as seen on day 3 were observed on day 15 post plating (Figure 21A lower). All four ES cell clones revealed a stable eGFP expression under undifferentiation conditions as also quantified by using flow cytometry (Figure 21B).

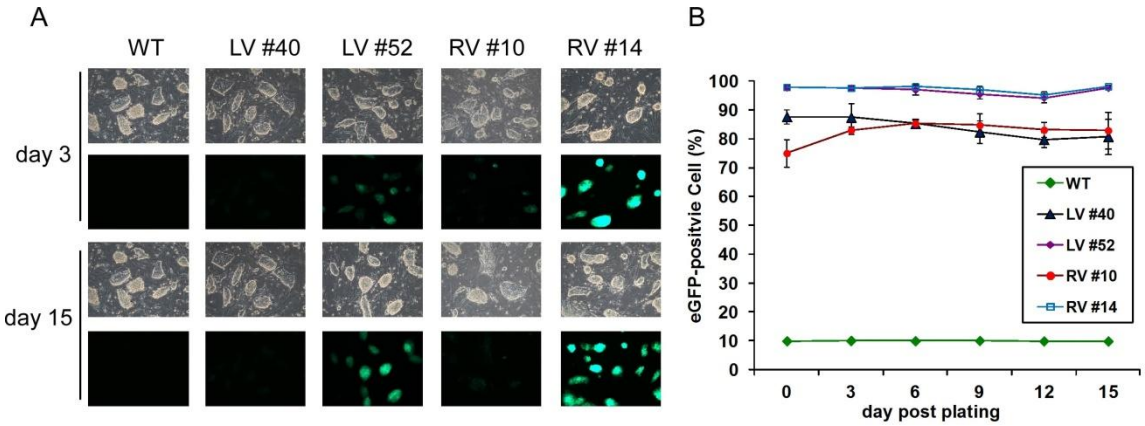

Figure 21: eGFP expression of single copy ES cell clones under undifferentiation conditions. (**A**) Brightfield and fluorescence images of untransduced (WT) ES cells and SIN-LV- and SIN-RV-transduced ES cell clones carrying a single integrant on day 3 and 15 post plating. (**B**) Percentage of eGFP-positive ES cells during cultivation under undifferentiation conditions for 15 days. Green rhombus: untransduced ES cells (WT). Black triangle: SIN-LV ES cell clone #40 (LV #40). Purple rhombus: SIN-LV ES cell clone 52 (LV #52). Red circle: SIN-RV ES cell clone #10 (RV #10). White square: SIN-RV ES cell clone #14 (RV #14) (n=3, Mean±SEM).

3.1.2.3. Single copy ES cell clones under differentiation conditions

Next, the single integrant ES cell clones were cultured under differentiation conditions and analyzed by fluorescence microscopy and flow cyctometry on day 5 and 14 post differentiation. eGFP expressions of the two low expressing cell clones (LV #40 and RV #10) could not be detected on day 5 and 14 post differentiation in fluorescence images (Figure 22A) and percentages of eGFP-positive cells decreased from around 80% to less than 20% on day 5 post differentiation. On day 14 post differentiation the percentages of eGFP-positive cells of these two clones were on the similar level as untransduced cells (WT) (Figure 22B) as already seen on fluorescence images (see also Figure 22A).

In contrast, eGFP expressions of high expressing LV #52 and RV #14 clones were still detectable in the fluorescence images on day 5 post differentiation (Figure 22A). Flow cytometer revealed 74.30±9.69% and 55.61±11.20% eGFP positive cells on day 5 post differentiation and still 50.21±4.18 and 40.32±3.22 % eGFP positive cells on day 14 post differentiation for clones LV #52 and RV #14, respectively (Figure 22B). In addition, the high eGFP expressing RV #14 clone showed a higher reduction of eGFP-positive cells as compared to LV #52 (Figure 22B).

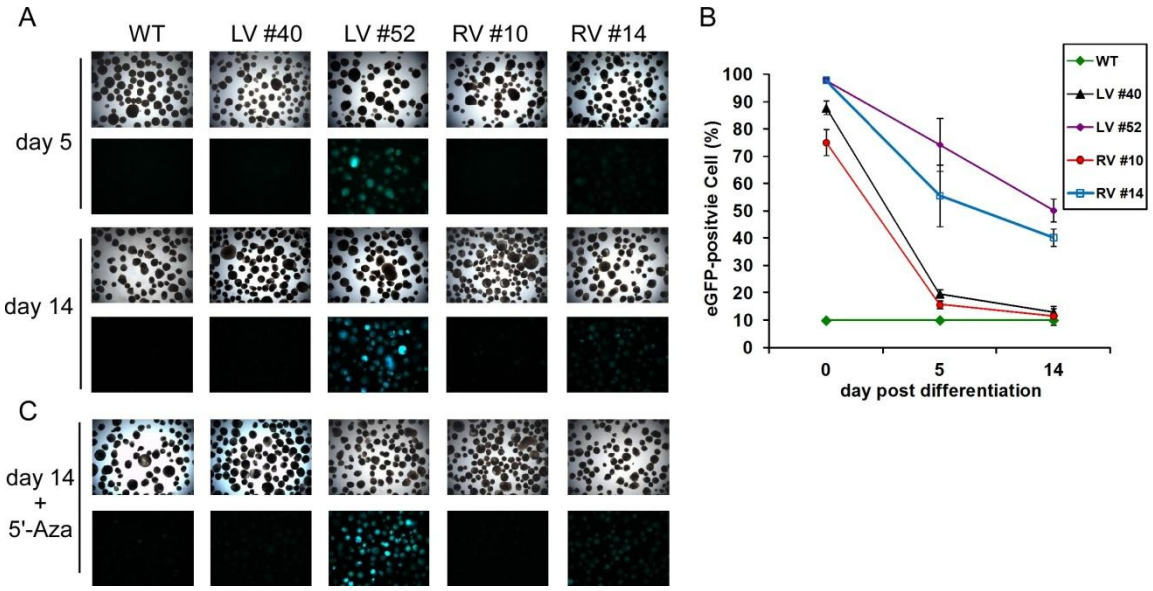

Figure 22: eGFP expression of single copy ES cell clones under differentiation conditions. (**A**) Brightfield and fluorescence images of EBs on day 5, 14 post differentiation of untransduced (WT) ES cells and SIN-LV- and SIN-RV-transduced ES cell clones carrying a single integrant. (**B**) Percentage of eGFP-positive cells during 2-week differentiation. Green rhombus: untransduced ES cells (WT). Black triangle: SIN-LV ES cell clone #40 (LV #40). Purple rhombus: SIN-LV ES cell clone 52 (LV #52). Red circle: SIN-RV ES cell clone #10 (RV #10). White square: SIN-RV ES cell clone #14 (RV #14) (n=3, Mean±SEM). (**C**) Brightfield and fluorescence images of EBs of untransduced (WT) ES cells and SIN-LV- and SIN-RV-transduced ES single copy clones on day14 post differentiation with 5'Aza treatment.

As already shown for mixed ES cell populations, the methylation status of provirus integration had a high impact on transgene silencing (see also Figure 18). Therefore, an inhibitor against DNA methyltransferases was applied in order to prevent methylation of integrated provirus DNA during differentiation. The inhibitor (5-aza-2' deoxycytidine (5'-Aza)) was applied on day 11 post differentiation on the cells and fluorescence images were taken three days later (Figure 22C). Unexpectedly, the 5'-Aza was not able to reactive the eGFP expression in these four ES cell clones.

When comparing images of eGFP fluorescence and percentage of eGFP-positive cells it can be clearly seen that intensity of eGFP expression is much higher for LV #52 as for RV

#14, although the number of transduced cells were both in a similar range. Therefore, in the next step, the eGFP intensity was also quantified (Figure 23): The two low expressing clones (LV #40 and RV #10) show a declining in eGFP intensity to WT level already within the first five days post differentiation. Although the high eGFP expressing RV #14 clone resulted in more than 50% eGFP-positive cells (see Figure 22B), the eGFP intensity decreased rapidly to a very lower level (Figure 23). In contrast to RV #14, the high eGFP expressing ES cell clone LV #52 showed a much lower decreasing in eGFP intensity although the intensity on day 0 (before differentiation) was lower as compared to RV #14 (Figure 23). Taken together, the ES cell clone LV #52 had the highest level of eGFP expression as observed on fluorescence images and by measuring the mean fluorescence intensity and also revealed the highest percentage of eGFP expressing cells under differentiation conditions.

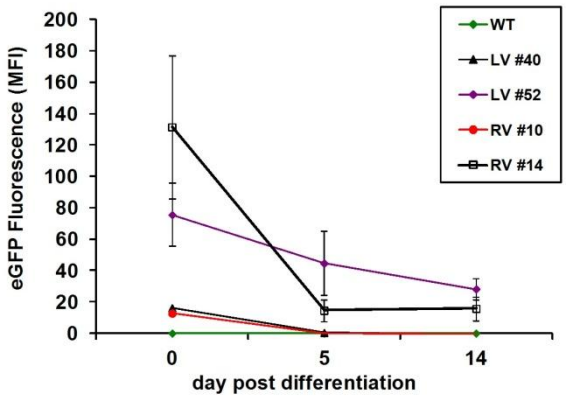

Figure 23: Mean fluorescence intensity (MFI) of single copy ES cell clones during 2-week differentiation. Green rhombus: untransduced ES cells (WT). Black triangle: SIN-LV ES cell clone #40 (LV #40). Purple rhombus: SIN-LV ES cell clone 52 (LV #52). Red circle: SIN-RV ES cell clone #10 (RV #10). White square: SIN-RV ES cell clone #14 (RV #14) (n=3, Mean±SEM).

3.1.2.4. Methylation-sensitive Southern blot analysis

To identify the reason for the decrease in eGFP intensity the methylation status of integrated provirus DNA was next investigated. Therefore, the four single copy ES cell clones were studied by using methylation-sensitive Southern blot analysis as already applied for the mixed ES cell populations. The four ES cell clones were cultured under undifferentiation conditions for 15 days and in parallel under differentiation conditions for 14 days. The gDNAs of LV clones #40 and #52 were each digested by *Eco*RI and *Asc*I

(methylation sensitive enzyme) leading to unmethylated (1.5 kb) and methylated bands (1.8 kb) (Figure 24A, B) and analyzed by Southern blot.

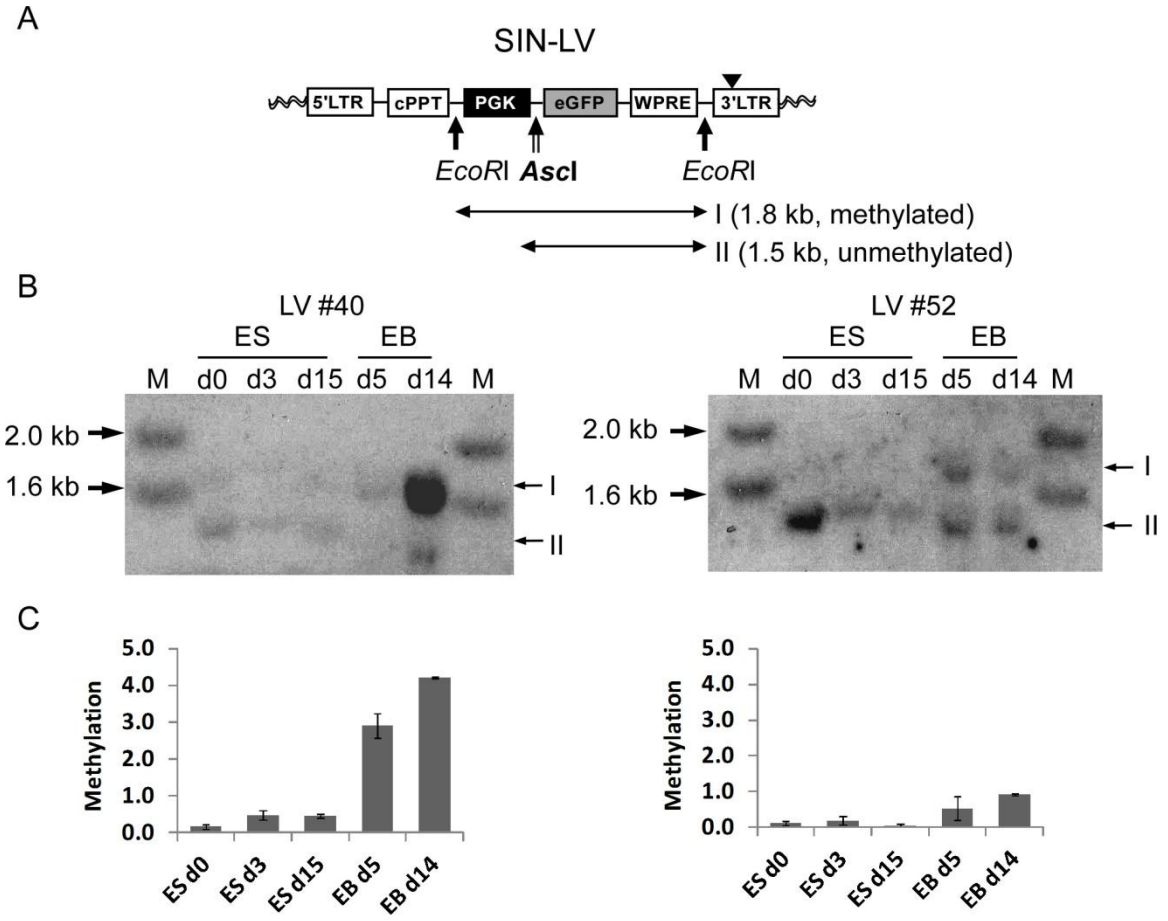

Figure 24: Analysis of the methylation status of SIN-LV ES cell clones using the methylation-sensitive restriction enzyme *Asc*I. (**A**) Methylation of the eGFP region was analyzed by digesting gDNA with the methylation-sensitive *Asc*I. Methylation of the CpG dinucleotide present in the *Asc*I site results in a 1.8-kb band in the Southern blot (band I). Unmethylated DNA yields a 1.5-kb band after *EcoR*I/*Asc*I digestion (band II). (**B**) Southern blot analysis of single copy ES cell clones SIN-LV #40 and #52 on day 0, 3 and 15 post plating and EBs on day 5 and 14 post differentiation. Radioactive labeled eGFP probe was used to detect digested fragments. (**C**) The methylation status of integrants were quantified by calculating the ratio of methylated (band I) to unmethylated bands (band II) (n=3, Mean±SEM).

The low eGFP expressing ES cell clone LV #40 showed mainly unmethylated bands under undifferentiation conditions (ES cells on day 0, 3 and 15 post plating) whereas only very weak methylated bands were observed (Figure 24B left). Under differentiation conditions (EBs on day 5 and 14 post differentiation) very strong methylated bands were observed and only weak unmethylated bands (Figure 24B left). In contrast, the eGFP high expressing ES cell clone LV #52 showed clearly unmethylated bands under both undifferentiation and differentiation conditions and under differentiation conditions (EBs

on day 5 and 14 post differentiation) only slight methylated bands were detected (Figure 24B right).

Taken together, these results indicate that the eGFP low expressing LV #40 had just a slight methylation before differentiation and a high methylation level during the differentiation. The provirus DNA of high expressing clone LV #52 maintained the unmethylation state until the end of the differentiation process. Taking the intensities of methylated and unmethylated bands into account it is clearly visible that LV #40 clone shows a much stronger methylation after differentiation (Figure 24B, C left). In contrast, ES cell clone LV #52 revealed a much slighter methylation under differentiation conditions (Figure 24 B, C right).

Next, the methylation status of low and high expressing RV ES cell clones #10 and # 14 were also analyzed by methylation–sensitive Southern blot using *Acc65*I and *Sal*I (methylation-sensitive enzyme) resulting in 2.6 kb (methylated) and 1.8 kb (unmethylated) bands (Figure 25A). The low eGFP expressing RV ES cell clone #10 showed only very weak unmethylated bands and mainly strong methylated bands under undifferentiation conditions (ES cells on day 0, 3 and 15 post plating) (Figure 25B left). Furthermore, under differentiation conditions (EB on day 5 and 14 post differentiation) the methylated bands of RV #10 were even stronger indicating a very high methylation level (Figure 25B left). In contrast, the high eGFP expressing RV ES cell clone #14 revealed obviously unmethylated bands only under undifferentiation conditions whereas methylated bands are mainly visible at differentiation stage (Figure 25 right). This suggests that a high methylation level of RV #14 occurs notably during the differentiation. Again, by quantifying intensity of unmethylated and methylated bands a high methylation has been obtained in RV #10 in both undifferentiation and differentiation stages (Figure 25C left). On the other hand, a high methylation was only observed in RV #14 under differentiation conditions (Figure 25C right).

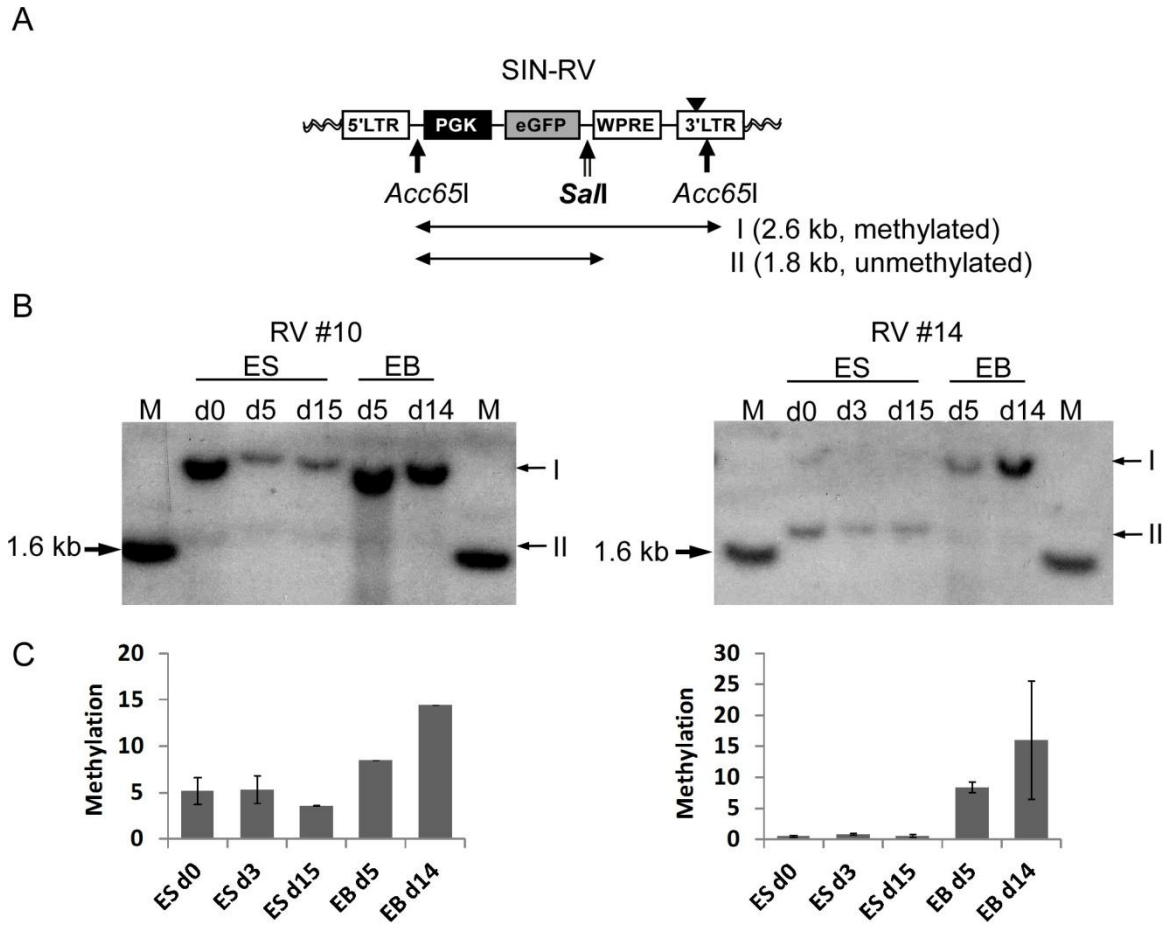

Figure 25: Analysis of the methylation status of SIN-RV ES cell clones using the methylation-sensitive restriction enzyme *Sal*I. Methylation of the eGFP region was analyzed by digesting gDNA with the methylation-sensitive *Sal*I. Methylation of the CpG dinucleotide present in the *Sal*I site results in a 2.6-kb band after *Acc65*I/*Sal*I digestion (band I). Unmethylated DNA yields a 1.8-kb band in the Southern blot (band II). (**B**) Southern blot analysis of single copy ES cell clones SIN-RV #10 and #14 on day 0, 3 and 15 post plating and EBs on day 5 and 14 post differentiation. Radioactive labeled eGFP probe was used to detect digested fragments. (**C**) The methylation status of integrants were quantified by calculating the ratio of methylated (band I) to unmethylated bands (band II) (n=3, Mean±SEM).

Next, the percentages of eGFP positive cells (see also Figures 21B and 22B) were correlated to the coefficients of methylation (see also Figures 24C and 25C) for the four different single integrated ES cell clones (Figure 26) for both undifferentiation and differentiation conditions. An inverse correlation between eGFP-positive cells and methylation coefficient has been observed for both low and high eGFP expressing LV ES cell clones (Figure 26A). The same is true for high eGFP expressing RV ES cell clone #14 (Figure 26B). In contrast, a lower correlation coefficient was obtained for low expressing clone RV #10 (Figure 26B). This may be due to the fact that already a very high methylation level was observed under undifferentiation conditions for RV#10.

