Aus dem Max von Pettenkofer-Institut für Hygiene und Medizinische Mikrobiologie
der Ludwig Maximilians-Universität München
Vorstand: Prof. Dr. Dr. h. c. Ulrich Koszinowski

Adenovirus-based gene therapy approaches for hemophilia B

Dissertation
zum Erwerb des Doktorgrades der Humanbiologie
an der Medizinischen Fakultät der
Ludwig-Maximilians-Universität zu München

vorgelegt von
Wenli Zhang
aus
Hebei, China

2012

Mit Genehmigung der Medizinischen Fakultät
der Universität München

Berichterstatter:	**Prof. Dr. Anja. Ehrhardt**
Mitberichterstatter:	**Prof. Dr. Dr. h. c. Wolfgang Schramm**
	Priv. Doz. Dr. Stefan Böck
Mitbetreuung durch den promovierten Mitarbeiter:	
Dekan:	**Prof. Dr. med. Dr. h.c. M. Reiser, FACR, FRCR**
Tag der mündlichen Prüfung:	**12. 06. 2012**

Table of contents

1. Introduction

1.1. Gene therapy: the novel medical concept of the future

1.1.1. Gene therapy and treated diseases

Gene therapy is the use of a genetic element to correct a deficiency in an individual's cells and biological tissues to treat disease. The common form of gene therapy is the introduction of therapeutic nucleotides into target cells via different vectors as delivery vehicle, resulting in the cure of diseases (Anderson, 1992). As a medical concept, gene therapy first emerged in the 1960s, when thousands of human diseases were distinguished to be genetically determined (McKusick, 1970; Friedmann and Roblin, 1972). Gene therapy has long been considered as a promising technique to treat both inherited and acquired diseases. Since the first human gene therapy trial was conducted in 1989 (Rosenberg *et al.*, 1990), up to now over 1700 clinical studies have been completed, are ongoing or have been approved (Wiley, 2011). These trials deal with different human diseases, involving for instance cancer, cardiovascular disease, inherited monogenic diseases and infectious disease **(Figure 1.1)**.

Cancer treatments took a good portion of these clinical trials, because of the enormous prevalence, impact and potentially fatal outcomes. So far, different types of cancer have been targeted, including head and neck, lung, skin and breast tumors, as well as nervous system and gastrointestinal tumors (Edelstein *et al.*, 2007; Bedognetti *et al.*, 2010). Distinct approaches are being investigated, mainly dealing with the delivery of tumour-suppressor genes, prodrug-activating genes and immunostimulatory genes into tumor cells

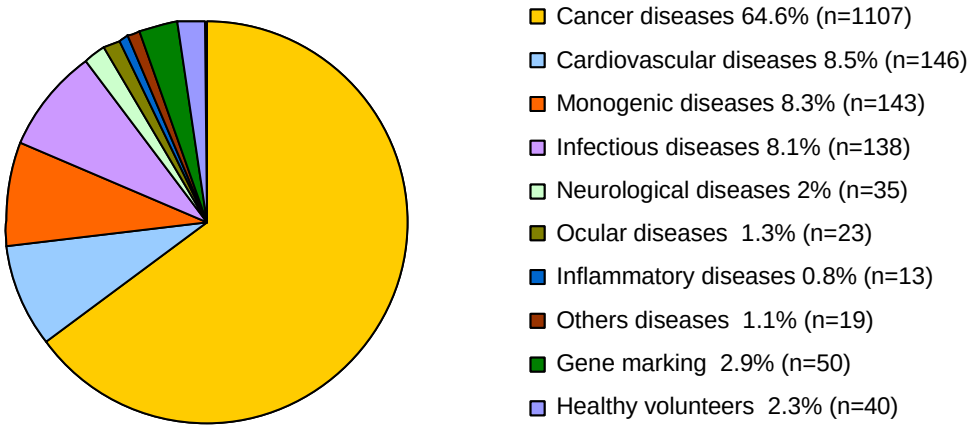


Figure 1.1: Overview of human diseases targeted by gene therapy clinical trials worldwide. As of June 2011, a total of 1714 gene therapy trials have been reported. A majority (64.6%) of trials were involved in the treatment of cancer, followed by cardiovascular disease (8.5%), inherited monogenic diseases (8.3%) and infectious diseases (8.1%). Modified from the Journal of gene Medicine, www.wiley.com/legacy/wileychi/genmed/clinical.

(McCormick, 2001; Wilson, 2002). An alternative option for cancer gene therapy is to harness the inherent ability of viruses to replicate and lyse cells, so called oncolytic viruses (Dalba *et al.*, 2005). The proposed future application of the oncolytic viruses is the safe tumour-specific replication, which is enabled by the deletion of viral genes that are necessary for replication in normal somatic cells. Thus the virus can only replicate in tumour cells in which the missing function is supplied (Van Den Wollenberg DJ *et al.*, 2009; Stanford *et al.*, 2010).

Further diseases targeted in human gene therapy clinical trials are cardiovascular disease, inherited monogenic diseases and infectious disease. As cardiovascular diseases is nowadays one major cause of morbidity and mortality in human population, gene therapy is emerging as a potential alternative for patients who are not candidates for traditional revascularization procedures (Jazwa *et al.*, 2007). The cardiovascular gene therapy is expected to show improvement in therapeutic angiogenesis, myocardial protection, regeneration and repair (Iverson *et al.*, 2008; Jones and Koch, 2005). However, most of cardiovascular gene therapy trials have addressed therapeutic angiogenesis to increase blood flow to ischemic regions (Barbato *et al.*, 2003; Edelstein *et al.*, 2007). Recently, the combination of different angiogenic growth factors or simultaneous applications of genes and progenitor cells in order to obtain stable and functional blood vessels in ischemic tissue have achieved promising success (Losordo and Dimmeler, 2004; Stamm *et al.*, 2008).

Gene therapy is being investigated as an alternative treatment for a wide range of infectious diseases that are not amenable to standard clinical management (Walter, 1995; Bunnell and Morgan, 1998). Gene therapy for infectious diseases requires the introduction of transgenes designed to specifically block or inhibit some particular virus gene expression or function of essential gene products, thus the replication of the infectious agent is blocked or limited. The therapeutic material for treatment could be nucleic acids like antisense DNA and RNA, protein as single-chain antibodies or even genetic vaccines, with the aim of inhibiting multiple stages of the viral life cycle (Lorenzi *et al.*, 2010; Liu, 2010). In clinical trials, human immunodeficiency virus (HIV) infected patients are the major research target (Gilboa and Smith, 1994; Sorg and Methali, 1997). However, most clinical trials aimed not at the treatment of AIDS or the HIV infection itself, but the secondary infection caused by tetanus, cytomegalovirus (CMV), hepatitis B/C and influenza virus (Edelstein *et al.*, 2007).

The treatment of monogenetic diseases, as an initial idea for gene therapy, is to correct the genetic disorder by replacing the defective gene with its functional counterpart (Kay and Woo, 1994). More than 20 different monogenic diseases have been targeted in clinical trials, mostly on cystic fibrosis, severe combined immunodeficiency syndromes (SCID), Duchenne muscular dystrophy and hemophilia (Edelstein *et al.*, 2007). SCID gene therapy studies for example, have been conducted in several countries by different research

groups, showing lasting and clinically meaningful therapeutic benefit (Aiuti *et al.*, 2002; Gaspar *et al.*, 2004; Fisher *et al.*, 1996). Another extensively studied monogenetic disease in the field of gene therapy is the clotting disorder- hemophilia, for which small and large animal models are available for preclinical testing (Evans *et al.*, 1989; Lin *et al.*, 1997; Jin *et al.*, 2004). Importantly, the efficiency of treatment can be easily quantified by well-defined and optimized assays. Therefore, hemophilia has been placed at the forefront of candidate diseases for gene therapy and also has been chosen by most researchers as a model disease for gene therapy studies (Kay, 1998; Herzog and High, 1998; Murphy and High, 2008).

1.1.2. Vectors used in gene therapy

The fast development of the gene delivery vectors is a prerequisite for gene therapy applications. A successful gene therapy system should have the following features: efficient gene delivery, specific targeting and expression and in many cases long-term transgene expression (Lundstrom, 2003). Major improvements have been achieved during the past decades. However, as it is obvious that there are no universally applicable ideal vector systems available, no single vector can be suitable for all applications. The various features of each vector and types of disease to be treated should be well studied and defined before the final decision is made as to which vector type is used for a certain disease.

So far, numerous vector systems have been developed, tested and applied in gene therapy clinical trials **(Figure 1.2)** (Wiley, 2011). They are usually divided into viral and non-viral vectors. Viral vectors have the advantage of the million-year biology evolution for

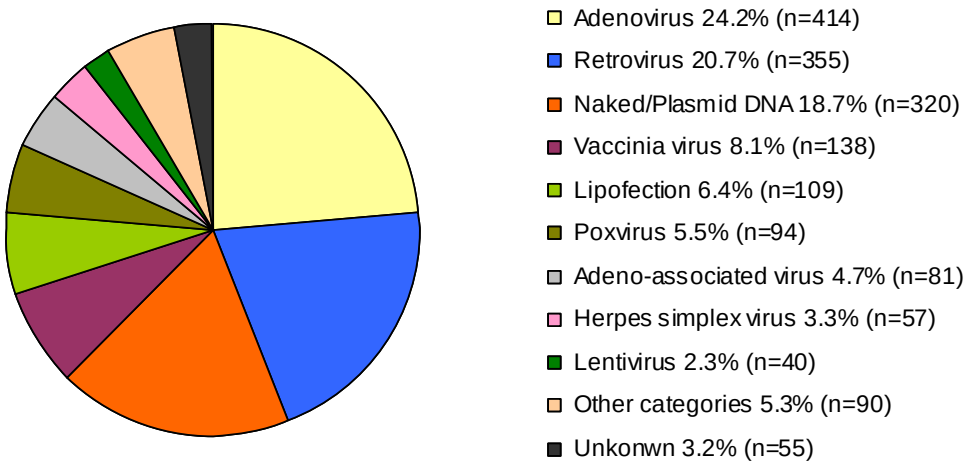


Figure 1.2: Overview of the vectors used in gene therapy clinical trials worldwide. Adenoviral (24.2%) and retroviral (20.7%) vectors were the most commonly used vectors in clinical gene therapy trials, while non-viral vectors, mainly naked/plasmid DNA (18.7%) and lipofection (6.4%), represent one quarter of all trials. Modified from the Journal of gene Medicine, www.wiley.com/legacy/wileychi/genmed/clinical.

efficient cellular delivery and long-term maintaining as an episome or integration in a chromosome (Walther and Stein, 2000; Young *et al.*, 2006). However, immune responses to viral particles and the risk of insertional mutagenesis raise major safety concerns for the use of viral vectors (Hacein-Bey-Abina *et al.*, 2003; Raper *et al.*, 2003; Pien *et al.*, 2009). As in gene therapy clinical trials, the most used viral vectors are adenoviral and retroviral vectors, followed by vaccinia virus, poxvirus, adeno-associated virus (AAV), herpes simplex virus (HSV) and lentivirus **(Figure 1.2)**. On the other hand, non-viral vectors emerged quickly as a safe alternative to viral vectors (Edelstein *et al.*, 2007; Edelstein *et al.*, 2004). The simplest non-viral vector system utilizes 'naked' DNA, which can be injected directly into certain tissues, particularly muscle, and can lead to significant levels of gene expression. However, the utility is limited by the absence of efficient cellular delivery and lack of long-term expression. Novel non-viral integration systems, such as transposons (Ivics and Izsvak, 2006; Hackett *et al.*, 2010; Claeys Bouuaert and Chalmers, 2010) and the bacteriophage integrases (Sauer, 2002; Calos, 2006; Smith *et al.*, 2010) are also under development as alternative tools. It is clear that each of these vectors is characterized by a set of different properties that make it suitable for some applications and unsuitable for others (**Table 1.1**) (Thomas *et al.*, 2003; Lundstrom, 2003).

Recombinant adeno-associated viral vector (rAAV) for example, is unique among viral vectors that are being developed for gene therapy because the wild-type virus has never been shown to cause human disease (Daya and Berns, 2008). The small size and simplicity of the vector particle make it possible to administer high doses of vector systemically without eliciting acute inflammatory responses or toxic side effects (Carter, 2005; Schultz and Chamberlain, 2008). However, from another point of view, the small capacity of the vector (<5 kb) makes them unsuitable for some other applications that require incorporation of larger transgenes (Grieger and Samulski, 2005; Lai *et al.*, 2010). Therefore, some larger viral vectors, as high-capacity adenoviral vectors (HC-AdVs) and herpes simplex virus-1 (HSV-1) (**Table 1.1**), with capacity to carry larger fragments of foreign DNA, are good alternatives.

Furthermore, the vector tropism also determines its application. Wild-type HSV-1 is a neurotropic virus that can establish life-long persistence in sensory neurons. This natural tropism has made neuropathological disorders the reasonable target choice for replication-defective HSV-1 vectors (Burton *et al.*, 2001). The tropism of other vectors, like AAV and AdV, depends on their serotypes (Davidson *et al.*, 2000). Thus, the serotype must be considered according to the target tissue/organ before therapeutic trials are initiated.

In addition, AdVs, which elicite a strong immunoreponse (especially the earlier vector generations), may be not suitable for some gene therapy applications. However, immunogenic AdVs will probably find its niche in the treatment of cancer in which cellular

toxicity and immunogenicity might enhance anti-tumour effects (Alemany and Zhang, 2000; Toth *et al.*, 2010).

The vector status after delivery is another important factor that influences the suitability of the vector for specific therapeutic applications. The integrating viral vectors such as oncoretroviruses and lentiviruses which insert their genomes into host cellular chromatin are the tools of choice if stable genetic alteration needs to be maintained in dividing cells. On the other hand, the normally non-integrating vectors that persist with their genomes in the cell nucleus predominantly as extra-chromosomal episomes (AAVs, AdVs and HSVs) (**Table 1.1**), can mediate persistent transgene expression in non-proliferating cells under certain circumstances. However, the insertional mutagenesis caused by oncoretroviruses are still of high concerns (Cavazzana-Calvo and Fischer, 2004; Kustikova *et al.*, 2010), thus, the development of non-viral integration machineries like Sleeping Beauty transposase (SB), bacteriophage integrases (phiC31) and zinc-finger nucleases (ZFNs) are of high interest.

Table 1.1: Gene therapy vectors and their main properties.

	Genetic material	Packaging capacity	Vector form	Tropism	Main limitations	Main advantages
viral vectors						
Retrovirus	RNA	10 kb	integrated	dividing cells	transduce only dividing cells, preference for integration into 5' ends of genes	persistent gene transfer in dividing cells
Lentivirus	RNA	10 kb	integrated	broad	preference for integration into active genes	persistent gene transfer in most tissues
AAV	ssDNA	5 kb	episomal (>90%) Integrated (<10%)	broad	small packaging capacity, transient expression	non-inflammatory, non-pathogenic
Adenovirus	dsDNA	36 kb	episomal	broad	immunogenetic	extremely efficient transduction of most tissues
HSV-1	dsDNA	40 kb* 150 kb#	episomal	strong for neurons	Inflammatory	Large packaging capacity, strong tropism for neurons
non-viral vectors						
Naked plasmid	dsDNA	plasmid size	Episomal/ integrated	depends on delivery	transient expression, absence of efficient cellular delivery	non-inflammatory
Sleeping beauty	dsDNA	10 kb	integrated	depends on delivery	random integration, absence of efficient cellular delivery	low toxicity, persistent gene transfer
Phic31 integrase	dsDNA	not limited	integrated	depends on delivery	induce deletions upon integration, chromosome rearrangements, absence of efficient cellular delivery	low toxicity, persistent gene transfer, site-specific integration (up to 15%)

*Replication defective. #Amplicon. AAV, adeno-associated viral vector; HSV-1, herpes simplex virus-1. Modified from Thomas *et al.*, 2003.

1.2. Adenovirus and its development as gene delivery vectors

1.2.1. Adenovirus

Adenovirus (Ad) was first discovered in 1953, isolated from cultures of human adenoid tissues (Rowe, 1953). Since then, 53 different serotypes of human adenoviruses have been isolated and characterized, and they are classified into six subgroups (A-F). Although human adenoviruses cause significant levels of respiratory, ocular, and gastrointestinal disease (Goncalves *et al.*, 2010; Louie *et al.*, 2008), highest incidence of severe diseases caused by Ads happens in immuno-compromised individuals (Ison, 2006; Echavarria, 2008). Among the general population these infections are usually resolved quickly and result in life long immunity to the virus (Lenaerts *et al.*, 2008). Historically, Ads have been popular as model systems to study viral entry, transcription and replication of the DNA genome, mRNA splicing, viral assembly, transformation of cells *in vitro*, and tumorigenesis (Babiss and Ginsberg, 1984; Carr, 1993; Benson *et al.*, 1999).

Adenoviruses are medium-sized (70–100 nm in diameter), nonenveloped icosahedral viruses composed of a nucleocapsid and a double-stranded linear DNA genome with an average length between 27 and 36 kb (Russell, 2000). The virus replication origins are present in the first 50 base pairs of the 100–140 bp inverted terminal repeats (ITRs), which are located at each end of the genome. A terminal protein (TP) is covalently linked to each 5' terminus of the viral genome. Both ITRs and TPs play an essential role in viral DNA replication (Hearing *et al.*, 1987; de Jong and van der Vliet, 1999). Adenoviral genes are encoded on both strands of the DNA in a series of overlapping transcription units **(Figure 1.3.a)** (Curiel and Douglas, 2002).

The current emphasis on therapeutic applications of adenoviruses has led to increased interest in the virion structure. In 2010, scientists achieved new insights into the structure of the human adenovirus by crystallization of a fiber-modified adenovirus type 5 (Ad5) and cryo–electron microscopy (cryo-EM) single-particle analysis of Ad5. This helps our research using adenovirus as vaccines and gene therapy vectors in a great degree (Liu *et al.*, 2010a; Reddy *et al.*, 2010).

1.2.2. Adenovirus as vector for gene delivery

Adenoviral vectors (AdVs) are widely used for gene transfer into a broad variety of cell types (Shirakawa, 2009). This is evidenced by the fact that adenovirus is currently being used in roughly one quarter of all gene therapy clinical trials, resulting in the biggest vector group currently being used **(Figure 1.2)** (Wiley, 2011).

To attain utility in patients, any gene therapy vector must deliver genes to the intended target organ and provide expression for an appropriate period of time to achieve a

therapeutic effect. When administered systemically it should infect only tissue in which gene delivery is desired, thus limiting toxicity to surrounding tissues and organs. Regarding AdVs, improvements along these lines have been obtained mostly through deletion of multiple adenovirus genes **(Figure 1.3.b)**, thereby preventing or reducing certain protein expression and vector replication and the subsequent immune destruction of transduced cells (Campos and Barry, 2007; McConnell and Imperiale, 2004).

1.2.3. Development of the early generation adenoviral vectors

Genes in the E1 region are necessary for the activation of viral promoters and expression of both early and late genes. Thus, removal of the E1 coding sequence results in viruses that are severely impaired in their ability to replicate. Replacement of the E1 region with transgenes was the initial strategy used for construction of adenoviral vectors, giving rise to the so-called first-generation vectors (Chinnadurai *et al.*, 1979). Removal of the E1 region and optional E3 allow insertion of foreign DNA of up to 7.5 kb **(Figure 1.3.b.i)** (Berkner, 1988), because adenovirus serotype 5 can package up to 38 kb (105% of its original size) without affecting viral titer and growth rate (Bett *et al.*, 1993).

First-generation AdVs have proven to be highly promising as vehicles for gene delivery, however, challenges do exist. The most troublesome limitation associated with the use of first-generation vectors is represented by acute toxicity and strong immune response towards leaky expressed adenoviral proteins, resulting in rapidly fading transgene expression (Lochmuller *et al.*, 1994). In addition, although the first-generation AdVs are generally considered replication defective, there is some low level expression of viral antigens, which eliminate the transduced cells by the cellular immune system, subsequently limiting the duration of transgene expression *in vivo* (McConnell and Imperiale, 2004). Another challenge is the recombination between the E1 region sequences in the producing cell line and the recombinant virus during vector production. This recombination gives rise to viral progeny with functional E1 genes that are replication competent (Yang *et al.*, 1994; Yang *et al.*, 1995; Dai *et al.*, 1995).

To prevent the immune response generated by low-level replication of E1-deleted viruses, vectors deleted for multiple genes have been created to inhibit viral gene expression more effectively. These so-called ‘second-generation vectors’ have been constructed primarily by the removal of E2 and/or E4 coding sequences additionally to E1 and E3 regions. These vectors show improved transgene persistence and decreased inflammatory response **(Figure 1.3.b.ii)** (Engelhardt *et al.*, 1994). They also provide the benefit of a larger capacity for transgene insertion. However, the yielded amounts of these vectors during production are considerably decreased and toxic side effects were still observed (Gao *et al.*, 1996; Gorziglia *et al.*, 1996).

1.2.4. High-capacity adenoviral vectors (HC-AdVs)

To avoid eliciting unwanted cellular immune responses, the third-generation adenoviral vector, helper-dependent adenoviral vectors (HD-AdVs), also called “gutless” vectors and high-capacity adenoviral vectors (HC-AdVs), have been developed (Kochanek *et al.*, 1996; Parks *et al.*, 1996; Parks and Graham, 1997; Ehrhardt and Kay, 2002). These HC-AdVs devoid of all viral coding sequences consist solely of the ITRs flanking the virus genome at its termini and the packaging signal (Ψ) required for encapsidation **(Figure 1.3.b.iii)** (Fisher *et al.*, 1996).

The elimination of Ad antigen expression in HC-AdVs permits long-term episomal expression of genes in quiescent cells by avoiding problems associated with the cellular immune response against viral gene products (Palmer and Ng, 2005; Brunetti-Pierri *et al.*, 2005). This type of vector has been shown to result in long-term transgene expression and phenotypic correction in animal models. Researchers have shown that after a single injection of HC-AdV, the vector genome can be maintained life-long in mice and for up to 2 years in rats (Ehrhardt and Kay, 2002; Toietta *et al.*, 2005). In dogs and non-human primates, HC-AdV liver transduction also led to long-term transgene expression (Brunetti-Pierri *et al.*, 2007). To date, the longest period of transgene expression (up to 964 days) after HC-AdV hepatic transduction was observed in baboons, using an improved balloon occlusion

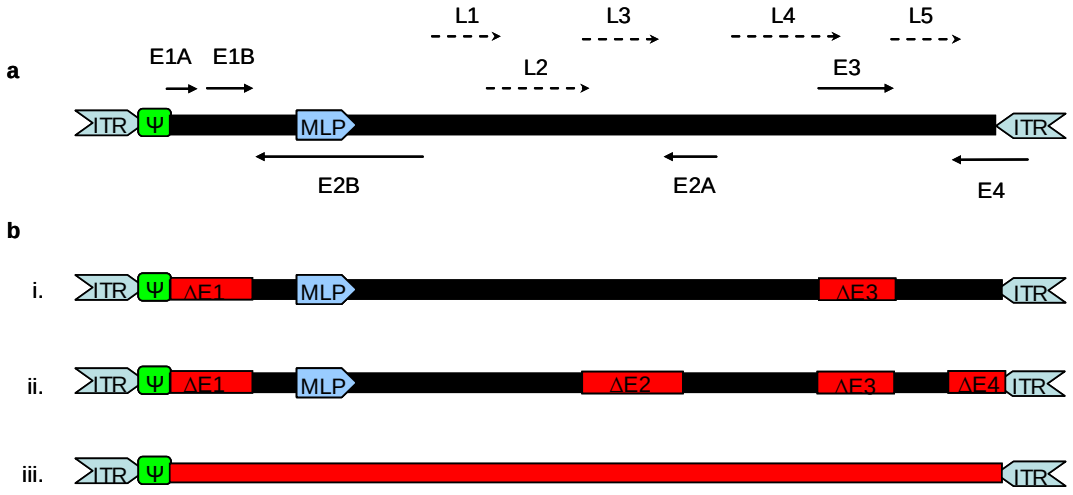


Figure 1.3: Schematic overview of wild type adenovirus and recombinant adenoviral vector genomes. (a) Map of the wild type adenovirus genome and its transcription units. The central, solid line represents the viral genome. Positions of the left and right inverted terminal repeats (ITRs), the packaging signal (Ψ), the early transcription units (E1A, E1B, E2A, E2B, E3, and E4 in solid arrow), and the late transcription units (major late promoter [MLP], L1–L5 in hatched arrow) are shown. Arrows indicate the direction of transcription. **(b) Different generations of recombinant adenoviral vectors used in gene therapy.** Elements shown in red represent deletions providing space for insertion of transgene cassette. (i) First-generation adenoviral vectors lacking E1and/or E3. (ii) Second-generation adenoviral vectors with multi-deletions. (iii) Helper-dependent adenoviral vectors with all viral coding regions deleted. Adopted from Curiel and Douglas, 2002.

catheter–based method which permits even greater hepatocyte transduction efficiency at clinically relevant low doses of vector infusion (1×10^{11}, 3×10^{10}, and even 1×10^{10} VPs/kg) (Brunetti-Pierri *et al.*, 2009).

A recent study demonstrated that HC-AdV genomes persist predominantly as replication-defective monomeric genomes (Jager and Ehrhardt, 2009). Moreover, the ability to grow recombinant viruses to high titers (Jager *et al.*, 2009; Palmer and Ng, 2003) facilitates its further use in a broader research area. In addition, these vectors have a much higher packaging capacity of foreign DNA (36 kb), enabling the expression of large transgenes or the inclusion of human genomic regulatory elements (Palmer and Ng, 2005; Palmer and Ng, 2008).

As adenoviral vectors are believed to integrate into host chromosomes at low frequencies (Harui *et al.*, 1999; Hillgenberg *et al.*, 2001; Stephen *et al.*, 2008; Jager and Ehrhardt, 2009), episomal genomes are not able to segregate to daughter cells during subsequent mitoses. As a result, adenoviral vectors are not applicable in gene therapy applications in which the target tissue consists of dividing cells. As shown in a previous study, transgene expression levels from HC-AdV *in vivo* declined by 95% over a period of one year in mice (Ehrhardt and Kay, 2002). Furthermore, it was shown that an HC-AdV containing a canine coagulation FIX (cFIX) expression cassette injected into hemophilia B dogs at a nontoxic dose (8.6×10^{11} viral particles per kilogram, VPs/kg), exhibited no hepatotoxicity as shown by liver enzymes and liver histology, and had no dropping platelet counts. However, only transient phenotypic correction was achieved (therapeutic levels of cFIX for about 2 months) and the cFIX levels declined over time to undetectable levels (Ehrhardt *et al.*, 2003). Another study with HC-AdV observed sustained cFIX expression for over a year at a high dose (3.57×10^{12}, and 1.3×10^{13} VPs/kg), but induced transient mild liver enzyme elevations and a decrease in platelet counts (Brunetti-Pierri *et al.*, 2005).

To prolong the therapeutic effect after adenoviral gene transfer and to stabilize persistence of the therapeutic DNA at a lower dose, especially in targeted cells with a high replicative potential, one promising approach is to combine HC-AdV with tools for somatic integration. A variety of adenoviral hybrid-vectors that lead to somatic integration of the transgene from the episomal adenoviral vector genome into the host chromosome have been developed. These vectors combine the highly efficient DNA delivery of adenoviral vectors with the integration machineries of transposons (HC-AdV/retrotransposon, HC-AdV/Sleeping Beauty transposon) (Yant *et al.*, 2002; Hausl *et al.*, 2010; Soifer *et al.*, 2001), phage integrases (HC-AdV/PhiC31) (Ehrhardt *et al.*, 2007), retroviruses/lentiviruses (E1-deleted AdV/RV) (Soifer *et al.*, 2002), or adeno-associated virus (HC-AdV/AAV) (Recchia *et al.*, 1999; Recchia *et al.*, 2004), and resulted in stable transgene expression by somatic integration.

1.3. Genetic disease and hemophilia B

1.3.1. Genetic diseases

Genetic factors play a crucial role in disease development. Genetic diseases, for example, are the disorders caused by abnormalities in an individual's genome. There are different types of genetic diseases, and the best studied ones among them are the monogenetic diseases, which are caused by changes or mutations that occur in a single gene. There are more than 6000 known single-gene disorders such as cystic fibrosis, sickle cell anemia, Marfan syndrome, Huntington's disease, hereditary hemochromatosis and hemophilia (Robin, 2008).

1.3.2. Hemophilia and the current medical treatment

Hemophilia A and B are blood clotting disorders caused by mutations in the genes encoding for blood coagulation factor VIII (FVIII gene) and factor IX (FIX gene). The human FVIII and FIX genes are located on the long arm of the X chromosome (chr Xq28 and chr Xq27) (Purrello *et al.*, 1985), therefore both hemophilia A and B are X-linked recessive diseases **(Figure 1.4)** (Bolton-Maggs, 2003; Cutler *et al.*, 2004).

The clinical features of hemophilia depend on the level of factor deficiency. Spontaneous hemorrhage occurs if factor levels are <1% of normal; hemorrhage and hemarthrosis occur following injuries if factor levels are 5%-30% of normal. There is usually no significant bleeding if factor levels are >30% of normal **(Table 1.2)** (Pagon RA, 2011). Current treatment for hemophilia involves protein (clotting factors) infusion therapy, which is based on intravenously injection of high-purity plasma-derived factors and recombinant products to hemophilia patients (Mannucci *et al.*, 2001). Although this treatment has markedly improved the life expectancy of patients suffering from hemophilia, and some studies even showed

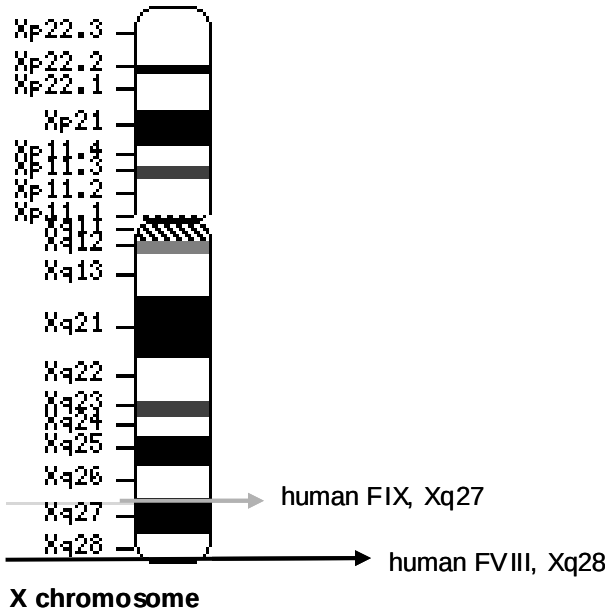


Figure 1.4: The chromosomal location of the human coagulation factor VIII (FVIII) and factor IX (FIX) genes on the X chromosome. The grey arrow shows the position of the FIX gene, while the black indicates the FVIII gene. The figure was modified from NCBI Map View.

correction of the hemophilic phenotype with continuous prophylactic administration of the clotting factors (Manco-Johnson *et al.*, 2007), most hemophilia patients worldwide are still at risk of life-threatening bleeding episodes and chronic joint damage (Darby *et al.*, 2007). This is partly due to the limited availability and high cost (>$100,000 per patient per year for prophylactic replacement therapy) (Pipe, 2010) of purified proteins. The protein infusion therapy is therefore mostly used to treat acute bleeding on demand rather than to provide continuous prophylactic coverage (Roosendaal and Lafeber, 2007). In addition, especially the short half-life of the coagulation factors (e.g., 8 to 23 hours for FVIII and 18 to 24 hours for FIX) make the protein infusion therapy more complicated (van den Berg *et al.*, 2007). An important side-effect is that some patients develop inhibitors, which are antibodies that block the function of a coagulation factor and make bleeding episodes difficult to treat (Lusher, 2000; Hay, 2006; DiMichele, 2007).

Table 1.2: Classification of hemophilia B based on *in vitro* clotting activity. Modified from Pagon RA, 2011.

Clinical Severity	Factor IX Clotting Activity	Clinical Bleeding Symptoms
Severe	<1%	Frequent spontaneous bleeding; excessive and/or prolonged bleeding after minor injuries, surgery, or tooth extractions
Moderate	1%-5%	Spontaneous bleeding rare; excessive and/or prolonged bleeding after minor injuries, surgery, or tooth extractions
Mild	>5%-30%	No spontaneous bleeding; excessive and/or prolonged bleeding after major injuries, surgery, or tooth extractions

1.3.3. Gene therapy as an alternative therapeutic approach for hemophilia B

As a technique for correcting defective genes responsible for genetic diseases, gene therapy is well suited for hemophilia treatment, not only because hemophilia is due to a single gene defect, but also a slight increase in clotting factor levels can potentially convert severe hemophilia to a mild version of the disease. As shown in table 1.2, hemophilia B is associated with severe disease with <1% normal circulating levels of FIX activity, thus improvement of FIX activity into the range of >1% results in a profound improvement in symptoms and is sufficient to prevent spontaneous and life-threatening bleeding episodes. Activity improvement to up to 10% may result in near elimination of the bleeding diathesis. (Lofqvist *et al.*, 1997; Chuah *et al.*, 2004). Another advantage of hemophilia B in the development of gene therapy strategies is the relatively small size of FIX cDNA (~1.4 kb of coding sequence), which makes it suitable to many different vector types and provides the

possibility to add more regulatory elements to improve transgene expression (Pierce *et al.*, 2007).

For these reasons, various vector systems such as retroviral (both gamma retroviral and lentiviral), recombinant adenoviral (AdV), recombinant adeno-associated viral (AAV), and non-viral vectors have been designed for expression of FIX in a gene therapy setting (Liras and Olmedillas, 2009; Matrai *et al.*, 2010).

1.3.4. The application of different vector systems for hemophilia B gene therapy

Retroviral vectors integrate into the target cell genome, which ensures stable maintenance of the DNA and allows long-term transgene expression, but can also result in insertional mutagenesis (Hacein-Bey-Abina *et al.*, 2003; Ott *et al.*, 2006). An early study using retroviral vectors encoding canine FIX resulted in a successful long-term gene therapy in a canine model of hemophilia B and low levels of plasma FIX (~2-8 ng/ml). Notably, the whole blood clotting time (WBCT) decreased from nearly 50 minutes to as low as 15 minutes, which led to a change in phenotype from severe hemophilia to a moderate form (Kay *et al.*, 1993). Another researchers injected a murine leukemia virus (MLV) - based retroviral vector into neonatal hemophilia B puppies which resulted in high levels of cFIX (500 ng/ml) and marked shortening of WBCT from over 60 minutes to 8-12 minutes (Xu *et al.*, 2003). A recent study using human immunodeficiency virus (HIV) – based lentiviral vectors expressing human FIX enabled sustained gene transfer in hemophilia B mice for more than 280 days, with over 10% of normal FIX activity in treated mice (Brown *et al.*, 2006). Although these preclinical results are very encouraging, enthusiasm has been damped by concerns about the risk of insertional mutagenesis with retroviruses (Hacein-Bey-Abina *et al.*, 2003). Researchers are now exploring some alternative designs for a safer vector form, like self-inactivating (SIN) retroviral or lentiviral vectors and integrase-defective lentiviral vectors (IDLVs) (Yu *et al.*, 1986; Olson *et al.*, 1994; Zufferey *et al.*, 1998; Wanisch and Yanez-Munoz, 2009), which hold great potential to further improve the gene therapy safety profile.

As one of the most used vector in human clinical trials (Wiley, 2011) **(Figure 1.2)**, adenoviral vectors were also explored in hemophilia B gene therapeutic studies. An earlier generation of adenovirus serotype 5 (Ad5) containing the canine FIX cDNA demonstrated the first successful somatic cell gene therapy approach in a canine model for hemophilia B, in which plasma cFIX levels were observed to be normal or super-physiological (>5000 ng/ml) for about a week, however, followed by a slow return to pre-treatment or baseline levels in 70 to 100 days (Kay and Woo, 1994). It turned out that the success of adenovirus-mediated gene therapy for hemophilia B was limited by the host immune response to the adenoviral proteins. The adenoviral vector activates specific cytotoxic T lymphocytes (CTLs), T helper cells (both Th1 and Th2 subsets), and B cells, leading to inflammation, the destruction of

infected tissues, and the cessation of transgene expression (Arruda, 2006). Later on, important advancements have been reported after the use of helper-dependent adenoviral constructs that encode no viral proteins.

Another vector that holds great promise for hemophilia gene therapy is represented by recombinant adeno-associated viral vector (rAAV). AAVs have a favorable safety profile because of their non-pathogenic nature, and are able to achieve persistent transgene expression by their predominately episomal maintained AAV genomes (Daya and Berns, 2008; Nakai *et al.*, 2001). Protocols to use rAAV–mediated gene therapy for hemophilia were established in several laboratories (Xiao *et al.*, 1996; Snyder *et al.*, 1997). The two main strategies utilizing muscle and liver as target organs showed promising results. Infusion of an adeno-associated viral vector serotype 2 (AAV2) into the muscle resulted in long-term (> 3 years) and high FIX expression (circulating levels of 4%-14%) in a canine model for hemophilia B (Arruda *et al.*, 2005), achieving complete correction of the bleeding disorder. Based on these results in animal models, a phase I clinical trial was performed in severe hemophilia B patients utilising intramuscular injection of AAV2-FIX vectors at multiple sites (Kay *et al.*, 2000). Although there was evidence of gene transfer and expression in the treated patients for more than 3 years (Manno *et al.*, 2003), however, the systemic FIX levels were below the therapeutic range. On the other hand, liver-directed AAV gene therapy resulted in sustained therapeutic FIX expression without inhibitor development in normal and hemophilic mice, hemophilia B dogs and nonhuman primates (Snyder *et al.*, 1999; Mount *et al.*, 2002; Nathwani *et al.*, 2007). Recently, an 8-year study in inhibitor prone null mutation hemophilia B dogs treated with liver directed AAV2-FIX demonstrated long-term correction of the hemophilic phenotype with FIX activity stably remained between 4% and 10%, and without inhibitor development (Hasbrouck and High, 2008). A phase I clinical trial was therefore initiated in severe hemophilia B patients, who received infusion of liver-directed AAV2 vectors, expressing FIX from a liver-specific promoter (Manno *et al.*, 2006).Therapeutic FIX levels of up to 10% were obtained in the high dose group, however, transgene expression was lost after one month, most likely as a result of immune response against the AAV2 capsids (Pien *et al.*, 2009). Therefore, as an alternative approach, scientists are trying to use alternative AAV serotypes (Wu *et al.*, 2006) and transient immunosuppressive regimens (Jiang *et al.*, 2006) to overcome these limitations.

Non-viral vectors have the advantage of being less immunogenic and less toxic than its viral counterparts (**Table 1.1**), because no viral proteins are involved. However, the efficiency of non-viral DNA delivery is generally quite low compared to viral vector-mediated gene transfer (Douglas, 2008). Different strategies have been developed to introduce DNA into target cells, like ultrasound, electroporation, cationic liposomes conjugated with polyethylene glycol (PEG), polymers such as polyethyleneimine (PEI) or hybrid polymeric

lipids, or hydrodynamic transfection. In a preclinical study, a plasmid encoding human factor IX (hFIX) formulated with a protective, interactive, noncondensing (PINC) polymer was injected into the skeletal muscle followed by administration of multiple electrical pulses (electroporation). In mice long-term expression was achieved and the ability to re-administrate formulated plasmid was also demonstrated. In normal dogs, expression of hFIX reached 0.5-1.0% of normal levels, however, only transient expression was observed due to the development of antibodies against hFIX (Fewell *et al.*, 2001). Similarly, a phase I clinical trial for hemophilia A was conducted with FVIII-transfected autologous fibroblasts (Roth *et al.*, 2001b). Although no long-term adverse effects occurred due to the transfected cells or to the implantation procedure, only a modest rise in FVIII activity was detected in seven out of 12 patients and the FVIII expression levels were transient. To overcome this limitation of transient transgene expression, researchers then tried to combine non-viral therapy with chromosomal integration mediated by Sleeping Beauty transposase (Mates *et al.*, 2009), or to increase episomal persistence by incorporating elements like matrix attachment regions (Harraghy *et al.*, 2008), or by removing redundant bacterial backbone sequences like minicircle DNA (Mayrhofer *et al.*, 2009). Furthermore, in combination with cell-based therapy as shown in the past (Tatsumi *et al.*, 2008), non-viral vectors could be a promising option in the future (Lillicrap *et al.*, 2006).

The collective results from these hemophilia gene therapy studies indicate that the current approaches and doses are effective and safe, and long-term expression of clotting factors have been achieved in small and large animal models using multiple gene therapy strategies. However, these results have not yet been successfully translated into human patients. More efforts are needed to better understand the basic biology of gene therapy, and to further develop and improve these vectors for treatment of hemophilia and other inborn genetic disorders.

1.3.5. Optimization with respect to the transgene encoding sequence

The goal of hemophilia gene therapy is to effectively prevent bleeding and its associated complications in a relatively long-term period. Therefore, the ideal treatment of hemophilia B by gene therapy, on the one hand, requires the use of a gene delivery system that is efficient, safe, non or less-immunogenic and allows for long-term transgene expression (Petrus *et al.*, 2010). On the other hand, the transgene itself also plays an essential role in therapeutic efficacy. For instance the improvement on the functional properties of the coagulation factors could not only enhance the therapeutic effect but can also reduce the vector dose needed, thereby significantly reduce the side effects caused by a high gene therapy vector dose infusion.

1.3.5.1 Bioengineering strategies to improve the functional properties of human coagulation factors

With the fast development of recombinant DNA technology and deep understanding of the protein structure, bioengineering strategies have been used to improve the functional properties the coagulation proteins. These improvements have been directed at overcoming the recombinant coagulation factors' inherent limitations, like biosynthesis and secretion, functional activity, half-life, and antigenicity/immunogenicity. Some of these strategies have already reached commercialization, several are in ongoing clinical trials, and a number of strategies are in advanced preclinical development (Pipe, 2010). For example, the B-domain–deleted (BDD)-FVIII cDNA, not only significantly improved the yield of recombinant FVIII production but also facilitated packaging within certain viral vectors facilitating its adoption for gene therapy strategies (Chao *et al.*, 2000). Until now, BDD-rFVIII remains the first and only modified human rFVIII molecule getting commercialized.

Another approach for improving properties of recombinant coagulation proteins would be the half-life extension. Prolonging the half-life of coagulation factors could greatly reduce the frequency and dose of infusions, thereby improving the efficacy, convenience and patient quality of life (Lillicrap, 2008). One strategy to extend the half-life of FIX is the fusion protein technology, which links FIX to another protein with a much longer plasma half-life. For instance, the constant region (Fc) of immunoglobulin G was fused to FIX (FIXFc). The presence of the Fc portion protects the fusion protein from catabolism through interaction with the neonatal Fc receptor (FcRn), thereby preventing the degradation in the lysosome. Preclinical data with FIXFc demonstrated a 3- to 4-fold longer terminal half-life in mice, rats, and cynomolgus monkeys (Peters *et al.*, 2010).

The most attractive area for improvements is modifying functional activity of human coagulation factor. For instance, the bioengineered FIX variants with increased catalytic activity may be useful for achieving therapeutic FIX activity with lower vector doses. Different strategies have been used to generate recombinant human FIX (rhFIX) variants with increased coagulating activity, for instance, by introduction of point mutations. By this approach a hyperactive variant was generated with a single point mutation changing arginine at 338 to alanine (hFIX-R338A), which showed a 3 times increased clotting activity than that of the wild type FIX (wt-hFIX) in the *in vitro* actived partial thromboplastin time assay (APPT) (Chang *et al.*, 1998). Importantly, application of this hFIX-R338A in hemophilia B mice delivered by AAVs displayed a 2 to 6-fold higher specific activity (Schuettrumpf *et al.*, 2005). Later on, based on this hFIX-R338A, more variants including some other mutants or domain exchange mutants demonstrated even higher clotting activities. When applied in *in vivo* gene therapy approaches, these further engineered variants resulted in up to 13-fold increased activity (Brunetti-Pierri *et al.*, 2009; Lin *et al.*, 2010; Kao *et al.*, 2010).

Moreover, a gain-of-function mutation in FIX gene was recently identified from a family with a potent X-linked thrombophilia. This mutant R338L makes the plasma FIX activity 800% of normal activity, and it exhibited a 5- to 10-fold higher specific activity *in vitro* (Simioni *et al.*, 2009).

The structure and function analyses offer a direct way to bioengineer novel and improved coagulation factors. From the sequence alignment and structural characteristics of the catalytic domain of hFIX with several homologous coagulation enzymes, researchers found that the segments exchange between FIX and FX can dramatically increase the activity up to 7000-fold based on a substrate selection assay (**Figure 1.5**)(Hopfner *et al.*, 1997; Sichler *et al.*, 2003).

These bioengineered FIX variants are not only valuable for recombinant FIX protein replacement therapy, but can also increase the therapeutic index of gene therapy vectors by permitting administration of lower vector doses to achieve the same therapeutic outcome. For instance, the hFIX mutants discovered by Hopfner (Hopfner *et al.*, 1997) with up to 7000-fold increased activity, are not been applied for gene therapy approaches so far.

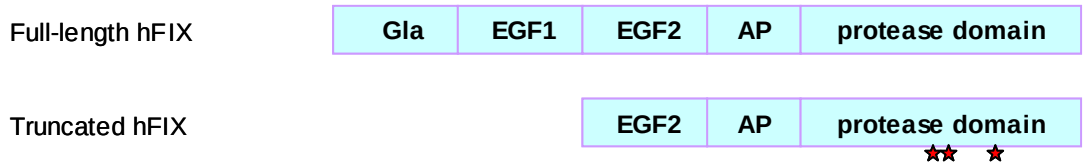


Figure 1.5: Schematic view of the human coagulation Factor IX protein. The gene for human FIX consists of eight exons and seven introns, is approximately 34 kb long, and is located on the long arm of the X-chromosome at Xq27.1. Structurally, FIX contains an N-terminal γ-carboxy glutamic acid (Gla) domain (residues 1–40), a short hydrophobic stack (residues 41–46), two epidermal growth factor (EGF)-like domains (EGF1: residues 47–83, and EGF2: residues 88–127, which are connected by linker residues 84–87), an activation peptide (AP, residues 146–180), and a C-terminal protease domain (residues 181– 415) (top panel) (Brandstetter *et al.*, 1995; Hopfner *et al.*, 1997). The truncated version used by Brandstetter et al. is depicted on the buttom panel. The red stars indicate the mutations to generate the hyperactive variants.

1.4. Adenovirus/Sleeping Beauty transposase hybrid-vectors

1.4.1. Vectors for somatic integration

To develop novel and safe vectors for therapeutic transgene expression in rapidly dividing cells is of great interest to the research community. Integrating viral vector systems based on retrovirus and on lentivirus are suitable tools, as the integration of viral vector genomes into the cellular chromosomal DNA allows for a more stable and long-lasting transgene expression than episomal gene-delivery models (Kustikova *et al.*, 2010). However, the integrating viral vectors tested in preclinical and clinical settings revealed that integration site preferences may cause genotoxicity by changing the expression profile and properties of

the transduced target cells due to insertional mutagenesis (Haviernik and Bunting, 2004). For instance, retroviruses prefer the regulatory sequences for integration and lentiviruses integrate predominantly into transcribed gene regions. Therefore, to obtain stable gene transfer for therapeutic applications, alternative approaches have been explored based on Sleeping Beauty (SB) transposon system, phiC31 integrase and zinc-finger nucleases (ZFNs) for somatic integration. In contrast to viruses, these non-viral integration machinery lack direct mechanisms to gain entry to the cell across the membrane. However, viral hybrid-vectors have been developed, by combining non-viral integration machineries with viruses for efficient delivery.

The integrase from the Streptomyces phage PhiC31 was first described in 1991 (Kuhstoss and Rao, 1991). It belongs to the serine recombinase family that includes the Tn3 resolvase and a number of transposases. In contrast to other integration machineries, PhiC31 was shown to mediate unidirectional site-specific recombination between two DNA recognition sequences, the phage attachment site (attP) and the bacterial attachment site (attB) (Rowley and Smith, 2008). Expressed under the control of a mammalian promoter, PhiC31 integrase mediates integration of extra-chromosomal plasmids bearing an attB site into a limited number of 'pseudo-attP' sites present in the native mammalian genomes (Groth *et al.*, 2000; Chalberg *et al.*, 2006). A remarkable feature of the PhiC31 integrase for gene therapy is the fact that integration is limited to ''hot spot'' sites with up to 15% specificity in the mammalian genome (Chalberg *et al.*, 2006; Ehrhardt *et al.*, 2006) potentially decreasing the risk of insertional mutagenesis. Recently, more effort was spent on understanding the molecular mechanism of integrase in mammalian cells (Keravala *et al.*, 2009; Liesner *et al.*, 2010; Liu *et al.*, 2010b). Due to the low delivery efficiency of PhiC31 integrase to target cells by non-viral DNA (**Table 1.1**), researchers have also focused on combining the PhiC31 system with viral vectors for high cell transduction efficiency. For instance, in a previous study, a hybrid-vector system that combines the high transduction efficiency of a HC-AdV and the integration machinery of the PhiC31 was developed for stable transduction and limited integration sites. Herein, the integration of the transgene expression cassette from the adenoviral vector resulted in 5-fold higher transgene expression levels in the active PhiC31 group compared to the control group, which received a mutated and inactive version of PhiC31. Moreover, the previously described mpsL1 hot spot of integration was confirmed (Ehrhardt *et al.*, 2007).

Zinc-finger nucleases (ZFNs) are artificial restriction enzymes generated by fusing a zinc finger DNA-binding domain to a DNA-cleavage domain. Zinc finger domains can be engineered to target desired DNA sequences, which enable zinc-finger nucleases to induce double strand breaks at the desired DNA sequences, subsequently been repaired by the cellular repair mechanism (Durai *et al.*, 2005). Therefore, in gene therapy application, ZFN

enables the replacement of the defective gene at its natural chromosomal location, which offers an ideal strategy for site-specific integration (Urnov *et al.*, 2010). To take advantage of the high efficiency of viral vectors for cell delivery, ZFNs were combined with AdVs and integrase-defective lentiviral vectors (IDLVs) (Lombardo *et al.*, 2007; Perez *et al.*, 2008). IDLVs were used to deliver ZFNs directed at the human IL-2Rγ gene, a combination vector that carried one ZFN and one copy of the donor DNA was produced to reduce the number of separate viruses that had to infect the same cell. However, the efficiency of gene targeting varied widely among different types of cells, for example this system achieved targeting frequencies up to 39% in K562 cells, but lower efficiencies in other cell types (Lombardo *et al.*, 2007).

