A novel vector for the gene therapy of the Wiskott-Aldrich syndrome

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Man cannot discover new oceans

unless he has the courage to lose sight of the shores.

André Gide (1869-1951)
The Nobel Price in Literature in 1947

I. SUMMARY

Gene therapy has proven as a very successful therapeutic approach to treat primary immunodeficiencies. Indeed, previous clinical hematopoietic gene therapy studies have reported sustained or temporary correction of lymphocytes (X-linked severe combined immunedeficiency disorder (XSCID), adrenoleukodystrophy (ALD)), myeloid cells (chronic granulomatous disease (CGD), ALD) and red blood cells (thalassemia). The clinical trial for the Wiskott-Aldrich Syndrome (WAS) that is currently ongoing at the Hannover Medical School (Germany) has recently reported the correction of all affected cell lineages of the hematopoietic system in the first treated patients. However, the occurrence of leukemia-like episodes in some XSCID patients as well as a premyelodysplastic syndrome in CGD patients treated with gene therapy has raised concerns about the drawbacks of this strategy, including the risk of insertional mutagenesis, thus reinforcing the need of developing safer vectors for gene transfer into HSC. To minimize the risk of genotoxicity while providing expression of the therapeutical transgene at physiological levels, we have developed a novel SIN-γ-retroviral vector with a split-packaging design, high titer production in human 293T cells and the potential of pseudotyping for important target populations, such as murine and human hematopoietic stem cells. In this newly designed vector, the wild-type *WASP* cDNA has been replaced by a novel codon optimized sequence, which allows to overcome the "weakness" of EFS, a physiologic promoter, thus obtaining similar mRNA level and transgene expression as with SFFV, a stronger promoter of viral origin. Importantly, with this vector it is possible to reconstitute *in vitro* the aberrant cytoskeleton of the myeloid progeny of hematopoietic stem cells of WAS patients, achieving the same efficiency as the earlier vector used in the ongoing WAS clinical trial in Hannover. This demonstrates in a clinically relevant scenario, that the novel vector presented here could be an effective tool for gene therapy, while circumventing the risk of insertional mutagenesis intrinsic to all transfer vectors used in clinical trials so far.

Key words: SIN-γ-retrovirus, codon optimized *WASP*, gene therapy, Wiskott-Aldrich syndrome.

II. ZUSAMMENFASSUNG

Die Gentherapie hat sich als ein sehr erfolgsversprechender Therapieansatz für primäre Immundefekte erwiesen. Es gibt verschiedene Beispiele für erfolgreiche klinische Gentherapiestudien im blutbildenden System. So konnte man eine komplette oder temporäre genetische Korrektur von Lymphozyten (X-linked severe combined immunedeficiency disorder (XSCID), adrenoleukodystrophy (ALD)), myeloischen Zellen (chronic granulomatous disease (CGD), ALD) und Erythrozyten (Thalassämie) erzielen. Die klinische Gentherapiestudie für das Wiskott-Aldrich Syndrom (WAS) an der Medizinischen Hochschule Hannover (Deutschland) zeigte die genetische Korrektur aller betroffenen Blutlinien in den ersten behandelten Patienten. Trotz dieser Erfolge wurden auch Nebenwirkungen beobachtet. So kam es in einigen gentherapierten XSCID Patienten zu Leukämie-ähnlichen Episoden und auch in der CGD Studie zu prämyelodysplastischen Syndromen. Diese Rückschläge und die damit verbundenen Bedenken im Kontext der sog. Insertionsmutagenese unterstreichen die Notwendigkeit für die Entwicklung sicherer Vektoren für die somatische Gentherapie. Um das Risiko der Genotoxizität zu minimieren – möglichst unter Beibehaltung der Expression des therapeutischen Transgens auf ein physiologisches Niveau -, wurde in dieser Arbeit ein neuer SIN-γ-retroviraler Vektor mit „split-packaging“ Design entwickelt. Dieser zeigte hohe Titer in humanen 293T-Produzenten-Zellen und konnte mit zahlreichen Hüllproteinen pseudotypisiert werden, um so wichtige Zielpopulationen, wie z.B. murine oder humane hämatopoetische Stammzellen, anzusteuern. In diesem neu entwickelten Vektor wurde die Wild-typ *WASP* cDNA gegen eine neue kodonoptimierte Form und bessere exprimierbare WAS Sequenz ausgetauscht, um so die Inkorporation von schwächeren zellulären Promotoren zu erlauben. Der Elongationsfaktor 1α „short“ (EFS) ist so ein zellulärer Promotor. Zusammen mit der Kodonoptimierung können so Expressionslevel im Bereich starker viraler Promotoren erreicht werden. Mit diesem neuen möglicherweise sicheren Vektor war es nun möglich den Phänotyp der WAS Erkrankung, das aberrante Zytoskelett, in myeloischen Vorläuferzellen von WAS Patienten *in vitro* zu rekonstruieren. Perspektivisch ist dieser Vektor ein vielversprechender Kandidat für zukünftige WAS-Gentherapiestudien, indem er bei

vergleichbarer Effizienz möglicherweise ein geringeres Risiko der insertionalen Mutagenese aufweist im Vergleich zu allen bis jetzt in der Klinik eingesetzten Vektoren.

Schlagworte: SIN-γ-Retrovirus, codon-optimiertes *WASP*, Gentherapie, Wiskott-Aldrich Syndrom.

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V. ABBREVIATIONS

ºC	Celsius degree
%	percentage
ul	microliter
ug	microgram
Δ	deletion
γc	common gamma chain
Ab	antibody
ADA	adenosine deaminase
Ag	antigen
AIDS	acquired immunodeficiency syndrome
ALD	adrenoleukodystrophy
Arp2/3	actin-related protein-2/3
BIV	bovine immunodeficiency virus
BMI1	*BMI1* polycomb ring finger oncogene
BMT	bone marrow transplantation
BSA	bovine serum albumin
CAI	codon adaptation index
CCND2	cyclin D2
CD	cluster of differentiation
Cdc42-GTP	cell division control protein 42-GTP
cDNA	complementary deoxyribonucleic acid
GBD	guanosine-triphosphatase- binding domain
CGD	chronic granulomatous disease
cGy	centiGray
CO_2	carbon dioxide
cPPT	central polypurine tract
Cy5	cyanine 5
DAPI	4´,6-diamidino-2-phenylidole
DC	dendritic cell
ddH_2O	bidistilled water
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
dNTPs	deoxynucleotide triphosphates
DMEM	Dulbecco´s modified Eagle medium
DMSO	dimethyl sulfoxide
EDTA	ethylenediamine-tetraacetic acid
EIAV	equine infectious anemia virus
env	envelope
EVI1	ecotropic viral integration site 1
F-actin	filament-actin
FACS	fluorescence activated cell sorting
FCS	foetal calf serum

FITC	fluoresceine isothiocyanate
FIV	feline immunodeficiency virus
Flt-3L	FMS-like tyrosine kinase 3 ligand
gag	group antigens
GALV	gibbon ape lymphosarcoma virus
GAPDH	glyceraldehyde 3- phophate dehydrogenase
G-CSF	granulocyte-colony stimulating factor
GeoM	geometrical mean intensity
GM-CSF	granulocyte macrophage-colony stimulating factor
GpC	cytosin-phosphatidyl-guanine
GTP	guanosine triphosphate
GTPase	guanosine triphosphatase
GV	γ-retrovirus
h	hour
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HIV	human immunodeficiency virus
HSC	hematopoietic stem cells
HSCT	hematopoietic stem cell transplantation
Ig	immunoglobulin
IL	interleukin
IL-2Rγc	interleukin-2 receptor gamma chain
IMDM	Iscove´s modified Dulbecco´s medium
kb	kilobase pair
kDa	kiloDalton
KO	knock-out
LAD	leukocyte adhesion deficiency
LMO-2	LIM domain only 2
LTR	long terminal repeat
LV	lentivirus
M	molar
mA	milliampere
MACS	magnetic cell sorting
MDS-1	myelodysplastic syndrome 1
MEM	minimum essential medium Eagle
mg	milligram
MHC	major histocompatibility complex
min	minutes
ml	milliliter
MLV	moloney leukemia virus
mRNA	messenger ribonucleic acid
NADPH	nicotinamide adenosine dinucleotide phosphate
ng	nanogram
NRE	negative regulatory element
NK	natural killer
N-WASP	neuronal WASP
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBMC	peripheral blood mononuclear cells

pfu	particle forming units
pH	power of hydrogen
PID	primary immunedeficiency
pol	polymerase
PRDM16	PR domain containing 16
PST-PIP	proline-serine-threonine phosphatase-interacting protein 1
RCR	replication competent retrovirus
RCL	replication competent lentivirus
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute
REV	responsive element
rpm	rotations per minute
RRE	rev-responsive element
RSV	Rous sarcoma virus
RT	room temperature
SCF	stem cell factor
SCID	severe combined immunedeficiency
SDS	sodium dodecylsulphate
SIN	self-inactivating
SETBP1	SET binding protein 1
TAE	Tris acetate EDTA
TPO	thrombopoietin
Tris	2-amino-2-hydroxymethyl-propan-1,3-diol
TRITC	tetramethyl rhodamine iso-thiocyanate
VCA	verprolin, cofilin, acidic
VSV-g	vesicular stomatitis virus glycoprotein
v/v	volume to volume
WAS	Wiskott-Aldrich Syndrome
WASP	Wiskott-Aldrich Syndrome protein
WH1	WASP-homology 1 motif
WIP	WASP interacting protein
WH2	WASP-homology 2 motif
XLN	X-linked neutropenia
XLT	X-linked thrombocytopenia
XSCID	X-linked SCID

1. INTRODUCTION

1.1. THE WISKOTT-ALDRICH SYNDROME

The Wiskott-Aldrich Syndrome (WAS) is an X-linked, complex primary immunodeficiency disorder with a characteristic clinical phenotype that includes thrombocytopenia with small platelets, eczema, recurrent infections caused by immunodeficiency, and an increased incidence of autoimmune manifestations and malignancies (1-3). The gene responsible for this disorder was identified in 1994 by positional cloning and designated as the *WAS protein* (*WASP*) gene (4). The clinical spectrum of the syndrome includes chronic or intermittent X-linked thrombocytopenia (XLT) (5, 6). Of note, patients with WASP mutations may also present with an unrelated clinical disease called an X-linked neutropenia (XLN) (7).

1.1.1. Clinical and pathological manifestations

Clinical manifestations due to WASP deficiency often occur in early infancy and consist of petechiae, bruising, and bloody diarrhea (1). Eczema is a frequent manifestation of classic WAS during infancy and childhood. The most consistent finding at diagnosis is thrombocytopenia and small platelets. Bacterial infections are also frequent symptoms during the first six months of life. The severity of the immune deficiency varies among families, depending on the mutation and its effects on protein expression (8,9).

1.1.1.1. Defects in the immune system

WASP-deficiency leads to T cell dysfunction including decreased proliferative response upon CD3 stimulation (10) and defective immunological synapse formation (11,12). WAS patients have reduced numbers of B lymphocytes as well as architectural abnormalities in their secondary lymphoid organs. Thus, absence of WASP in B lymphocytes (13) is at least partly responsible of an impaired humoral immune response (14). The cytolytic activity of WASP-

deficient NK cells is significantly reduced (15). A striking defect in both WASP-deficient monocytes/macrophages and dendritic cells is their inability to form podosomes (16,17). WASP-deficient macrophages and DCs show impaired IgG-mediated phagocytosis (18) and defective antigen presentation (19), respectively. A common defect in WASP-deficient leukocytes is their defective chemotaxis (20-22).

1.1.1.2. Platelets abnormalities

A consistent finding in WAS patients is thrombocytopenia associated with small platelet volume (7). After splenectomy, platelet counts and platelet volume may increase but are still less than those of healthy control subjects (23,24).

1.1.1.3. Other manifestations

Eczema is one of the characteristic findings in WAS patients. Patients with a milder form of WAS have either mild and transient eczema or none at all (8,9). Autoimmune diseases are present in 40% of the patients (3). Malignant tumors, typically associated with oncogenic viruses, can occur during childhood but are more frequent in adolescents and young adults with classic WAS phenotype (3). WAS patients also display histological abnormalities that affect the thymus and lymphoid organs (25).

1.1.2. Molecular basis

WAS is caused by mutations in the *WASP* gene, located in the region Xp11.22-Xp11.3 (26). It encodes a 502-amino-acid intracellular protein (WASP) that is expressed exclusively in nucleated hematopoietic cells. WASP is a member of a distinct family of proteins that participate in the transduction of signals from the cell surface to the actin cytoskeleton (10). Other members of this family include the more widely expressed neuronal WASP (N-WASP) and WASP family verprolin-homologous protein.

WASP has a C-terminal VCA region, comprising a WASP-homology 2 motif (WH2; also called a verprolin- homology domain) that binds actin monomer; a cofilin- homology motif that binds and activates the Arp2/3 complex, a potent nucleator of actin polymerization (28), and an acidic motif that also participates in Arp2/3 binding (Figure 1). WASP presents actin monomers to preformed Arp2/Arp3 dimers, catalyzing the formation of new actin filaments

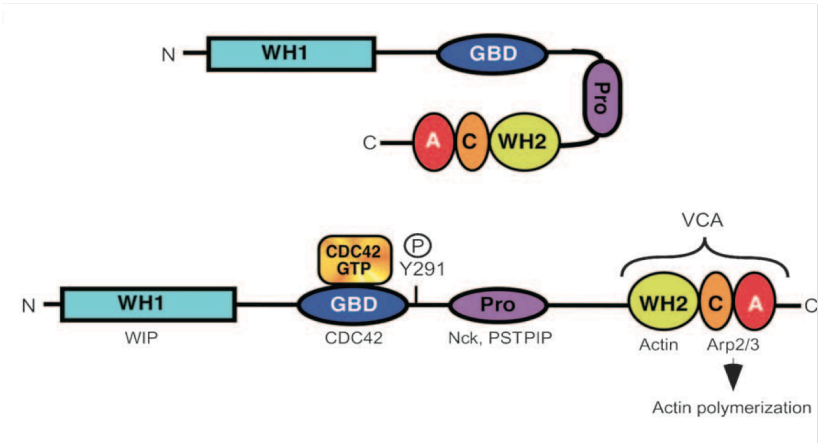

Figure 1.1. Domain structure and conformations of WASP. In unstimulated cells, WASP adopts an autoinhibited conformation (upper figure). Binding of Cdc42-GTP to GBD induces the conformational change that allows WASP to activate Arp2/3 complex function (bottom figure). *WH1*, WASP-homology 1 motif; *GBD*, guanosine-triphosphatase-binding domain; *Pro*, proline-rich region; *WH2*, verprolin-homology domain; *C*, cofilin-homology motif; *A*, acidic motif. (From Billadeau DD and Jurkhardt JK. *Traffic* 2006; 7: 1451-1460)

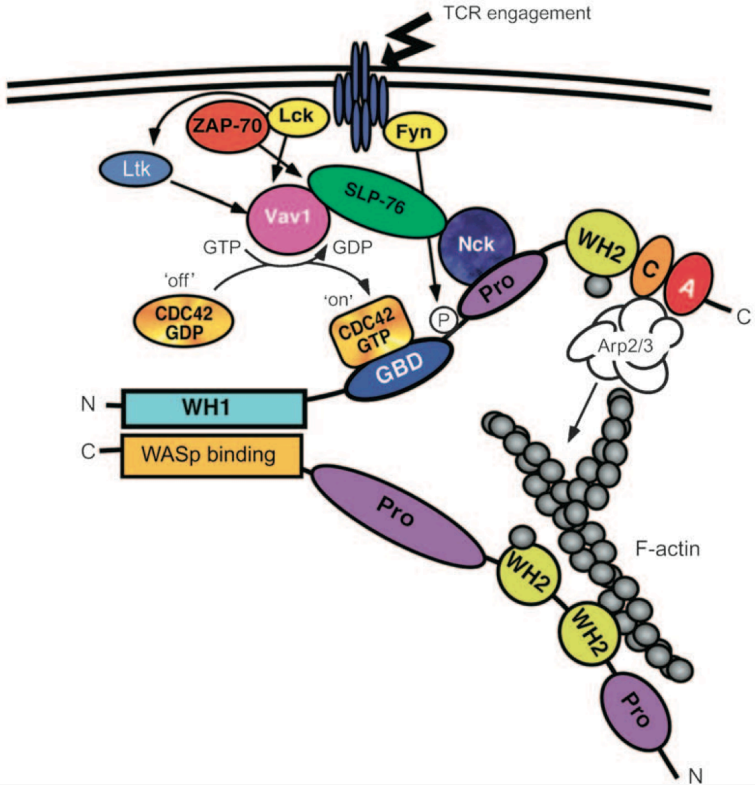

Figure 1.2. Model of WASP/WIP function at the immunological synapse. WASP and WASP interacting protein (WIP) function coordinately, with WASP activating Arp2/3-complex-induced actin polymerization and WIP stabilizing actin filaments and perhaps also facilitating polymerization. (From Billadeau DD and Jurkhardt JK. *Traffic* 2006; 7: 1451-1460).

on the sides of the pre-existing filaments (29) (Figure 2). WASP interacts with Cdc42-GTP through its guanosine-triphosphatase- binding domain (GBD) (30). In the absence of Cdc42, the VCA region folds back on the GBD, inhibiting WASP activity (31). Binding of Cdc42-GTP releases the VCA region, allowing Arp2/3 complex activation. Between the GBD and the VCA domains lies a proline-rich region that targets WASP to the immunological synapse by means of interactions with Nck and proline-serine-threonine phosphatase-interacting protein 1 (PST-PIP) (32-34). The WASP-homology 1 motif (WH1) domain at the N-terminus of WASP mediates binding to WASP-interacting protein (WIP) (35).