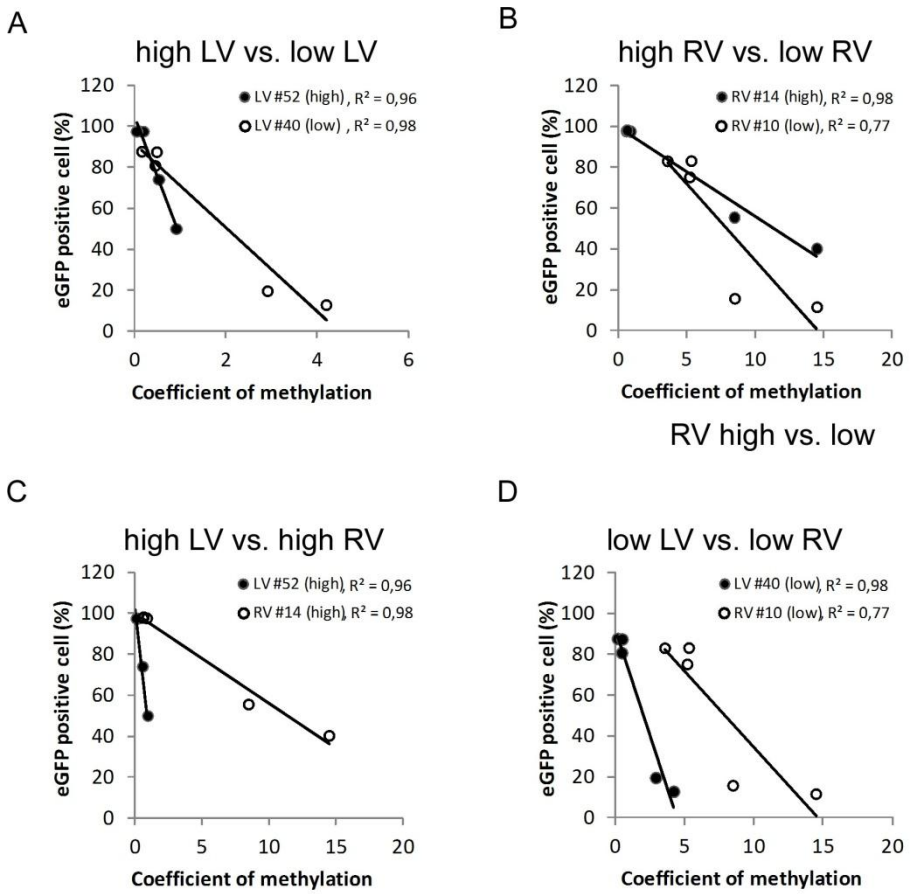

Figure 26: Correlation between percentage of eGFP-positive cells and methylation levels in the eGFP region of SIN-LV and SIN-RV ES cell clones. (**A**) Comparison of SIN-LV ES cell clones: LV #40 (low) vs. LV #52 (high). (**B**) Comparison of SIN-RV ES cell clones: RV #10 (low) vs. RV #14 (high) (**C**) Comparison between high eGFP expressing clones: LV #52 vs. RV #14. (**D**) Comparison of low eGFP expressing clones: LV #40 vs. RV #10. Data were obtained from Figures 21B, 22B, 24C and 25C.

Comparing high eGFP expressing ES cell clone LV #52 with its retroviral counterpart (RV #14) shows a lower slope of the regression curve for RV#14 (Figure 26C) indicating a higher methylation level as already seen on methylation sensitive southern blot (see also Figures 24C and 25C, right). Taking the fluorescence images (Figure 22A) and the mean fluorescence intensities (Figure 23) into account, a clear relation between methylation pattern and transgene silencing can be drawn as e.g. clone LV#52 also showed high eGFP expression under both differentiation and undifferentiation conditions. This indicates that methylation plays an important role on high eGFP expressing clones. In contrast, both low eGFP expressing ES cell clones (LV#40, RV#10) show different levels of methylation on undifferentiation and differentiation stage with no particular difference among the two clones (Figure 26D). Compared to the high expressing clones a generally higher methylation level can be observed (see also Figures 24C and 25C). This is also reflected in the low eGFP expressions (see also Figures 21 and 22).

Taken together, the methylation mechanisms indeed dramatically reduce the transgene expressions.

3.1.2.5. Methylation analysis by bisulfite sequencing

Besides methylation-sensitive Southern blot analysis, bisulfite sequencing represents a versatile approach for a more precise methylation analysis (Hayatsu et al, 1970; Laird, 2003) (2.10). Therefore, DNA is treated with bisulfite, amplified by PCR and the PCR products are subcloned for subsequent sequencing. Bisulfite treatment leads to conversion of unmethylated cytosines of DNAs into uracil whereas methylated cytosines can not be affected (Figure 27). During PCR, uracils are amplified as thymidines and the unaffected methylated cytosines are amplified again as cytosines (Figure 27). After subcloning of the amplified fragments in plasmids, the DNA is sequenced and compared to the sequence of untreated DNA. The methylation levels can then be evaluated by the percentage of methylated cytosines as compared to unmethylated ones.

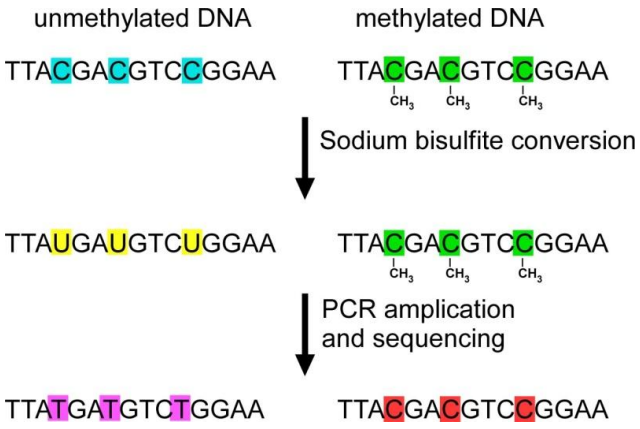

Figure 27: Schematic illustration of bisulfite sequencing. DNA is treated with sodium bisulfite solution resulting in conversion of unmethylated cytosine to uracil whereas 5-Methyl-cytsoine is not affected. PCR amplication results finally in replacement of uracil to thymidine and replacement of methylated cytosine to cytosine.

The four single copy LV and RV ES cell clones (LV #40, LV #52, RV #10, RV #14) were cultured under differentiation conditions for 14 days and the EBs on day 14 post differentiation were used for gDNA isolation. After bisulfite conversions of gDNAs, the eGFP region (255 bp) containing 18 CpG islands was amplified by using non-methylation primers (Laird, 2003) and the resulting PCR products were subcloned into plasmids. Figure 28 shows the sequencing results of several plasmids that were finally obtained from the ES cell clones with one row representing the 18 CpG islands, respectively. Several rows for

one clone show different plasmids that were used for sequencing. Closed circles indicate methylated CpGs, open circles indicate unmethylated CpGs. Except for one plasmid of low expressing clone LV #40, all plasmids demonstrated almost complete methylation of CpG islands with an average CpG methylation of 78.89±16.51% (n=6, Mean±SEM). In contrast, only the half of CpG islands in high expressing LV #52 was methylated (53.33±17.18%, n=4, Mean±SEM). Interestingly, both low and high expressing RV ES cell clones (#10 and #14) showed a high methylation level of CpG islands (94.44±2.97% and 88.89±3.93%, n=7 and n=4, Mean±SEM, respectively) indicating an almost complete methylation under differentiation conditions for both clones.

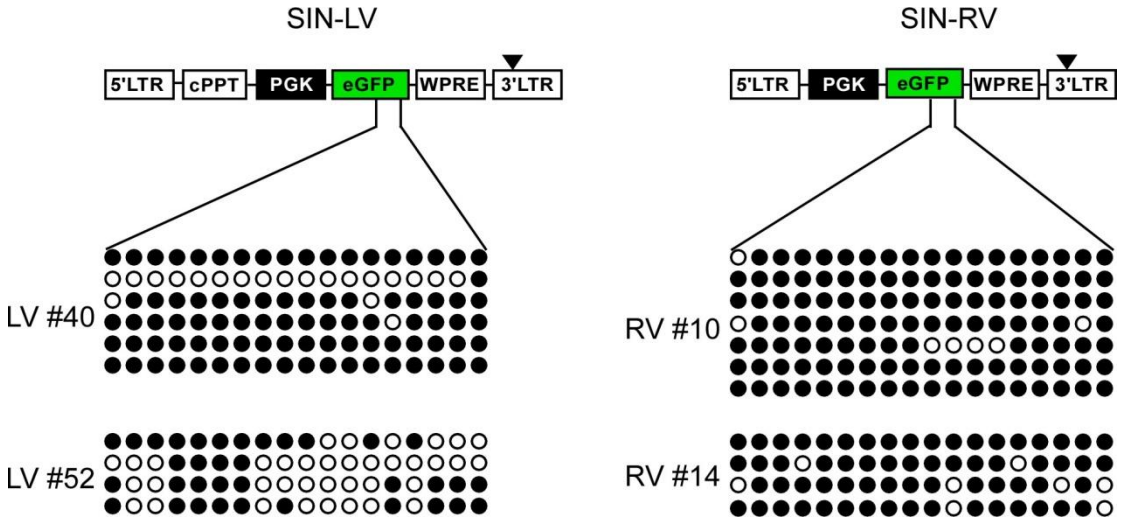

Figure 28: High-resolution DNA methylation analysis of CpG dinucleotides of the eGFP gene by bisulfite sequencing. Methylation pattern of a 255-bp region (18 CpG islands) in the eGFP region of SIN-LV clones (left) and SIN-RV clones (right). Low expressing LV #40 exhibits 78.89±16.51% (n=6) CpG methylations. High expressing LV #52 53.33±17.18%, (n=4) CpG methylations. Low expressing RV #10 exhibits 94.44±2.97% (n=7) CpG methylations. High expressing RV #52 exhibits 88.89±3.93% (n=4) CpG methylations. Closed circles indicate methylated CpGs, open circles indicate unmethylated CpGs. (Mean±SEM). (2.10)

The bisulfite sequencing results of the four clones corresponds nicely with the results obtained from the methylation-sensitive Southern blot analyses of differentiated clones (see also Figures 24C and 25C): these quantifications revealed a methylation ratio of:4:1 (=80% methylation) for low eGFP expressing LV #40, 1:1 (=50% methylation) for high eGFP expressing LV #52, 15:1 (=93% methylation) for low eGFP expressing RV #10) and 15:1 (=93% methylation) for high eGFP expressing RV #14.

3.1.3 SIN-LV and SIN-RV transgenic mouse

Finally, transgenic mice were generated to study the eGFP expression *in vivo* when using SIN-LVs or SIN-RVs. For transgenesis, the subzonal virus injection method was used in which the viral vectors are directly injected in the perivitelline space of fertilized oocytes (Figure 29) (Fassler, 2004; Hofmann et al, 2004). The injected zygotes were further incubated until reaching morulae or blastocyst state and were then transferred into pseudopregnant foster mice.

Around 10 to 20 embryos were used for transfer into the uterus of one foster animal. Around two weeks after the uterine transfer pups were born. These pups were further screened by using genotyping PCR and Western blot analysis for detection of viral integration and eGFP expression, respectively.

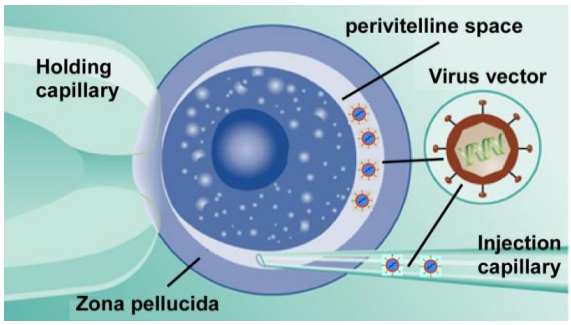

Figure 29: Subzonal virus injection of embryos. The schematic illustration was modified from Faessler, 2004 (Fassler, 2004) and shows the procedure of generation of transgenic mice by injection of viral vectors into the perivitelline space through the zona pellucida of a fertilized oocyte.

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gDNA for genotyping PCR was isolated from pup's tail and specific eGFP primers were chosen to amplify the eGFP region of integrated proviruses. PKG-1 primers were used as loading control of gDNA (Figure 30A) (2.6.1). In order to check whether these integrated proviruses also express eGFP protein, Western blot analysis of proteins, isolated from pup's tail, was used by applying eGFP antibody and β-actin antibody as loading control, respectively (Figure 30B).

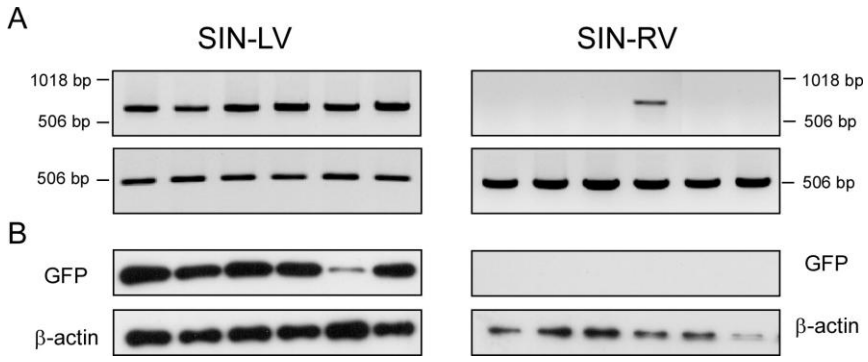

Figure 30: Analysis of newborn mice generated by subzonal virus injection of embryos by genotyping PCR and Western blot. The tail end of pups resulting from subzonal injection of SIN-LVs or SIN-RVs was used for gDNA and protein isolation and further analyzed using PCR or Western blot, respectively. (**A**) PCR using GFP primers was applied to detect cDNA of GFP from integrated provirus in SIN-LV/RV transgenic mice leading to a 716 bp band. AP18 and KISAX primers were used as PCR loading control targeting the housekeeping gene Protein Kinase G 1 (PKG-1) leading to a 529 bp band. (**B**) Western blot analysis using GFP antibody resulted in a 55 kD band and β-actin antibody was used as loading control resulting in a 42 kD band. Gels and blots are representative examples; at least PCR analysis was performed for all pups, Western blot analysis was done for proteins of all transgenic pups.

Performing two subzonal SIN-LVs injections thirteen pups were born from eight foster animals (Table 2). Six out of the thirteen pups were transgenic as shown by PCR analysis (Figure 30A left, only transgenic pups are shown, Table 2). All of these six SIN-LV transgenic mice showed eGFP expression as analyzed using Western blot (Figure 30B left, Table 2). In contrast, from nine subzonal SIN-RVs injections only two out of fifty three pups born were transgenic as proven by PCR analysis (Figure 30A right, Table 2). Interestingly, both transgenic mice showed no eGFP expression (see also Figure 30B right; one of the two transgenic mice is shown).

Table 2: Summary of transgenic mice generated by subzonal injections.

Virus	numbers of SI's	number of fosters	number of injected embryos	sum pups	transgenic pups (PCR)	GFP positive pups (WB)
SIN-LV	2	8	71	13	6	6
SIN-RV	9	33	438	53	2	0

Taken together, the efficiency in generation of transgenic mice by applying subzonal injection is much higher for SIN-LVs as compared to SIN-RVs (Figure 31). In addition, all of the transgenic mice resulting from SIN-LV injection revealed eGFP expression. In contrast, SIN-RV injection showed not only low efficiency in generating transgenic pups but also resulted in no eGFP expressing mouse (Figure 31).

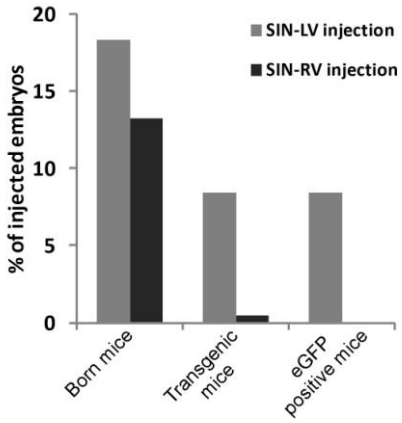

Figure 31: Statistic analysis of SIN-LV and SIN-RV gene transfer in mice. Data were used from Table 2.

Another part of the present work beside the analysis of the epigenetic regulation of SIN-RVs and SIN-LVs in ES cells was the application of SIN-LVs for efficient transgene expression for an approach in regenerative medicine.

3.2. LVs-mediated TK expression in pluripotent stem cells for regenerative medicine

In the second part of this work, the focus relied on the specific elimination of pluripotent stem cells. Cell populations that were differentiated from pluripotent stem cells carry a potential risk of tumor formation due to the contamination with residual undifferentiated cells. Therefore, the HSV-TK/GCV system was applied on pluripotent stem cells in order to remove potential tumor-forming cells. The system relies on expression of the suicide gene thymidine kinase (TK, from Herpes simplex virus) that converts the prodrug Ganciclovir (GCV) into a toxic metabolite leading to cell death. For the genetic modification of cells, SIN-LVs were chosen as a gene transfer tool to mediate TK expression. For this purpose the murine ES cell line α-pig was used which carries the genes for puromycin resistance and green fluorescence protein (eGFP) connected via the IRES (internal ribosomal entry site) element under control of the cardiac specific α-myosin heavy chain (α-MHC) promoter (Figure 32). This leads to transgene expression only in ES cell-derived cardiomyocytes (Kolossov et al, 2005). Within the context of *in vitro* ES cell transduction two different approaches were applied: lentiviral transduction of ES cells and GCV treatment of these mixed cell populations without further selection or picking of ES cell clones that were used for further treatment and analysis.

Figure 32: Schematic drawing of transgene cassette of ES cell line used (α-pig ES cells). Murine ES cells (D3 cell line) carry the expression cassette for puromycin resistance connected with eGFP gene via an IRES (internal ribosomal entry site) element driven by mouse cardiac specific α-myosin heavy chain (α-MHC) promoter..

3.2.1. LVs-mediated TK expression in ES cells

In order to generate ES cells stably expressing TK, LVs carrying HSV-TK cDNA under the control of the promoter of pluripotency genes Nanog or Oct-3/4 (NT and OT) (Figure 33A) were applied leading to TK expression only in undifferentiated ES cells (Chambers et al, 2003; Mitsui et al, 2003; Niwa et al, 2000; Ovitt & Scholer, 1998). Treatment of the transduced cells with GCV for 72 hours had a high killing effect on the ES cells under undifferentiation conditions (Figure 33B). Using different concentrations of GCV in preliminary experiments revealed that treatment with 20 µM GCV was sufficient to eliminate undifferentiated cells in mixed ES cell populations. Moreover, higher GCV concentrations led to cellular toxicity to untransduced ES cells (Figure 33B). Therefore, 20 µM GCV was used in all further *in vitro* experiments.

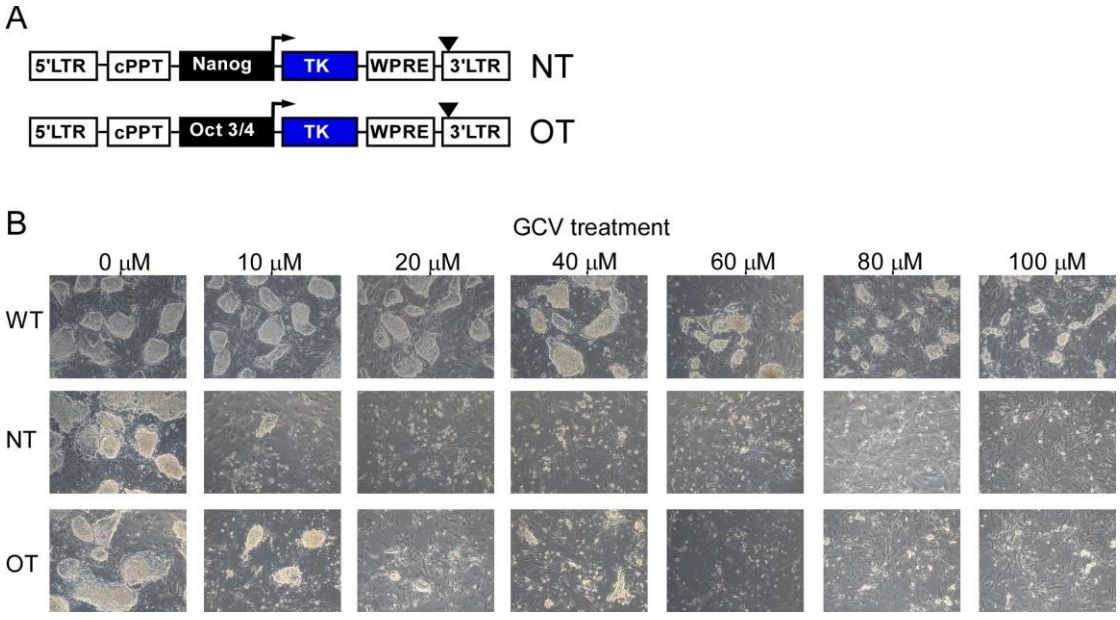

Figure 33: Constructs of SIN-LVs carrying TK expression cassette and analysis of NT- or OT-transduced ES cells *in vitro*. (**A**) Constructs of LVs carrying thymidine kinase gene from herpes simplex virus driven by pluripotent promoter Nanog (NT) or Oct-3/4 (OT). (**B**) ES cells were transduced with LVs (NT and OT, 200 ng of reverse transcriptase) or not transduced (WT) and further treated with 0, 10, 20, 40, 60, 80 and 100 µM GCV for 72 hours. Representative brightfield images are shown (n=3).

Next, the effect of GCV in LV-transduced ES cells under differentiation conditions was investigated. For differentiation, untransduced (WT) and NT- or OT-transduced ES cells were cultured as embryoid bodies (EBs) by using the mass culture protocol (Doetschman et al, 1985) for 14 days. If EBs contained ES cell-derived cardiomyocytes, eGFP expressions driven by cardiac specific promoter α-MHC were detectable using fluorescence microscopy (Figure 34A). Fluorescence images of untransduced and NT- or OT-transduced EBs (with or without 20µM GCV treatment) revealed eGFP expression, respectively indicating formations of ES cell-derived cardiomyocytes in EBs (Figure 34B).

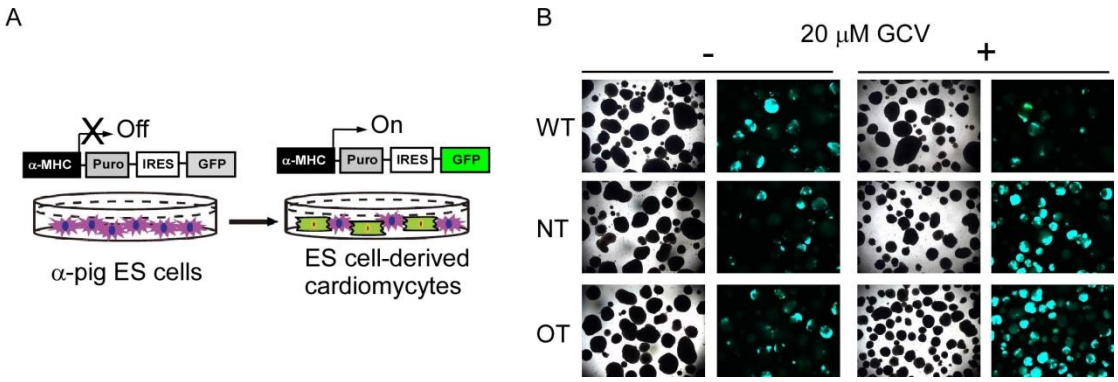

Figure 34: Differentiation of untransduced (WT) and NT- or OT-transduced ES cells. (**A**) Schematic representation of the strategy to detect the formation of cardiomyocytes by eGFP expression (modified form Ieda et al, 2010). (**B**) Brightfield and fluorescence images of EBs derived from untransduced (WT) and NT- or OT-transduced ES cells on day 14 post differentiation. eGFP expression driven by cardiac specific promoter α-MHC indicates formation of ES cell-derived cardiomyocytes.