1.4.2. The Sleeping Beauty (SB) transposon system

The Sleeping Beauty (SB) transposon system was first molecularly reconstructed from ancient inactive copies of Tc1/mariner-like elements found in several fish genomes in 1997 (Ivics *et al.*, 1997). The wild-type transposon consists of the SB transposase and inverted terminal repeats (IRs) flanking the transposase encoding sequence. For transposition, SB transposase recognizes the IRs and mediates the insertion into a TA site of the host genome by a 'cut-and-paste' mechanism **(Figure 1.6)**. In gene therapy approaches, SB components are split into an artificial transposon containing the therapeutic cargo sequence flanked by the IRs and the transposase provided in trans for mobilization of the

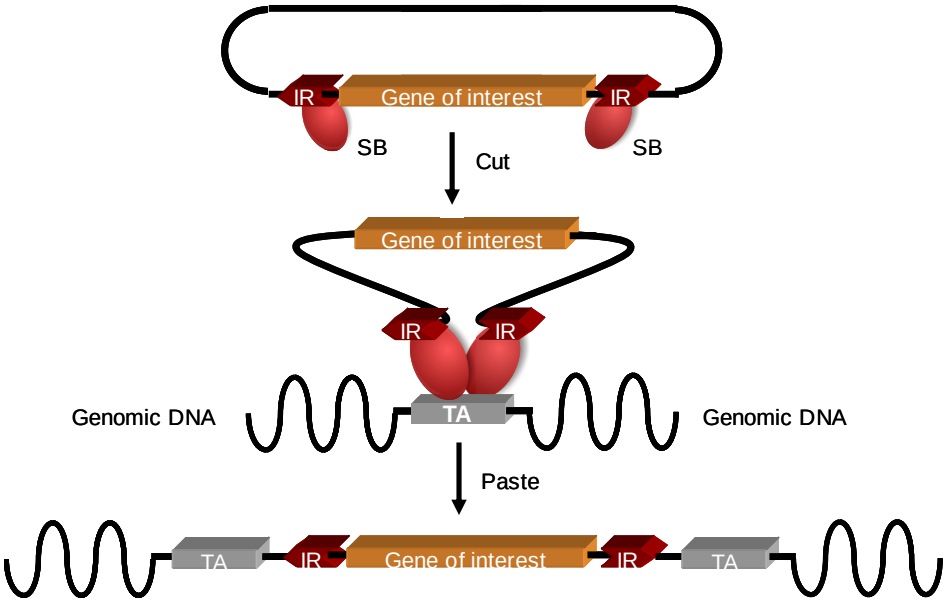


Figure 1.6: Sleeping beauty transposase-mediated somatic integration. A two-component system is shown in which a gene of interest flanked by transposon-derived inverted repeats (IRs) is mobilized by the transposase protein provided in trans. For gene transfer application, the tranposon is usually excised from a plasmid or a viral vector and integrated into a genomic target site (TA). This system is commonly referred to as 'cut-and-paste' mechanism.

transposon (Izsvak and Ivics, 2004). Due to the high transpositional activity in vertebrates, SB transposase is an attractive tool for human gene therapy. It has been shown to provide long-term transgene expression *in vitro* with a random integration profile in the context of plasmid design (Yant *et al.*, 2000; Yant *et al.*, 2005). However, also for the SB system, delivery presents a major hurdle and methods such as hydrodynamic delivery (Bell *et al.*, 2007) are not suitable in humans. Therefore, much effort was spent on designing virus/SB hybrid-vector systems to deliver the SB transposon system efficiently. Until now, the SB transposase machinery was combined with several different viral vectors, like AdV, herpes simplex virus (HSV), lenti- and retroviral vectors (Yant *et al.*, 2002; Peterson *et al.*, 2007; Vink *et al.*, 2009; de Silva *et al.*, 2010; Heinz *et al.*, 2011), and showed efficient delivery and long-term transgene expression.

1.4.3. Previous work on adenovirus/SB transposase hybrid-vectors

To combine efficient delivery and high transduction efficiency of adenoviral vectors with the long-term expression of a transgene expression cassette after SB-mediated somatic integration, the first AdV/SB hybrid-vector system was generated in 2002 (Yant *et al.*, 2002). This hybrid-vector system consisted of two independent HC-AdVs, in which one contained the integration machinery and the other encoded the transposon. This particular molecular design was called “two-vector-system”. Herein, one adenoviral vector contained SB transposase and Flp recombinase encoding sequences, and the second vector contains the transposon which encompasses the transgene expression cassette bordered by inverted repeats (IRs) for SB recognition and the FRT sites for Flp recognition **(Figure 1.7)**

Therefore, after co-transduction of a single cell with both HC-AdVs the transgene expression cassette integrates into the cellular genome by a two-step mechanism. Initially, Flp recombinase interacts with the FRT sites and excises the transposon from the HC-AdV genome generating a circular intermediate. Subsequently, the SB transposase recognizes the IRs in the excised circular DNA and integrates the transposon by a ‘cut-and-paste’ mechanism into the genomic DNA **(Figure 1.7)**. In that study, stable FIX expression levels were obtained in murine liver even after inducing rapid cell cycling of hepatocytes with carbon tetrachloride (CCl_4) (Yant *et al.*, 2002). However, further optimization of the vector design and multi-experiments setting are needed before it is ready for clinical application.

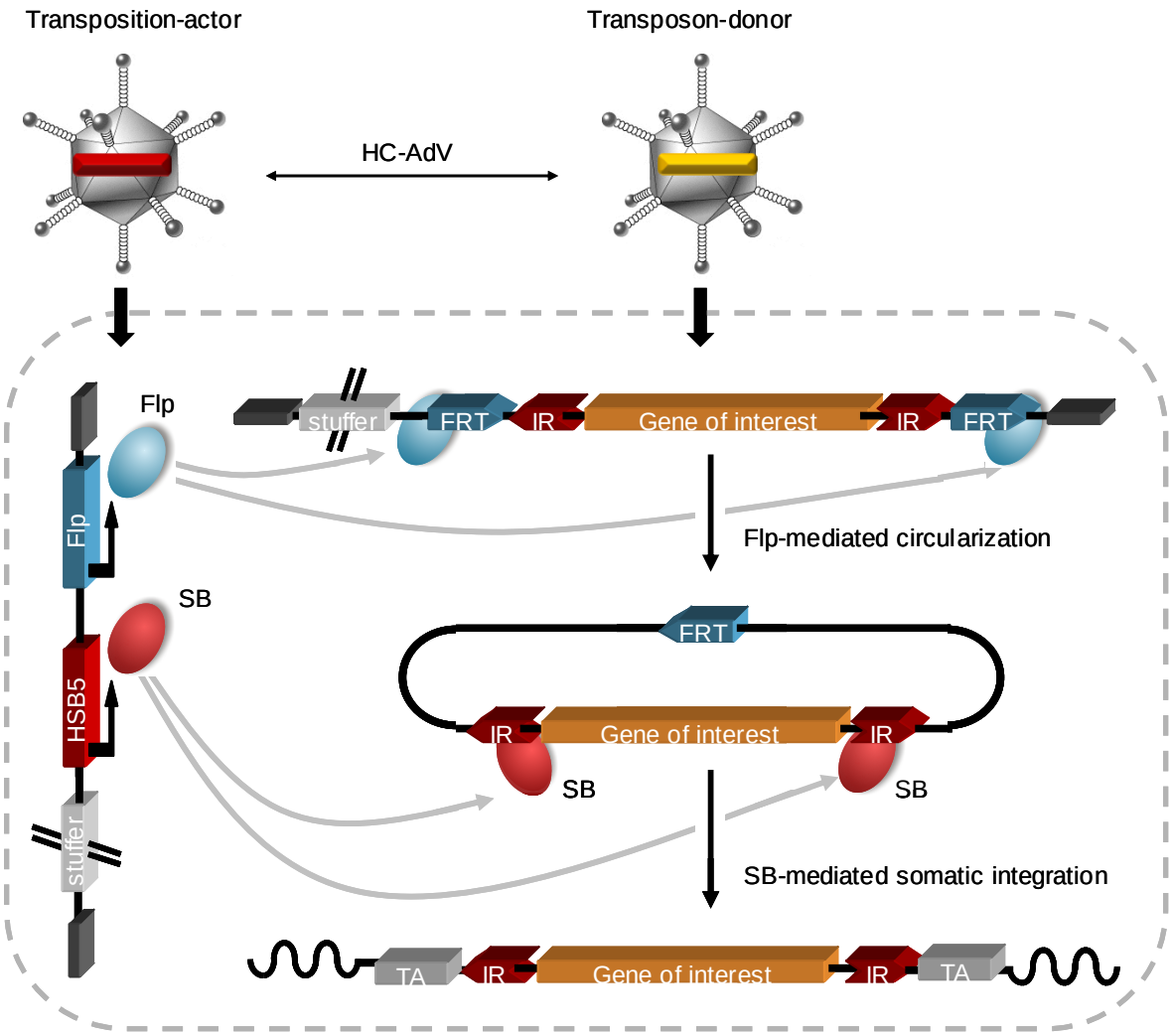


Figure 1.7: Principle of the adenovirus/transposase hybrid vector system. Schematically shown is a "two-vector strategy" based on two HC-AdVs co-infecting one cell. The first HC-AdV acts as transposon-donor vector and encodes the transgene flanked by transposon-derived inverted repeats (IR) and FRT sites for Flp recombinase mediated excision; the other HC-AdV provides SB transposase and Flp recombinase and plays the role as a 'Transposition actor', initializing circularization by Flp-FRT recognition essential for SB functionality and transgene insertion into TA sites of the host genome by the 'cut-and-paste' mechanism. HC-AdV, high-capacity adenoviral vectors; SB, Sleeping Beauty.

1.5. Potential improvements of the adenovirus/transposase hybrid-vector system and future challenges

The previous study which introduced an adenovirus/SB transposase (AdV/SB) hybrid-vector system (Yant *et al.*, 2002) provided a potential new mean to treat genetic diseases. However, also this system has some limitations to be overcome before considering clinical studies.

For instance, the conventional system was wild type SB for somatic integration. However, the intrinsic activity of the SB system has been enhanced through a variety of measures including the creation of a series of hyperactive SB transposases and engineering

of transposons with identical 3´ and 5´ inverted repeats (IR). For instance, the hyperactive version of SB transposases like SB11, HSB5 and SB100x (Zayed *et al.*, 2004; Yant *et al.*, 2004; Mates *et al.*, 2009) which showed increasing enzymatic activities *in vitro*. These could be introduced into the AdV/SB hybrid-vector system to increase the integration efficiency and potentially enhance the therapeutic effect.

Furthermore, it is essential to explore the dose effects of the AdV/SB hybrid-vectors. In almost all clinical trials, multiple ascending dose studies need to be conducted to better understand the pharmacokinetics and pharmacodynamics of the drug. Therefore, multi-dose settings in animals would be beneficial to better understand the AdV/SB hybrid-vector system, and this had not been performed in the previous study.

In addition, the development of gene transfer strategies, similar as the traditional research procedure for any new medication, usually begins with evaluation of the gene transfer effect in tissue culture. Then the efficacy and safety of the vector system need to be addressed in small animals (usually mice, rats and rabbits), in large animals (dogs, sheep, pig and non-human primates), and finally in humans. The direct translation from mice to human is usually challenging, mainly due to the huge body size difference (approximately 3500-fold body weight scale-up from mice to human), necessary changes of the administration route, target tissue or organ and more importantly vector amount **(Figure 1.8)** (Hausl *et al.*, 2011; High, 2005). Moreover, the short life span of mice compared to human and the less predictive immune responses of inbred mice make the outcome in humans less predictable. Therefore, up-scaling approaches from treating small animal models to large animal models are essential before going into clinical studies. It is of note that at the beginning of this study, the adenovirus/SB transposase hybrid-vectors had not been explored in large animals.

Importantly, safety is an essential issue when new medical agents are evaluated in a pre-clinical or any clinical setting. As to gene therapy, there are several aspects to be considered. Firstly, high doses of any vector type can result in acute inflammatory responses with potentially lethal consequences (Brunetti-Pierri *et al.*, 2004). Therefore, the vector load is one primary issue to be considered. Secondly, with respect to immune responses, immune-toxicity caused by both transgene product and vector protein need to be monitored as the immune-response against the transgene product counteracts the therapeutic effect due to the vector elimination (Manno *et al.*, 2006). Additionally, this kind of host immune-response results in inflammation at the injection sites or the target organ leading to the final failure of the therapy (Raper *et al.*, 2003). Furthermore, the over-production of the transgene product can sometimes also be toxic. For example, some enzyme products may cause unwanted side effects such as over-activity of coagulation factors which may turn hemophilia to thrombosis (Dargaud *et al.*, 2005). Considering the genome level, potential genotoxicity

needs to be analyzed, especially for integrating vector systems. After somatic integration of the transgene, stable transgene expression can be obtained and long-term therapy effect is achievable. However, any insertion of foreign segments could change the genetic composition of the cell. This kind of toxicity has raised researchers' attention since the cases of leukemia happened to the patients who received oncoretroviral vectors transduced stem cells (Hacein-Bey-Abina *et al.*, 2003; Ott *et al.*, 2006; Howe *et al.*, 2008). A recent clinical study showed a monosomy of chromosome 7 after retroviral vector therapy (Stephen *et al.*, 2010) and even more frequently toxicity caused by the insertion of the transgene was observed after retroviral infection (Li *et al.*, 2002; Kustikova *et al.*, 2005; Donsante *et al.*, 2007). There are also other types of genotoxicity, such as genome deletions and other rearrangements observed after PhiC31-mediated integration (Ehrhardt *et al.*, 2006), and in AAV integration (Miller *et al.*, 2002). Therefore, the evaluation of genotoxicity is of high importance. Notablely, SB-mediated integrations have been shown to cause neglectable genotoxicity with a fairly random integration profile in plasmid context (Liu *et al.*, 2005; Yant *et al.*, 2005). However, the SB integration profile via adenoviral vector is still need to be characterized.

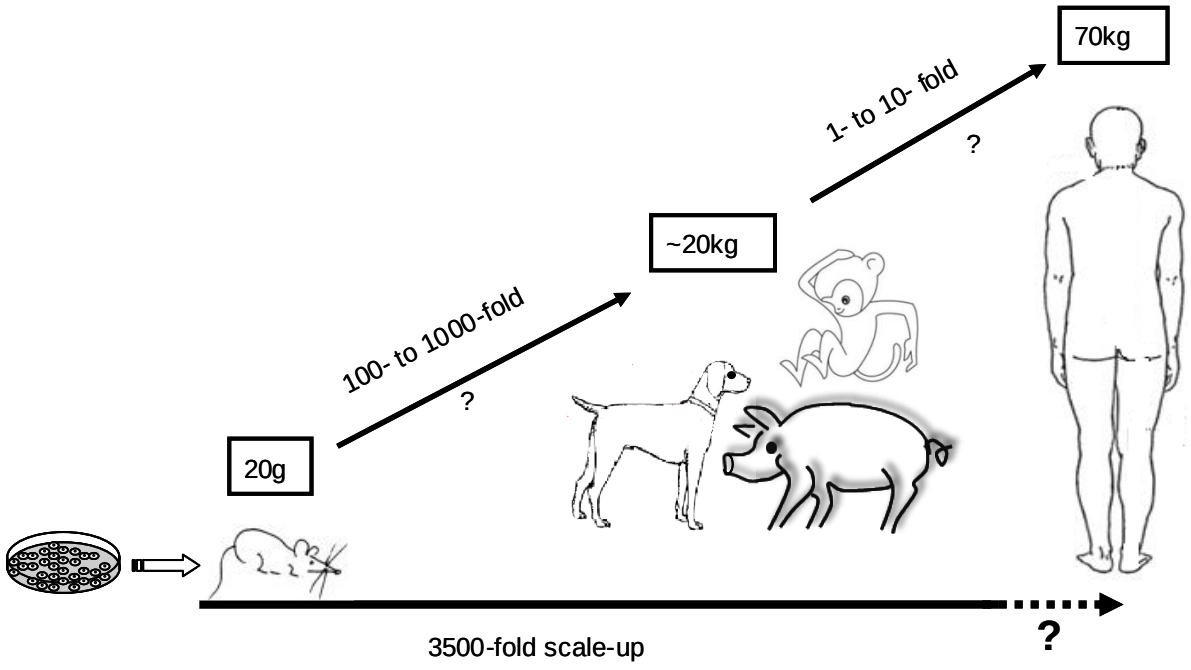


Figure 1.8: Translation of the medicines from tissue culture to clinical trials. The figure shows the procedure of drug discovery. It may begin with tissue culture, and then go on to different animal models, finally achieve in human beings. For preclinical studies, translation into the clinic and research on human diseases especially in the area of gene therapy, large animals (cat, dog, pig, primate) are preferred models compared to mice. One important aspect to consider is the increasing body weight which automatically requires a scale-up of the required vector dose. For instance from a large animal to human the scale-up would be approximately 1- to10-fold, whereas the scale-up from mouse to human could be as much as 3500-fold.

Aims of this work

One of the best studied and the most utilized vectors in gene therapy are adenoviral vectors (AdVs). Especially the advanced version represented by high-capacity adenoviral vectors (HC-AdVs) attracted researchers, because these vectors display a low toxicity profile and result in long-term transgene expression in quiescent cells. In proliferating cells, the combination of HC-AdV with the integration machinery of Sleeping Beauty transposase (SB) demonstrated also great potential in gene therapeutic applications. The aim of this study was to evaluate this novel adenovirus/Sleeping Beauty transposase (AdV/SB) hybrid-vector system with respect to the following aspects:

1. The first aim was to evaluate the dose effect of this novel AdV/SB hybrid-vector system in mice in order to define the appropriate amount, which is sufficient to obtain long-term transgene expression and only with limited side-effects in regenerating tissue. Towards this end, in depth viral vector titration and characterization are required. Also, focusing vector dose dependency, *in vivo* studies in male and female mice should be performed.

2. The second aim was to evaluate the safety profile of this novel AdV/SB hybrid-vector system in detail. Therefore, the potential genotoxicity caused by SB-mediated integration as well as the acute liver toxicity after *in vivo* application of the AdV/SB hybrid-vectors need to be analyzed. With respect to genotoxicity, the integration profile of the AdV/SB hybrid-vectors should be determined.

3. As a further step, this novel adenovirus/SB transposase hybrid-vector system was explored in a canine model for hemophilia B. Herein, gene transfer efficiencies, the somatic integration pattern after transposition and anti-adenoviral immune responses needed to be evaluated.

4. To further improve adenovirus-based gene therapy approaches for hemophilia, it was planned to explore the potential of a previously described hyperactive variant of human coagulation factor IX (hFIX-K265A/Y345A), which was generated from structure comparison and sequence alignment.

In total, this work will provide important information about the underlying mechanisms and the features of this novel AdV/SB hybrid-vector system. Thus, this system can be optimized to be more suitable for human applications.

2. Material and methods

2.1. Material

2.1.1. Plastic ware

All sterile plastic wares were supplied by Peske and Falcon, if not stated otherwise.

2.1.2. Chemicals and enzymes

All chemicals were obtained from Roth or Sigma, respectively. All enzymes were purchased from New England Biolabs, if not stated otherwise.

2.1.3. Equipment

Bacteria incubator	wtc Binder
Bench top centrifuge	Biofuge fresco, Heraeus
Chemistry bench	Kottermann
Clinical centrifuge	Rotanta 460, Hettich
Coagulation analyser	MC 1, ABW Medizin und Technik GmbH
Electronic balance	Kern
Electroporator	Gene Pulser II, Biorad
ELISA reader	Sunrise, Tecan
Humidified incubator	Thermo electron
Isofluran machine	Eickemeyer
Magnetic stirrer	Bellco
Microscope	Zeiss Axiovert-25, Spectra
PCR cycler	T professional basic, Biometra
PIPETBOY acu	IBS - INTEGRA Biosciences
Phosphoimager	FLA-3000, Fujifilm
pH Meter	pH 526, Bachofer
Refrigerator	Liebherr
Rotor for ultracentrifuge	SW 41, Beckman Coulter
Spectrophotometer	Ultrospec 3000, Pharmacia
Taqman-Cycler	7500 Fast Real-time PCR System, Applied Biosystems
Thermomixer comfort	Eppendorf
Tissue culture hood	LaminAir HBB 2448, Heraeus
Ultracentrifuge	Optima LE-80K, Beckman Coulter
Vortexer	MS1 Minishaker, Ika

2.1.4. Kits

Advantage™ 2 PCR Kit	BD Biosciences Clontech
ALT Kit	Randox
APPT liquid reagent	MediRox AB
FastStart Universal Probe Master (ROX)	Roche
FastStart Universal SYBR Green Master (ROX)	Roche
FuGENE® HD Transfection Reagent	Roche
FuGENE® 6 Transfection Reagent	Roche
GenomeWalker™ Universal Kit	BD Biosciences Clontech
KOD Hot Start DNA Polymerase	Novagen
Phase Lock Gel light/heavy	5 Prime
PureYield™ Plasmid Midiprep System	Promega
ProtoScript® First Strand cDNA Synthesis Kit	NEB
QIAquick Gel Extraction Kit	QIAGEN
SuperFect Transfection Reagent	QIAGEN
TRIZOL Reagent	Invitrogen
Vivapure AdenoPACK 20	Sartorius Stedim Biotech
Zero Blunt® TOPO® PCR Cloning Kit	Invitrogen

2.2. Viruses, viral vectors, oligonucleotides and plasmids

2.2.1. Viruses and viral vectors

Table 2.1: Overview over viruses and viral vectors used

Virus/vector	Relevant characteristics	Reference	Figure
Adenovirus serotype 5 wild type (Ad5 wt)	Wild type virus occurring in the human population	(Garnett *et al.*, 2002)	Figure 1.3 A
Ad-RSV/lacZ	First generation adenovirus expressing LacZ with Rous sarcoma virus (RSV) long terminal repeat promoter	(Kay and Woo, 1994)	
Helper virus (AdNG163R-2)	Ad5-based, inverted packaging signal Ψ is flanked by loxP-sites; promotes replication of HC-AdVs	(Palmer and Ng, 2003)	
HC-AdV-HSB5	High-capacity adenoviral vector (HC-AdV) containing a transgene expression cassette for the HSB5 and Flp	(Hausl *et al.*, 2010)	Figure 1.7; 3.1a
HC-AdV-mSB	The control vector of HC-AdV-HSB5 containing the inactive version of SB (mSB)	(Hausl *et al.*, 2010)	
HC-AdV-TcFIX	HC-AdV containing a transposon determined by inverted repeats (IRs) of SB at each end of transgene cFIX, the transposon is flanked by two FRT sites	(Hausl *et al.*, 2010)	
HC-AdV-luciferase	HC-AdV containing a luciferase expression cassette under the control of hAAT promoter	(Jager and Ehrhardt,	

		2009)	
HC-AdV-wt-hFIX	HC-AdV containing a wild type human coagulation factor IX (wt-hFIX) expression cassette under the control of hAAT promoter	This study	Figure 3.21
HC-AdV-hy-hFIX	HC-AdV containing a hyperactive human coagulation factor IX (hy-hFIX) expression cassette under the control of hAAT promoter	This study	

2.2.2. List of oligonucleotides

Table 2.2: Overview over the oligonucleotides used in this study. All primers were obtained from operon, while all probes were from Ella Biotech.

Target/Name	Orientation	Sequence 5' to 3'	Reference
ASP1	Forward	GTAATACGACTCACTATAGGGC	BD
ASP2	Reverse	ACTATAGGGCACGCGTGGT	
Dog B2M-F	Forward	GGATGAGTTTAGCTGCCGTG	This study
Dog B2M-R	Reverse	TATCTGAGCACAGGCACAGC	
E1-F	Forward	GGGTGAGGAGTTTGTGTTAGATTATG	(Puntel *et al.*, 2006)
E1-R	Reverse	TCCTCCGGTGATAATGACAAGA	
E1-specific probe		[6-FAM] AGC ACC CCG GGC ACG GTTG [TAMAR]	
GSP1	Forward	CCTTAAGACAGGGAATCTTTACTCGGA	This study
GSP2	Reverse	GGCTAAGGTGTATGTAAACTTCCGACT	
hAAT-cFIX f	Forward	CTG ACC TGG GAC AGT GAA TGA T	
hAAT-cFIX r	Reverse	GCC TGG TGA TTC TGC CAT GAT	
hFIX-forw	Forward	ATGCAGCGCGTGAACATGATCA	
hFIX-OL1	Reverse	ACTGCTGGTTCACAGGACTTCTG	
hFIX-OL2	Forward	CAGAAGTCCTGTGAACCAGCAGT	
hFIX-rev	Reverse	AGCTCACTTAATGAAAGCTTGCGG	
hFIX qPCR f		TGGAAGCAGTATGTTGATGGAGA	
hFIX qPCR r		CCTTGCAACTGCCGCCATTT	
Human B2M f	Forward	TGCTGTCTCCATGTTTGATGTATCT	(Vandesompele *et al.*, 2002)
Human B2M r	Reverse	TCTCTGCTCCCCACCTCTAAGT	
L3-F	Forward	GAG TTG GCA CCC CTA TTC GA	(Puntel *et al.*, 2006)
L3-R	Reverse	ATG CCA CAT CCG TTG ACT TG	
L3-specific probe		[6-FAM] CCA CCC GTG TGT ACC TGG TGG ACA [TAMAR]	
Linker for genome walker PCR		GTAATACGACTCACTATAGGGCACGCG TGGTCGACGGCCCGGGCTGGT–3' 3'–H2N-CCCGACCA-PO4–5'	BD
Mouse TBP f	Forward	CCCCACAACTCTTCCATTCT	This study
Mouse TBP r	Reverse	GCAGGAGTGATAGGGGTCAT	
SB-L	Forward	GGTGGCAGCATCATGTTGTG	This study
SB-R	Reverse	CCTTCCTCATGATGCCATCTATT	

DB, BD GenomeWalker™ Kit from BD Biosciences

2.2.3. Plasmids

Table 2.3: List of plasmids used in this study

Plasmids name	Characteristics	Reference
pAAV-EF1a-wthFIX	human elongation factor 1a (EF1a) gene enhancer/promoter-driven wild type (wt) hFIX expression cassette	(Nakai *et al.*, 2001)
pAdFTC	HC-AdV cloning plasmid, Amp^R	(Ehrhardt and Kay, 2002)
pAdFTC-hAAT-(wt/hy)-hFIX	pAdFTC containing wt or hy hFIX under the control of hAAT promoter	This study
pAdFTC-hAAT-cFIX	pAdFTC containing cFIX under the control of hAAT promoter	This study; (Hausl *et al.*, 2010)
pAdFTC-PGK-HSB5	pAdFTC containing HSB5 under the control of the phospho-glycerate kinase promoter (PGK)	
pAdFTC-PGK-mSB	pAdFTC containing mSB under the control of the PGK promoter	
pCMV-HSB5	A helper plasmid encoding a hyperactive transposase (HSB5) under the control of CMV promoter	(Yant *et al.*, 2007)
pCMV-mSB	A helper plasmid encoding inactive transposase (mSB) under the control of CMV promoter	(Yant *et al.*, 2000)
pCI-hAAT-2xMAR	pCI vector with hAAT promoter and two stuffer DNA fragments (ChMAR: matrix attachment region from chicken lysozyme gene, 2.8 kb of each), Amp^R	Generated by Hausl M
pCI-hAAT-(wt/hy)-hFIX	pCI-hAAT-2xMAR containing wt or hy hFIX under the control of hAAT promoter	This study
pCR-Blunt II-TOPO	for the cloning of blunt end PCR fragments, Kan^R	Invitrogen
pET22b-(K265T/Y345T) hFIX	pET22b vector containing the truncated human coagulation factor IX (hFIX) variant (K265T/Y345T)	(Hopfner *et al.*, 1997)
pGL3-Control	Luciferase expression, SV40 promoter, Amp^R	Promega
pGL3-hAAT (ΔSV40 and Luc)	pGL3-Control with liver specific human alpha-1-antitrypsin promoter (hAAT) promoter, w/o ΔSV40 promoter and Luciferase expression	This study
pGL3-hAAT-(wt/hy)-hFIX	pGL3 vector containing wt or hy hFIX under the control of hAAT promoter	This study
pHM5	Shuttle vector, Kan^R	(Mizuguchi and Kay, 1999)
pHM5-hAAT-cFIX	pHM5 containing canine coagulation factor IX (cFIX) under the control of hAAT promoter	(Hausl *et al.*, 2010)
pHM5-hAAT-(wt/hy)-hFIX	pHM5 containing wt or hy hFIX under the control of hAAT promoter	This study
pTnori	Kan^R/Neo^R- marked transposon ori to permit recovery of transposon insertions	(Yant *et al.*, 2000) Figure 3.6
pTOPO-(wt/hy)-hFIX	pTOPO vector containing the full-length wild type (wt) or hyperactive (hy) hFIX	This study
pTOPO-mTBP	pTOPO vector containing mouse TBP gene (TATA box binding protein) fragment	
pTOPO-dB2M	pTOPO vector containing dog B2M gene (Beta-2-microgloblin) fragment	
pTOPO-hB2M	pTOPO vector containing human B2M gene (Beta-2-microgloblin) fragment	

pTOPO-SB	pTOPO vector containing SB gene (Sleeping Beauty transposase) fragment	
pTOPO-L3	pTOPO vector containing L3 gene fragment of Ad 5	
pZAC2.1	For AAV2/8 production, ITR, 4543 bp, Amp^R	Wilson Lab
pZAC-CMV-(wt/hy)-hFIX	pZAC vector containing the full-length wt or hy hFIX under the control of cytomegalovirus (CMV) immediate early promoter	This study

2.3. Molecular genetic methods

2.3.1. Plasmid preparation

For cloning selection and integration site analysis, a small amount (3~5 ml) of bacteria was cultured over night in Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl). To isolate plasmid DNA, 1.5~3 ml bacterial culture were spun down at 15.000 x g for 2 min in a bench top centrifuge. The supernatant was discarded and the bacterial cell pellet was resuspended with 100 µl of resuspension buffer (50 mM Tris-HCl, pH 7.5; 10 mM EDTA, pH 8.0; 100 µg/ml RNase A). Then 100 µl of lysis buffer (0.2 M NaOH, 1 % SDS) were added and the samples were mixed thoroughly and incubated at room temperature for 3 min. After addition of 100 µl of neutralisation buffer (4.09 M guanidine hydrochloride, pH 4.8; 759 mM potassium acetate; 2.12 M glacial acetic acid), the samples were mixed and centrifuged for 5 min at 15.000 x g. The supernatant was transferred to a new Eppendorf tube, which contains 30 µl of sodium acetate (3 M NaAc, pH 5.0) and 900 µl of ice cold 100% ethanol were added. The solution was mixed and the tube was centrifuged at 15.000 x g for 8 min. Then the supernatant was removed and the pellet was washed once with 500 µl of 70% ethanol. After completely removing the supernatant, the DNA pellet was dried and resuspended in 100 µl dH_2O.

For a large amount of plasmid DNA preparation, a plasmid midiprep kit from Promega was used, according to the manufacturer's instructions.

The concentration of DNA was determined using spectrophotometry. 2 µl of DNA solution were measured in 98 µl dH_2O using the Ultrospec 3000 spectrophotometer (Pharmacia Biotech). The concentration of the DNA was calculated using Lambert Beers Law which states concentration of double-stranded DNA in µg/ml = A_{260} x 50 x dilution factor, whereby A_{260} is the adsorption coefficient of DNA at 260 nm. The solution was also measured to determine the absorption at 280 nm for proteins. Only DNA samples with an A_{260}/A_{280} ratio of 1.8-2.0 were used for experiments.

2.3.2. Manipulation of plasmid DNA

2.3.2.1. Restriction digestion of DNA and agarose gel electrophoresis

Plasmid DNA or DNA fragments generated by PCR were digested with the respective restriction endonuclease in a total volume of 20 µl for analytic digests or 100 µl for preparative digests. Digests were performed for 1 h or overnight at the respective cleaving temperature. Cleaved DNA samples were loaded on to 1 % agarose gels containing ethidium bromide (EtBr) for visualization and separation.

2.3.2.2. Isolation of DNA fragments from agarose gels

After running the 1% agarose gel, the respective band of DNA was cut out under UV light. Exposure of the DNA to UV light was reduced to a minimum to avoid mutations. The cut out gel slices were transferred to Eppendorf tubes and the DNA was isolated using a gel extraction kit (Qiagen) according to the manufacturer's instructions.

2.3.2.3. Ligation of DNA fragments and plasmids

To prevent self-ligation, the digested vector DNA was dephorphorylated. 1U of calf intestinal phosphatase (CIP) was used and incubated at 37ºC for 1 h. CIP was then inactivated by incubation at 75 ºC for 10 min. The dephosphorylated DNA was subsequently extracted with phenol: chloroform: isoamylalcohol and ethanol precipitation. The precipitated DNA was re-suspended in 20 µl of dH_2O.

According to the size of the respective fragments/plasmids used in the ligation reaction, an excess of insert DNA was added (plasmid: insert = 1:4). 400 units of T4 DNA ligase (NEB) were used in an as small as possible reaction volume. Ligation was performed for overnight at 16 ºC. A control ligation without insert was always included.

2.3.3. Transformation of bacteria

2.3.3.1. Electrotransformation

Electrocompetent E. coli cells were thawed on ice, and then the DNA was added and gently mixed. For re-transformations, 1 µl of the plasmid DNA were used. For newly ligated plasmids, up to 2 µl of the ligation mixture were used. 50 µl of competent cells containing the plasmid DNA were then transferred to 0.2 cm cuvettes. Samples were electroporated with a BioRad GenePulser® II electroporator using the following conditions: 2.5 kV, 200 Ω, 25 µF. 600 µl of room temperature S.O.C. medium (2% tryptone, 0.5% yeast extract, 8.56 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, and 20 mM glucose) were immediately added after transformation and the suspension was transferred to a 1.5 ml Eppi. The transformed

bacteria were shaken for 1 h at 37°C , subsequently 10-50 µl were plated on the appropriate solid agar, flowing by a over night incubation at 37°C. For further culture and analysis, ~10 colonies were picked.

2.3.3.2. Chemical transformation

Chemically competent E. coli cells were thawed on ice, and then the DNA was added, followed by 5 to 30 minutes incubation on ice. For re-transformations, 1 µl of the plasmid DNA were used. For newly ligated plasmids, up to 5 µl of the ligation mixture were used. Cells were heat-shocked for 30 seconds at 42°C without shaking. Then the tubes were transferred immediately to ice. After addition of 250 µl room temperature S.O.C. medium, the transformed bacteria were shaken for 1 h at 37°C, followed by a similar procedure as in the Electrotransformation (2.3.3.1).

2.3.4. DNA-sequencing and bioinformatic analysis

To control the correctness of cloned DNA fragments and to obtain DNA-sequences for the design of oligonucleotides, respective plasmids were sequenced by Eurofins (MWG Operon, Ebersberg). Obtained sequences were uploaded in VectorDesigner Database browser (Invitrogen) for further analyses. Briefly, the sequences were analyzed by sequence alignments to known DNA sequences and/or certain species genome database. All programmes were provided by the National Center for Biotechnology Information (NCBI). The database and the respective algorithms are available at http://blast.ncbi.nlm.nih.gov/Blast.cgi.

In case of integration sites location in genomic DNA and genomic strand decision, the genomic data base and the BLAT search program for mouse and dog from the Center for Biomolecular Science & Engineering (UCSC) was used (http://genome.ucsc.edu/cgi-bin/hgBlat) (Fujita *et al.*, 2011). For evaluation of the potential cancer formation of the integration sites, all genes located in and near the integration sites were checked to one Cancer Gene Expression Database (CGED, http://lifesciencedb.jp/cged/) (Kato *et al.*, 2005).

2.3.5. Isolation of DNA from mammalian cells

2.3.5.1. Isolation of genomic DNA from cell pellets

The cell pellet was resuspended in 200 µl DPBS and vortexed for complete resuspension. Then 200 µl lysis buffer (10 mM Tris, 10 mM EDTA, 0.5% SDS) were added, supplemented with 30 µl 10% SDS and 15 µl proteinase K (Qiagen, 600 mAU/ml = 20 mg/ml). The suspension was mixed thoroughly and shaken over night at 55°C. On the next day, 2 µl RNase A (10 mg/ml) were added and the suspension was incubated for 30 min at

37°C. Genomic DNA was then purified by phenol: chloroform: isoamylalcohol (PCI, 25:24:1) extraction. Herein, the suspension was mixed thoroughly with PCI and subsequently centrifuged for 2 min at high speed in a bench top centrifuge. Then the supernatant was transferred to a fresh tube. The PCI extraction was repeated once as described above. The DNA was then precipitated by addition of 50 µl NaAc (pH 5.0, 3 M) and 1 ml ice cold 100% ethanol. The DNA was pelleted by centrifugation for 10 min at high speed in a bench top centrifuge. The supernatant was removed and the pellet was washed twice with 500 µl of 70% ethanol. Afterwards, the supernatant was discarded, and the pellet was dried and subsequently dissolved in 120 µl of dH_2O. For determination of DNA concentration and purity a test gel was run and the A_{260}/A_{280} was measured.

2.3.5.2. Isolation of genomic DNA from liver

2 ml lysis buffer (40 mM NaCl, 10 mM Tris pH 7.5, 10 mM EDTA) were filled into 50 ml tubes (Falcon) and 300 µl of 10% SDS were added. Approximately 0.1 g of liver tissue was used. Small pieces of tissue were immediately transferred to the prepared lysis buffer and homogenized with a syringe (needle gauge: 20G). Then 10 µl of Proteinase K (20 mg/ml) were added and the samples were incubated at 55°C for 2 h with permanent gentle shaking. Subsequently, 15 µl RNase A (10 mg/ml) were added and the samples were incubated over night at 37°C on a shaking platform. On the next day the samples were extracted twice with 500 µl phenol: chloroform: isoamylalcohol (25:24:1). After centrifugation at 3000 rpm for 2 min, the supernatant was transferred to a 15 ml Falcon tube. For precipitation of the DNA, three volumes of ice cold 100% ethanol were added and the samples were mixed thoroughly. Subsequently, the samples were centrifuged at 2000 rpm for 10 min. Afterwards, the supernatant was discarded and the DNA-pellet was briefly air-dried. To solve the DNA 600 µl freshly prepared TE buffer (10 mM Tris pH 7.5, 1 mM EDTA, 10 µg/ml RNase A) were added and the samples were gently shaken for 2 h at room temperature. For long-time storage the samples were frozen at -20°C.

2.3.6. Isolation of RNA from mammalian cells

2.3.6.1. Isolation of RNA from cells grown in monolayer

Cells from 6-well plates were resuspended in 200 µl TRIZOL Reagent (Invitrogen), homogenized with a 21-gauge needle (or pipette) and RNA was isolated following the manufacture's instructions. The concentration of isolated RNA was determined using a spectrophotometer (Pharmacia). The integrity of the isolated RNA was evaluated by agarose gel electrophoresis. All work was done on ice. Isolated RNA was stored at -80°C.

2.3.6.2. Isolation of RNA from liver

Liver tissue was stored at -80°C and was transferred in liquid nitrogen. Approximately 0.05~0.1 g of liver tissue was used. 1 ml TRIZOL Reagent (Invitrogen) was added directly into the store tube before the tissue sample was thawed. The sample was homogenized with the help of glass balls in mixer mill MM 200 (Retsch) and RNA was isolated following the manufacture's instructions.

2.3.6.3. Reverse transcription of mRNA into cDNA

Transcription of mRNA into cDNA was done according to the NEB First Strand Synthesis Protocol using 2 µg of purified total RNA. 5 µl of cDNA were directly used for PCR.

2.3.7. Polymerase chain reactions (PCR)

2.3.7.1. Standard PCR

Reaction components

Components	Amount	Final concentration
10 x KOD buffer	5 µl	1 x
MgSO4 (25 mM)	3 µl	1.5 mM
dNTPs (2 mM)	5 µl	0.2 mM (each)
Oligonucleotide 1 (10 µM)	1.5 µl	0.3 µM
Oligonucleotide 2 (10 µM)	1.5 µl	0.3 µM
Template DNA	10-100 ng	
KOD Hot Start DNA Polymerase (1 U/µl)	1 µl	0.02 U/µl
deionized H_2O	add to 50 µl	
Total	50 µl	

Cycling parameters:

Parameters	Temperature	Time	Cycles
Initial Denaturation	95°C	2 min	1 x
Denaturation	95°C	20 sec	30-35 x
Annealing	x°C	10 sec	
Elongation	70°C	y sec	
Final extension	70°C	10 min	1 x

x: Annealing temperature is dependent on the Tm (melting temperature) of oligonucleotides.

y: Elongation is typically 30 second per kb of amplified DNA using KOD polymerase.

2.3.7.2. PCR for GenomeWalker™ DNA walking

1) First PCR

Reaction components

Components	Amount
Template DNA	1 µl of each DNA library
AP1 (10 µM)	1 µl
GSP1 (10 µM)	1 µl
dNTPs (10 mM)	1 µl
10X Advantage 2 PCR buffer	5 µl
Advantage 2 Polymerase Mix (50X)	1 µl
deionized H_2O	40 µl
Total	50 µl

Cycling parameters:

Parameters	Temperature	Time	Cycles
First Denaturation	94°C	25 sec	7 x
First Elongation	72°C	3 min	
Second Denaturation	94°C	25 sec	32 x
Second Elongation	67°C	3 min	
Final extension	67°C	7 min	1 x

2) Nested PCR

Reaction components

Components	Amount
Template DNA	1 µl of 10 x diluted primary PCR product
AP2 (10 µM)	1 µl
GSP2 (10 µM)	1 µl
dNTPs (10 mM)	1 µl
10X Advantage 2 PCR Buffer	5 µl
Advantage 2 Polymerase Mix (50X)	1 µl
deionized H_2O	40 µl
Total	50 µl

Cycling parameters:

Parameters	Temperature	Time	Cycles
First Denaturation	94°C	25 sec	5 x
First Elongation	72°C	3 min	
Second Denaturation	94°C	25 sec	20 x
Second Elongation	67°C	3 min	
Final extension	67°C	7 min	1 x

2.3.7.3. Quantitative real-time PCR (qRT-PCR)

Up to 100 ng of genomic DNA or 20 ng of plasmid DNA was used in a final volume of 20 µl. Oligonucleotides were designed with primerExpress3.0 software (Applied Biosystems) and obtained from Operon. The Taqman Fast Universal PCR Master Mix (Applied Biosystems) was used in corporation with a Taqman 7500 Fast Real Time PCR System (Applied Biosystems) with 7500 Standard program. Table 2.2 shows all oligonucleotides, which were used for qPCR.

(1) Without probe

Components	Volume	Final Conc.
Template DNA	5 µl	Up to 100 ng of genomic DNA or 20 ng of plasmid DNA
FastStart Universal SYBR Green Master (ROX)	10 µl	1 x
Forward primer (10 µM)	0.6 µl	300 nM
Reverse primer (10 µM)	0.6 µl	300 nM
deionized H_2O	3.8 µl	
Total	20 µl	

Cycling parameters:

Parameters	Temperature	Time	Cycles
AmpErase UNG reaction	50°C	2 min	1 x
DNA Polymerase activation	95°C	10 min	1 x
Amplification and real-time analysis	95°C	15 sec	40 x
	60C	1 min	
Dissociation	95°C	15 sec	1 x
	60°C	1 min	
	95°C	15 sec	
	60°C	15 sec	

Amplification and data collection were at 60 °C for 1 min

(2) With probe

Components	Volume	Final Conc.
Template DNA	5 µl	Up to 100 ng of genomic DNA or 20 ng of plasmid DNA
FastStart Universal Probe Master (ROX)	10 µl	1 x
Hydrolysis probe (10µM)	0.625 µl	250 nM
Forward primer (40 µM)	0.5625 µl	900 nM
Reverse primer (40 µM)	0.5625 µl	900 nM
deionized H_2O	3.25 µl	
Total	20 µl	

Cycling parameters:

Parameters	Temperature	Time	Cycles
AmpErase UNG reaction	50°C	2 min	1 x
DNA Polymerase activation	95°C	10 min	1 x
Amplification and real-time analysis	95°C	15 sec	40 x
	60°C	1 min	

Amplification and data collection were at 60°C for 1 min

2.4. Enzyme activity assays

2.4.1. Measurement of alanine aminotransferase (ALT)

For the quantitative determination of ALT in murine serum as signal for liver toxicity, the ALT kit (Randox) was used according to the manufacturer's instructions. 10 µl of fresh murine serum were applied to each reaction. The measurement was conducted in the Ultrospec 3000 spectrophotometer (Pharmacia Biotech).

2.4.2. Enzyme linked immunosorbent assay (ELISA)

2.4.2.1. Detection of canine factor IX (cFIX) in murine serum

Detection of cFIX in murine serum was performed in a sandwich ELISA. 96-well plates (Greiner Bio-One) were coated with an anti-cFIX antibody (Affinity Biologicals). The anti-cFIX antibody was 1:500 diluted in coating buffer (0.1 M $NaHCO_3$, pH 9.4). The plates

were incubated using 50 µl per well at 4°C over night. For blocking, plates were washed with 200 µl TBST (10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20) per well. After removal of the TBST solution, 200 µl dilution buffer (TBST + 5% FBS) per well were added and the plates were incubated 1 h at room temperature. Following twice washes with TBST, samples were diluted with dilution buffer (TBST + 5% FBS) in appropriate relations. For generation of a standard curve, normal dog plasma was used. 50 µl of standard or detected sample per well were loaded and the plates were incubated for 2 h at 37°C. Afterward, the plates were washed 3 times with TBST. For detection, an anti-cFIX-HRPO antibody (Affinity Biologicals) was used. The anti-cFIX-HRPO antibody was applied in a 1:1000 dilution, and loaded 50 µl per well followed by incubate for 1 h at 37°C and 4 times washes with TBST. For development, 100 µl substrate solution (Sigma Fast OPD) per well were used and the plates were incubated for 10 min at room temperature. To stop and enhance the reaction, 50 µl of 2 M H_2SO_4 were added to each well. The samples were finally read at 492 nm in a plate reader (Tecan, Magellan3 software).

2.4.2.2. Detection of human factor IX (hFIX) in tissue culture supernatant and murine plasma

Detection of hFIX was performed in a similar procedure as cFIX. Briefly, an anti-hFIX antibody (Sigma) was used for coating with 1:1000 dilution, purified hFIX (ProSpec-Tany Technogene) was used for generation of a standard curve and an anti-hFIX-HRPO antibody (1:1000 diluted) (Biozol) was used for detection.

2.4.3. Detection of human coagulation factor IX (hFIX) activity by activated partial thromboplastin time (APPT)

The APTT tests were performed on the Merlin MC 1 table coagulation analyser (ABW Medizin und Technik GmbH), which is a semi-automatic mechanical coagulation detection system. The sample was thawed at 37°C immediately prior to performing the assays, Owren's buffer (11 mM sodium citrate, 132 mM NaCl, pH7.3, with 4mM Sodium pyrophosphate as a conservative. MediRox AB) was used for plasma dilution when needed. 50 µl (diluted) sample were mixed with an equal volume of human FIX-deficient plasma (Hart Biologicals) without shaking. This mixture was added in the 12 o'clock position of the cuvette on the machine. After two-minute incubation at 37°C, 100 µl 37°C pre-warmed APPT liquid reagent (MediRox AB) were added to the cuvette in the same position. After another incubate at 37°C for 5 minutes, the clotting was induced by dispensing 100 µl 37°C pre-warmed Calcium Chloride (0.025 mol/L, MediRox AB) in the 9 o'clock position of the cuvette. The clotting time and activity can then be recorded and calculated. For generation of a standard curve, normal human plasma (Hart Biologicals) was used.

2.5. Cell culture

2.5.1. Prokaryotic cells

All bacteria (**Table 2.4**) were routinely grown in Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl). Liquid media were sterilised by autoclaving (121 °C at 1 bar for 20 min). For solid agar, 1.5% agar was added. If required, media were supplemented with the respective antibiotic (**Table 2.5**). Bacteria on LB-agar were cultivated over night at 37°C, if not stated otherwise. Bacteria in liquid culture were cultivated over night at 37°C on a shaking platform.

Table 2.4: Bacterial strains

***E. coli* strain**	**Characteristics**	**Reference**
DH10B	F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139 Δ(*ara, leu*)7697 *gal*U *gal*K λ- *rps*L *nup*G	Invitrogen
DH5α	*end*A1 *hsd*R17(rk-mk+) *supE44 thi*-1 *recA1 gyrA relA1* Δ(*lacZYAargF*) U169 (φ80*lacZ*Δ*M15*)	(Hanahan, 1983)
TOP10	F- mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(araleu) 7697 galU galK rpsL (StrR) endA1 nupG	Invitrogen
Stabl-2	F- endA1 glnV44 thi-1 recA1 gyrA96 relA1 Δ(lac-proAB) mcrA Δ(mcrBC-hsdRMS-mrr) λ^-	Invitrogen

Table 2.5: Antibiotics used for selection of prokaryotic and eukaryotic cells

Antibiotic	**Abbreviation**	**Dissolved in**	**Working concentration**
Ampicillin	Amp	H_2O	100 µg/ml
Kanamycin	Kan	H_2O	20 µg/ml
Hygromycin B	Hyg	H_2O	100 µg/ml
Geneticin	G418	H_2O	500 µg/ml

2.5.2. Eukaryotic cells

2.5.2.1. Cell culture media

1) **Growth medium:**
   Modified eagle medium (MEM, PAA) or Dulbecco's modified eagle medium (DMEM, PAA), supplemented with 10% (v/v) fetal bovine serum (FBS, PAA). For liver cell lines, non-essential amino acids (PAA) were added.
2) **Freezing medium:**
   DMEM or MEM supplemented with 20% (v/v) FBS and 5% (v/v) filter sterilised dimethylsulfoxide (DMSO).

2.5.2.2. Cultivation of eukaryotic cell lines

All cells were grown in a humidified incubator at 37°C and 5% CO_2.

Human embryonic kidney 293 cells (ATCC CRL-1573) and HeLa cells (ATCC CCL-2) were grown in DMEM supplemented with 10% FBS.

Huh7, Sk-Hep-1 (ATCC HTB 52) cells were kept in DMEM containing 10% FBS and 0.1 mM non-essential amino acids.

116 cells (Palmer and Ng, 2003) were used for production of HC-AdVs. This cell line carries a hygromycin resistance gene, expresses Cre recombinase, and was grown in MEM supplemented with 10% FBS and 100 µg/ml hygromycin B.

2.5.2.3. Establishment of cell cultures from cryostocks

The cells were thawed at 37°C and transferred immediately into a 15 ml tubes (Falcon) containing 5 ml of growth medium. Cells were collected by centrifugation at 200 g for 3 min at room temperature. After removing the supernatant, the cell pellet was re-suspended with 5 ml of fresh growth medium and transferred to cell culture dishes containing the appropriate growth medium.

2.5.2.4. Preparation of permanent cell culture stocks in liquid nitrogen

80% confluency cells were trypsinized and collected by centrifugation at 200 g for 3 min at room temperature. After removing the supernatant, the cell pellet was re-suspended in freezing medium. 500 µl of the cell suspension were aliquoted into cryovials and kept in liquid nitrogen for long-term storage.

2.5.2.5. Passaging of eukaryotic cell lines

At a confluence of 50-80%, cells were split at a ratio of 1:4. Cells were detached from the dish with trypsin-EDTA solution (PAA). Trypsinised cells were immediately transferred to new cell culture plates containing the appropriate growth medium.