1.1.3. The spectrum of *WASP* mutations

Sequencing studies compiled from single centers in the United States, Italy and Japan in a large cohort of 270 unrelated families with patients with WAS/XLT have identified 158 unique WASP mutations (Figure 1.3.). The most frequent mutations were missense mutations in exons 1-3, splice-site mutations, short deletions and nonsense mutations. Insertions, complex mutations and large lesions were less frequent. Six mutational hotspots have been detected, which accounted for 25% of the cohort. Three of this mutations were consistently found in WASP positive patients with mild phenotype (XLT), whereas the three other mutations were mainly WASP negative and had a severe WAS phenotype (36, 37).

1.1.4. Genotype-phenotype correlation

Mutations of the *WASP* gene result in at least four distinct phenotypes: 1) the classic WAS (the most severe phenotype), characterized by thrombocytopenia with small platelets, recurrent infections and eczema; 2) the milder XLT variant (5); 3) intermittent thrombocytopenia (6); and 4) congenital X-linked neutropenia (XLN), caused by mutations in the GTPase-binding domain of WASP, without any of the clinical findings characteristic of WAS/XLT (7, 38).

The most consistent phenotype-genotype correlation was observed when patients were divided into two categories: WASP positive (if a mutated protein is expressed at normal size) and WASP negative (if the protein is absent or truncated) (9,8). The former usually present XLT with a milder phenotype, whereas the latter suffer from the classic and most severe WAS phenotype (30).

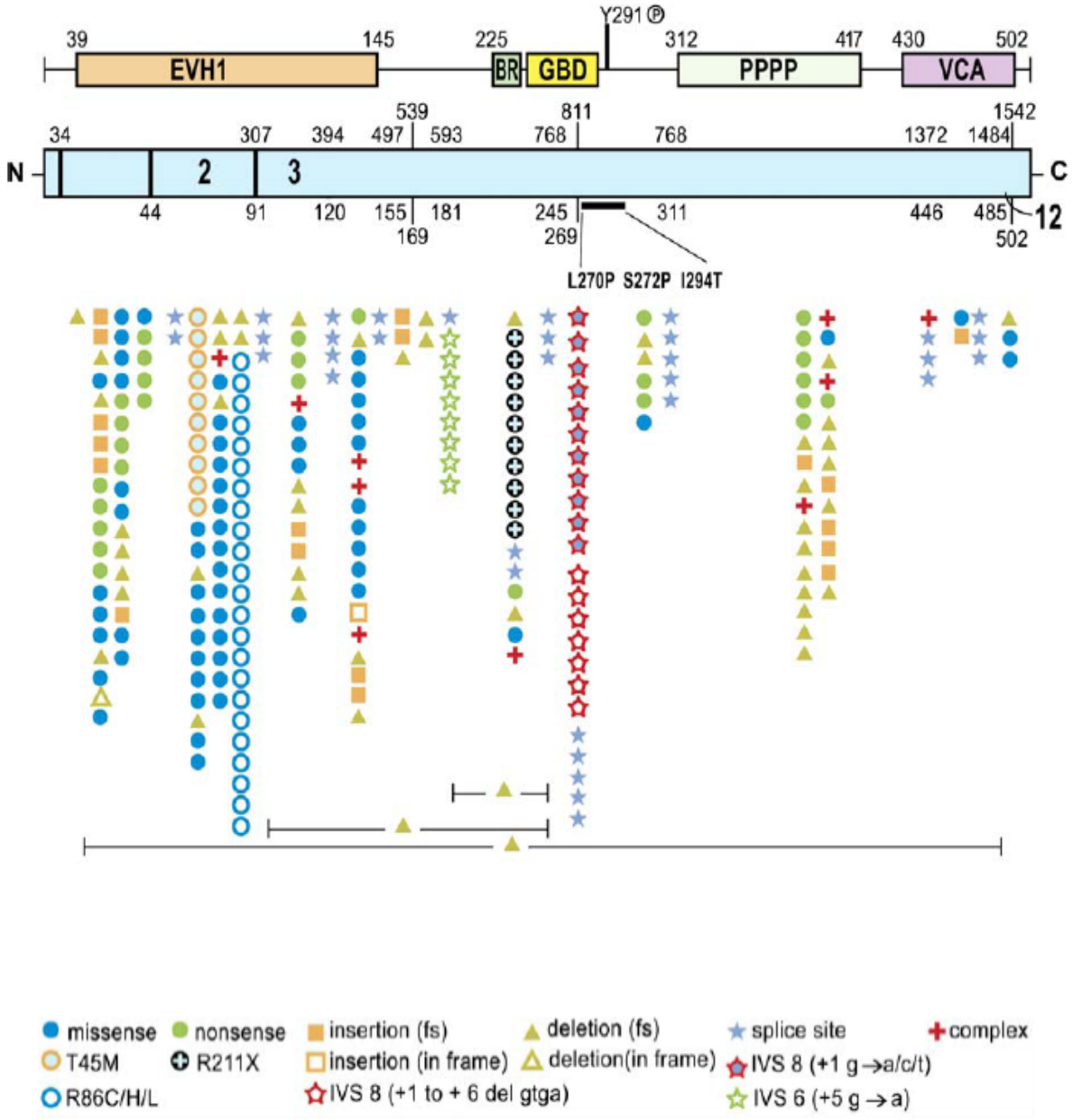

Figure 1.3. Schematic representation of mutations in the *WASP* gene. *WASP* encodes a protein with 12 exons and 5 major functional domains. The mutations identified in 270 unrelated WAS families are visualized according to their location in the exons and the exon-intron junctions. Each symbol represents a single family with *WASP* mutation. Missense mutations are located mostly in exons 1 through 4. Deletions, insertions, and nonsense mutations are distributed throughout the *WASP* gene. Splice-site mutations are found predominantly in introns 6, 8, 9, and 10. *EVH1*, Ena/WASP homology 1 domain; *BR*, basic region; *GBD*, GTPase binding domain; *PPPP*, proline-rich region; *VCA*, verprolin cofilin homology domains/acidic region. *From* (37).

1.1.5. Somatic reversion of the *WASP* gene and mosaic formation

Spontaneous reversion of an inherited disease-causing genetic mutation results in somatic mosaicism (40). Such a mechanism has been observed in several WAS patients, resulting in the restoration of the reading frame and expression of normal WASP (41, 42). While the majority of cases of somatic mosaicism in WAS is caused by true back reversions, more rare scenarios of second-site mutations leading to altered but functional gene products have also been documented (43, 44). This demonstrates that a normal copy of the WASP allele confers clear survival or growth advantage. Additionally, reversion events restore function and might result in significant improvement of clinical phenotype, if a sufficient proportion and diversity of WASP expressing cells reconstitutes the individual´s immune system. The study of somatic mosaicism due to secondary somatic mutations is of considerable interest for the prospect of gene therapy, as it supports the concept of a potential proliferative advantage of gene-corrected cells at least for T lymphocytes and NK cells (45, 46).

1.1.6. Current therapeutic approaches and perspectives for gene therapy in WAS

The standard curative therapy for WAS patients at present is allogeneic hematopoietic stem cell transplantation (HSCT) – a term used to encompass several possible clinical sources of HSC, including bone marrow, mobilized peripheral blood and umbilical cord blood. If a related human leukocyte antigen-identical donor is available, HSCT leads to more than 80% survival rate (47-50) but this percentage decreases drastically when using the bone marrow of a mismatched related donor (47-50). Bone marrow or cord blood transplantation from a matched unrelated donor is a valid therapeutic option, leading to a 71% to 81% survival rate (48, 49, 51). Unfortunately, allogeneic HSCT is associated with an elevated risk of developing life-threatening EBV^+ lymphoproliferative syndrome, infections, autoimmunity, and graft-versus-host disease (48). In addition, allogeneic HSCT should be performed early in life, in patients younger than five years of age (48, 49). Given the side effects and the poor outcome associated to HSCT and the lack of matched unrelated donors for most of the patients, the development of new alternative therapeutic strategies is highly desirable. The transplantation of autologous gene-corrected HSC could circumvent all these limitations

because it would avoid rejection and graft-versus-host disease and could be applicable to all WAS patients lacking a suitable bone marrow donor. The rational for gene therapy is also supported by the observation of frequent spontaneous somatic revertants conferring selective advantage to WASP-expressing cells, in an „auto-gene-therapy“ fashion (52). In these patients, somatic chimerism may result in a milder clinical phenotype (41-43, 52).

Various lines of evidence support the hypothesis that retrovirus-mediated *WASP* expresion in WASP-deficient cells may also confer a selective advantage. The inactivation of the X-chromosome in female conductors of this disease occurs in a non-random fashion (53). The notion that the reversion of the genetic defect in a single cell leads to partial restoration of the clinical phenotype and an accumulation of WASP-expressing cells strongly supports the hypothesis that WASP expression confers a selective advantage (54).

1. 2. GENE THERAPY

Gene therapy can be defined as any therapy involving the modification or insertion of genes into the patient´s somatic cells in order to treat inherited or acquired diseases.

1.2.1. The main strategies in gene therapy

Gene therapy protocols for inherited disorders can be generally classified into three strategies (Figure 1.4.): gene addition, targeted integration and gene correction (55).

1.2.1.1. Gene addition

„Gene addition“ consists of the insertion and expression of a wild-type copy of the disease-causing gene, leading to the correction of the phenotype at the cellular level. The therapeutic transgene is composed of a full transcription unit, including promoter and termination signals, which ensure a certain level of transgene expression depending on the site of insertion.

1.2.1.2. Targeted integration

In order to compensate for a genetic defect in a given gene, a transgene expression cassette flanked by sequences homologous to a certain „safe harbor“ is embedded in a donor vector.

A double-strand break stimulates homologous recombination between the donor DNA and the „safe harbor“.

1.2.1.3. Gene correction

A gene fragment encompassing wild-type sequences homologous to the mutant gene is used to transduce the target cells. A double-strand break stimulates homologous recombination between the donor DNA and the defect gene to generate an entirely refurbished locus.

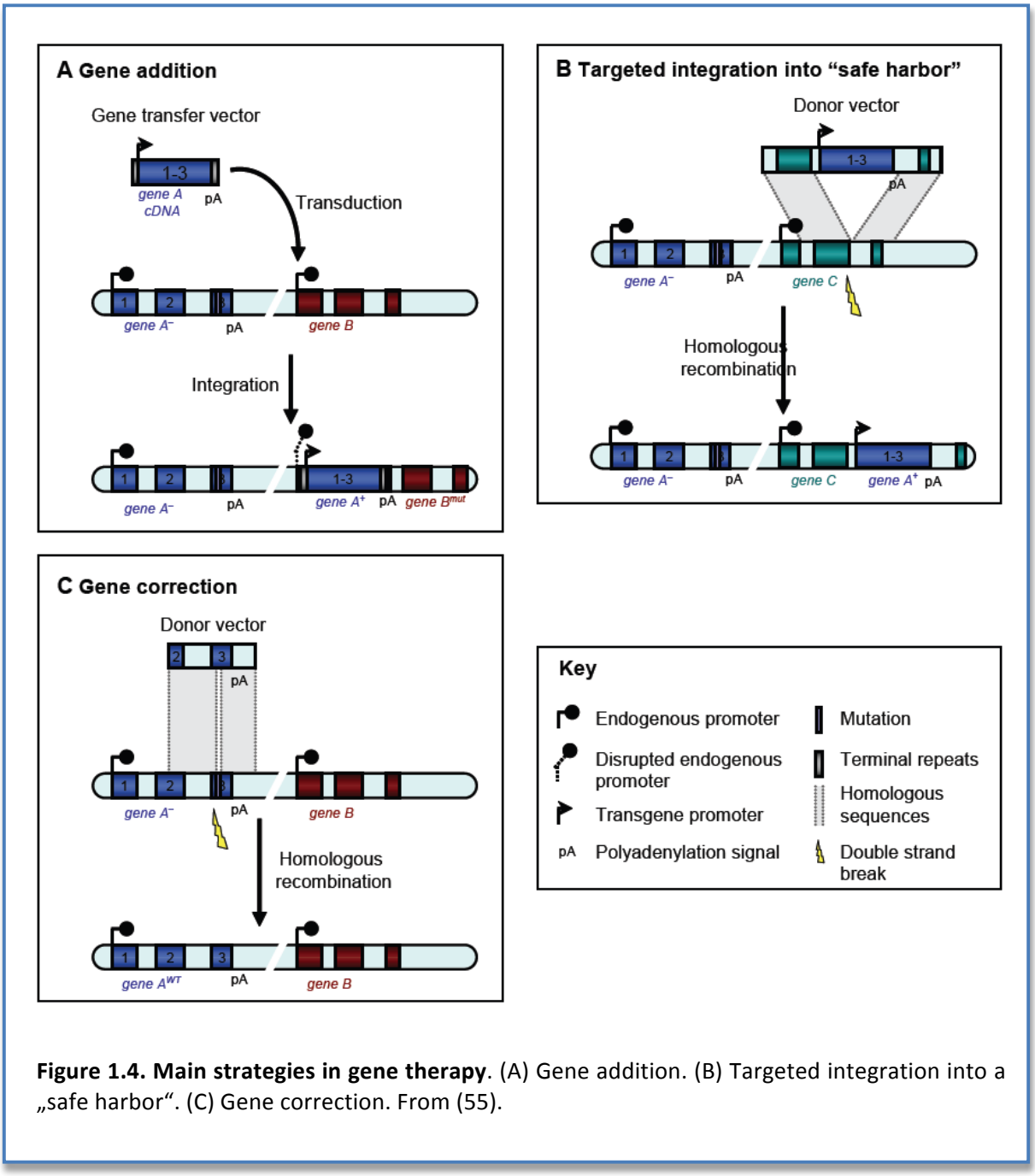

Figure 1.4. Main strategies in gene therapy. (A) Gene addition. (B) Targeted integration into a „safe harbor“. (C) Gene correction. From (55).

Gene addition is the conventional and, to date, most successful gene therapy for inherited disorders (56-59), however, this strategy has some drawbacks including the risk of insertional mutagenesis (60) (discussed later). More sophisticated methods, such as gene correction or targeted insertion, may displace gene-addition-type gene therapy but the first is challenging and lacks clinical validation and the target frequency of the latter and its off-target effects still pose a challenge. In summary, despite the side effects observed, conventional gene therapy remains the most successful strategy. In the meantime, it is important to increase the safety of the current protocols.

1.2.2. Gene therapy in primary immunodeficiencies: Clinical trials and outcome

Inherited monogenetic diseases may be viewed as the „natural target“ for gene therapy, since in principle correction of the defective gene underlying the disease should be sufficient to cure a given patient (53, 61). This is particularly true if the respective disease is pathophysiologically linked to one defined organ system. In the latter case, replacement therapy in the given organ should allow to induce disease remission or, if possible even full recovery. Due to its accessibility with relative ease for in vitro manipulation and subsequent reinfusion (62), the hematopoietic system is one of the organs in the human body that is of special interest for gene therapeutic interventions. Moreover, the blood system clearly reveals a hierarchic structure with HSC being the origin of any mature blood cell (63). It has been calculated that human beings as well as other mammals do have a relatively limited number (in the range of about 10 000 to 30 000) of long-term repopulating HSC (64, 65), thus, correction of a small number of cells might be sufficient to eradicate a genetic disease in the blood system. In line with these theoretical considerations, it has already been shown that transplantation of allogeneic (from a major histocompatibility complex-matched donor), healthy HSC contained in the bone marrow is sufficient to cure primary immune deficiencies (PID) (66, 67-70). However, as discussed in Section 1.1.6., allogeneic HSCT is associated with severe morbidity and mortality secondary to infections, Graft-versus-Host-Disesase (GvHD), non-engraftment and post-transplant lymphoproliferative syndrome (71), especially in patients transplanted with HSC from mismatched donors (48).

The rationale underlying gene therapy for PID is that gene correction and re-transplantation of the patient's own HSC could lead to the same clinical benefits as matched allogeneic HSCT, but without the immunologic complications. Efforts to perform gene therapy for PID began in the 1980s as gene delivery vectors were derived from murine retroviruses. Studies performed at that time demonstrated the ability of retroviral vectors to introduce genes into the multipotent HSC from murine bone marrow (72, 73). Based on these results, an initial wave of enthusiasm emerged for the prospects of gene therapy to be used to treat patients, spreading from the inherited blood cell diseases to other genetic diseases and acquired conditions including cancer, cardiovascular disorders, neurological diseases, and others (74).

1.2.2.1. Adenosine Deaminase (ADA)-Deficiency SCID – Initial trials

ADA-deficiency was the first genetic cause of human severe combined immune deficiency (SCID) to be characterized at the biochemical and genetic basis (75). In the 1990s, a retroviral vector carrying a normal human ADA cDNA was used to transduce T cells from the peripheral blood of four patients with ADA-deficient SCID who were receiving ADA enzyme replacement therapy, which partially restored the numbers of circulating T lymphocytes. The protocol called for repeated cycles of leukopheresis, ADA gene transduction and cell reinfusion. There were no complications from the procedures and the presence of circulating T cells expressing ADA enzyme activity was demonstrated (76). However, it is not apparent whether any clinical benefit was derived from the procedure, as the patients remained on the ADA enzyme replacement therapy per protocol design.

Subsequent studies have primarily focused on the correction of the phenotype by gene addition in HSC, which may lead to long-term production of a broad repertoire of functioning lymphocytes. To this aim, retroviral-mediated gene transfer was applied to hematopoietic stem cells, isolating them from the patients' bone marrow, culturing them for a few days for gene addition, and then re-infusing the cells into the patients (77). Again, while the procedure did not have complications, there was no evidence of clinical benefit and only very low levels of gene-containing peripheral blood leukocytes were produced.

In retrospect, the lack of success from these early trials is not surprising. The methods used for culturing the HSC are now known to be suboptimal both for gene transfer and for survival of the HSC. Additionally, the continued administration of ADA enzyme replacement therapy may have blunted the putative selective advantage conferred to ADA-corrected

lymphocytes, impeding the potential amplification of initial gene correction efficiency. The methods for gene transfer to HSC were still „in its infancy“ and the retroviral vectors were produced at suboptimal titers (78). Further developments such as the identification of new hematopoietic growth factors that were able to induce cycling of primitive long-lived HSC, highly improved retroviral gene transfer (56).