The resulting EBs were further dissociated and characterized by immunostaining with antibodies against Oct-3/4 (ES cell marker) and Hoechst (nucleus staining) (Figure 35A). By counting of Oct-3/4-positive cells without GCV treatment, 21.76%±1.27, 21.14%±2.39 and 22.12%±2.07 (Mean±SEM) Oct-3/4-positive cells were observed in the untransduced and NT- or OT-transduced ES cells, respectively, indicating that these cells remained undifferentiated and maintained their pluripotent status (Figure 35B). In the untransduced ES cells with GCV treatment, 20.95%±1.96 Oct-3/4-positive cells were observed indicating that GCV alone had no influence on the vitality of the cells (Figure 35B). In contrast, only 4.68%±1.36 and 3.75%±2.24 (Mean±SEM) of the NT- or OT-transduced ES cells were quantified as Oct-3/4-positive when treated with GCV (Figure 35B). Thus, most of the undifferentiated ES cells were eliminated during differentiation by TK mediated GCV conversion leading to cell death. Neither the LV transduction nor the GCV treatment alone influenced differentiation of ES cells (Figure 35B).

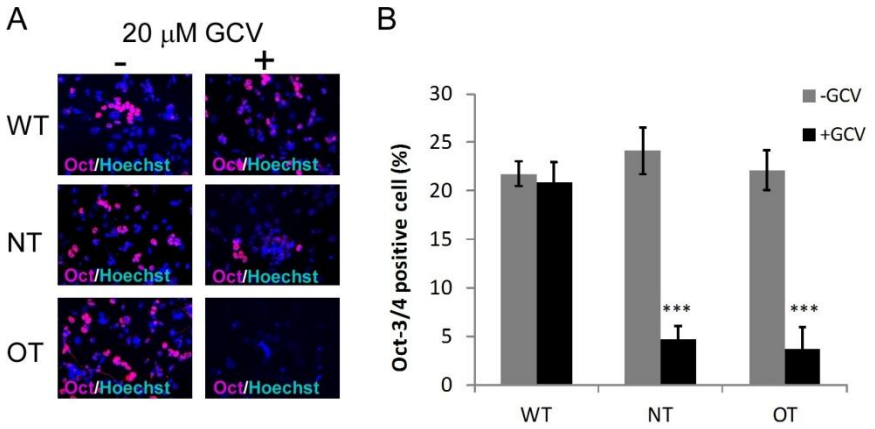

Figure 35: Analysis of dissociated EBs derived from untransduced (WT) and NT- or OT-transduced ES cells using immunochemistry. (**A**) NT- or OT-transduced ES cells and untransduced (WT) ES cells (+/- GCV) were differentiated and immunostaining of dissociated EBs on day 14 with Oct-3/4 (red) and Hoechst (blue) was performed indicating undifferentiated ES cells and nucleus, respectively. Representative images are shown (n=3). (**B**) Percentage of Oct-3/4-positive cells on day 14 post differentiation analyzed by cell counting of images representatively shown in (A) (n=5, Mean±SEM, ***P<0.001, ANOVA, analysis of variance).

Immunostaining of the dissociated EBs using antibody against α-actinin (sarcomere marker) showed that untransduced (WT) as well as the transduced ES cells (NT and OT) were able to differentiate into ES cell-derived cardiomyocytes as also shown by cardiac-specific α-MHC promoter driven eGFP expression (Figure 36).

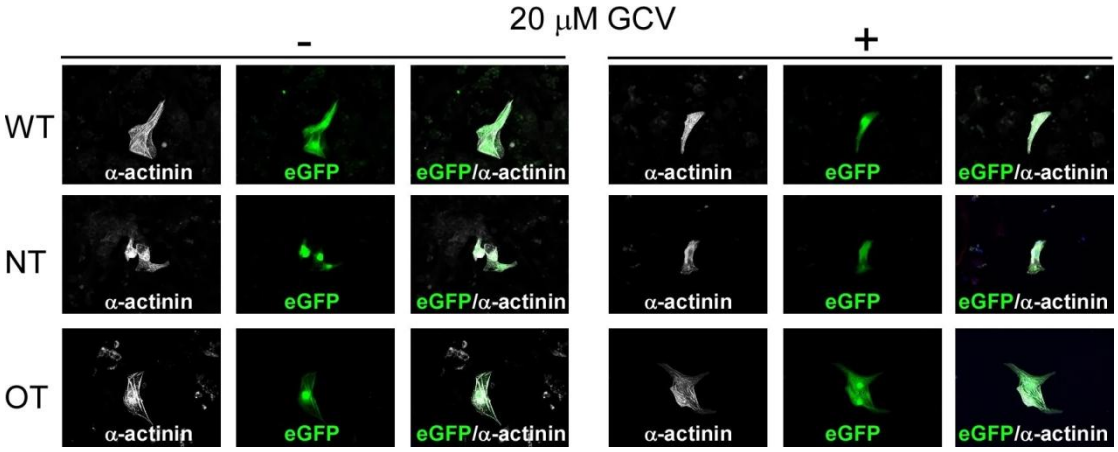

Figure 36: Immunostaining of dissociated EBs derived from untransduced (WT) and NT- or OT-transduced ES cells. Immunostaining of dissociated EBs on day 14 post differentiation with antibody against skeletal α-actinin (sarcomere) indicating the formation of ES cell-derived cardiomyocytes (white); green fluorescence indicates eGFP expression driven by cardiac specific promoter, α-MHC.

3.2.2. LVs-mediated TK expression in induced pluripotent stem (iPS) cells

Like ES cells, iPS cells also carry the potential risk of tumor formation in *in vivo* applications. Thus, removal of iPS cells that did not undergo differentiation is an important task which could bring iPS cells closer to clinical applications. The LVs NT and OT

(Figure 33A) were applied on two different iPS cell lines: iPS and iPS-Oct-GFP, the latter containing an eGFP expression cassette under the control of the pluripotent Oct-3/4 promoter. Upon GCV administration, almost no survival of NT- or OT-transduced iPS-cells could be observed under undifferentiation conditions (Figure 37A).

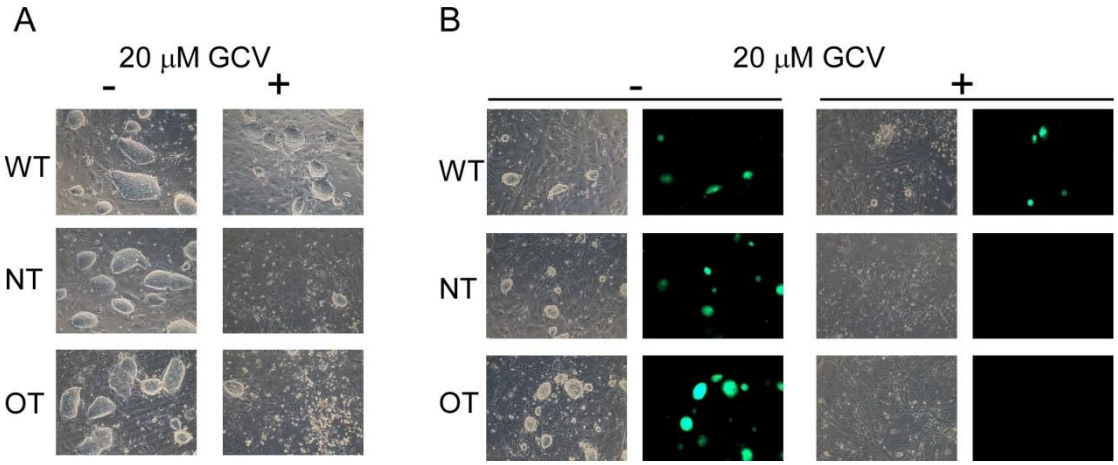

Figure 37: Analysis of NT- or OT-transduced iPS cells *in vitro*. (**A**) iPS cells were transduced with LVs (NT and OT, 300 ng of reverse transcriptase) or not transduced (WT) and further treated without/with (-/+) 20 µM GCV under undifferentiation conditions. Representative brightfield images are shown (n=3). (**B**) iPS-Oct-GFP cells were transduced by NT and OT (300 ng of reverse transcriptase) or not transduced (WT) and treated without/with (-/+) 20 µM GCV under undifferentiation conditions. Representative brightfield and fluorescence images are shown (n=3).

Brightfield and fluorescence images of iPS-Oct-GFP cells also revealed a high killing effect of the TK/GCV system on these cells as no eGFP-positive cells survived in the NT- or OT-transduced cells upon GCV treatment (Figure 37B). Taken together, besides murine ES cells, the TK/GCV system also led to an efficient elimination of undifferentiated iPS cells upon GCV treatment *in vitro*. Further experiments using iPS cells were not performed within the present study.

3.2.3. LVs-mediated TK expression in ES cells with low copy numbers

3.2.3.1 NT- or OT-transduced mixed ES cell populations

The initial experiments clearly demonstrated the usability of the TK/GCV system in pluripotent stem cells. In these experiments LVs with a certain physical titer (displayed as ng reverse transcriptase per µl) were used. However, for clinical applications, it is worthwhile to use low copy numbers of LVs to minimize the potential risk of insertional mutagenesis by LV integrations (Zielske et al, 2004). Therefore, ES cell populations with a low average copy number of approximately 1.5 provirus integrations per genome were

investigated. Recent reports showed that quantitative real time PCR (qPCR) can be used as a reliable method to determine the copy number of transgenic mice (Pfeifer et al, 2010). Hence, ES cell populations by transducing ES cells with different LV concentrations were generated and analyzed using qPCR (Figure 38A). These LV-transduced ES cell populations without additional selections are further referred to as mixed ES cell populations. qPCR data were validated by comparison with samples of known number of integrants (one and two LV-integrants) that have been proven by Southern Blot analysis (Figure 38B). ES cell populations with an average copy number of 1.5 provirus integrations per genome were chosen for further analysis (1.60±0.09 (NT) and 1.38±0.24 (OT) (Mean±SEM)) (Figure 38C).

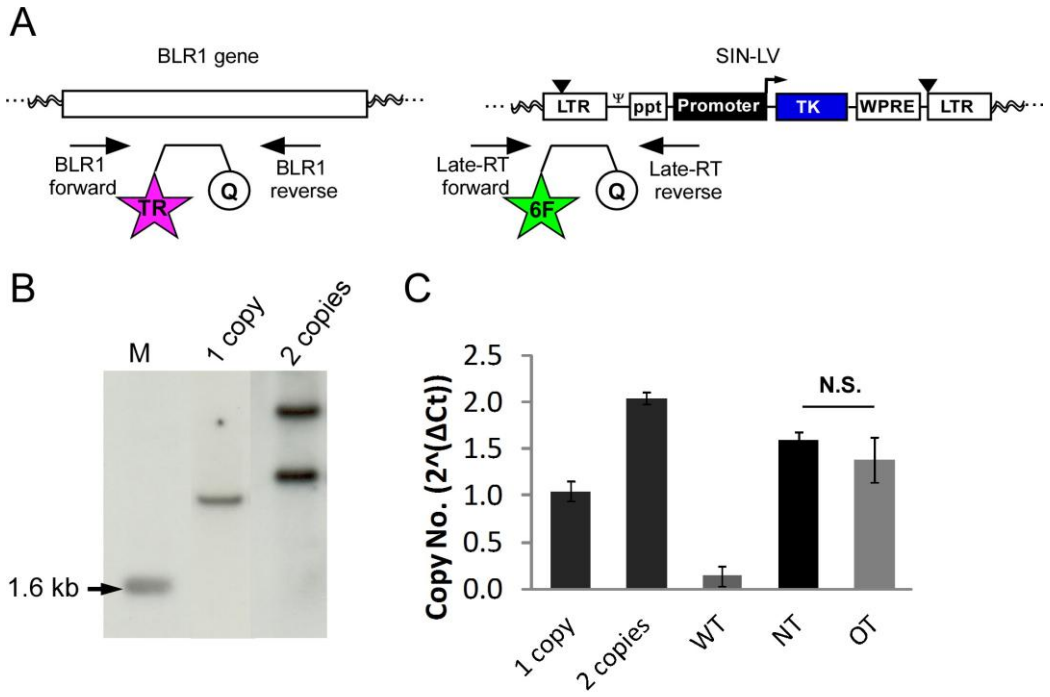

Figure 38: Copy number analysis of NT- or OT-transduced ES cells (mixed population). (**A**) Schematic illustration of qPCR-primers and probes for detection of house-keeping gene Burkitt lymphoma receptor 1 (BLR1) and long terminal repeat (LTR) of integrated LVs for determination of number of provirus integrations in ES cells. Copy number of LV per genome is determined by the means of the cycle threshold (Ct) values by using following formulas: copy number=$2^{\Delta Ct}$ with ΔCt=Ct(BLR1)-Ct(Late-RT). (**B**) Southern blot analysis of LV-mediated transgenic mice carrying one or two integrants. The gDNA of these two mice were used as control in qPCR analysis. (**C**) ES cells were not transduced (WT) or transduced with different amount of NT or OT. Mixed ES cell populations were tested using qPCR. Shown are the results of mixed ES cell populations carrying 1.5 provirus integrants per genome in average (n=3, Mean±SEM, N.S., not significant difference, ANOVA, analysis of variance).

GCV treatment of these undifferentiated mixed ES cell populations strongly inhibited cell survival as shown on bright field images (Figure 39A).

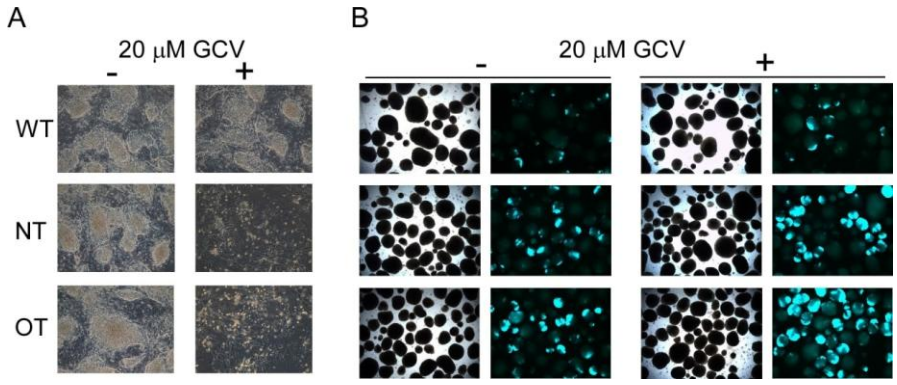

Figure 39: Analysis of NT- or OT-transduced ES cells (mixed population) with an average copy number of 1.5 per genome. (**A**) Brightfield images of mixed ES cell populations without (WT) or with (NT, OT) LV transduction with (+) or without (-) 20µM GCV treatment under undifferentiation conditions. Representative brightfield images are shown (n=3). (**B**) Brightfield and fluorescence images of EBs derived from untransduced (WT) and NT- or OT-transduced ES cells (mixed populations) carrying 1.5 copy numbers per genome in average on day 14 post differentiation with (+) or without (-) 20µM GCV. eGFP expression driven by cardiac specific promoter α-MHC indicates formation of ES cell-derived cardiomyocytes.

Fluorescence images of dissociated EBs after differentiation demonstrated that untransduced and NT- or OT-transduced EBs (with or without GCV) expressed eGFP, indicating a normal formation of ES cell-derived cardiomyocytes and no influence of GCV treatment (Figure 39B).

Next, immunostaining of differentiated cells was performed using antibody against Oct-3/4 and Hoechst (Figure 40A).

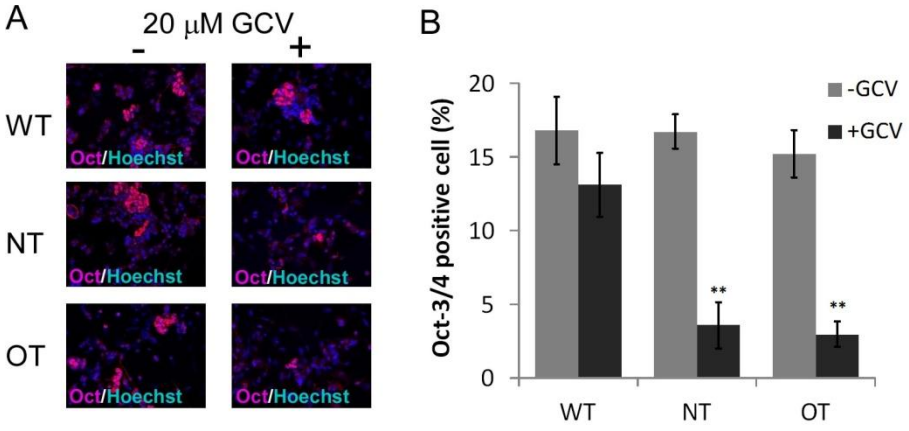

Figure 40: Analysis of dissociated EBs derived from untransduced (WT) and NT- or OT-transduced ES cells with 1.5 copy numbers per genome in average using immunochemistry. (**A**) Untransduced (WT) or NT- or OT-transduced ES cells were differentiated (+/- 20 µM GCV) and immunostaining of dissociated EBs on day 14 with Oct-3/4 (red) and Hoechst (blue) was performed indicating undifferentiated ES cells and nucleus, respectively. Representative fluorescence images are shown (n=3). (**B**) Percentage of Oct-3/4-positive cells of dissociated EBs on day 14 post differentiation with (+) and without (-) GCV treatment as analyzed by cell counting of images representatively shown in (A) (n=5, Mean±SEM, **P<0.001, ANOVA, analysis of variance).

Images were taken using fluorescence microscopy (Figure 40A) and Oct-3/4-positive cells were counted (Figure 40B). Importantly, GCV treatment of differentiated NT- or OT-transduced ES cells significantly reduced the survival of Oct-3/4-positive cells as compared to untransduced cells (3.58%±1.58 (NT) and 2.97%±0.88 (OT) vs. 13.12%±2.20 (WT)) (Figure 40B).

Next, dissociated EBs were again analyzed by using immunostaining against α-actinin and by fluorescence detection of cardiac specific promoter driven eGFP expression (Figure 41). Again, formations of ES cell-derived cardiomyocytes suggested that the TK/GCV system allows normal ES cell differentiation of NT- or OT-transduced ES cells carrying 1.5 copy numbers in average per genome and has no influence on the cell development.

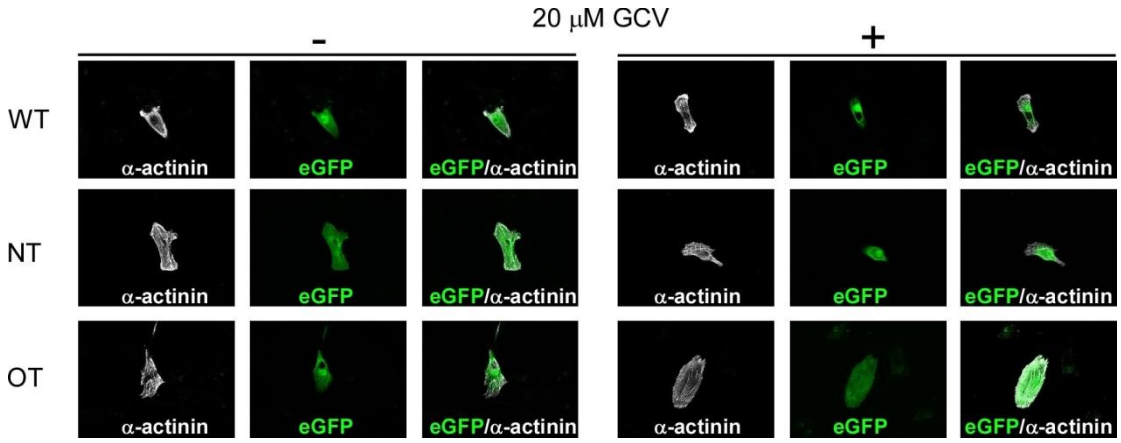

Figure 41: Immunostaining of dissociated EBs derived from untransduced (WT) and NT- or OT-transduced ES cells with 1.5 copy numbers per genome in average. Dissociated EBs were stained with antibody against skeletal α-actinin (sarcomere) indicating the successful formation of ES cell-derived cardiomyocytes (white); green fluorescence indicates eGFP expression driven by cardiac specific promoter, α-MHC.

Taken together, LV-transduced mixed ES cell populations with low copy numbers revealed a significant killing efficiency upon GCV treatment. Nevertheless, the mixed populations still contained around 3 to 4% Oct-3/4-positive cells on day 14 post differentiation (see also Figure 40B) suggesting a potential risk of teratoma formation *in vivo* (Gropp et al, 2012). This presence of Oct-3/4-positive cells could result from an insufficient TK expression level of OT- or NT-transduced ES cells or due to remaining untransduced ES cells. Thus, both approaches were investigated in detail to improve the efficiency to eliminate undifferentiated cells.

3.2.3.2. Use of EOS promoters to drive TK expression

In order to achieve higher levels of TK expression, the promoter of pluripotency genes EOS-C3 and EOS-S4 were applied that have been shown be stronger than Nanog or Oct in human and murine ES and iPS cells (Hotta et al, 2009). These two promoters were cloned into the LV backbone leading to LV-constructs EOS-C3-TK (CT) and EOS-S4-TK (ST) (Figure 42A). The four LVs (NT, OT, CT, ST) were then used in parallel for transduction of ES cells and LV integrations were each determined using qPCR. ES cell populations with an average copy number of approximately 1.5 per genome were further investigated (Figure 42B).