2.5.2.6. Transfection of eukaryotic cells

For transfection of eukaryotic cells, commercial transfection agents (FuGENE® HD/6 Transfection Reagent, SuperFect Transfection Reagent) were used according to the manufacturer's instructions. Briefly, one day before the transfection experiment, the cells were trypsinized and seeded in the chosen cell-culture vessel. On the day of transfection, plasmids DNA to be transfected were diluted in OPTI-MEM I reduced serum medium (Invitrogen), mixed with respective transfection agent and added to the cells. After a certain

length of incubation period (normally 48 hrs), the respective cells and supernatant were collected and measured.

2.6. Adenovirus production, purification and titration

2.6.1. Production and purification of viral vectors and viruses

2.6.1.1. Production of helper virus

AdNG163R-2 helper virus was amplified in adherent 293 cells (Palmer and Ng, 2003; Jager *et al.*, 2009). Three serial passages using 60-mm (one in total), 100-mm (two in total) and 150-mm (six in total) tissue culture dishes were first performed as pre-amplification. Afterward, a large-scale amplification of the virus was produced on 30 confluent 15 cm cell culture plates. 48 hours after each infection, a cytopathic effect (CPE) should be observed because of helper virus replication (cells are rounded up and loosely or completely detached from the tissue culture dish). Then the cells were harvested for virus purification (see also 2.6.1.3).

2.6.1.2. Production of high-capacity adenoviral vectors (HC-AdVs)

To produce of high-capacity adenoviral vectors, the linearised FTC plasmid DNA was transfected with Superfect (Qiagen) into the producer cell line 116 according to the manufacturer's instructions. 16 to 18 h post transfection, the cells were co-infected with helper virus AdNG163R-2 (HV) (Palmer and Ng, 2003) applying 5 transducing units (TUs) per cell. Cells and supernatant (= passage P0) were harvested 48 h post-infection and frozen at -80°C. Then, 3 to 5 serial passaging steps (P1 - P5) on 6 cm, 10 cm and 15 cm dishes are required for pre-amplification of HC-AdV. Usually, two third of the three-time frozen-thawed lysate of the previous passage was applied to infect the next plate. Co-infection with HV was always at 2 TUs/cell. Lysate was generally harvested when complete cytopathic effect was observed.

After the pre-amplification steps, large-scale HC-AdV production was performed with a 3000 ml spinner flask (Bellco) as described (**Figure 2.1**) (Parks *et al.*, 1996; Palmer and Ng, 2003; Jager *et al.*, 2009). Herein, 116 cells were flushed off with 10 ml fresh media from 10 confluent 15 cm dishes and transferred into a spinner flask containing 900 ml of pre-warmed fresh MEM (Gibco) supplemented with 10% FBS (Sigma) and 100 µg/ml hygromycin B (Invitrogen). Over the next days, the same growth medium with all necessary supplementals was added to a final volume of 3 litres (500 ml on days 2 and 3, 1000 ml on day 4). On day five, 3 litres of 116 cells at 3 to $4x10^5$ cells/ml were harvested by centrifugation (8 min at 1500 rpm in a clinical centrifuge), resuspended in 120 ml volume medium and co-infected with 100% of the crude lysate from the 15 cm dishes of the last

serial passaging step and HV at an MOI of 1 TU/cell. In an alternative simplified protocol, when an HC-AdV is to be re-amplified from excited stock, purified HC-AdV instead of crude lysate was used as inoculum at an MOI of 100 for co-infection of 3 litres of 116 cells. Virus adsorption was performed at 37°C on a magnetic stir plate (Bellco) for 2 h, after which medium (MEM supplemented with 5% FBS, Sigma) was added to a final volume of 2 litres. Co-infected cells were harvested 48 h later for adenoviral vector purification.

2.6.1.3. Purification of adenoviruses and adenoviral vectors

At the end of virus amplification, cells and supernatant of infected dishes or suspend cells from spinner flask were collected in conic 500 ml bottles. Cells containing the virus were spun down for 10 min at 200 x g. Supernatant was removed and the cell pellet was resuspended in 28 ml DPBS. This suspension can be stored at -80°C. To release the virus from the cells, the suspension was four times frozen in liquid nitrogen and thawed in a 37°C water bath. The lysate was spun down for 8 min at 200 x g and the supernatant containing the virus was collected.

For purification of adenovirus and adenoviral vectors, the suspensions obtained above were loaded onto one step and one continuous gradient. The step gradient was established by pipetting cesium chloride (CsCl) solutions with different densities in the following order:

0.5 ml	1.5 g/cm^3 CsCl
3 ml	1.35 g/cm^3 CsCl
3.5 ml	1.25 g/cm^3 CsCl
5 ml	virus/vector

This step gradient was spun for 1.5 h at 35000 rpm (226000 x g)/ 12°C in an ultracentrifuge (Beckman Coulter). The lower bluish band containing the virus was collected and mixed with 1.35 g/cm^3 CsCl (final volume 12 ml). This continuous gradient was spun for at least 20 h at 35000 rpm (226000 x g)/ 12°C. The virus band was collected in an as little volume as possible. The purified virus was dialysed over night at 4°C in 2 l dialysis solution (10 mM Tris pH 7.5, 10% Glycerol, 1 mM $MgCl_2$) at very low stir speed and then aliquotted.

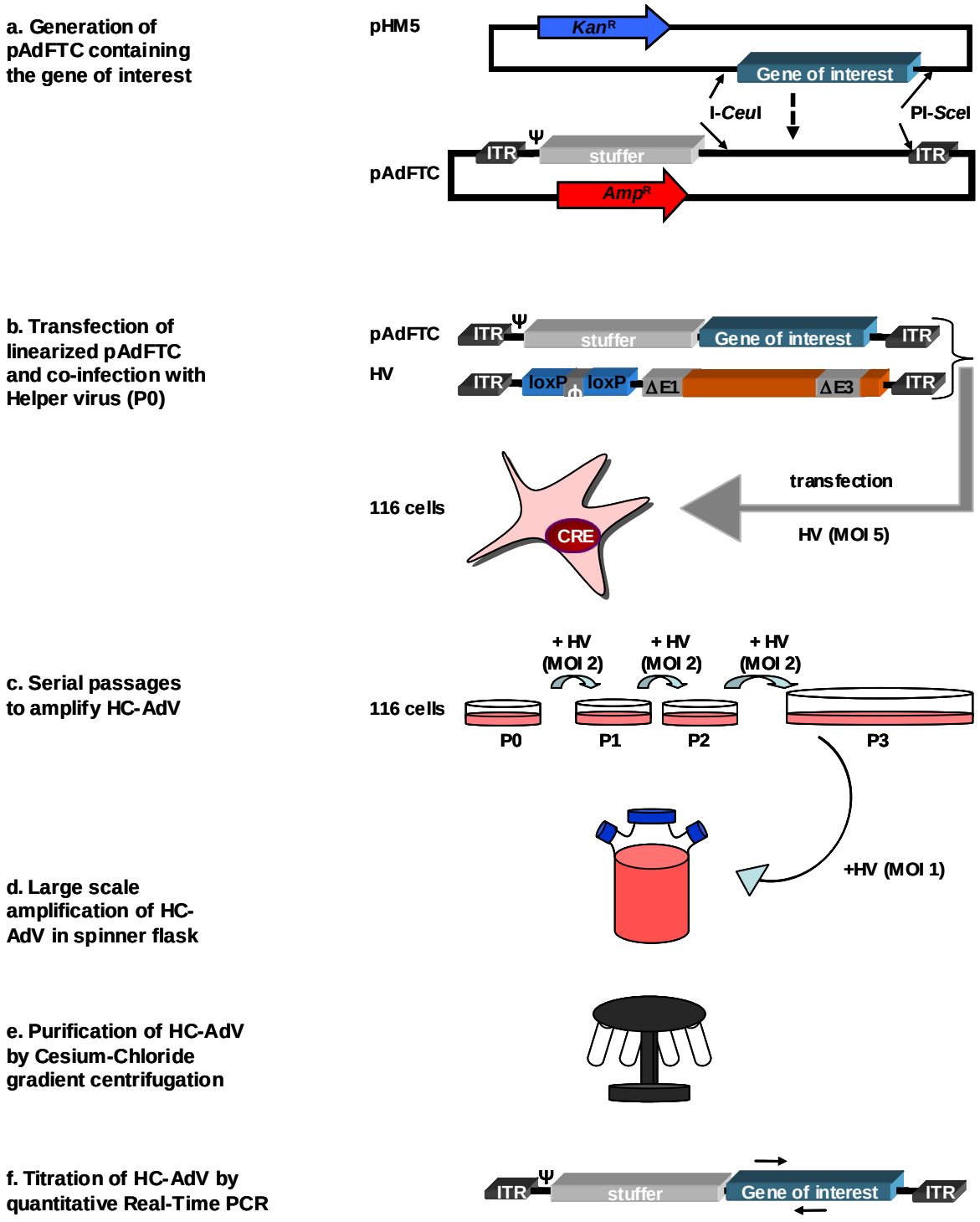


Figure 2.1: Schematic outline for the production of HC-AdV. The transgene is usually cloned from a shuttle vector pHM5 into pAdFTC. The linearized pAdFTC with transgene is then transfected into 116 cells and is co-infected with helper virus AdNG163R-2 at a multiplicity of infection (MOI) of 5. Helper virus provides all necessary gene products for HC-AdV production, while its own genome is not efficiently packaged due to excision of their packaging signal Ψ in Cre-expressing 116 cells. 3-5 serial passages are used to pre-amplify HC-AdV in adherent 116 cells before going to suspend 116 cells in a 3-liter spinner flask. HC-AdV is purified by cesium chloride centrifugation, dialysed and titrated by quantitative Real-Time PCR with primers specifically detecting each transgene (black arrows). ITR: Inverted terminal repeat; HV: helper virus; HC-AdV: High-capacity adenoviral vector; Ψ: packaging signal. Modified from Jager *et al*. 2009.

2.6.2. Adenovirus DNA isolation

50 µl CsCl-purified virus were incubated in 150 µl lysis buffer (10 mM Tris, 10 mM EDTA, 0,5% SDS) supplemented with 10 µl proteinase K (Qiagen, 600 mAU/ml = 20 mg/ml) for 2 h at 55°C. Subsequently, the solutions were purified with phenol: chloroform: isoamylalcohol extraction. The DNA was precipitated with ice cold 100% ethanol for 20 min at -20°C and centrifuged with full speed at 4°C for 10 min. Precipitated DNA was resuspended in 50 µl dH_2O.

2.6.3. Titration of adenoviral vector preparations

2.6.3.1. Determination of the physical titer

Viral DNA was released from virions obtained from CsCl gradients in dilution buffer (100 mM Tris, 10 mM EDTA, 0.1% SDS). The A_{260} was measured to determine the viral titer. The viral particle concentration was determined by the well-characterized absorption of pure adenovirus DNA at 260 nm (Maizel *et al.*, 1968), an OD_{260nm} unit corresponds to of 1.1 x 10^{12} VPs/ml based on a 36 kb size of the wild type particle genome. Thus, the optical particle units (OPU) for HC-AdV and other adenoviruses were calculated using the following formula: OPU/ml = (absorbance at 260 nm) × (dilution factor) × (1.1×10^{12}) × (36)/(size of HC-AdV in kb).

In brief, 475 µl of dilution buffer were added to a 25 µl aliquot of the CsCl purified virus. Suspensions were vigorously shaken at 1200 rpm for 15 min in a table top shaker. Subsequently, samples were briefly centrifuged and subjected to OD_{260nm} measurement. 100 µl of each 5 aliquots were used for determination.

2.6.3.2. Determination of the infectious titer by quantitative real-time PCR

The number of infectious genomes was determined by quantitative real-time PCR (qPCR) employing the oligonucleotides described in table 2.2. Infectious particles were determined by analyzing two different diluted (10^{-2} and 10^{-3}) genomic DNA of infected (2 µl, 0.4 µl or 0.08 µl virus infect 6-cm diches) 293 cells in a total volume of 20 µl. These infected 293 cells were harvested with trypsin after 4 h of incubation, and washed twice with PBS to remove unabsorbed virions. Genomic DNA was isolated as described (2.3.5.1). The Taqman 7500 Fast Real Time PCR System (Applied Biosystems) was used for detection. The programme of the Taqman 7500 was set as described in 2.3.8.3. Universal Fast PCR Master Mix (Applied Biosystems) was used for Taqman qPCR. Primers were designed by Primer3 when not found from literature.

Each sample was normalized to genomic hB2M gene level. Quantification was performed by calculating known hB2M levels from the plasmid pTOPO-hB2M by the

following term:

Copies/g plasmid= (6.02 x 10^{23} copies/mol) / [plasmid size (bp) x (330 Da (g/mol)/nucleotide) x 2 nucleotide/bp]

2.6.3.3. Determination of helper virus contamination and back-recombination of E1 sequences

The determination of the helper virus contamination in HC-AdV preparations was performed using two differently diluted (10^{-2} and 10^{-3}) genomic DNA preparations isolated from virus infected cells. While the detection of E1 back-recombination was analyzed using purified adenoviral DNA in three different dilutions per assay: 10^{-1} to 10^{-3}. For generation of a standard curve for qPCR, 10^{2}–10^{8} genome copies of a purified wild-type adenovirus serotype 5 with known plaque-forming units were used. Adenovirus DNA was isolated according to step 2.6.2. Universal Fast PCR Master Mix (Applied Biosystems) was used for Taqman qPCR. Oligonucleotides, probes and protocol parameters were chosen according to Puntel *et al.* 2006. In a total volume of 20 µl per reaction, 5 µl of the template DNA was added.

Quantitative real-time PCR was carried out by the Taqman 7500 Fast Real Time PCR System (Applied Biosystems) amplifying a 67 bp area of the adenoviral late gene 3 (L3) and a 436 bp area of the adenoviral early gene 1 (E1), respectively. For L3 quantification, the oligonucleotides L3 forward and L3 reverse together with an L3 specific probe were used. The PCR was run with the program described in 2.3.8.3. For E1 quantification, the same program was used but with its own relative primers and probe, as shown in table 2.2.

2.7. Animal studies

2.7.1. Mice studies

All mice (C57Bl/6) were kept and treated according to the regulations of the Government of Upper Bavaria. 6- to 8-week old C57Bl/6 mice were used for all studies.

2.7.1.1. Transduction of C57Bl/6 mice with viral vectors and plasmid vectors

For viral vectors delivery, all mice were injected via the tail vein with a total volume of 200 µl containing different transducing units of HC-AdVs diluted in Dulbecco´s phosphate buffered saline (DPBS, Gibco).

For delivery of plasmids to murine liver, the hydrodynamic injection procedure was utilized. Herein, all mice were injected via the tail vein with 20-80 µg of plasmid DNA in a total volume of 2 ml PBS within 5 to 8 seconds (Bell *et al.*, 2007).

Rapid cell cycling of murine liver cells was induced by intraperitoneal administration of 50 µl carbon tetrachloride (CCl_4, Sigma) solution (1:1 dilution in mineral oil, Sigma).

2.7.1.2. Blood sample collection from mice

For serum sample, blood was collected via periorbital puncture, using heparin coated glass capillaries (micro-haematocrit-tubes, Assistent). Serum was subsequently separated from blood cells by centrifuge for 15 min at 4°C and 9000 rpm. All serum samples were stored in -80ºC before use.

For plasma collection, it's important not to damage the coagulation factors. For this purpose, the mice were first anesthetized with isofluran (Eickemeyer), and then pre-warmed for 5 min by a red light lamp. It is important that the head is covered with one or more tissues while pre-warming. The eye was stitch with a normal glass capillary without soaking in the blood, after discarding the first drop of blood the following blood drops were directly collected into a tube with 3.8% of sodium citrate solution at a ratio of 1:10. After the collection, the samples were mixed and put immediately on ice. Finally the plasma is separated by centrifugation for 15 min at 4°C and 9000 rpm. The plasma can be stored in -80ºC for months.

2.7.2. Dog studies

Inbred hemophilia B dogs were obtained from the University of North Carolina at Chapel Hill (Chapel Hill, NC). These hemophilia B dogs carry a missense mutation in the catalytic domain of the FIX-coding sequence resulting in undetectable FIX protein product and activity (Evans *et al.*, 1989). Dog studies were performed under the guidelines of the University of North Carolina. For vector administration, dogs were sedated with Domitor (750 µg/m^2 body surface area) and the vector dilution (~18.5 ml) followed by ~10 ml PBS for vector wash was infused by peripheral vein injection at 0.5 ml/minute into the right foreleg. During injection heart rate, blood pressure, and body temperature were monitored. Routine laboratory measurements before and after adenoviral vector administration were performed.

2.7.2.1. Detection of neutralizing anti-adenoviral antibodies in canine serum

The principle of this test is to analyze at which dilution the canine serum potentially containing anti-adenoviral antibodies is able to inhibit 50% of the transduction of a reporter virus into 293 cells.

293 cells were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% FBS. One day before performing the assay, 293 cells were seeded into 96-well tissue culture plates containing 100 µl of medium. To destroy heat-sensitive complement components, both dog serum and all FBS used for cell culture were incubated at 56°C for 40 minutes. Briefly, the serum samples were diluted in 2-fold steps in DMEM with 10% heat-inactivated FBS in a total volume of 50 µl. Then 50 µl of the lacZ-expressing reporter virus

Ad-RSV/lacZ were added to the diluted serum samples at a concentration of 1 x 10^9 plaque-forming units/mL (diluted in DMEM with 10% heat-inactivated FBS). After an incubation of 1 hour at 37°C, the serum-virus mixture was applied to 80% confluent monolayer of 293 cells. Cells were incubated for 24 hours, and then fixed with 0.5% glutaraldehyde for 5 minutes, washed with PBS, and stained in PBS containing 3 mM potassium ferricyanide, 3 mM potassium ferrocyanide, 2 mM MgCl2, and 1 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). As a negative control untreated dog serum and PBS were used for the experiment, respectively. The neutralizing anti-adenovirus antibody titer was defined as the reciprocal of the highest dilution of serum at which the infectivity of the reporter virus was decreased by at least 50% (**Figure 2.2**).

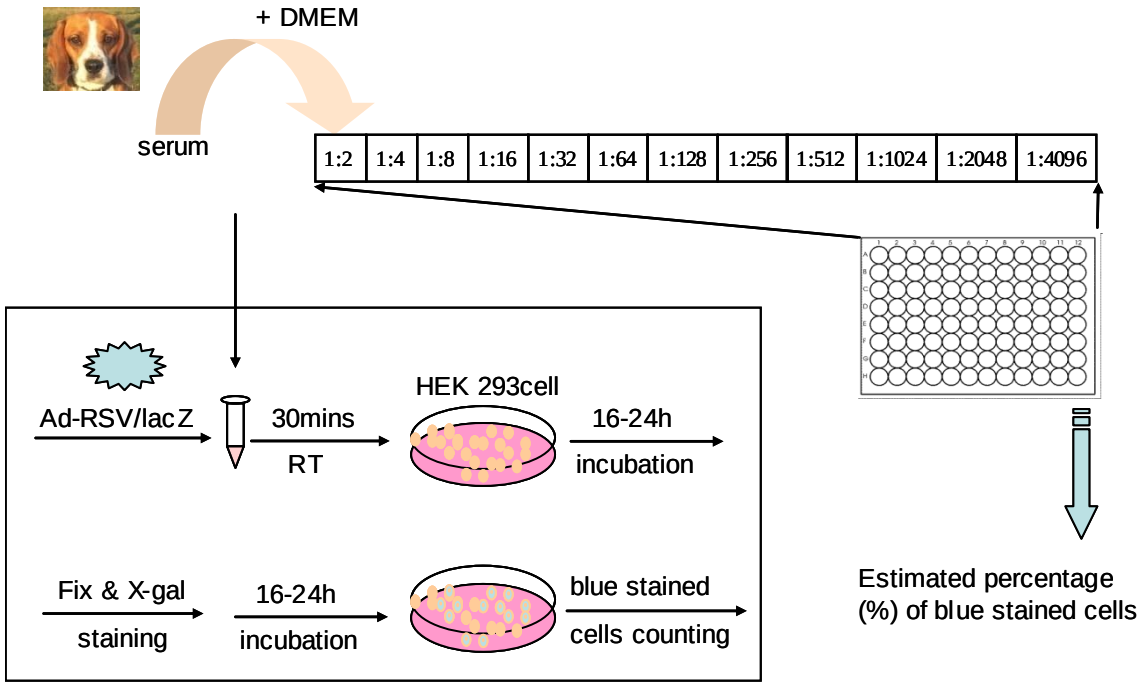


Figure 2.2: Schematic outline for the detection of neutralizing anti-adenoviral antibodies in treated dogs. The anti-adenoviral antibodies can recognize the LacZ expressing virus (Ad-RSV/lacZ) and inhibit its infection and LacZ expression. Therefore, the inhibition rate is determined by which dilution the antibody contained in blood serum can inhibit the infectivity of the reporter virus by 50%. The neutralizing-antibody titer was defined as the reciprocal of this dilution.

3. Results

3.1. Virus characterization and titration

To evaluate the gene-transfer effect and safety profile of adenovirus/Sleeping Beauty (SB) transposase hybrid-vectors after systemic application, high-quality viral vector productions are needed and the viral vectors need to be titrated for their transducing ability. Moreover, some other unwanted side effects from the virus production, like helper virus contamination and back-recombination of the deleted viral genome region, should be carefully checked and excluded.

3.1.1. Production of high-capacity adenoviral vectors (HC-AdVs)

Three HC-AdVs were generated for the adenovirus/Sleeping Beauty (SB) transposase hybrid-vector system **(Figure 3.1.a).** The transposon-donor vector HC-AdV-TcFIX contains a transposon, which encodes a cassette for liver-specific expression of canine factor IX (cFIX). The transposon is flanked by SB specific inverted repeats (IRs) and Flp recognized FRT sites. The transposon-actor vector HC-AdV-HSB5 provides the components of the integration machinery, which include the cDNA for Flp recombinase and the hyperactive SB transposase mutant HSB5. HSB5 demonstrated 10-fold higher activity in cell culture in comparison to the wild type transposase (Yant *et al.*, 2007). As a negative control for the integration machinery, the vector HC-AdV-mSB containing an inactive version of the Sleeping Beauty transposase (mSB) (Yant *et al.*, 2004) was used. Several independent viral preparations of each HC-AdV were produced and amplified following a protocol for large-scale production of HC-AdV in spinner flasks and subsequent purification by cesium chloride (CsCl) ultracentrifugation (**Figure 2.1**) (Palmer and Ng, 2003; Jager *et al.*, 2009). The viral vectors used for the dog study and in part viruses for mouse studies were prepared by Martin Hausl.

3.1.2. Determination of infectious titers and total physical titers of high-capacity adenoviral vectors (HC-AdVs)

All virus preparations were characterized with respect to total physical titers and infectious titers. It was found in previous studies that the ratios of physical titer compared to infectious titer (virus particle/transducing unit, VP/TU) vary for different HC-AdV preparations (Palmer and Ng, 2003; Crettaz *et al.*, 2008). Therefore, it is important to carefully measure both parameters.

Infectious titers of final vector preparations for animal studies were determined by quantitative real-time PCR (qPCR) with the primer pairs specific for the respective transgenes (HSB5/mSB and cFIX) described in Table 2.2 and Figure 3.1a with horizontal arrows. The genomic DNA isolated from virus infected 293 cells was used as templates. The

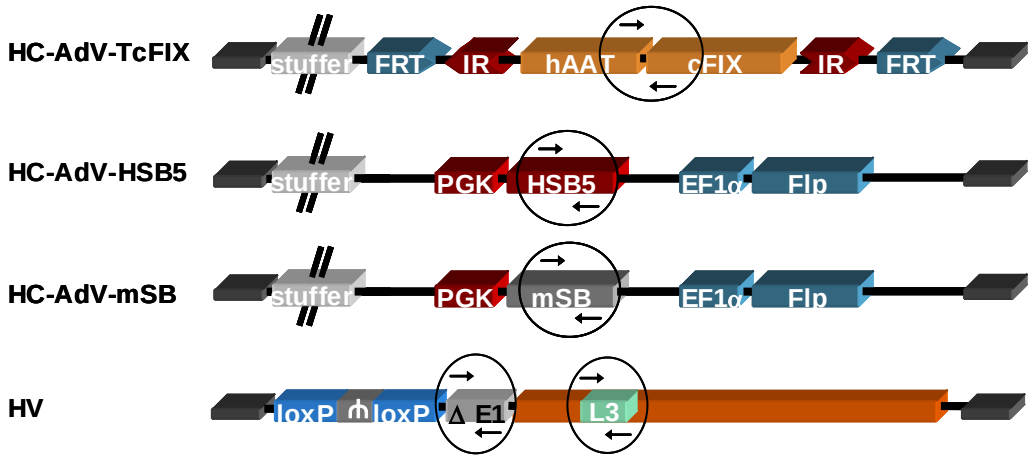
human B2M (Beta-2-microgloblin) gene was used as internal control (Vandesompele *et al.*, 2002). Herein, it was found that the infectious titers measured by qPCR were generally non-equal to the physical titers quantified by OD_{260nm} measurements for all three viruses (HC-AdV-TcFIX, HC-AdV-HSB5 and HC-AdV-mSB) (**Figure 3.1.b**). Figure 3.1 b shows representative virus preparations that were used for murine studies. The VP/TU ratios were 13.6 and 10.1 for the two cFIX viruses, 4.2 and 4.6 for the two HSB5 viruses; 18.9 and 47.8 for the two mSB viruses. The second mSB virus with the VP/TU ratio 47.8 was excluded from further studies, because a high VP/TU ratio means low percentage of infectious HC-AdV virions.

3.1.3. Determination of the helper virus (HV) contamination and exclusion of replication competent adenovirus (RCA)

In the HC-AdV production system, the Cre/loxP system is used to excise the packaging signal (Ψ) from the helper virus (HV) genome, resulting in unpackaging of the HV genome (Palmer and Ng, 2003). However, the excision efficiency of Cre/loxP is not 100%, thus the HVs are packaged to some extent. The physical separation of the HC-AdV from the HV by CsCl ultracentrifugation can significantly reduce the level of HV contamination but is still not 100% efficient. Therefore, it is important to figure out the level of HV contamination in each HC-AdV preparation. The level of helper virus contamination was determined by quantitative real-time PCR using the genomic DNA isolated from virus infected cells. The PCR specifically detected the helper virus genome by primers and probe binding to the late protein 3 (L3) of adenovirus serotype 5 (**Figure 3.1.a**). The helper virus (infectious units) contamination levels of purified HC-AdVs were lower than 0.03% compared to the infectious titers of HC-AdV. For example, the infectious units ratio of HV to HC-AdV were 0.013% and 0.011% for the two cFIX viruses, 0.006% and 0.011% for the two HSB5 viruses and 0.021% and 0.0266% for the two mSB viruses (**Figure 3.1.b**).

Homologous recombination between the genomes of the helper virus and producing cell line which expresses the complementary E1 sequence during vector amplification has been reported to yield replication competent adenovirus (RCA)(Sandig *et al.*, 2000; Palmer and Ng, 2003). Therefore, it is important to rule out whether replication competent adenovirus is present in the HC-AdV preparations and the helper virus stocks (Puntel *et al.*, 2006). The E1 back-recombination was checked by quantitative real-time PCR using isolated virus genomic DNA from purified virus preparations. The PCR primers and probe specifically detected the E1 region (**Figure 3.1.a**). As expected, the E1 sequence was undetectable in all virus preparations, indicating that there were no replication competent adenovirus present in the purified HC-AdV particles and the helper virus stock (data not shown).

a



b

Virus	HC-AdV-TcFIX		HC-AdV-HSB5		HC-AdV-mSB	
	Prep 1	Prep 2	Prep 1	Prep 2	Prep 1	Prep 2
VP/µl	$3.5x10^8$	$7.9x10^8$	$4.2x10^9$	$2.9x10^9$	$6.6x10^8$	$6.5x10^8$
TU/µl	$2.6x10^7$	$7.9x10^7$	$9.9x10^8$	$6.4x10^8$	$3.5x10^7$	$1.4x10^7$
VP/TU	13.6	10.1	4.2	4.6	18.9	47.8
% HV	0.013	0.011	0.006	0.011	0.021	0.026

Figure 3.1: Characterization of the viruses for *in vivo* application. (a) Schematic outline of the viral vector constructs and location of real-time PCR primers. HC-AdV-TcFIX represents the transposon-donor vector, expressing canine coagulation factor IX under control of the liver specific human alpha-1-antitrypsin promoter (hAAT) including two liver specific enhancers (HCR: hepatocyte control region; ApoE: Apolipoprotein E). It contains a transposon determined by inverted repeats (IRs) at the ends and is flanked by two FRT sites for Flp-mediated excision. The HC-AdV-HSB5 contains a transgene expression cassette for the hyperactive Sleeping Beauty (SB) transposase (HSB5) under the control of the phospho-glycerate kinase promoter (PGK) and an expression-cassette for the Flp recombinase driven by the elongation factor-1-alpha promoter (EF1α). The control vector HC-AdV-mSB contains the inactive version of SB (mSB). All HC-AdVs include 22 kb stuffer DNA derived from human/mouse chromosomal DNA to optimize packaging of viral vectors. (A detailed description for the principle of the adenovirus/transposase hybrid-vector system can be found in Figure 1.7 from introduction part). The helper virus (HV) AdNG163R-2 used for the HV-AdV production is an E1-deleted first generation adenoviral vector. Arrows inside the circles show the real-time PCR primers binding sites. For the HC-AdV-TcFIX vector, one 150 bp PCR product was amplified; for vectors HC-AdV-HSB5 and HC-AdV-mSB, the same primer sets were used to amplify a 141 bp PCR product, for HV, a primer pair and probe binding to L3 gene was used to amplify and detect a 67 bp PCR product, and for the detection of E1 back-recombination, primers and probe specific to E1 gene were used to amplify and detect a 436 bp PCR product. (**b) Titer and purity of the final adenoviral vector preparations.** The table summarizes the titration results and contamination levels with the helper virus used for vector production. Shown are six representative preparations of the HC-AdV which were used for hepatic infusion into C57Bl/6 mice. Physical titers were determined by OD_{260nm} measurements. Molecular titration using qPCR was carried out to measure infectious titers and HV contamination by analyzing genomic DNA of virus infected 293 cells. The level of helper-virus contamination was defined as the percentage of transducing helper-virus particles contained in the total amount of transducing HC vector units.

After the titration and characterization, most of the HC-AdV preparations were found to contain high transducing unit numbers (10^7-10^8 TUs/µl), low helper virus contamination levels (<0.03%) and undetectable RCA. Otherwise the virus would be excluded from the further *in vivo* studies (e.g. the second mSB virus preparation, Prep2).

3.2. *In vivo* application of the novel adenovirus/transposase hybrid-vectors

3.2.1. Application of the hybrid-vector system in small animals

After systemic administration of high-capacity adenoviral vectors (HC-AdVs), the severity of the acute toxicity is vector dose dependent (Brunetti-Pierri *et al.*, 2004). This phenomenon represents the most essential obstacle currently hindering the usefulness of this otherwise promising gene therapy technology. Therefore, it is important to optimize the vector dose in a pre-clinical setting. Herein three different vector doses were chosen for C57Bl/6 mice: a low dose with $1.6x10^8$ transducing units (TUs), a medium dose with $8x10^8$ TUs or a high dose with $4x10^9$ TUs per mouse, respectively. The two vectors HC-AdV-TcFIX and HC-AdV-HSB5 were co-injected at a ratio of 3:1 **(Figure 3.2.a)**. As negative controls for somatic integration, mice of the control group were treated with HC-AdV-mSB encoding an inactive version of SB or using HC-AdV-Luc as an irrelative vector control instead of HC-AdV-HSB5 at medium and high vector doses ($8x10^8$ or $4x10^9$ TUs per mouse) **(Figure 3.2.a).**

In groups which received the highest dose ($4x10^9$ TUs per mouse), high levels of transgene expression were obtained (~10000 ng/ml cFIX measured by ELISA). However, this dose was accompanied by cases of death (9 mice died from 15) one week post vector administration. And the reasons for these fatalities remain to be elucidated. At the lowest dose, only low levels of serum cFIX were detected (~100 ng/ml). This was in sharp contrast to the medium-dose group, in which serum cFIX levels ~1000 ng/ml were measured until 4 weeks post-vector infusion **(Figure 3.2.b).**

3.2.1.1. Stable transgene expression after the hybrid-vectors administration into mice

To test in which extent SB-mediated integration from the episomal adenoviral genome can facilitate the stability of transgene expression levels *in vivo*, mice with different vector infusions **(Figure 3.2.a)** were further treated intraperitoneally with carbon tetrachloride (CCl_4). CCl_4 is a liver-toxic substance and leads to death of affected hepatocytes by necrosis (Weber *et al.*, 2003). Therefore, the total size of the liver and number of hepatocytes will be reduced. The amount of CCl_4 injected (25µl) leads to loss of about 70% of the whole liver (Das *et al.*, 2007). To reconstruct a normal liver size, the hepatocytes need to divide. When the hepatocytes are actively dividing, only genes which integrate stably into chromosomal DNA

can be replicated under the control of the parental chromosome. Following each cell division, the integrated gene will be stably inherited to both daughter cells (Strachan *et al.*, 1999). As a result, all hepatocytes that descend from a single cell in which stable integration took place will contain the integrated gene; thereby the transgene can continue its stable expression after CCl_4 injection. In contrast, episomal genes which do not integrate can not segregate to all daughter cells during subsequent mitoses. As a result, the non-integrated transposon forms will be diluted and get lost quickly after CCl_4 treatment and cell division, resulting in the decrease of transgene expression levels.

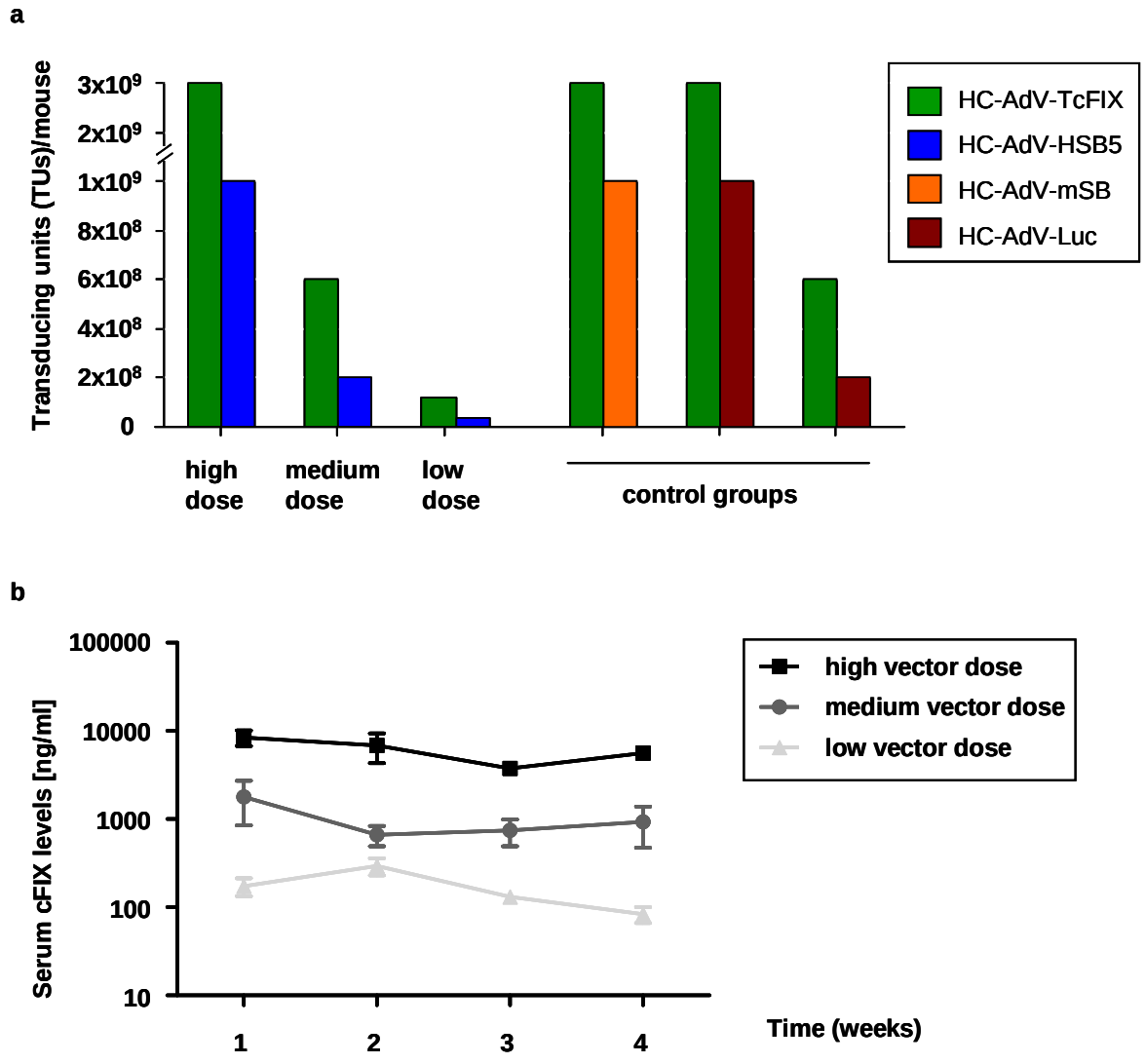


Figure 3.2: *In vivo* application of the AdV/SB hybrid-vector system in mice. (**a) Vector administration**. Female C57Bl/6 mice were co-injected with HC-AdV-TcFIX and HC-AdV-HSB5 at a ratio of 3:1 with three different vector doses ($1.6x10^8$, $8x10^8$ or $4x10^9$ TU per mouse). Mice of the control groups were treated with HC-AdV-mSB (expressing inactive SB) or HC-AdV-Luc (as an irrelevant vector control) instead of HC-AdV-HSB5 with the vector doses ($8x10^8$ or $4x10^9$ TU per mouse). (**b) Dose-effect of the AdV/SB hybrid-vector in mouse**. Dose-dependent transgene expression levels of the AdV/SB hybrid-vector were found in mouse (n=5 for medium and low dose; n=3 for high dose). Canine FIX expression levels were measured weekly by ELISA.

All experimental groups (HC-AdV-TcFIX co-injected with HC-AdV-HSB5 at three doses: $1.6x10^8$, $8x10^8$ or $4x10^9$ TUs per mouse; HC-AdV-TcFIX co-injected with HC-AdV-mSB or HC-AdV-Luc at two doses: $8x10^8$ or $4x10^9$ TUs per mouse) were monitored for transgene (cFIX) expression levels for more than three months. Canine FIX expression levels in blood circulation were measured by ELISA one day before CCl_4 injection and at later time points every other week. Although the transgene expression levels were slightly decreased after the first CCl_4 injection, mice which received the high dose ($4x10^9$ TUs per mouse) of the HC-AdV-TcFIX/HSB5 remained stable at physiological cFIX levels (3000~5000 ng/ml) during the whole time course of the experiment. This was in contrast to the control group which received the same dose of vectors but instead of HSB5 a vector expressing inactive SB (mSB). In this group the transgene expression levels were most likely derived from non-integrating vectors, and therefore the cFIX levels dropped quickly after induction of cell division by CCl_4 treatment (**Figure 3.3.a**). However, probably due to the toxicity caused by the high vector dose, many infused mice died due to dose-dependent toxicity one week post-injection. Therefore, this high viral load ($4x10^9$ TUs per mouse, referring to $2x\ 10^{11}$ TUs per kilogram body weight) could be a limitation for *in vivo* application.

In the medium vector dose group ($8x10^8$ TUs per mouse), the same stability of transgene expression as in the high vector dose group was observed after adenovirus/transposon hybrid-vectors infusion and CCl_4 treatment. Although the cFIX levels decreased after the first CCl_4 injection, stable levels of cFIX (300-800 ng/ml) were detected throughout the whole experiment. As a control group, mice received the same dose of the cFIX vector but instead an irrelative vector (HC-AdV-Luc) was co-delivered. As expected, in this group the serum cFIX expression levels decreased quickly after induction of cell cycling **(Figure 3.3.b).**

Regarding the group which received the lowest dose of vector infusion ($1.6x10^8$ TUs per mouse), low serum cFIX levels (~100 ng/ml) were observed in the first few weeks, which dropped to undetectable levels after CCl_4 administration (data not shown).

To compare stability of transgene expression from episomal HC-AdV (TcFIX/luc), HC-AdVs from which the transgene is excised (TcFIX/mSB), and HC-AdVs from which the transgene is excised and subsequently integrated into the host genome (TcFIX/HSB5), transgene expression levels before and after CCl_4 administration were determined. The loss rates of transgene expression levels for all systems with and without induction of rapid cell cycling in murine liver were determined. The integrating HC-AdV system utilizing HSB5 for somatic integration was found to be superior in cycling cells compared to episomal HC-AdVs (**Figure 3.3.c**). Moreover, comparable levels of relative transgene expression were observed in the HC-AdV- TcFIX/mSB group and HC-AdV- TcFIX/Luc group after CCl_4 treatment, indicating that the transgene expression is independent of excision of the transposon from

the adenoviral vector (**Figure 3.3.c**) (Hausl *et al.*, 2010). These results are in concordance with historical data utilizing AdV/SB hybrid-vectors (Yant *et al.*, 2002). In both studies stable transgene expression levels were obtained after CCl_4 administration.

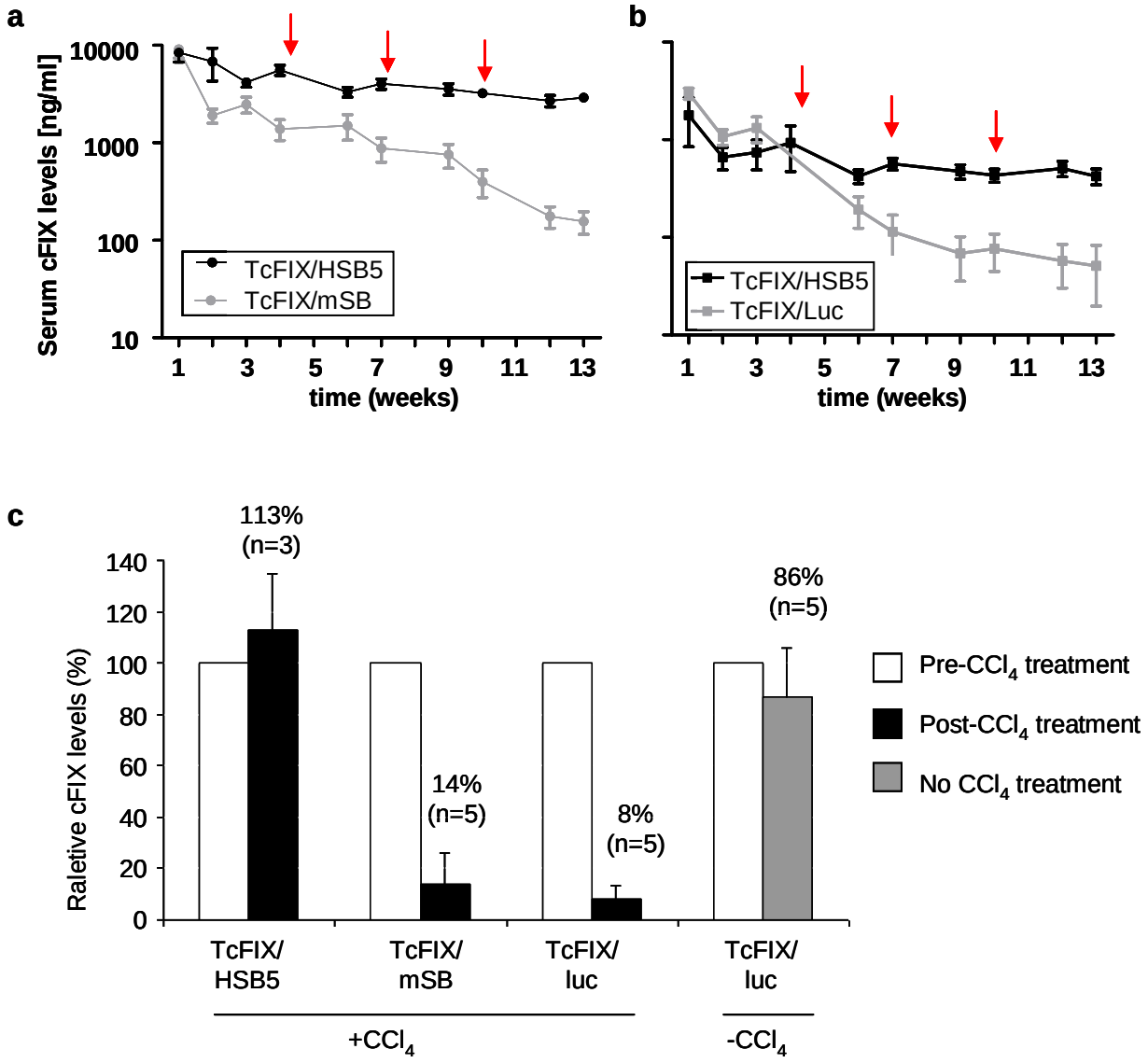


Figure 3.3: *In vivo* cFIX expression levels in actively dividing mouse hepatocytes. HC-AdV-TcFIX was co-delivered with either HC-AdV-HSB5 (HSB5), HC-AdV-mSB (mSB) or the irrelevant vector HC-AdV-luc (Luc) at a ratio of 3:1. Persisting transgene expression levels were found in mouse which received TcFIX/HSB5 hybrid-vector, compared to mice that received inactive SB (TcFIX/mSB) or irrelevant vector (TcFIX/Luc). (n=5 for all group, except for high dose HSB5 n=3). Canine FIX expression levels were measured weekly by ELISA. Arrows indicate when animals received intraperitoneal injections of CCl_4 to promote hepatic cell cycling. **(a) Mice received high dose ($4x10^9$ TU per mouse) of vector infusion. (b) Mice received medium dose ($8x10^8$ TU per mouse) of vector infusion. (c) Relative transgene expression levels.** Mice were treated three times with CCl_4 and serum levels of cFIX before CCl_4 treatment (white bar) were defined as 100% for each group. Black bar, post-CCl_4 treatment; gray bar: no CCl_4 treatment. Serum cFIX levels were determined one day before and 3 weeks after CCl_4 treatment for all groups.

3.2.2. Application of the adenovirus/transposase hybrid-vectors in large animals

Several biological differences exit between mouse and human, such as the body size, life span and immune responses (**Figure 1.8**). All these elements make it necessary to address the treatment effect in large animals before going to human clinical trials. For the present study using the novel adenovirus/transposase (HSB5) hybrid-vector system, after verifying its efficiency in mice, it is of importance to check whether transposition can also enable somatic integration and subsequent phenotypic correction in a preclinical animal model for hemophilia B.

Therefore, three hemophilia B dogs carrying a missense mutation in the catalytic domain of the genomic cFIX coding sequence (Evans *et al.*, 1989) were intravenously injected with the same vector system (**Figure 3.1.a**)**.** All dogs (D1, D2 and D3) were co-injected with the canine factor IX transposon donor vector and the transposase delivery vector at a 2:1 ratio. For the experimental setting, dog D1 received a total dose of $9.4x10^{11}$ VPs/kg of the TcFIX/HSB5 system while dog D2 received a 2.6-fold increased dose ($2.4x10^{12}$ VPs/kg). As a control, dog D3 was injected with the inactive version of the transposase system (TcFIX/mSB) at a total dose of 1.2 $x10^{12}$ VPs/kg, which was equivalent to the virus dose applied in dog D1 (Hausl *et al.*, 2010).

3.2.2.1. Phenotypic correction after administration of the hybrid-vectors in hemophilia B dogs

For dog D1, which received the TcFIX/HSB5 hybrid-vector system, the whole blood clotting time (WBCT) was shortened to 18.5 minutes and stabilized for the total study length of 960 days (**Figure. 3.4.a**). This clearly demonstrated that phenotypic correction of the coagulation factor IX deficiency occurred and suggested that in contrast to the historical study utilizing a comparable dose of the cFIX encoding vector (Ehrhardt *et al.*, 2003), stabilization of transgene expression occurred. In sharp contrast, systemic administration of the inactive transposase system (TcFIX/mSB) in dog D3 at a similar viral dose resulted in transient phenotypic correction (**Figure. 3.4.b**) (Hausl *et al.*, 2010). Surprisingly, dog D2 which was also treated with the TcFIX/HSB5 hybrid-vector system but with an increased dose showed only transient phenotypic correction (**Figure. 3.4.b**). This may indicate that toxic side effects associated with the incoming viral proteins or a robust immunological response against incoming viral particles or against one of the transgene products may have occurred.

In order to monitor transgene expression, cFIX levels in canine plasma samples were monitored. Supraphysiological plasma cFIX levels of up to 40 000 ng/ml were detected for dog D1 on days 3-9 post-injection (**Figure. 3.4.a**). During 9-27 days post-injection, a rapid decline of canine factor IX (cFIX) plasma levels was observed. However, after 4 weeks,

plasma cFIX levels stabilized within a therapeutic range (60 ng/ml) for up to 295 days (latest time point analyzed by cFIX ELISA; this ELISA experiment was performed by Christina Rauschhuber). For dogs D2 and D3 high physiological cFIX plasma levels were observed 5-10 days post-injection, which declined to undetectable levels 40 and 20 days post-injection for dogs D2 (**Figure 3.4.b**) and D3 (**Figure 3.4.b**), respectively (Hausl *et al.*, 2010).