The presence of either a feeder layer of marrow stromal cells or the extracellular matrix protein fibronectin was shown to both enhance gene transfer to human HSC as well as to support their survival during *ex vivo* culture (79). These improved techniques were applied for a second generation of clinical trials of gene therapy for PID begun in the late 1990s.

1.2.2.2. ADA-deficiency SCID – Second generation trials

In the year 2000, A. Aiuti and colleagues at the San Raffaele Telethon Institute for Gene Therapy, Milan, began a trial of retroviral-mediated gene transfer for ADA-deficient SCID in two patients using the improved transduction methods. Two extra major changes from the prior clinical approach were adopted: patients did not recieve ADA enzyme replacement therapy prior to gene therapy (56), which would allow the selective advantage for the gene-corrected cells to be manifested. Additionally, patients were treated with pretransplant conditioning by administration of the chemotherapy agent busulfan, in approximately one fourth of the full dosage typically used in clinical BMT to achieve complete marrow ablation. Busulfan is an alkylating agent that is highly specific for eradicating HSC, and acts to “make space” in the bone marrow to facilitate engraftment of transplanted HSC. Both subjects have had recovery of their immune function; one has had sustained protective immunity, whereas the second who had a smaller HSC dosage only achieved partial immune reconstitution. These results represent a major milestone in the treatment of PID. The same group recently reported extended outcome results in a total of 10 subjects treated under this protocol, with the majority of the patients achieving clinically beneficial immune reconstitution that has allowed them to lead essentially normal lives without the need for ADA enzyme replacement therapy (80, 81). Similar studies by A. J. Thrasher and collaborators at University College London, Institute of Child Health in the United Kingdom and by K. L. Shaw and collaborators in the U.S. at the University of California, Los Angeles and the National Institutes of Health are obtaining similar results (82, 83). Immune reconstitution was observed in the majority of the treated subjects who received busulfan and were not on ADA enzyme replacement.

1.2.2.3. X-linked SCID (XSCID)

The X-linked form of SCID (XSCID) is caused by mutations in the gene encoding the common cytokine receptor gamma chain (γc), a component of the receptor for multiple cytokines acting to promote lymphocyte development and function (84). A strong selective advantage for lymphoid progenitor cells in XSCID patients was demonstrated by a patient who underwent a spontaneous reversion of a γc mutation in a single lymphoid progenitor cell, leading to some immunologic reconstitution. Preclinical studies showed that retroviral-mediated transfer of a normal human γc cDNA into cells from XSCID patients or murine models of the disease restored lymphocyte production and activity (85). Based on these results, a clinical trial of gene therapy for XSCID was initiated at the Hôpital Necker Enfant-Malade in Paris, France. Again, HSC from the patients' bone marrow were cultured for retroviral-mediated gene transfer and then reinfused into the patients. In this trial, no chemotherapy conditioning was administered, with the expectation that the gene-corrected lymphoid cells would have a very high selective survival in the profoundly lymphopenic patients, and marrow cytoreduction to facilitate higher engraftment of HSC would not be needed. The results from the first two subjects were reported in 2000, showing rapid and robust production of T lymphocytes, with lesser improvements in the numbers of circulating B and NK cells (57). Subsequent reports demonstrated similar responses in 9 of 10 subjects, with the exception being a patient who had marked splenomegaly at the time of treatment, which may have led to consumption of the infused cells (86). A trial at UCL Institute of Child Health in London, using similar techniques achieved immune reconstitution in another 10 subjects (87). However, 2–5 years after the treatment, a serious complication developed in a total of 5 of the 20 subjects treated in the two trials, with a leukemia-like T lymphoproliferative disorder arising (88-91). Those patients were treated with chemotherapy and four have remained in complete remission with continued restored immunity, but one succumbed from the leukemia, despite therapy.

Investigations into the mechanisms found that, in each subject, there was an outgrowth of a clonal population of T cells containing the retroviral vector integrated adjacent to one or more cellular proto-oncogenes (*LMO-2* in 4 cases) (90, 91). Retroviruses are capable of activating the expression of cellular genes in the vicinity of their integration site by the

action of the viral enhancers contained in the viral long-terminal repeats (LTR). If the cellular genes activated are ones that control cellular growth (proto-oncogenes), the result can be stimulated proliferation that can lead to accumulation of additional growth-promoting mutations and eventually malignant transformation. This, in fact, is the mechanism by which the oncogenic leukemia-causing retroviruses cause disease in susceptible animal hosts when capable of unrestricted replication. Presumably, the initial gene transfer to the patients' HSPC led to thousands or even millions of vector integrants at sites across the genome in individual cells; rare integrants that activated cellular proto-oncogenes induced increased cell proliferation which then led to a cascade of cellular events culminating in the malignant transformation. The reasons why this complication has occurred in 5 out of 20 XSCID patients, but none of more than 20 ADA-deficient SCID patients are not clear. The role of the γc gene product (a trans-membrane protein capable of providing intracellular signaling), the nature of the XSCID patients' marrow stem cells, effects of the γc -deficiency on the susceptibility to transformation and the rapidity of immune reconstitution in the XSCID subjects have all been speculated as involved, but no definitive explanation has yet emerged.

Another clinical trial for XSCID has been reported that treated three teenage boys with XSCID who had prior unsuccessful allogeneic HSCT and had poor immunity and other chronic complications (92). Only one of these subjects had any evidence of improvement in immune function, suggesting that this gene therapy approach may be most effective in younger subjects with potential for robust thymic function.

1.2.2.4. Chronic Granulomatous Disease (CGD)

The chronic granulomatous disease (CGD) is another monogenic PID candidate for gene therapy. It is characterized by the inability to fight bacterial and fungal infections due to mutations in the NADPH oxidase complex, which is necessary for neutrophil function (93). The first trial was conducted in the mid-late 1990s, at the National Institutes of Health and Indiana University School of Medicine. Peripheral blood stem cells (PBSC) mobilized by granulocyte-colony stimulating factor (G-CSF) were transduced *ex vivo* with γ-retroviral vectors and reinfused without prior treatment with chemotherapeutic agents (94-96). Only low frequencies of corrected granulocytes could be detected in the first months and then no

long-lasting effects could be observed. Subsequently, another trial was performed by M. Grez and his collaborators in Germany in which the patients, two young men, were conditioned with a moderate dosage of busulfan (8 mg/kg) prior to reinfusion of the transduced PBSC (97). In this case, the levels of corrected leukocytes detected in peripheral blood increased up to 80% over the first year. For the first time, the integration sites of the transfer vector were studied in the CGD treated patients, revealing a highly restricted pattern, with the majority of vector integrants in the engrafted stem cells being near one of a few genes known to be involved in myeloid cell proliferation (*MDS-1*, *PRDM16* or *SETBP1*). These two patients went on to develop myelodysplasia, a preleukemic condition; one subject has had a bone marrow transplant and the other died of an acute infection with a loss of the restored neutrophil function. In a recent study (98), S. Stein and coworkers report progressive decline in blood counts – including platelets, red blood cells and neutrophils – in the two CGD subjects at 15 and 28 months, respectively, after gene therapy. Bone marow examination showed myelodysplasia, a preleukemic clonal marrow failure syndrome characterized by ineffective and disordered myeloid maturation. Despite the persistent high frequency of vector-corrected neutrophils, expression of NADPH oxidase dropped drastically in both patients over time. They found that the silencing of NADPH oxidase occurred through progressive CpG methylation of the promoter contained in the LTR of the vector. Beginning 15 months after gene threrapy, one of the patients developed progressive severe cytopenias, evidence for myelodysplasia on bone marrow examination and increasing dominance of an MDS1-EVI1 clone that also developed monosomy 7, and eventually succumbed to sepsis. The second subject was referred for unrelated donor stem cell transplantation while still infection free (98). As with the XSCID trials, it will be vital to understand how to avoid this unwanted complication while retaining the clear-cut clinical benefits that can be achieved.

1.2.2.5. Leukocyte Adhesion Deficiency (LAD)

The leukocyte adhesion deficiency (LAD), due to deficiency of the CD18 adhesion protein, is another monogenetic PID candidate for gene therapy. Two patients were treated using a retroviral vector and no chemotherapy conditioning (99). Essentially, no gene-transduced lymphocytes were produced in the patients. More recently, these investigators have reported promising results in a canine model of LAD, using a foamy virus vector and low

dose total-body irradiation (200 cGy) as conditioning, with excellent restoration of lymphocyte function (100).

1.2.2.6. The Wiskott-Aldrich Syndrome

Another PID that can be treated with allogeneic HSCT is the Wiskott-Aldrich Syndrome (101). WAS is a promising candidate disease for gene therapeutic approaches, since WASP expression is restricted to cells of the hematopoietic system and grants a proliferative advantage over WASP-negative cells (45, 102). A clinical trial using a retroviral vector carrying a wild-type human WASP cDNA has started in Hannover (Germany) in October, 2006 (103). Prior to gene therapy, patients have received a partially myeloablative conditioning regime using busulfan. Up to 2.5 years after gene therapy, the patients´ clinical conditions have markedly improved with respect to their susceptibility to infection, hemorrhagic diathesis, eczema, autoimmunity and predisposition to severe infections and sustained WASP expression has been observed in HSPCs, lymphoid and myeloid cells and platelets. The study of the clonal inventory reveals that *LMO2* and *CCND2* were targeted preferentially in lymphoid cells, whereas *MDS1/EVI1* was almost exclusively hit in myeloid cells, however, no persistent clonal imbalance has been observed so far (103). This study provides first proof-of-principle that gene therapy for WAS is feasible and safe up to three years after gene therapy, indicating that early HSPC contributing quantitatively to all cell lineages of blood, were successfully corrected. It also confirms for the first time correction of platelets upon transplantation of retrovirus-transduced HSC in humans, thus achieving complete immunological correction of treated patients (103).

1.3. ADVERSE REACTIONS IN GENE THERAPY

1.3.1. Gene transfer

Gene therapy of genetic blood disorders requires stable genetic modification of hematopoietic stem cells. Retroviral (including lentiviral) vectors are the preferred vehicles for gene transfer (104), for they integrate into the cellular genome, providing stable gene transfer (105). The basic principle of turning a pathogenic retrovirus into a delivery system

relies on the ability to separate the components needed for replication from those capable of causing disease (Fig. 1.5.). Briefly, the viral genome is deleted of any dispensable genetic information and plasmids that encode only genes required for essential functions in *trans* are generated. Ideally, a vector encodes only the transgene and the *cis*-regulatory elements necessary for packaging and transduction (Fig. 1.6.).

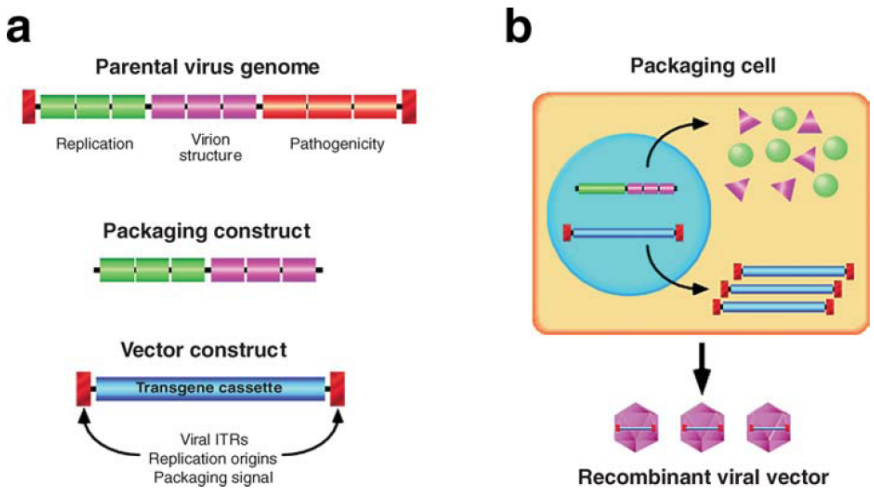

Figure 1.5. Principles for the generating retroviral vectors. (a) Converting a virus into a recombinant vector. The packaging construct contains only genes that encode functions required for replication and structural proteins. The vector construct contains the essential *cis*-acting sequences and the transgene cassette that contains the required transcriptional regulatory elements. (b) The packaging and vector constructs are introduced into the packaging cell. Proteins required for replication and assembly of the virion are expressed from the packaging construct, and the replicated vector genomes are encapsidated into virus particles to generate the recombinant vector. From (106).

Because of their integrative nature, transduction of target cells with retroviral vectors is inevitably associated with the risk of genotoxicity (107). It is important for retroviruses to ensure high expression of their genome independent of the integration locus, therefore, most retroviral promoters consist of condensed enhancer/promoter sequences (108, 109) which induce high transgene expression, and a duplication of the enhancer/promoter region in the long terminal repeat (LTR), which may affect expression of neighboring genes by a process called insertional mutagenesis (110).

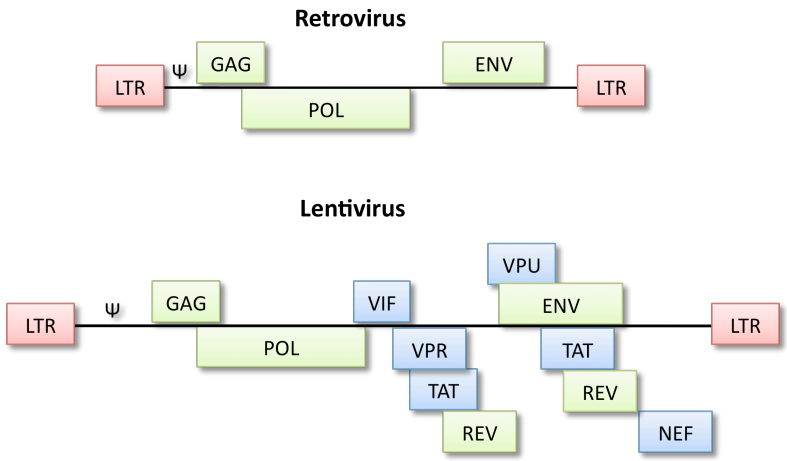

Figure 1.6. Commonly used retroviruses converted into recombinant viral vectors. The coloured boxes indicate genes or *cis*-acting elements that are either essential [and therefore retained in vectors (*red*) or supplied by packaging constructs or cell lines (*green*)] or that are nonessential and often deleted (*blue*). Only the major genetic elements are shown, and viruses are not drawn to scale. Modified from (106).

1.3.2. Genotoxicity associated with gene transfer vectors: insertional mutagenesis.

Genotoxicity can be exerted by anything that affects DNA integrity or leads to DNA or chromosomal damage, or both, classically chemicals or radiation that modify DNA (111). Nonetheless, insertion of genetic elements or expression of proteins that act on DNA can also cause genotoxicity, by means of insertional mutagenesis (55). The term *insertional mutagenesis* refers to the mutagenic effects of the insertion of any piece of DNA (110).

The mechanisms of insertional mutagenesis caused by retroviral integrations (Figure 1.5.) are:

a) Generation of an aberrant transcript. The viral long terminal repeat, containing promoter/enhancer sequences, is sometimes not properly terminated leading to long read-through transcripts, which can lead to increased or ectopic expression of the cellular protein.

b) Retroviral promoter acting as promoter/enhancer for cellular gene, for instance a proto-oncogene such as *LMO2*. Thus, the transactivation activity of the LTR extends

beyond the viral cassette to influence expression of cellular genes. This is one of the main mechanisms having led to aberrant expression of *LMO2* in the X-SCID gene therapy trials.

c) Disruption of a negative regulatory element or silencer element. The viral genome integrated into the host genome could disrupt negative regulatory control of the expression of a cellular gene, leading to increased expression of the cellular gene. This explains in part, this is what happened in the *LMO2* cases.
d) Integration of viral genome leading to an aberrant transcript encoding an aberrant protein that lacks regulatory domains, thereby generating a constitutively active or dominant negative form of the protein.

Through these different mechanisms, insertional mutagenesis can cause: (a) transcriptional upregulation or temporal deregulation fo nearby genes (112), (b) generation of fusion or read-through transcripts from the inserted promoter into cellular genes (113); and (c) gene disruption by the destruction of open reading frames. The latter event is thought to be frequent, but its phenotypic effects are predicted to be small because of the diploid nature of the mammalian genome (114). Therefore, the major side effect of retroviral vectors is transcriptional deregulation as the insertion of an enhancer/promoter can lead to a drastic upregulation of neighboring genes (115).

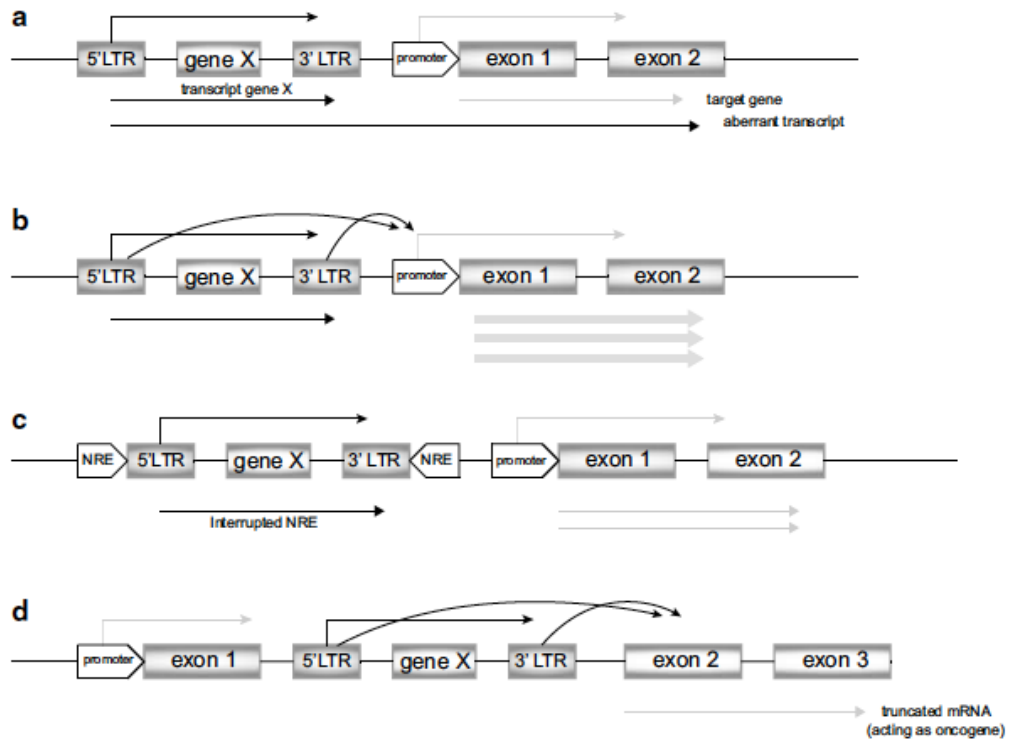

Figure 1.7. **Mechanisms of insertional mutagenesis.** (a) Generation of an aberrant transcript. (b) Expression of cellular genes by transactivation activity of the LTR. (c) Disruption of a negative regulatory element (NRE) or silencer element. (d) Generation of a constitutively active form of a protein. From (116).