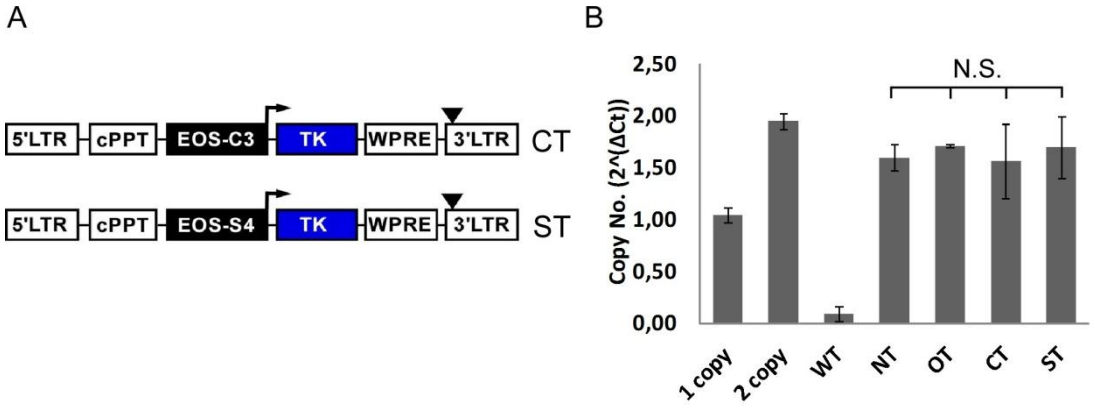

Figure 42: Use of different promoters of pluripotency genes EOS-C3 and EOS-S4 in LVs to drive TK expression. (**A**) Constructs of LVs carrying TK cDNA driven by promoters EOS-C3 or EOS-S4 led to LVs EOS-C3-TK (CT) or EOS-S4-TK (ST). (**B**) Copy number of NT-, OT-, CT- and ST-transduced ES cells as analyzed by qPCR (n=3, Mean±SEM, N.S., not significant, ANOVA, analysis of variance). As control, DNA of two transgenic mice was used that were previously analyzed by Southern Blot to have one or two LV integrants.

Next, the mixed ES cell populations carrying 1.5 copy numbers per genome in average were analyzed concerning their TK expression on mRNA level (Figure 43A) and protein level (Figure 43B) that was detected using qPCR and Western Blot analysis, respectively. These experiments revealed that both promoters of pluripotency genes, EOS-C3 and EOS-S4, gave rise to more mRNA and subsequently even more TK protein compared to Nanog and Oct promoters in murine ES cells (Figure 43A, B). Using EOS-S4 promoter to drive TK expression, the highest TK abundance was obtained (Figure 43A, B).

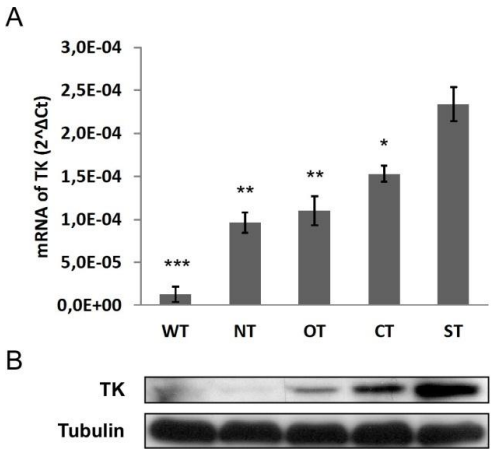

Figure 43: Analysis of TK expression of NT-, OT-, CT- and ST-transduced ES cells carrying 1.5 copy numbers per genome in average. (**A**) TK expression on mRNA level of NT-, OT-, CT- and ST-transduced ES cells as analyzed by qPCR and normalized to GAPDH (n=3, Mean±SEM, *P<0.05, **P<0, ANOVA). (**B**) Western Blot of NT-, OT-, CT- and ST-transduced ES cells with antibody against TK. As loading control Tubulin was used.

The four mixed ES cell populations were then analyzed concerning the efficiency of the TK/GCV system under undifferentiation conditions. Brightfield images (Figure 44A) showed no obvious difference in the killing efficiency. In addition, a Lactate Dehydrogenase (LDH) assay was applied.

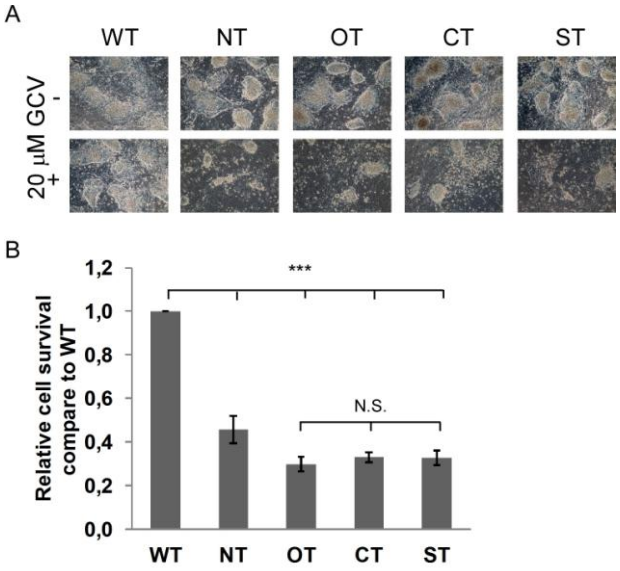

Figure 44: Analysis of ES cells transduced with LVs using different promoters of pluripotency genes. (**A**) Brightfield images of NT-, OT-, CT- and ST-transduced (1.5 copies per genome in average) and not transduced (WT) ES cells after 72 hours treatment with 20µM GCV under undifferentiation conditions are shown. (Representative images of n=3). (**B**) NT, OT, CT, ST transduced (1.5 copies per genome in average) or not transduced (WT) ES cells were treated for 72 hours with (+) or without (-) 20µM GCV. Cell culture supernatants were either directly analyzed using LDH assay or LDH was measured after complete lysis of cells of each set of samples. The differences of extinction with and without lysis were determined representing the number of ES cells that survived GCV treatment. Feeder cell derived LDH release was each substracted. Relative survivals of GCV treated NT, OT, ST and CT cells as compared to WT are shown. (n=3, Mean±SEM, ***P<0.001, compared to WT, N.S., not significant, ANOVA, analysis of variance).

The assay measures LDH that is released by dead cells into the cell culture supernatant. Interestingly, a higher TK expression level did only slightly increase the effect of GCV on ES cells (Figure 44B) demonstrating that the portion of surviving cells is rather the consequence of incomplete transduction of the ES cell population than of low TK expression.

3.2.3.3. LVs-mediated TK expression in single copy ES cell clones

The application of the TK/GCV system in mixed ES cell populations with 1.5 copy numbers per genome in average resulted in a survival of around 3-4% Oct-3/4-positive cells after GCV treatment of NT- or OT-transduced cells (see also Figure 40B). Furthermore, an increase of TK expression (as shown on mRNA or protein level) had no additional effect on the survival of undifferentiated cells after GCV treatment (Figure 44B).

However, a complete elimination of undifferentiated cells should be reached, since already a few pluripotent cells could lead to formation of teratomas (Gropp et al, 2012). Transducing ES cells with LVs carrying the TK cassette without further selection (mixed ES cell populations) would always leave a certain percentage of cells remaining untransduced. These cells will not be eliminated by GCV treatment. Therefore, NT or OT expressing ES cell lines derived from picked clones were generated and ES cell clones carrying only one integrant per genome were chosen for further analysis to improve the efficiency of the TK/GCV system. LV integrations of those ES cell lines were proven by Southern blot analysis and two different single-integrant clones of both NT- or OT-transduced ES cells were used for further investigations (NT #8, NT #11, OT #4, OT #11) (Figure 45A, B).

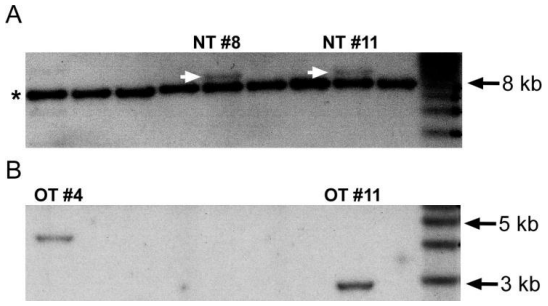

Figure 45: Southern blot analysis of NT- or OT-transduced ES cell clones. ES cells were transduced with NT (A) or OT (B) and picked clones were analyzed by Southern Blot. Marked are the results of the single LV integrant ES cell clones. NT transduced ES cell clones are highlighted by white arrows. Unspecific bands are indicated by asterisk (*). (**A**) Single copy NT-transduced ES cell clones: NT #8 and NT #11 (white arrows). (**B**) Single copy OT-transduced ES cell clones: OT #4 and OT #11.

Next, the four ES cell clones were cultured under undifferentiation and differentiation conditions with or without GCV treatment. This showed that the cells of all four clones were completely eliminated upon GCV treatment under undifferentiation conditions (Figure 46A). Under differentiation conditions, untransduced and NT- or OT-transduced EBs expressed eGFP indicating the successful formation of ES cell-derived cardiomyocytes (Figure 46B).

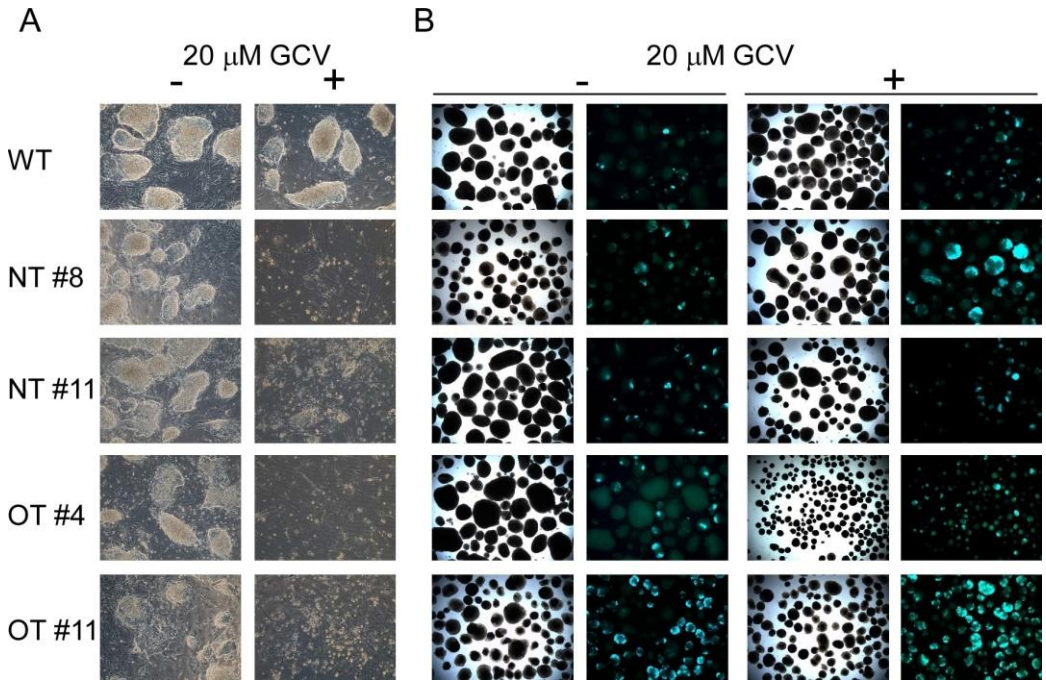

Figure 46: Analysis of single copy NT- or OT-transduced ES cell clones *in vitro*. (**A**) ES cells were transduced with NT or OT or not transduced (WT) and single ES cell clones carrying each one integrant were further cultured upon treatment with (+) or without (-) 20 µM GCV. Representative brightfield images of n=3 are shown. (**B**) ES cell lines from (A) were cultured under differentiation conditions for 14 days. Brightfield and fluorescence images of EBs derived from untransduced (WT) and NT or OT single copy ES cell clones (NT #8, NT #11, OT#4 and OT #11) on day 14 post differentiation are shown. eGFP expression driven by cardiac specific promoter α-MHC indicates formation of ES cell-derived cardiomyocytes.

The EBs derived from cultivation under differentiation condition were further dissociated and immunostained using antibodies againstOct-3/4 and Hoechst (Figure 47A).

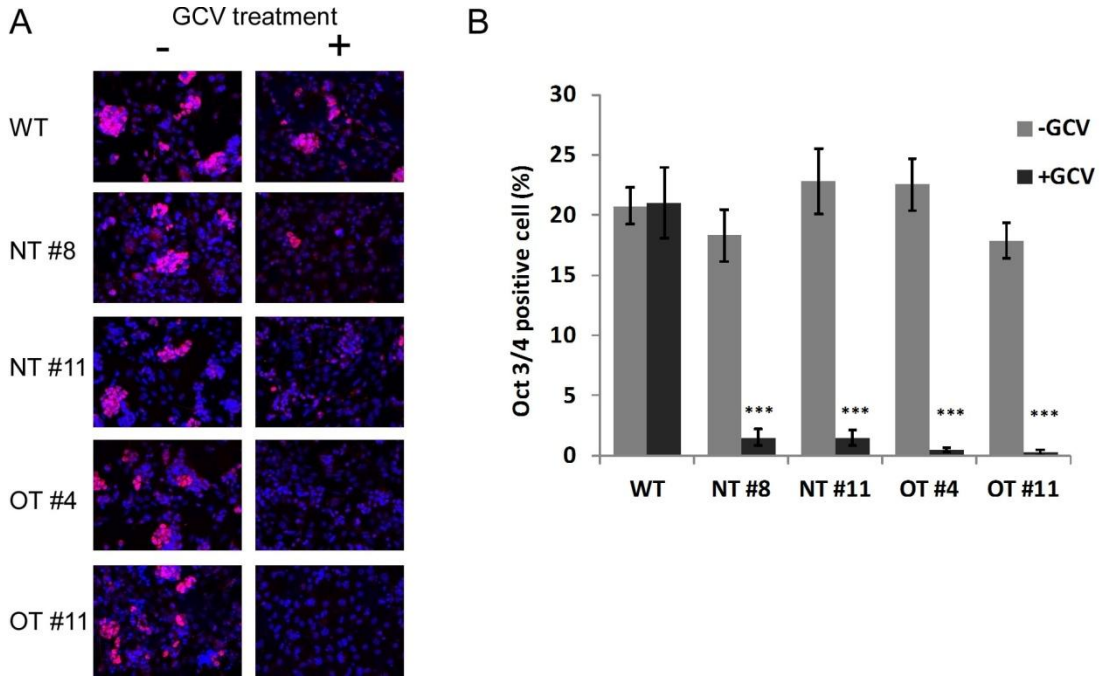

Figure 47: Analysis of dissociated EBs derived from untransduced (WT) and single copy NT- or OT-transduced ES cell clones using immunostaining. (**A**) Untransduced or single copy NT or OT ES cell clones (+/- 20 µM GCV) were differentiated and immunostaining of dissociated EBs on day 14 with Oct-3/4 (red) and Hoechst (blue) was performed indicating undifferentiated ES cells and nucleus, respectively. Representative images are shown (n=3). (**B**) Percentage of Oct-3/4-positive cells of dissociated EBs on day 14 post differentiation with (+) and without (-) GCV treatment as analyzed by cell counting of images representatively shown in (A) (n=3, Mean±SEM, ***P<0.001, ANOVA, analysis of variance).

Importantly, the four ES cell clones revealed only 1.51%±0.72 (NT #8), 1.51%±0.64 (NT #11), 0.45%±0.16 (OT #4) and 0.26%±0.26 (OT #11) (Mean±SEM) surviving Oct-3/4-positive cells present on day 14 post differentiation when treated with GCV (Figure 47B).

Again, the differentiation of the ES cells of all four clones into ES cell-derived cardiomyocytes was not influenced by the LV transduction or the GCV treatment as shown by immunostaining against skeletal α-actinin and fluorescence detection of cardiac specific promoter driven eGFP expression (Figure 48).

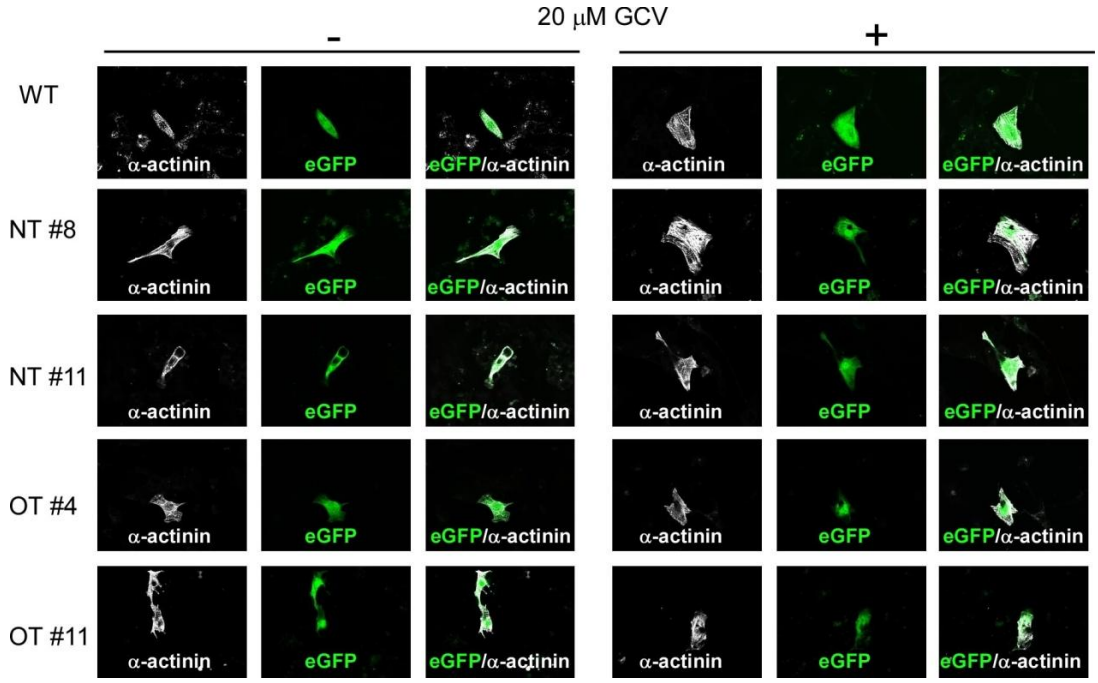

Figure 48: Immunostaining of dissociated EBs derived from untransduced (WT) or single copy NT- or OT-transduced ES cell clones. Dissociated EBs on day 14 post differentiation were stained with antibody against skeletal α-actinin (sarcomere) indicating the successful formation of ES cell-derived cardiomyocytes (white); green fluorescence indicates eGFP expression driven by cardiac specific promoter, α-MHC.

3.2.3.4. LV-mediated TK expression in mixed ES cell population using Hygromycin pre-selection

As compared to mixed ES cell populations (see also Figure 40B), the use of single integrant NT- and OT-transduced ES cell clones led to a more efficient elimination of undifferentiated cells after GCV treatment (see also figure 47B). However, these two approaches can not abolish undifferentiated cells completely and are not efficient enough for future clinical applications. Importantly, all ES cells for clinical use should be transduced by LVs containing TK cDNA to enable a complete elimination of undifferentiated cells upon GCV treatment. Therefore, for excluding the untransduced ES cells from mixed populations an antibiotic pre-selection scheme was chosen: A hygromycin resistance gene expression cassette was incorporated in the ST construct leading to construct EOS-S4-TK-PGK-Hygromycin (STPH) (Figure 49). The hygromycin resistance gene was under the control of the ubiquitous PGK promoter enabling hygromycin selection of successfully transduced ES cells of each state. For incorporating the selection cassette the construct ST was chosen, as this LV resulted in the highest TK abundance although no higher efficiency of TK/GCV system was obtained (see also Figures 43 and 44).

Figure 49: Construct of LV double cassette containing a hygromycin resistance gene driven by PGK promoter and TK cDNA under the control of EOS-S4 promoter (STPH).

The STPH construct was then used for transduction of ES cells and mixed ES cell populations with an average copy number per genome of 1.5 were further investigated (data not shown). The cells were treated with GCV with or without hygromycin pre-selection under undifferentiation conditions (Figure 50).

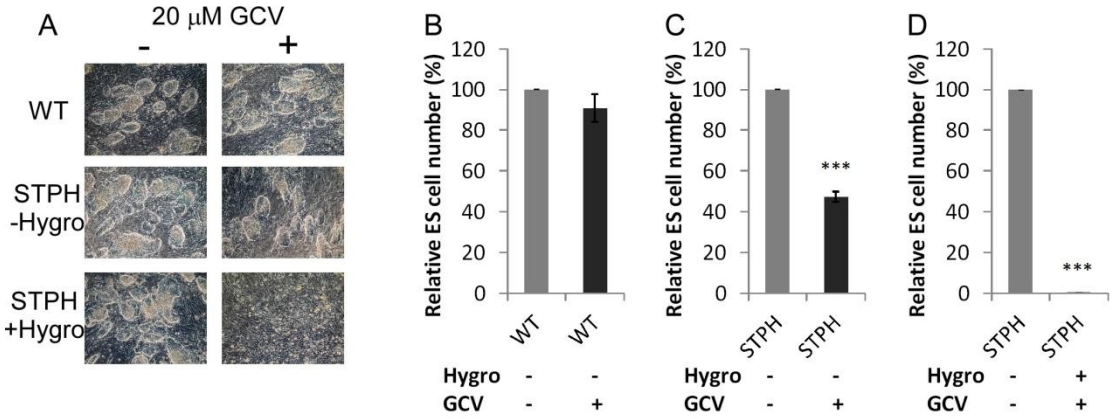

Figure 50: Analysis of ES cells transduced with STPH with 1.5 copy numbers per genome in average under undifferentiation conditions. (**A**) Brightfield images of untransduced (WT) or STPH-transduced ES cells with (+Hygro) or without hygromycin (-Hygro) pre-selection and with (+) or without (-) 20 µM GCV treatment under undifferentiation conditions. Representative brightfield images are shown (n=3). (**B**), (**C**) and (**D**) Relative cell survival of untransduced ES cells (WT) (B), STPH-transduced ES cells without pre-selection (C) and STPH-transduced ES cells with pre-selection (D) with (+) or without (-) 20 µM GCV treatment as analyzed by cell counting of images representatively shown in (A) (n=3, Mean±SEM, ***P<0.001 compared to without GCV treatment, respectively, t-test).