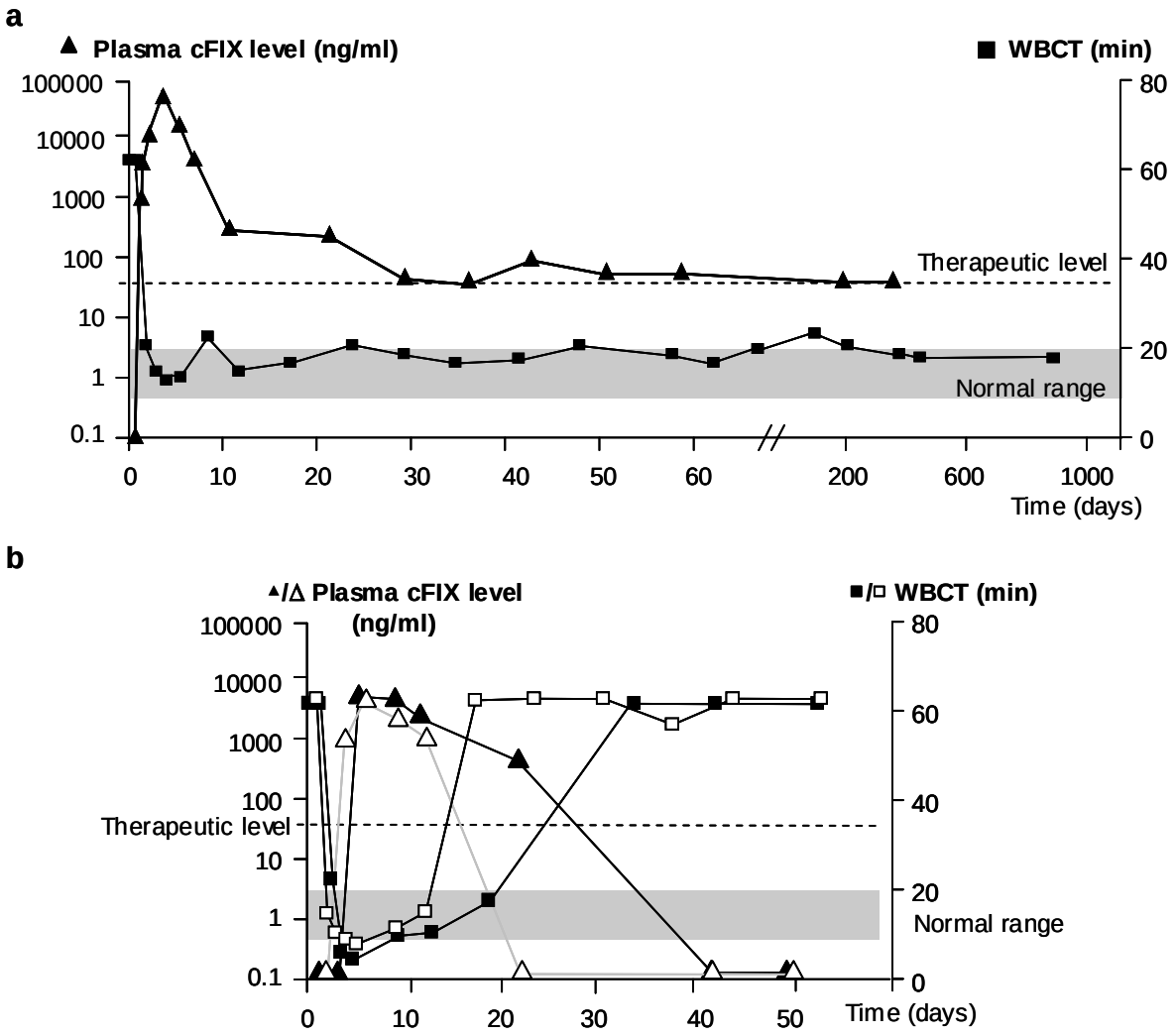


Figure 3.4: Summary of the therapeutic effect of the adenovirus/transposase hybrid-vectors in a canine model for hemophilia B. The whole blood clotting time (WBCT) is an indicator for phenotypic correction in treated hemophilia B dogs. The WBCT for hemophilia B dogs is longer than 60 min. The grey shadow shows the WBCT range for normal dogs. The expression level of canine coagulation factor IX (cFIX) was measured by ELISA. Normal levels of healthy dogs are between 500 and 12000 ng/ml, but even a cFIX level of about 50 ng/ml is sufficient for effective coagulation (therapeutic level). **(a) Therapeutic effect in a hemophilia B dog (D1) receiving low dose vector infusion (9.4 x 10^{11} viral particles per kilogram body weight [VPs/kg]). (b) The therapeutic effect in a hemophilia B dog (D2) receiving high dose vector infusion (2.4x10^{12} VPs/kg) and the control dog (D3) receiving inactive transposase system (TcFIX/mSB).** ▲/△, cFIX expression levels of the dog D2 and D3, respectively; ■/□, the WBCT of the dog D2 and D3, respectively.

3.2.2.2. Detection of neutralizing anti-adenoviral antibodies

To further investigate the potential reasons for the transient phenotypic correction in dogs D2 and D3, as well as the rapid decrease of cFIX plasma levels in dog D1 during the first weeks post injection, I searched for anti-cFIX inhibitors and measured neutralizing anti-adenoviral antibody levels. The Bethesda inhibitor assay was used to detect anti-cFIX inhibitors in dog plasma before and after injection, but no antibodies against the transgene-encoded product were detected in either dog (data not shown). Thus, the decrease in cFIX transgene expression was not caused by a humoral immune response directed against the recombinant cFIX protein.

For analysis of the humoral immune response directed against the adenoviral vector, dog serum at various time points was examined for the presence of anti-adenoviral neutralizing antibodies. For this purpose, a neutralization assay was used, measuring the inhibitory effect of anti-adenoviral antibodies contained in canine serum samples by infection of 293 cells with an adenoviral vector encoding a reporter gene (see also **Figure 2.2**). Neutralizing antibodies for dog D1 were monitored until the end of the experiment (960 days post injection) and for dogs D2 and D3 up to 80 days post injection (latest time points measured). Highest levels were measured 2–3 weeks post-treatment (**Figure 3.5**). Although the biological relevance of this finding is not clear at the time, injection of HC-AdVs at a similar dose resulted in comparable or even higher levels of neutralizing antibodies in a previous study (Ehrhardt *et al.*, 2003), indicating that these neutralizing antibodies levels are normal for this injected vector dose.

The efficient phenotypic corrections in these hemophilia B dogs (especially dog D1) hold promising potential of this novel AdV/SB hybrid-vectors for hemophilia B gene therapy. The hemophilia B dog study serves also as an example for translation from a small animal to a large animal model.

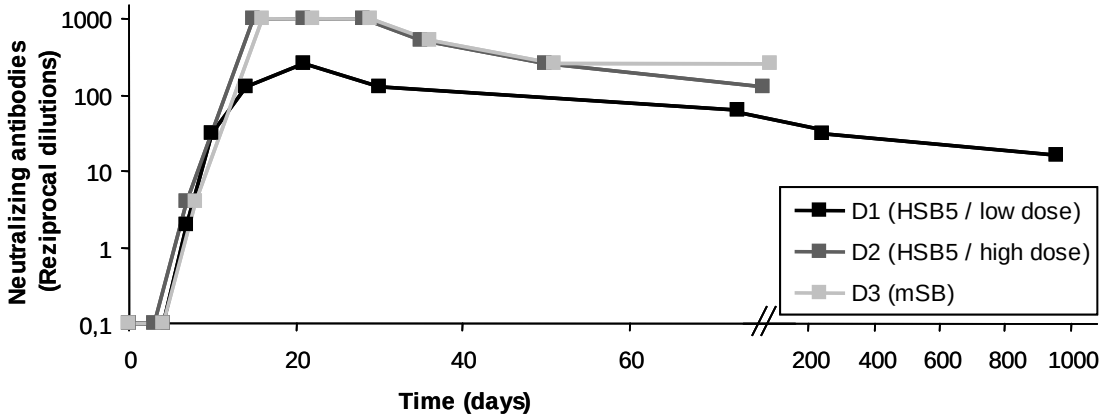


Figure 3.5: Neutralizing antibodies directed against adenoviral vectors in treated hemophilia B dogs. As an indicator for the adaptive immune response challenged by the HC-AdVs, neutralizing antibodies directed against the vector were measured. Therefore the highest dilution of canine serum was determined, which is able to inhibit 50% transduction efficiencies of a reporter virus in 293 cells. The neutralizing-antibody titer was defined as the reciprocal of this dilution.

3.3. Analyzing safety issues of the adenovirus/Sleeping Beauty transposase hybrid-vectors

Safety is an essential issue to be considered for gene therapy when analyzing its efficacy in a pre-clinical or any clinical setting. Therefore, after *in vivo* application of the adenovirus/transposase hybrid-vectors genotoxicity related to insertional mutagenesis was analyzed in detail, as well as the acute liver toxicity.

3.3.1. Integration site analyses

After stable transgene expression was obtained from the AdV/SB hybrid-vector system for *in vivo* application in mice and dogs (Chapter 3.2), the next step was to confirm the existence of somatic integration by identification of integration sites. Moreover, the genotoxicity found in the other vector systems make it a requirement to characterize the genomic insertion pattern for each vector system being explored. Thus, it was of great importance to carefully analyze the integration sites after SB-mediated transposition from the adenoviral vector context.

3.3.1.1. Establishment of a GenomeWalker-based method to determine integration sites

The BD GenomeWalker™ Kit from BD Biosciences was adopted to detect integration sites. In a previous study this strategy was used to analyze integration sites after PhiC31-mediated integration from an adenoviral vector (Ehrhardt *et al.*, 2007). In the current study, the BD GenomeWalker™ Kit was adjusted to analyze the integration sites after Sleeping Beauty transposase-mediated integration. To establish the protocol, a tissue culture based study was first initiated **(Figure 3.6)**, which detected SB-mediated integration sites in a

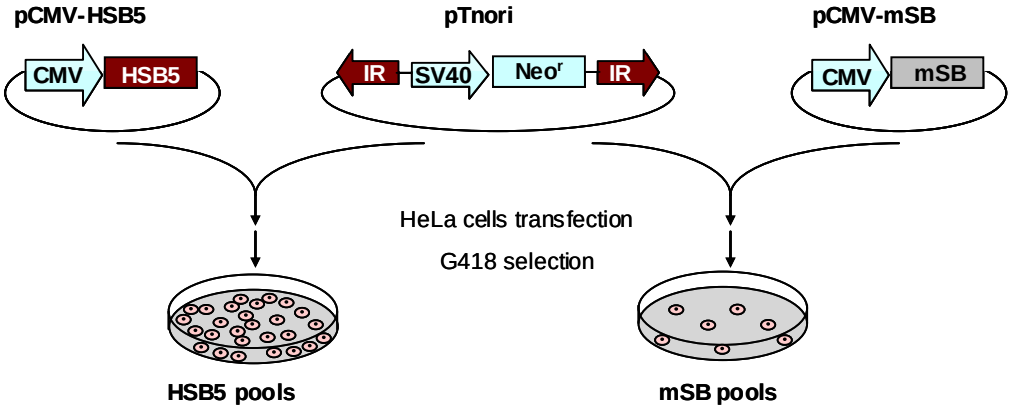


Figure 3.6: Overview of the transposition from plasmids in cultured mammalian cells. pTnori, the transposon donor plasmid; pCMV-HSB5, the helper plasmid encoding hyperactive transposase; pCMV-mSB, control vector encoding an inactive transposase. IR, inverted repeat; SV40, simian virus promoter; neo, neomycin-phosphotransferase gene; CMV, cytomegalovirus promoter.

plasmid-based context. Herein the donor plasmid pTnori carrying a transposon for neomycin-phosphotransferase gene expression was co-transfected with a second plasmid containing either hyperactive SB transposase (pCMV-HSB5) or a control vector with inactive SB (pCMV-mSB). The transfected Hela cells were then selected with G418 for two weeks to get pools of drug resistant cells **(Figure 3.6).** Subsequently, cell genomic DNA was isolated and integration sites were characterized **(Figure 3.7).**

The GenomeWalker-based method for integration site analysis was established and evaluated at each single step **(Figure 3.7 and 3.8).** The construction of GenomeWalker DNA libraries should begin with clean, high-molecular weight genomic DNA which requires a high quality DNA preparation. To ensure that the genomic DNA is of adequate quality, the genomic DNA was visualized on a 0.6% agarose/EtBr gel **(Figure 3.8.a)**. To avoid potential biases for detection of integration sites, 4 blunt-end digestions with different restriction enzymes were set up **(Figure 3.7.a and 3.8.b).** Importantly, these enzymes should not digest the sequence from the first PCR primer to the 3'-end of the IR. After phase lock gel

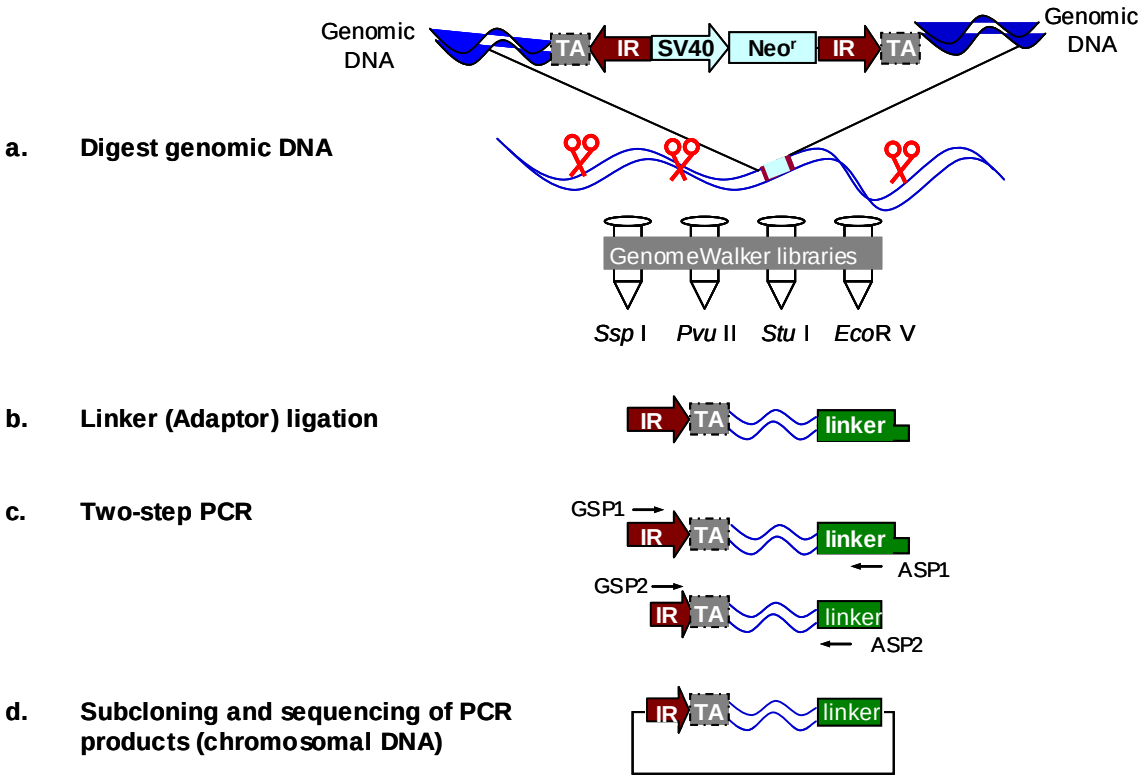


Figure 3.7: Schematic outline of the GenomeWalker-based method to determine integration sites. (a) DNA digest: highly purified genomic DNA was digested with four blunt-ended restriction enzyme nucleases: *Ssp*I, *Pvu*II, *Stu*I, *Eco*RV. **(b) Linker ligation:** linker from BD GenomeWalker™ kit was ligated to both ends of the genomic DNA fragments to create GenomeWalker libraries. **(c) Two-step PCR:** a two-step PCR was performed with gene-specific primers (GSPs) binding to the transposon flanked sequence IR, and adaptor primers which specifically amplify from the 5' end of the adaptor-ligated genomic fragments. **(d) Subcloning and sequencing of PCR products (chromosomal DNA):** the PCR products were subcloned into pCR-blunt II-TOPO vector, after restriction enzyme digestion screening, the genomic-transposon complex was sequenced and analyzed.

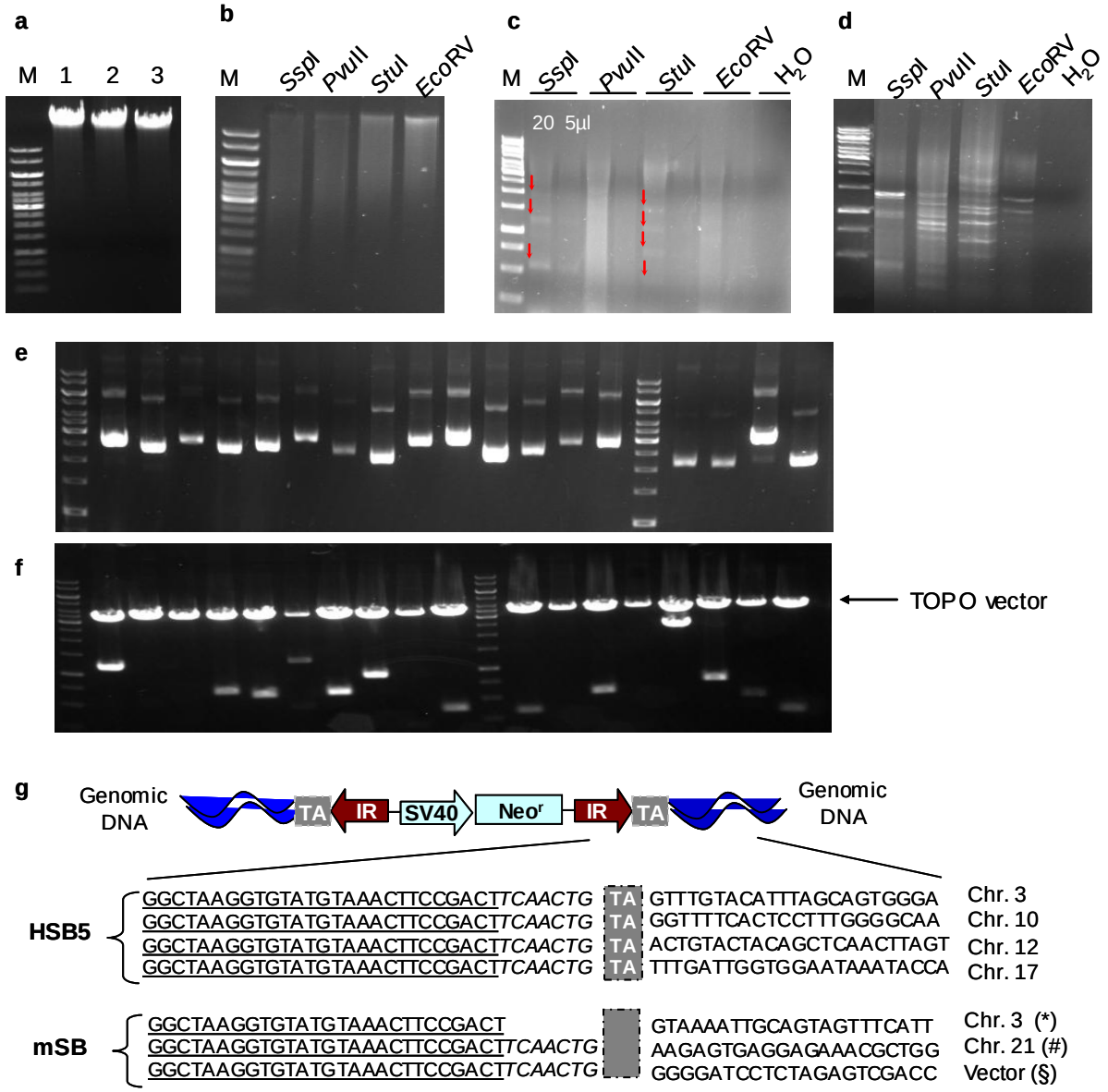


Figure 3.8: Control Gels to check quality of the GenomeWalker procedure. (a) Quality of Genomic DNA: the size of genomic DNA was checked by loading on a 0.6% agarose/EtBr gel, genomic DNA should be bigger than 50 kb with minimum smearing. M, 1kb DNA marker; Lanes 1-3, genomic DNA from three independent dishes for pools of transgene integrated Hela cells. **(b) Completed digestion of genomic DNA**: digestion was controlled by loading on a 0.6% agarose/EtBr gel, a smooth smear in each lane shows that the genomic DNA can be completely digested by the restriction enzymes (*Ssp*I, *Pvu*II, *Stu*I, *Eco*RV). **(c) Results of primary PCR:** 20µl and 5 µl of the primary PCR products were loaded on a 1.5% agarose/EtBr gel, in all lanes except for negative controls, a multiple banding or a smearing pattern could be observed, the size ranging are from about 500 bp to 5 kb. The red arrows indicate the bands. **(d) Results of secondary PCR:** 5 µl of the secondary PCR products were loaded on a 1.5% agarose/EtBr gel, products of secondary PCR ranged from 200 bp to 6 kb with some strong major bands. **(e) Integration site library visualized on agarose gel.** The size differences of these plasmids containing the sub-cloned PCR products indicate the different integration events. **(f) Integration site library from restriction enzyme digest screening**. As is shown, in the digest library, unique bands were identified to be DNA segments of different sizes visualized on agarose gel (Valid PCR products should be bigger than 0.2 kb). **(g) Analysis of Sleeping Beauty mediated transposition events in the genome of Hela cells.** The nested primer sequences are underlined, sequences at the end of the inverted-repeat (IR) are in italic letters, the target sites property (TA) is in the central shaded box. The sequences analyzed in HSB5 transposed cells contained all these elements. ***** non-specific PCR product; # random integration; § back ground.

purification, the BD GenomeWalker™ adaptors were ligated to the blunt ends of the genomic DNA fragments to create GenomeWalker libraries **(Figure 3.7.b).** To facilitate the PCR-walker reaction, two gene-specific primers, one for the primary PCR (GSP1) and one for the nested PCR (GSP2) were designed. These primers bind specifically to the left inverted repeat sequences of Sleeping Beauty transposon (see also in **Table 2.2**). The primary and nested PCRs were performed according to the two-step PCR program described in the method part **(Figure 3.7.c).** The efficiencies of PCR reactions were visualized on a 1.5% agarose/EtBr gel **(Figure 3.8.c, 3.8.d),** where a multiple banding or a smearing pattern could be observed. The PCR products were finally subcloned using the Zero Blunt™ TOPO™ PCR Cloning Kit (Invitrogen) **(Figure 3.7.d)** and pre-screened by EcoRI restricted enzyme digestion **(Figure 3.8.e, 3.8.f)**. The EcoRI double digest can excise from the pTOPO vector, and only plasmids containing a unique insert bigger than 100bp were sequenced by T7 and/or SP6 primers.

3.3.1.2. Features of rescued Sleeping Beauty transposase-mediated integration events

The first step to analyze the sequences contained in the Topo vector is to identify and remove vector- and linker-related sequences. The PCR products subcloned into the TOPO vector consist of three parts: the IR sequence from the nested primer GSP2, the genomic DNA sequence flanking the vector integration sites and the linker sequence from the other nested primer ASP2 **(Figure 3.8.g, 3.9.a).** Since the sequence primers bind to the TOPO vector, the integration site sequences include also part of the sequence from pTOPO vector **(Figure 3.9.b).**

Integration events were considered authentic only if they (1) contained the sequence from the nested primer GSP2 (underlined) until the end of the inverted-repeat (IR) (bold) (5' GGCT AAGGT GTATG TAAAC TTCCG ACT **TC AACTG** 3'), (2) matched a genomic location starting immediately after the end of IR (TCAACTG) and starting with a TA-dinucleotide sequence, (3) showed >95% identity to the genomic sequence, and (4) matched no more than one genomic locus with the highest identity. Only the sequences having these features described were used for further analyses. Some examples of sequences rescued from HSB5 or mSB transduced Hela cells are shown in Figure 3.8.g. These sequences from HSB5 transduced cells contained all the required elements, therefore were identified as Sleeping Beauty transposase-mediated integrations. As a contrast, the sequences from mSB transduced cells are shown. They either lack the TA site or have an incomplete IR sequence or show no homology to the chromosomal DNA. These events are therefore not considered SB-mediated integrations.

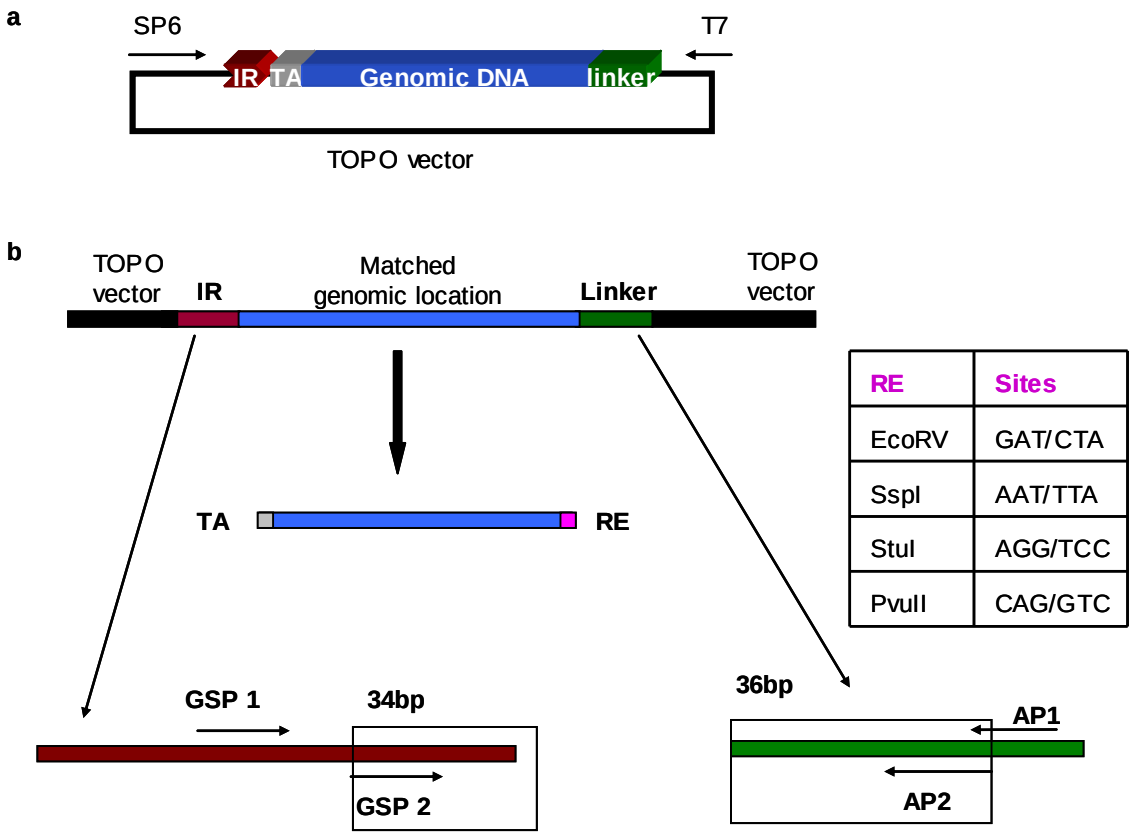


Figure 3.9: Features of rescued sequences after SB-mediated integration. (a) DNA sequences in the rescued integration library contained in the pTOPO vector. SP6/T7: sequence primers binding to TOPO vector; IR, inverted repeat; TA, dinucleotide sequence, genomic target site. **(b) The normal pattern of an integration site (IS) sequence.** The genomic DNA sequences are flanked by the vector sequence, the IR and the Linker sequence. The IR sequence is a 34-bp fragment containing the sequence from the nested primer GSP2 to the end of the IR. The sequence immediately after the end of the 5' IR matches a genomic location and normally begins with a TA-dinucleotide sequence. The linker, which demonstrates a 36-bp fragment containing the sequence from the nested primer ASP2 to the end of the linker, the genomic sequence connects to the linker shows part of a restriction enzyme (RE) site from the digestion step during the build-up of the integration library.

3.3.1.3. Integration site analysis in female C57Bl/6 mice

After the GenomeWalker-based integration site analyzing method was established with plasmid-based transposition, the major focus was to characterize the transposition profile from the adenovirus/transposase hybrid-vectors *in vivo*. Therefore, the SB-mediated integration site analysis was performed in female C57Bl/6 mice. The group which received a high dose of the AdV/SB hybrid-vectors ($4x10^9$ TUs per mouse), high transgene expression levels were obtained (Chapter 3.2). After the second CCl_4 administration, serum cFIX levels of around 4 500 ng/ml were detected in mice which received the HSB5 expressing virus, while the cFIX levels decreased to 750 ng/ml in the control group that received the mSB expressing virus. One mouse from each group, HSB5 mouse 1 (f1) with serum cFIX levels of

4800 ng/ml and mSB mouse 1 (mSB) with serum cFIX levels of 700 ng/ml were sacrificed 9 weeks post-vector injection to isolate liver genomic DNA for integration site analysis (**Figure 3.10.a**). After an additional CCl_4 administration at week 13 post-vector injection, integration sites from a second mouse (female mouse 2, f2) of the HSB5 group with serum cFIX levels of about 3100 ng/ml was analyzed. Notably, at this time the control group which received the mSB expressing virus had serum cFIX levels that decreased to 150 ng/ml (**Figure 3.10.a**). In addition, one mouse (female mouse 3, f3) from the medium vector dose group ($8x10^8$ TUs per mouse) (**Figure 3.3.b**), with serum cFIX levels of 520 ng/ml was scarified at 14 weeks post-vector infusion for integration site analysis. Serum cFIX levels in the control group (TcFIX /Luc) of the same vector dose were around 70 ng/ml (**Figure 3.10.a**).

Integration site analysis was then performed with the method described above (**Figure 3.7**). Briefly, adaptors for genome walker was ligated to digest genomic DNA isolated from respective mouse liver, PCR reaction was performed and PCR products were subcloned. Before each unique clone was sequenced, they were pre-screened by restriction enzyme digestion with EcoRI, which rescue the PCR products with a double digest from the Topo vector at the multiple cloning site. Thus the sizes of the DNA bands shown on the gel refer to the sizes of the cloned PCR products. As shown, in the library derived from the mouse (f1) that received the TcFIX/HSB5 viruses, more unique bands could be identified compared to the mSB group (valid PCR products range from 0.2 to 6 kb) (**Figure 3.10.b**). 92 SB-mediated unique integration sites (ISs) were identified from female C57Bl/6 mice which received high dose and medium dose of the AdV/SB hybrid-vectors infusion (38 ISs from female mouse 1, f1; 35 ISs from female mouse 2, f2; 19 ISs from female mouse 3, f3).

In a previous study, insertional patterns and preferences of the SB transposon in mouse liver have been analyzed in plasmid-based approaches, which showed a random-like distribution among chromosomes (Yant *et al.*, 2005). In order to obtain insight into the target site properties of AdV/SB hybrid-vector system *in vivo* in the present study, all 94 integration sites (92 unique ISs) were mapped in the murine chromosomes (**Appendix Figure 2.a, 2.b and 2.c**). Unlike the evenly chromosomal distribution of ISs after plasmid-based SB transposition, the SB-mediated integration from AdV/SB hybrid-vector system showed a bias towards the X-chromosome for all investigated mice (**Figure 3.11**). There were 18 (18/38) integration sites located in the X-chromosome in mouse f1, 13 (13/35) integration sites located in the X-chromosome in mouse f2 and 5 (5/19) integration sites located in the X-chromosome in mouse f3 (**Figure 3.11**). Although a large amount of integration sites remains to be analyzed to draw clear conclusions with respect to the genome-wide integration profile, the high percentage of X-chromosome integrations was different compared to the plasmid based studies.

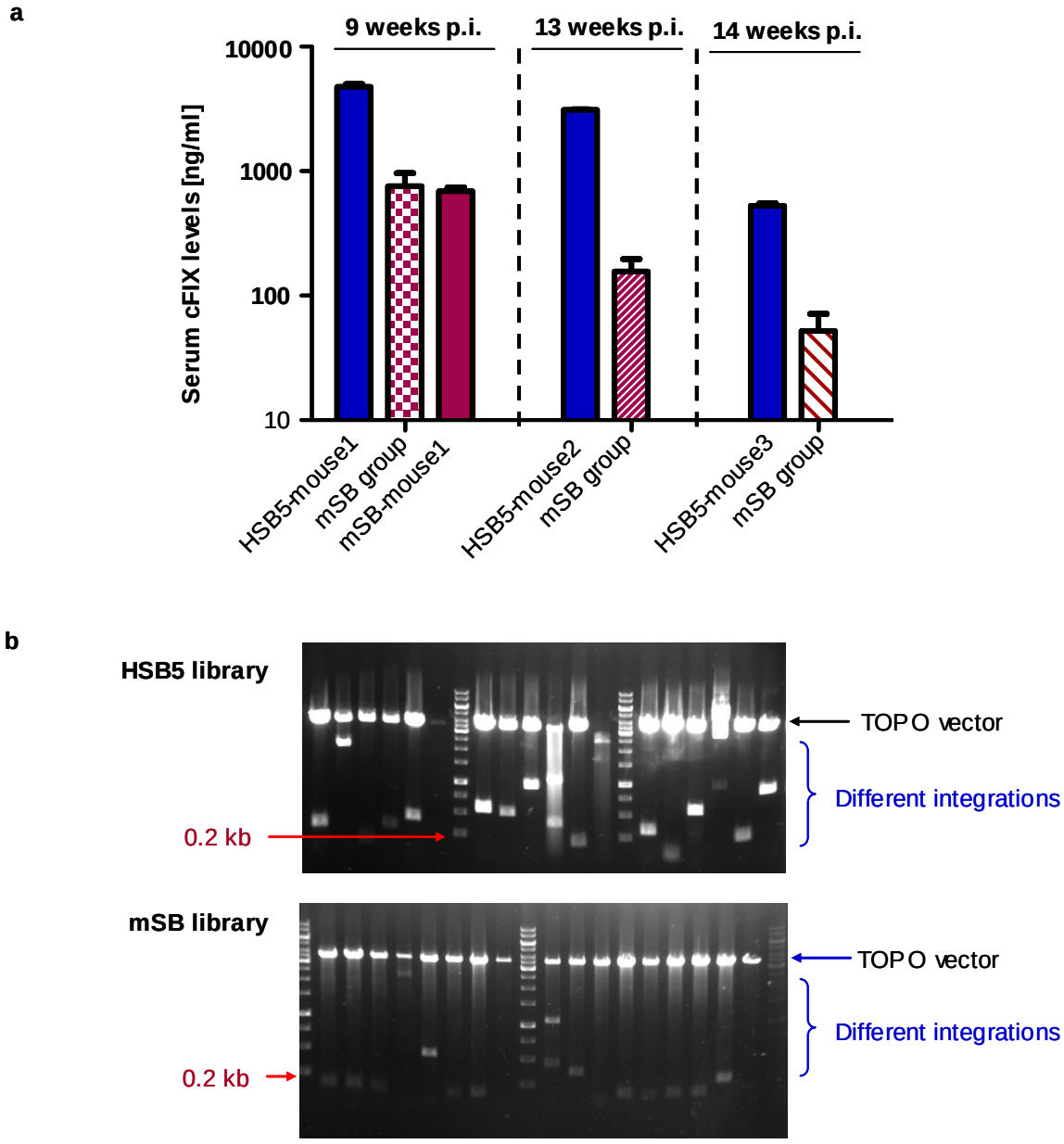


Figure 3.10: SB-mediated integration sites in female C57Bl/6 mice. (a) Serum cFIX levels of the mice used for integration site analysis. From the high vector dose group (Figure 3.3a), at 9 weeks post vector infusion, one mouse from HSB5 group (HSB5-mouse1) with serum cFIX level of 4800 ng/ml was sacrificed, liver genomic DNA was used for setting up the first integration site library (female mouse 1, f1). At the same time point, the serum cFIX levels of the control group (mSB group) were around 750 ng/ml, one mouse with serum cFIX level of 700 ng/ml was then used as control. At a second time point, 13 weeks post vector infusion another mouse from HSB5 group (HSB5-mouse2) with serum cFIX level of 3100 ng/ml was sacrificed for the second integration site library (female mouse 2, f2), while the serum cFIX levels of the control group (mSB group) was around 150 ng/ml. From the medium vector dose group (Figure 3.3b), at 14 weeks post vector infusion, one mouse from HSB5 group with serum cFIX level of 520 ng/ml was sacrificed for the third integration site library (female mouse 3, f3), while the serum cFIX levels of the control group (Luc group) was around 70 ng/ml. **(b) Integration site library after restriction enzyme digestion.** As is shown, in the HSB5 library, more unique bands were identified compared to the mSB group (Valid PCR products should be bigger than 0.2 kb).

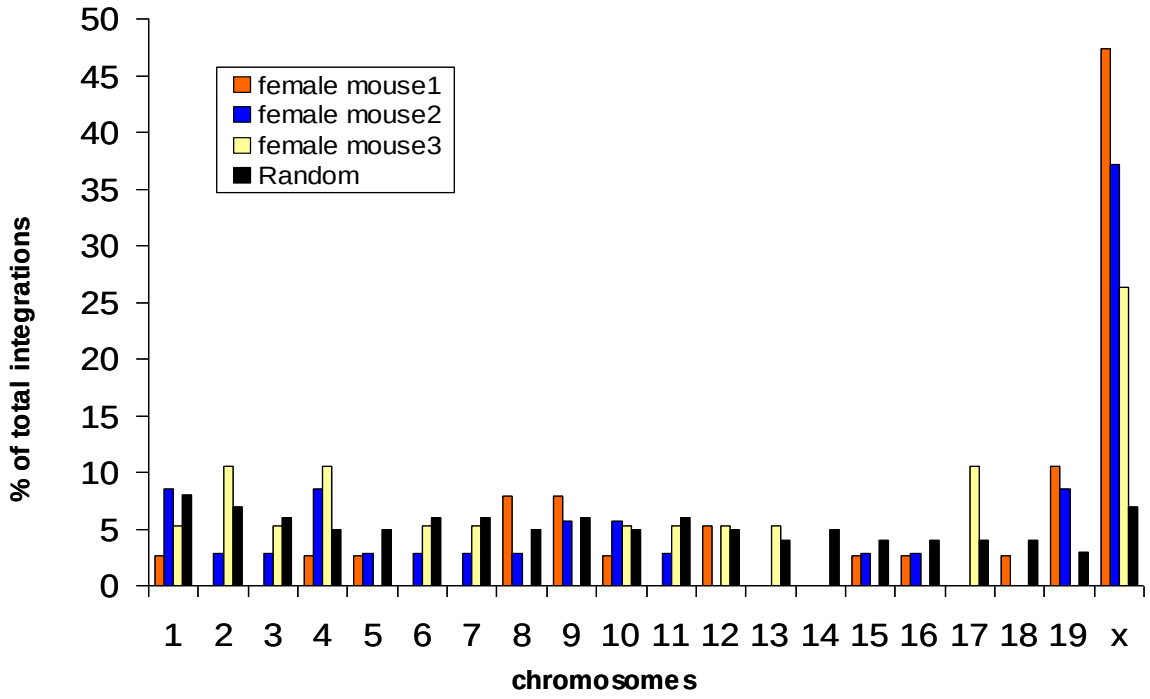


Figure 3.11: Genome-wide distributions of SB-mediated integration events in female C57Bl/6 mice. Integration sites analyzed from three HSB5 co-delivered mice. Mouse 1 was sacrificed 9 weeks post-vector infusion, while mouse 2 and 3 was 13 and 14 weeks post-vector infusion (Figure 3.10a). The distribution of SB-mediated integration events was compared to that of a computer-simulated random integration profile (Yant *et al.*, 2005). The Y axis shows the percentage of integration events from each individual chromosome.

3.3.1.4. Integration site analysis in male C57Bl/6 mice

Due to the distinct bias of integration events towards the X-chromosome found in female mice after AdV/SB hybrid-vectors administration (**Figure 3.11**), it is of great interest to check the chromosomal distribution pattern also in male mice containing only one X-chromosome. Therefore, male mice were infused with the same vector system (HC-AdV-TcFIX co-injected with HC-AdV-HSB5 at the ratio of 3:1) at both high dose ($4x10^9$ TUs per mouse) and medium dose ($8x10^8$ TUs per mouse). Mice which received the medium vector dose showed stable transgene expression, although the serum cFIX level decreased after the first CCl_4 administration, stable transgene expression levels were observed after the second and third CCl_4 administration (**Figure 3.12**). However, as for the high dose group in previous experiments all mice died probably due to dose-dependent toxicity. To build up an integration site library, two mice with serum cFIX levels of ~460 and ~850 ng/ml from the medium vector dose group were sacrificed at 14 weeks post-vector infusion.

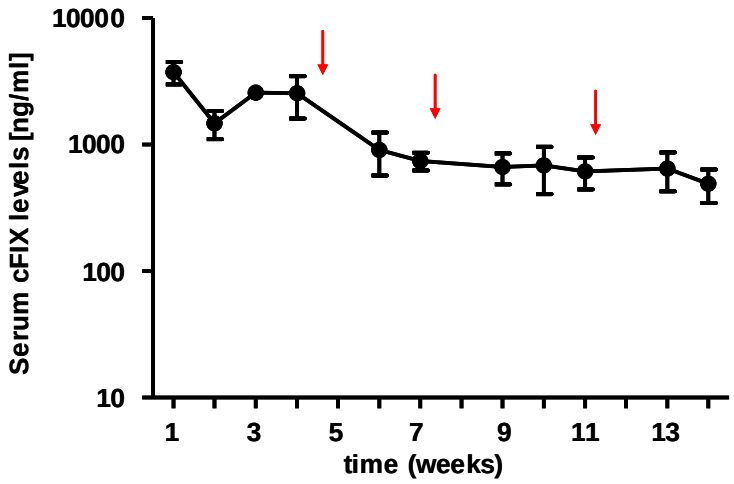


Figure 3.12: Stability of cFIX expression in male mice which received the AdV/SB hybrid-vector. Male C57Bl/6 mice were co-injected with HC-AdV-TcFIX and HC-AdV-HSB5 at a ratio of 3:1 with two different vector doses ($8x10^8$ or $4x10^9$ transducing units (TU) per mouse). For the high dose group ($4x10^9$ TU/mouse, n=5) all mice died within 2-3 weeks post vector infusion. The transgene expression levels in the medium vector dose group ($8x10^8$ TU/mouse, n=5) are shown. Red vertical arrows indicate carbon tetrachloride (CCl4) injection.

Of the 44 unique transposon insertions found in male mice, three X-chromosomal integrations were identified from male mouse 1 (m1) and two were from male mouse 2 (m2). This preference is almost similar to random integration. Notably, although there was a Y chromosome present in these male mice, no integration site was located there (**Appendix Figure 2.d, 2.e**). With a total of 44 integration sites, it is not surprising that some chromosomes may be hit more than once, while some may not be hit at all (e.g., chromosomes 9, 18 and 19 were not hit) (**Appendix Figure 2.d, 2.e**).

3.3.1.5. Summary of the chromosomal distribution profile on X-chromosome

To show the relative position of all integration sites on a chromosomal level, all 136 integration sites from 5 mice were mapped on the respective mouse chromosomes (**Appendix Figure 2.a, 2.b, 2.c, 2.d and 2.e**).

In order to look inside the chromosomal distribution profile on the X-chromosome in more detail, all 41 independent integration sites from all 5 mice on the X-chromosome are schematically shown in different colors in **Figure 3.13**. The X-chromosome band XA1.1 was found to be the most preferred location with 6 integration sites in total, followed by band XE3 with 4 events. No integration site was observed on bands A7.2, A7.3, B, C3, E2, and F5. The average distribution profile was found to be not related to the location of the mouse FIX gene (Band XA6), although the canine FIX cDNA is highly homologous to it, which may lead to homologous recombination.

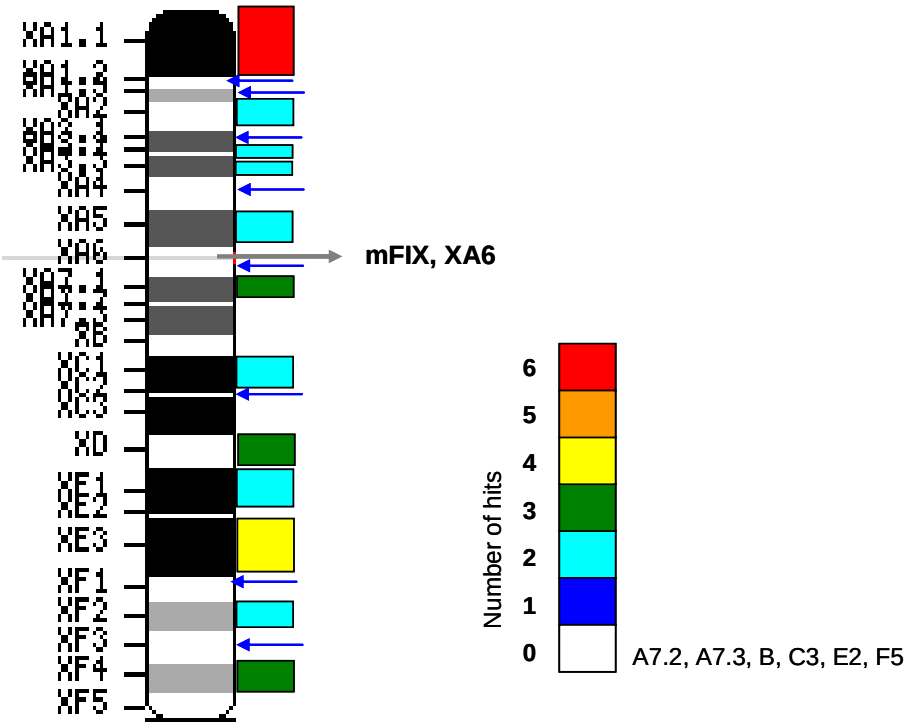


Figure 3.13: SB transposition site mapping of the X-chromosome. The relative positions of 42 independent integration sites from all 5 mice for SB-mediated transposition from the adenoviral vectors (AdV/SB hybrid-vector system) are shown. The colored squares next to the chromosome figure indicate the number of integration sites that located in the corresponding chromosomal bands. The blue arrows indicate single integration events; the gray arrow indicates the location of the murine coagulation factor IX (mFIX) gene. No integration site located in bands A7.2, A7.3, B, C3, E2, and F5.

3.3.1.6. Frequencies of transposon insertions within or outside genes

The mutagenic potential of any given integrating vector system is one of the most essential considerations for gene therapy applications. Normally, the integrations in genes display a higher risk to cause genotoxicity than those which hit an intergenic region. Thus, the relation of transposon insertions to transcriptional units was analyzed, aiming to determine the mutagenic potential associated with AdV/SB hybrid-vectors mediated integrations isolated from the three female mice and the two male mice. 31 of all the 136 transposon integrations in mice liver (23%) were mapped within at least 1 of the 18,090 mouse RefSep genes (**Figure 3.14.a**). When compared to the computer-simulated random integrations (26%) (Yant *et al.*, 2005), these data revealed a similar or lightly less preference for SB-mediated integration into mouse genes. Notably, this frequency is much lower when compared to murine leukemia virus (MLV, retroviral vectors) with 50.7% and human immunodeficiency virus derived vectors (lentiviral vectors) with 83.4% (Wu *et al.*, 2003; Schroder *et al.*, 2002). Among the 31 total integrations that occurred in genes, which were distributed evenly amongst the transcript units, 27 mapped within intron sequences, only 4

were on exons (**Figure 3.14.a**). The bias towards introns is probably due to the larger overall size of introns when compared to exon sequences.

As to the 105 integration sites in intergenic sequences, 8 (7.6%) were found near genes (±5kb), while >70% were located 50kb far away from genes (**Figure 3.14.b**). This is in sharp contrast to AAV vectors, for which over 38.3% of integrations were found near genes (Nakai *et al.*, 2005).

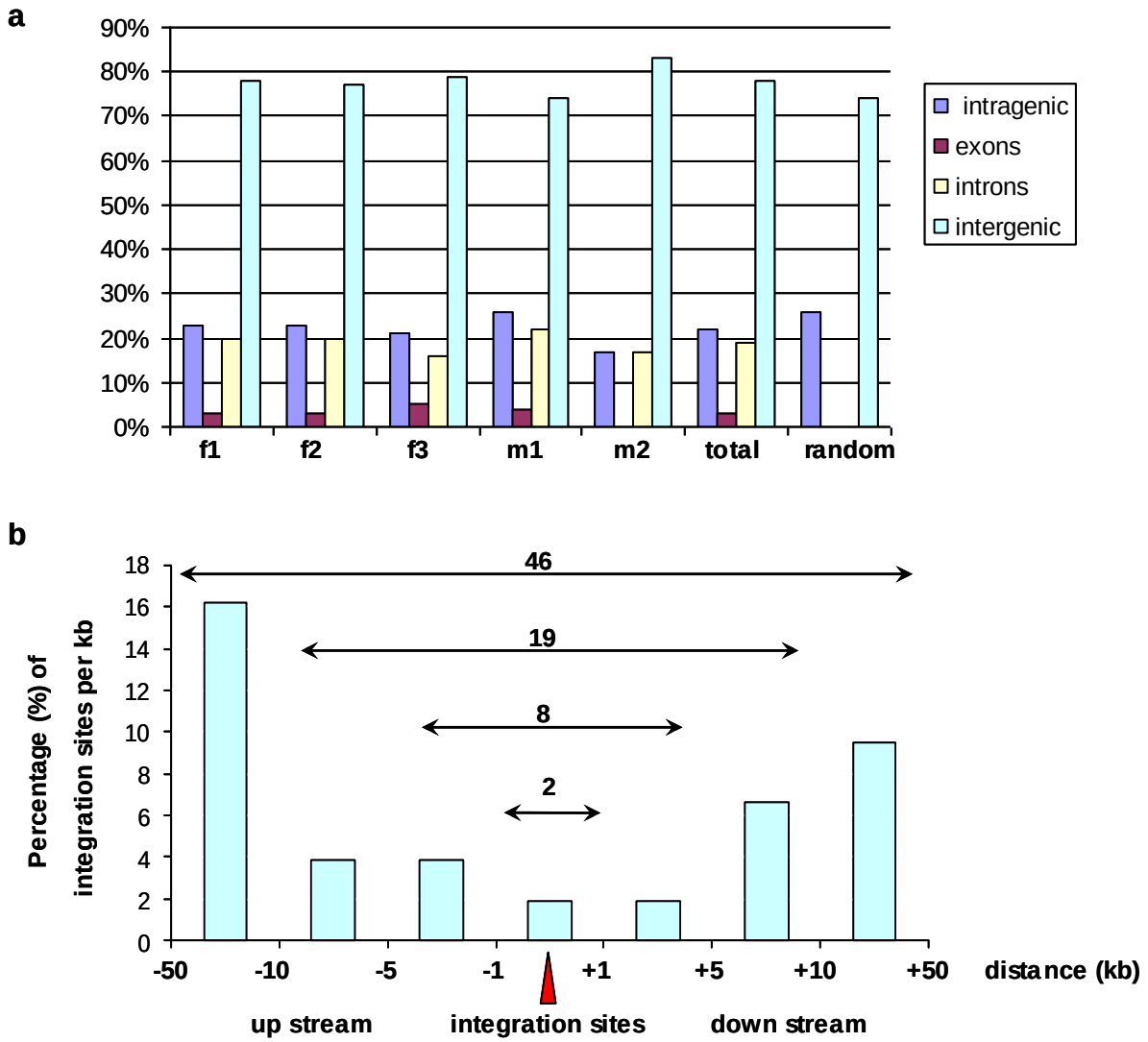


Figure 3.14: Frequencies of insertions within or outside of genes. (a) Percentage of integration sites. f1: female mouse 1; f2: female mouse 2; f3: female mouse 3; m1: male mouse 1; m2: male mouse 2; total: all integration sites from five mice; random: computer predicted data. **(b) Proximity of integration sites to genes.**

3.3.1.7. Transposon insertions in or near cancer related genes

To evaluate the risk of neoplastic changes that could be potentially induced by the integrating vector system, those genes which were hit by integration events or near (±5kb) the integration sites were checked in Cancer Gene Expression Databases (CGED, http://lifesciencedb.jp/cged/) (Kato *et al.*, 2005)

Although no neoplastic change was observed macroscopically in all treated animals, 10 cancer related genes were found to be affected by transpositions from the AdV/SB hybrid-vectors (**Table 3.1**). Three cancer related genes were identified in the treated female mouse 1 (SCARB2, GPC3 and CASK). SCARB2 locates in chromosome 5 and relates to gastric cancer, while both GPC3 and CASK locate in chromosome X and relate to hepatocellular cancer. Of the integration events in female mouse 2, two located in the cancer related genes ITCH (relate to hepatocellular cancer) and ACSL4 (breast cancer), one located near to the CUTC gene (gastric cancer). No cancer related gene was found in the transposition events of female mouse 3.

In the male mouse 1, two integrations were identified in the cancer related genes MGAT4A (thyroid cancer) and EHBP1 (gastric cancer) and one was near the ARAF gene (colorectal cancer). Moreover, one integration site was found to locate to the ABLIM2 gene (gastric cancer) in male mouse 2.