1.3.3. Observations from clinical trials and animal models.

Long-term observational studies of primates that received BMT and chemotherapy revealed leukemic expansion caused by insertional mutagenesis (117). Interestingly, a large fraction of the integration sites in these animal models were located in genes involved in proliferation and stem cell renewal (118), a phenomenon also observed in clinical gene therapy trials (119, 120). In successful gene therapy trials that restored the immune system in children suffering from XSCID (57, 58), four patients showed signs of T-cell leukemia in the Paris cohort (88, 121). All four cases appear to be causally linked to an insertion of the retroviral vector into known proto-oncogenes (*LMO2*, *BMI1* and *CCND2*) (89). Initially it was believed that the accumulation of adverse events in Paris was the result of protocol-specific cofactors, however, the occurrence of malignant T-cell expansion in one patient treated in the XSCID trial in London, probably also caused by an insertion near the *LMO2* locus (120-122), ruled

out this possibility: the discrepancy between the two trials could no longer be explained by chance but rather by attendant circumstance. In fact, these cases hint at a complex interplay between vector insertion, the IL-2Rγc transgene and the proto-oncogene *LMO2* in this particular disease setting. In a clinical trial to treat CGD, an expansion of gene-modified cells and clonal dominance was observed after transduction with a retroviral expression vector (97). Integration-site analysis revealed activating vector insertions into gene loci, which could have induced expanded myelopoiesis (97). However, the death of a patient 2.5 years after gene transfer was caused by severe sepsis and gene therapy failure rather than leukemia (123). Analysis of transduced cells at the time of death revealed that, despite the fact that many hematopoietic cells contained the vector, expression of the transgene was almost undetectable, suggestive of gene silencing (123).

It is now well accepted that the insertion of enhancers next to proto-oncogenes constitutes the first step toward leukemogenesis. Moreover, if the transgene is an oncogene itself, the insertion site may synergize with its oncogenic nature and lead to accelerated tumor development (124, 204).

A synopsis of the above-mentioned studies leads to the following observation and conclusion: all cases of premalignant or malignant clonal outgrowth associated with insertional mutagenesis reported to date have been observed following the use of simple MLV or HIV-derived vectors (205) that harbor strong enhancer-promoter sequences in their long terminal repeats. Therefore, it could be possible to drastically decrease the risk for clonal dominance or leukemia induction by further studying the impact of vector design and consequently modifying the vector architecture or the nature of the *cis*-elements contained therein.

1.4. TOWARDS SAFER GENE THERAPY: SAFER METHODS AND SAFER VECTORS

Long-lasting phenotype correction of hematopoietic stem/progenitor cells (HSPC) requires persistence of the corrective gene for it to be passed on to all the progeny blood cells. At least six methods that ensure such persistance in ways that are safer for patients, are under study (Figure 1.7.).

1.4.1. Self-inactivating (SIN) vectors

Vectors lacking the promoter-enhancer sequences of the LTR are called self-inactivating (SIN). The LTR sequences at the ends of retroviral-vector sequences possess strong enhancer activity that may play a key role in the activation of adjacent cellular proto-oncogenes. Vector designs that lead to "self-inactivation" of these LTR enhancer sequences have significantly reduced transactivation activity (125).

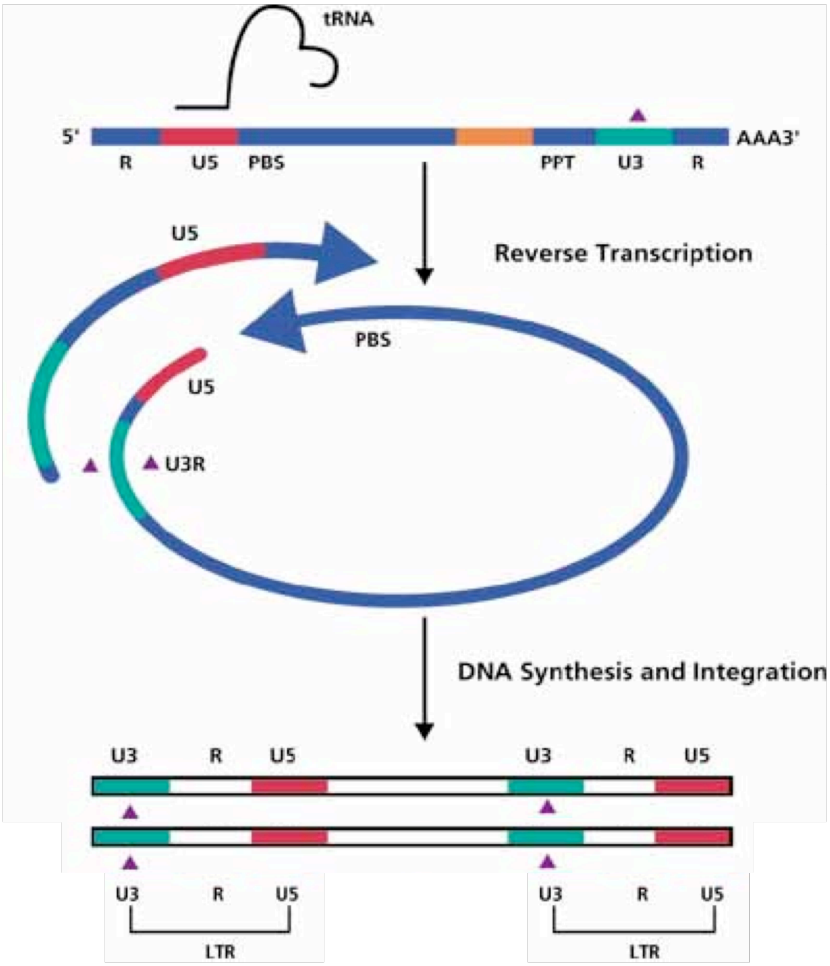

Figure 1.8. Self-inactivating vector design. SIN-vectors are constructed by deleting the enhancer and/or the promoter in the U3 region of the 3' LTR. During reverse transcription, a circular intermediate is formed that transfers the deletion to the 5' LTR of the proviral DNA. The deletion abolishes any transcriptional activity driven by the LTR so that no full-length vector RNA is produced in transduced cells. Following a single round of replication, the changes are copied into both 5' and 3' LTRs resulting in inactive provirus. (*Modified from* Martin *et al.*, 2000 BioTechniques 28:702–708)

This type of vectors has several major advantages. The self-inactivation of the retroviral vector minimizes the risk that RCRs (Replication Competent Retrovirus) will emerge. It also reduces the likelihood that cellular coding sequences located adjacent to the vector integration site will be aberrantly expressed, either due to the promoter activity of the 3' LTR or through an enhancer effect. These vectors are a significant development in gene therapy research where a particular concern is preventing incidental activation of an endogenous oncogene. Finally, a potential transcriptional interference between the LTR and the internal promoter (used for expression in tissue/cells of interest) driving the transgene is prevented by the SIN-vector design.

1.4.2. Cellular promoters

Promoters of cellular genes rather than the strong viral promoters often used in viral vectors, allow increased physiologic regulation of gene expression of the therapeutic gene and reduce the risk of activating adjacent cellular genes (126). Promoters with relatively low activity in HSPC are expected to be less likely to upregulate proto-oncogenes in this cell fraction, which is considered to be the major origin for the initiation of leukemias (127, 206). Other promoters, such as inducible promoters (e.g., tetracycline-inducible) (128) or lineage specific promoters (e.g., globin promoter) (129, 130) can be incorporated and may further reduce the likelihood of activation of cellular genes. If a cellular promoter fragment is too weak to express the transgene above the therapeutic threshold, improving RNA processing of the vector is an important option. Any attempts that enhance RNA export, stability, and translation would be useful (131-136). Increasing protein stability represents another approach that might reduce the need for strong enhancers in the vector backbone (137).

1.4.3. Boundary elements

DNA sequences present throughout the genome act to block interactions between adjacent transcriptional units. The role of these boundary elements (including insulators and matrix-attachment regions) would be to preserve the functional identity of the expression domain, counteracting any interference that could be transmitted from neighbouring sequences and also, avoiding the spread of endogenous enhancer function outside the domain (138). They have repeatedly been proposed for their inclusion in standard constructs, in order to protect

them from chromosomal position effects and to allow high level of expression (139). However, in spite of some successful experiments in transgenic animals (140, 141), other reports do not seem to agree with this simple model (139, 140), and therefore claim for a more cautious explanation has been forwarded, until we gain more knowledge regarding the mechanism of action of insulators (143, 144). Alternatively, or in addition to, scaffold attachment regions can be introduced into the vector (146-148). Although a relative reduction of enhancer interactions would be expected to translate into a gain of safety, detailed studies may be required to derive optimal vector configurations with high titers.

1.4.4. Gene transfer vectors with better integration-site choices

All gene vectors exhibit a semi-random insertion pattern, with varying degrees of preference for actively transcribed genes and distinct *cis*-regulatory regions such as GpC islands and DNAse 1 hypersensitive sites. Differences between the vectors studied may be profound, depending on the window chosen for the biostatistical analysis (149-150). The most pronounced differences have been noted in the immediate proximity of the transcriptional start site and a small window surrounding DNAse 1 hypersensitive sites, which coincide with gene-regulatory regions. In these areas, MLV-based vectors exhibit a preference for insertion, as compared with other types of viral and nonviral vector. In contrast, HIV-derived vectors are more likely to integrate in, and thus potentially disrupt, active transcription units. The differences observed between the various vectors are mostly relative, not absolute. Moreover, it is important to examine details of the bioinformatics approach taken by the various investigators. For instance, the predilection of MLV for insertion around DNAse 1 hypersensitive sites is only markedly different from that of HIV if the region of interest is kept relatively small (152, 153).

1.4.5. Direct *in situ* gene repair

A more sophisticated alternative method would be the direct *in situ* gene repair to obviate random gene insertion. Methods to perform gene repair by means of efficient homologous recombination are being developed, aided by sequence-specific endonucleases that facilitate the process. Gene correction, rather than gene addition, would not lead to random insertion of transgene sequences (154). However, this method still remains a challenge rather than a promise for gene transfer in gene therapy.

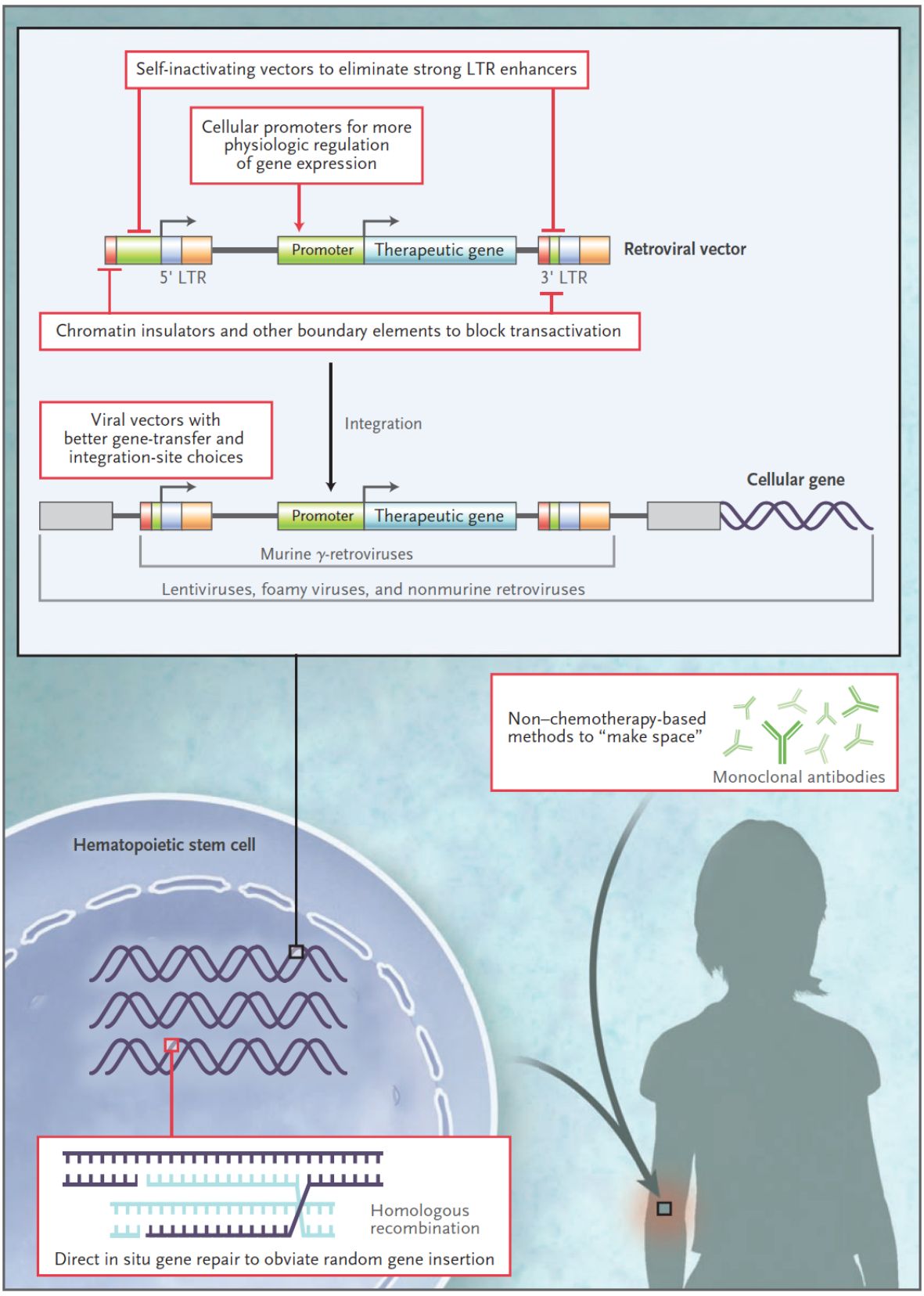

Figure 1.9. Strategies to increase the safety and efficacy in hematopoietic stem cell based gene therapy. From (139)

1.4.6. Non-chemotherapy-based approaches

The use of non-chemotherapy-based approaches has arised as a less aggressive alternative conditioning treatment to favor safer engraftment of gene-corrected stem cells. For instance, in the study by Aiuti *et al* (80), a chemotherapeutic agent, busulfan, was administered to patients to „create niches“ in their bone marrow for the genetically modified HSC to engraft. Methods that use less-toxic agents, such as monoclonal antibodies that bind to and deplete stem cells, may be able to facilitate stem-cell engraftment with fewer potential short- and long-term side effects (155). However, this strategy might not be feasible in a clinical scenario, due to the extremely large amount of antibodies required, the difficulty of actually deplete all stem cells, and the incertain specificity of the antibodies for hematopoietic cells.

Besides changing the vector backbone, redesigning the architecture of the transgene is an important option for improving both safety and efficiency (156-159). Considering that the majority of insertional side effects described to date appear to be related to gene activation (162), a safer transgene architecture should convert a vector from a potential “dominant” mutagen (a mutagen that causes gene activations) into a “recessive” one (a mutagen that causes loss of function of a single allele, which often will not be sufficient to change cell fate). The ongoing approaches to improve vector design can be grouped into three major fields: (1) replacing constitutively active enhancer-promoter cassettes with more physiological promoters, (2) introducing boundary sequences (as discussed before), and (3) improving transcriptional termination of the transgene cassette. Transcriptional termination is leaky in retroviruses, and even more so in the SIN vectors, which lack the majority of their U3 region. A study that compared HIV- and MLV-based SIN vectors indicated that both contain poor termination signals (163). This not only increases the risk of activating a downstream cellular proto-oncogene, but also potentially triggers “transductive recombination” (164), a process that allows a retroviral transcript to pick up cellular sequences before particle formation and thus has led to the generation of transforming retroviruses transferring cell-derived oncogenes. Termination enhancers potentially useful for vector construction could be derived from cellular or viral resources.

Although improving vector design appears to be the most straightforward approach to increase safety, retroviral (including lentiviral) vector configurations combining all these safety modifications remain to be described.

1.5. AIMS OF THIS STUDY

The overall goal of this study was to develop a novel vector for gene transfer into hematopoietic stem/progenitor cells for stem cell-based gene therapy for the Wiskott-Aldrich Syndrome, that combines safety modifications and yet supports physiological levels of transgene expression.

This study specifically aimed at:

1. Designing a novel vector that incorporates the following safety features:
    a. A split-packaging design
    b. The potential of pseudotyping for important target populations
    c. A self-inactivating configuration
    d. An internal promoter of cellular origin

2. Improving the expression of the therapeutical transgene by:
    a. Selecting the most suitable vector type (γ-retro- or lentivirus)
    b. Redesigning the sequence of the wild-type *WASP* cDNA.

3. Assessing the functionality of the novel vector by gene transfer into WASP-deficient human hematopoietic stem/progenitor cells.

2. MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Antibodies

All antibodies were aliquoted and storaged at -20°C until used. After thawing, aliquotes were kept at 4°C for short-term storage.

- Intracellular staining of WASP

Antibody	Dilution	Company
Anti-human WASP monoclonal IgG2a	1/100	Santa Cruz Biotechnology (Santa Cruz, USA)
Mouse IgG2a isotype	1/100	Becton Dickinson (Heidelberg, Germany)
Goat F(ab0)2 fragment rat IgG-phycoerythrin	1/100	Immunotech (Vaudreuil-Dorion, Canada)

- Western-blot

Antibody	Dilution	Company
Anti-human WASP monoclonal antibody	1/400	Santa Cruz Biotechnology
Anti-human GAPDH monoclonal antibody	1/10000	Santa Cruz Biotechnology
HRP-conjugated goat anti-mouse antibody	1/10000	Becton Dickinson

- Staining of podosomes

Antibody	Dilution	Company
Anti-vinculin	1/50	Sigma (Dorset, UK)
Cy5-conjugated rabbit anti-mouse IgG F(ab0)2	1/100	Jackson Immuno Research (Soham, UK)
Phalloidin-TRITC	0.1 mg/ml	Sigma (Dorset, UK)
DAPI	1/100	Invitrogen (Karlsruhe, Germany)

- Cell depletion

Components	Dilution	Company
Microbeads anti-CD34	100 ul per 10^8 total cells	Miltenyi Biotech (Berglisch Gladbach, Germany)
Microbeads anti-CD14	20 ul per 10^7 total cells	Miltenyi Biotech

Storage: 4°C

2.1.2. Cytokines

All recombinant cytokines were reconstituted with ddH_2O, aliquoted and storaged at -20°C until used. After thawing, aliquotes were kept at 4°C for short-term storage.

- Culture and expansion of human CD34+ cells

Cytokine	Concentration	Company
human Flt-3 ligand	100 ng/ml	CellSystems Biotechnology (St. Katharinen, Germany)
human SCF	100 ng/ml	CellSystems Biotechnology
human TPO	20 ng/ml	CellSystems Biotechnology

- Differentiation in CD14+ cells

Cytokine	Concentration	Company
human Flt-3 ligand	50 ng/ml	CellSystems Biotechnology
human SCF	50 ng/ml	CellSystems Biotechnology
human TPO	20 ng/ml	CellSystems Biotechnology
human G-CSF	10 ng/ml	CellSystems Biotechnology
human GM-CSF	10 ng/ml	CellSystems Biotechnology

- Culture and expansion of murine HSPC

Cytokine	Concentration	Company
human Flt-3 ligand	100 ng/ml	CellSystems Biotechnology
murine SCF	50 ng/ml	CellSystems Biotechnology
murine IL-3	10 ng/ml	CellSystems Biotechnology
human IL-11	100 ng/ml	CellSystems Biotechnology

2.1.3. Enzymes

All enzymes employed in molecular biology were purchased from New England Biolabs (Bad Schwalbach, Germany), Fermentas (Neu-Ulm, Germany) and Promega (Mannheim, Germany).

2.1.4. Buffers and solutions

All buffers and solutions were diluted in ddH_2O and stored at room temperature unless specified otherwise.

- (30%) Acrylamide

Components	Concentration	Company
Acrylamide	292 g/l	Carl-Roth (Karlsruhe, Germany)
Bis-acrylamide	8 g/l	Carl-Roth

Storage: 4°C

- (1%) Agarose gel

Components	Concentration	Company
TAE-buffer	diluent	(see composition bellow)
Agarose	1%	Biozym (Oldendorf, Germany)
Ethidium bromide	2 ug/ml	AppliChem

Preparation: agarose was added to TAE-buffer and warmed to get diluted. Ethidium bromide was added shortly before use.

- Blocking solution

Components	Concentration	Company
Non-fat milk	5%	Sigma-Aldrich
Tween-20	0.1%	Sigma-Aldrich

Preparation: All components were diluted in PBS
Storage: 4°C

- (5x) Bradford reagent

Components	Concentration	Company
Coomassie brilliant blue	1 mg/ml	Sigma-Aldrich
Ethanol	25%	Sigma-Aldrich
H_3PO_4	50%	Sigma-Aldrich

- DNA loadying dye

Components	Concentration	Company
Bromophenol blue	0.15%	Sigma-Aldrich
Xylenecyanol	0.10%	Nunc (Wiesbaden, Germany)
EDTA	50 mM	Armin Baack (Schwerin, Germany)
Glycerol	50%	Sigma-Aldrich

Storage: 4°C

- 2x Hepes-bufferd saline (2xHBS)

Components	Concentration	Company
NaCl	0.3 M	Qiagen (Hilden, Germany)
Hepes	50 mM	Gibco (Karlsruhe, Germany)
Na_2HPO_4	1.5 mM	Operon (Köln, Germany)

pH was adjusted to 7.2.

- MACS buffer

Components	Concentration	Company
BSA	0.5%	Merck
EDTA	2 mM	Armin Baack

Preparation: All components were diluted in PBS and pH was adjusted to 7.6.
Storage: 4°C

- Hypotonic lysis buffer

Components	Concentration	Company
Hepes	20 mM	Gibco
KCl	10 mM	Carl-Roth (Karlsruhe, Germany)
$MgCl_2$	1 mM	Sigma-Aldrich
Glycerin	20%	Sigma-Aldrich
Triton X-100	0.1%	Sigma-Aldrich
Dithiothriol	0.5 M	Roche (Mannheim, Germany)
Na_3VO_4	1 mM	Merck (Darmstadt, Germany)
Protease inhibitor cocktail	70 ul/ml	Sigma-Aldrich

pH was adjusted to 7.6

- Slide mounting solution (microscopy)

Components	Concentration	Company
MOWIOL 4-88	0.133 g/ml	Calbiochem, Merk Eurolab GmbH (Darmstadt, Germany)
DABCO	250 mM	Sigma-Aldrich
Glycerol	0.3 g/ml	Carl-Roth
Tris-HCl	0.13 M	Carl-Roth

Preparation: glycerol and mowiol were dissolved in ddH_2O with agitation for 1h at RT. Tris-HCl was added and incubated 2h at 50°C under periodical stirring. DABCO was dissolved by stirring at RT. The mounting solution was aliquoted and stored until use.
Storage: -20°C

- (1x) Phosphate buffered saline (PBS)

Components	Concentration	Company
NaCl	160 g/l	Qiagen
$Na_2HPO_4 \bullet 2H_2O$	23.6 g/l	Sigma-Aldrich
KH_2PO_4	4 g/l	Operon
KCl	4 g/l	Carl-Roth

pH was adjusted to 7.2.

- (5x) Protein loadying dye

Components	Concentration	Company
Tris-base	60 mM	Carl-Roth
Glycerol	25%	Sigma-Aldrich
SDS	2%	Sigma-Aldrich
Bromophenol blue	0.1%	Sigma-Aldrich
β-mercaptoethanol	50 ul/ml	Sigma-Aldrich

- Running buffer

Components	Concentration	Company
Tris-base	2.9 g/l	Carl-Roth
Glycine	14.4 g/l	Sigma-Aldrich
SDS	1 g/l	Sigma-Aldrich

pH was adjusted to 8.2

- SDS-PAGE buffer

Components	Concentration	Company
Tris-base	25 mM	Carl-Roth
Glycine	192 mM	Sigma-Aldrich
SDS	0.1%	Sigma-Aldrich

- (1x) TAE-Buffer

Components	Concentration	Company
Tris-base	40 mM	Carl-Roth
Acetic acid	20 mM	Carl-Roth
EDTA	1 mM	Armin Baack

- Transfer buffer

Components	Concentration	Company
Glycine	14.4 g/l	Sigma-Aldrich
Tris-base	3 g/l	Carl-Roth
Methanol	20%	Sarstedt (Nümbrecht, Germany)

- Trypsin/EDTA

Components	Concentration	Company
Trypsin	500 mg/l	Sigma-Aldrich
EDTA	200 mg/l	Armin Baack

Preparation: All components were diluted in PBS
Storage: -20°C

- Washing solution

Components	Concentration	Company
Non-fat milk	0.5%	Sigma-Aldrich
Tween-20	0.1%	Sigma-Aldrich

Preparation: All components were diluted in PBS
Storage: 4°C

- Other solutions

Solution	Concentration	Storage	Company
$CaCl_2$	200 mM	RT	Merck
Chloroquine	25 mM	-20°C	ICN Biomedicals (Aurora, USA)
Cyclosporin A	1 mg/ml	-20°C	Sigma-Aldrich
Fibronectin	10 mg/ml	-20°C	Roche (Mannheim, Germany)
Ficoll Paque		RT	GE Healthcare Bio-Sciences AB (Uppsala, Sweden)
Mitomycin C	2 mg/ml	-20°C	Sarstedt
Polybrene	8 ug/ml	-20°C	Sigma-Aldrich
Retronectin	10 mg/cm^2	-20°C	Takara Bio Europe (Gennevilliers, France)
Türks solution		RT	Sigma-Aldrich
Trypan blue Stain		RT	Merck

2.1.5. Commercial kits

Kit	Company
ECL Plus Western blotting detection reagents	Genovac (Freiburg, Germany)
Fix & perm Kit	Caltag-Medsystems (Buckingham, UK)
Lineage Cell Depletion Kit	Miltenyi Biotech
Plasmid maxi kit	Qiagen
Qia Gel Extraction Kit	Qiagen
Qia Miniprep Kit	Qiagen

2.1.6. Bacterial culture

Media for growing bacteria were prepared diluting all components in ddH_2O and sterilized by autoclaving. Prior to use, ampicillin was always added to a final concentration of 100 mg/ml. LB medium and agar plates were stored at 4°C.

- Agar plates

Components	Concentration	Company
LB medium	diluent	(see below)
Agar	4g /l	Carl-Roth
Ampicillin	100 mg/l	Sigma-Aldrich

- LB medium

Components	Concentration	Company
Tryptone	10 g/l	Carl-Roth
Yeast extract	10 g/l	Carl-Roth
NaCl	5 g/l	Carl-Roth

- Freezing medium for bacteria

Components	Concentration	Company
LB medium	diluent	
Glycerol	10%	Carl-Roth

2.1.7. Cell culture media

All cell culture media were stored at 4°C and prewarmed at 37°C prior to use. Only the freezing medium was used without prewarming.

- Supplemented alfa-MEM

Components	Concentration	Company
alfa-MEM	diluent	Gibco
Horse serum	20%	StemCell Technologies (Vancouver, Canada)
Penicillin	100 U/ml	Gibco
Streptomycin	0.1 mg/ml	Gibco
L-glutamine	0.3 mg/ml	Gibco

- Supplemented DMEM-medium

Components	Concentration	Company
DMEM-medium	diluent	Sigma-Aldrich
FCS	10%	StemCell Technologies
Penicillin	100 U/ml	Gibco
Streptomycin	0.1 mg/ml	Gibco
L-glutamine	0.3 mg/ml	Gibco

- Supplemented IMDM-medium

Components	Concentration	Company
IMDM medium	diluent	Sigma-Aldrich
FCS	10%	StemCell Technologies
Penicillin	100 U/ml	Gibco
Streptomycin	0.1 mg/ml	Gibco
L-glutamine	0.3 mg/ml	Gibco

- Supplemented RPMI-medium

Components	Concentration	Company
RPMI-1640 medium	diluent	Sigma-Aldrich
FCS	10%	StemCell Technologies
Penicillin	100 U/ml	Gibco
Streptomycin	0.1 mg/ml	Gibco
L-glutamine	0.3 mg/ml	Gibco

- Supplemented Stemspan serum-free

Components	Concentration	Company
Stemspan HS2000	diluent	StemCell Technologies
Penicillin	100 U/ml	Gibco
Streptomycin	0.1 mg/ml	Gibco
L-glutamine	0.3 mg/ml	Gibco

- Transfection medium

Components	Concentration	Company
DMEM-medium	diluent	Gibco
FCS	10%	StemCell Technologies
Penicillin	100 U/ml	Gibco
Streptomycin	0.1 mg/ml	Gibco
L-glutamine	0.3 mg/ml	Gibco
HEPES	20 mM	Gibco

- Freezing medium for eukaryotic cells

Components	Concentration	Company
FCS	diluent	StemCell Technologies
DMSO	10%	Sigma-Aldrich

2.1.8 Cell lines, primary cells and culture conditions

- Cell lines

Name	Description	Culture medium
293T	human renal epithelial cell line	Supplemented DMEM
A549	carcinomic human alveolar basal epithelial cells	Supplemented DMEM
B95-8	marmoset blood leukocytes cell line	Supplemented RPMI without antibiotica
CR-1	human hematopoietic progenitor cell line	Supplemented IMDM with 5ng/ml rmIL-3
EBV-LCL	Epstein-Barr virus immortalized lymphoblastoid cell line	Supplemented RPMI
HT1080	human fibrocarcinoma-derived cell line	Supplemented DMEM
MS-5	murine stromal cell line	Supplemented alfa-MEM
SC-1	murine embryo fibroblast cell line	Supplemented DMEM

- Primary cells

Name	Description	Culture medium
CD34+ cells	human hematopoietic stem cells	Supplemented Stemspan serum-free with cytokines
Lin-	murine hematopoietic stem and progenitor cells	Supplemented Stemspan serum-free with cytokines
PBMC	(human) peripheral blood mononuclear cells	

2.1.9. Patient samples

All experiments were performed upon informed consent of patients or legal representatives. The experiments presented in this study were approved by the institutional review boards at Hannover Medical School. CD34+ cells from WAS patients and healthy individual donors were purified from bone marrow or granulocyte colony-stimulating factor (G-CSF) mobilized peripheral blood.

2.1.10. Animals

All mice were maintained and bred under specific pathogen free conditions in the central animal facility at Hannover Medical School, in agreement with local and national guidelines.

- Mause strains

Strain	Description	Origin
129 SvEv	wild-type	Taconics(Southampton, USA)
129 S6 / SvEv Tac@Bom	WASP-/-	Provided by Dr Snapper (Massachusetts General Hospital, Boston, USA)

2.1.11. Disposable wares

Item	Company
Cuvettes for DNA	UVette®, Eppendorf (Hamburg, Germany)
Cuvettes for Bradford	Becton Dickinson (Heidelberg, Germany)
Cell culture flasks	25 cm^2, 75 cm^2 and 175 cm^2, Sarstedt (Nümbrecht, Germany)
Cell culture plates	6-, 24-well plates, Sarstedt (Nümbrecht, Germany)
Conical tubes	15, 50 ml, Sarstedt (Nümbrecht, Germany)
Cryotube vials	Nunc (Roskilde, Denmark)
Flow cytometry tubes	Sarstedt (Nümbrecht, Germany)
Micropipete tips	Terumo Europe (Leuven, Belgien)
Microscope slides	Engelbrecht (Germany)
Microtubes	1.5 ml easy cup microtubes, Sarstedt (Nümbrecht, Germany)
Pipetes	2, 5, 10 and 25 ml, Sarstedt (Nümbrecht, Germany)
Sterile filters	0.22 um, JT Backer (Griesheim, Germany)
Syringes	10 ml B. Braun Melsungen (Melsungen, Germany)

2.1.12. Equipment

Device	Name, company (City, Country)
AutoMACS device	AutoMACS Separator, Miltenyi Biotech
Cell counter	Neubauer, BRAND (Wertheim, Germany)
Cell incubator	Steri-Cycle CO_2 incubator, Thermo Life Sciences (Egelsbach, Germany)
Cell sorter	MoFlo Cell Sorter (Glostrup, Denmark)
Centrifuges	Centrifuge 5415D, Eppendorf (Hamburg, Germany) Megafuge 1.0R, Heraeus (Osterode, Germany)
Electophoresis chamber	Sub-Cell GT Agarose Gel Electrophoresis System, BIO-RAD (Munich, Germany)
Flow cytometer	FACScan, BD biosciences (Heidelberg, Germany)
Fluorescence microscope	Axiovert 200 (Carl Zeiss, Göttingen, Germany)
Heat block	Thermomixer comfort, Eppendorf (Hamburg, Germany)
Image analyzer	Imaging Station, BIO-RAD (Munich, Germany)

Device	Name, company (City, Country)
Laminar flow	S1/S2 HERAsafe HS, Heraeus (Osterode, Germany)
Microscope	Carl Zeiss (Göttingen, Germany)
Mini Protean® System	BIO-RAD
Photometer	BioPhotometer, Eppendorf (Hamburg, Germany)
Pipetus	Pipetus®-akku, Hirschmann Laborgeräte (Eberstadt, Germany)
Phosphor-imaging Storm B20 Scanner	Amersham Biosciences (Freiburg, Germany)
Power supplier	POWER PAC 300, BIO-RAD (Munich, Germany)
Transilluminator	302nm, Bachofer (Reutlingen, Germany)
Vortex	REAX control, Heidolph (Schwabach, Germany)
Water bad	GFL (Burgwedel, Germany)

2.1.13. Computer software

Action	Program
Data processing	Excel 2007
Cloning	DNA Star, Clone Manager 6, VectorNTI 7
Sequencing	Flinch TV
FACS analysis	CellQuest, FlowJo
Protein detection	Quantity One
Microscopy	Openlab 3.1.7 imaging software

2.2. METHODS

2.2.1. Molecular biology

2.2.1.1. Bacterial culture and storage

Molecular cloning was performed in the *Escherichia coli* strain XL-1Gold (Stratagene, Heidelberg, Germany), which is characterized by a low frequency of recombination and a high susceptibility to transformation. All plasmids used in this study were ampicillin-resistent and bacteria were grown in Luria Bertani (LB) medium either liquid or in agar plates, always in the presence of 100 mg/ml ampicillin.

Culture of *E. coli* started out of a single colony picked up from an ampicillin containing agar plate. Bacterial colonies were grown in LB medium in the presence of ampicillin in a shaker at 220 rpm during 16 hours at 37^{o}C for amplification. For long-term storage, transformed bacteria were resuspended in bacterial freezing medium and kept at -80^{o}C.