In addition to taking brightfield images (Figure 50A) ES cells were also counted (Figures 50 B to D). The GCV treatment of the unselected STPH-transduced ES cells resulted in a significant reduction of undifferentiated cells (47.30%±0.37, Mean±SEM) as compared to unselected STPH transduced cells without GCV application (Figure 50A, C). Untransduced cells showed no difference with or without GCV (Figure 50A, B). Importantly, with hygromycin pre-selection, only 0.37%±0.29 undifferentiated STPH-transduced ES cells were observed in the presence of GCV indicating that pre-selection is necessary for complete abolishment of ES cells *in vitro* (Figure 50A, D).

In the following, the STPH-transduced ES cells with low copy numbers were differentiated into EBs. Fluorescence analysis of α-MHC driven eGFP expression in the EBs showed the eGFP expression in all cell populations indicating successful formation of ES cell-derived cardiomyocytes (Figure 51A). Immunostaining of Oct-3/4-positive cells on day 14 post

differentiation revealed no Oct-3/4-positive cells after GCV treatment of pre-selected cells indicating successful elimination of undifferentiated cells (Figure 51B).

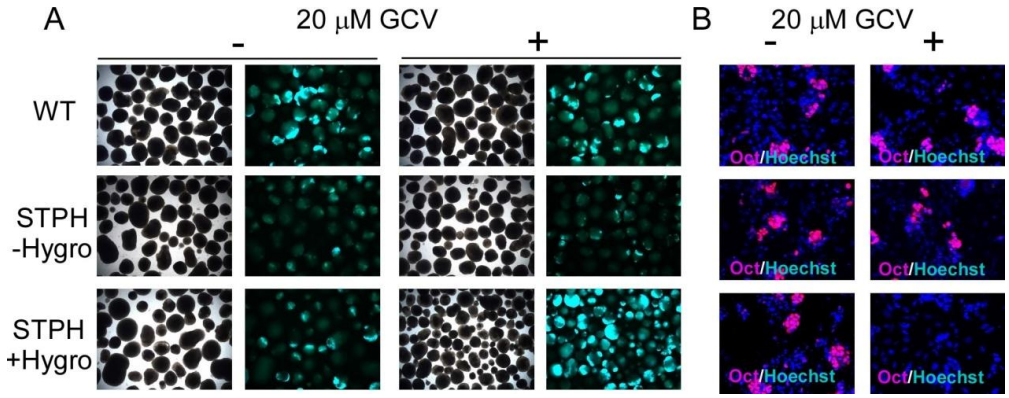

Figure 51: Differentiation of STPH-transduced ES cells. (**A**) Brightfield and fluorescence images of EBs derived from untransduced (WT) or STPH-transduced mixed ES cell populations carrying 1.5 copy number per genome in average on day 14 post differentiation and treated with (+) or without (-) 20 µM GCV after pre-selection with (+Hygro) or without hygromycin (-Hygro). Representative images are shown (n=3). eGFP expression driven by cardiac specific promoter α-MHC imdicates formation of ES cell-derived cardiomyocytes. (**B**) Immunostaining of dissociated EBs form (A) with Oct-3/4 (red) and Hoechst (blue) indicating Oct-3/4-positive cells (ES cells) and nucleus, respectively. Representative images are shown (n=3).

Again, formation of ES cell-derived cardiomyocytes from these STPH-transduced ES cells was analyzed and revealed again no influence of TK/GCV on the differentiation process as shown by immunostaining against skeletal α-actinin and fluorescence detection of cardiac specific promoter driven eGFP expression (Figure 52).

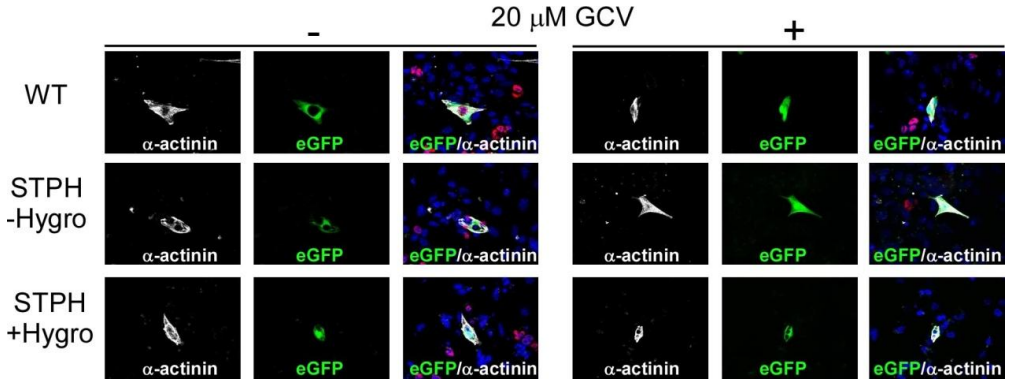

Figure 52: Immunostaining of dissociated EBs of STPH-transduced ES cells. Dissociated EBs from untransduced (WT) or STPH-transduced mixed ES cells populations with (+) or without (-) 20 µM GCV treatment after pre-selection with (+Hygro) or without hygromycin (-Hygro) were immunostained with skeletal α-actinin (sarcomere) indicating the successful formation of ES cell-derived cardiomyocytes (white); green fluorescence indicates eGFP expression driven by cardiac specific promoter, α-MHC.

However, for a more convenient use of ES cells in clinical applications renouncement of additional pre-selection would be worthwhile. Therefore, higher lentiviral copy numbers in ES cells without pre-selection might result in a similar efficiency. Thus, ES cells were transduced with higher LV concentrations of STPH leading to an average copy number of 3.8 as analyzed by qPCR (data not shown). GCV treatment of these cells carrying high copy numbers without pre-selection showed *in vitro* a strong elimination of undifferentiated cells (35.43%±2.00, Mean±SEM) as shown by brightfield images (Figure 53A) and cell counting (Figure 53B, C).

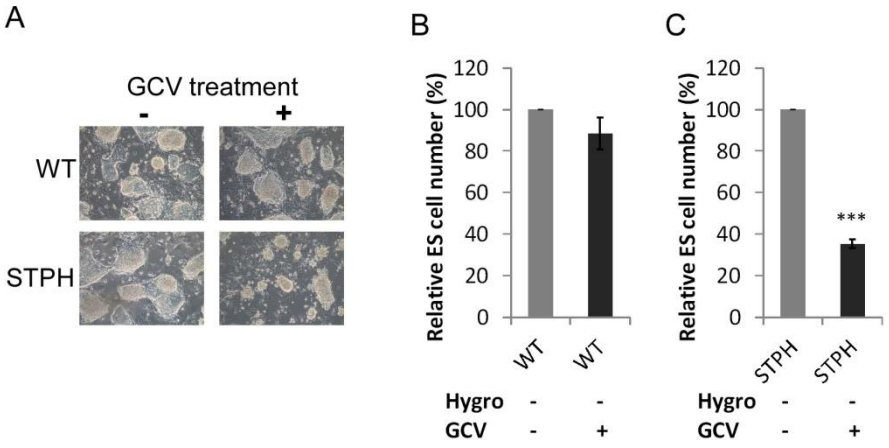

Figure 53: Analysis of STPH-transduced ES cells with high copy number. (**A**) ES cells were not transduced (WT) or were transduced with different concentrations of LV STPH leading to 3.8 copy number (high copy number) per genome in average and treated with (+) or without (-) 20 µM GCV without (-) Hygromycin. Representative brightfield images are shown (n=3). (**B**) and (**C**) Relative cell survival of untransduced ES cells (WT) (B) or STPH-transduced ES cells (C) with (+) or without (-) 20 µM GCV treatment as analyzed by cell counting of images representatively shown in (A) (n=3, Mean±SEM; ***P<0.001 compared to without GCV treatment, respectively, Student's *t*-test).

These data demonstrate that increasing the copy number significantly increases the elimination efficiency of TK/GCV system as compared to low copy number STPH transduced ES cells (see also Figure 50C). Unfortunately, complete killing of undifferentiated cells was not achieved as observed for pre-selected low copy number STPH transduced ES cells (see also Figure 50D).

Taken together, hygromycin application on STPH-transduced ES cells of low copy number resulted in successful selection of only transduced ES cells and thus, an extremely efficient elimination of undifferentiated cells was achieved upon GCV treatment. However, increasing the lentiviral copy number showed lower cell survival of undifferentiated cells without pre-selection. But the complete elimination of ES cells was not achieved and thus, pre-selection seems to be necessary.

3.2.4. *in vivo* application of TK expressing ES cells

3.2.4.1. *in vivo* application of STPH-transduced ES cells with low copy numbers

Since *in vitro* data revealed an efficient elimination of undifferentiated ES cells after STPH transduction, hygromycin pre-selection and GCV treatment, the system was also tested *in vivo*. Therefore, $1x10^6$ untransduced or STPH-transduced ES cells with hygromycin pre-selection were injected into the left and right hind limbs of 8-week old SCID® mice, respectively (Figure 54). Three hours after ES cell injection, the mice were administered with either saline solution (0.9% (w/v) NaCl) or with 20 mg/kg GCV per day for 12 days.

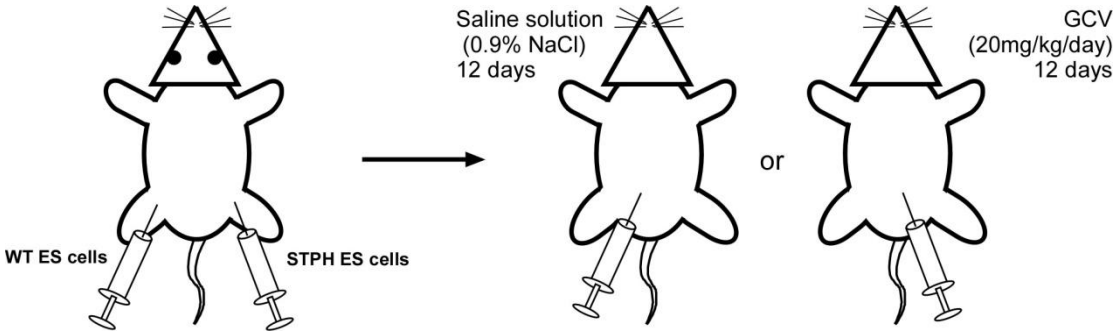

Figure 54: Schematic illustration of *in vivo* application of ES cells. $1x10^6$ ES cells (untransduced (WT) or STPH transduced) were injected into hind limbs of SCID mice. Saline solution or GCV (20mg/kg/day) were administrated by intraperitoneal injections (i.p.) for 12 days.

Three weeks after ES cells injection the mice were sacrificed for analysis of potential teratoma formation. Teratomas were weighed, measured concerning their dimensions and used for H&E staining. No significant difference in the weights of teratomas was detected in mice treated with saline solution after injection of both untransduced and pre-selected STPH-transduced ES cells (Figure 55A, B). Importantly, applying STPH-transduced ES cells with pre-selection and subsequent GCV administration resulted in only one out of six mice that revealed the formation of only a small tissue (Figure 55B, C and Table 3). This clearly indicates the prevention of teratoma formation.

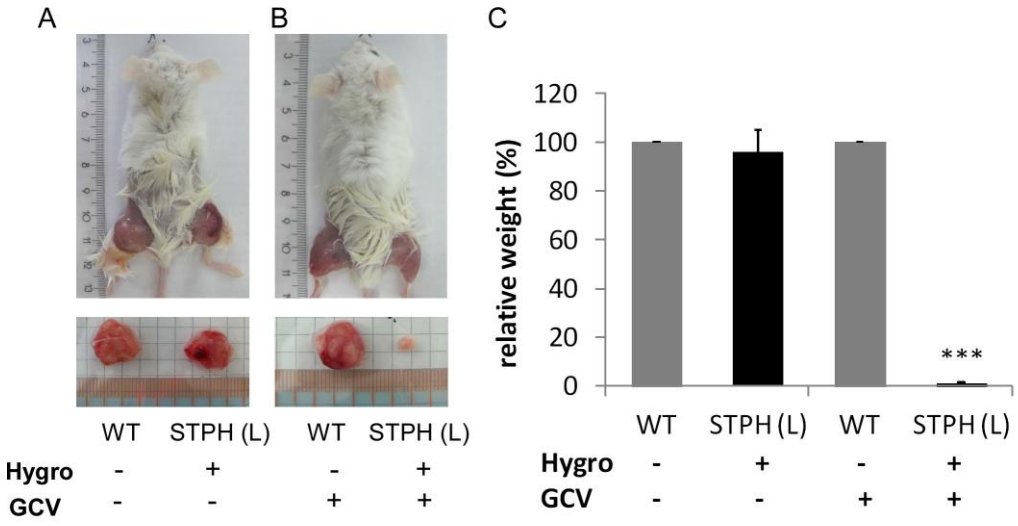

Figure 55: *in vivo* application of STPH-transduced ES cells (low copy) with hygromycin pre-selection. Three weeks after injections of $1x10^6$ ES cells (STPH-transduced with 1.5 copies per genome in average or not transduced (WT)) into hind limbs of SCID/beige mice and administration with Saline solution (0.9% (w/v) NaCl) or GCV (20mg/kg/day) for 12 days, the mice were sacrificed for analysis of teratoma formation. (**A**) and (**B**) Images of ES cell injected mice and dissected teratomas. (**C**) Relative weight of teratoma from engraftment of WT and hygromycin pre-selected STPH-transduced ES cells with low copy number (L (1.5 copies per genome in average)) with (+) or without (-) GCV treatment (n=4 or 6, Mean±SEM; ***P<0.001, ANOVA, analysis of variance).

Teratomas derived from injection of STPH-transduced ES cells with both saline and GCV treatment were further investigated by applying H&E staining (2.13). Expectedly, stained teratoma sections exhibited tissues from all of the three germ layers in case of saline treatment (Figure 56A). In contrast, the small tissue obtained from one out of six mice that all received STPH transduced pre-selected ES cells and GCV treatment showed only muscle tissue without any other germ layer tissue (Figure 56 B).

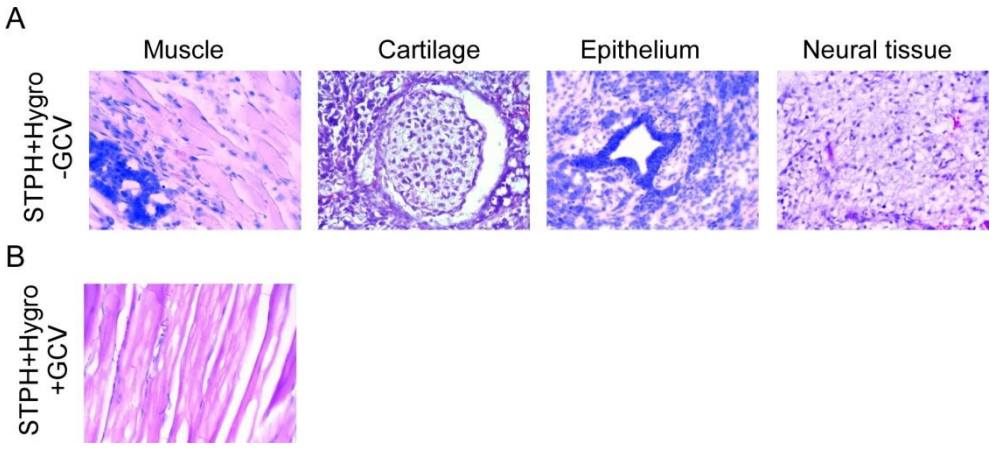

Figure 56: H&E staining of teratomas derived from *in vivo* application of STPH-transduced ES cells with hygromycin pre-selection. (**A**) and (**B**) H&E stained sections of explanted teratomas emerging from injections of pre-selected STPH-transduced ES cells without GCV (A) or with GCV treatment (B) demonstrating muscle (left), cartilage, glandular epithelium and neural tissue (right) as indicated.

In addition, the average size of teratomas (maximal dimensions) was measured (Table 3). If untransduced (WT) cells were injected, teratoma dimensions of more than 1cm were observed for both saline and GCV treatment indicating no influence of GCV alone (Table 3). Similar teratoma dimensions were detected after injection of STPH-transduced ES cells (low copy numbers) with pre-selection in absence of GCV (Table 3). In contrast, injection of STPH-transduced ES cells with pre-selection upon GCV application exhibited only muscle tissue (see also Figure 56B) with a diameter of only ~0.3 cm (Table 3). No teratoma or tissue was detected in all other mice of this group (Table 3).

Table 3: Summary of teratoma dimensions formed from ES cells injected in hind limbs of SCID/beige mice

Cell line[a]	Hygromycin selection[b]	GCV Treatment[c]	n	Teratoma > 1 cm	Teratoma < 1 cm	No teratoma
ES w/o virus	-	-	9	8/9	1/9	-
ES w/o virus	-	+	11	11/11	-	-
ES + STPH (H)	-	-	5	5/5	-	-
ES + STPH (H)	-	+	5	1/5	4/5	-
ES + STPH (L)	+	-	4	4/4	-	-
ES + STPH (L)	+	+	6	-	1 [d] /6	5/6

The table summarizes the ES cell injections into hind limbs of SCID/beige mice and teratomas were analyzed 3 weeks later. Teratoma size (>1 cm or <1 cm) indicates maximum dimension.

[a]ES cells were transduced with STPH (+STPH) or not transduced (w/o virus), respectively.

H and L indicate ES cells carrying high (3.8) or low (1.5) copy number per genome in average.

[b]Hygromycin pre-selected ES cells were cultured with 300ng/ml hygromycin for 9 days before ES cell injection.

[c]ES cells were injected and mice were treated with (+) or without (-) 20mg/kg/day GCV for 12 days. GCV-untreated mice received the same volume of 0.9% (w/v) NaCl.

[d]Teratoma's diameter is less than 0.3 cm and exhibit only muscle tissue (see also Figure 56B).

In conclusion, ES cells that have teratoma-forming potential seemed to be completely eliminated due to GCV application *in vivo* if a pre-selection of LV-transduced ES cells is performed. These results confirmed the *in vitro* findings (see also Figure 50D). . Importantly, these results were obtained with cells carrying only 1.5 provirus integrations per genome in average. Mixed ES cell populations were applied for hygromycin treatment without the need of further clone picking or additional screening, e.g. by southern blot analysis. This suggests promising future for use in regenerative medicine approaches

3.2.4.2. *in vivo* application of STPH-transduced ES cells with high copy numbers

In addition, STPH-transduced ES cells carrying high copy numbers without pre-selection were also investigated *in vivo*. When treating the mice with saline, similar teratoma weights were observed for both injections of untransduced and of STPH-transduced cells without pre-selection (Figure 57A, C).

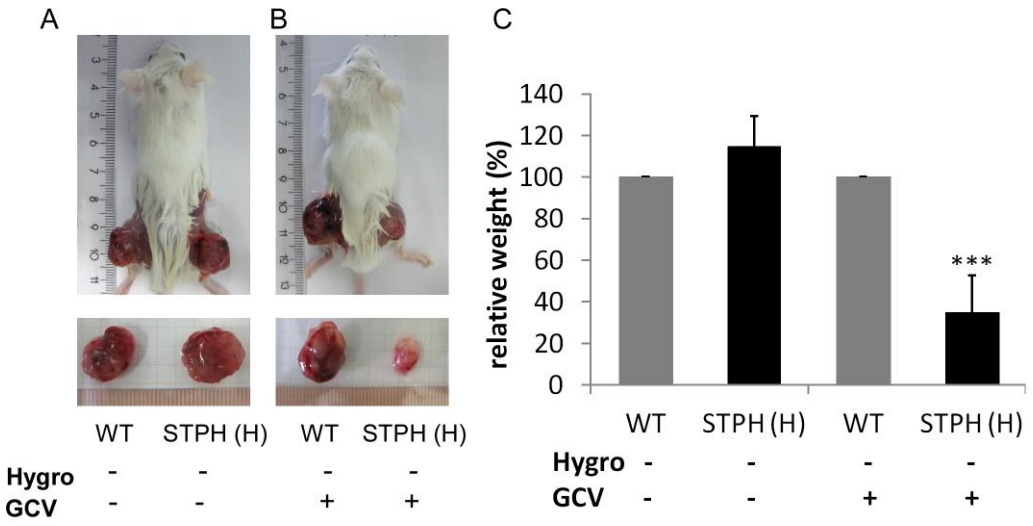

Figure 57: *in vivo* application of STPH-transduced ES cells (high copy) without hygromycin pre-selection. Three weeks after injections of $1x10^6$ ES cells (STPH-transduced with 3.8 copies per genome in average or not transduced (WT)) into hind limbs of SCID/beige mice and administration with Saline solution (0.9% (w/v) NaCl) or GCV (20mg/kg/day) for 12 days, the mice were sacrificed for analysis of teratoma formation. (**A**) and (**B**) Images of ES cell injected mice and dissected teratomas. (**C**) Relative weight of teratoma from engraftment of WT and non-selected (-Hygro) STPH-transduced ES cells with high copy number (H (3.8 copies per genome in average)) with (+) or without (-) GCV treatment (n=5, Mean±SEM; ***P<0.001, ANOVA, analysis of variance).

All of these mice developed teratomas with maximum dimensions of more than 1 cm (Table 3). Compared to the control group that received untransduced cells, a significant reduction in teratoma weight (around 65%) was observed for injection of high copy number STPH-transduced ES cells without pre-selection when GCV was applied (Figure 57B, C). Furthermore, four out of five mice showed formation of only small teratomas upon GCV treatment (Table 3). However, higher LV copy number of STPH-transduced ES cells without pre-selection could not result in a complete absence of teratomas as observed for injection of pre-selected low copy number STPH-transduced ES cells (see also Figure 55B, C).

These *in vivo* results confirmed the *in vitro* findings (see also Figure 53C) where also no complete elimination of undifferentiated ES cells was achieved that have teratoma-forming potential. It was figured out, that pre-selection of LV-transduced ES cells is much more

favorable than increasing LV doses. Although, the hygromycin pre-selection represents an additional step in cell preparing before injection, it is an easy-to-handle method. Importantly, the risk of insertional mutagenesis due to integrating LVs can be reduced as only low copy numbers are sufficient to avoid tumor formation.

4. Discussion

Within the first part of the work epigenetic regulation of the transgene cassette in ES cells was analyzed after viral transduction with either LVs or RVs. Both viral vectors belong to the family of *Retroviridae* and are either based on HIV-1 or on MLV.