Table 3.1: Transposon insertions in or near cancer related genes.

mouse	chromosomal location	Band	gene symbol & name	cancer type
	chr5: 92880283	5 E2	SCARB2: scavenger receptor class B, member 2	gastric (GC)
f1	chrx: 49859131	XA5	GPC3: glypican 3	hepatocellular (HCC)
	chrx: 13341506	XA1.1	CASK: calcium/calmodulin-dependent serine protein kinase	hepatocellular (HCC)
	chr2: 154999131	2H1	ITCH: itchy, E3 ubiquitin protein ligase	hepatocellular (HCC)
f2	chrx: 138774886	XF2	ACSL4: acyl-CoA synthetase long-chain family member 4	breast (BC)
	chr19: 43843935	19C3	1.1kb up to CUTC: cutC copper transporter homolog	gastric (GC)
	chr1: 37535572	1B	MGAT4A: mannoside acetylglucosaminyltransferase 4, isoenzyme A	thyroid (TC)
m1	chr11: 21917357	11A3.1	EHBP1: EH domain binding protein 1	gastric (GC)
	chrx: 20425116	XA1.3	1.99kb down after ARAF: v-raf murine sarcoma 3611 viral oncogene homolog	colorectal (CC)
m2	chr5: 36106310	5B3	ABLIM2: actin-binding LIM protein 2	gastric (GC)

3.3.1.8. Transposition into extra-chromosomal target sequences

In addition to the 136 incidences of genomic integration, 27 transposon integration events (with 12 unique sites) were found to localize within the HC-AdV genome used in this study. These insertion sites were evaluated in more detail and it turned out, that they were located in the stuffer DNA contained in the HC-AdV genome (IgkMAR and alphoid repeat DNA) and the transgene. Multiple insertions were observed at two of these sites. Four were located at one site of the IgkMAR sequence (location: mouse chromosome 6, 70675584) and 8 were within human FIX gene intron 1 (location: human chromosome x, 138612987), which was added to the transgene region in the expression cassette and located before the cFIX cDNA sequence. Extra-chromosomal targets for SB transposes were also described in a plasmid-based study (Yant et al. 2005), in which the promoter region and the polyadenylation signal region were found to be a “hot spot” including 85% of all extra-chromosomal transposition events. The present study, however, showed no bias towards promoter or signal regions (**Figure 3.15**).

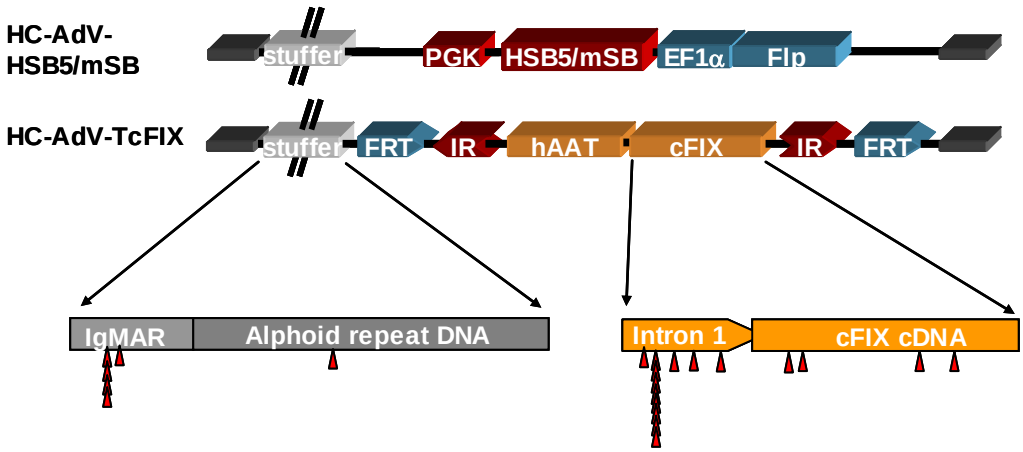


Figure 3.15: Transposition into extra-chromosomal target sequences. The upper panel schematically shows the vector constructs of the transposition-actor vectors HC-AdV-HSB5/mSB and the transposon-donor vector HC-AdV-TcFIX. A detailed description of these vectors can be found in Figure 3.1. The lower panel shows a detailed map of the stuffer DNA and the transgene. The stuffer DNA consists of two parts: IgkMAR (the matrix attachment region of the murine immunoglobulin κ locus) and alphoid repeat DNA (alphoid repeat DNA sequences from human chromosome 17). The core transgene represented by the canine coagulation factor IX cDNA (cFIX) and contained a portion of the first intron from the human FIX gene. The red triangles show the relative positions of the extra-chromosomal integrations identified in mouse liver genomic DNA.

3.3.1.9. Summary of all integration events

For all the five SB transposed mice (f1, f2, f3, m1 and m2), 60~80 clones from each library were analyzed, of which ~40 clones were shown to contain unique PCR products visualized by unique DNA bands (**Figure 3.16**). Briefly, amongst these sequence results, the

majority of sequences obtained from the two female mice (f1 and f2), which received a high dose of vector infusion ($4x10^9$ TUs per mouse), were identified to be real transposon integrations. This was in contrast to the other three treated mice (f3, m1 and m2) that received a medium dose vector infusion ($8x10^8$ TUs per mouse), in which more than half of the clones sequenced were not real transposition events. Extra-chromosomal transposition events and non-specific PCR products were obtained from all treated groups, especially in the mouse receiving mSB encoding virus, in which no real transposition event was observed.

Interestingly, I observed that those mice in which most of the transposon insertion sites could be identified, relatively high transgene expression levels were detected. For female mouse 1 and 2, which had high levels of serum cFIX (4800 and 3100 ng/ml) (**Figure 3.10.a**), 40 of 47 and 35 of 50 clones sequenced turned out to be real transposition events. In contrast, in the other three mice (female mouse 3, male mouse 1 and 2) which had relative low transgene expression levels (less than 1000 ng/ml) (**Figure 3.10, 3.12**), the real transposition events also take low percentage of all clones that were sequenced (**Figure 3.16**).

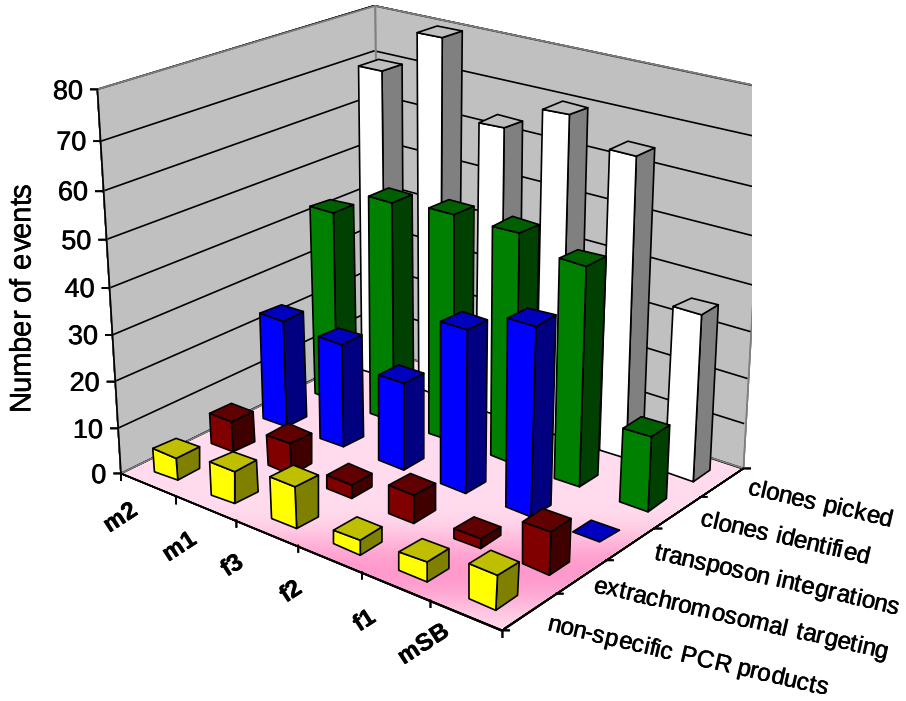


Figure 3.16: Number of different transposition events in all five mice. mSB: female mouse which received inactive transposase (TcFIX/mSB); f1: female mouse 1; f2: female mouse 2; f3: female mouse 3; m1: male mouse 1; m2: male mouse 2; f1, f2, f3, m1 and m2 received the hyperactive transposase HSB5 (TcFIX/HSB5). Clones identified referred to the number of clones that were screened by restriction enzyme (EcoRI) digestion. Transposon integrations are the confirmed integration events mediated by Sleeping Beauty transposase. Non-specific PCR products are those sequences, which did not contain an intact IR or TA. Extra-chromosomal target events were those transposition events which mapped to the vector genome used for delivery (Figure 3.15).

3.3.1.10. Detection of transposition events from treated canine liver genomic DNA

To demonstrate that the cFIX transposon underwent transposition in canine liver and to analyze the fate of transduced vector DNA, detailed molecular analysis of liver-derived canine genomic DNA was performed with dog D1. The GenomeWalker-based integration site analyzing method was used to determine integration sites (**Figure 3.7**). Briefly, an adaptor for the GenomeWalker method was ligated to pre-digested genomic DNA, PCR reaction was performed and PCR products were subcloned. From more than 100 clones analyzed, 40 were identified to contain unique bands after performing a restriction enzyme digest screen. Analyzing of the sequences rescued, 4 unique transposon insertions were identified (**Appendix Table**), although one integration site in the synaptophysin gene was found more than once. Similar to the observation in mice (**Figure 3.15**), five transposon insertions were found in extra-chromosomal target sequences. Two were in the vector stuffer DNA containing alphoid repeat DNA sequence, one was in the FRT sequence, and other two were in the cFIX cDNA.

All the other sequences were catalogued to be non-specific PCR products due to their lack of an intact IR. Since the dog-specific genomic DNA sequences database is not as complete as human and mouse at the moment, the number of insertion sites may be not representative. However, these transposon insertions identified evidenced the fact that the novel adenovirus/transposase hybrid-vectors can also facilitate somatic integration in large animals.

3.3.2. Genome status of the hybrid-vectors in different animals

To further understand the transposon mechanism related to this novel adenovirus/transposase hybrid-vector system, the genome copy numbers of each vector from liver genomic DNA of all animals (mice f1-f3, m1, m2 and mSB virus treated mice, and dog D1) was checked by real-time PCR.

For analyses of cFIX and SB encoding sequences analyses, the same primer pairs as for vector titration (**Figure 3.1.a**) were used. To normalize the different samples, the same amount of genomic DNA was analyzed by real-time PCR using a house keeping gene as an internal control. For mouse samples, mouse TBP gene (TATA box binding protein) was used and for dog samples the dog B2M gene (Beta-2-microgloblin) was determined.

In contrast to the results obtained from a semi-quantification PCR (Hausl *et al.*, 2010), the SB transposase gene was detectable by real-time PCR in all animals (**Figure 3.17**) ranging from 0.004 to 0.106 copies per cell. However, in all AdV/SB transposase hybrid-vectors treated animals except for the mouse which received the inactive version of transposase (mSB), the cFIX gene copy numbers per cell were one log higher than the

measured SB gene copies. Moreover, in the two female mice f1 and f2, which received a high dose vector infusion ($4x10^9$ transducing units (TUs) per mouse, equals to $2x10^{11}$ TUs/kg), high genome copy numbers (0.811 and 0.581 copies/cell for transposon-encoding sequence, 0.106 and 0.080 copies/cell for transposase-encoding sequence), as well as high cFIX expression levels (4500 and 3100 ng/ml at the last time point measured) could be measured. In contrast, in mice which received a medium vector dose (f3, m1 and m2, $8x10^8$ TUs per mouse, equals to $4x10^{10}$ TUs/kg) low genome copy numbers and transgene expression levels were detected (**Figure 3.17**). Regarding dog D1, which received a similar vector dose as the medium vector dose used in mice, revealed comparable genome copy

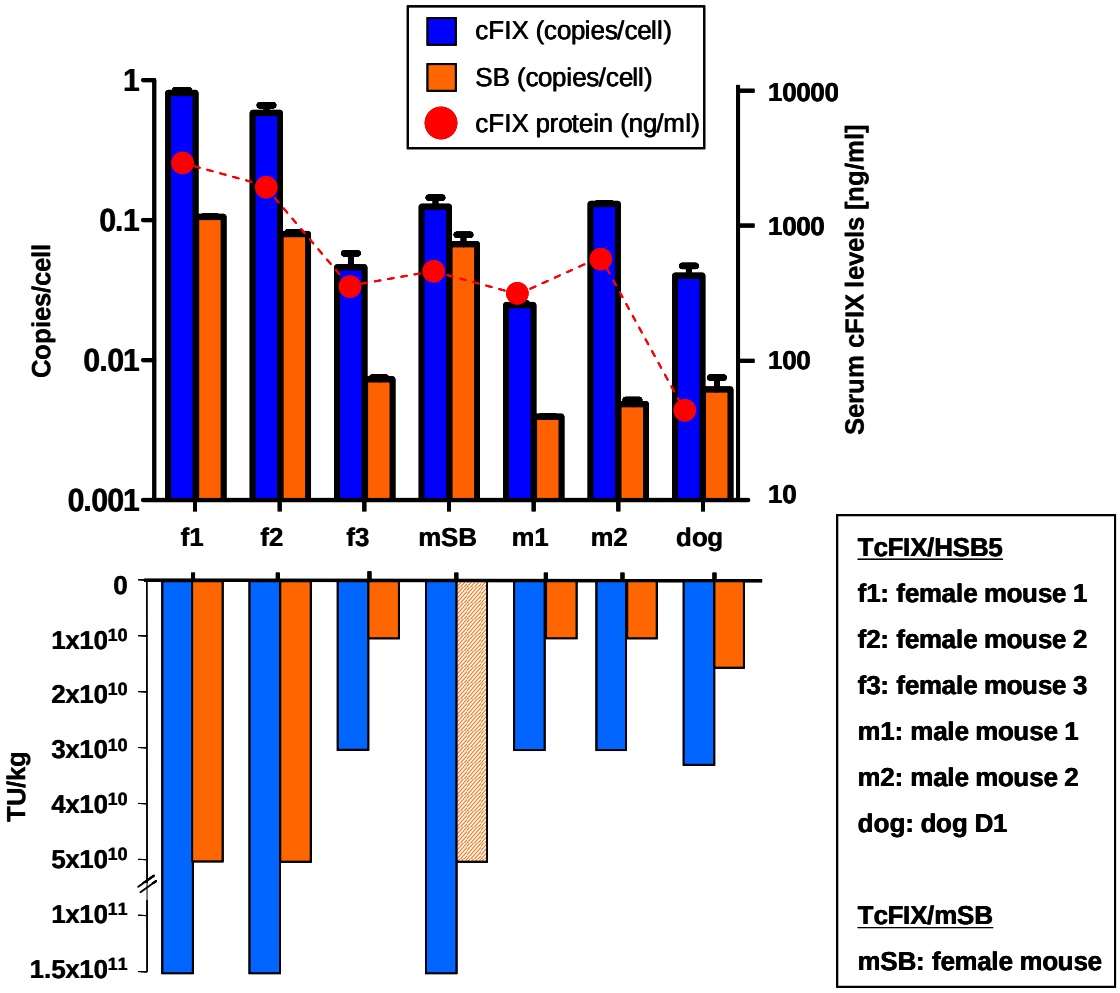


Figure 3.17: Vector genome copy numbers in liver after *in vivo* application. The upper figure shows the vector genome copy numbers and serum cFIX levels of each treated subject. Liver genomic DNA was analysed by real-time PCR using primers detecting cDNAs of the two main functional genes cFIX and SB, and the house keeping genes (TBP for mouse, B2M for dog) were used as internal control. The lower figure indicates the dose of each vector infused. mSB: female mouse receiving inactive transposase (TcFIX/mSB); f1: female mouse 1; f2: female mouse 2; f3: female mouse 3; m1: male mouse 1; m2: male mouse 2; f1, f2, f3, m1 and m2 received hyperactive transposase HSB5 (TcFIX/HSB5) (Figure 3.10 and 3.12). Dog indicates dog D1 which received a low dose ($9.4x10^{11}$ vps/kg, equals to $4.7x10^{10}$ TU/kg) vector infusion.

numbers, but the transgene expression levels measured by ELISA were 10-times lower than that in mice. This is probably due to the difference of the biological properties between these two species. Thus, the physiological cFIX levels can not be directly compared. Although the exact genome status (e.g. percentage of episomal/circular/integrative molecules) of each vector is not clear at the time, the relative higher levels of cFIX cDNA compared to SB cDNA can be another indirect evidence for somatic integration, as the original vector infusion in mice was with a ratio of 3 to 1 between the two vectors HC-AdV-cFIX and HC-AdV-SB and 2 to 1 between the two vectors in treated dogs.

3.3.3. Acute liver toxicity

It has been shown that similar to early generation adenoviral vector, systemic administration of HC-AdV also resulted in activation of an acute inflammatory response indicated by the dramatic and prolonged increases in transaminases (Muruve et al., 1999; Schnell et al., 2001; Brunetti-Pierri et al., 2005; Brunetti-Pierri et al., 2004). Therefore, in the present study, several markers for activation of the acute inflammatory response were measured at various time points post injection.

Limited toxicity studies were performed by monitoring alanine aminotransferase (ALT) levels in mouse serum samples of treated mice to detect acute toxicity associated with administration of HC-AdVs. For all study groups, ALT levels were measured one day before injection and one day post-injection. ALT levels in these mice were slightly elevated compared to the levels before injection, especially for mice which received the high dose virus infusion (**Figure 3.18.a**), but remained in the normal range for all treated mice (20–80 U/l). To conduct a long term survey for liver toxicity, ALT levels in one group were measured for one week post-vector injection and the levels decreased to similar levels as before vector injection (**Figure 3.18.b**).

To monitor indirectly hepatocytes' damage, and to measure liver toxicity caused by CCl4 administration, ALT levels before and after CCl4 injection were measured. Serum transaminase levels dramatically increased to up to 600 U/l at day 1 after CCl4 administration but declined to a normal range one week later (**Figure 3.18.b**).

To investigate whether vector related toxicity occurred in the hybrid-vectors treated dogs, several laboratory measurements were performed. Aspartate aminotransferase (AST, normal range, 12–118 U/l) and ALT (normal range, 15–66 U/l) levels in plasma of dogs D1, D2, and D3 were monitored, which are used as the markers for acute liver toxicity. It was found that ALT and AST levels were elevated in dog D2, whereas transaminase levels for dogs D1 and D3 remained in a normal range (**Figure 3.19.a-b**). Quantification of alkaline phosphatase levels (ALP) revealed that this parameter was increased in dog D2, whereas levels stayed in a normal range for dogs D1 and D3 (**Figure 3.19.c**). Notably, elevated ALP

levels have been shown to be associated with certain medical conditions, like drug intoxication and tissue damage. In concordance with these results, the most significant drop of platelets was observed in dog D2 (**Figure 3.19.d**). Furthermore, all other monitored parameters like hematocrit, hemoglobin and total protein in blood remained in a normal range for dogs D1 and D3 (data not shown).

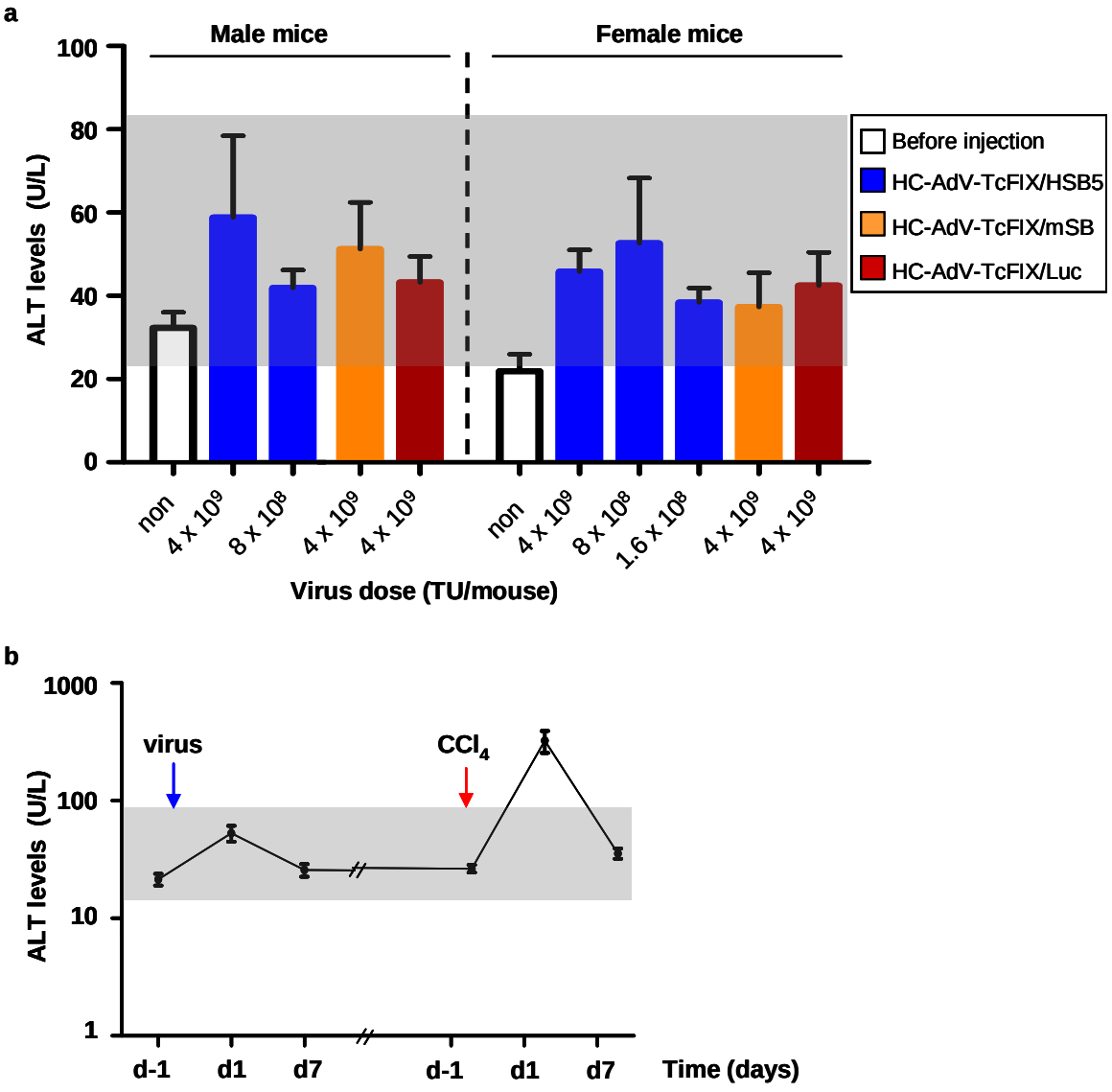


Figure 3.18: Alanine transaminase (ALT) levels. (a) ALT levels in serum samples of mice from different experiment groups. Samples were measured 1 day before- and 1 day post-virus injection (n=5). **(b) ALT levels in long term observation.** Serum samples were measured 1 day before-, 1 day post- and 7days post-virus injection and the time points around CCl_4 administration (n=5). The grey shadow shows the normal ALT range for mice (20–80 U/l). (U/l)=units per liter.

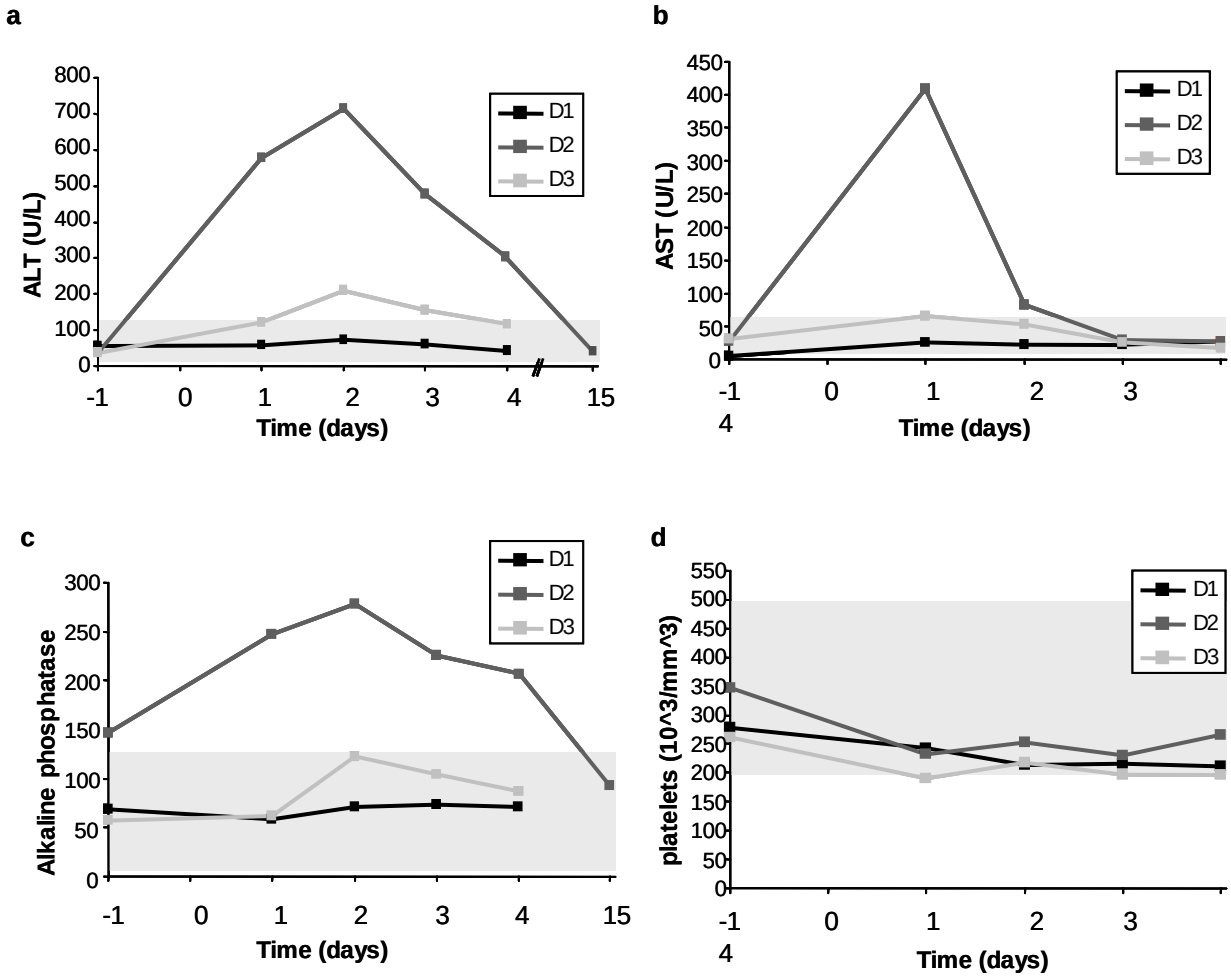


Figure 3.19: Acute toxicity profiles of treated hemophilia B dogs. Plasma samples of dogs D1 (HSB5 / low dose), D2 (HSB5 / high dose) and D3 (mSB) were collected at indicated time points and analyzed for markers related with acute liver toxicity. **(a) Alanine aminotransferase levels** (ALT; normal range: 12 – 118 U/L) were measured as well as **(b) aspartate aminotransferase levels** (AST; normal range: 15 – 66 U/L) and **(c) alkaline phosphatase levels** (normal range: 5 – 131 U/L). In addition **(d) the number of platelets** (normal range: 200,000 – 500,000 / mm^3) was quantified for all three dogs. HSB5, hyperactive Sleeping Beauty transposase; mSB, inactive transposase.

3.4. Exploration of the potential of a hyperactive human coagulation factor IX variant (hy-hFIX) in gene therapy application

Gene therapy has shown great potential for hemophilia treatment in preclinical and clinical studies. However, the spontaneously potent innate inflammatory and immune responses caused by the high vector dose that is necessary for an efficient therapeutic effect, remain an obstacle for successful treatment in patients (Manno *et al.*, 2006). The discovery and application of coagulation factors with enhanced activity hold a promise to overcome this limitation, because it would permit administration of lower vector doses and the therapeutic index could be increased (Dooriss *et al.*, 2009; Pipe, 2010).

Based on the structural homologies of human coagulation factor IX and factor X genes, Brandstetter group generated several hyperactive FIX variants. These recombinant FIX (rhFIX) proteins expressed in E. coli revealed 10^3-fold increased amidolytic activity in *in vitro* FIX substrate binding assays (Hopfner *et al.*, 1997; Sichler *et al.*, 2003). However, these rhFIX variants used were truncated because they only encoded the second epidermal growth factor domain, the activation peptide, and the catalytic domain of human FIX. Therefore, I wanted to generate a full-length hFIX cDNA for one hyperactive variant (K265T/Y345T) to evaluate its therapeutic potential (**Figure 1.5**).

3.4.1. Investigation of the hyperactive coagulation variant in a plasmid-based context

3.4.1.1. Subcloning of a full-length hyperactive coagulation factor encoding sequence

A full length hFIX was generated using an over-lapping PCR strategy with the primer pairs (hFIX-forw, hFIX-OL1, hFIX-OL2 and hFIX-rev) described in Table 2.2. Two plasmids, pET22b which contained the original truncated hFIX variant (K265T/Y345T) (Hopfner *et al.*, 1997) and a pAAV-EF1a-FIX plasmid containing the wild-type hFIX (Nakai *et al.*, 2001) were used for the over-lapping PCR amplification. The 1.4kb full-length hFIX cDNA of hyperactive variant (hy-hFIX) and wild-type (wt-hFIX) were then extracted from an agarose gel and subcloned into the pTOPO vector. These pTOPO plasmids containing wt- or hy-hFIX were checked by DNA-sequencing and restriction enzyme analysis.

3.4.1.2. Examination of transcription and expression in tissue culture

To initially determine the transcription and expression of the newly-generated hFIX variants, the full length hy-hFIX and the wt-hFIX encoding sequences were cloned into the pZAC vector carrying a CMV promoter resulting in pZAC-CMV-(wt/hy)-hFIX. These plasmids together with the pAAV-EF1a-FIX plasmid which served as a positive control were transfected into a human hepatoma cell line (Huh7) using FuGENE 6 transfection reagent. The transfected cells were incubated for 48 hours prior to measuring mRNA transcription and protein expression. Herein, the supernatant was collected to analyze hFIX expression by ELISA, and the cell pellets were treated with TRIZOL reagent for RNA isolation and reverse transcription PCR (RT-PCR). As shown in **Figure 3.20.a**, transcription of the hFIX transgene was detectable from both wt- and hy-hFIX containing pZAC plasmids as well as the positive control plasmid pAAV-EF1a-FIX using the primer pair hFIX-forw and hFIX-OL1. For protein detection as measured by ELISA, 60-80 ng/ml hFIX level in cell culture supernatant was detected (**Figure 3.20.b**).

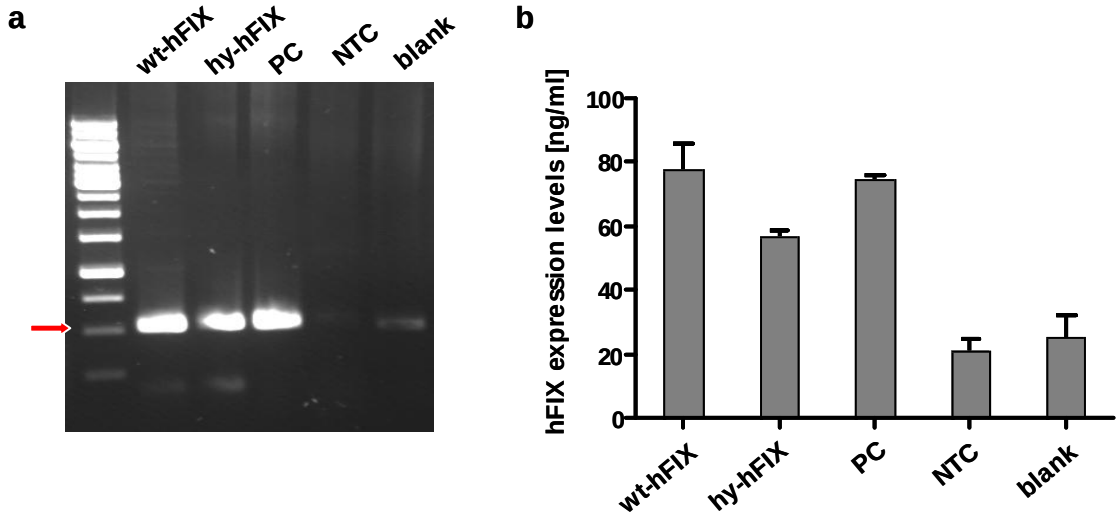


Figure 3.20: Transcription and expression of hFIX in tissue culture. (a) Reverse transcription PCR to check transcription of hFIX. Primer pairs hFIX-forw and hFIX-OL1 were used to amplify a 500 bp DNA fragment. The red arrow indicates the correct PCR products (~500 bp). (**b) ELISA to check transgene expression.** wt-hFIX: supernatant from the cells transfected with the plasmid pZAC-CMV-wt-hFIX; hy-hFIX: supernatant from the cells transfected with the plasmid pZAC-CMV-hy-hFIX; PC: positive control, supernatant from the cells transfected with the pAAV-EF1a-FIX plasmid; NTC: negative control, supernatant from the cells transfected with empty pZAC plasmid; blank: supernatant from the cells without transfection.

3.4.2. Evaluation of the hyperactive coagulation FIX variant in the context of an adenovirus

After the transcription and expression of the hyperactive hFIX variant was confirmed, it is important to measure its functional activity. Furthermore, the transgene expression levels in tissue culture from the plasmids were too low for detection using the actived partial thromboplastin time assay (APPT) (data not shown). As the adenoviral vector is more efficient in cellular transduction and most likely results in higher transgene expression levels compared to non-viral vectors, I produced high-capacity adenoviral vectors containing the wt-hFIX or hy-hFIX encoding sequences.

3.4.2.1. Generation of high-capacity adenoviral vectors (HC-AdVs) containing the human coagulation factor variants

The pAdFTC vectors containing the hFIX expression cassettes were generated according to a standard protocol (Jaeger et al. 2009). Briefly, the 1.4kb wt-hFIX and hy-hFIX segments were first sub-cloned into the vector pCI-hAAT-2xMAR, which contains the liver-specific promoter and enhancer region (ApoE HCR hAAT; 1.3 kb), and stuffer DNA fragments (2 x ChMAR: matrix attachment region from chicken lysozyme gene, 2.8 kb of each). Subsequently the complete expression cassette and the ChMAR stuffer DNA

fragments (9.7 kb) together were cut out from the pCI vector and cloned by the restriction enzymes PI-SceI and I-CeuI into the pHM5 shuttle plasmid. The same restriction enzymes were then used to clone the hFIX constructs into the adenovirus production plasmid pAdFTC, resulting in pAdFTC-hAAT-wtFIX and pAdFTC-hAAT-hyFIX (**Figure 3.21**).

The two HC-AdV-hFIX viruses were produced and amplified following a protocol for large-scale production of HC-AdV in spinner flasks and subsequent purification by cesium chloride (CsCl) ultracentrifugation (**Figure 2.1**)(Palmer & Ng 2003; Jager et al. 2009). Both virus preparations were characterized with respect to total physical titers and infectious titers. The physical titers were quantified by OD_{260nm} measurements, while infectious titers were determined by quantitative real-time PCR (qPCR) with the primer pairs specific for the hFIX cDNA (**Table 2.2**). For the PCR reaction, the genomic DNA isolated from virus infected 293 cells was used as template and the human B2M (Beta-2-microgloblin) gene was used as internal control.

3.4.2.2. Examination of transgene expression levels in tissue culture

Transgene expression from the adenoviral vectors HC-AdV-wt-hFIX and HC-AdV-hy-hFIX was examined in tissue culture. Herein, Huh7 cells were infected with MOIs 10, 100 and 1000, supernatant was collected 48 hours post-infection and measured for transgene expression by ELISA. Unfortunately, hFIX expression (5-24 ng/ml) was only observed after infection with HC-AdV-hy-hFIX at all three MOIs. From the HC-AdV-wt-hFIX infected cells, transgene expression was undetectable (data not shown).

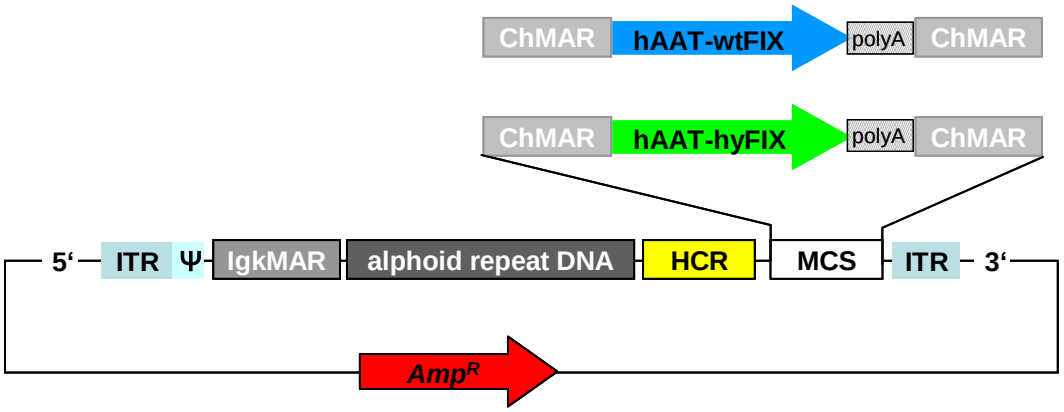


Figure 3.21: Plasmid constructs to study *in vivo* efficacy of a hyperactive hFIX expressing virus. Plasmids pAdFTC-hAAT-wtFIX and pAdFTC-hAAT-hyFIX were used for production of the respective viruses HC-AdV-wt-hFIX and HC-AdV-hy-hFIX. The plasmids carry an ampicillin resistance gene (AmpR). 5′- and 3′-ITR: human adenovirus serotype 5 inverted terminal repeats; Ψ: packaging signal; IgkMAR: the matrix attachment region of the murine immunoglobulin κ locus; HCR: alphoid repeat DNA sequences from human chromosome 17 and a liver-specific enhancer (hepatic control region); ChMAR: matrix attachment region from chicken lysozyme gene; hAAT-wtFIX and hAAT-hyFIX: human alpha-1-antitrypsin-promoter, wtFIX and hyFIX as transgenes, wtFIX is the wild type human coagulation factor IX gene, while hyFIX contains two point mutations (K265T/Y345T); polyA: SV40 late polyadenylation signal.

3.4.2.3. Examination of transgene expression and functional activity in hemophilia B mice

Due to the undetectable transgene expression of the HC-AdV containing wt-hFIX, only the HC-AdV-hy-hFIX vector was further examined for functional activity. Briefly, FIX knock-out mice were transduced at three different vector doses ($2x10^7$, $2x10^8$ and $2x10^9$ transducing units (TUs) per mouse) via tail vein injection. Plasma samples were collected at weeks 1, 2, 4 and 6 post-injection for factor IX (FIX) antigen (Ag) and coagulation FIX activity (C) determination. FIX antigen was quantified by ELISA while the coagulation activity was measured by the actived partial thromboplastin time assay (APPT). Only from the mouse which received a vector dose of $2x10^9$ TUs, FIX antigen and activity were detectable (700-1600 ng/ml and 2.6-8.3%, respectively) (**Figure 3.22**). Although it shows dose-dependency between the hFIX antigen and hFIX activity, the hyperactivity of this hFIX variant (K265T/Y345T) still can not be determined from these primary data. This part of the experiment related to hemophilia B mice was conducted by our cooperation partner Kazuo Ohashi (Institute of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University, Tokyo, Japan).

Time:week p.i	1	2	4	6
FIX:Ag ng/ml	1656	713	852	874
FIX:C ng/ml	8.3	2.6	3.2	8.1

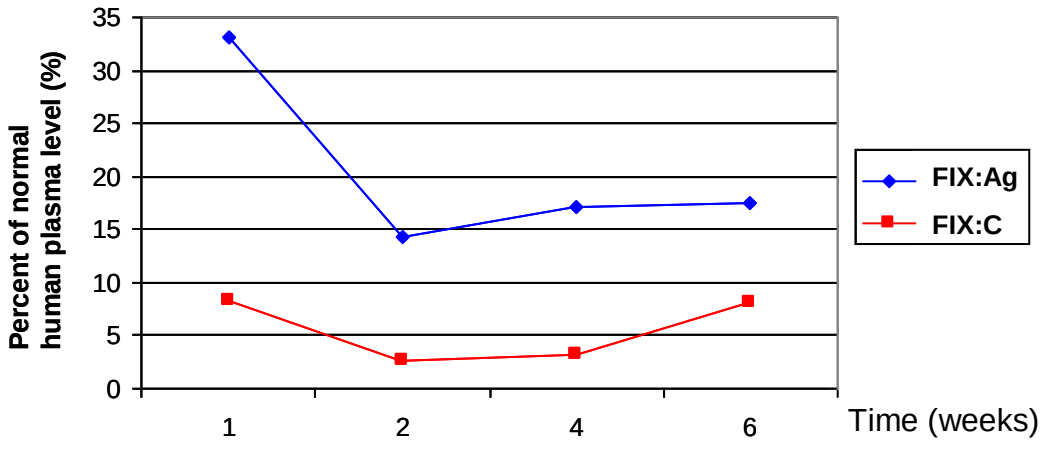


Figure 3.22: Tansgene expression levels and functional activities in one hemophilia B mouse. One FIX knock out mouse was transduced with HC-AdV-hy-hFIX encoding mutated hFIX at vector doses of $2x10^9$ transducing units (TUs). Plasma samples were collected in 0.1 volume of 3.8% sodium citrate at weeks 1, 2, 4 and 6 post-injection. The table shows the plasma hFIX levels at each time point, while the figure shows the percentage of FIX antigen (Ag) and activity (C) compared to normal human plasma (%).

4. Discussion

Novel developments in medicine played a crucial role in history, because therapeutic advances and successful applications of modern medicine resulted in reduced mortality, cured diseases, improved quality of life, and prolonged life span. For instance, emerging chemical substances as drugs, such as antibiotics have inhibited many life threatening bacterial infections. Later on, with the deeper understanding of many human diseases, other molecular forms, such as proteins and peptides were developed as a modern medical concept. For instance, the application of insulin for diabetic patients started a new chapter in human medicine. Similarly, diseases like hemophilia, a lifelong bleeding disorder which was well known as a European royal disease, could be treated by protein replacement therapies because plasma concentrates of coagulation factors became available in the 1970s. However, soon afterward, the contamination of infectious viruses as hepatitis B and C in the concentrates manufactured from pooled plasma obtained from thousands of donors raised the risk of this infusion therapy. To be free from blood-transmitted pathogens and to increase the worldwide capacity of replacement therapy, the production of recombinant coagulation factors was tested and began to be used in the 1990s (Schwartz *et al.*, 1990; Lusher *et al.*, 1993; White *et al.*, 1997; Roth *et al.*, 2001a). Nevertheless, inhibitors, high costs of the products, repeated infusions due to the short half life of the coagulation factors, and the limited availability are still factors which raise concerns about this treatment option. Therefore, the use of somatic gene therapy for hemophilia attracted researchers' attention and turned out to be a promising alternative to protein replacement therapy since the early 1990s (Mannucci and Tuddenham, 2001; Nichols *et al.*, 2009).

So far, the existing pre-clinical and clinical hemophilia gene therapy trials revealed the contradiction in terms of efficiency and safety. For instance, in a recent liver directed hemophilia B gene therapy trial utilizing adeno-associated virus serotype 2 (AAV2), the therapeutic effects were only observed in patients which received a high vector dose infusion, but the successful transduction turned out to be transient, probably due to an immune response directed against protein structures of the gene transfer vehicle (Manno *et al.*, 2006; Herzog, 2010). In concordance with these studies also in some other pre-clinical studies, the application of adenoviral vectors for hemophilia B treatment showed vector dose-dependent therapeutic effects (Brunetti-Pierri *et al.*, 2005; Brunetti-Pierri *et al.*, 2004; Ehrhardt and Kay, 2002; McCormack *et al.*, 2006).

For viral vectors, there is usually a narrow therapeutic window in which optimal effectiveness can be achieved. On the one hand, a high dose vector infusion can lead to an improved result, as more recombinant protein is formed. However, this is also associated with a higher risk of vector-related toxicity. In addition, a high dose can lead to transduction of undesired cells or organs, which could lead to serious safety concerns. On the other hand,

side effects after injection of a low vector dose are most likely negligible, but the effects may not be therapeutic, because not enough cells were transduced and thus sub-therapeutic amounts of the protein are formed (Tao *et al.*, 2001; Morral *et al.*, 2002; Brunetti-Pierri and Ng, 2011). Therefore, it is essential to develop other strategies, which enable therapeutic effects inside this therapeutic window.

The effectiveness of the gene-transfer vector is determined by the vector itself as well as the transgene to be delivered. In the present study, both determination factors to optimize the therapeutic effect have been explored in gene therapeutic studies for hemophilia B.

4.1. Therapeutic effects of the novel adenovirus/transposase hybrid-vectors

The aim of gene therapy for hemophilia is to deliver the functional transgene (cDNA of coagulation factor) into the target tissue of patients for achieving stable and therapeutic effects. In the present study, the novel adenovirus/transposase hybrid-vectors showed effective therapeutic effects in both small animal (mouse) and large animal (dog) models.

The adenovirus/transposase hybrid-vector system was initially evaluated in C57Bl/6 mice in a dose-dependent study. It was found that the serum transgene expression levels behaved in a non-linear vector dose-dependent manner (**Figure 3.2**). When quantifying the genome copy numbers of each vector in the treated animals by quantitative real-time PCR, it was shown that the genome copies of each vector directly correlated with the infused vector doses and transgene expression levels (**Figure 3.17**). These expression profiles bear similarities to the results obtained in previous studies, in which increasing doses of adenoviral vectors resulted in a nonlinear increase in transgene expression (Ehrhardt and Kay, 2002; Morral *et al.*, 2002; Morral *et al.*, 1998; Tao *et al.*, 2001).

It is also important to point out that there was toxicity accompanied with the high vector dose infusion. In particular, severe toxicity caused the death of mice from the transposase group (TcFIX/HSB5) and the control group (TcFIX/Luc) in the high vector dose setting ($4x10^9$ TUs per mouse). The reasons for this are unclear for the moment. It could be speculated that one potential reason may be the helper virus contamination. An important feature of these high-capacity adenoviral vectors is the need of helper virus in the vector preparation (Jager *et al.*, 2009; Palmer and Ng, 2003). Although Cre recombinase-mediated excision of the packaging signal from the helper virus genome is highly efficient, a small number of helper virus genomes with retained packaging signals are still packaged into capsids, and the helper viruses are not 100% separated from the high-capacity adenoviral vector preparations (Palmer and Ng, 2003; Parks *et al.*, 1996; Umana *et al.*, 2001). The HC-AdVs preparations used in this study, however, only contained a level of <0.03% helper virus contamination in the final vector preparations (**Figure 3.1**). The helper virus (HV, AdNG163R-2) used in our high-capacity adenoviral vectors production is a first generation

adenoviral vector (FGAdV), which was generated by deletion of the early adenoviral genes E1 and E3 (Jager *et al.*, 2009; Palmer and Ng, 2003). Therefore, the high dose mice received helper virus at $1.2x10^6$ TUs per mouse (equals to $6x\ 10^8$ TUs/kg). Such low doses of FGAdV are expected to cause no toxicity in mice and large animals (Morral *et al.*, 2002; Nunes *et al.*, 1999). Therefore, we believe it is unlikely that the toxicity observed in this study was attributable to the contaminating helper virus. Besides helper virus contamination, existence of replication-competent adenovirus (RCA) was also excluded from these virus preparations.

Notably, no abnormality was observed in mice which received the high dose in the first few days post-injection including the liver alanine aminotransferase (ALT) levels. The ALT levels in these mice were slightly elevated compared to the levels before injection (**Figure 3.18a**), but remained in the normal range for all treated mice (20–80 IU/l). Notably, the lethal toxicity of the high vector dose infusion caused the death of nine mice on day 5-9 post-injection, and three mice even died 2 weeks post-injection. This time period was probably after transgene expression or activation of an adaptive immune response. C57Bl/6 mice at 6-8 weeks of age were used for this study, which were in a good health status at the experiment time period. We do not know whether these mice had pre-existing anti-human Ad5 antibodies or whether any foreign gene products (SB, Flp, cFIX and Luciferase) caused toxicity. This needs to be further analyzed. In contrast to the toxicity observed in this study, numerous previous studies have shown that systemic administration of HC-AdVs led to dose-dependent acute toxicity (Hartman *et al.*, 2008). Those toxicities were observed shortly after vector injection (<48hrs) consisting of activation of the innate inflammatory immune responses (Brunetti-Pierri *et al.*, 2004; Schiedner *et al.*, 2003; Zang *et al.*, 2001; Croyle *et al.*, 2005). The single clinical trial of intravascular administration of HC-AdV into a human patient with a dose of 4.3×10^{11} VPs/kg resulted in very low FVIII levels (approximately 1%), and was also accompanied with transient inflammatory response with hematologic and liver abnormalities (Chuah *et al.*, 2004).

Furthermore, the dose chosen in my study, even the high dose ($4x10^9$ TUs per mouse, equals to $2x10^{11}$ TUs/kg), is comparable to or even lower than those used in the other studies. For instance, $5.6x10^{12}$ VPs/kg of the gutless adenovirus HC-AdV-LacZ was a safe dose in baboons, and 7.5×10^{12} VPs/kg of the apoE expressing HC-AdV or $3x10^{11}$ particles per mouse of a VLDLR expressing HC-AdV were safe doses in mice (Brunetti-Pierri *et al.*, 2005; Oka *et al.*, 2001; Kim *et al.*, 2001). However, the methods used to titrate the high-capacity adenoviral vectors vary in different laboratories (Kreppel *et al.*, 2002; Puntel *et al.*, 2006). In fact, most studies determined the viral titer expressed in viral particles (VPs) and not transducing units (TUs), making the issue more complicated. VPs present the titer in the number of viral genomes, while TUs indicate the number of active viruses and their

transducing ability (Crettaz *et al.*, 2008). Nevertheless, it is clear from this study that a dose of ≥2x10^{11} TUs/kg of the HC-AdV/SB hybrid-vectors is above the maximum tolerable dose in mice.