2.2.1.2. Preparation and transformation of competent bacteria

The preparation of competent bacteria was performed following standard procedures (165). Transformation of bacteria with plasmid DNA was carried out as described in Sambrock *et al* (1989).

2.2.1.3. Separation of fragments of DNA by electrophoresis

Separation of fragments of DNA was carried out in a 0.6% to 2% agarose gel, depending on the length of the expected fragments. To this aim, the appropriate amount of agarose was added to 1xTAE-buffer and melted in a microwave. Subsequently, 2ug/ml ethidium bromide was added and the mixture was cast in the gel chamber. After solidification, DNA samples were added together with DNA-loading dye. Electrophoresis was carried out in 1xTAE buffer at 80-100 Volt during 20-40min and DNA fragments were detected using UV-light. Fragments size was determined by comparison with DNA marker, i.e., with HindIII digested Lambda Phage DNA or with 1kb marker (Fermentas, St. Leon-Rot, Germany). The presence of DNA was determined by means of the fluorescence emitted by the ethidiumbromide bound to DNA in a UV transilluminator (254nm).

2.2.1.4. Gel extraction and purification of fragments of DNA

Pieces of agarose gel containing the target fragment of DNA were separated electrophoretically and visualized using UV-light (wave length 366 nm) for extraction. Purification of DNA from the piece of agarose gel was carried out using the Gel Extraction Kit (Qiagen) following the manufacturer´s instructions.

2.2.1.5. Digestion of DNA with restriction endonucleases

Digestion of DNA was performed with the appropriate restriction enzyme following the indications provided by the manufacturer (New England Biolabs, Fermentas, Promega) in the presence of the corresponding specific buffer.

2.2.1.6. Fill-in reaction of sticky-end sites

Fragments of DNA whose end sites are sticky and not compatible must be converted to blunt-ends to allow the ligation of the plasmid. This reaction, called fill-in reaction, was carried out using the enzyme Klenow (Fermentas) in the presence of dNTPs and its specific buffer, following the manufacturer´s instructions.

2.2.1.7. Dephosphorilation of DNA

The removal of the 5'-phosphate group of a DNA fragment, process known as dephosphorilation, is an essential process to avoid religation of this fragment, when incubated in the presence of a ligase (see below). This reaction was carried out using the enzyme antarctic-phosphatase (New England Biolabs, Bad Schwalbach, Germany) and its specific buffer, following the indications provided by the manufacturer.

2*.2.1.8. Ligation of of DNA fragments*

The ligation of two fragments of DNA, a vector and a particular insert, was performed using the T4-polymerase (Fermentas), ratio 1:6, in the conditions indicated by the company.

2.2.1.9. Mini-preparation of plasmid DNA

The isolation of plasmid DNA from transformed *E. coli* was performed using the Qiaprep MiniPrep-Kit (Qiagen), following the manufacturer´s instructions.

2.2.1.10. Maxi-preparation of plasmid DNA

The isolation of plasmid DNA from large culture of transformed *E. coli* was performed using the Qiaprep MaxiPrep-Kit (Qiagen), following the manufacturer´s instructions.

2.2.2. Cell culture

All cell lines and primary cells used in this study were grown in the appropriate culture medium at 37^{o}C in a humidified 5% CO_2 incubator, unless specified otherwise. Medium was changed regularly and cell density was adjusted when needed.

2.2.2.1. Generation of γ-retroviral and lentiviral vectors

All cell-free viral supernatants were generated by tripartite transient cotransfection of 293T packaging cells with each transfer vector, together with packaging constructs coding for the gag-pol proteins and the envelope plasmid. To generate lentiviruses, a plasmid containing RSV-rev was aditionally cotransfected. The amount of DNA used for each transfection was: 8ug of transfer vector, 12ug of gag/pol plasmid, 5ug of envelope plasmid and 5ug of RSV-rev plasmid. Briefly, six to seven million cells per 10cm tissue culture dish were seeded in supplemented DMEM. Next day, medium was replaced by 8ml of transfection medium containing 25uM chloroquine per dish. Subsequently, plasmids were diluted in bi-distilled

water containing 50ul of 2M $CaCl_2$ in a final volume of 500ul. The DNA/$CaCl_2$-mixture was added dropwise to 500ul of 2xHBS and inmediately vortexed shortly. The DNA-mixture with calcium phosphate was added to the cells and plates were kept at 37°C in a humidified 5% CO_2 incubator. Medium with chloroquine was discarded and replaced by equal volume of transfection medium 10 to 16h later. Culture medium was collected at 24, 48 and 72h after transfection, filtered through a 0.22um-filter, aliquoted and stored at –80°C until used.

2.2.2.2. Titration of viral supernatants

The viral titers were determined by infecting SC-1 (ecotropic viruses) or HT1080 cells (if viruses were GalV- or VSV-G-pseudotyped, except R-WAS-W (see Figure 3.2.A), which was titered in CR-1 cells) with serial dilutions of thawed vector stock, in the presence of 8ug of polybrene per mililiter of medium. Three days later, transduced cells were harvested, resuspended in PBS and analyzed by flow cytometry to determine the percentage of GFP-expressing cells and calculate the corresponding viral titer.

2.2.2.3. Isolation of mononuclear cells from peripheral blood

Heparinized peripheral blood was mixed with equal volume of PBS and carefully layed over 10ml of Ficoll Paque in a 50ml-conical tube. After centrifuging the sample during 30min at 450g without brake, the buffy coat interface containing the peripheral blood mononuclear cells (PBMC) was removed and transferred into a new 50ml-conical tube, washed twice with PBS and resuspended for cell count using the trypan blue exclusion method. Cells were aliquoted and kept at –80°C in freezing medium until used.

2.2.2.4. Generation of Epstein-Barr Virus (EBV)-immortalized B cell lines (EBV-LCL)

The procedure to immortalize B lymphocytes by EBV can be divided into two stages:

I. Preparation of EBV-containing culture supernatants: one million exponentially growing B95-8 cells were incubated in complete RPMI during three days. Subsequently, cells were centrifuged at 4°C during 10min at 300g and the EBV-containing culture supernatant was separated, filtered through a 0.45-um filter, aliquoted and stored at -80°C.

II. Infection of target cells: 10^7 PBMC resuspended in 2.5ml of complete RPMI were cultured together with 2.5ml of EBV-supernatant in a 50ml-conical tube in a 37°C water bath. After incubating two hours, 5ml of complete RPMI supplemented with 1ug/ml

cyclosporin A were added and the whole cell suspension was transferred to a 25-cm^2 tissue culture flask and incubated for three weeks. At this time, the cell line can be cryopreserved at –80°C in freezing medium or maintained in long-term culture.

2.2.2.5. Isolation and purification of bone marrow (BM)-derived Lineage negative (Lin-) cells

Donor mice were sacrified and tibiae and femurs were obtained. Total bone marrow was flushed from bones with a syringe (0.4 x 12mm) with supplemented IMDM and always kept on ice in the same medium. Then, cells were passed through another syringe (0.6 x 33mm) and expelled into a new tube to obtain a single cell suspension. Leukocytes were counted using Türks solution, which selectively stains living leucocytes, usually yielding aprox. $50x10^6$ BM white cells per animal. Subsequently, cells were centrifuged 10 min at 1000rpm to discard the supernatant completely and resuspended in MACS buffer to a density of 10^7cells in 40ul. Lineage negative (Lin-) cells were separated from total bone marrow using the Lineage Cell Depletion Kit (Miltenyi Biotech) following the instructions provided by the manufacturer. Briefly, total BM cells were magnetically labeled with a cocktail of biotinylated antibodies against a panel of lineage antigens (CD3, CD45R (B220), CD11b, Gr-1 (Ly-6G/C), and Ter-119) and anti-biotin MicroBeads. Using an AutoMACS device (Miltenyi Biotech), Lin- cells were automatically separated, whereas mature hematopoietic cells such as T cells, B cells, monocytes, macrophages, granulocytes and erythrocytes as well as their committed precursors were depleted. This method usually yielded $5x10^5$ Lin- cells per animal. Finally, cells were aliquoted and stored at –80°C in freezing medium until use or directly cultured for expansion.

2.2.3. Viral transduction

2.2.3.1. Retronectin pre-coating of plates for viral preloading

For precoating, 24 well-plates were incubated with 10mg of RetroNectin per cm^2 either two hous at room temperature or overnight at 4°C. Subsequently, RetroNectin was discarded and wells were blocked 30min with PBS+2%BSA at room temperature before washing three times with PBS+2,5% (v/v) 1M HEPES. This washing solution was discarded before freshly thawed viral supernatants were added.

2.2.3.2. Transduction of non-hematopoietic human and murine cell lines

Freshly thawed viral supernatants were loaded onto 6 well-plates and centrifuged at 1000g, during 30min at 4°C. Then, supernatants were discarded and 10^5 cells were seeded in each well. Twenty-four hours later, the proceadure was repeated, exactly in the same conditions as the day before. Transduced cells were incubated for expansion 48h to 72h, before starting further experiments.

2.2.3.3. Transduction of human EBV-LCL

Viral supernatant was loaded on retronectin-precoated 24-well plates and centrifuged at 800g during 15min, at 4°C. After centrifugation, supernatant was discarded and $2x10^6$ cells in supplemented IMDM were seeded per well. Cells were incubated 48h to 72h for expansion before performing further experiments.

2.2.3.4. Ex vivo *transduction of murine BM-derived Lin- cells*

Prior to transduction, Lin- cells were prestimulated for two days in supplemented Stemspan serum-free medium with cytokines (see Section 2.1.2.) at a density of 1 to 5 $x10^5$ cells per mililiter. On day three, viral supernatant was loaded on RetroNectin-precoated 24-well plates and centrifuged at 800g during 15min, at 4°C. After centrifugation, supernatant was discarded and $2x10^5$ cells resuspended in 250ul fresh culture medium with cytokines were seeded per well. On day four, cells were transferred to freshly prepared plates, preloaded with RetroNectin and viral vector for a second round of transduction. Afterwards, they were incubated 48h to 72h for expansion before performing further experiments. Culture medium was changed and fresh cytokines were provided regularly.

2.2.3.5. Ex vivo *transduction of human CD34+ cells*

Cells were expanded for two days prior to transduction. On day three, cells were transduced with GalV-pseudotyped viruses at MOI=5 by incubation on suspension culture dishes previously loaded with viral supernatant. Virus preloading was carried out on RetroNectin-coated (10 g/cm^2; TaKaRa) dishes, as indicated in Section 2.2.3.1.. Transduced cells were incubated for expansion 48h to 72h before proceeding to *in vitro* differentiation.

2.2.4. Laboratory methods

2.2.4.1. *MACS separation*

MACS separation of samples was always carried out following the instructions provided by the manufacturers.

2.2.4.2. Flow cytometry and cell sorting

For fluorescence-activated cell sorting (FACS) analysis, at least fifty thousand viable cells per sample (as determined by trypan blue exclusion) were harvested and resuspended in PBS. Data were collected with a FACSCalibur (BD-Pharmingen, SD, CA) equipped with the standard fluorescein filter set and the fluorescence distribution was analized using CELLQuest software (BD Biosciences, San Jose, CA). For cell sorting, cells were harvested, washed with PBS and resuspended in PBS supplemented with 10% FCS before being sorted in a MoFlo cell sorter.

2.2.4.3. RNA isolation and northern-blot

RNA was isolated (from 3-5x10^6 cells) using RNAzol (WAK Chemicals, Steinbach, Germany) according to the manufacturer´s instructions. The RNA pellet was resuspended in ultra pure water supplemented with 1ul RiboLock Rnase inhibitor (Fermentas) and stored at -20^{o}C. To analyze isolated RNA, samples were quantified photometrically and 10ug of each RNA were separated in denaturing formaldehyde gels. RNA was blotted to Biodyne-B membranes (Pall Corp., Pensacola, FL, USA) by capillary transfer and heat fixed for 2h at 80^{o}C. Hybridization was performed with probes that corresponded either to the PRE fragment or to 18S rRNA following standard procedures (158). The PRE-specific probe corresponds to the PRE cDNA and was generated by digestion for the corresponding plasmid with SalI and Acc65I. To detect 18S rRNA, a genomic fragment was PCR-amplified and subcloned into pCR2.1 (Invitrogen, Karlsruhe, Germany). The probes were radiolabelled using the DecaLabel Kit (Fermentas) and quantified with a phosphor-imaging Storm B20 Scanner (Amersham Biosciences, Freiburg, Germany).

2.2.4.4. Protein isolation and western-blot.

Cells were washed with cold PBS, resuspended in cold hypotonic lysis buffer and incubated on ice during 5 min. Then, lysates were centrifuged for 5 min at 4000 rpm at 4^{o}C to collect supernatants. A small aliquote was separated from each sample to quantify the protein

content and samples were stored at –80°C until used. The protein concentration was calculated with a standard curve, established with different concentrations of BSA, using the Bradford method. All samples and standards were measured in a spectrophotometer (Eppendorf, Germany). For Western blot analysis, 10 to 15 ug of protein were loaded on 10% SDS-polyacrylamide gels, separated by electrophoresis and blotted onto nitrocellulose membrane. The membrane was exposed to anti-WASP monoclonal (clone D-1) primary antibody. As secondary antibody, horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody was used. Protein detection was performed with the Amersham ECL Plus Western blotting detection reagents (Genovac, Freiburg, Germany). Membranes were reprobed with GAPDH monoclonal antibody, followed by HRP-conjugated goat anti-mouse antibody. The hybridization and detection were done according to the instructions provided by the antibody manufacturer.

2.2.4.5. Intracellular staining and detection

Intracellular WASP was stained using the Fix & Perm kit (Caltag Laboratories, Burlingame, CA, USA), following the protocol provided by the manufacturer. Anti-human WASP monoclonal IgG2a and mouse IgG2a isotype (control) were used as primary antibodies. As a secondary antibody goat F(ab0)2 fragment rat IgG-phycoerythrin was used.

2.2.4.6. In vitro *differentiation of CD34+ cells into myeloid progeny*

Fifty thousand MS-5 feeder cells were seeded per well in a 24 well-plate in 1ml supplemented alfa-MEM and incubated for 12-16h. After incubation, the supernatant was discarded and replaced with 500 ul of fresh medium containing 10 mg/ml mitomycin C and the cells were incubated 2 hours. Subsequently, cells were washed three times with prewarmed sterile PBS and incubated 2 hours with fresh medium at 37°C. Finally, ten thousand CD34+ cells resuspended in supplemented IMDM-medium with cytokines for differentiation were seeded in each well and incubated for two additional weeks.

2.2.4.7. Staining and detection of podosomes

Fifty thousand cells were plated on 25mm^2 coverslips in six-well plates previously coated with 10 mg/mL fibronectin and incubated for 2 hours at 37°C. Cells were fixed for 20 minutes in 4% paraformaldehyde, permeabilized for 5 minutes in 0.2% Triton-X-100 in PBS and blocked with 1% BSA for 30 minutes. Cells were stained with anti-vinculin antibody for 30

minutes followed by staining with Cy5-conjugated rabbit anti-mouse IgG F(ab0)2 fragment and phalloidin-TRITC to detect F-actin. Coverslips containing cells were transferred to slides with mounting solution and examined using a fluorescence microscope Axiovert 200 equipped with an Axioplan 2 imaging system (Carl Zeiss, Göttingen, Germany). Images were captured with a PLAN-APOCHROMAT 63X/1,4 oil objective at room temperature and acquired with the Openlab 3.1.7 imaging software (Improvision Ltd., Coventry, UK).

3. RESULTS

3.1. Generation of viral vectors

3.1.1. γ-Retroviral vectors

To minimize the risk of formation of RCR and to reduce the likelihood of activation of cellular genes, an advanced generation, self-inactivating (SIN) γ-retroviral vector (GV), was constructed. In this vector, named SRS11.EFS.WASP.iresGFP.pre (R-EFS-W), the full-length human *WASP* cDNA was cloned through XbaI and SalI unique restriction sites downstream the human elongation factor 1-α (EFS) short promoter (Fig. 3.1.A). To make the vector bicistronic, *iresGFP* was cloned into a unique BamHI restriction site downstream the therapeutic transgene. By replacing the EFS internal promoter with the phosphoglycerate kinase (PGK) promoter, the Wiskott-Aldrich Syndrome (WAS) promoter and spleen focus forming virus (SFFV) U3 promoter/enhancer, three new additional SIN-GV, named SRS11.PGK.WASP.iresGFP.pre (R-PGK-W), SRS11.WAS.WASP.iresGFP.pre (R-WAS-W) and SRS11.SFFV.WASP.iresGFP.pre (R-SF-W) respectively, were constructed. All these constructs carry the woodchuck hepatitis virus post-transcriptional regulatory element (PRE) downstream the marker gene (Fig.3.1.A).

The substitution of *WASP* cDNA by a codon-optimized *WASP* (*coWASP*) sequence (Epoch Biolabs Inc., Texas, USA, with minor modifications) was carried out in R-EFS-W and R-SF-V through AgeI/XbaI and SalI unique restriction sites, thus generating SRS11.EFS.coWASP.iresGFP.pre (R-EFS-coW) and SRS11.SFFV.coWASP.iresGFP.pre (R-SF-coW) respectively, which constituted the second series of SIN γ-retroviral vectors (Fig. 3.1.B).

The cloning of the CMMP.WASP.iresGFP (CMMP) vector, which contains an intact myeloproliferative sarcoma virus (MPSV) long terminal repeat (LTR), was performed inserting the *iresGFP* sequence in the BamHI unique restriction site in the CMMP.WASP vector. This vector is currently being used in the Hannover clinical trial for WAS and has been previously described (87) (Fig.3.2.).