4.1. RVs and LVs transgenesis and gene therapy

In the early 1950's it has been discovered that mouse leukemia is transmitted to newborn mice by an agent that is still acting after filtration (Gross, 1951a; Gross, 1951b). It turned out that the causer was a virus. Starting from these studies deeper insights in the life cycle of retroviruses have been revealed and retroviral vector system have been developed. RVs have even been used as vehicles in the context of gene therapy (Rein, 2011) as they are able to stably integrate into the genome of target cells, which leads to persistent expression of the transgene (Pfeifer & Verma, 2001a). In contrast to a simple retroviruses such as MLV, a complex retrovirus like the lentivirus human immunodeficiency virus HIV-1 contains additional regulatory and accessory genes (Coffin, 1992; Vogt, 1997). These two viruses differ also in their infectivity: HIV-1 can efficiently infect nondividing cells (Naldini et al, 1996) whereas MLV generally does not (Lewis & Emerman, 1994; Yamashita & Emerman, 2006). This distinctive feature provides broad spectrum toward many applications of lentivirus-derived vectors for which MLV are not favorable. However, it has been reported that very few nondividing cells including human macrophages or post-mitotic neural cells could be transduced with simple retrovirus-derived vectors (Jarrosson-Wuilleme et al, 2006; Liu et al, 2011).

The integration site of HIV-1 and MLV is not purely random, but both viruses have different preferences (Bushman, 2002; Schroder et al, 2002; Wu et al, 2003). The integration site of HIV-1 is influenced by a verity of factors including specific cellular protein binding to viral integrase (Lewinski et al, 2006). HIV-1 prefers to integrate in transcriptionally active regions of the host genome (Mitchell et al, 2004; Schroder et al, 2002). For MLVs, cellular factors including higher order chromatin structures are likely to affect accessibility to target DNA (Rohdewohld et al, 1987; Vijaya et al, 1986). In detail, MLV particularly favors transcriptional start sites, regions near DNase I hypersensitivity sites (Rohdewohld et al, 1987; Vijaya et al, 1986) as well as CpG islands (Qasim et al, 2010; Wu et al, 2003). Around 17% of MLV integrate within human CpG islands (± 1 kb) whereas only 2% of HIVs integrate within these sites (Wu et al, 2003).

Compared to RVs, LVs can accommodate larger transgene expression cassettes (up to ~ 8 kb) (De Meyer et al, 2006; Zufferey et al, 1998). In addition, compared to other gene transfer vehicles like adenovirus or adeno-associated virus vectors (Chirmule et al, 1999; Mingozzi et al, 2007), LVs showed a decreased immunogenicity in *in vivo* applications (Abordo-Adesida et al, 2005). So far, LVs have been already proved to be safe in *ex vivo* gene therapy in the context of human clinical trials (Cartier et al, 2009; Cavazzana-Calvo et al, 2010; Huang et al, 2006).

RVs have been also applied for different clinical approaches. However, a serious drawback related to the risk of retrovirus-mediated insertional mutagenesis leading to aberrant gene expressions was observed in a clinical trial for X-linked severe combined immunodeficiency disease (SCID) (Hacein-Bey-Abina et al, 2003). It has recently been shown that autologous stem cell gene therapy by using RVs for X-linked SCID treatment also caused T cell leukemiagenesis (Hacein-Bey-Abina et al, 2008; Howe et al, 2008). Furthermore, transactivation of retroviral insertion led to clonal expansion of myeloid cells when RVs were used for genetic modification of hematopoietic stem cells (HSCs) (Ott et al, 2006). In contrast, there are two early reports showing no insertional mutagenesis in patients that were treated with autologous T cells modified *ex vivo* by using LVs transduction (Levine et al, 2006; Wang et al, 2009).

Prototypic 3’ LTR enhancer/promoter elements of integrated wildtype RVs or wildtype LVs in host genome could cause the aberrant activation of downstream flanking genes. Therefore, the wildtype 3’ LTR enhancer/promoter elements by applying self-inactivation (SIN) of the viral vectors (refer to 1.4 for detailed information) leading to SIN-LVs (Zufferey et al, 1998) as well as SIN-RVs (Yu et al, 1986). Importantly, SIN-LVs and SIN-RVs lacking of wildtype 3’ LTR enhancers/promoters were considered to be safe to be used as gene transfer vehicles (Lever et al, 2004; Yu et al, 1986). Nevertheless, strong residual promoter activity was still observed in the 3′ LTR of many currently used SIN-RVs, which may raise the potential risk of insertional mutagenesis (Xu et al, 2012). However, this same phenomenon was so far not found in SIN-LVs (Xu et al, 2012; Zufferey et al, 1998). Some other reports also suggested that SIN-LVs transduction are more favorable compared to applying retroviral MLV backbone with the same SIN configuration due to the higher risk of insertional mutagenesis of SIN-RVs (Modlich et al, 2009; Qasim et al, 2010).

4.2. Epigenetic regulation of SIN-RVs and SIN-LVs *in vitro*

As there are some differences between SIN-LVs and SIN-RVs the goal of the first part of the present work was the investigation of epigenetic regulation of SIN-LVs and SIN-RVs in transduced ES cells. Early studies demonstrated that *de nova* methylation of CpG nucleotides appeared to be a major reason for the transcriptional inactivation of retrovirus during mouse embryogenesis (Challita & Kohn, 1994; Cherry et al, 2000; Jahner et al, 1982; Laker et al, 1998). However, *de nova* methylase independent silencing of LVs in ES cells had also been reported (Pannell et al, 2000). In addition, hypoacetylation of histone H3 and H1 in ES cells played an important role in transgene silencing (Yao et al, 2004). To minimize transgene silencing of viral vectors due to methylation of provirus in host cells, SIN vectors have been developed lacking a number of viral elements that are potential targets for the host cellular silencing machinery (Yu et al, 1986; Zufferey et al, 1998). Indeed, most wildtype viral vectors were silenced in host cells, but in case of SIN vectors only half of the integration sites were silenced (Osborne et al, 1999; Pannell et al, 2000). A study comparing the activity of SIN-LVs and wildtype RVs in murine ES cells showed that transgene expression in SIN-LV-transduced ES cells was still observed during cell differentiation (Hamaguchi et al, 2000). In contrast, the transgene expression dramatically declined in RVs-transduced ES cells (Hamaguchi et al, 2000). Although, methylation and histone modification of SIN-LVs were still found in murine embryonic carcinoma cells, SIN-LVs generally displayed a significantly better performance as compared to the prototypic retroviruses (He et al, 2005).

However, Schambach et al have reported that SIN-RVs showed the similar properties as SIN-LVs in hematopoietic stem cells (Schambach et al, 2006a) when using identical enhancer, promoter and transgene cassette sequences. In that study they did not investigate the fate of SIN-RVs in ES cells under differentiation conditions or in *in vivo* experiments. Therefore, in the present study, SIN-LVs and SIN-RVs were applied in ES cells under undifferentiation as well as differentiation conditions. Under undifferentiation conditions, these two vectors exhibited the same stable expression of transgene in mixed ES cell populations (i. e. without further selection) as already reported by Schambach et al. Surprisingly, under differentiation conditions, the transgene expression in both cases declined at day 5 post differentiation (see also Figure 13) due to provirus methylation (see also Figure 16 and 17).

For further analysis of SIN-LV- and SIN-RV-mediated gene expression, single copy clones of transduced ES cells were picked and SIN-LV and SIN-RV ES cell clones with "high expression level" or with "low expression level" were investigated, respectively. These single clones all showed a stable transgene expression under undifferentiation conditions as already observed for mixed ES cell populations. Nevertheless, only the high expressing SIN-LV ES cell clone maintained transgene expression when cells underwent differentiation, whereas the low expressing SIN-LV clone and both low or high expressing SIN-RV clones did not. The analysis of methylation status was performed using methylation-sensitive Southern Blot as well as the high-resolution method of sodium bisulfite sequencing. These data revealed that a much higher level of methylation occur in the low expressing SIN-LV clone and the low and high expressing SIN-RV clones as compared to the high expressing SIN-LV clone. This indicates that provirus methylation is one of the main reasons for the transgene silencing. A former study has proposed that application of the methyltransferase inhibitor 5'-Aza in differentiated ES cells could partially reactivate the silenced transgene if silencing is due to cytosine methylation (Yao et al, 2004). However, application of 5'-Aza in differentiated ES cell clones did not reverse transgene silencing in the present study (see also Figure 22C). Moreover, high concentrations of 5'-Aza showed cellular toxicity in ES cells.

The main reason for transgene silencing in the four clones under differentiation conditions seems to be the *de novo* methylation of CpG nucleotides. However, the role of histone acetylation could not be excluded. This could be further investigated by either applying the histone deacetylase inhibitors Trichostatin A (TSA) on silenced ES cell clones (Yao et al, 2004) or by use of the *de novo* methylase-null [dnmt3a-/-; dnmt3b-/-] ES cell line (Pannell et al, 2000). Transgene silencing resulting from histone modification in single copy SIN-LVs or SIN-RVs ES cell clones were not investigated in the scope of the present work and require further investigation.

4.3. Epigenetic regulation of SIN-RVs and SIN-LVs *in vivo*

To investigate the fate of viral vectors *in vivo* generation of transgenic animals is a suitable method. It enables steady monitoring of the activity of viral vectors during embryogenesis and of the transgene expression in the transgenic livestock. Previous studies showed that lentiviral vectors are able to efficiently transduce human ES cells and pre-implantation

embryos of mice and rats (Lois et al, 2002; Pfeifer et al, 2002). Additionally, farm transgenic livestock were also successfully generated by applying subzonal injections of LVs, i.e. transgenic pigs (Hofmann et al, 2003) or cattles (Hofmann et al, 2004). By now SIN-LVs are well accepted as efficient vehicles for gene delivery *in vitro* as well as *in vivo*. Their potential for the generation of transgenic animals has been recognized as a promising alternative to the conventional DNA-microinjection method (Chan et al, 1998; Wells et al, 1999). In contrast, using wildtype RVs as vehicles for gene transfer into murine embryos a strong inactivation of the transgene is observed due to *de nova* methylation during embryogenesis (Jahner et al, 1982) although a successful transduction is achieved (Jaenisch et al, 1976). SIN vectors lacking the 5' LTR promoter were shown to overcome the CpG methylation in comparison to RVs carrying wildtype 5' LTR promoter as reported for embryonic carcinoma cells (Osborne et al, 1999).

In the present study, the subzonal virus injection method was used (see also Figure 29). Almost 20% of implanted embryos injected with SIN-LVs resulted in born pups by applying only 2 subzonal injections. As compared to SIN-LVs injection, only 12% of implanted SIN-RVs injected embryos developed to born pups and 9 SIs were performed (see also Table 2). Interestingly, 50 % of the born pups were transgenic when using SIN-LVs, whereas less than 1 % of them were transgenic when applying SIN-RVs (see also Figure 31). This extremely low efficiency to generate transgenic mice when using SIN-RV might result from low transduction efficiency of SIN-RVs in embryos or the poor survival of transgenic mice after birth when SIN-RVs subzonal injection was used.

4.4. Application of LVs in pluripotent stem cells

In the last decade LVs have been reported as an efficient tool for gene transfer in pluripotent stem cells (Hamaguchi et al, 2000; Pfeifer et al, 2002). Moreover, LVs seemed to be a favorable vehicle for an efficient production of transgenic mice by implantation of LV-transduced pluripotent stem cells in preimplantation embryos (Lois et al, 2002; Okada et al, 2007; Rubinson et al, 2003). Chimeric mice that were generated in that way by using siRNA expressing LVs showed a strong knockdown of the prion protein, which enabled a prolongation of survival of scrapie-infected mice (Pfeifer et al, 2006). Further reports demonstrated that lentiviral transduction of somatic cells allowed a successful creation of genetically modified disease-specific iPS cells (Lee et al, 2009; Thatava et al, 2011).

Additionally, using LV transduction of iPS cells enabled the generation of disease-corrected, patient-specific haematopietic progenitor cells, which demonstrated its potential in cell therapy applications (Raya et al, 2009). Taken together, LVs represent a suitable tool for the genetic modification of pluripotent stem cells. As already shown within the present study LVs give also rise to be more suitable for use in pluripotent cells as compared to e.g. RVs.

4.5. Removal of undifferentiated pluripotent stem cells

Embryonic as well as induced pluripotent stem cells with their characteristic of pluripotency have become promising tools for regenerative medicine since they have been isolated (Takahashi et al, 2007b; Wobus & Boheler, 2005). However, there is a potential risk of teratoma development if implanted pluripotent stem cell populations are not completely differentiated and contaminated with undifferentiated ES or iPS cells (Blum & Benvenisty, 2008). Thus, before the technology can be turned into clinical applications, this major safety obstacle has to be solved. For minimization of tumorigenesis due to undifferentiated pluripotent cells, different approaches have already been developed to remove the undifferentiated cells e.g. by using cytotoxic antibodies against undifferentiated ES cells (Choo et al, 2008; Tan et al, 2009) or by applying an antibody against a surface antigen of ES cells in combination with FACS sorting-based separation (Tang et al, 2011). Another method is the use of a suicide strategy based on a TK/GCV system to eliminate undifferentiated pluripotent cells (Jung et al, 2007; Naujok et al, 2010; Schuldiner et al, 2003). The principle of TK/GCV systems is the conversion of the prodrug GCV by the TK (thymidine kinase) from Herpes simplex virus leading to a toxic metabolite that eliminates TK expressing cells upon GCV treatment. Importantly, the TK/GCV-based treatment has already been proven for its general safety *in vivo* and is already in use in clinical trials for cancer therapy (Kubo et al, 2003; Li et al, 2006; Sterman et al, 1998; Wildner et al, 1999). Furthermore, it was also stated as a "safety tool" in adaptive immunotherapy (Bonini et al, 2007; Georgoudaki et al, 2010; Lupo-Stanghellini et al, 2010).

Genetic modification of ES cell lines to express the HSV-TK gene has already been achieved by several groups (Goodwin et al, 2001; Jung et al, 2007; Naujok et al, 2010; Schuldiner et al, 2003). Various routes to deliver the transgene have been studied (Jung et al, 2007; Schuldiner et al, 2003). In previous studies, TK expression in ES cells was for

example achieved by plasmid transfection (Naujok et al, 2010; Schuldiner et al, 2003). However, to obtain a stable transgene expression, LVs represent highly efficient vehicles for the genetic modification of pluripotent stem cells *in vitro* as well as *in vivo* (Pfeifer et al, 2002). Using ubiquitous promoters like PGK (Schuldiner et al, 2003) or CMV (Jung et al, 2007) to drive TK expression led to elimination of all transplanted cells (Schuldiner et al, 2003), whereas using promoter of pluripotency genes for TK expression resulted in the death of only undifferentiated ES cells (Naujok et al, 2010). Hence, a combination of LVs expressing TK under the control of promoters of pluripotency genes represents a promising strategy to achieve a stable and specific elimination of pluripotent stem cells upon GCV treatment.

4.6. TK/GCV system in pluripotent stem cells for regenerative medicine

In the present study, LVs carrying promoters of different pluripotency genes (Nanog, Oct-3/4, EOS-C3, EOS-S4) were used to establish pluripotent cells stably expressing TK gene. In order to minimize the risk of insertional mutagenesis caused by integrating LVs, cells transduced at low copy number were investigated that were treated with GCV.

Preliminary *in vitro* experiments revealed a high killing effect of LV-transduced ES and iPS cells upon GCV treatment using Nanog- and Oct3/4 promoters. However, a complete elimination of undifferentiated cells was not achieved (see also Figure 35). An incomplete elimination might result from an insufficient TK expression level of transduced cells. Therefore, the EOS promoters, that have been proven to achieve higher transgene expression in pluripotent stem cells, were used to increase TK expression (Hotta et al, 2009). Although the EOS-S4 promoter led to the highest TK expression in cells, a much higher killing efficiency of undifferentiated cells was still not observed. This suggests that survival of undifferentiated cells is more likely the consequence of untransduced cells that can not be eliminated upon GCV treatment. To overcome this circumstance, single copy LV-transduced ES cell clones were therefore picked and further investigated to ensure TK expression in the whole cell populations. Unfortunately, in differentiated single ES cell clones (NT #8 and NT #11) more than 1% of Oct-3/4-positive cells (undifferentiated ES cells) were still observed upon GCV treatment (see also Figure 47B). This might result from a contamination with untransduced cells when picking the single cell clones. The problem can be solved through further subcloning to achieve genuine single ES cell clones.

This was not performed within the scope of the present work. Another explanation for the survival of undifferentiated cells after GCV treatment might be the epigenetic silencing of integrated LV provirus in the picked single copy ES cell clones (Hofmann et al, 2006). The CpG methylation-related transgene silencing in LV-transduced ES cell clones has already been shown in the first part of the work (3.1.2).

However, neither by raising TK expression using stronger EOS promoters nor by analyzing single copy ES cell clones seemed to enable a complete elimination of undifferentiated cells upon GCV treatment. Another approach to obtain pure cell populations is the use of a pre-selection strategy that was also investigated in the present study. This pre-selection method needs neither the additional clonal selection nor a screening using Southern blot analysis that is needed for the identification of single copy ES cell clones. As pre-selection tool a hygromycin resistance gene was used to select for the LV-transduced ES cells. The resistance gene was driven by the ubiquitous promoter PKG enabling selection of cells of each differentiation state. The PGK-hygromycin cassette was inserted into the LV-construct EOS-S4-TK (ST) resulting in the LV double-cassette STPH. As expected, STPH-transduced ES cells carrying low copy number of LVs without hygromycin selection showed a significant higher but not complete elimination of undifferentiated ES cells upon GCV treatment as compared to untransduced cells. Importantly, GCV treatment of STPH-transduced ES cells after hygromycin pre-selection indeed resulted in a complete elimination of undifferentiated cells *in vitro*. Finally, these pre-selected cell populations were also applied *in vivo* in a mouse teratoma model. Importantly, when applying pre-selected STPH-transduced ES cells with low copy number, no teratomas were observed upon GCV administration. In contrast, tumor formation was detected when using untransduced ES cells and GCV application or when applying STPH transduced ES cells but using saline solution instead of GCV. These teratomas exhibited all three germ layers. Similar findings have been also reported in the work of Cheng et al (Cheng et al, 2012). They showed that LV-mediated TK expression in ES cells resulted in selectively ablation of tumors derived from differentiated cell populations *in vivo* when GCV was applied (Cheng et al, 2012).

To simplify the manipulation of LV-transduced ES cells, it was furthermore tested if a similar killing efficiency can be achieved by avoiding the initial selection at the same time using just higher LV doses for ES cell transduction resulting in higher copy number of LVs. Thus, STPH-transduced ES cell populations with high copy number of LVs (~4 copies per

genome in average) without pre-selection were tested *in vitro* as well as *in vivo*. Due to the risk of insertional mutagennesis higher LV doses were not applied. Compared to the control group, a significant reduction in teratoma weight (around 65%) was observed for STPH-transduced ES cells with high copy number without pre-selection when treated with GCV. However, the complete absence of teratoma was not achieved. These findings suggest that pre-selection of the transduced ES cells is required to achieve complete elimination of undifferentiated ES cells using the TK/GCV strategy. This might facilitate the development of clinical approaches in regenerative medicine.

5. Summary

Embryonic stem cells are characterized by their self-renewal capability and pluripotency. They represent an unlimited cell source for regenerative medicine. In order to genetically modify ES cells for gene therapy, self-inactivating lentiviral vectors (SIN-LVs) have been proposed as versatile tools for the gene transfer *in vitro*, *ex vivo* or *in vivo* leading to stable transgene expression (Matrai et al, 2010; Pfeifer et al, 2002). Although retroviral vectors (RVs) were also used for the genetic modifications of ES cells, a major limitation in using RVs is the epigenetic silencing (Jahner et al, 1982). However, the performance of SIN-RVs as well as SIN-LVs has been reported in HSCs (Schambach et al, 2006a) but not in ES cells. Therefore, in the first part of this work the focus lied on the comparison between SIN-LV and SIN-RV transduction of ES cells. The data obtained showed a similar transgene expression level of both SIN-LVs and SIN-RVs transduced ES cells under undifferentiation conditions. Under differentiation conditions transgene silencing in SIN-LVs as well as SIN-RVs transduced ES cells was observed that mainly was due to CpG methylation. For both vectors the contribution of CpG methylation to transgene silencing was of the same extent. For a more accurate analysis of SIN-LV- and SIN-RV-mediated transgene expression, transduced ES cell clones with either high or low expression level were picked for further analysis. In case of SIN-RV ES cell clones, both clones (low and high expressing) showed strong transgene silencing under differentiation conditions due to CpG methylation. In contrast, only the low expressing SIN-LV ES cell clone showed high CpG methylation level under differentiation conditions. Importantly, the high expressing LV ES cell clone revealed a lower CpG methylation level and transgene expression was still observed under differentiation conditions. Moreover, using subzonal injection for the generation of transgenic mice, injection of SIN-LVs in preimplantation embryos resulted in around 50 % transgenic mice of born pups whereas SIN-RVs subzonal injections led to less than 1 %. This data indicated the successful and efficient usage of SIN-LV transduction of murine embryos whereas SIN-RVs showed very low efficient transduction. Taken together, SIN-LVs seem to be the proper vehicle for gene transfer either in ES cells or in embryos for the generation of transgenic mice.

Using pluripotent stem cell derived cells for regenerative medicine carries the potential risk of tumor formation due to the contamination with residual undifferentiated cells (Blum & Benvenisty, 2008; Nussbaum et al, 2007; Wakitani et al, 2003). Therefore, the specific

elimination of pluripotent stem cells was investigated in the second part of this work. Pluripotent stem cells including ES and iPS cells were transduced with LVs carrying thymidine kinase (TK) from Herpes simplex virus under control of promoters of pluripotency genes. TK expressing cells are in turn sensitive to ganciclovir (GCV) as TK converts GCV into a toxic metabolite leading to cell death. Treatment of TK transduced pluripotent cells in not further selected (mixed) cell populations with GCV led indeed to a high elimination of undifferentiated cells. Although picked single LV-transduced ES cell clones showed a higher killing efficiency upon GCV treatment complete elimination of undifferentiated cells was not observed. However, raising TK expression by using the stronger EOS promoters in mixed ES cell populations did also not result in a significant reduction of undifferentiated cells. In order to obtain complete transduced cell populations, a pre-selection strategy with hygromycin resistance gene was thus applied. These STPH (LV-EOS-S4-TK-PGK-Hygromycin)-transduced ES cell populations carrying only low copy number of LVs demonstrated a complete elimination of undifferentiated cells after Hygromycin pre-selection upon GCV administration *in vitro*. More importantly, no teratoma was observed when pre-selected cells were injected into mice that were treated with GCV. These *in vivo* data confirmed the *in vitro* findings and suggested that STPH-transduced ES cells with pre-selection seemed to be completely eliminated upon GCV treatment if they did not undergo differentiation. This gives rise to possible promising applications in future clinical approaches.