On the other hand, as shown in the preclinical model in this study, one hemophilia B dog injected with a relatively low vector dose of 9.4 x 10^{11} VPs/kg (equals to about 4.7x 10^{10} TUs/kg) showed long-term phenotypic correction of the clotting disorder (3 years) and persistent expression of cFIX **(Figure 3.4.a**). It is of note, that the adenovirus/transposase hybrid-vectors mediated gene transfer in hemophilia B dogs was not as efficient as that in mice with respect to cFIX protein expression levels **(Figure 3.3**). However, long-term (three years) phenotypic correction was observed in dog D1, demonstrating an efficient therapeutic effect in a large animal model. Moreover, the HC-AdV/SB hybrid-vector system has proved to be superior to the episomal adenoviral vectors, in terms of long-term transgene expression. For instance, in our historical study systemic administration of a HC-AdV-TcFIX at a nontoxic dose (8.6 × 10^{11} VPs/kg) into hemophilia B dogs only achieved transient phenotypic correction (therapeutic levels of cFIX for about 2 months) (Ehrhardt *et al.*, 2003). While another study with HC-AdV showed sustained cFIX expression for over a year at a high dose (3 × 10^{12} VPs/kg), but transient mild liver enzyme elevations and a decrease in platelet counts were observed (Brunetti-Pierri *et al.*, 2005). In summary, these results demonstrated the advanced features of the adenovirus/transposase hybrid-vector system, which facilitates the long-term therapeutic effect by stably inserting the transgene expression cassette into the host chromosomes.

The study performed in large animal models is particularly important because large animals like dogs and non-human primates have more biological similarities to human patients than mice. This includes inherit diversity, immune response, life span, body weight and vector delivery route. Dogs have long been used as a preclinical model for hemophilia gene therapy utilizing different viral vector systems. For instance, retroviral and lentiviral vectors were explored for hemophilia gene therapy in canine models. Although correction of the phenotype and expression of coagulation factors were observed (Kay *et al.*, 1993; Xu *et al.*, 2003), further development has been damped by concerns about the risk of insertional mutagenesis (Hacein-Bey-Abina *et al.*, 2003). As one of the best studied vectors, adeno-associated viral vector (AAV) has also shown its promising potential in hemophilia therapy. Protocols to use AAV for hemophilia gene therapy were established in several laboratories (Snyder *et al.*, 1997; McCown *et al.*, 1996). These approaches were based on two main strategies utilizing muscle and liver as target tissues. In case of muscle targeting, infusion of an AAV2 throughout the muscle resulted in long-term (> 3 years) FIX expression (circulating levels of 4%-14%) in a canine model for hemophilia B, achieving complete correction of the bleeding disorder (Arruda *et al.*, 2005). These results were similar to the therapeutic effect

observed in the dog D1 of my study. Recently, an 8-year study in inhibitor prone null mutation hemophilia B dogs treated with liver directed AAV2-FIX demonstrated long-term correction of the hemophilic phenotype with stable FIX activity remained between 4% and 10% (Niemeyer *et al.*, 2009). However, unlike that in my study, the stable transgene expression in these dogs was most likely due to the extra-chromosomal form of AAV, which was proved by the AAV integration sites analysis utilizing linear amplification–mediated polymerase chain reaction (LAM-PCR) (Niemeyer *et al.*, 2009). Notably, both muscle and liver directed AAV deliver strategies have been applied in clinical trials. Briefly, the muscle directed hemophilia clinical trial did not reveal detectable coagulation expression, while the liver directed gene transfer resulted in transgene expression, but the therapeutic effect was transient (Kay *et al.*, 2000; Manno *et al.*, 2006).

Development of a new medical agent follows the transition steps first from tissue culture to a small animal model, then to large animal or preclinical models, subsequently clinical trials and finally into clinical application (**Figure 1.8**). These transitions are always difficult as they force our knowledge from the predictably known into the uncertain. As to gene therapy, the successful translation rates from each step turned out to be even lower than the traditional medicine (Lowenstein and Castro, 2009). A variety of vectors, which demonstrated significant effects in cell culture can not be applied *in vivo* and a number of vectors and approaches that showed promising gene transfer efficiencies in small animals, failed to translate the results to large animals as dogs and non-human primates. Given the relatively small numbers of hemophilia clinical trials, it is not unexpected that the success rate from preclinical model to human beings is marginal. For example, in the case of AAV-mediated FIX gene transfer to liver, there were completely different outcomes in humans (Manno *et al.*, 2006) compared to experimental animals such as mice, rats, hemophilic dogs and nonhuman primates. All of these animals treated showed long-term expression of the transgene after AAV-mediated gene transfer (Mount *et al.*, 2002; Snyder *et al.*, 1999; Niemeyer *et al.*, 2009; Nathwani *et al.*, 2002). Notably, in that clinical trial, detailed analyses of the humoral and cellular immune responses found that the AAV capsid, rather than wild-type FIX was the antigen that was targeted by the host immune response (Manno *et al.*, 2006). Unlike experimental animals, humans are naturally infected by AAV2 during childhood. Therefore, the prior exposure to the virus capsid probably results in the difference in the immune responses.

As speculated already in other studies performed in canines, this study emphasize that only a small therapeutic window for HC-AdV may exist. As shown, application of a twofold higher viral dose (2.4×10^{12} VPs/kg body weight) resulted in transient albeit complete correction of the hereditary genetic disease in dog D2 (**Figure 3.4.b**). Moreover, increasing the viral dose resulted in transient liver toxicity (**Figure 3.19**) and may present one potential

reason for the strong decrease of plasma cFIX levels observed in dog D2. This dose threshold effect, for which a linear increase in viral vector dose does not correlate with protein expression, was also shown in several other studies in mice (Bristol *et al.*, 2000) and non-human primates (Brunetti-Pierri *et al.*, 2005; Brunetti-Pierri *et al.*, 2004). However, the minimal dose in mice for achieving long-term transgene expression seems to be lower compared to canines. This underscores the species-dependent differences after systemic administration of adenoviral vectors and that direct extrapolation across species may not be possible.

In summary, the adenoviral/SB hybrid-vector system was shown to be a promising tool for long-term gene-transfer in small animals and in a large animal model. Notably, it was the first trial to apply the SB system in a larger animal model. For stable transduction and treatment of tissues with a high-cell turn-over rate, our system represents a valuable tool for treatment. For instance, treatment of diseases affecting the skin or ex vivo correction of hematopoietic stem cells may be feasible utilizing our vector system.

4.2. Integration profile of the novel adenovirus/transposase hybrid-vectors

Like all medications, viral vectors carry risks, including potential harmful inflammatory reactions, germline transmission and insertional mutagenesis. Relevant preclinical tests for assessment of these and other potential adverse outcomes play an important role and will help us to better understand the potential risks of the respective gene therapy vector.

Regarding integrating vectors, on the one hand, these vectors can insert the transgene expression cassette into the host genomic DNA, thereby facilitating long-term stable transgene expression especially in rapidly dividing cells and regenerating tissues. On the other hand, any insertion of a foreign DNA fragment into the chromosome could result in genotoxicity. From preclinical tests and clinical trials, we learned that the potential risks of the integrating vectors depend on their integration profiles (Baum, 2007; Kustikova *et al.*, 2010). For instance, gamma retroviruses and derived vectors, which preferentially target regulatory sequences, were also shown to be associated with insertional mutagenesis. Transducing hematopoietic stem/progenitor cells with gamma retroviral vectors had released most of the treated patients (17/20, 75%) from severe immunodeficiency. However, these gamma retroviral vectors, which were designed to express the therapeutic transgene interleukin 2 receptor-γ (IL2RG) caused the case of leukemia in five of the treated patients (Antoine *et al.*, 2003; Cavazzana-Calvo and Fischer, 2004; Hacein-Bey-Abina *et al.*, 2010).

To analyze the integration events in the present study, I used a simple method (GenomeWalker-based method), which was adopted from a commercially available kit. This method was first used for integration site analysis after PhiC31-mediated integration from an adenovirus (Ehrhardt *et al.*, 2007). Briefly, this strategy involved a linker ligation-mediated

PCR and can be applied for samples isolated from different sources such as cells and tissues. Since it does not require any special equipment, it is applicable for every laboratory, especially when there is only a relatively small amount of integration events to be analyzed. By this method, the transposition events and integration profile after systemic administration of the adenovirus/transposase hybrid-vectors *in vivo* were carefully evaluated. Herein, I found that the transposase-mediated integration events from the adenovirus demonstrated a near random integration profile among gene and non-gene regions (**Figure 3.14**). However, the chromosomal distribution of these integration events was uneven and was shown to be bias towards the X-chromosome in female mice (**Figure 3.11**).

4.2.1. Sleeping Beauty transposase mediated integration events distributed randomly at genomic level

SB transposase-mediated integration profile has been extensively studied in previous studies. In one plasmid-based study, 1336 SB insertions were identified in primary and cultured mammalian cells (Yant *et al.*, 2005). As also shown in my study, the SB integrations were found to be widely distributed. Although target site preferences showed a small bias towards genes and their upstream regulatory sequences, it was much less pronounced and was not significantly influenced by transcriptional activity compared to those of most integrating viruses, like retroviral and lentiviral vectors (Hematti *et al.*, 2004; Mitchell *et al.*, 2004), and AAV (Nakai *et al.*, 2005; Nakai *et al.*, 2003). The same random distribution profile of SB with respect to genes and intergenic regions was confirmed by another group (Liu *et al.*, 2005). Therefore, regarding the integration profile there seems to be no difference in plasmid transfection and viral transduction approaches. However, SB has been clearly shown to be less effective in terms of cell entry compared to viral vector delivery systems. Therefore, it has been proposed that combining the cell entry properties of viruses and the integration machinery of SB could be a potential useful toolkit to generate novel vectors for sustained transgene expression (Yant *et al.*, 2002; Vink *et al.*, 2009; Peterson *et al.*, 2007; Heinz *et al.*, 2011).

Towards this, several studies have been performed in the past. Adenovirus, herpes simplex virus (HSV), retrovirus and lentivirus have been designed as a platform for gene insertion by SB transposase. In the HSV/SB hybrid-vector system, only few SB-mediated integration sites were identified (Peterson *et al.*, 2007; de Silva *et al.*, 2010). The SB-mediated integration profile from integrase defective lentivirus (IDLVs) has been analyzed in detail, and revealed a profile mimicking SB-plasmid integration. Briefly, in contrast to 70-80% frequency of integrase-proficient lentiviral vectors (ILVs) integration to active transcription units, the integration profile of SB transposition from IDLVs ranged from 30-50% within RefSeq genes (Vink *et al.*, 2009; Staunstrup *et al.*, 2009; Moldt *et al.*, 2011).

In contrast to the lentiviral delivery system, which has an integrative nature, the genomes of the adenoviral vectors have been shown to integrate into host chromosomes at low frequencies (Harui *et al.*, 1999; Hillgenberg *et al.*, 2001; Stephen *et al.*, 2008). It can be speculated that the SB transposon plays the major role in the target-site selection in the AdV/SB system. Therefore, in the present study, it is not surprising that the integration profile of SB transposition from HC-AdV is still the same or quite similar to that of the plasmid-based approaches (**Figure 3.14**).

In addition to these chromosomal integration events, 27 extra-chromosomal integrations were characterized, which were found to be located in the HC-AdV genome, mainly the stuffer DNA sequence and the transgene region (**Figure 3.15**). In the 30kb HC-AdV genome, approximately several thousand potential TA sites are present, and only 12 of them were hit by transposon integrations. Notably, these sites were located within two hot spots: 4 were in the IgkMAR sequence of the stuffer DNA and 8 were within the intron 1 which was included in the cFIX transgene. In previous studies based on the adenovirus/transposase and adenovirus/integrase hybrid-vector systems, integration events identified from HC-AdV administrated mice liver also showed transposon jumping into the Ad vector itself (Ehrhardt *et al.*, 2007; Yant *et al.*, 2002).

Extra-chromosomal integrations have also been described earlier in SB-mediated integration from plasmids (Yant *et al.*, 2005). Herein, 41 extra-chromosomal integrations were identified from mouse liver and human cell lines after SB transposition in the plasmid context. These events were located in two small regions of the target plasmid. One corresponded to the promoters while the other was the polyadenylation signal region (Yant *et al.*, 2005). In other integrating vector systems like retroviral and lentiviral vectors, or the combining of self-inactivating retroviral vector (SIN-RVs) and integrase-defective lentiviral vectors (IDLVs) with SB, although >1000 of integrations have been identified, extra-chromosomal integrations have not been reported (Beard *et al.*, 2007; Hematti *et al.*, 2004; Wu *et al.*, 2003; Deichmann *et al.*, 2007; Gabriel *et al.*, 2009; Paruzynski *et al.*, 2010). Nevertheless, findings in this study from AdV systems emphasize that the integration not only takes place in the host chromosomes but it can also occur between the vectors, even at high frequencies. For instance, a likelihood of 20% for the adenovirus/transposase hybrid-vectors was observed in the present study. How and when these integrations happened in the AdV/SB system is at the moment unknown. However, it can be speculated that these extra-chromosomal integrations will probably influence the therapeutic efficacy of these vectors by decreasing the effective chromosomal integration.

4.2.2. Sleeping Beauty transposase mediated integration events distributed unevenly at chromosomal level

One major feature identified for the AdV/SB hybrid-vectors is that the chromosomal distribution of integrations was biased towards the X-chromosome in female mice. In total three female mice were analyzed, two with high vector dose infusion (f1 and f2, $4x10^9$ TUs per mouse) and one with medium dose (f3, $8x10^8$ TUs per mouse). High percentages of integrations were found to be located in the X-chromosome in these mice (47% for f1, 37% for f2 and 26% for f3) (**Figure 3.11**). Interestingly, when I tried to analyze the integration profile in male mice, which received a medium vector dose (m1 and m2, $8x10^8$ TUs per mouse), no clear chromosomal preferences were observed (**Appendix Figure 2.d, 2.e**). Whether the integration pattern differs because of the fact that female mice contain two X-chromosomes while male mice only contain one, is not clear, at the time.

The data of the present study is not in line with results obtained by Yant and colleagues. In that study it was found that plasmid-based SB insertions were distributed evenly at the chromosomal level, and the X-chromosome even seemed to be somewhat disfavored (Yant *et al.*, 2005). Some other studies analyzed the integration profile of SB transposition from integrase defective lentiviral vectors (IDLVs) and self-inactivating retroviral vectors (SIN-RVs). These analyses in human cell lines did not demonstrate any chromosomal preferences at a higher resolution of more than 1000 events (Moldt *et al.*, 2011; Staunstrup *et al.*, 2009; Vink *et al.*, 2009). However, these vector systems were not checked with respect for the integration profile *in vivo*. Therefore, it could be speculated that the differences in integration profiles may be due to the experimental settings. It may be possible that SB-mediated integration combined with adenoviral transduction of hepatocytes and coexpression of Flp recombinase in the target cell might have caused a difference in the integration mechanism compared to non-viral or *in vitro* approaches. In contrast, in another study using an adeno/AAV hybrid-vector system, three (12 in total) X-chromosomal integration events were identified by ligation-mediated PCR (LM-PCR) from mice (Recchia *et al.*, 2004). However, the authors did not further characterize whether the integrase protein (Rep) or the AdV itself caused the bias. Moreover, the small amount of integration events was not convincing enough to make decisions on the chromosomal distribution.

Another factor that may influence the chromosomal location is the stuffer DNA contained in the HC-AdV genome. Many HC-AdVs contain noncoding “stuffer” DNA to maintain the size of the vector DNA within appropriate virion packaging limits. Previous studies showed that the nature of the stuffer sequence not only affects the replication and production of the HC-AdVs, but also has a significant effect on the function of the vector (Sandig *et al.*, 2000). An HC-AdV vector containing 22 kb of eukaryotic DNA has been shown to express a transgene to a higher level and for a longer duration than a vector containing 22

kb of prokaryotic DNA, both *in vitro* and *in vivo* (Parks *et al.*, 1999). A HC-AdV containing a 21.5-kb human β-globin locus control region (LCR) derived from the β-globin minilocus revealed a 25% integration frequency within the genomic globin LCR (Wang and Lieber, 2006). As to the stuffer DNAs used in the HC-AdVs of this study, it includes a 16.2-kb human centromeric fragment and a murine IgκMAR. Although this centromeric region and the IgkMAR are not presented on the murine X-chromosome, and sequence homology was not found either, it is likely that the vector backbone DNA might influence the integration pattern. However, it remains to be determined whether these elements caused the bias towards X-chromosomal distribution of integration events in the present study.

It is of note, that the integration sites obtained in other studies were analyzed by different methods. For example, in the plasmid-based SB integration study, a plasmid rescue method was used (Yant *et al.*, 2005). That method relies on self-ligation of the digested flanking DNA, and depends on antibiotic selection in bacteria (Rutledge and Russell, 1997). Utilizing this strategy, AAV integration junctions were first analyzed from AAV vector transduced Hela cells (Miller *et al.*, 2002; Rutledge and Russell, 1997). Recently, SB and PhiC31 mediated integration profile were also determined by plasmid rescue as well as integration of AAV in mouse liver (Ehrhardt *et al.*, 2006; Nakai *et al.*, 2005; Yant *et al.*, 2005). Compared to the GenomeWalker-based method used in my study, this method has the advantage to track the flanking genomic DNA from both sides of the integrated cargo in parallel. Thereby, it is possible to analyze the chromosomal deletions and potential rearrangements. However, as the selection depends on the antibiotic resistance gene expressed from the transgene cargo, this method is not applicable for most kind of gene transfer applications, when the transgene is a functional gene like a coagulation factor gene (cFIX) used in my study.

Another method called linear amplification–mediated PCR (LAM-PCR) is the most advanced and widely used approach especially in clinical studies. So far, it has been used for most of lentiviral and retroviral vector studies. This method combines the linear amplification of the target DNA with solid-phase second-strand synthesis. Therefore, the sensitivity, specificity and efficacy is considered to be very high (Schmidt *et al.*, 2009; Schmidt *et al.*, 2002). It was shown that LAM-PCR has the ability to characterize highly complex samples with multiple target sequences and sensitivity down to the single-cell level (Schmidt *et al.*, 2007). Recently, Schmidt and colleagues further developed this linear amplification–mediated PCR to nonrestrictive LAM-PCR (nrLAMPCR), which allows the comprehensive genome-wide detection of existing integration sites without the use of any restriction enzyme (Gabriel *et al.*, 2009; Paruzynski *et al.*, 2010). These modifications ensure a unidirectional ligation of the linker to the PCR products. Importantly, this approach solved the problem of bias of the integration sites caused by the choice of the restriction enzyme(s)

(Gabriel *et al.*, 2009; Paruzynski *et al.*, 2010). Although not as sensitive as LAM-PCR, nrLAMPCR obviously increased the integration sites retrieval, and identifies variable amplicon sizes of sequences around integration sites.

In summary, the integration events identified might be method-dependent. For example, in the present study, due to the restriction enzymes used, only the sites that located near certain enzymes could be rescued by the GenomeWalker-based method. Moreover, this GenomeWalker-based method may not be sensitive enough to identify a high amount of events compared to a method like LAM-PCR.

4.2.3. The potential genotoxicity associated with the novel adenovirus/transposase hybrid-vectors

Cancer caused by insertional mutagenesis has been one major concern. Animal models and unfortunately also clinical trials have revealed that the genomic risk associated with insertional mutagenesis originating from integrating vectors can be fatal (Li *et al.*, 2002; Baum *et al.*, 2006; Ott *et al.*, 2006; Lai *et al.*, 2010). Therefore, I have begun to evaluate the cancer-causing-potential of the novel HC-AdV/SB hybrid-vector system.

From all 136 integration events identified in mice, 10 were located in or near (±5kb) cancer-related genes (CRGs) (**Table 3.1**). Of these CRGs, three genes (GPC3 and CASK from f1; ITCH from f2) which can be related to hepatocellular carcinoma (HCC) are of high consideration. GPC3 is member of the glypican-related integral membrane proteoglycan family (GRIPS). Proteins of this family may play a role in the control of cell division and growth regulation (Zittermann *et al.*, 2010; Suzuki *et al.*, 2010). Therefore, the insertional hit in this gene would not be the 'original sin' to develop cancer, but may change the regulation function of this cancer-suppressing gene. The CASK gene encodes a calcium/calmodulin-dependent serine protein kinase, and belongs to the membrane-associated guanylate kinase (MAGUK) family of scaffolding molecules, which are associated with intercellular junctions (Hsueh, 2009; Hsueh *et al.*, 2000). Since the CASK encoding protein is essential for gene ontology features (cell adhesion and protein phosphorylation), it may be speculated that the insertion of foreign DNA may affect the cellular fate. The ITCH gene encoded protein is a closely related member of the NEDD4-like protein family. This family of proteins are E3 ubiquitin-ligase molecules and regulate key trafficking decisions, including targeting of proteins to proteosomes or lysosomes (Fang *et al.*, 2002). Moreover, E3 ligase ITCH is upregulated in anergic T cells (Gronski *et al.*, 2004). Development of cancer is seen as a failure of the immune system to control tumor growth. Therefore, the loss-of-function mutation in ITCH could decrease the T cells viability.

Integration events in or near cancer-related genes have been found from integrating viral vectors like retroviral vectors (Li *et al.*, 2002; Hacein-Bey-Abina *et al.*, 2003) and

lentiviral vectors (Themis *et al.*, 2005), as well as AAV (Donsante *et al.*, 2007), and have been proven to be related to cancer induction. A recent study analyzed the integration profile from three mostly used non-viral integrating vectors, Sleeping Beauty (SB), Tol2, and piggyBac (PB) transposon systems. After transfection of human primary T cells, 10 cancer-related genes were found as targets from the 96 selected T cell colonies. Interestingly, these cancer-related genes were only found from Tol2 and PB mediated integrations, none from SB-mediated integration events. The authors concluded that Tol2 and PB more likely promote clonal expansion than SB (Huang *et al.*, 2010a). Also in other studies, which analyzed SB-mediated integrations (Yant *et al.*, 2005; Staunstrup *et al.*, 2009; Moldt *et al.*, 2011), cancer-related gene targeting was not yet reported.

Since at least 1% of the gene repertoire may be involved in the various forms of cancer, transgene integration has a relatively high potential to affect genes regulating cell growth and survival (Kustikova *et al.*, 2010). However, the induction of cancer by a single insertional mutagenesis may not have a high impact, as cancer is a multi-step process that requires quite specific forms of oncogene collaboration (Hanahan and Weinberg, 2000). This assumption was supported by a recent study, which assessed the potential for AAV vector genotoxicity in a murine model. After 18 months of follow-up, AAV injected mice did not show a significantly higher rate of hepatocellular carcinoma compared with controls. Although 1029 integrants were identified, no integration events were identified as causing increased oncogene expression (Li *et al.*, 2011).

Another concern from this study is that high genome copy numbers of transposase encoding vector (Hc-AdV-HSB5/mSB) were detected in the hybrid-vectors administrated mice (**Figure 3.17**). Long-term high level SB transposase expression may cause transposon moving, potentially resulting in an increase of the cancer forming potential. However, the frequency of SB mobility from mammalian cell chromosomes was turned out to be very low (10^{-4} to 10^{-6} events per transfected cell) (Luo *et al.*, 1998; Yant and Kay, 2003; Yant *et al.*, 2004). Meanwhile, in the vector design, a phospho-glycerate kinase promoter (PGK) is used to control the expression of SB transposase (**Figure 3.1**). This PGK promoter is relatively weak but constitutively expressed. Therefore, the expression of transposase is probably not high but still existing. Of cause this prediction needs to be confirmed by the analyses of transcription and expression levels of SB. A recent study examined Sleeping Beauty transposase after trans delivery in primary human T cells. They also found an unexpectedly high copy number of Sleeping Beauty transposase but low frequency of persistent expression (Huang *et al.*, 2010b).

Nevertheless, the present analyses gave the primary insight to the cancer-causing-potential of the adenovirus/transposase hybrid-vectors in liver. Data from this study showed integration events into cancer-related genes, but it remains unknown whether this may alter

the expression of these genes or neighboring cancer-related genes. Therefore, it would be of high interest to investigate whether the cancer-related genes in or near the integration sites are transcriptional activated or suppressed.

4.3. Improving gene therapy efficiency through engineered transgene expression cassettes

In this study, I aimed at increasing the efficacy and safety of hemophilia B gene therapy by using gene transfer vectors that express a catalytically enhanced FIX molecule. The rationale was that such variant could increase the therapeutic index of the vector by permitting a therapeutic outcome at lower viral vector doses. This is important because viral vector-mediated toxicity is dose dependent, as shown in the present and other studies (Brunetti-Pierri *et al.*, 2004).

In this study I evaluated vectors expressing one previously described bioengineered FIX molecule (K265T/Y345T) in both tissue culture and in a murine model for hemophilia B. Although transgene expression has been observed in a plasmid context in transfected Huh7 cells, this recombinant coagulation factor was not able to show higher clotting activity in FIX-deleted plasma compared to the wild type protein (data not shown). In a hemophilia B mouse, the tail vein injection of $2x10^9$ transducing units of a high-capacity adenoviral vector containing this human coagulation factor variant (HC-AdV-hyhFIX) resulted in 700-1600 ng/ml FIX antigen levels and 2.6-8.3% clotting activity (**Figure 3.22**).

The initial study showed that Y94F, K98T and Y177T substitutions in a Gla-EGF1 domain-truncated FIXa increased the cleavage rate of the FXa-specific chromogenic substrate d-Nle-Gly-Arg-pNA 7000-fold (Sichler *et al.*, 2003). However, the effects of the mutations on FIX activity in the model systems are different from those observed in plasma. One recent study constructed five pre-described full-length FIX mutants with amino acid exchanges in the catalytic domain of FIX and characterized their activity in FX activation in model systems and in plasma (Hartmann *et al.*, 2009). Similar to our result, it was found that these mutants showed no or marginally improved catalytic properties in FX activation by the intrinsic tenase complex (FIXa-FVIIIa-Ca (2+)-phospholipid). As to the activation of the natural substrate FX, the Y94F and K98T substitutions (chymotrypsinogen numbering Y94F/K98T equals to Y259F/K265T by the normally used FIXa numbering) enhanced the catalytic efficiency of FX activation approximately two-fold (only in the absence of FVIIIa). Unexpectedly, the catalytic efficiency even decreased (both with and without FVIIIa) when the Y177F (equals to Y345T in our study) substitution was introduced into FIXa-Y94F/K98T. The activity of this FIX mutant (FIXa-Y94F/K98T/Y177F) was even lower than that of plasma-derived FIXa (Hartmann *et al.*, 2009). A potential explanation could be that these hyperactive mutants evaluated from model systems may impair their FVIII binding activity. Therefore, the

enhancement of FXIa-catalyzed activation of the FIX mutants determined in model systems are not representative of those clotting activities measured in plasma when FVIII is presented. This could be one reason for the fact that I did not observe expected higher clotting activity in plasma assay from the pre-described hyperactive-hFIX mutant.

There are recent studies which applied hyperactive factor IX in a gene therapy setting, such as FIX-R338A (Schuettrumpf *et al.*, 2005), R338A+$FIX_{VIIEGF1}$ (Brunetti-Pierri *et al.*, 2009) and FIX-V86A/E277A/R338A (Lin *et al.*, 2010). These *in vitro* screen-generated mutants with improved catalytic activity were shown to increase the therapeutic index of hemophilia B gene therapy. Importantly, all these mutants included an essential mutation at amino acid position 338. This residue change has not only been shown to increase the catalytic activity from recombinant FIX (R338A) (Chang *et al.*, 1998), but also in a clinical study. In that study, a thrombophilia patient with FIX containing a gain-of-function mutation (R338L), revealed approximately eight times increased clotting activity compared to the normal factor IX protein (Simioni *et al.*, 2009). Arginine at position 338 and these in the near region are highly conserved in factor IX from mammals (Czapinska and Otlewski, 1999), and this region of the protein is important for substrate (factor X) binding. Stop-codon mutations at R338 are common in hemophilia B, but there is only one missense mutation (R338P) at this position in hemophilia B identified (Bottema *et al.*, 1993; Ketterling *et al.*, 1994; P.M. Green, 2004) (The Haemophilia B Mutation Database – version 13, http://www.kcl.ac.uk/ip/petergreen/haemBdatabase.html). Therefore, the R338 substitution is an essential candidate when developing hyperactive FIX mutants. My primary evaluation of a hFIX variant which combining the R338A substitution to the hFIX-K265T/Y345T mutant (result in hFIX-K265T/Y345T/ R338A) showed enhance activity than that without R338A in tissue culture (data not shown).

Last but not least, although our study needs further investigation, it is believed that the idea to use bioengineered FIX molecules with enhanced activity still holds great promise. In the gene therapy context, use of such molecules would greatly improve safety and efficacy by permitting the administration of lower vector doses for achieving a therapeutic benefit. For example, the innate inflammatory immune response directed towards to Ad-based vectors is strongly dose dependent (Morral *et al.*, 2002; Brunetti-Pierri *et al.*, 2004) and therefore, any reduction in vector dose would greatly increase the vectors' therapeutic index.

4.4. Outlook and future perspectives

The adenovirus/transposase hybrid-vector system is a valuable tool for direct *in vivo* gene therapy based on the SB transposase. However, the treatment of hemophilia B dogs revealed a narrow therapeutic window for this system. To face the challenge to translate from preclinical studies to the clinics, the system needs to be further optimized. In general there

are two directions for improving the system. On the one hand, the efficiency of each single component of the hybrid-vector system could be improved. For instance, the integrating machinery (SB) used in this hybrid-vector system could be optimized with respect to activity. Although this HSB5 has been shown to be hyperactive compared to the former version SB11 (Yant *et al.*, 2004), even more advanced versions emerged in the last years, like SB100X that displayed 100 times higher transposase efficiency in tissue culture and SB100Xo, a codon-optimized version of SB100X that adapted to optimal tRNA usage for Homo sapiens (Mates *et al.*, 2009; Galla *et al.*, 2011). It may be speculated that the application of the more efficient transposases will significantly increase the integration efficiency as well as the therapeutic effect. Besides the efficiency, the specifity of the transposase could be another important aspect to be improved. Most of the unwanted side effects are due to the off-target integrations, therefore, a transposon designed to integrate specifically into 'safe' harbors, could decrease the risk of genotoxicity. On the other hand, the transgene expression cassettes could be optimized. As discussed, the utility of a hyperactive enzyme (FIX) may represent a potential solution to increase the therapy effect. For instance, it would be interesting to further explore the effect of the single mutant R338A/L (Chang *et al.*, 1998; Simioni *et al.*, 2009) in the functional improvement of the current hFIX variants (K265T/Y345T), or even in the cFIX context. Furthermore, the selection of different introns could highly increase the transgene expression levels (Kurachi S Fau - Furukawa *et al.*, 1995; Sam *et al.*, 2010). Also, the stuffer DNA contained in the HC-AdV genome has been shown to play a critical role in the virus production and transgene expression, as well as in integration.

Another important challenge which needs to be addressed in the future is the complexity of the design of this adenovirus/SB transposase hybrid-vector system. The co-delivery of the transposon-donor and -actor vectors makes the efficiency of somatic integration highly dependent on entry of these two vectors into one cell. In absence of either FLP or SB, the system does not mediate integration. Therefore, the ratio between transposons and SB transposase proteins should be optimized including the ratio between vectors and the promoters driving the transcription of SB transposase and Flp recombinase. Alternatively, utilizing an inducible system for expression of the integration machinery might reduce genotoxic and immunogenic side effects and may decrease an overproduction inhibition effect. In the further, it could be attractive to explore a "one vector-strategy" including all components for somatic integration (therapeutic DNA and the respective recombinase) in one vector genome is an option to overcome the necessity of co-transduction of one cell with two vectors.

Furthermore, to characterize the AdV/SB integration profile in more detail, it would be beneficial to apply a more sensitive method, like nonrestrictive linear amplification–mediated

PCR (nrLAM-PCR) (Gabriel *et al.*, 2009; Paruzynski *et al.*, 2010)(Gabriel, R, 2009; Paruzynski A. 2010) to analyze a large amount of integration events. The present study was the first to show an unevenly chromosomal distribution after SB-mediated integration, and this X-chromosome biased distribution needs to be further explored at a higher resolution of integration events. Furthermore, it would be helpful to compare the different methods of integration site analyses, to evaluate whether the integration preference for the X-chromosome was method-dependent. Moreover, cancer-related genes in or near integration sites were identified and raise concern about the cancer-causing-potential of SB transposase. Thus, it will be of interest to analyze the transcription and expression levels of these cancer-related genes, to determine at which degree the integrations events influences the respective target site. In addition, regarding the extra-chromosomal integrations, we do not know the mechanism of these unwanted vector rearrangements. It would be interesting to further evaluate these integrations, to get a clearer view of the adenovirus/transposase hybrid-vectors for *in vivo* application.

Summary

Gene therapy can be used to treat devastating inherited diseases, especially diseases and patients that are not suitable for a conventional cure. The blood clotting disorder hemophilia is one of the most extensively studied monogenetic diseases in gene therapeutic approaches. Several viral vectors were tested for the treatment of hemophilia B. The administration of an episomal adenoviral vector at non-toxic dose showed effective phenotypic correction, but the therapeutic effect was only transient. Therefore, the combination of non-viral integration machineries for somatic integration with adenoviral vectors for efficient delivery offers a promising alternative for achieving persistent transgene expression. Towards this end, the delivery of the Sleeping Beauty transposase (SB) integration machinery via high-capacity adenoviral vectors (HC-AdVs) has demonstrated efficient hepatocyte-directed gene transfer and long-term coagulation factor IX expression *in vivo.*

However, the safety issues of this adenoviral vector/Sleeping Beauty transposase (AdV/SB) hybrid-vector system, especially the vector dose-effect and genotoxicity were not addressed yet. Thus, I evaluated this hybrid-vector system in both mice and a canine model for hemophilia B with different vector dose settings, and analyzed the integration profile in respect to genotoxicity after systemic administration.

First of all, the viral vector preparations involved in the AdV/SB hybrid-vector studies were analyzed regarding physical and infectious titers. To avoid toxic side effects, the helper virus (HV) contamination levels were quantified and the potential existence of replication-competent adenovirus (RCA) in final vector preparations was excluded by quantitative real-time PCR. Only the HC-AdV preparations with high amounts of transducing units (10^7-10^8 TUs/µl), low HV contamination levels (<0.03%) and undetectable RCA were subsequently used for *in vivo* studies.

My study showed that this AdV/SB hybrid-vector system can result in significantly stabilized transgene expression in rapidly dividing hepatocytes in both male and female mice, as well as in a canine model for hemophilia B (three years). Notably, I demonstrated that the efficiency of the hybrid-vector system in terms of long-term transgene expression occurred in a dose-dependent manner. This phenomenon was confirmed at the molecular level by determining vector genome copy numbers of the AdV/SB hybrid-vectors in liver of treated animals. Furthermore, it was found that the toxicity profile of the hybrid-vector was also dose-dependent.

Regarding genotoxicity, the SB transposition events from the adenoviral vector were identified and analyzed at chromosomal level. For integration site analysis, I first established a PCR-based method for high-throughput analysis of integration events. Utilizing this method, I identified a total of 163 SB transposase-mediated integration events from five

transduced mice liver and 9 events from dog. Herein, similar to previously published plasmid-based studies, a fairly random integration pattern with respect to genes (exons and introns) and intergenic regions was observed for the adenovirus hybrid-vector. Unexpectedly, the chromosomal distribution displayed a bias towards the X-chromosome in female mice. Moreover, some extra-chromosomal integration events were observed, most likely due to vector rearrangements. Furthermore, 10 integration events were located in and near cancer-related genes, but the cancer-causing-potential of SB remains to be analyzed.

Besides the vector itself, the transgene can significantly influence the outcome of a gene therapeutic approach. To increase the therapeutic index of hemophilia B gene therapy utilizing adenoviral vectors, a previously described hyperactive human coagulation factor IX variant (hFIX-K265T/Y345T) was evaluated. However, the exploration did not reveal the expected hyperactive effect. Nevertheless, the utility of an improved transgene expression cassette represents a promising strategy for optimized gene therapeutic approaches.

In summary, this thesis provides novel insights into the adenovirus/SB transposase hybrid-vector system and demonstrates that this integrating adenoviral vector system presents a promising tool for gene therapy especially in regenerating tissues.

Zusammenfassung

Gentherapie ist eine vielversprechende Methode um schwerwiegende Erbkrankheiten zu behandeln, vor allem jene, die nicht durch eine konventionelle Behandlung therapiert werden können. Eine der am meisten untersuchten monogenetischen Erkrankungen in gentherapeutischen Ansätzen ist die Hämophilie B, die auf einer Störung der Blutgerinnung basiert. Diverse virale Vektoren wurden bereits für die Behandlung von Hämophilie B getestet. Die Verabreichung einer nicht toxischen Dosis eines episomalen, adenoviralen Vektors zeigte hierbei eine signifikante phänotypische Verbesserung, der therapeutische Effekt war jedoch nur transient. Deshalb bietet die Kombination aus nicht-viralen Integrationssystemen für somatische Integration zusammen mit einem adenoviralen Vektor für einen effizienten Transport in die Zelle eine vielversprechende Alternative für anhaltende Transgenexpression. Diesbezüglich demonstrierte die Integrationsmaschinerie der Sleeping Beauty Transposase (SB) in Kombination mit einem gen-deletierten, adenoviralen Vektor (HC-ADV) effizienten Hepatozyten-Gentransfer und langfristige Faktor IX Expression *in vivo*.

Allerdings wurde bisher die Eigenschaft dieses adenoviralen Vektor/Sleeping Beauty Transposase (AdV/SB)-Hybrid-Vektor-Systems, vor allem im Hinblick auf die Vektor-Dosis und Genotoxizität nicht untersucht. Im Rahmen dieser Arbeit wurde dieses Hybrid-Vektor-System sowohl in Mäusen als auch im Hunde-Modell für Hämophilie B angewandt. Dabei wurde die Vektor-Dosis variiert und das Integrationsprofil in Bezug auf Induktion von Genotoxizität nach systemischer Applikation analysiert.

Zunächst wurden die viralen Vektoren der AdV/SB-Hybrid-Vektor-Studien hinsichtlich physischem- und infektiösem Titer untersucht. Um toxische Nebenwirkungen zu vermeiden wurde zudem die Helfer-Virus (HV) Kontamination quantifiziert und die Existenz von replikationskompetenten Adenoviren (RCA) in dem endgültigen Vektor-Präparat wurde ausgeschlossen. Die HC-AdV Präparate mit hohen Mengen an transduzierenden Einheiten (10^7-10^8 TUs/µl), geringer HV Kontamination (<0.03%) und nicht nachweisbaren RCA wurden anschließend in den *in vivo*-Studien eingesetzt.

Weiterhin konnte gezeigt werden, dass das AdV/SB-Hybrid-Vektor-System die Transgenexpression in sich schnell teilenden Hepatozyten in männlichen und weiblichen Mäusen, sowie in einem Hunde-Modell für die Hämophilie B signifikant steigert. Die Dosisabhängigkeit des Hybrid-Vektors konnte im Bezug auf langfristige Transgenexpression in dieser Arbeit demonstriert werden. Dieses konnte außerdem auf molekularer Ebene durch die Bestimmung der Kopienzahl der Vektorgenome in der Leber der behandelten Tiere bestätigt werden. Darüber hinaus wurde gezeigt, dass auch das Toxizitätsprofil des Hybrid-Vektors dosisabhängig ist.

In Bezug auf Genotoxizität wurden die SB vermittelten Transpositionsereignisse ausgehend von dem adenoviralen Vektor identifiziert und auf chromosomaler Ebene

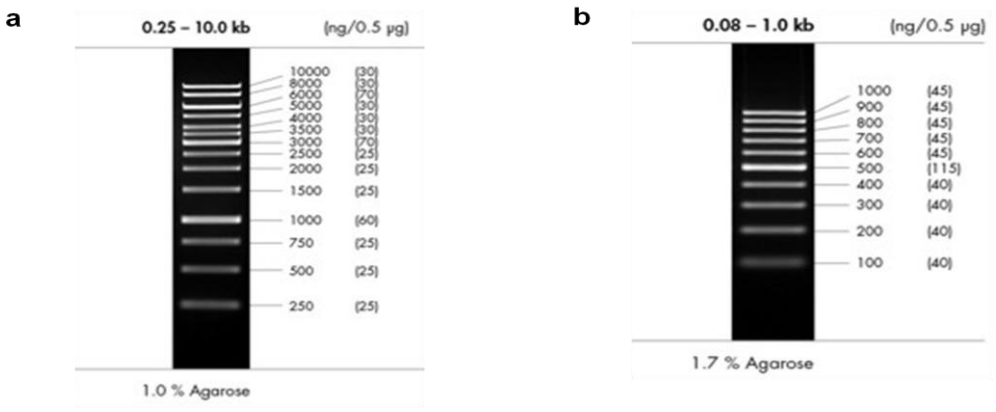
untersucht. Die Analyse der Integrationsorte erforderte die Etablierung einer PCR-basierten Methode für eine Hochdurchsatz-Analyse. Mit Hilfe dieser Methode konnten insgesamt 163 SB Transposase-vermittelte Integrationsereignisse aus fünf Mäusen und neun Ereignisse in der transduzierten Hundeleber identifiziert werden. Hierbei konnte, vergleichbar mit den zuvor veröffentlichten Plasmid-basierten Studien, ein zufälliges Integrationsmuster in Bezug auf Gene (Exons und Introns) und intergenische Regionen beobachtet werden. Dennoch zeigte die chromosomale Verteilung eine Tendenz zu dem X-Chromosom in weiblichen Mäusen. Darüber hinaus wurden einige extra-chromosomale Integrationsereignisse beobachtet, vermutlich aufgrund von Vektorumlagerungen. Außerdem konnten 10 Integrationsereignisse in der Nähe von Krebs-Genen lokalisiert werden. Das tumorigene Potential dieser Integrationen bleibt zu untersuchen.

Neben dem Vektor kann auch das Transgen selbst das Ergebnis einer Gentherapie beeinträchtigen. Zur Optimierung der Hämophilie B-Gentherapie, wurde im Rahmen dieser Arbeit eine zuvor beschriebene hyperaktive Faktor IX-Variante (hFIX-K265T/Y345T) charakterisiert. Die Analysen zeigten jedoch nicht die erwartete hyperaktive Wirkung des Blutgerinnungsfaktors. Dennoch stellt die Verbesserung der Transgen-Expressionskassette eine vielversprechende Strategie für eine optimierte gentherapeutische Anwendung dar.

Zusammenfassend vermittelt diese Arbeit neue Einblicke in das Adenovirus/SB Transposase Hybrid-Vektor-System und zeigt, dass ein integrierendes System basierend auf einem adenoviralen Vektor besonders in regenerativen Geweben ein vielversprechendes Werkzeug für die Gentherapie ist.

5. Appendix

5.1. Molecular size marker



Appendix Figure 1: DNA markers for agarose gel. (a) peqGOLD 1kb DNA marker. This molecular marker was used for approximate determination of DNA fragments on 1% agarose gels. The sizes of the single bands are indicated. Size range: 250bp to 10000bp. (**b) peqGOLD 100bb DNA marker.** This molecular marker was used for approximate determination of DNA fragments on 1.5-2% agarose gels. The sizes of the single bands are indicated. Size range: 100bp to 1000bp. Pictures obtained from peqlab Biotechnology GmbH.

5.2. Abbreviations

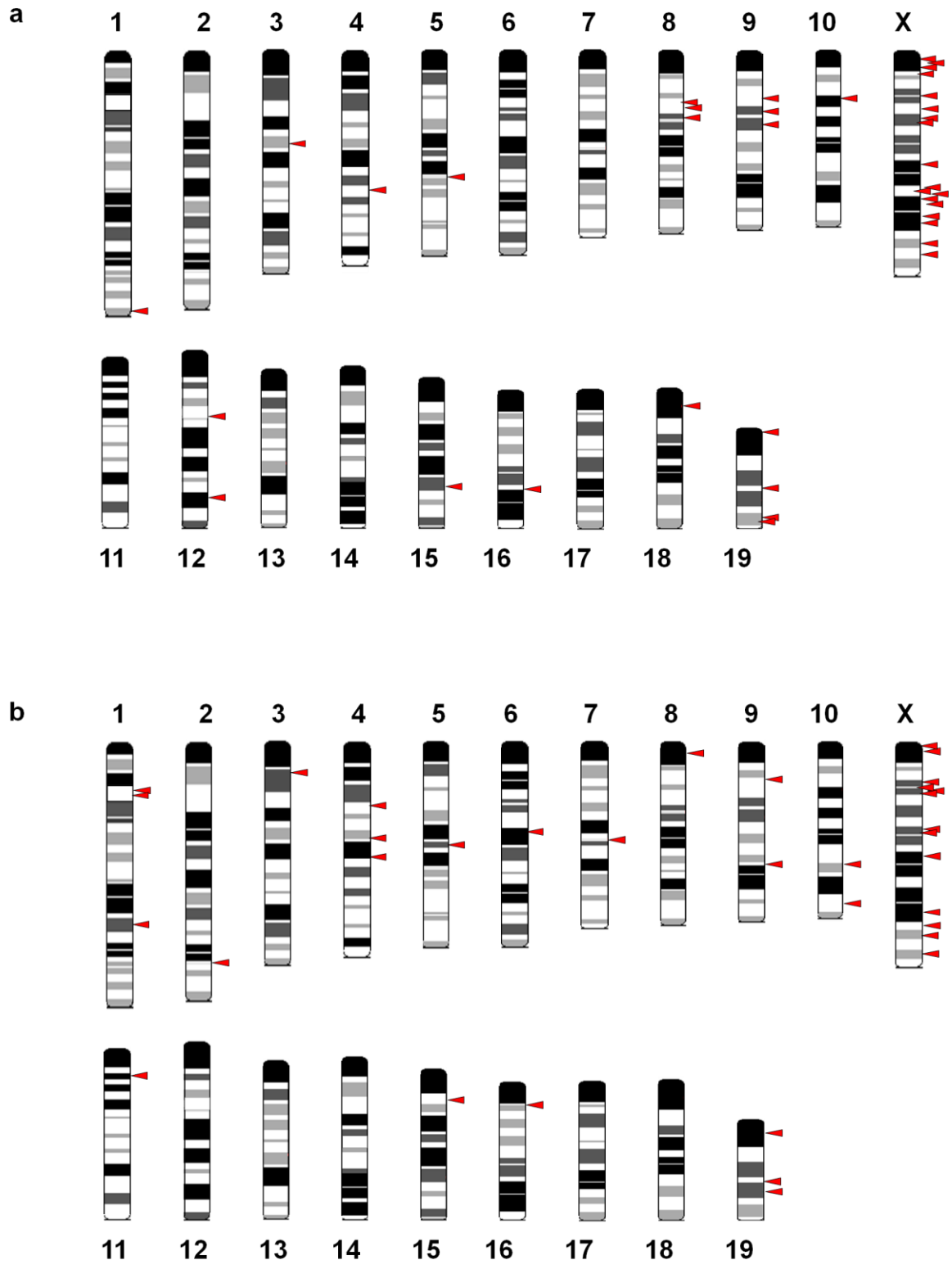
AAV	Adeno-associated virus
Ad5 wt	Adenovirus serotype 5 wild type
Ad	Adenovirus
AdV	Adenoviral vector
ALT	Alanine transferase
Amp^R	Resistance against ampicillin
ApoE	Apolipoprotein E
APPT	Actived partial thromboplastin time assay
bp	Base pairs
cDNA	Complementary DNA
cFIX	Canine blood coagulation factor IX
CIP	Calf intestine phosphatase
CMV	Cytomegalovirus
CsCl	Cesium chloride
DNA	Desoxy ribonucleic acid
dNTPs	Desoxy nucleoside triphosphates (dATP, dCTP, dTTP, dGTP)
dATP	Desoxy adenosine triphosphate
dCTP	Desoxy cytidine triphosphate
dGTP	Desoxy guanosine triphosphate
dTTP	Desoxy thymidine triphosphate

DMSO	Dimethyl sulfoxide
ds	Double-stranded
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme Linked Immunosorbent Assay
EtBr	Ethidium bromide
FBS	Foetal bovine serum
Flp	Flippase recombination enzyme
FRT	Flippase Recognition Target
Gen^R	Resistance against geneticin
hAAT	Human alpha-1-antitrypsin
HC-AdV	High-capacity adenoviral vector
HCR	Hepatic control region
hFIX	Human blood coagulation factor IX
hFVIII	Human blood coagulation factor VIII
HIV	Human immunodeficiency virus
Hyg^R	Resistance against hygromycin B
HSV	Herpes simplex virus
IDLVs	Integrase-defective lentiviral vectors
IRs	Inverted repeats
ITR	Inverted terminal repeat
Kan^R	Resistance against kanamycin
kb	Kilo base pairs
LB	Luria Bertani
MAR	Matrix attachment region
MCS	Multiple cloning site
mg	milligram
ml	millilitre
MLV	Murine leukemia virus
MOI	Multiplicity of infection (VPs/cell)
mRNA	Messenger RNA
ng	nanogram
nM	nanomolar
OD_{260nm}	Optical density at 260 nm
ORF	Open reading frame
ori	Origin of replication
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PGK	Phospho-glycerate kinase promoter
phiC31	Bacteriophage integrase
p.i.	post infection
polyA	Polyadenylation signal

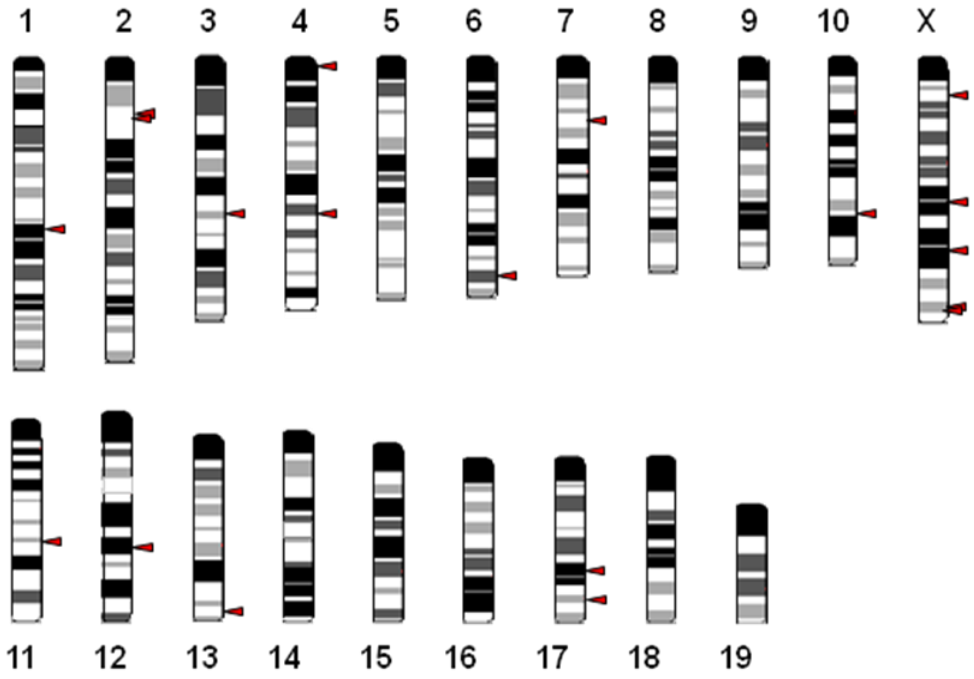
rAdV	Recombinant adenoviral vectors
RNA	Ribonucleic acid
RT	Room temperature
SB	Sleeping Beauty transposase
SCID	Severe combined immunodeficiency syndromes
SDS	Sodium dodecyl sulfate
SV40	Simian virus 40
TA	Tyrosin and adenosine dinucleotide
TAE	Tris-acetate-EDTA
TBS	Tris-buffered saline
TBS-T	TBS-Tween 20
TE	Tris-EDTA
TP	Terminal protein
TRIS	Tris-(hydroxymethyl)-ammonium methane
TUs	Transducing units
VPs	Viral particles
WBCT	Whole blood clotting time
ZFNs	Zinc-finger nucleases
Ψ	Packaging signal
°C	Degree Celsius