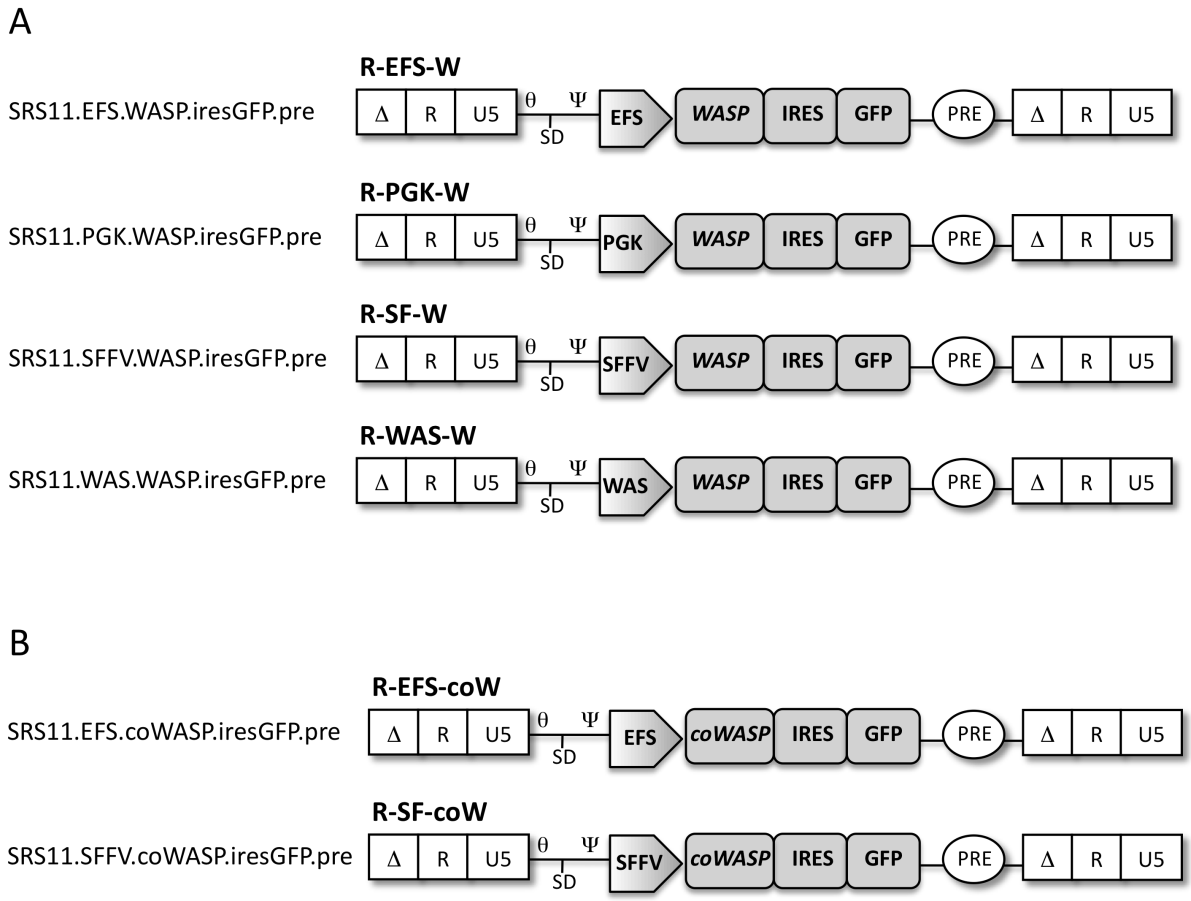

Figure 3.1. Design of Self-Inactivating (SIN) gammaretroviruses (GV) used in this study. Deleted viral enhancer/promoter in U3 (Δ), R region (R), U5 region of the LTR (U5), elongation factor 1-α (EFS) promoter, *WAS protein* (*WASP*) gene, codon optimized WASP (*coWASP*), internal ribosomal entry site (IRES), green fluorescent protein (GFP), woodchuck hepatitis virus post-transcriptional regulatory element (PRE), splice donor (SD), primer binding site (Θ), packaging signal (Ψ). (A) GVs containing WASP wild-type cDNA as transgene (B) GVs containing the codon optimized-WASP (*coWASP*) as transgene.

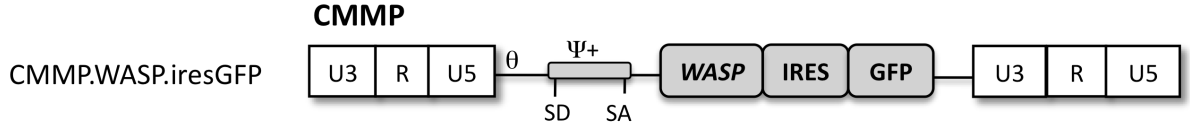

Figure 3.2. Modified LTR-driven GV used in the Hannover clinical trial for WAS. For this study, the *iresGFP* sequence was cloned downstream *WASP* in the LTR-driven GV used in the Hannover clinical trial. U3 region derived from MPSV (U3), splice acceptor (SA), extended packaging signal (Ψ+). See other abbreviations in figure 3.1.

3.1.2. SIN-lentiviral vectors

To generate the lentiviral counterpart of R-EFS-W, the same expression cassette was cloned through a NotI (blunted by Klenow polymerase) / SpeI restriction into the XhoI (blunted by Klenow polymerase) / SpeI sites of pRRL.PPT.SF.GFP.pre, a standard 3rd generation lentiviral vector, also harbouring PRE downstream *GFP*. The resulting vector was named pRRL.PPT.EFS.WASP.iresGFP.pre (L-EFS-W) (Fig.3.3.A). By substituting the EFS promoter by SFFV, we generated pRRL.PPT.SFFV.WASP.iresGFP.pre (L-SF-W), thus constituting the first batch of SIN-lentiviral vectors (LV) (Fig. 3.3.A).

The replacement of *WASP* cDNA by the codon-optimized *WASP* (*coWASP*) sequence was carried out in L-EFS-W and L-SF-V through AgeI/XbaI and SalI unique restriction sites, thus generating pRRL.PPT.EFS.coWASP.iresGFP.pre (L-EFS-coW) and pRRL.PPT.SFFV.coWASP.iresGFP.pre (L-SF-coW) respectively, which constituted the second series of SIN-lentiviral vectors (Fig. 3.3.B).

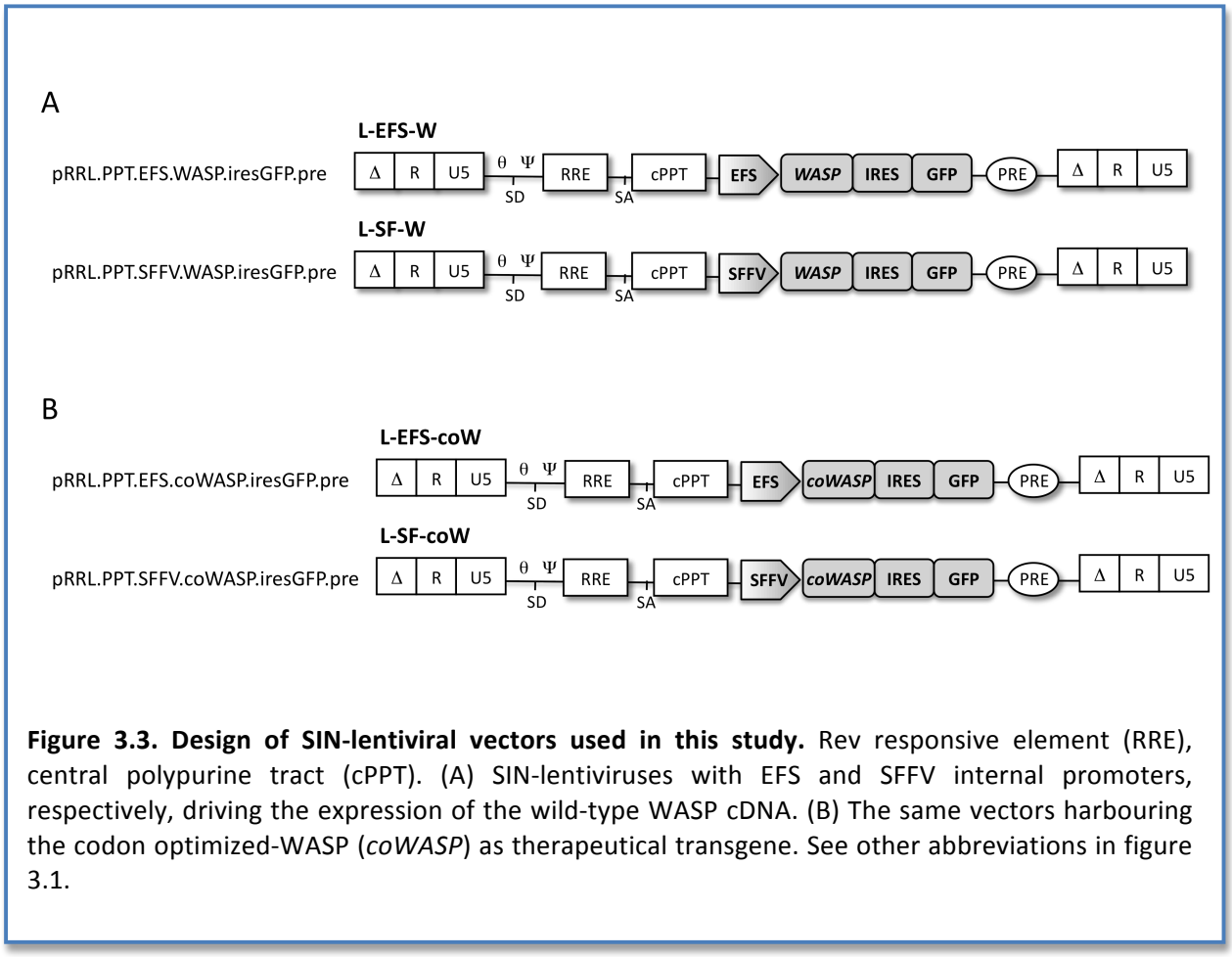

Figure 3.3. Design of SIN-lentiviral vectors used in this study. Rev responsive element (RRE), central polypurine tract (cPPT). (A) SIN-lentiviruses with EFS and SFFV internal promoters, respectively, driving the expression of the wild-type WASP cDNA. (B) The same vectors harbouring the codon optimized-WASP (*coWASP*) as therapeutical transgene. See other abbreviations in figure 3.1.

3.1.3. Titration of viral supernatants

After transfection of 293T, supernatants containing viral particles were collected, stored and titered separatedly every 12h from 24h to 60h after transfection. The titers of the viral supernatants were calculated on the basis of GFP expression measured by flow cytometry, as described in Materials and Methods. Average titers at these time points are summarized in Figure 3.4.

CONSTRUCT	TIME POST-TRANSFECTION			
	24h	36h	48h	60h
CMMP	2,5	3,8	2,1	1,4
L-EFS-W	2,8	6,2	1,5	0,6
L-EFS-coW	3,0	5,9	1,4	0,7
L-SF-W	3,3	6,7	1,8	0,2
L-SF-coW	3,2	5,5	1,1	0,2
R-EFS-W	2,0	4,5	1,8	0,4
R-EFS-coW	3,6	6,7	3,4	0,9
R-PGK-W	1,9	3,9	0,9	0,2
R-SF-W	3,0	5,9	3,5	0,5
R-SF-coW	3,6	7,1	2,9	0,3
R-WAS-W	1	2,2	0,7	0,1

Figure 3.4. Table of viral titers. Titers are expressed in 10^6 pfu/ml.

3.1.4. Kinetics of production of viral particles

The use of SIN vectors has prompted a shift in vector production methodology from the traditional use of stable packaging cell lines to the use of transient production by transfection. This is firstly due to the fact that transfection-based methodology offers greater flexibility as changes to plasmids can be made without having to generate another stable producer cell line. Secondly and more importantly, while LTR vectors allow for efficient generation of clones of vector producing cells using transduction, the same approach cannot be used for SIN vectors as the SIN U3 deletion prevents genomic RNA to be packaged after transduction (see Section 1.4.1.). All viral supernatants used in this study have been generated using the 293T cell line, which is well characterized, is highly transfectable, and expresses viral restriction factors only at low levels (166). To reduce the chance of generating RCR or RCL, vector and packaging sequences have been introduced into the producer cell using a split-(genome) packaging system (Figure 1.6.) (167, 169, 170).

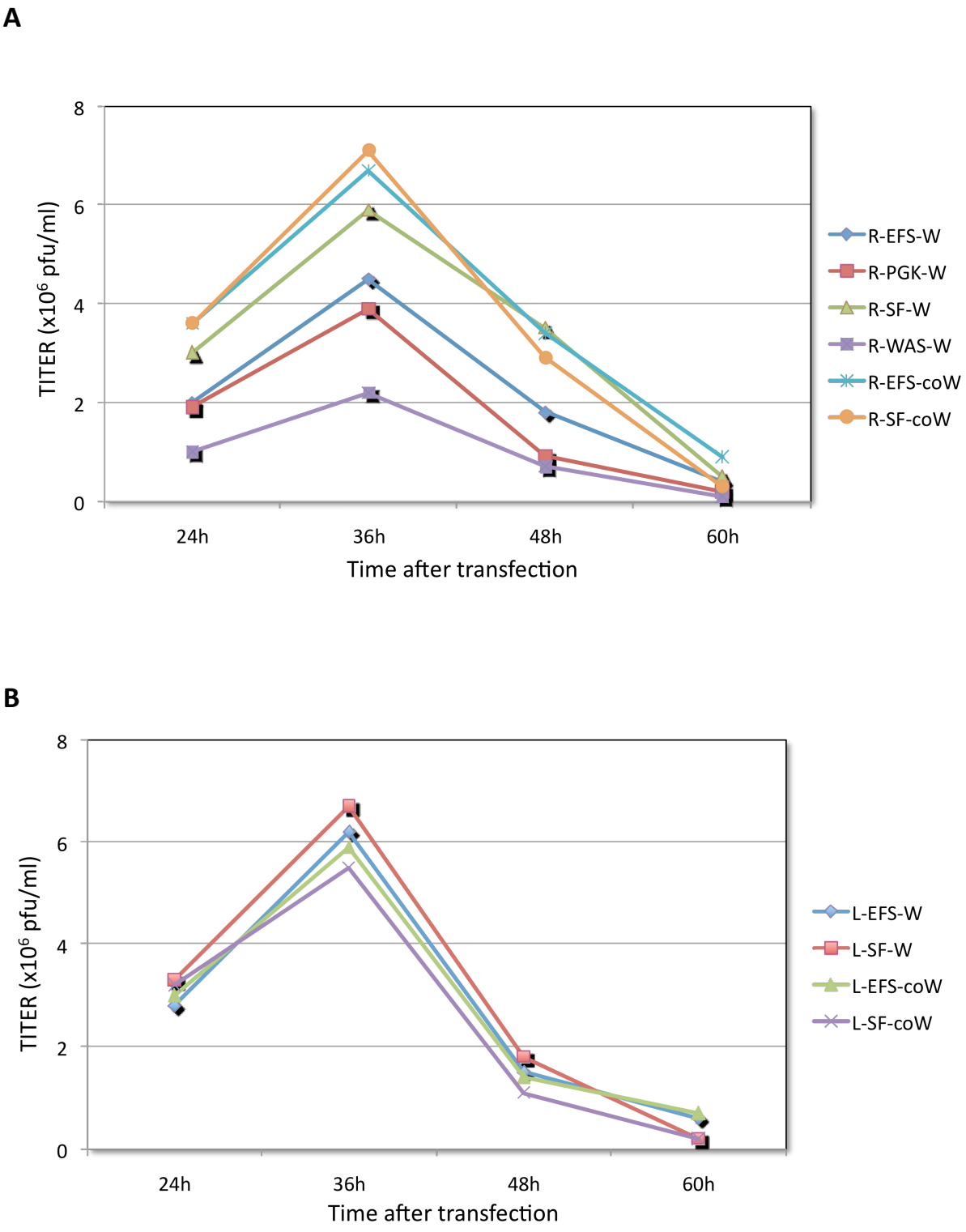

Figure 3.5. Kinectics of production of viral particles. (A) Follow-up of generation of viral particles of GVs carrying (A) *WASP* cDNA and (B) *coWASP* as transgene. (B) Follow-up of generation of viral particles of LVs harbouring (A) *WASP* cDNA and (B) *coWASP* as transgene.

To precisely determine the time point in which the maximal viral production occurrs, all supernatants were collected and titered separatedly every 12h, during 60h after transfection. In all cases, the highest production of viral particles was observed 36h post transfection, decreasing to undetectable levels at 60h (Figure 3.5.). The codon optimization of WASP tended to improve titers of GVs, but not of LVs. Importantly, all titers are high enough for clinical applications if data can be reproduced at large scale.