6. Curriculum vitae

7. Publications

Parts of the results of this thesis were compiled to a manuscript and submitted for publication (see below).

Tiong-Ti Lim, Caroline Geisen, Michael Hesse, Bernd K. Fleischmann, Katrin Zimmermann, Alexander Pfeifer (2013).

Lentiviral vector mediated thymidine kinase expression in pluripotent stem cells enables removal of tumorogenic cells.

submitted

Pfeifer, A., **Lim, T**., Zimmermann, K. (2010).

Lentivirus transgenesis.

Methods in Enzymology 477, 3-15

Reichenbach M, **Lim T**, Reichenbach HD, Guengoer T, Habermann FA, Matthiesen M, Hofmann A, Weber F, Zerbe H, Grupp T, Sinowatz F, Pfeifer A, Wolf E. (2009)

Germ-line transmission of lentiviral PGK-EGFP integrants in transgenic cattle: new perspectives for experimental embryology.

Transgenic Res 19, 549-556

8. References

Abordo-Adesida E, Follenzi A, Barcia C, Sciascia S, Castro MG, Naldini L, Lowenstein PR (2005) Stability of lentiviral vector-mediated transgene expression in the brain in the presence of systemic antivector immune responses. *Hum Gene Ther* **16**(6)**:** 741-751

Assady S, Maor G, Amit M, Itskovitz-Eldor J, Skorecki KL, Tzukerman M (2001) Insulin production by human embryonic stem cells. *Diabetes* **50**(8)**:** 1691-1697

Bednarik DP, Cook JA, Pitha PM (1990) Inactivation of the HIV LTR by DNA CpG methylation: evidence for a role in latency. *EMBO J* **9**(4)**:** 1157-1164

Berger EA, Murphy PM, Farber JM (1999) Chemokine receptors as HIV-1 coreceptors: roles in viral entry, tropism, and disease. *Annu Rev Immunol* **17:** 657-700

Bird AP, Wolffe AP (1999) Methylation-induced repression--belts, braces, and chromatin. *Cell* **99**(5)**:** 451-454

Bjorklund LM, Sanchez-Pernaute R, Chung S, Andersson T, Chen IY, McNaught KS, Brownell AL, Jenkins BG, Wahlestedt C, Kim KS, Isacson O (2002) Embryonic stem cells develop into functional dopaminergic neurons after transplantation in a Parkinson rat model. *Proc Natl Acad Sci U S A* **99**(4)**:** 2344-2349

Blum B, Benvenisty N (2008) The tumorigenicity of human embryonic stem cells. *Adv Cancer Res* **100:** 133-158

Boer B, Kopp J, Mallanna S, Desler M, Chakravarthy H, Wilder PJ, Bernadt C, Rizzino A (2007) Elevating the levels of Sox2 in embryonal carcinoma cells and embryonic stem cells inhibits the expression of Sox2:Oct-3/4 target genes. *Nucleic Acids Res* **35**(6)**:** 1773-1786

Boheler KR, Czyz J, Tweedie D, Yang HT, Anisimov SV, Wobus AM (2002) Differentiation of pluripotent embryonic stem cells into cardiomyocytes. *Circ Res* **91**(3)**:** 189-201

Bonini C, Bondanza A, Perna SK, Kaneko S, Traversari C, Ciceri F, Bordignon C (2007) The suicide gene therapy challenge: how to improve a successful gene therapy approach. *Mol Ther* **15**(7)**:** 1248-1252

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:** 248-254

Burnette WN (1981) "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate--polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal Biochem* **112**(2)**:** 195-203

Bushman F (2002) Targeting retroviral integration? *Mol Ther* **6**(5)**:** 570-571

Butler SL, Hansen MS, Bushman FD (2001) A quantitative assay for HIV DNA integration in vivo. *Nat Med* **7**(5)**:** 631-634

Carpenter MK, Inokuma MS, Denham J, Mujtaba T, Chiu CP, Rao MS (2001) Enrichment of neurons and neural precursors from human embryonic stem cells. *Exp Neurol* **172**(2)**:** 383-397

Cartier N, Hacein-Bey-Abina S, Bartholomae CC, Veres G, Schmidt M, Kutschera I, Vidaud M, Abel U, Dal-Cortivo L, Caccavelli L, Mahlaoui N, Kiermer V, Mittelstaedt D, Bellesme C, Lahlou N, Lefrere F, Blanche S, Audit M, Payen E, Leboulch P, l'Homme B, Bougneres P, Von Kalle C, Fischer A, Cavazzana-Calvo M, Aubourg P (2009) Hematopoietic stem cell gene therapy with a lentiviral vector in X-linked adrenoleukodystrophy. *Science* **326**(5954)**:** 818-823

Cavazzana-Calvo M, Payen E, Negre O, Wang G, Hehir K, Fusil F, Down J, Denaro M, Brady T, Westerman K, Cavallesco R, Gillet-Legrand B, Caccavelli L, Sgarra R, Maouche-Chretien L, Bernaudin F, Girot R, Dorazio R, Mulder GJ, Polack A, Bank A, Soulier J, Larghero J, Kabbara N, Dalle B, Gourmel B, Socie G, Chretien S, Cartier N, Aubourg P, Fischer A, Cornetta K, Galacteros F, Beuzard Y, Gluckman E, Bushman F, Hacein-Bey-Abina S, Leboulch P (2010) Transfusion independence and HMGA2 activation after gene therapy of human beta-thalassaemia. *Nature* **467**(7313)**:** 318-322

Challita PM, Kohn DB (1994) Lack of expression from a retroviral vector after transduction of murine hematopoietic stem cells is associated with methylation in vivo. *Proc Natl Acad Sci U S A* **91**(7)**:** 2567-2571

Chambers I, Colby D, Robertson M, Nichols J, Lee S, Tweedie S, Smith A (2003) Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. *Cell* **113**(5)**:** 643-655

Chan AW, Homan EJ, Ballou LU, Burns JC, Bremel RD (1998) Transgenic cattle produced by reverse-transcribed gene transfer in oocytes. *Proc Natl Acad Sci U S A* **95**(24)**:** 14028-14033

Cheng F, Ke Q, Chen F, Cai B, Gao Y, Ye C, Wang D, Zhang L, Lahn BT, Li W, Xiang AP (2012) Protecting against wayward human induced pluripotent stem cells with a suicide gene. *Biomaterials* **33**(11)**:** 3195-3204

Cherry SR, Biniszkiewicz D, van Parijs L, Baltimore D, Jaenisch R (2000) Retroviral expression in embryonic stem cells and hematopoietic stem cells. *Mol Cell Biol* **20**(20)**:** 7419-7426

Chirmule N, Propert K, Magosin S, Qian Y, Qian R, Wilson J (1999) Immune responses to adenovirus and adeno-associated virus in humans. *Gene Ther* **6**(9)**:** 1574-1583

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* **162**(1)**:** 156-159

Choo AB, Tan HL, Ang SN, Fong WJ, Chin A, Lo J, Zheng L, Hentze H, Philp RJ, Oh SK, Yap M (2008) Selection against undifferentiated human embryonic stem cells by a cytotoxic antibody recognizing podocalyxin-like protein-1. *Stem Cells* **26**(6)**:** 1454-1463

Clark SJ, Harrison J, Paul CL, Frommer M (1994) High sensitivity mapping of methylated cytosines. *Nucleic Acids Res* **22**(15)**:** 2990-2997

Clever JL, Taplitz RA, Lochrie MA, Polisky B, Parslow TG (2000) A heterologous, high-affinity RNA ligand for human immunodeficiency virus Gag protein has RNA packaging activity. *J Virol* **74**(1)**:** 541-546

Coffin J (1990) Retroviridae and their replication. In *Virology*, Fields B, Knipe D (eds), 2nd edn, pp 1437-1500. *Raven Press*

Coffin JM (1992) Structure and Classification of Retroviruses. In *The Retroviridae*, Levy J (ed), pp 19-50. New York: Plenum Press

De Meyer SF, Vanhoorelbeke K, Chuah MK, Pareyn I, Gillijns V, Hebbel RP, Collen D, Deckmyn H, VandenDriessche T (2006) Phenotypic correction of von Willebrand disease type 3 blood-derived endothelial cells with lentiviral vectors expressing von Willebrand factor. *Blood* **107**(12)**:** 4728-4736

Delenda C (2004) Lentiviral vectors: optimization of packaging, transduction and gene expression. *J Gene Med* **6 Suppl 1:** S125-138

Doetschman TC, Eistetter H, Katz M, Schmidt W, Kemler R (1985) The in vitro development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium. *J Embryol Exp Morphol* **87:** 27-45

Dull T, Zufferey R, Kelly M, Mandel RJ, Nguyen M, Trono D, Naldini L (1998) A third-generation lentivirus vector with a conditional packaging system. *J Virol* **72**(11)**:** 8463-8471

Evans MJ, Kaufman MH (1981) Establishment in culture of pluripotential cells from mouse embryos. *Nature* **292**(5819)**:** 154-156

Fassati A (2006) HIV infection of non-dividing cells: a divisive problem. *Retrovirology* **3:** 74

Fassler R (2004) Lentiviral transgene vectors. *EMBO Rep* **5**(1)**:** 28-29

Fischer U, Meyer S, Teufel M, Heckel C, Luhrmann R, Rautmann G (1994) Evidence that HIV-1 Rev directly promotes the nuclear export of unspliced RNA. *EMBO J* **13**(17)**:** 4105-4112

Follenzi A, Ailles LE, Bakovic S, Geuna M, Naldini L (2000) Gene transfer by lentiviral vectors is limited by nuclear translocation and rescued by HIV-1 pol sequences. *Nat Genet* **25**(2)**:** 217-222

Frommer M, McDonald LE, Millar DS, Collis CM, Watt F, Grigg GW, Molloy PL, Paul CL (1992) A genomic sequencing protocol that yields a positive display of 5-

methylcytosine residues in individual DNA strands. *Proc Natl Acad Sci U S A* **89**(5)**:** 1827-1831

Fuks F, Hurd PJ, Wolf D, Nan X, Bird AP, Kouzarides T (2003) The methyl-CpG-binding protein MeCP2 links DNA methylation to histone methylation. *J Biol Chem* **278**(6)**:** 4035-4040

Gallay P, Hope T, Chin D, Trono D (1997) HIV-1 infection of nondividing cells through the recognition of integrase by the importin/karyopherin pathway. *Proc Natl Acad Sci U S A* **94**(18)**:** 9825-9830

Georgoudaki AM, Sutlu T, Alici E (2010) Suicide gene therapy for graft-versus-host disease. *Immunotherapy* **2**(4)**:** 521-537

Geraghty RJ, Talbot KJ, Callahan M, Harper W, Panganiban AT (1994) Cell type-dependence for Vpu function. *J Med Primatol* **23**(2-3)**:** 146-150

Goodwin NC, Ishida Y, Hartford S, Wnek C, Bergstrom RA, Leder P, Schimenti JC (2001) DelBank: a mouse ES-cell resource for generating deletions. *Nat Genet* **28**(4)**:** 310-311

Gropp M, Shilo V, Vainer G, Gov M, Gil Y, Khaner H, Matzrafi L, Idelson M, Kopolovic J, Zak NB, Reubinoff BE (2012) Standardization of the Teratoma Assay for Analysis of Pluripotency of Human ES Cells and Biosafety of Their Differentiated Progeny. *PLoS One* **7**(9)**:** e45532

Gross L (1951a) Pathogenic properties, and "vertical" transmission of the mouse leukemia agent. *Proc Soc Exp Biol Med* **78**(1)**:** 342-348

Gross L (1951b) "Spontaneous" leukemia developing in C3H mice following inoculation in infancy, with AK-leukemic extracts, or AK-embrvos. *Proc Soc Exp Biol Med* **76**(1)**:** 27-32

Hacein-Bey-Abina S, Garrigue A, Wang GP, Soulier J, Lim A, Morillon E, Clappier E, Caccavelli L, Delabesse E, Beldjord K, Asnafi V, MacIntyre E, Dal Cortivo L, Radford I, Brousse N, Sigaux F, Moshous D, Hauer J, Borkhardt A, Belohradsky BH, Wintergerst U, Velez MC, Leiva L, Sorensen R, Wulffraat N, Blanche S, Bushman FD, Fischer A, Cavazzana-Calvo M (2008) Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1. *J Clin Invest* **118**(9)**:** 3132-3142

Hacein-Bey-Abina S, von Kalle C, Schmidt M, Le Deist F, Wulffraat N, McIntyre E, Radford I, Villeval JL, Fraser CC, Cavazzana-Calvo M, Fischer A (2003) A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. *N Engl J Med* **348**(3)**:** 255-256

Hamaguchi I, Woods NB, Panagopoulos I, Andersson E, Mikkola H, Fahlman C, Zufferey R, Carlsson L, Trono D, Karlsson S (2000) Lentivirus vector gene expression during ES cell-derived hematopoietic development in vitro. *J Virol* **74**(22)**:** 10778-10784

Hayatsu H, Wataya Y, Kai K, Iida S (1970) Reaction of sodium bisulfite with uracil, cytosine, and their derivatives. *Biochemistry* **9**(14)**:** 2858-2865

He J, Yang Q, Chang LJ (2005) Dynamic DNA methylation and histone modifications contribute to lentiviral transgene silencing in murine embryonic carcinoma cells. *J Virol* **79**(21)**:** 13497-13508

Hirsch MS, Curran J. (1996) Human immunodeficience viruese. In Fields B, Knipe D, Howley PM (eds.). Lipincott-Raven, Philadelphia, pp. 953–1975.

Hofmann A, Kessler B, Ewerling S, Kabermann A, Brem G, Wolf E, Pfeifer A (2006) Epigenetic regulation of lentiviral transgene vectors in a large animal model. *Mol Ther* **13**(1)**:** 59-66

Hofmann A, Kessler B, Ewerling S, Weppert M, Vogg B, Ludwig H, Stojkovic M, Boelhauve M, Brem G, Wolf E, Pfeifer A (2003) Efficient transgenesis in farm animals by lentiviral vectors. *EMBO Rep* **4**(11)**:** 1054-1060

Hofmann A, Zakhartchenko V, Weppert M, Sebald H, Wenigerkind H, Brem G, Wolf E, Pfeifer A (2004) Generation of transgenic cattle by lentiviral gene transfer into oocytes. *Biol Reprod* **71**(2)**:** 405-409

Hotta A, Cheung AY, Farra N, Vijayaragavan K, Seguin CA, Draper JS, Pasceri P, Maksakova IA, Mager DL, Rossant J, Bhatia M, Ellis J (2009) Isolation of human iPS cells using EOS lentiviral vectors to select for pluripotency. *Nat Methods* **6**(5)**:** 370-376

Howe SJ, Mansour MR, Schwarzwaelder K, Bartholomae C, Hubank M, Kempski H, Brugman MH, Pike-Overzet K, Chatters SJ, de Ridder D, Gilmour KC, Adams S, Thornhill SI, Parsley KL, Staal FJ, Gale RE, Linch DC, Bayford J, Brown L, Quaye M, Kinnon C, Ancliff P, Webb DK, Schmidt M, von Kalle C, Gaspar HB, Thrasher AJ (2008) Insertional mutagenesis combined with acquired somatic mutations causes leukemogenesis following gene therapy of SCID-X1 patients. *J Clin Invest* **118**(9)**:** 3143-3150

Huang X, Wilber AC, Bao L, Tuong D, Tolar J, Orchard PJ, Levine BL, June CH, McIvor RS, Blazar BR, Zhou X (2006) Stable gene transfer and expression in human primary T cells by the Sleeping Beauty transposon system. *Blood* **107**(2)**:** 483-491

Ikawa M, Tanaka N, Kao WW, Verma IM (2003) Generation of transgenic mice using lentiviral vectors: a novel preclinical assessment of lentiviral vectors for gene therapy. *Mol Ther* **8**(4)**:** 666-673

Jaenisch R, Dausman J, Cox V, Fan H (1976) Infection of developing mouse embryos with murine leukemia virus: tissue specificity and genetic transmission of the virus. *Hamatol Bluttransfus* **19:** 341-356

Jaenisch R, Fan H, Croker B (1975) Infection of preimplantation mouse embryos and of newborn mice with leukemia virus: tissue distribution of viral DNA and RNA and leukemogenesis in the adult animal. *Proc Natl Acad Sci U S A* **72**(10)**:** 4008-4012

Jaenisch R, Harbers K, Jahner D, Stewart C, Stuhlmann H (1982) DNA methylation, retroviruses, and embryogenesis. *J Cell Biochem* **20**(4)**:** 331-336

Jahner D, Stuhlmann H, Stewart CL, Harbers K, Lohler J, Simon I, Jaenisch R (1982) De novo methylation and expression of retroviral genomes during mouse embryogenesis. *Nature* **298**(5875)**:** 623-628

Jarrosson-Wuilleme L, Goujon C, Bernaud J, Rigal D, Darlix JL, Cimarelli A (2006) Transduction of nondividing human macrophages with gammaretrovirus-derived vectors. *J Virol* **80**(3)**:** 1152-1159

Jones KA (1997) Taking a new TAK on tat transactivation. *Genes Dev* **11**(20)**:** 2593-2599

Jones PA, Baylin SB (2002) The fundamental role of epigenetic events in cancer. *Nat Rev Genet* **3**(6)**:** 415-428

Jones PL, Veenstra GJ, Wade PA, Vermaak D, Kass SU, Landsberger N, Strouboulis J, Wolffe AP (1998) Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. *Nat Genet* **19**(2)**:** 187-191

Jung J, Hackett NR, Pergolizzi RG, Pierre-Destine L, Krause A, Crystal RG (2007) Ablation of tumor-derived stem cells transplanted to the central nervous system by genetic modification of embryonic stem cells with a suicide gene. *Hum Gene Ther* **18**(12)**:** 1182-1192

Kao SY, Calman AF, Luciw PA, Peterlin BM (1987) Anti-termination of transcription within the long terminal repeat of HIV-1 by tat gene product. *Nature* **330**(6147)**:** 489-493

Kaufman DS, Hanson ET, Lewis RL, Auerbach R, Thomson JA (2001) Hematopoietic colony-forming cells derived from human embryonic stem cells. *Proc Natl Acad Sci U S A* **98**(19)**:** 10716-10721

Keller G (2005) Embryonic stem cell differentiation: emergence of a new era in biology and medicine. *Genes Dev* **19**(10)**:** 1129-1155

Kim JB, Sebastiano V, Wu G, Arauzo-Bravo MJ, Sasse P, Gentile L, Ko K, Ruau D, Ehrich M, van den Boom D, Meyer J, Hubner K, Bernemann C, Ortmeier C, Zenke M, Fleischmann BK, Zaehres H, Scholer HR (2009) Oct4-induced pluripotency in adult neural stem cells. *Cell* **136**(3)**:** 411-419

Kim VN, Mitrophanous K, Kingsman SM, Kingsman AJ (1998) Minimal requirement for a lentivirus vector based on human immunodeficiency virus type 1. *J Virol* **72**(1)**:** 811-816

Klug MG, Soonpaa MH, Koh GY, Field LJ (1996) Genetically selected cardiomyocytes from differentiating embronic stem cells form stable intracardiac grafts. *J Clin Invest* **98**(1)**:** 216-224

Kolossov E, Lu Z, Drobinskaya I, Gassanov N, Duan Y, Sauer H, Manzke O, Bloch W, Bohlen H, Hescheler J, Fleischmann BK (2005) Identification and characterization of embryonic stem cell-derived pacemaker and atrial cardiomyocytes. *FASEB J* **19**(6)**:** 577-579

Kubo H, Gardner TA, Wada Y, Koeneman KS, Gotoh A, Yang L, Kao C, Lim SD, Amin MB, Yang H, Black ME, Matsubara S, Nakagawa M, Gillenwater JY, Zhau HE, Chung

LW (2003) Phase I dose escalation clinical trial of adenovirus vector carrying osteocalcin promoter-driven herpes simplex virus thymidine kinase in localized and metastatic hormone-refractory prostate cancer. *Hum Gene Ther* **14**(3)**:** 227-241

Kulinski T, Olejniczak M, Huthoff H, Bielecki L, Pachulska-Wieczorek K, Das AT, Berkhout B, Adamiak RW (2003) The apical loop of the HIV-1 TAR RNA hairpin is stabilized by a cross-loop base pair. *J Biol Chem* **278**(40)**:** 38892-38901

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**(5259)**:** 680-685

Laird PW (2003) The power and the promise of DNA methylation markers. *Nat Rev Cancer* **3**(4)**:** 253-266

Laker C, Meyer J, Schopen A, Friel J, Heberlein C, Ostertag W, Stocking C (1998) Host cis-mediated extinction of a retrovirus permissive for expression in embryonal stem cells during differentiation. *J Virol* **72**(1)**:** 339-348

Lee G, Papapetrou EP, Kim H, Chambers SM, Tomishima MJ, Fasano CA, Ganat YM, Menon J, Shimizu F, Viale A, Tabar V, Sadelain M, Studer L (2009) Modelling pathogenesis and treatment of familial dysautonomia using patient-specific iPSCs. *Nature* **461**(7262)**:** 402-406

Lei H, Oh SP, Okano M, Juttermann R, Goss KA, Jaenisch R, Li E (1996) De novo DNA cytosine methyltransferase activities in mouse embryonic stem cells. *Development* **122**(10)**:** 3195-3205

Levenberg S, Golub JS, Amit M, Itskovitz-Eldor J, Langer R (2002) Endothelial cells derived from human embryonic stem cells. *Proc Natl Acad Sci U S A* **99**(7)**:** 4391-4396

Lever AM, Strappe PM, Zhao J (2004) Lentiviral vectors. *J Biomed Sci* **11**(4)**:** 439-449

Levine BL, Humeau LM, Boyer J, MacGregor RR, Rebello T, Lu X, Binder GK, Slepushkin V, Lemiale F, Mascola JR, Bushman FD, Dropulic B, June CH (2006) Gene transfer in humans using a conditionally replicating lentiviral vector. *Proc Natl Acad Sci U S A* **103**(46)**:** 17372-17377