5.3. SB insertion site mapping in mouse genome

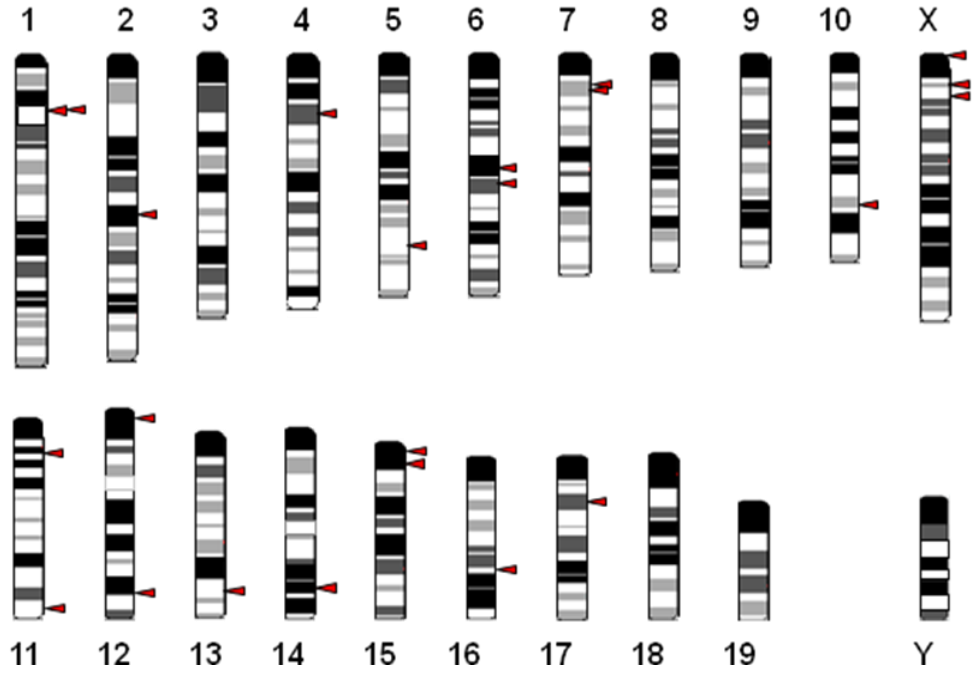
Appendix Figure 2: SB insertion site mapping in mouse genome. The relative positions of 136 independent integration sites from all 5 mice for SB-mediated transposition from adenovirus construct (AdV/SB hybrid-vector system) within the mouse genome were mapped according to BLAST results against NCBI mouse genomic DNA database. The red triangles indicate the relative positions of the chromosomal transposon integrations observed from mice liver genomic DNA. Figures (a-e) indicate each single mouse. (a) f1: female mouse 1. (b) f2: female mouse 2. (c) f3: female mouse 3. (d) m1: male mouse 1. (e) m2: male mouse 2. (f) The summary of all integration sites from these 5 mice.


a
1
2
3
4
5
6
7
8
9
10
X
11
12
13
14
15
16
17
18
19
b
1
2
3
4
5
6
7
8
9
10
X
11
12
13
14
15
16
17
18
19

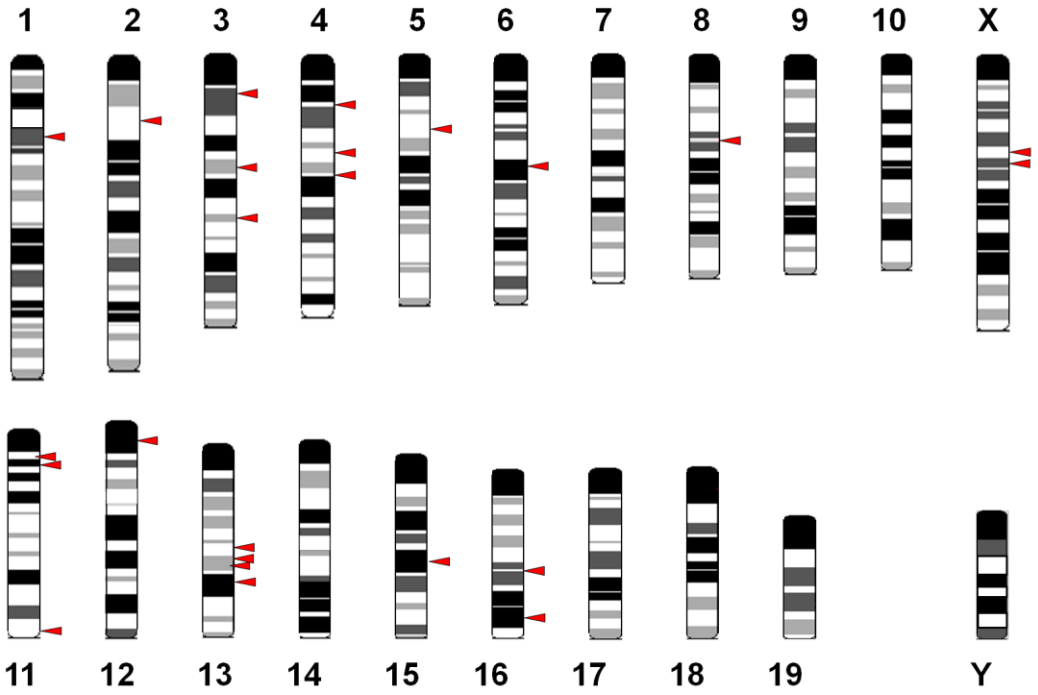
c


1
2
3
4
5
6
7
8
9
10
X
11
12
13
14
15
16
17
18
19

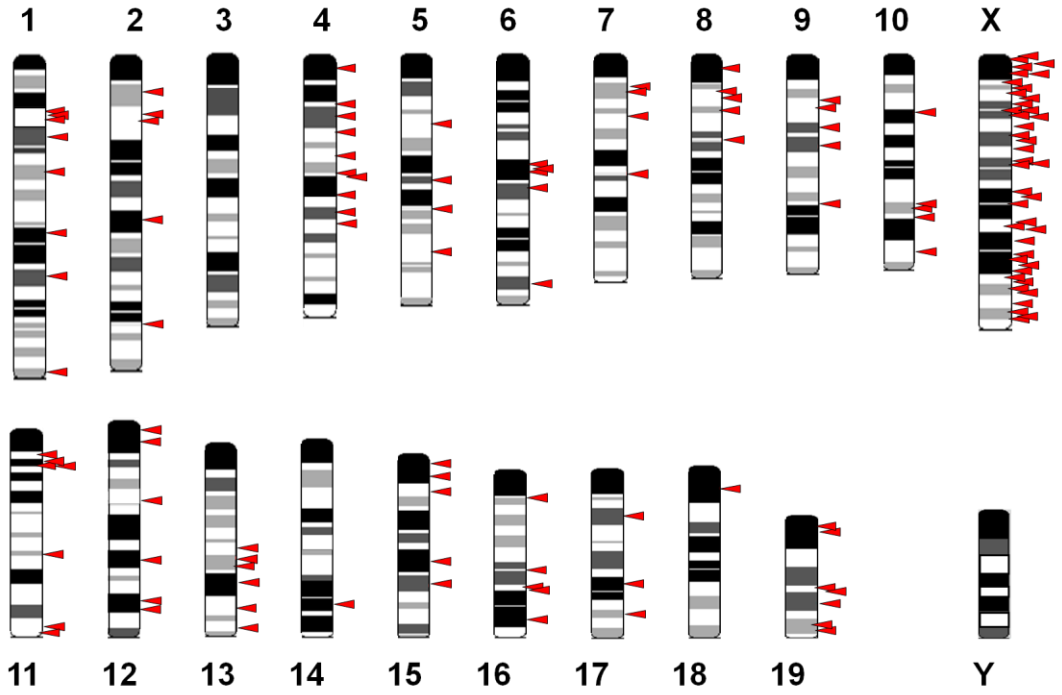
d


1
2
3
4
5
6
7
8
9
10
X
11
12
13
14
15
16
17
18
19
Y

e


1
2
3
4
5
6
7
8
9
10
X
11
12
13
14
15
16
17
18
19
Y

f


1
2
3
4
5
6
7
8
9
10
X
11
12
13
14
15
16
17
18
19
Y

5.4. Detailed description of transposition sites in mouse and dog liver genomic DNA

Appendix Table

Chromosome location: hit from	Band	located gene	gene symbol	orient-ation	cancer-related gene	distance to genes	
						Upstream	downstream
female mouse 1							
chr1: 194422060	1H6	Intragenic	Hhat	(+)	no		
chr3: 65662292	3 E1	intergenic	N/A	(-)	N/A	>>	>>
chr4: 104929873	4C6	intergenic	N/A	(+)	N/A	25 kb	>>
chr5: 92880283	5 E2	Intragenic	Scarb2	(+)	yes		
chr8: 36919710	8A4	intergenic	N/A	(-)	N/A	30 kb	>>
chr8: 38395562	8A4	intergenic	N/A	(+)	N/A	>>	>>
chr8: 50820216	8B1.2	intergenic	N/A	(+)	N/A	>>	>>
chr9: 3020270	9A4	intergenic	N/A	(+)	N/A	>>	>>
chr9: 42533603	9A5.1	Intragenic	Grik4	(+)	no		
*chr9: 52086874	9A5.3	intergenic	N/A	(-)	N/A	>>	>>
chr10: 35140724	10B1	intergenic	N/A	(-)	N/A	17 kb	9.8 kb down after Pm20d1
chr12: 46830855	12B3	intergenic	N/A	(+)	N/A	>>	>>
chr12: 98173577	12E	intergenic	N/A	(+)	N/A	>>	>>
chr15: 74257858	15D3	intergenic	N/A	(+)	N/A	>>	>>
chr16: 70579425	16C2	intergenic	N/A	(+)	N/A	10.1 kb	>>
chr18: 11755160	18A1	intergenic	N/A	(+)	N/A	>>	>>
chr19: 8282601	19A	Intragenic	D63000-2G06Rik	(-)	no		
chr19: 36673880	19C2	Intragenic	Hectd2	(-)	no		
*chr19: 57747837	19D2	Intragenic	Atrnl1	(+)	no		
chr19: 58649662	19D2	intergenic	N/A	(-)	N/A	21 kb	>>
chrx: 3833873	XA1.1	intergenic	N/A	(+)	N/A	>>	>>
chrx: 10225114	XA1.1	intergenic	N/A	(+)	N/A	>>	>>
chrx: 13341506	XA1.1	Intragenic	Cask	(-)	yes		
chrx: 15843807	XA1.2	intergenic	N/A	(-)	N/A	>>	>>
chrx: 33107224	XA3.2	intergenic	N/A	(+)	N/A	>>	>>
chrx: 44119865	XA4	intergenic	N/A	(+)	N/A	>>	>>
chrx: 49859131	XA5	Intragenic	Gpc3	(-)	yes		
chrx: 53163914	XA5	Intragenic	Gm8015	(-)	no		
chrx: 89237584	XC1	intergenic	N/A	(-)	N/A	>>	22.9 kb
chrx: 105294595	XD	intergenic	N/A	(+)	N/A	>>	>>
chrx: 105740425	XD	intergenic	N/A	(-)	N/A	>>	>>
chrx: 106723407	XD	intergenic	N/A	(+)	N/A	>>	>>
chrx: 108319959	XE1	intergenic	N/A	(+)	N/A	513 bp up to Cylc1	>>
chrx: 115229175	XE1	intergenic	N/A	(+)	N/A	>>	>>
chrx: 121295716	XE3	intergenic	N/A	(+)	N/A	46 kb	>>
chrx: 130860559	XE3	intergenic	N/A	(-)	N/A	9.3 kb up to Arl13a	7.3 kb down after Trmt2b
chrx: 139540810	XF2	intergenic	N/A	(+)	N/A	1.58 kb up to Rgag1	>>
chrx: 146195524	XF3	intergenic	N/A	(-)	N/A	>>	29 kb
female mouse 2							
chr1: 40405475	1B	Intragenic	Il1rl2	(+)	no		

Appendix Table continued

Chromosome location: hit from	**Band**	**located gene**	**gene symbol**	**orient-ation**	**cancer-related gene**	**distance to genes**	
chr1:40405964	1B	Intragenic	Il1rl2	(-)	no		
chr1: 133682925	1 E4	intergenic	N/A	(-)	N/A	>>	>>
chr2: 154999131	2H1	Intragenic	Itch	(-)	yes		
chr3: 23027811	3A3	intergenic	N/A	(+)	N/A	>>	30 kb
chr4: 45756871	4B1	intergenic	N/A	(+)	N/A	2 kb up to Igkj5	845bp down to Igk-C
chr4: 71120349	4C2	intergenic	N/A	(-)	N/A	24 kb	>>
chr4:83033499	4C3	intergenic	N/A	(-)	N/A	>>	>>
chr5: 75314112	5C3.3	intergenic	N/A	(+)	N/A	>>	>>
chr6: 70675170	6C1	intergenic	N/A	(-)	N/A	>>	>>
chr7: 79596448	7D1	intergenic	N/A	(+)	N/A	9.8 kb up to 60S ribosomal protein L21-like	12kb
chr8: 7133849	8A1.1	intergenic	N/A	(+)	N/A	>>	>>
chr9: 3000960	9A4	intergenic	N/A	(+)	N/A	>>	>>
chr9: 84714017	9E 2	intergenic	N/A	(-)	N/A	>>	>>
chr10:89748283	10C2	Intragenic	Anks1b	(-)	no		
chr10: 120789794	10D2	intergenic	N/A	(-)	N/A	>>	>>
chr11: 18486121	11A3.1	intergenic	N/A	(+)	N/A	>>	>>
chr15: 20903343	15A2	intergenic	N/A	(-)	N/A	>>	>>
chr16: 16052282	16A2	Intragenic	231000-8H04Rik	(-)	no		
chr19: 14809979	19A	intergenic	N/A	(-)	N/A	>>	>>
chr19: 37444874	19C2	intergenic	N/A	(+)	N/A	35 kb	6.2 kb down after Kif11
chr19: 43843935	19C3	intergenic	N/A	(-)	N/A	1.1 kb up to Cutc (cancer relative gene)	12 kb
chrx: 7224956	XA1.1	intragenic	Syp	(+)	no		
chrx: 12660407	XA1.1	intergenic	N/A	(-)	N/A	>>	11 kb
chrx: 27868001, 27207021, 26778136	XA3.1	intergenic	N/A	(+)	N/A	38 kb	>>
chrx: 32650536, 29173628, 24543339	XA3.2	intergenic	N/A	(-)	N/A	21 kb	>>
chrx: 35713786	XA3.3	intergenic	N/A	(-)	N/A	26 kb	43 kb
chrx: 37563854	XA3.3	intergenic	N/A	(-)	N/A	>>	>>
chrx: 66650755	XA7.1	Intragenic	Aff2	(-)	no		
chrx: 67224297	XA7.1	intergenic	N/A	(-)	N/A	25 kb	>>
chrx: 83829163	XC1	intergenic	N/A	(+)	N/A	>>	>>
chrx: 128596826	XE3	intergenic	N/A	(-)	N/A	>>	>>
chrx: 138187413	XF1	intergenic	N/A	(+)	N/A	27 kb	>>
chrx: 138774886	XF2	Intragenic	Acsl4	(-)	yes		
chrx: 153941500	XF4	intergenic	N/A	(-)	N/A	11 kb	47 kb
female mouse 3							
chr1: 109939552	1E2.1	intergenic	N/A	(+)	N/A	>>	>>
chr2: 46430608	2B	intergenic	N/A	(+)	N/A	>>	>>
chr2: 48181018	2B	intergenic	N/A	(+)	N/A	>>	>>

Appendix Table continued

Chromosome location: hit from	Band	located gene	gene symbol	orient-ation	cancer-related gene	distance to genes	
chr3: 96293888	3F2.1	intergenic	N/A	(-)	N/A	>>	>>
chr4: 92665103	4C5	intergenic	N/A	(-)	N/A	>>	>>
chr4: 9790374	4A1	intragenic	Gdf6	(+)	no		
chr6: 139086159	6G1	intergenic	N/A	(-)	N/A	>>	>>
chr7: 48894699	7B3	intragenic	Zfp788	(+)	no		
chr10: 96805748	10C3	intergenic	N/A	(-)	N/A	>>	>>
chr11: 72029791	11B4	intragenic	4930563-E22Rik	(+)	no		
chr12: 76703967	12C3	intergenic	N/A	(-)	N/A	8.5 kb up to Ppp2r5c	29 kb
chr13: 115413189	13D2.2	intergenic	N/A	(-)	N/A	>>	>>
chr17:65504778	17E1.1	intergenic	N/A	(-)	N/A	>>	>>
chr17: 81325464	17 E3	Intragenic	C230072-F16Rik	(-)	no		
chrx: 21056881	XA2	intergenic	N/A	(-)	N/A	>>	6.1 kb down after Agtr2
chrx: 90090876	XC2	intergenic	N/A	(+)	N/A	>>	>>
chrx: 119198273	XE3	intergenic	N/A	(+)	N/A	>>	>>
chrx: 152507426	XF4	intergenic	N/A	(+)	N/A	>>	>>
chrx: 152683688	XF4	intergenic	N/A	(-)	N/A	>>	>>
male mouse1							
*chr1: 37535572	1B	intragenic	Mgat4a	(+)	yes		
chr2: 98502694	2E 1	intergenic	N/A	(+)	N/A	>>	9.8 kb down after Rngtt
chr4: 33387605	4A5	intergenic	N/A	(-)	N/A	>>	>>
chr5: 21504508	5A3	intragenic	Reln	(+)	no		
chr5: 114227669	5F	intragenic	Iscu	(+)	no		
chr6: 70675372	6C1	intergenic	N/A	(-)	N/A	>>	>>
chr6: 81394562	6C3	intergenic	N/A	(+)	N/A	>>	>>
chr7:22185492	7A3	intergenic	N/A	(-)	N/A	>>	5.9 kb down after Tmcc3
chr7: 22668522	7A3	intergenic	N/A	(-)	N/A	>>	>>
chr10: 93971778	10C2	intergenic	N/A	(-)	N/A	>>	>>
chr11: 116280651	11 E2	intergenic	N/A	(-)	N/A	6.5 kb up to Rnf157	14 kb
chr11: 21917357	11A3.1	intragenic	Ehbp1	(-)	yes		
chr12: 8130076	12A1.1	intergenic	N/A	(+)	N/A	>>	>>
chr12: 102730920	12E	intergenic	N/A	(-)	N/A	>>	>>
chr13: 95763763	13D1	intragenic	Wdr41	(+)	no		
chr14: 106831507	14E2.3	intergenic	N/A	(+)	N/A	15 kb	>>
chr15: 11026633	15A1	intergenic	N/A	(-)	N/A	>>	>>
chr15: 11540100	15A1	intragenic	Adamts12	(+)	no		
chr16: 67498961	16C2	intergenic	N/A	(-)	N/A	>>	>>
chr17: 26693467	17A3.3	intergenic	N/A	(+)	N/A	>>	4.57 kb down after Ergic1
chrx: 8673141	XA1.1	intergenic	N/A	(+)	N/A	>>	8.3 kb down after 4930402K13Rik

Appendix Table continued

Chromosome location: hit from	Band	located gene	gene symbol	orient-ation	cancer-related gene	distance to genes	
chrx: 20425116	XA1.3	intergenic	N/A	(-)	N/A	>>	1.99 kb down after Araf (cancer relative gene)
chrx: 22444327	XA2	intergenic	N/A	(-)	N/A	>>	>>
male mouse 2							
chr1: 48600054	1C1.1	intergenic	N/A	(-)	N/A	>>	>>
chr2: 21992479	2A3	intergenic	N/A	(-)	N/A	1.5 kb after Gm13337	>>
*chr4: 29830686	4A4	intergenic	N/A	(-)	N/A	>>	>>
*chr4: 61508632	4B3	intergenic	N/A	(-)	N/A	>>	>>
chr4: 71120369	4C2	intergenic	N/A	(-)	N/A	>>	12 kb
chr5: 36106310	5B3	intragenic	Ablim2	(+)	yes		
chr6: 65335553	6C1	Intragenic	C130060-K24Rik	(-)	no		
chr8: 18575969	8A1.2	intergenic	N/A	(-)	N/A	>>	>>
chr11: 18486436	11A2	intergenic	N/A	(+)	N/A	>>	
chr11: 18486585	11A3.1	intergenic	N/A	(-)	N/A	>>	>>
chr11: 118496485	11 E2	intragenic	D11Bw-g0517e	(+)	no		
chr12: 10642739	12A1.1	intergenic	N/A	(-)	N/A	>>	>>
chr13: 63684106	13B3	intergenic	N/A	(-)	N/A	>>	17 kb
*chr13: 71245313	13C1	intergenic	N/A	(+)	N/A	>>	>>
chr13: 71245898	13C1	intergenic	N/A	(-)	N/A	>>	>>
chr13: 82419479	13C3	intergenic	N/A	(-)	N/A	>>	>>
chr15: 56690742	15D1	intergenic	N/A	(-)	N/A	>>	>>
chr16: 58651148	16C1.2	intergenic	N/A	(-)	N/A	>>	>>
chr16: 85890787	16C3.3	Intragenic	Adamts5	(-)	no		
chrx: 57950898	XA6	intergenic	N/A	(-)	N/A	>>	>>
chrx: 63637490	XA7.1	intergenic	N/A	(-)	N/A	>>	>>
Dog D1							
chr4: 65779013	N/A	intergenic	N/A	(-)	N/A	>>	>>
chr5: 13684258	N/A	intergenic	N/A	(-)	N/A	28 kb	36 kb
#chrx: 42244951	N/A	intragenic	Synap-tophysin	(+)	no		
chrUn: 59183592	N/A	intergenic	N/A	(+)	N/A	>>	>>

*integration events were found twice; # integration events were found several times.

>> Integration events were more than 50 kb way from genes.

Only genes that were <10 kb from integration events were checked for cancer relation.

References

Aiuti, A., Slavin, S., Aker, M., Ficara, F., Deola, S., Mortellaro, A., Morecki, S., Andolfi, G., Tabucchi, A., Carlucci, F., Marinello, E., Cattaneo, F., Vai, S., Servida, P., Miniero, R., Roncarolo, M. G. &Bordignon, C. (2002). Correction of ADA-SCID by stem cell gene therapy combined with nonmyeloablative conditioning. *Science* 296(5577): 2410-2413.

Alemany, R. &Zhang, W. W. (2000). Oncolytic adenoviral vectors. *Methods Mol Med* 35: 395-412.

Anderson, C. (1992). Gene therapy researcher under fire over controversial cancer trials. *Nature* 360(6403): 399-400.

Antoine, C., Muller, S., Cant, A., Cavazzana-Calvo, M., Veys, P., Vossen, J., Fasth, A., Heilmann, C., Wulffraat, N., Seger, R., Blanche, S., Friedrich, W., Abinun, M., Davies, G., Bredius, R., Schulz, A., Landais, P. &Fischer, A. (2003). Long-term survival and transplantation of haemopoietic stem cells for immunodeficiencies: report of the European experience 1968-99. *Lancet* 361(9357): 553-560.

Arruda, V. R. (2006). Toward gene therapy for hemophilia A with novel adenoviral vectors: successes and limitations in canine models. *J Thromb Haemost* 4(6): 1215-1217.

Arruda, V. R., Stedman, H. H., Nichols, T. C., Haskins, M. E., Nicholson, M., Herzog, R. W., Couto, L. B. &High, K. A. (2005). Regional intravascular delivery of AAV-2-F.IX to skeletal muscle achieves long-term correction of hemophilia B in a large animal model. *Blood* 105(9): 3458-3464.

Babiss, L. E. &Ginsberg, H. S. (1984). Adenovirus type 5 early region 1b gene product is required for efficient shutoff of host protein synthesis. *J Virol* 50(1): 202-212.

Barbato, J. E., Kibbe, M. R. &Tzeng, E. (2003). The emerging role of gene therapy in the treatment of cardiovascular diseases. *Crit Rev Clin Lab Sci* 40(5): 499-545.

Baum, C. (2007). Insertional mutagenesis in gene therapy and stem cell biology. *Curr Opin Hematol* 14(4): 337-342.

Baum, C., Kustikova, O., Modlich, U., Li, Z. &Fehse, B. (2006). Mutagenesis and oncogenesis by chromosomal insertion of gene transfer vectors. *Hum Gene Ther* 17(3): 253-263.

Beard, B. C., Keyser, K. A., Trobridge, G. D., Peterson, L. J., Miller, D. G., Jacobs, M., Kaul, R. &Kiem, H. P. (2007). Unique integration profiles in a canine model of long-term repopulating cells transduced with gammaretrovirus, lentivirus, or foamy virus. *Hum Gene Ther* 18(5): 423-434.

Bedognetti, D., Wang, E., Sertoli, M. R. &Marincola, F. M. (2010). Gene-expression profiling in vaccine therapy and immunotherapy for cancer. *Expert Rev Vaccines* 9(6): 555-565.

Bell, J. B., Podetz-Pedersen, K. M., Aronovich, E. L., Belur, L. R., McIvor, R. S. &Hackett, P. B. (2007). Preferential delivery of the Sleeping Beauty transposon system to livers of mice by hydrodynamic injection. *Nat Protoc* 2(12): 3153-3165.

Benson, S. D., Bamford, J. K., Bamford, D. H. &Burnett, R. M. (1999). Viral evolution revealed by bacteriophage PRD1 and human adenovirus coat protein structures. *Cell* 98(6): 825-833.

Berkner, K. L. (1988). Development of adenovirus vectors for the expression of heterologous genes. *Biotechniques* 6(7): 616-629.

Bett, A. J., Prevec, L. &Graham, F. L. (1993). Packaging capacity and stability of human adenovirus type 5 vectors. *J Virol* 67(10): 5911-5921.

Bolton-Maggs, P. (2003). Severe bleeding in idiopathic thrombocytopenic purpura. *J Pediatr Hematol Oncol* 25 Suppl 1: S47-51.

Bottema, C. D., Ketterling, R. P., Vielhaber, E., Yoon, H. S., Gostout, B., Jacobson, D. P., Shapiro, A. &Sommer, S. S. (1993). The pattern of spontaneous germ-line mutation: relative rates of mutation at or near CpG dinucleotides in the factor IX gene. *Hum Genet* 91(5): 496-503.

Brandstetter, H., Bauer, M., Huber, R., Lollar, P. &Bode, W. (1995). X-ray structure of clotting factor IXa: active site and module structure related to Xase activity and hemophilia B. *Proc Natl Acad Sci U S A* 92(21): 9796-9800.

Bristol, J. A., Shirley, P., Idamakanti, N., Kaleko, M. &Connelly, S. (2000). In vivo dose threshold effect of adenovirus-mediated factor VIII gene therapy in hemophiliac mice. *Mol Ther* 2(3): 223-232.

Brown, B. D., Venneri, M. A., Zingale, A., Sergi Sergi, L. &Naldini, L. (2006). Endogenous microRNA regulation suppresses transgene expression in hematopoietic lineages and enables stable gene transfer. *Nat Med* 12(5): 585-591.

Brunetti-Pierri, N., Grove, N. C., Zuo, Y., Edwards, R., Palmer, D., Cerullo, V., Teruya, J. &Ng, P. (2009). Bioengineered factor IX molecules with increased catalytic activity improve the therapeutic index of gene therapy vectors for hemophilia B. *Hum Gene Ther* 20(5): 479-485.

Brunetti-Pierri, N. &Ng, P. (2011). Helper-dependent adenoviral vectors for liver-directed gene therapy. *Hum Mol Genet* 20(R1): R7-13.

Brunetti-Pierri, N., Nichols, T. C., McCorquodale, S., Merricks, E., Palmer, D. J., Beaudet, A. L. &Ng, P. (2005). Sustained phenotypic correction of canine hemophilia B after systemic administration of helper-dependent adenoviral vector. *Hum Gene Ther* 16(7): 811-820.

Brunetti-Pierri, N., Palmer, D. J., Beaudet, A. L., Carey, K. D., Finegold, M. &Ng, P. (2004). Acute toxicity after high-dose systemic injection of helper-dependent adenoviral vectors into nonhuman primates. *Hum Gene Ther* 15(1): 35-46.

Brunetti-Pierri, N., Stapleton, G. E., Palmer, D. J., Zuo, Y., Mane, V. P., Finegold, M. J., Beaudet, A. L., Leland, M. M., Mullins, C. E. &Ng, P. (2007). Pseudo-hydrodynamic delivery of helper-dependent adenoviral vectors into non-human primates for liver-directed gene therapy. *Mol Ther* 15(4): 732-740.

Bunnell, B. A. &Morgan, R. A. (1998). Gene therapy for infectious diseases. *Clin Microbiol Rev* 11(1): 42-56.

Burton, E. A., Bai, Q., Goins, W. F. &Glorioso, J. C. (2001). Targeting gene expression using HSV vectors. *Adv Drug Deliv Rev* 53(2): 155-170.

Calos, M. P. (2006). The phiC31 integrase system for gene therapy. *Curr Gene Ther* 6(6): 633-645.

Campos, S. K. &Barry, M. A. (2007). Current advances and future challenges in Adenoviral vector biology and targeting. *Curr Gene Ther* 7(3): 189-204.

Carr, K. (1993). Nobel goes to discoverers of 'split genes'. *Nature* 365(6447): 597.

Carter, B. J. (2005). Adeno-associated virus vectors in clinical trials. *Hum Gene Ther* 16(5): 541-550.

Cavazzana-Calvo, M. &Fischer, A. (2004). Efficacy of gene therapy for SCID is being confirmed. *Lancet* 364(9452): 2155-2156.

Chalberg, T. W., Portlock, J. L., Olivares, E. C., Thyagarajan, B., Kirby, P. J., Hillman, R. T., Hoelters, J. &Calos, M. P. (2006). Integration specificity of phage phiC31 integrase in the human genome. *J Mol Biol* 357(1): 28-48.

Chang, J., Jin, J., Lollar, P., Bode, W., Brandstetter, H., Hamaguchi, N., Straight, D. L. &Stafford, D. W. (1998). Changing residue 338 in human factor IX from arginine to alanine causes an increase in catalytic activity. *J Biol Chem* 273(20): 12089-12094.

Chao, H., Mao, L., Bruce, A. T. &Walsh, C. E. (2000). Sustained expression of human factor VIII in mice using a parvovirus-based vector. *Blood* 95(5): 1594-1599.

Chinnadurai, G., Chinnadurai, S. &Brusca, J. (1979). Physical mapping of a large-plaque mutation of adenovirus type 2. *J Virol* 32(2): 623-628.

Chuah, M. K., Collen, D. &VandenDriessche, T. (2004). Clinical gene transfer studies for hemophilia A. *Semin Thromb Hemost* 30(2): 249-256.

Claeys Bouuaert, C. &Chalmers, R. (2010). Transposition of the human Hsmar1 transposon: rate-limiting steps and the importance of the flanking TA dinucleotide in second strand cleavage. *Nucleic Acids Res* 38(1): 190-202.

Crettaz, J., Olague, C., Vales, A., Aurrekoetxea, I., Berraondo, P., Otano, I., Kochanek, S., Prieto, J. &Gonzalez-Aseguinolaza, G. (2008). Characterization of high-capacity adenovirus production by the quantitative real-time polymerase chain reaction: a comparative study of different titration methods. *J Gene Med* 10(10): 1092-1101.

Croyle, M. A., Le, H. T., Linse, K. D., Cerullo, V., Toietta, G., Beaudet, A. &Pastore, L. (2005). PEGylated helper-dependent adenoviral vectors: highly efficient vectors with an enhanced safety profile. *Gene Ther* 12(7): 579-587.

Curiel, D. &Douglas, J. T. (2002). *Adenoviral vectors for gene therapy.* Amsterdam ; Boston: Academic Press.

Cutler, J. A., Mitchell, M. J., Smith, M. P. &Savidge, G. F. (2004). Germline mosaicism resulting in the transmission of severe hemophilia B from a grandfather with a mild deficiency. *Am J Med Genet A* 129A(1): 13-15.

Czapinska, H. &Otlewski, J. (1999). Structural and energetic determinants of the S1-site specificity in serine proteases. *Eur J Biochem* 260(3): 571-595.

Dai, Y., Schwarz, E. M., Gu, D., Zhang, W. W., Sarvetnick, N. &Verma, I. M. (1995). Cellular and humoral immune responses to adenoviral vectors containing factor IX gene: tolerization of factor IX and vector antigens allows for long-term expression. *Proc Natl Acad Sci U S A* 92(5): 1401-1405.

Dalba, C., Klatzmann, D., Logg, C. R. &Kasahara, N. (2005). Beyond oncolytic virotherapy: replication-competent retrovirus vectors for selective and stable transduction of tumors. *Curr Gene Ther* 5(6): 655-667.

Darby, S. C., Kan, S. W., Spooner, R. J., Giangrande, P. L., Hill, F. G., Hay, C. R., Lee, C. A., Ludlam, C. A. &Williams, M. (2007). Mortality rates, life expectancy, and causes of death in people with hemophilia A or B in the United Kingdom who were not infected with HIV. *Blood* 110(3): 815-825.

Dargaud, Y., Beguin, S., Lienhart, A., Al Dieri, R., Trzeciak, C., Bordet, J. C., Hemker, H. C. &Negrier, C. (2005). Evaluation of thrombin generating capacity in plasma from patients with haemophilia A and B. *Thromb Haemost* 93(3): 475-480.

Das, R. K., Hossain, S. U. &Bhattacharya, S. (2007). Protective effect of diphenylmethyl selenocyanate against CCl4-induced hepatic injury. *J Appl Toxicol* 27(6): 527-537.

Davidson, B. L., Stein, C. S., Heth, J. A., Martins, I., Kotin, R. M., Derksen, T. A., Zabner, J., Ghodsi, A. &Chiorini, J. A. (2000). Recombinant adeno-associated virus type 2, 4, and 5 vectors: transduction of variant cell types and regions in the mammalian central nervous system. *Proc Natl Acad Sci U S A* 97(7): 3428-3432.

Daya, S. &Berns, K. I. (2008). Gene therapy using adeno-associated virus vectors. *Clin Microbiol Rev* 21(4): 583-593.

de Jong, R. N. &van der Vliet, P. C. (1999). Mechanism of DNA replication in eukaryotic cells: cellular host factors stimulating adenovirus DNA replication. *Gene* 236(1): 1-12.

de Silva, S., Mastrangelo, M. A., Lotta, L. T., Jr., Burris, C. A., Federoff, H. J. &Bowers, W. J. (2010). Extending the transposable payload limit of Sleeping Beauty (SB) using the Herpes Simplex Virus (HSV)/SB amplicon-vector platform. *Gene Ther* 17(3): 424-431.

Deichmann, A., Hacein-Bey-Abina, S., Schmidt, M., Garrigue, A., Brugman, M. H., Hu, J., Glimm, H., Gyapay, G., Prum, B., Fraser, C. C., Fischer, N., Schwarzwaelder, K., Siegler, M. L., de Ridder, D., Pike-Overzet, K., Howe, S. J., Thrasher, A. J., Wagemaker, G., Abel, U., Staal, F. J., Delabesse, E., Villeval, J. L., Aronow, B., Hue, C., Prinz, C., Wissler, M., Klanke, C., Weissenbach, J., Alexander, I., Fischer, A., von Kalle, C. &Cavazzana-Calvo, M. (2007). Vector integration is nonrandom and clustered and influences the fate of lymphopoiesis in SCID-X1 gene therapy. *J Clin Invest* 117(8): 2225-2232.

DiMichele, D. (2007). Inhibitor development in haemophilia B: an orphan disease in need of attention. *Br J Haematol* 138(3): 305-315.

Donsante, A., Miller, D. G., Li, Y., Vogler, C., Brunt, E. M., Russell, D. W. &Sands, M. S. (2007). AAV vector integration sites in mouse hepatocellular carcinoma. *Science* 317(5837): 477.

Dooriss, K. L., Denning, G., Gangadharan, B., Javazon, E. H., McCarty, D. A., Spencer, H. T. &Doering, C. B. (2009). Comparison of factor VIII transgenes bioengineered for improved expression in gene therapy of hemophilia A. *Hum Gene Ther* 20(5): 465-478.

Douglas, K. L. (2008). Toward development of artificial viruses for gene therapy: a comparative evaluation of viral and non-viral transfection. *Biotechnol Prog* 24(4): 871-883.

Durai, S., Mani, M., Kandavelou, K., Wu, J., Porteus, M. H. &Chandrasegaran, S. (2005). Zinc finger nucleases: custom-designed molecular scissors for genome engineering of plant and mammalian cells. *Nucleic Acids Res* 33(18): 5978-5990.

Echavarria, M. (2008). Adenoviruses in immunocompromised hosts. *Clin Microbiol Rev* 21(4): 704-715.

Edelstein, M. L., Abedi, M. R. &Wixon, J. (2007). Gene therapy clinical trials worldwide to 2007--an update. *J Gene Med* 9(10): 833-842.

Edelstein, M. L., Abedi, M. R., Wixon, J. &Edelstein, R. M. (2004). Gene therapy clinical trials worldwide 1989-2004-an overview. *J Gene Med* 6(6): 597-602.

Ehrhardt, A., Engler, J. A., Xu, H., Cherry, A. M. &Kay, M. A. (2006). Molecular analysis of chromosomal rearrangements in mammalian cells after phiC31-mediated integration. *Hum Gene Ther* 17(11): 1077-1094.

Ehrhardt, A. &Kay, M. A. (2002). A new adenoviral helper-dependent vector results in long-term therapeutic levels of human coagulation factor IX at low doses in vivo. *Blood* 99(11): 3923-3930.

Ehrhardt, A., Xu, H., Dillow, A. M., Bellinger, D. A., Nichols, T. C. &Kay, M. A. (2003). A gene-deleted adenoviral vector results in phenotypic correction of canine hemophilia B without liver toxicity or thrombocytopenia. *Blood* 102(7): 2403-2411.

Ehrhardt, A., Yant, S. R., Giering, J. C., Xu, H., Engler, J. A. &Kay, M. A. (2007). Somatic integration from an adenoviral hybrid vector into a hot spot in mouse liver results in persistent transgene expression levels in vivo. *Mol Ther* 15(1): 146-156.

Engelhardt, J. F., Ye, X., Doranz, B. &Wilson, J. M. (1994). Ablation of E2A in recombinant adenoviruses improves transgene persistence and decreases inflammatory response in mouse liver. *Proc Natl Acad Sci U S A* 91(13): 6196-6200.

Evans, J. P., Brinkhous, K. M., Brayer, G. D., Reisner, H. M. &High, K. A. (1989). Canine hemophilia B resulting from a point mutation with unusual consequences. *Proc Natl Acad Sci U S A* 86(24): 10095-10099.

Fang, D., Elly, C., Gao, B., Fang, N., Altman, Y., Joazeiro, C., Hunter, T., Copeland, N., Jenkins, N. &Liu, Y. C. (2002). Dysregulation of T lymphocyte function in itchy mice: a role for Itch in TH2 differentiation. *Nat Immunol* 3(3): 281-287.

Fewell, J. G., MacLaughlin, F., Mehta, V., Gondo, M., Nicol, F., Wilson, E. &Smith, L. C. (2001). Gene therapy for the treatment of hemophilia B using PINC-formulated plasmid delivered to muscle with electroporation. *Mol Ther* 3(4): 574-583.

Fisher, K. J., Choi, H., Burda, J., Chen, S. J. &Wilson, J. M. (1996). Recombinant adenovirus deleted of all viral genes for gene therapy of cystic fibrosis. *Virology* 217(1): 11-22.

Friedmann, T. &Roblin, R. (1972). Gene therapy for human genetic disease? *Science* 175(25): 949-955.

Fujita, P. A., Rhead, B., Zweig, A. S., Hinrichs, A. S., Karolchik, D., Cline, M. S., Goldman, M., Barber, G. P., Clawson, H., Coelho, A., Diekhans, M., Dreszer, T. R., Giardine, B. M., Harte, R. A., Hillman-Jackson, J., Hsu, F., Kirkup, V., Kuhn, R. M., Learned, K., Li, C. H., Meyer, L. R., Pohl, A., Raney, B. J., Rosenbloom, K. R., Smith, K. E., Haussler, D. &Kent, W. J. (2011). The UCSC Genome Browser database: update 2011. *Nucleic Acids Res* 39(Database issue): D876-882.

Gabriel, R., Eckenberg, R., Paruzynski, A., Bartholomae, C. C., Nowrouzi, A., Arens, A., Howe, S. J., Recchia, A., Cattoglio, C., Wang, W., Faber, K., Schwarzwaelder, K., Kirsten, R., Deichmann, A., Ball, C. R., Balaggan, K. S., Yanez-Munoz, R. J., Ali, R. R., Gaspar, H. B., Biasco, L., Aiuti, A., Cesana, D., Montini, E., Naldini, L., Cohen-Haguenauer, O., Mavilio, F., Thrasher, A. J., Glimm, H., von Kalle, C., Saurin, W. &Schmidt, M. (2009). Comprehensive genomic access to vector integration in clinical gene therapy. *Nat Med* 15(12): 1431-1436.

Galla, M., Schambach, A., Falk, C. S., Maetzig, T., Kuehle, J., Lange, K., Zychlinski, D., Heinz, N., Brugman, M. H., Gohring, G., Izsvak, Z., Ivics, Z. &Baum, C. (2011). Avoiding cytotoxicity of transposases by dose-controlled mRNA delivery. *Nucleic Acids Res*.

Gao, G. P., Yang, Y. &Wilson, J. M. (1996). Biology of adenovirus vectors with E1 and E4 deletions for liver-directed gene therapy. *J Virol* 70(12): 8934-8943.

Garnett, C. T., Erdman, D., Xu, W. &Gooding, L. R. (2002). Prevalence and quantitation of species C adenovirus DNA in human mucosal lymphocytes. *J Virol* 76(21): 10608-10616.

Gaspar, H. B., Parsley, K. L., Howe, S., King, D., Gilmour, K. C., Sinclair, J., Brouns, G., Schmidt, M., Von Kalle, C., Barington, T., Jakobsen, M. A., Christensen, H. O., Al Ghonaium, A., White, H. N., Smith, J. L., Levinsky, R. J., Ali, R. R., Kinnon, C. &Thrasher, A. J. (2004). Gene therapy of X-linked severe combined immunodeficiency by use of a pseudotyped gammaretroviral vector. *Lancet* 364(9452): 2181-2187.

Gilboa, E. &Smith, C. (1994). Gene therapy for infectious diseases: the AIDS model. *Trends Genet* 10(4): 139-144.

Goncalves, G., Gouveia, E., Mesquita, J. R., Almeida, A., Ribeiro, A., Rocha-Pereira, J. &Sao Jose Nascimento, M. (2010). Outbreak of acute gastroenteritis caused by adenovirus type 41 in a kindergarten. *Epidemiol Infect*: 1-4.

Gorziglia, M. I., Kadan, M. J., Yei, S., Lim, J., Lee, G. M., Luthra, R. &Trapnell, B. C. (1996). Elimination of both E1 and E2 from adenovirus vectors further improves prospects for in vivo human gene therapy. *J Virol* 70(6): 4173-4178.

Grieger, J. C. &Samulski, R. J. (2005). Packaging capacity of adeno-associated virus serotypes: impact of larger genomes on infectivity and postentry steps. *J Virol* 79(15): 9933-9944.

Gronski, M. A., Boulter, J. M., Moskophidis, D., Nguyen, L. T., Holmberg, K., Elford, A. R., Deenick, E. K., Kim, H. O., Penninger, J. M., Odermatt, B., Gallimore, A., Gascoigne, N. R. &Ohashi, P. S. (2004). TCR affinity and negative regulation limit autoimmunity. *Nat Med* 10(11): 1234-1239.

Groth, A. C., Olivares, E. C., Thyagarajan, B. &Calos, M. P. (2000). A phage integrase directs efficient site-specific integration in human cells. *Proc Natl Acad Sci U S A* 97(11): 5995-6000.

Hacein-Bey-Abina, S., Hauer, J., Lim, A., Picard, C., Wang, G. P., Berry, C. C., Martinache, C., Rieux-Laucat, F., Latour, S., Belohradsky, B. H., Leiva, L., Sorensen, R., Debre, M., Casanova, J. L., Blanche, S., Durandy, A., Bushman, F. D., Fischer, A. &Cavazzana-Calvo, M. (2010). Efficacy of gene therapy for X-linked severe combined immunodeficiency. *N Engl J Med* 363(4): 355-364.

Hacein-Bey-Abina, S., Von Kalle, C., Schmidt, M., McCormack, M. P., Wulffraat, N., Leboulch, P., Lim, A., Osborne, C. S., Pawliuk, R., Morillon, E., Sorensen, R., Forster, A., Fraser, P., Cohen, J. I., de Saint Basile, G., Alexander, I., Wintergerst, U., Frebourg, T., Aurias, A., Stoppa-Lyonnet, D., Romana, S., Radford-Weiss, I., Gross, F., Valensi, F., Delabesse, E., Macintyre, E., Sigaux, F., Soulier, J., Leiva, L. E., Wissler, M., Prinz, C., Rabbitts, T. H., Le Deist, F., Fischer, A. &Cavazzana-Calvo, M. (2003). LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science* 302(5644): 415-419.

Hackett, P. B., Largaespada, D. A. &Cooper, L. J. (2010). A transposon and transposase system for human application. *Mol Ther* 18(4): 674-683.

Hanahan, D. (1983). Studies on transformation of Escherichia coli with plasmids. *J Mol Biol* 166(4): 557-580.

Hanahan, D. &Weinberg, R. A. (2000). The hallmarks of cancer. *Cell* 100(1): 57-70.

Harraghy, N., Gaussin, A. &Mermod, N. (2008). Sustained transgene expression using MAR elements. *Curr Gene Ther* 8(5): 353-366.

Hartman, Z. C., Appledorn, D. M. &Amalfitano, A. (2008). Adenovirus vector induced innate immune responses: impact upon efficacy and toxicity in gene therapy and vaccine applications. *Virus Res* 132(1-2): 1-14.

Hartmann, R., Dockal, M., Kammlander, W., Panholzer, E., Nicolaes, G. A., Fiedler, C., Rosing, J. &Scheiflinger, F. (2009). Factor IX mutants with enhanced catalytic activity. *J Thromb Haemost* 7(10): 1656-1662.

Harui, A., Suzuki, S., Kochanek, S. &Mitani, K. (1999). Frequency and stability of chromosomal integration of adenovirus vectors. *J Virol* 73(7): 6141-6146.

Hasbrouck, N. C. &High, K. A. (2008). AAV-mediated gene transfer for the treatment of hemophilia B: problems and prospects. *Gene Ther* 15(11): 870-875.

Hausl, M. A., Zhang, W., Muther, N., Rauschhuber, C., Franck, H. G., Merricks, E. P., Nichols, T. C., Kay, M. A. &Ehrhardt, A. (2010). Hyperactive sleeping beauty transposase enables persistent phenotypic correction in mice and a canine model for hemophilia B. *Mol Ther* 18(11): 1896-1906.

Hausl, M. A., Zhang, W., Muther, N., Voigtländer, R., Rauschhuber, C. &Ehrhardt, A. (2011). Development of adenovirus hybrid vectors for Sleeping Beauty transposition in large mammals. *Current Gene Therapy*.

Haviernik, P. &Bunting, K. D. (2004). Safety concerns related to hematopoietic stem cell gene transfer using retroviral vectors. *Curr Gene Ther* 4(3): 263-276.

Hay, C. R. (2006). The epidemiology of factor VIII inhibitors. *Haemophilia* 12 Suppl 6: 23-28; discussion 28-29.

Hearing, P., Samulski, R. J., Wishart, W. L. &Shenk, T. (1987). Identification of a repeated sequence element required for efficient encapsidation of the adenovirus type 5 chromosome. *J Virol* 61(8): 2555-2558.

Heinz, N., Schambach, A., Galla, M., Maetzig, T., Baum, C., Loew, R. &Schiedlmeier, B. (2011). Retroviral and transposon-based tet-regulated all-in-one vectors with reduced background expression and improved dynamic range. *Hum Gene Ther* 22(2): 166-176.

Hematti, P., Hong, B. K., Ferguson, C., Adler, R., Hanawa, H., Sellers, S., Holt, I. E., Eckfeldt, C. E., Sharma, Y., Schmidt, M., von Kalle, C., Persons, D. A., Billings, E. M., Verfaillie, C. M., Nienhuis, A. W., Wolfsberg, T. G., Dunbar, C. E. &Calmels, B. (2004). Distinct genomic integration of MLV and SIV vectors in primate hematopoietic stem and progenitor cells. *PLoS Biol* 2(12): e423.

Herzog, R. W. (2010). Hepatic AAV gene transfer and the immune system: friends or foes? *Mol Ther* 18(6): 1063-1066.

Herzog, R. W. &High, K. A. (1998). Problems and prospects in gene therapy for hemophilia. *Curr Opin Hematol* 5(5): 321-326.

High, K. (2005). Gene transfer for hemophilia: can therapeutic efficacy in large animals be safely translated to patients? *J Thromb Haemost* 3(8): 1682-1691.

Hillgenberg, M., Tonnies, H. &Strauss, M. (2001). Chromosomal integration pattern of a helper-dependent minimal adenovirus vector with a selectable marker inserted into a 27.4-kilobase genomic stuffer. *J Virol* 75(20): 9896-9908.

Hopfner, K. P., Brandstetter, H., Karcher, A., Kopetzki, E., Huber, R., Engh, R. A. &Bode, W. (1997). Converting blood coagulation factor IXa into factor Xa: dramatic increase in amidolytic activity identifies important active site determinants. *EMBO J* 16(22): 6626-6635.

Howe, S. J., Mansour, M. R., Schwarzwaelder, K., Bartholomae, C., Hubank, M., Kempski, H., Brugman, M. H., Pike-Overzet, K., Chatters, S. J., de Ridder, D., Gilmour, K. C., Adams, S., Thornhill, S. I., Parsley, K. L., Staal, F. J., Gale, R. E., Linch, D. C., Bayford, J., Brown, L., Quaye, M., Kinnon, C., Ancliff, P., Webb, D. K., Schmidt, M., von Kalle, C., Gaspar, H. B. &Thrasher, A. J. (2008). Insertional mutagenesis combined with acquired somatic mutations causes leukemogenesis following gene therapy of SCID-X1 patients. *J Clin Invest* 118(9): 3143-3150.

Hsueh, Y. P. (2009). Calcium/calmodulin-dependent serine protein kinase and mental retardation. *Ann Neurol* 66(4): 438-443.

Hsueh, Y. P., Wang, T. F., Yang, F. C. &Sheng, M. (2000). Nuclear translocation and transcription regulation by the membrane-associated guanylate kinase CASK/LIN-2. *Nature* 404(6775): 298-302.

Huang, X., Guo, H., Tammana, S., Jung, Y. C., Mellgren, E., Bassi, P., Cao, Q., Tu, Z. J., Kim, Y. C., Ekker, S. C., Wu, X., Wang, S. M. &Zhou, X. (2010a). Gene transfer efficiency and genome-

wide integration profiling of Sleeping Beauty, Tol2, and piggyBac transposons in human primary T cells. *Mol Ther* 18(10): 1803-1813.

Huang, X., Haley, K., Wong, M., Guo, H., Lu, C., Wilber, A. &Zhou, X. (2010b). Unexpectedly high copy number of random integration but low frequency of persistent expression of the Sleeping Beauty transposase after trans delivery in primary human T cells. *Hum Gene Ther* 21(11): 1577-1590.

Ison, M. G. (2006). Adenovirus infections in transplant recipients. *Clin Infect Dis* 43(3): 331-339.

Iverson, N., Plourde, N., Chnari, E., Nackman, G. B. &Moghe, P. V. (2008). Convergence of nanotechnology and cardiovascular medicine : progress and emerging prospects. *BioDrugs* 22(1): 1-10.

Ivics, Z., Hackett, P. B., Plasterk, R. H. &Izsvak, Z. (1997). Molecular reconstruction of Sleeping Beauty, a Tc1-like transposon from fish, and its transposition in human cells. *Cell* 91(4): 501-510.

Ivics, Z. &Izsvak, Z. (2006). Transposons for gene therapy! *Curr Gene Ther* 6(5): 593-607.

Izsvak, Z. &Ivics, Z. (2004). Sleeping beauty transposition: biology and applications for molecular therapy. *Mol Ther* 9(2): 147-156.

Jager, L. &Ehrhardt, A. (2009). Persistence of high-capacity adenoviral vectors as replication-defective monomeric genomes in vitro and in murine liver. *Hum Gene Ther* 20(8): 883-896.

Jager, L., Hausl, M. A., Rauschhuber, C., Wolf, N. M., Kay, M. A. &Ehrhardt, A. (2009). A rapid protocol for construction and production of high-capacity adenoviral vectors. *Nat Protoc* 4(4): 547-564.

Jazwa, A., Jozkowicz, A. &Dulak, J. (2007). New vectors and strategies for cardiovascular gene therapy. *Curr Gene Ther* 7(1): 7-23.

Jiang, H., Couto, L. B., Patarroyo-White, S., Liu, T., Nagy, D., Vargas, J. A., Zhou, S., Scallan, C. D., Sommer, J., Vijay, S., Mingozzi, F., High, K. A. &Pierce, G. F. (2006). Effects of transient immunosuppression on adenoassociated, virus-mediated, liver-directed gene transfer in rhesus macaques and implications for human gene therapy. *Blood* 108(10): 3321-3328.

Jin, D. Y., Zhang, T. P., Gui, T., Stafford, D. W. &Monahan, P. E. (2004). Creation of a mouse expressing defective human factor IX. *Blood* 104(6): 1733-1739.

Jones, J. M. &Koch, W. J. (2005). Gene therapy approaches to cardiovascular disease. *Methods Mol Med* 112: 15-35.

Kao, C. Y., Lin, C. N., Yu, I. S., Tao, M. H., Wu, H. L., Shi, G. Y., Yang, Y. L., Kao, J. T. &Lin, S. W. (2010). FIX-Triple, a gain-of-function factor IX variant, improves haemostasis in mouse models without increased risk of thrombosis. *Thromb Haemost* 104(2): 355-365.

Kato, K., Yamashita, R., Matoba, R., Monden, M., Noguchi, S., Takagi, T. &Nakai, K. (2005). Cancer gene expression database (CGED): a database for gene expression profiling with accompanying clinical information of human cancer tissues. *Nucleic Acids Res* 33(Database issue): D533-536.

Kay, M. A. (1998). Hepatic gene therapy for haemophilia B. *Haemophilia* 4(4): 389-392.

Kay, M. A., Manno, C. S., Ragni, M. V., Larson, P. J., Couto, L. B., McClelland, A., Glader, B., Chew, A. J., Tai, S. J., Herzog, R. W., Arruda, V., Johnson, F., Scallan, C., Skarsgard, E., Flake, A. W. &High, K. A. (2000). Evidence for gene transfer and expression of factor IX in haemophilia B patients treated with an AAV vector. *Nat Genet* 24(3): 257-261.

Kay, M. A., Rothenberg, S., Landen, C. N., Bellinger, D. A., Leland, F., Toman, C., Finegold, M., Thompson, A. R., Read, M. S., Brinkhous, K. M. &et al. (1993). In vivo gene therapy of hemophilia B: sustained partial correction in factor IX-deficient dogs. *Science* 262(5130): 117-119.

Kay, M. A. &Woo, S. L. (1994). Gene therapy for metabolic disorders. *Trends Genet* 10(7): 253-257.

Keravala, A., Lee, S., Thyagarajan, B., Olivares, E. C., Gabrovsky, V. E., Woodard, L. E. &Calos, M. P. (2009). Mutational derivatives of PhiC31 integrase with increased efficiency and specificity. *Mol Ther* 17(1): 112-120.

Ketterling, R. P., Vielhaber, E. &Sommer, S. S. (1994). The rates of G:C-->T:A and G:C-->C:G transversions at CpG dinucleotides in the human factor IX gene. *Am J Hum Genet* 54(5): 831-835.

Kim, I. H., Jozkowicz, A., Piedra, P. A., Oka, K. &Chan, L. (2001). Lifetime correction of genetic deficiency in mice with a single injection of helper-dependent adenoviral vector. *Proc Natl Acad Sci U S A* 98(23): 13282-13287.

Kochanek, S., Clemens, P. R., Mitani, K., Chen, H. H., Chan, S. &Caskey, C. T. (1996). A new adenoviral vector: Replacement of all viral coding sequences with 28 kb of DNA independently expressing both full-length dystrophin and beta-galactosidase. *Proc Natl Acad Sci U S A* 93(12): 5731-5736.

Kreppel, F., Biermann, V., Kochanek, S. &Schiedner, G. (2002). A DNA-based method to assay total and infectious particle contents and helper virus contamination in high-capacity adenoviral vector preparations. *Hum Gene Ther* 13(10): 1151-1156.

Kuhstoss, S. &Rao, R. N. (1991). Analysis of the integration function of the streptomycete bacteriophage phi C31. *J Mol Biol* 222(4): 897-908.

Kurachi S Fau - Furukawa, M., Furukawa M Fau - Salier, J. P., Salier Jp Fau - Wu, C. T., Wu Ct Fau - Wilson, E. J., Wilson Ej Fau - French, F. S., French Fs Fau - Kurachi, K. &Kurachi, K. (1995). Regulatory mechanism of human factor IX gene: protein binding at the Leyden-specific region. (0006-2960 (Print)).

Kustikova, O., Brugman, M. &Baum, C. (2010). The genomic risk of somatic gene therapy. *Semin Cancer Biol* 20(4): 269-278.

Kustikova, O., Fehse, B., Modlich, U., Yang, M., Dullmann, J., Kamino, K., von Neuhoff, N., Schlegelberger, B., Li, Z. &Baum, C. (2005). Clonal dominance of hematopoietic stem cells triggered by retroviral gene marking. *Science* 308(5725): 1171-1174.

Lai, Y., Yue, Y. &Duan, D. (2010). Evidence for the failure of adeno-associated virus serotype 5 to package a viral genome > or = 8.2 kb. *Mol Ther* 18(1): 75-79.

Lenaerts, L., Kelchtermans, H., Geboes, L., Matthys, P., Verbeken, E., De Clercq, E. &Naesens, L. (2008). Recovery of humoral immunity is critical for successful antiviral therapy in disseminated mouse adenovirus type 1 infection. *Antimicrob Agents Chemother* 52(4): 1462-1471.

Li, H., Malani, N., Hamilton, S. R., Schlachterman, A., Bussadori, G., Edmonson, S. E., Shah, R., Arruda, V. R., Mingozzi, F., Wright, J. F., Bushman, F. D. &High, K. A. (2011). Assessing the potential for AAV vector genotoxicity in a murine model. *Blood* 117(12): 3311-3319.

Li, Z., Dullmann, J., Schiedlmeier, B., Schmidt, M., von Kalle, C., Meyer, J., Forster, M., Stocking, C., Wahlers, A., Frank, O., Ostertag, W., Kuhlcke, K., Eckert, H. G., Fehse, B. &Baum, C. (2002). Murine leukemia induced by retroviral gene marking. *Science* 296(5567): 497.

Liesner, R., Zhang, W., Noske, N. &Ehrhardt, A. (2010). Critical amino acid residues within the phiC31 integrase DNA-binding domain affect recombination activities in mammalian cells. *Hum Gene Ther* 21(9): 1104-1118.

Lillicrap, D. (2008). Extending half-life in coagulation factors: where do we stand? *Thromb Res* 122 Suppl 4: S2-8.

Lillicrap, D., VandenDriessche, T. &High, K. (2006). Cellular and genetic therapies for haemophilia. *Haemophilia* 12 Suppl 3: 36-41.

Lin, C. N., Kao, C. Y., Miao, C. H., Hamaguchi, N., Wu, H. L., Shi, G. Y., Liu, Y. L., High, K. A. &Lin, S. W. (2010). Generation of a novel factor IX with augmented clotting activities in vitro and in vivo. *J Thromb Haemost* 8(8): 1773-1783.

Lin, H. F., Maeda, N., Smithies, O., Straight, D. L. &Stafford, D. W. (1997). A coagulation factor IX-deficient mouse model for human hemophilia B. *Blood* 90(10): 3962-3966.

Liras, A. &Olmedillas, S. (2009). Gene therapy for haemophilia...yes, but...with non-viral vectors? *Haemophilia* 15(3): 811-816.

Liu, G., Geurts, A. M., Yae, K., Srinivasan, A. R., Fahrenkrug, S. C., Largaespada, D. A., Takeda, J., Horie, K., Olson, W. K. &Hackett, P. B. (2005). Target-site preferences of Sleeping Beauty transposons. *J Mol Biol* 346(1): 161-173.

Liu, H., Jin, L., Koh, S. B., Atanasov, I., Schein, S., Wu, L. &Zhou, Z. H. (2010a). Atomic structure of human adenovirus by cryo-EM reveals interactions among protein networks. *Science* 329(5995): 1038-1043.

Liu, M. A. (2010). DNA vaccines: an historical perspective and view to the future. *Immunol Rev* 239(1600-065X (Electronic)): 62-84.

Liu, S., Ma, J., Wang, W., Zhang, M., Xin, Q., Peng, S., Li, R. &Zhu, H. (2010b). Mutational analysis of highly conserved residues in the phage phiC31 integrase reveals key amino acids necessary for the DNA recombination. *PLoS One* 5(1): e8863.

Lochmuller, H., Jani, A., Huard, J., Prescott, S., Simoneau, M., Massie, B., Karpati, G. &Acsadi, G. (1994). Emergence of early region 1-containing replication-competent adenovirus in stocks of replication-defective adenovirus recombinants (delta E1 + delta E3) during multiple passages in 293 cells. *Hum Gene Ther* 5(12): 1485-1491.

Lofqvist, T., Nilsson, I. M., Berntorp, E. &Pettersson, H. (1997). Haemophilia prophylaxis in young patients--a long-term follow-up. *J Intern Med* 241(5): 395-400.

Lombardo, A., Genovese, P., Beausejour, C. M., Colleoni, S., Lee, Y. L., Kim, K. A., Ando, D., Urnov, F. D., Galli, C., Gregory, P. D., Holmes, M. C. &Naldini, L. (2007). Gene editing in human stem cells using zinc finger nucleases and integrase-defective lentiviral vector delivery. *Nat Biotechnol* 25(11): 1298-1306.

Lorenzi, J. C., Trombone, A. P., Rocha, C. D., Almeida, L. P., Lousada, R. L., Malardo, T., Fontoura, I. C., Rossetti, R. A., Gembre, A. F., Silva, A. M., Silva, C. L. &Coelho-Castelo, A. A. (2010). Intranasal vaccination with messenger RNA as a new approach in gene therapy: use against tuberculosis. *BMC Biotechnol* 10: 77.

Losordo, D. W. &Dimmeler, S. (2004). Therapeutic angiogenesis and vasculogenesis for ischemic disease: part II: cell-based therapies. *Circulation* 109(22): 2692-2697.

Louie, J. K., Kajon, A. E., Holodniy, M., Guardia-LaBar, L., Lee, B., Petru, A. M., Hacker, J. K. &Schnurr, D. P. (2008). Severe pneumonia due to adenovirus serotype 14: a new respiratory threat? *Clin Infect Dis* 46(3): 421-425.

Lowenstein, P. R. &Castro, M. G. (2009). Uncertainty in the translation of preclinical experiments to clinical trials. Why do most phase III clinical trials fail? *Curr Gene Ther* 9(5): 368-374.

Lundstrom, K. (2003). Latest development in viral vectors for gene therapy. *Trends Biotechnol* 21(3): 117-122.

Luo, G., Ivics, Z., Izsvak, Z. &Bradley, A. (1998). Chromosomal transposition of a Tc1/mariner-like element in mouse embryonic stem cells. *Proc Natl Acad Sci U S A* 95(18): 10769-10773.

Lusher, J. M. (2000). Inhibitor antibodies to factor VIII and factor IX: management. *Semin Thromb Hemost* 26(2): 179-188.

Lusher, J. M., Arkin, S., Abildgaard, C. F. &Schwartz, R. S. (1993). Recombinant factor VIII for the treatment of previously untreated patients with hemophilia A. Safety, efficacy, and development of inhibitors. Kogenate Previously Untreated Patient Study Group. *N Engl J Med* 328(7): 453-459.

Maizel, J. V., Jr., White, D. O. &Scharff, M. D. (1968). The polypeptides of adenovirus. I. Evidence for multiple protein components in the virion and a comparison of types 2, 7A, and 12. *Virology* 36(1): 115-125.

Manco-Johnson, M. J., Abshire, T. C., Shapiro, A. D., Riske, B., Hacker, M. R., Kilcoyne, R., Ingram, J. D., Manco-Johnson, M. L., Funk, S., Jacobson, L., Valentino, L. A., Hoots, W. K., Buchanan, G. R., DiMichele, D., Recht, M., Brown, D., Leissinger, C., Bleak, S., Cohen, A., Mathew, P., Matsunaga, A., Medeiros, D., Nugent, D., Thomas, G. A., Thompson, A. A., McRedmond, K., Soucie, J. M., Austin, H. &Evatt, B. L. (2007). Prophylaxis versus episodic treatment to prevent joint disease in boys with severe hemophilia. *N Engl J Med* 357(6): 535-544.

Manno, C. S., Chew, A. J., Hutchison, S., Larson, P. J., Herzog, R. W., Arruda, V. R., Tai, S. J., Ragni, M. V., Thompson, A., Ozelo, M., Couto, L. B., Leonard, D. G., Johnson, F. A., McClelland, A., Scallan, C., Skarsgard, E., Flake, A. W., Kay, M. A., High, K. A. &Glader, B. (2003). AAV-mediated factor IX gene transfer to skeletal muscle in patients with severe hemophilia B. *Blood* 101(8): 2963-2972.

Manno, C. S., Pierce, G. F., Arruda, V. R., Glader, B., Ragni, M., Rasko, J. J., Ozelo, M. C., Hoots, K., Blatt, P., Konkle, B., Dake, M., Kaye, R., Razavi, M., Zajko, A., Zehnder, J., Rustagi, P. K., Nakai, H., Chew, A., Leonard, D., Wright, J. F., Lessard, R. R., Sommer, J. M., Tigges, M., Sabatino, D., Luk, A., Jiang, H., Mingozzi, F., Couto, L., Ertl, H. C., High, K. A. &Kay, M. A. (2006). Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response. *Nat Med* 12(3): 342-347.

Mannucci, P. M., Mendolicchio, L. &Gringeri, A. (2001). Use of prophylaxis to prevent complications of hemophilia. *Adv Exp Med Biol* 489: 59-64.

Mannucci, P. M. &Tuddenham, E. G. (2001). The hemophilias--from royal genes to gene therapy. *N Engl J Med* 344(23): 1773-1779.

Mates, L., Chuah, M. K., Belay, E., Jerchow, B., Manoj, N., Acosta-Sanchez, A., Grzela, D. P., Schmitt, A., Becker, K., Matrai, J., Ma, L., Samara-Kuko, E., Gysemans, C., Pryputniewicz, D., Miskey, C., Fletcher, B., VandenDriessche, T., Ivics, Z. &Izsvak, Z. (2009). Molecular evolution of a novel hyperactive Sleeping Beauty transposase enables robust stable gene transfer in vertebrates. *Nat Genet* 41(6): 753-761.

Matrai, J., Chuah, M. K. &VandenDriessche, T. (2010). Preclinical and clinical progress in hemophilia gene therapy. *Curr Opin Hematol* 17(5): 387-392.

Mayrhofer, P., Schleef, M. &Jechlinger, W. (2009). Use of minicircle plasmids for gene therapy. *Methods Mol Biol* 542: 87-104.

McConnell, M. J. &Imperiale, M. J. (2004). Biology of adenovirus and its use as a vector for gene therapy. *Hum Gene Ther* 15(11): 1022-1033.

McCormack, W. M., Jr., Seiler, M. P., Bertin, T. K., Ubhayakar, K., Palmer, D. J., Ng, P., Nichols, T. C. &Lee, B. (2006). Helper-dependent adenoviral gene therapy mediates long-term correction of the clotting defect in the canine hemophilia A model. *J Thromb Haemost* 4(6): 1218-1225.

McCormick, F. (2001). Cancer gene therapy: fringe or cutting edge? *Nat Rev Cancer* 1(2): 130-141.

McCown, T. J., Xiao, X., Li, J., Breese, G. R. &Samulski, R. J. (1996). Differential and persistent expression patterns of CNS gene transfer by an adeno-associated virus (AAV) vector. *Brain Res* 713(1-2): 99-107.

McKusick, V. A. (1970). Human genetics. *Annu Rev Genet* 4: 1-46.

Miller, D. G., Rutledge, E. A. &Russell, D. W. (2002). Chromosomal effects of adeno-associated virus vector integration. *Nat Genet* 30(2): 147-148.

Mitchell, R. S., Beitzel, B. F., Schroder, A. R., Shinn, P., Chen, H., Berry, C. C., Ecker, J. R. &Bushman, F. D. (2004). Retroviral DNA integration: ASLV, HIV, and MLV show distinct target site preferences. *PLoS Biol* 2(8): E234.

Mizuguchi, H. &Kay, M. A. (1999). A simple method for constructing E1- and E1/E4-deleted recombinant adenoviral vectors. *Hum Gene Ther* 10(12): 2013-2017.

Moldt, B., Miskey, C., Staunstrup, N. H., Gogol-Doring, A., Bak, R. O., Sharma, N., Mates, L., Izsvak, Z., Chen, W., Ivics, Z. &Mikkelsen, J. G. (2011). Comparative Genomic Integration Profiling of Sleeping Beauty Transposons Mobilized With High Efficacy From Integrase-defective Lentiviral Vectors in Primary Human Cells. *Mol Ther*.

Morral, N., McEvoy, R., Dong, H., Meseck, M., Altomonte, J., Thung, S. &Woo, S. L. (2002). Adenovirus-mediated expression of glucokinase in the liver as an adjuvant treatment for type 1 diabetes. *Hum Gene Ther* 13(13): 1561-1570.

Morral, N., Parks, R. J., Zhou, H., Langston, C., Schiedner, G., Quinones, J., Graham, F. L., Kochanek, S. &Beaudet, A. L. (1998). High doses of a helper-dependent adenoviral vector yield supraphysiological levels of alpha1-antitrypsin with negligible toxicity. *Hum Gene Ther* 9(18): 2709-2716.

Mount, J. D., Herzog, R. W., Tillson, D. M., Goodman, S. A., Robinson, N., McCleland, M. L., Bellinger, D., Nichols, T. C., Arruda, V. R., Lothrop, C. D., Jr. &High, K. A. (2002). Sustained phenotypic correction of hemophilia B dogs with a factor IX null mutation by liver-directed gene therapy. *Blood* 99(8): 2670-2676.

Murphy, S. L. &High, K. A. (2008). Gene therapy for haemophilia. *Br J Haematol* 140(5): 479-487.

Muruve, D. A., Barnes, M. J., Stillman, I. E. &Libermann, T. A. (1999). Adenoviral gene therapy leads to rapid induction of multiple chemokines and acute neutrophil-dependent hepatic injury in vivo. *Hum Gene Ther* 10(6): 965-976.

Nakai, H., Montini, E., Fuess, S., Storm, T. A., Grompe, M. &Kay, M. A. (2003). AAV serotype 2 vectors preferentially integrate into active genes in mice. *Nat Genet* 34(3): 297-302.

Nakai, H., Wu, X., Fuess, S., Storm, T. A., Munroe, D., Montini, E., Burgess, S. M., Grompe, M. &Kay, M. A. (2005). Large-scale molecular characterization of adeno-associated virus vector integration in mouse liver. *J Virol* 79(6): 3606-3614.

Nakai, H., Yant, S. R., Storm, T. A., Fuess, S., Meuse, L. &Kay, M. A. (2001). Extrachromosomal recombinant adeno-associated virus vector genomes are primarily responsible for stable liver transduction in vivo. *J Virol* 75(15): 6969-6976.

Nathwani, A. C., Davidoff, A. M., Hanawa, H., Hu, Y., Hoffer, F. A., Nikanorov, A., Slaughter, C., Ng, C. Y., Zhou, J., Lozier, J. N., Mandrell, T. D., Vanin, E. F. &Nienhuis, A. W. (2002). Sustained high-level expression of human factor IX (hFIX) after liver-targeted delivery of recombinant adeno-associated virus encoding the hFIX gene in rhesus macaques. *Blood* 100(5): 1662-1669.

Nathwani, A. C., Gray, J. T., McIntosh, J., Ng, C. Y., Zhou, J., Spence, Y., Cochrane, M., Gray, E., Tuddenham, E. G. &Davidoff, A. M. (2007). Safe and efficient transduction of the liver after peripheral vein infusion of self-complementary AAV vector results in stable therapeutic expression of human FIX in nonhuman primates. *Blood* 109(4): 1414-1421.

Nichols, T. C., Dillow, A. M., Franck, H. W., Merricks, E. P., Raymer, R. A., Bellinger, D. A., Arruda, V. R. &High, K. A. (2009). Protein replacement therapy and gene transfer in canine models of hemophilia A, hemophilia B, von willebrand disease, and factor VII deficiency. *ILAR J* 50(2): 144-167.

Niemeyer, G. P., Herzog, R. W., Mount, J., Arruda, V. R., Tillson, D. M., Hathcock, J., van Ginkel, F. W., High, K. A. &Lothrop, C. D., Jr. (2009). Long-term correction of inhibitor-prone hemophilia B dogs treated with liver-directed AAV2-mediated factor IX gene therapy. *Blood* 113(4): 797-806.

Nunes, F. A., Furth, E. E., Wilson, J. M. &Raper, S. E. (1999). Gene transfer into the liver of nonhuman primates with E1-deleted recombinant adenoviral vectors: safety of readministration. *Hum Gene Ther* 10(15): 2515-2526.

Oka, K., Pastore, L., Kim, I. H., Merched, A., Nomura, S., Lee, H. J., Merched-Sauvage, M., Arden-Riley, C., Lee, B., Finegold, M., Beaudet, A. &Chan, L. (2001). Long-term stable correction of low-density lipoprotein receptor-deficient mice with a helper-dependent adenoviral vector expressing the very low-density lipoprotein receptor. *Circulation* 103(9): 1274-1281.

Olson, P., Nelson, S. &Dornburg, R. (1994). Improved self-inactivating retroviral vectors derived from spleen necrosis virus. *J Virol* 68(11): 7060-7066.

Ott, M. G., 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, N. A., Copeland, N. G., Luthi, U., Hassan, M., Thrasher, A. J., 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.

P.M. Green, F. G., S.S. Sommer, M-C. Poon, M. Ludwig, R. Schwaab, P.H. Reitsma, M. Goossens, A. Yoshioka, M.S. Figueiredo, G. Tagariello and G.G. Brownlee (2004). The Haemophilia B Mutation Database – version 13.

Pagon RA, B. T., Dolan CR, Stephens K, Cheryl L Brower and Arthur R Thompson (2011). Hemophilia A, hemophiliaB. *GeneReviews* (0140-6736 (Print)).

Palmer, D. &Ng, P. (2003). Improved system for helper-dependent adenoviral vector production. *Mol Ther* 8(5): 846-852.

Palmer, D. J. &Ng, P. (2005). Helper-dependent adenoviral vectors for gene therapy. *Hum Gene Ther* 16(1): 1-16.

Palmer, D. J. &Ng, P. (2008). Methods for the production of helper-dependent adenoviral vectors. *Methods Mol Biol* 433: 33-53.

Parks, R. J., Bramson, J. L., Wan, Y., Addison, C. L. &Graham, F. L. (1999). Effects of stuffer DNA on transgene expression from helper-dependent adenovirus vectors. *J Virol* 73(10): 8027-8034.

Parks, R. J., Chen, L., Anton, M., Sankar, U., Rudnicki, M. A. &Graham, F. L. (1996). A helper-dependent adenovirus vector system: removal of helper virus by Cre-mediated excision of the viral packaging signal. *Proc Natl Acad Sci U S A* 93(24): 13565-13570.

Parks, R. J. &Graham, F. L. (1997). A helper-dependent system for adenovirus vector production helps define a lower limit for efficient DNA packaging. *J Virol* 71(4): 3293-3298.

Paruzynski, A., Arens, A., Gabriel, R., Bartholomae, C. C., Scholz, S., Wang, W., Wolf, S., Glimm, H., Schmidt, M. &von Kalle, C. (2010). Genome-wide high-throughput integrome analyses by nrLAM-PCR and next-generation sequencing. *Nat Protoc* 5(8): 1379-1395.

Perez, E. E., Wang, J., Miller, J. C., Jouvenot, Y., Kim, K. A., Liu, O., Wang, N., Lee, G., Bartsevich, V. V., Lee, Y. L., Guschin, D. Y., Rupniewski, I., Waite, A. J., Carpenito, C., Carroll, R. G., Orange, J. S., Urnov, F. D., Rebar, E. J., Ando, D., Gregory, P. D., Riley, J. L., Holmes, M. C. &June, C. H. (2008). Establishment of HIV-1 resistance in CD4+ T cells by genome editing using zinc-finger nucleases. *Nat Biotechnol* 26(7): 808-816.

Peters, R. T., Low, S. C., Kamphaus, G. D., Dumont, J. A., Amari, J. V., Lu, Q., Zarbis-Papastoitsis, G., Reidy, T. J., Merricks, E. P., Nichols, T. C. &Bitonti, A. J. (2010). Prolonged activity of factor IX as a monomeric Fc fusion protein. *Blood* 115(10): 2057-2064.

Peterson, E. B., Mastrangelo, M. A., Federoff, H. J. &Bowers, W. J. (2007). Neuronal specificity of HSV/sleeping beauty amplicon transduction in utero is driven primarily by tropism and cell type composition. *Mol Ther* 15(10): 1848-1855.

Petrus, I., Chuah, M. &VandenDriessche, T. (2010). Gene therapy strategies for hemophilia: benefits versus risks. *J Gene Med* 12(10): 797-809.

Pien, G. C., Basner-Tschakarjan, E., Hui, D. J., Mentlik, A. N., Finn, J. D., Hasbrouck, N. C., Zhou, S., Murphy, S. L., Maus, M. V., Mingozzi, F., Orange, J. S. &High, K. A. (2009). Capsid antigen presentation flags human hepatocytes for destruction after transduction by adeno-associated viral vectors. *J Clin Invest* 119(6): 1688-1695.

Pierce, G. F., Lillicrap, D., Pipe, S. W. &Vandendriessche, T. (2007). Gene therapy, bioengineered clotting factors and novel technologies for hemophilia treatment. *J Thromb Haemost* 5(5): 901-906.

Pipe, S. W. (2010). Hemophilia: new protein therapeutics. *Hematology Am Soc Hematol Educ Program* 2010: 203-209.

Puntel, M., Curtin, J. F., Zirger, J. M., Muhammad, A. K., Xiong, W., Liu, C., Hu, J., Kroeger, K. M., Czer, P., Sciascia, S., Mondkar, S., Lowenstein, P. R. &Castro, M. G. (2006). Quantification of high-capacity helper-dependent adenoviral vector genomes in vitro and in vivo, using quantitative TaqMan real-time polymerase chain reaction. *Hum Gene Ther* 17(5): 531-544.

Purrello, M., Alhadeff, B., Esposito, D., Szabo, P., Rocchi, M., Truett, M., Masiarz, F. &Siniscalco, M. (1985). The human genes for hemophilia A and hemophilia B flank the X chromosome fragile site at Xq27.3. *EMBO J* 4(3): 725-729.

Raper, S. E., Chirmule, N., Lee, F. S., Wivel, N. A., Bagg, A., Gao, G. P., Wilson, J. M. &Batshaw, M. L. (2003). Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer. *Mol Genet Metab* 80(1-2): 148-158.

Recchia, A., Parks, R. J., Lamartina, S., Toniatti, C., Pieroni, L., Palombo, F., Ciliberto, G., Graham, F. L., Cortese, R., La Monica, N. &Colloca, S. (1999). Site-specific integration mediated by a hybrid adenovirus/adeno-associated virus vector. *Proc Natl Acad Sci U S A* 96(6): 2615-2620.

Recchia, A., Perani, L., Sartori, D., Olgiati, C. &Mavilio, F. (2004). Site-specific integration of functional transgenes into the human genome by adeno/AAV hybrid vectors. *Mol Ther* 10(4): 660-670.

Reddy, V. S., Natchiar, S. K., Stewart, P. L. &Nemerow, G. R. (2010). Crystal structure of human adenovirus at 3.5 A resolution. *Science* 329(5995): 1071-1075.

Robin, N. H. (2008). Treatment for genetic diseases. *Curr Opin Pediatr* 20(1531-698X (Electronic)): 625-627.

Roosendaal, G. &Lafeber, F. (2007). Prophylactic treatment for prevention of joint disease in hemophilia--cost versus benefit. *N Engl J Med* 357(6): 603-605.

Rosenberg, S. A., Aebersold, P., Cornetta, K., Kasid, A., Morgan, R. A., Moen, R., Karson, E. M., Lotze, M. T., Yang, J. C., Topalian, S. L. &et al. (1990). Gene transfer into humans--immunotherapy of patients with advanced melanoma, using tumor-infiltrating lymphocytes modified by retroviral gene transduction. *N Engl J Med* 323(9): 570-578.

Roth, D. A., Kessler, C. M., Pasi, K. J., Rup, B., Courter, S. G. &Tubridy, K. L. (2001a). Human recombinant factor IX: safety and efficacy studies in hemophilia B patients previously treated with plasma-derived factor IX concentrates. *Blood* 98(13): 3600-3606.

Roth, D. A., Tawa, N. E., Jr., O'Brien, J. M., Treco, D. A. &Selden, R. F. (2001b). Nonviral transfer of the gene encoding coagulation factor VIII in patients with severe hemophilia A. *N Engl J Med* 344(23): 1735-1742.

Rowe, W. P. (1953). Propagation of group A Coxsackie viruses in denervated adult mouse muscle. *Science* 117(3052): 710.

Rowley, P. A. &Smith, M. C. (2008). Role of the N-terminal domain of phiC31 integrase in attB-attP synapsis. *J Bacteriol* 190(20): 6918-6921.

Russell, W. C. (2000). Update on adenovirus and its vectors. *J Gen Virol* 81(0022-1317 (Print)): 2573-2604.

Rutledge, E. A. &Russell, D. W. (1997). Adeno-associated virus vector integration junctions. *J Virol* 71(11): 8429-8436.

Sam, M. R., Zomorodipour, A., Shokrgozar, M. A., Ataei, F., Haddad-Mashadrizeh, A. &Amanzadeh, A. (2010). Enhancement of the human factor IX expression, mediated by an intron derived fragment from the rat aldolase B gene in cultured hepatoma cells. *Biotechnol Lett* 32(10): 1385-1392.

Sandig, V., Youil, R., Bett, A. J., Franlin, L. L., Oshima, M., Maione, D., Wang, F., Metzker, M. L., Savino, R. &Caskey, C. T. (2000). Optimization of the helper-dependent adenovirus system for production and potency in vivo. *Proc Natl Acad Sci U S A* 97(3): 1002-1007.

Sauer, B. (2002). Cre/lox: one more step in the taming of the genome. *Endocrine* 19(3): 221-228.

Schiedner, G., Hertel, S., Johnston, M., Dries, V., van Rooijen, N. &Kochanek, S. (2003). Selective depletion or blockade of Kupffer cells leads to enhanced and prolonged hepatic transgene expression using high-capacity adenoviral vectors. *Mol Ther* 7(1): 35-43.

Schmidt, M., Schwarzwaelder, K., Bartholomae, C., Zaoui, K., Ball, C., Pilz, I., Braun, S., Glimm, H. &von Kalle, C. (2007). High-resolution insertion-site analysis by linear amplification-mediated PCR (LAM-PCR). *Nat Methods* 4(12): 1051-1057.

Schmidt, M., Schwarzwaelder, K., Bartholomae, C. C., Glimm, H. &von Kalle, C. (2009). Detection of retroviral integration sites by linear amplification-mediated PCR and tracking of individual integration clones in different samples. *Methods Mol Biol* 506: 363-372.

Schmidt, M., Zickler, P., Hoffmann, G., Haas, S., Wissler, M., Muessig, A., Tisdale, J. F., Kuramoto, K., Andrews, R. G., Wu, T., Kiem, H. P., Dunbar, C. E. &von Kalle, C. (2002). Polyclonal long-term repopulating stem cell clones in a primate model. *Blood* 100(8): 2737-2743.

Schnell, M. A., Zhang, Y., Tazelaar, J., Gao, G. P., Yu, Q. C., Qian, R., Chen, S. J., Varnavski, A. N., LeClair, C., Raper, S. E. &Wilson, J. M. (2001). Activation of innate immunity in nonhuman primates following intraportal administration of adenoviral vectors. *Mol Ther* 3(5 Pt 1): 708-722.

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

Schuettrumpf, J., Herzog, R. W., Schlachterman, A., Kaufhold, A., Stafford, D. W. &Arruda, V. R. (2005). Factor IX variants improve gene therapy efficacy for hemophilia B. *Blood* 105(6): 2316-2323.

Schultz, B. R. &Chamberlain, J. S. (2008). Recombinant adeno-associated virus transduction and integration. *Mol Ther* 16(7): 1189-1199.

Schwartz, R. S., Abildgaard, C. F., Aledort, L. M., Arkin, S., Bloom, A. L., Brackmann, H. H., Brettler, D. B., Fukui, H., Hilgartner, M. W., Inwood, M. J. &et al. (1990). Human recombinant DNA-derived antihemophilic factor (factor VIII) in the treatment of hemophilia A. recombinant Factor VIII Study Group. *N Engl J Med* 323(26): 1800-1805.

Shirakawa, T. (2009). Clinical trial design for adenoviral gene therapy products. *Drug News Perspect* 22(3): 140-145.

Sichler, K., Kopetzki, E., Huber, R., Bode, W., Hopfner, K. P. &Brandstetter, H. (2003). Physiological fIXa activation involves a cooperative conformational rearrangement of the 99-loop. *J Biol Chem* 278(6): 4121-4126.

Simioni, P., Tormene, D., Tognin, G., Gavasso, S., Bulato, C., Iacobelli, N. P., Finn, J. D., Spiezia, L., Radu, C. &Arruda, V. R. (2009). X-linked thrombophilia with a mutant factor IX (factor IX Padua). *N Engl J Med* 361(17): 1671-1675.

Smith, M. L., Avanigadda, L. N., Liddell, P. W., Kenwright, K. M. &Howe, M. M. (2010). Identification of the J and K genes in the bacteriophage Mu genome sequence. *FEMS Microbiol Lett* 313(1): 29-32.

Snyder, R. O., Miao, C., Meuse, L., Tubb, J., Donahue, B. A., Lin, H. F., Stafford, D. W., Patel, S., Thompson, A. R., Nichols, T., Read, M. S., Bellinger, D. A., Brinkhous, K. M. &Kay, M. A. (1999). Correction of hemophilia B in canine and murine models using recombinant adeno-associated viral vectors. *Nat Med* 5(1): 64-70.

Snyder, R. O., Miao, C. H., Patijn, G. A., Spratt, S. K., Danos, O., Nagy, D., Gown, A. M., Winther, B., Meuse, L., Cohen, L. K., Thompson, A. R. &Kay, M. A. (1997). Persistent and therapeutic concentrations of human factor IX in mice after hepatic gene transfer of recombinant AAV vectors. *Nat Genet* 16(3): 270-276.

Soifer, H., Higo, C., Kazazian, H. H., Jr., Moran, J. V., Mitani, K. &Kasahara, N. (2001). Stable integration of transgenes delivered by a retrotransposon-adenovirus hybrid vector. *Hum Gene Ther* 12(11): 1417-1428.

Soifer, H., Higo, C., Logg, C. R., Jih, L. J., Shichinohe, T., Harboe-Schmidt, E., Mitani, K. &Kasahara, N. (2002). A novel, helper-dependent, adenovirus-retrovirus hybrid vector: stable transduction by a two-stage mechanism. *Mol Ther* 5(5 Pt 1): 599-608.

Sorg, T. &Methali, M. (1997). Gene therapy for AIDS. *Transfus Sci* 18(2): 277-289.

Stamm, C., Nasseri, B., Drews, T. &Hetzer, R. (2008). Cardiac cell therapy: a realistic concept for elderly patients? *Exp Gerontol* 43(7): 679-690.

Stanford, M. M., Bell, J. C. &Vaha-Koskela, M. J. (2010). Novel oncolytic viruses: riding high on the next wave? *Cytokine Growth Factor Rev* 21(2-3): 177-183.

Staunstrup, N. H., Moldt, B., Mates, L., Villesen, P., Jakobsen, M., Ivics, Z., Izsvak, Z. &Mikkelsen, J. G. (2009). Hybrid lentivirus-transposon vectors with a random integration profile in human cells. *Mol Ther* 17(7): 1205-1214.

Stephen, S. L., Montini, E., Sivanandam, V. G., Al-Dhalimy, M., Kestler, H. A., Finegold, M., Grompe, M. &Kochanek, S. (2010). Chromosomal integration of adenoviral vector DNA in vivo. *J Virol* 84(19): 9987-9994.

Stephen, S. L., Sivanandam, V. G. &Kochanek, S. (2008). Homologous and heterologous recombination between adenovirus vector DNA and chromosomal DNA. *J Gene Med* 10(11): 1176-1189.

Strachan, G., Williams, S., Moyle, S. P., Harris, W. J. &Porter, A. J. (1999). Reduced toxicity of expression, in Escherichia coli, of antipollutant antibody fragments and their use as sensitive diagnostic molecules. *J Appl Microbiol* 87(3): 410-417.

Suzuki, M., Sugimoto, K., Tanaka, J., Tameda, M., Inagaki, Y., Kusagawa, S., Nojiri, K., Beppu, T., Yoneda, K., Yamamoto, N., Ito, M., Yoneda, M., Uchida, K., Takase, K. &Shiraki, K. (2010). Up-regulation of glypican-3 in human hepatocellular carcinoma. *Anticancer Res* 30(12): 5055-5061.

Tao, N., Gao, G. P., Parr, M., Johnston, J., Baradet, T., Wilson, J. M., Barsoum, J. &Fawell, S. E. (2001). Sequestration of adenoviral vector by Kupffer cells leads to a nonlinear dose response of transduction in liver. *Mol Ther* 3(1): 28-35.

Tatsumi, K., Ohashi, K., Kataoka, M., Tateno, C., Shibata, M., Naka, H., Shima, M., Hisanaga, M., Kanehiro, H., Okano, T., Yoshizato, K., Nakajima, Y. &Yoshioka, A. (2008). Successful in vivo propagation of factor IX-producing hepatocytes in mice: potential for cell-based therapy in haemophilia B. *Thromb Haemost* 99(5): 883-891.

Themis, M., Waddington, S. N., Schmidt, M., von Kalle, C., Wang, Y., Al-Allaf, F., Gregory, L. G., Nivsarkar, M., Holder, M. V., Buckley, S. M., Dighe, N., Ruthe, A. T., Mistry, A., Bigger, B., Rahim, A., Nguyen, T. H., Trono, D., Thrasher, A. J. &Coutelle, C. (2005). Oncogenesis following delivery of a nonprimate lentiviral gene therapy vector to fetal and neonatal mice. *Mol Ther* 12(4): 763-771.

Thomas, C. E., Ehrhardt, A. &Kay, M. A. (2003). Progress and problems with the use of viral vectors for gene therapy. *Nat Rev Genet* 4(5): 346-358.

Toietta, G., Mane, V. P., Norona, W. S., Finegold, M. J., Ng, P., McDonagh, A. F., Beaudet, A. L. &Lee, B. (2005). Lifelong elimination of hyperbilirubinemia in the Gunn rat with a single injection of helper-dependent adenoviral vector. *Proc Natl Acad Sci U S A* 102(11): 3930-3935.

Toth, K., Dhar, D. &Wold, W. S. (2010). Oncolytic (replication-competent) adenoviruses as anticancer agents. *Expert Opin Biol Ther* 10(3): 353-368.

Umana, P., Gerdes, C. A., Stone, D., Davis, J. R., Ward, D., Castro, M. G. &Lowenstein, P. R. (2001). Efficient FLPe recombinase enables scalable production of helper-dependent adenoviral vectors with negligible helper-virus contamination. *Nat Biotechnol* 19(6): 582-585.

Urnov, F. D., Rebar, E. J., Holmes, M. C., Zhang, H. S. &Gregory, P. D. (2010). Genome editing with engineered zinc finger nucleases. *Nat Rev Genet* 11(9): 636-646.

van den Berg, H. M., De Groot, P. H. &Fischer, K. (2007). Phenotypic heterogeneity in severe hemophilia. *J Thromb Haemost* 5 Suppl 1: 151-156.

Van Den Wollenberg DJ, Van Den Hengel SK, Dautzenberg IJ, Kranenburg O, Hoeben RC. (2009). Modification of mammalian reoviruses for use as oncolytic agents. *Expert Opin Biol Ther* 9(1744-7682 (Electronic)): 1509-1520.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A. &Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 3(7): RESEARCH0034.

Vink, C. A., Gaspar, H. B., Gabriel, R., Schmidt, M., McIvor, R. S., Thrasher, A. J. &Qasim, W. (2009). Sleeping beauty transposition from nonintegrating lentivirus. *Mol Ther* 17(7): 1197-1204.

Walter, N. G. (1995). Modelling viral evolution in vitro using exo- Klenow polymerase: continuous selection of strand displacement amplified DNA that binds an oligodeoxynucleotide to form a triple-helix. *J Mol Biol* 254(0022-2836 (Print)): 856-868.

Walther, W. &Stein, U. (2000). Viral vectors for gene transfer: a review of their use in the treatment of human diseases. *Drugs* 60(2): 249-271.

Wang, H. &Lieber, A. (2006). A helper-dependent capsid-modified adenovirus vector expressing adeno-associated virus rep78 mediates site-specific integration of a 27-kilobase transgene cassette. *J Virol* 80(23): 11699-11709.

Wanisch, K. &Yanez-Munoz, R. J. (2009). Integration-deficient lentiviral vectors: a slow coming of age. *Mol Ther* 17(8): 1316-1332.

Weber, L. W., Boll, M. &Stampfl, A. (2003). Hepatotoxicity and mechanism of action of haloalkanes: carbon tetrachloride as a toxicological model. *Crit Rev Toxicol* 33(2): 105-136.

White, G. C., 2nd, Beebe, A. &Nielsen, B. (1997). Recombinant factor IX. *Thromb Haemost* 78(1): 261-265.

Wiley, J. M. (2011).Gene Therapy Clinical Trials Worldwide. Journal of Gene Medicine.

Wilson, D. R. (2002). Viral-mediated gene transfer for cancer treatment. *Curr Pharm Biotechnol* 3(2): 151-164.

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

Wu, Z., Asokan, A. &Samulski, R. J. (2006). Adeno-associated virus serotypes: vector toolkit for human gene therapy. *Mol Ther* 14(3): 316-327.

Xiao, X., Li, J. &Samulski, R. J. (1996). Efficient long-term gene transfer into muscle tissue of immunocompetent mice by adeno-associated virus vector. *J Virol* 70(11): 8098-8108.

Xu, L., Gao, C., Sands, M. S., Cai, S. R., Nichols, T. C., Bellinger, D. A., Raymer, R. A., McCorquodale, S. &Ponder, K. P. (2003). Neonatal or hepatocyte growth factor-potentiated adult gene therapy with a retroviral vector results in therapeutic levels of canine factor IX for hemophilia B. *Blood* 101(10): 3924-3932.

Yang, Y., Ertl, H. C. &Wilson, J. M. (1994). MHC class I-restricted cytotoxic T lymphocytes to viral antigens destroy hepatocytes in mice infected with E1-deleted recombinant adenoviruses. *Immunity* 1(5): 433-442.

Yang, Y., Li, Q., Ertl, H. C. &Wilson, J. M. (1995). Cellular and humoral immune responses to viral antigens create barriers to lung-directed gene therapy with recombinant adenoviruses. *J Virol* 69(4): 2004-2015.

Yant, S. R., Ehrhardt, A., Mikkelsen, J. G., Meuse, L., Pham, T. &Kay, M. A. (2002). Transposition from a gutless adeno-transposon vector stabilizes transgene expression in vivo. *Nat Biotechnol* 20(10): 999-1005.

Yant, S. R., Huang, Y., Akache, B. &Kay, M. A. (2007). Site-directed transposon integration in human cells. *Nucleic Acids Res* 35(7): e50.

Yant, S. R. &Kay, M. A. (2003). Nonhomologous-end-joining factors regulate DNA repair fidelity during Sleeping Beauty element transposition in mammalian cells. *Mol Cell Biol* 23(23): 8505-8518.

Yant, S. R., Meuse, L., Chiu, W., Ivics, Z., Izsvak, Z. &Kay, M. A. (2000). Somatic integration and long-term transgene expression in normal and haemophilic mice using a DNA transposon system. *Nat Genet* 25(1): 35-41.

Yant, S. R., Park, J., Huang, Y., Mikkelsen, J. G. &Kay, M. A. (2004). Mutational analysis of the N-terminal DNA-binding domain of sleeping beauty transposase: critical residues for DNA binding and hyperactivity in mammalian cells. *Mol Cell Biol* 24(20): 9239-9247.

Yant, S. R., Wu, X., Huang, Y., Garrison, B., Burgess, S. M. &Kay, M. A. (2005). High-resolution genome-wide mapping of transposon integration in mammals. *Mol Cell Biol* 25(6): 2085-2094.

Young, L. S., Searle, P. F., Onion, D. &Mautner, V. (2006). Viral gene therapy strategies: from basic science to clinical application. *J Pathol* 208(2): 299-318.

Yu, S. F., von Ruden, T., Kantoff, P. W., Garber, C., Seiberg, M., Ruther, U., Anderson, W. F., Wagner, E. F. &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.

Zang, R. Y., Shi, D. R., Lu, H. J., Cai, S. M., Lu, D. R., Zhang, Y. J. &Qin, H. L. (2001). Adenovirus 5 E1a-mediated gene therapy for human ovarian cancer cells in vitro and in vivo. *Int J Gynecol Cancer* 11(1): 18-23.

Zayed, H., Izsvak, Z., Walisko, O. &Ivics, Z. (2004). Development of hyperactive sleeping beauty transposon vectors by mutational analysis. *Mol Ther* 9(2): 292-304.

Zittermann, S. I., Capurro, M. I., Shi, W. &Filmus, J. (2010). Soluble glypican 3 inhibits the growth of hepatocellular carcinoma in vitro and in vivo. *Int J Cancer* 126(6): 1291-1301.

Zufferey, R., Dull, T., Mandel, R. J., 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.

Parts of this study were presented at the following conferences and published or prepared for publication in the following journals:

Conferences:

October 2010	17th Annual Meeting from the German Society for Gene Therapy Munich, Germany
May 2010	13th Annual Meeting from the American Society of Gene & Cell Therapy (ASGCT) Washington, DC, USA
November 2009	The Combined Meeting of: XVIIth Annual Congress of the European Society of Gene and Cell Therapy (ESGCT), 4th Annual Congress of the German Society for Stem Cell Research (GSZ), 16th Annual meeting of the German Society for Gene Therapy (DG-GT), Hannove, Germany

Publications:

Martin Häusl, **Wenli Zhang**, Nadine Müther, Christina Rauschhuber, H. W. Franck, Timothy C. Nichols, Mark A. Kay and Anja Ehrhardt (2010). A hyperactive transposase system enables persistent phenotypic correction in large animals. *Molecular Therapy*.

Raphael Liesner*, **Wenli Zhang***, Nadja Noske*, and Anja Ehrhardt (2010). Critical amino acid residues within the PhiC31 integrase DNA binding domain affect recombination activities in mammalian cells. (* these authors contributed equally to this work). *Human Gene Therapy*.

Martin Häusl, **Wenli Zhang**, Nadine Müther, Voigtländer Richard, Christina Rauschhuber and Anja Ehrhardt (2011) Development of adenovirus hybrid vectors for Sleeping Beauty transposition in large mammals. *Current Gene Therapy.*

Wenli Zhang, Martin Häusl and Anja Ehrhardt. Novel approach for mapping of transposon integration sites after transposition from an adenoviral hybrid vector. (Manuscript in preparation)

Acknowledgements

First of all, I would like to sincerely thank my supervisor and doctor mother Professor Dr. Anja Ehrhardt, who gave me the chance to study and work in her laboratory. I can never overstate my gratitude to Anja. With her encouragement, guidance, and great efforts I have been growing up from layman to a ‘clergyman’ in the last four years. Her clear and simple explain of research concepts always help me to develop my own understanding and new ideas of the project. Throughout my thesis-writing period, she provided so many encouragements, sound advice, careful correction, and lots of good ideas. I would have been lost without her!

It is a pleasure to thank my laboratory members, Tina, Nadja, Martin, Ricki, and Manisch, as well as the people who used to work with me, Raphi, Nicki, Lorenz, Ines, Nadine and Nina, thanks for all your help in my PhD work, especially Nadja and Lorenz for carefully reading through and nice suggestion of my thesis. Also thank Tina a lot for help me with the German Summary and Martin for the many nice technique discussions. It was so many funs to work with you. I will never forget, my first Thursday seminar, my first Mass of Bier, my first Cocktail---

Thanks Dr. Wo, Dr. Mü, Dr. No, Dr. Rau, Dr. Ra, Dr. Ja, Dr. Hau---

I am grateful to the China Scholarship Council (CSC), who gave me the chance and supports me to study abroad.

Many thanks to the international office of LMU, Dr, Hadesback, Ms. Esnof, Ms. Schmidt, Meng Lingqi and Qiqi, who organized many nice education and retreat programs and helped us so much in our beginning time here!

Importantly, it is my greatest luck to thank Laolunsi, 友谊之舟; I will never forget the most beautiful time we had. I also want to thank Weihua, 老秦, for all the helps from the first Power Adaptor---

Last but not least, I owe my deepest gratitude to my husband Xuehui, 老邢, thank you for all your support, trust and patient, thank you for your accompany in Germany.