Lewinski MK, Yamashita M, Emerman M, Ciuffi A, Marshall H, Crawford G, Collins F, Shinn P, Leipzig J, Hannenhalli S, Berry CC, Ecker JR, Bushman FD (2006) Retroviral DNA integration: viral and cellular determinants of target-site selection. *PLoS Pathog* **2**(6)**:** e60

Lewis PF, Emerman M (1994) Passage through mitosis is required for oncoretroviruses but not for the human immunodeficiency virus. *J Virol* **68**(1)**:** 510-516

Li M, Pevny L, Lovell-Badge R, Smith A (1998) Generation of purified neural precursors from embryonic stem cells by lineage selection. *Curr Biol* **8**(17)**:** 971-974

Li W, Wei W, Zhu S, Zhu J, Shi Y, Lin T, Hao E, Hayek A, Deng H, Ding S (2009) Generation of rat and human induced pluripotent stem cells by combining genetic reprogramming and chemical inhibitors. *Cell Stem Cell* **4**(1)**:** 16-19

Li ZB, Zeng ZJ, Chen Q, Luo SQ, Hu WX (2006) Recombinant AAV-mediated HSVtk gene transfer with direct intratumoral injections and Tet-On regulation for implanted human breast cancer. *BMC Cancer* **6:** 66

Liu XH, Xu W, Russ J, Eiden LE, Eiden MV (2011) The host range of gammaretroviruses and gammaretroviral vectors includes post-mitotic neural cells. *PLoS One* **6**(3)**:** e18072

Lois C, Hong EJ, Pease S, Brown EJ, Baltimore D (2002) Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. *Science* **295**(5556)**:** 868-872

Lorincz MC, Schubeler D, Goeke SC, Walters M, Groudine M, Martin DI (2000) Dynamic analysis of proviral induction and De Novo methylation: implications for a histone deacetylase-independent, methylation density-dependent mechanism of transcriptional repression. *Mol Cell Biol* **20**(3)**:** 842-850

Lupo-Stanghellini MT, Provasi E, Bondanza A, Ciceri F, Bordignon C, Bonini C (2010) Clinical impact of suicide gene therapy in allogeneic hematopoietic stem cell transplantation. *Hum Gene Ther* **21**(3)**:** 241-250

Maehr R, Chen S, Snitow M, Ludwig T, Yagasaki L, Goland R, Leibel RL, Melton DA (2009) Generation of pluripotent stem cells from patients with type 1 diabetes. *Proc Natl Acad Sci U S A* **106**(37)**:** 15768-15773

Marin M, Rose KM, Kozak SL, Kabat D (2003) HIV-1 Vif protein binds the editing enzyme APOBEC3G and induces its degradation. *Nat Med* **9**(11)**:** 1398-1403

Matrai J, Chuah MK, VandenDriessche T (2010) Recent advances in lentiviral vector development and applications. *Mol Ther* **18**(3)**:** 477-490

Mauritz C, Schwanke K, Reppel M, Neef S, Katsirntaki K, Maier LS, Nguemo F, Menke S, Haustein M, Hescheler J, Hasenfuss G, Martin U (2008) Generation of functional murine cardiac myocytes from induced pluripotent stem cells. *Circulation* **118**(5)**:** 507-517

May C, Rivella S, Callegari J, Heller G, Gaensler KM, Luzzatto L, Sadelain M (2000) Therapeutic haemoglobin synthesis in beta-thalassaemic mice expressing lentivirus-encoded human beta-globin. *Nature* **406**(6791)**:** 82-86

Mingozzi F, Maus MV, Hui DJ, Sabatino DE, Murphy SL, Rasko JE, Ragni MV, Manno CS, Sommer J, Jiang H, Pierce GF, Ertl HC, High KA (2007) CD8(+) T-cell responses to adeno-associated virus capsid in humans. *Nat Med* **13**(4)**:** 419-422

Mitchell RS, Beitzel BF, Schroder AR, Shinn P, Chen H, Berry CC, Ecker JR, Bushman FD (2004) Retroviral DNA integration: ASLV, HIV, and MLV show distinct target site preferences. *PLoS Biol* **2**(8)**:** E234

Mitsui K, Tokuzawa Y, Itoh H, Segawa K, Murakami M, Takahashi K, Maruyama M, Maeda M, Yamanaka S (2003) The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. *Cell* **113**(5)**:** 631-642

Miyoshi H, Blomer U, Takahashi M, Gage FH, Verma IM (1998) Development of a self-inactivating lentivirus vector. *J Virol* **72**(10)**:** 8150-8157

Modlich U, Navarro S, Zychlinski D, Maetzig T, Knoess S, Brugman MH, Schambach A, Charrier S, Galy A, Thrasher AJ, Bueren J, Baum C (2009) Insertional transformation of hematopoietic cells by self-inactivating lentiviral and gammaretroviral vectors. *Mol Ther* **17**(11)**:** 1919-1928

Modrow S, Falke D, Truyen U, Schätzl H (2010) *Molekulare Virologie*, Vol. 3. Auflage Heiderberg: Spektrum Akademischer Verlag.

Nagy A, Rossant J, Nagy R, Abramow-Newerly W, Roder JC (1993) Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. *Proc Natl Acad Sci U S A* **90**(18)**:** 8424-8428

Naldini L, Blomer U, Gallay P, Ory D, Mulligan R, Gage FH, Verma IM, Trono D (1996) In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* **272**(5259)**:** 263-267

Nan X, Ng HH, Johnson CA, Laherty CD, Turner BM, Eisenman RN, Bird A (1998) Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. *Nature* **393**(6683)**:** 386-389

Naujok O, Kaldrack J, Taivankhuu T, Jorns A, Lenzen S (2010) Selective removal of undifferentiated embryonic stem cells from differentiation cultures through HSV1 thymidine kinase and ganciclovir treatment. *Stem Cell Rev* **6**(3)**:** 450-461

Naviaux RK, Costanzi E, Haas M, Verma IM (1996) The pCL vector system: rapid production of helper-free, high-titer, recombinant retroviruses. *J Virol* **70**(8)**:** 5701-5705

Niwa H, Miyazaki J, Smith AG (2000) Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nat Genet* **24**(4)**:** 372-376

Nussbaum J, Minami E, Laflamme MA, Virag JA, Ware CB, Masino A, Muskheli V, Pabon L, Reinecke H, Murry CE (2007) Transplantation of undifferentiated murine embryonic stem cells in the heart: teratoma formation and immune response. *FASEB J* **21**(7)**:** 1345-1357

Okada Y, Ueshin Y, Isotani A, Saito-Fujita T, Nakashima H, Kimura K, Mizoguchi A, Oh-Hora M, Mori Y, Ogata M, Oshima RG, Okabe M, Ikawa M (2007) Complementation of placental defects and embryonic lethality by trophoblast-specific lentiviral gene transfer. *Nat Biotechnol* **25**(2)**:** 233-237

Okano M, Bell DW, Haber DA, Li E (1999) DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell* **99**(3)**:** 247-257

Osborne CS, Pasceri P, Singal R, Sukonnik T, Ginder GD, Ellis J (1999) Amelioration of retroviral vector silencing in locus control region beta-globin-transgenic mice and transduced F9 embryonic cells. *J Virol* **73**(7)**:** 5490-5496

Ott MG, Schmidt M, Schwarzwaelder K, Stein S, Siler U, Koehl U, Glimm H, Kuhlcke K, Schilz A, Kunkel H, Naundorf S, Brinkmann A, Deichmann A, Fischer M, Ball C, Pilz I, Dunbar C, Du Y, Jenkins NA, Copeland NG, Luthi U, Hassan M, Thrasher AJ, Hoelzer D, von Kalle C, Seger R, Grez M (2006) Correction of X-linked chronic granulomatous disease by gene therapy, augmented by insertional activation of MDS1-EVI1, PRDM16 or SETBP1. *Nat Med* **12**(4)**:** 401-409

Ovitt CE, Scholer HR (1998) The molecular biology of Oct-4 in the early mouse embryo. *Mol Hum Reprod* **4**(11)**:** 1021-1031

Pannell D, Osborne CS, Yao S, Sukonnik T, Pasceri P, Karaiskakis A, Okano M, Li E, Lipshitz HD, Ellis J (2000) Retrovirus vector silencing is de novo methylase independent and marked by a repressive histone code. *EMBO J* **19**(21)**:** 5884-5894

Pfeifer A, Eigenbrod S, Al-Khadra S, Hofmann A, Mitteregger G, Moser M, Bertsch U, Kretzschmar H (2006) Lentivector-mediated RNAi efficiently suppresses prion protein and prolongs survival of scrapie-infected mice. *J Clin Invest* **116**(12)**:** 3204-3210

Pfeifer A, Hofmann A (2009) Lentiviral transgenesis. *Methods Mol Biol* **530:** 391-405

Pfeifer A, Ikawa M, Dayn Y, Verma IM (2002) Transgenesis by lentiviral vectors: lack of gene silencing in mammalian embryonic stem cells and preimplantation embryos. *Proc Natl Acad Sci U S A* **99**(4)**:** 2140-2145

Pfeifer A, Lim T, Zimmermann K (2010) Lentivirus transgenesis. *Methods Enzymol* **477:** 3-15

Pfeifer A, Verma IM (2001a) Gene therapy: promises and problems. *Annu Rev Genomics Hum Genet* **2:** 177-211

Pfeifer A, Verma IM (2001b) Virus vectors and their application. PM H, DM K, D G, RA L, A M, B R, SE S (eds), Vol. 1, Fourth edn, pp 469-491. Philadelphia, Lippincott-Raven Publishers

Pollard VW, Malim MH (1998) The HIV-1 Rev protein. *Annu Rev Microbiol* **52:** 491-532

Portsmouth D, Hlavaty J, Renner M (2007) Suicide genes for cancer therapy. *Mol Aspects Med* **28**(1)**:** 4-41

Qasim W, Vink CA, Thrasher AJ (2010) Hybrid lentiviral vectors. *Mol Ther* **18**(7)**:** 1263-1267

Quinn G, Ochiya T, Terada M, Yoshida T (2000) Mouse flt-1 promoter directs endothelial-specific expression in the embyroid body model of embryogenesis. *Biochem Biophys Res Commun* **276**(3)**:** 1089-1099

Raya A, Rodriguez-Piza I, Guenechea G, Vassena R, Navarro S, Barrero MJ, Consiglio A, Castella M, Rio P, Sleep E, Gonzalez F, Tiscornia G, Garreta E, Aasen T, Veiga A, Verma IM, Surralles J, Bueren J, Izpisua Belmonte JC (2009) Disease-corrected haematopoietic progenitors from Fanconi anaemia induced pluripotent stem cells. *Nature* **460**(7251)**:** 53-59

Rein A (2011) Murine leukemia viruses: objects and organisms. *Adv Virol* **2011:** 403419

Reiser J, Harmison G, Kluepfel-Stahl S, Brady RO, Karlsson S, Schubert M (1996) Transduction of nondividing cells using pseudotyped defective high-titer HIV type 1 particles. *Proc Natl Acad Sci U S A* **93**(26)**:** 15266-15271

Robinton DA, Daley GQ (2012) The promise of induced pluripotent stem cells in research and therapy. *Nature* **481**(7381)**:** 295-305

Rohdewohld H, Weiher H, Reik W, Jaenisch R, Breindl M (1987) Retrovirus integration and chromatin structure: Moloney murine leukemia proviral integration sites map near DNase I-hypersensitive sites. *J Virol* **61**(2)**:** 336-343

Rong Z, Fu X, Wang M, Xu Y (2012) A scalable approach to prevent teratoma formation of human embryonic stem cells. *J Biol Chem*

Rubinson DA, Dillon CP, Kwiatkowski AV, Sievers C, Yang L, Kopinja J, Rooney DL, Zhang M, Ihrig MM, McManus MT, Gertler FB, Scott ML, Van Parijs L (2003) A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. *Nat Genet* **33**(3)**:** 401-406

Russell DW, Miller AD (1996) Foamy virus vectors. *J Virol* **70**(1)**:** 217-222

Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**(4839)**:** 487-491

Sambrook J, Russell D (2001) *Molecular Cloning: A Laboratory Manual.*
, 3 edn. New York.: Spring Harbor Laboratory Press.

Schambach A, Bohne J, Chandra S, Will E, Margison GP, Williams DA, Baum C (2006a) Equal potency of gammaretroviral and lentiviral SIN vectors for expression of O6-methylguanine-DNA methyltransferase in hematopoietic cells. *Mol Ther* **13**(2)**:** 391-400

Schambach A, Mueller D, Galla M, Verstegen MM, Wagemaker G, Loew R, Baum C, Bohne J (2006b) Overcoming promoter competition in packaging cells improves production of self-inactivating retroviral vectors. *Gene Ther* **13**(21)**:** 1524-1533

Schroder AR, Shinn P, Chen H, Berry C, Ecker JR, Bushman F (2002) HIV-1 integration in the human genome favors active genes and local hotspots. *Cell* **110**(4)**:** 521-529

Schubert U, Bour S, Ferrer-Montiel AV, Montal M, Maldarell F, Strebel K (1996a) The two biological activities of human immunodeficiency virus type 1 Vpu protein involve two separable structural domains. *J Virol* **70**(2)**:** 809-819

Schubert U, Ferrer-Montiel AV, Oblatt-Montal M, Henklein P, Strebel K, Montal M (1996b) Identification of an ion channel activity of the Vpu transmembrane domain and its involvement in the regulation of virus release from HIV-1-infected cells. *FEBS Lett* **398**(1)**:** 12-18

Schuldiner M, Itskovitz-Eldor J, Benvenisty N (2003) Selective ablation of human embryonic stem cells expressing a "suicide" gene. *Stem Cells* **21**(3)**:** 257-265

Schwartz SD, Hubschman JP, Heilwell G, Franco-Cardenas V, Pan CK, Ostrick RM, Mickunas E, Gay R, Klimanskaya I, Lanza R (2012) Embryonic stem cell trials for macular degeneration: a preliminary report. *Lancet* **379**(9817)**:** 713-720

Soldner F, Hockemeyer D, Beard C, Gao Q, Bell GW, Cook EG, Hargus G, Blak A, Cooper O, Mitalipova M, Isacson O, Jaenisch R (2009) Parkinson's disease patient-derived induced pluripotent stem cells free of viral reprogramming factors. *Cell* **136**(5)**:** 964-977

Soria B, Roche E, Berna G, Leon-Quinto T, Reig JA, Martin F (2000) Insulin-secreting cells derived from embryonic stem cells normalize glycemia in streptozotocin-induced diabetic mice. *Diabetes* **49**(2)**:** 157-162

Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol* **98**(3)**:** 503-517

Speers WC, Gautsch JW, Dixon FJ (1980) Silent infection of murine embryonal carcinoma cells by Moloney murine leukemia virus. *Virology* **105**(1)**:** 241-244

Starcich B, Ratner L, Josephs SF, Okamoto T, Gallo RC, Wong-Staal F (1985) Characterization of long terminal repeat sequences of HTLV-III. *Science* **227**(4686)**:** 538-540

Sterman DH, Treat J, Litzky LA, Amin KM, Coonrod L, Molnar-Kimber K, Recio A, Knox L, Wilson JM, Albelda SM, Kaiser LR (1998) Adenovirus-mediated herpes simplex virus thymidine kinase/ganciclovir gene therapy in patients with localized malignancy: results of a phase I clinical trial in malignant mesothelioma. *Hum Gene Ther* **9**(7)**:** 1083-1092

Takahashi K, Okita K, Nakagawa M, Yamanaka S (2007a) Induction of pluripotent stem cells from fibroblast cultures. *Nat Protoc* **2**(12)**:** 3081-3089

Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S (2007b) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* **131**(5)**:** 861-872

Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* **126**(4)**:** 663-676

Tan HL, Fong WJ, Lee EH, Yap M, Choo A (2009) mAb 84, a cytotoxic antibody that kills undifferentiated human embryonic stem cells via oncosis. *Stem Cells* **27**(8)**:** 1792-1801

Tanaka M, Hadjantonakis AK, Vintersten K, Nagy A (2009) Aggregation chimeras: combining ES cells, diploid, and tetraploid embryos. *Methods Mol Biol* **530:** 287-309

Tang C, Lee AS, Volkmer JP, Sahoo D, Nag D, Mosley AR, Inlay MA, Ardehali R, Chavez SL, Pera RR, Behr B, Wu JC, Weissman IL, Drukker M (2011) An antibody against SSEA-5 glycan on human pluripotent stem cells enables removal of teratoma-forming cells. *Nat Biotechnol* **29**(9)**:** 829-834

Thatava T, Armstrong AS, De Lamo JG, Edukulla R, Khan YK, Sakuma T, Ohmine S, Sundsbak JL, Harris PC, Kudva YC, Ikeda Y (2011) Successful disease-specific induced pluripotent stem cell generation from patients with kidney transplantation. *Stem Cell Res Ther* **2**(6)**:** 48

Thomsen DR, Stenberg RM, Goins WF, Stinski MF (1984) Promoter-regulatory region of the major immediate early gene of human cytomegalovirus. *Proc Natl Acad Sci U S A* **81**(3)**:** 659-663

Van Maele B, De Rijck J, De Clercq E, Debyser Z (2003) Impact of the central polypurine tract on the kinetics of human immunodeficiency virus type 1 vector transduction. *J Virol* **77**(8)**:** 4685-4694

Vijaya S, Steffen DL, Robinson HL (1986) Acceptor sites for retroviral integrations map near DNase I-hypersensitive sites in chromatin. *J Virol* **60**(2)**:** 683-692

Vogt P (1997) Historical Introduction to the General Properties of Retroviruses. In *Retroviruses*, Coffin J, Hughes S, Varmus H (eds), pp 1-25. New York: Cold Spring Harbor Laboratory Press

Wakitani S, Takaoka K, Hattori T, Miyazawa N, Iwanaga T, Takeda S, Watanabe TK, Tanigami A (2003) Embryonic stem cells injected into the mouse knee joint form teratomas and subsequently destroy the joint. *Rheumatology (Oxford)* **42**(1)**:** 162-165

Wang GP, Levine BL, Binder GK, Berry CC, Malani N, McGarrity G, Tebas P, June CH, Bushman FD (2009) Analysis of lentiviral vector integration in HIV+ study subjects receiving autologous infusions of gene modified CD4+ T cells. *Mol Ther* **17**(5)**:** 844-850

Warren L, Manos PD, Ahfeldt T, Loh YH, Li H, Lau F, Ebina W, Mandal PK, Smith ZD, Meissner A, Daley GQ, Brack AS, Collins JJ, Cowan C, Schlaeger TM, Rossi DJ (2010) Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. *Cell Stem Cell* **7**(5)**:** 618-630

Wells K, Moore K, Wall R (1999) Transgene vectors go retro. *Nat Biotechnol* **17**(1)**:** 25-26

Wernig M, Zhao JP, Pruszak J, Hedlund E, Fu D, Soldner F, Broccoli V, Constantine-Paton M, Isacson O, Jaenisch R (2008) Neurons derived from reprogrammed fibroblasts functionally integrate into the fetal brain and improve symptoms of rats with Parkinson's disease. *Proc Natl Acad Sci U S A* **105**(15)**:** 5856-5861

Wildner O, Morris JC, Vahanian NN, Ford H, Jr., Ramsey WJ, Blaese RM (1999) Adenoviral vectors capable of replication improve the efficacy of HSVtk/GCV suicide gene therapy of cancer. *Gene Ther* **6**(1)**:** 57-62

Wobus AM, Boheler KR (2005) Embryonic stem cells: prospects for developmental biology and cell therapy. *Physiol Rev* **85**(2)**:** 635-678

Wu X, Li Y, Crise B, Burgess SM (2003) Transcription start regions in the human genome are favored targets for MLV integration. *Science* **300**(5626)**:** 1749-1751

Wyatt R, Sodroski J (1998) The HIV-1 envelope glycoproteins: fusogens, antigens, and immunogens. *Science* **280**(5371)**:** 1884-1888

Xu W, Russ JL, Eiden MV (2012) Evaluation of residual promoter activity in gamma-retroviral self-inactivating (SIN) vectors. *Mol Ther* **20**(1)**:** 84-90

Yamanaka S, Blau HM (2010) Nuclear reprogramming to a pluripotent state by three approaches. *Nature* **465**(7299)**:** 704-712

Yamashita M, Emerman M (2004) Capsid is a dominant determinant of retrovirus infectivity in nondividing cells. *J Virol* **78**(11)**:** 5670-5678

Yamashita M, Emerman M (2006) Retroviral infection of non-dividing cells: old and new perspectives. *Virology* **344**(1)**:** 88-93

Yao S, Sukonnik T, Kean T, Bharadwaj RR, Pasceri P, Ellis J (2004) Retrovirus silencing, variegation, extinction, and memory are controlled by a dynamic interplay of multiple epigenetic modifications. *Mol Ther* **10**(1)**:** 27-36

Yu SF, von Ruden T, Kantoff PW, Garber C, Seiberg M, Ruther U, Anderson WF, Wagner EF, Gilboa E (1986) Self-inactivating retroviral vectors designed for transfer of whole genes into mammalian cells. *Proc Natl Acad Sci U S A* **83**(10)**:** 3194-3198

Zennou V, Petit C, Guetard D, Nerhbass U, Montagnier L, Charneau P (2000) HIV-1 genome nuclear import is mediated by a central DNA flap. *Cell* **101**(2)**:** 173-185

Zhou H, Wu S, Joo JY, Zhu S, Han DW, Lin T, Trauger S, Bien G, Yao S, Zhu Y, Siuzdak G, Scholer HR, Duan L, Ding S (2009) Generation of induced pluripotent stem cells using recombinant proteins. *Cell Stem Cell* **4**(5)**:** 381-384

Zielske SP, Lingas KT, Li Y, Gerson SL (2004) Limited lentiviral transgene expression with increasing copy number in an MGMT selection model: lack of copy number selection by drug treatment. *Mol Ther* **9**(6)**:** 923-931

Zufferey R, Donello JE, Trono D, Hope TJ (1999) Woodchuck hepatitis virus posttranscriptional regulatory element enhances expression of transgenes delivered by retroviral vectors. *J Virol* **73**(4)**:** 2886-2892

Zufferey R, Dull T, Mandel RJ, Bukovsky A, Quiroz D, Naldini L, Trono D (1998) Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery. *J Virol* **72**(12)**:** 9873-9880

Zufferey R, Nagy D, Mandel RJ, Naldini L, Trono D (1997) Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. *Nat Biotechnol* **15**(9)**:** 871-875