3.2. The choice of the internal promoter

To select the most suitable internal promoter (IP) to drive the expression of WASP transgene, three different cell lines (two non-hematoopoietic: murine SC-1 and human HT1080 cells and two hematopoietic: EBV-LCL from two WAS patients) were transduced with each GV containing the wild-type *WASP* cDNA as transgene. Transduction of human cells was performed with GALV-pseudotyped vectors whereas ecotropic vectors were used to transduce murine cells. All experiments were carried out using a very low multiplicity of infection (MOI=1), to avoid saturating conditions (168), intensify the differences among the viruses, and also to observe the behaviour of these vectors in conditions that partially mimick those used in preclinical and clinical assays, where the lowest number of insertions per genome is desired (ideally one copy per genome) to reduce the risk of insertional mutagenesis. Three days after transduction, samples were examined by flow cytometry. The expression of GFP was used as readout parameter to determine the transduction efficiency achieved with each vector and the geometrical mean intensity (GeoM) of the emitted fluorescence was used to quantify the strength of the promoter. CMMP, representing the vector that is currently being used in the Hannover clinical trial, was included as well and results obtained with SIN-GV were referred to it. The primary data obtained after flow cytometry is shown in Figure 3.6. As expected, R-WAS-W containing the tissue-specific promoter was active only in hematopoietic (EBV-LCL) cells, yielding 9.0% GFP+ cells. In this scenario, all other SIN-GV behaved very similarly, yielding between 9.0 and 13.0% GFP+ cells, whereas CMMP yielded 4.0% GFP+ cells. In the context of the murine non-hematopoietic (SC-1) cells, efficiency achieved by SIN-GV was lower and yielded nearly the same percentages of GFP-expressing cells as CMMP, ranging from 1.0 to 1.5% of the total sample. The same results were observed in human non-hematopoietic (HT1080) cells, where the percentages of GFP+ cells were slightly higher but did not

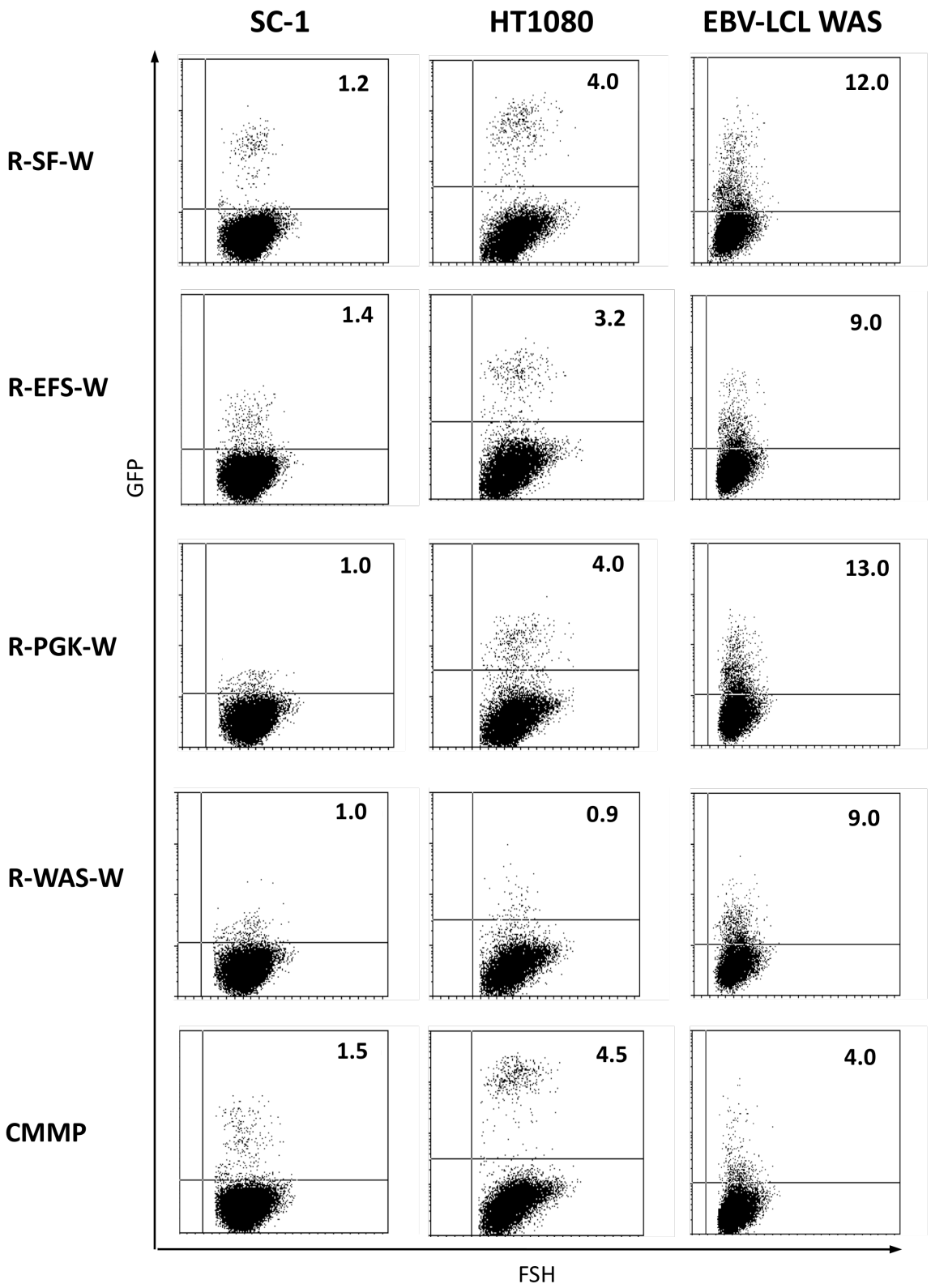

Figure 3.6. Comparative transduction of different cell lines. Fluorescence-activated cell sorting (FACS) analysis of SC-1, HT1080 and EBV-LCL upon transduction with SIN-GV compared to CMMP. Numbers in the upper right quadrant of each dot plot indicate the percentage of GFP positive cells.

significantly vary among vectors, ranging from 3.2 to 4.5% of the total, except R-WAS-W, as explained above. A more detailed analysis of these results is displayed in Figure 3.7., where expression of GFP+ cells (bars) and GeoM intensity (bounded points) for each vector and cell line were quantified.

All SIN-GV yielded very similar percentages of GFP+ cells in comparison with CMMP, especially in non-hematopoietic cells.

The comparative analysis of the GeoM values showed that R-SF-W led to the most intense expression of fluorescence, thus being the strongest promoter. On the contrary, R-WAS-W appeared to be the weakest of all four promoters, driving the lowest expression of fluorescence even in hematopoietic cells. EFS and PGK led intermediate values of GeoM intensity of GFP expression. In SC-1 and HT1080 cells, GeoM of EFS was higher than PGK and more similar to SFFV whereas in EBV-LCL, PGK and EFS yielded the same result.

Taken all together, these preliminary results showed that SFFV and EFS were the most efficient promoters in driving the expression of GFP.

Figure 3.7. Comparative analysis of different internal promoters in various cell lines (*see next page*). Bars indicate the percentage of GFP+ cells (left Y-axes) and bound points indicate the GeoM (right Y-axes) of the fluorescence emitted by GFP-expressing cells. The same analysis was performed on each cell line for comparison purposes. (A) SC-1 cells. (B) HT1080. (C) WASP-deficient EBV-LCL cells. C, CMMP; S, R-SF-W; E, R-EFS-W; P, R-PGK-W; W, R-WAS-W; UT, untransduced cells.

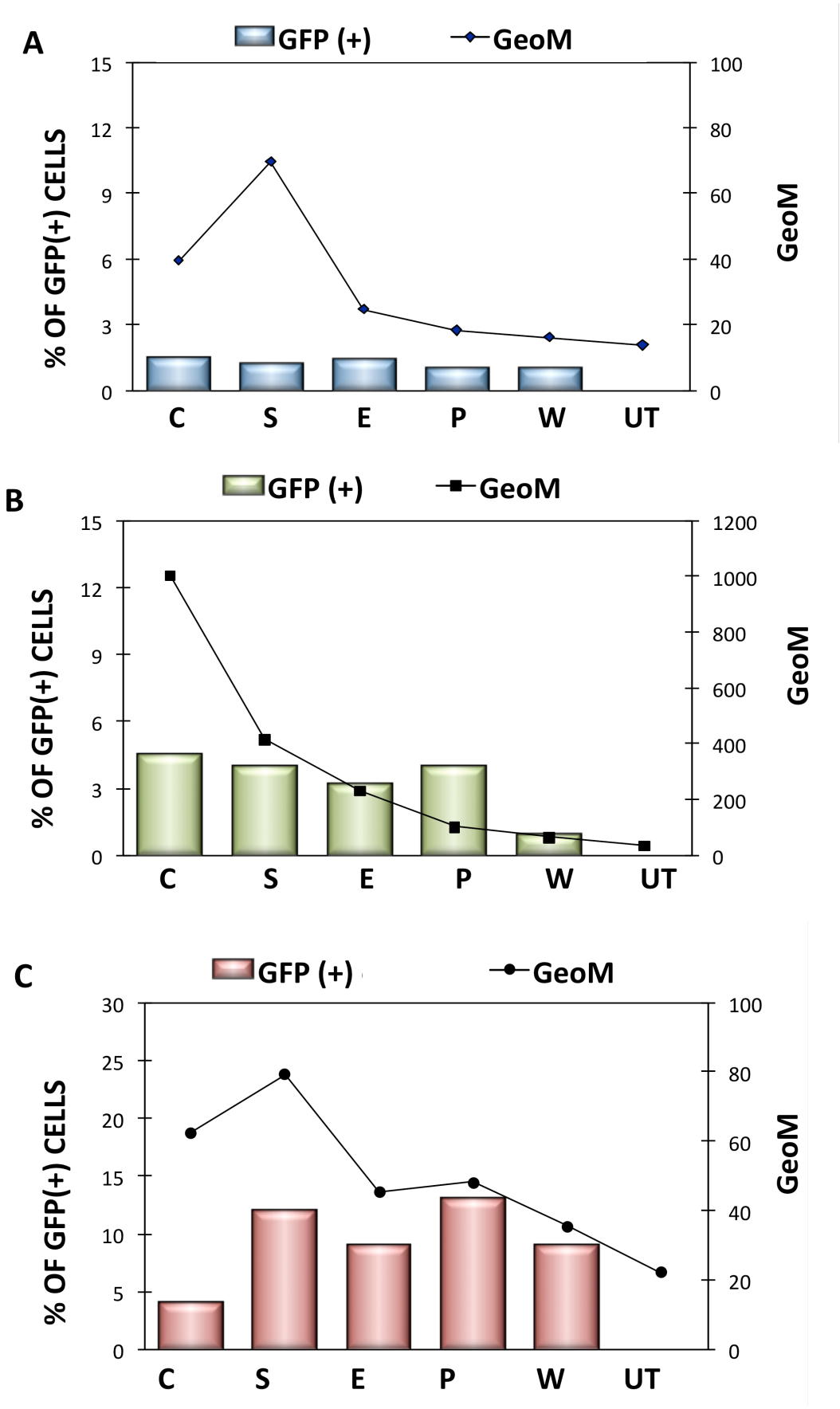

Figure 3.7. Comparative analysis of different internal promoters in various cell lines (*See previous page*).

3.3. Reconstitution of WASP expression in *WASP*-deficient B cells from two patients.

To confirm the efficiency of R-EFS-W to rescue the expression of WASP in hematopoietic cells, two EBV-LCL were established using PBMCs of two independent WAS patients (WAS1 and WAS2), and after checking their complete abscence of WASP by western-blot (Fig. 3.8.A), both cell lines were transduced at MOI=1 with this vector and with CMMP. To further work with highly pure transduced populations, cells were sorted on the basis of GFP expression to a purity of 90-96%, three days after transduction. Cell sorting after transduction with R-EFS-W yielded 9% WAS1 and 7% WAS2 GFP+ cells, respectively, whereas transduction with CMMP yielded 12% WAS1 and 8% WAS2 GFP+ cells respectively (Fig. 3.8.B), which shows that the transduction rates achieved by the SIN-GV are very similar to those achived by the CMMP vector with intact LTR.

Finally, the presence of WASP was detected by intracellular staining followed by flow cytometry in sorted GFP+ populations, untransduced cells and healthy donor cells. WASP content was assessed using the GeoM of the emitted fluorescence as readout parameter, determined with the software FlowJo (Fig. 3.8.C). The GeoM of the mock transduced cells from WAS1 and WAS2 was 3,56 and 3,25, respectively, in clear contrast with the healthy donor´s GeoM value, 7,35. As expected, the GeoM upon transduction with CMMP was higher (5,70 and 7,01, respectively) indicating that WASP is expressed upon transduction. Transduction with R-EFS-W yielded 6,96 in WAS1 and 7,29 in WAS2 GFP+ cells, values that are even closer to the healthy donor GeoM. These results indicate that R-EFS-W is able to rescue the expression of WASP in B cells from patients as well as CMMP, confirming that the new SIN-GV containing an internal promoter of cellular origin showed similar or even better expression capability in comparison with the clinically used LTR-driven vector.

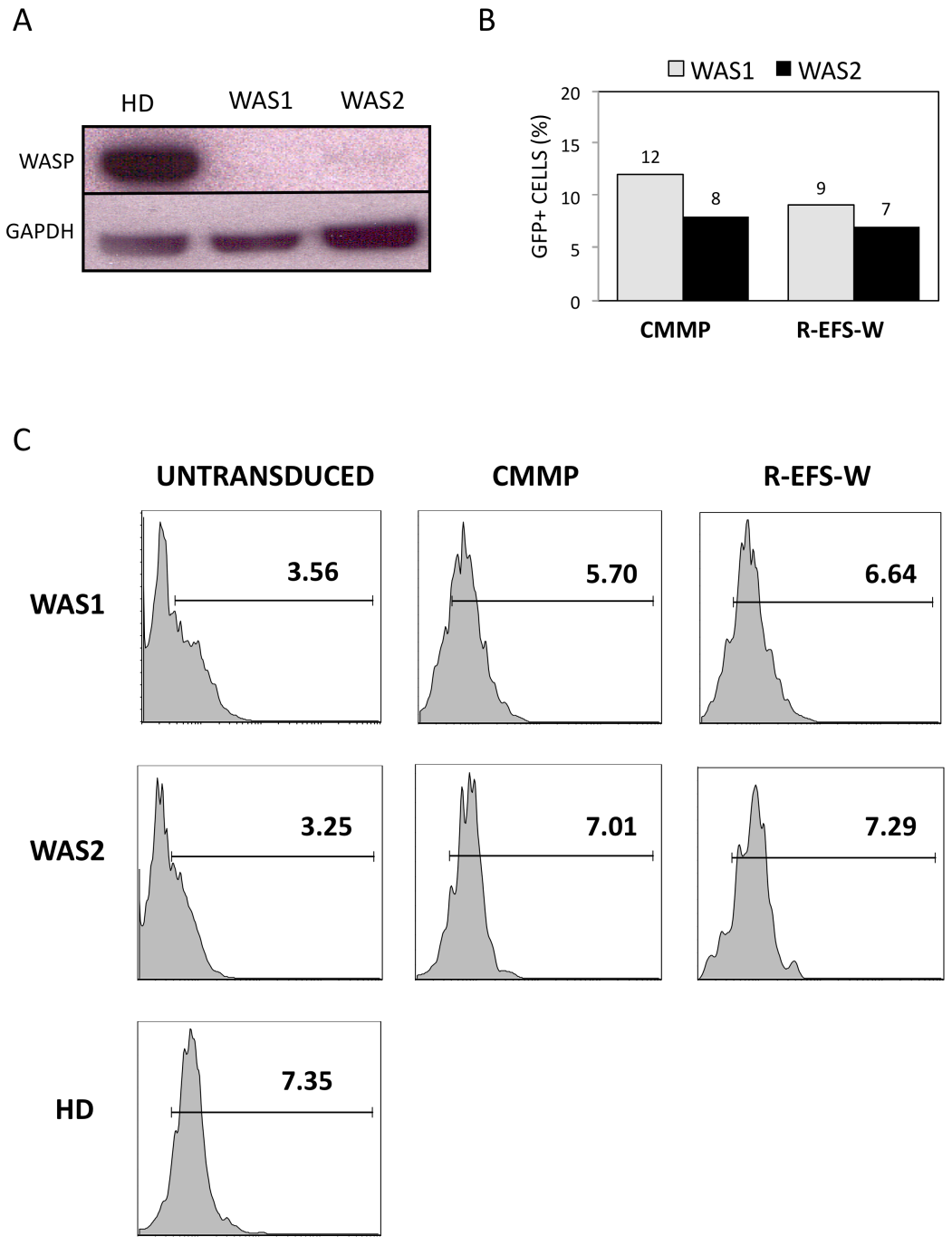

Figure 3.8. Reconstitution of WASP expression in B cells from two patients. (A) Western-blot analysis of immortalized B cells from a healthy donor and patients WAS1 and WAS2 prior to transduction. Total protein extracts were analyzed for *WASP* and GAPDH content. (B) Percentage of GFP+ cells 72h after transduction, determined by cell sorting. (C) FACS analysis for detection of intracellularly stained WASP in sorted GFP+ cells, and untransduced cells (from patients and a healthy donor). Y-axes indicate cell counts and X-axes show the expression of WASP. Numbers correspond to the GeoM of gated cells. The clear shift to the right in transduced samples is suggestive for WASP correction, as also seen for the healthy donor (HD).

3.4. Comparative analysis of transgene expression in the context of SIN-GV and SIN-LV

To assess whether transgene expression could be further improved using SIN-LV, L-EFS-W and L-SFFV-W (Fig. 3.3.B) were comparatively analyzed *in vitro* to R-EFS-W and R-SFFV-W, the two GV that yielded the best expression of GFP in Section 3.2. To this aim, replicating HT1080, SC-1 and WAS-/- Lin- cells were transduced separately with each vector pseudotyped with VSV-G using MOI=20. Three days after transduction, the expression of GFP was meassured by flow cytometry and the expression of WASP was detected by western-blot. This complete set of experiments was performed twice.

In SC-1 cells, the percentages of GFP+ cells observed by FACS in one representative experiment were 14.5% with L-SF-W, 1.2% with R-SF-W, 1.8% with L-EFS-W and 0,3% with R-EFS-W (Fig. 3.9. A, C and D). The expression of WASP did not directly correlate with the percentage of transduced cells: although R-SF-W yielded less GFP+ cells than L-EFS-W, the expression of WASP was much higher in the first than in the latter, despite the very low transduction rates (below 2%) displayed by both vectors (Fig. 3.9. B).

In HT1080 cells, the percentages of GFP+ cells observed by FACS in one representative experiment were 8.8% with L-SF-W, 2.7% with R-SF-W, 2.0% with L-EFS-W and 2.0% with R-EFS-W (Fig. 3.10. A, C and D). In this scenario, expression of WASP could be detected only in those samples transduced with SFFV-containing vectors as a very faint band (Fig. 3.10. B).

Transduction of WASP-/- Lin- cells yielded the following percentages of GFP+ cells: 2.9% with L-SF-W, 9.0% with R-SF-W, 0.5% with L-EFS-W and 7.7% with R-EFS-W (Fig. 3.11. A, C and D). In this case, Western-blot analysis showed the presence of WASP only in as a faint band in samples transduced with GV.

So far, these results suggest that vectors containing the SFFV promoter, especially the SIN-LV, would be more efficient for transduction of non-hematopoietic cells.

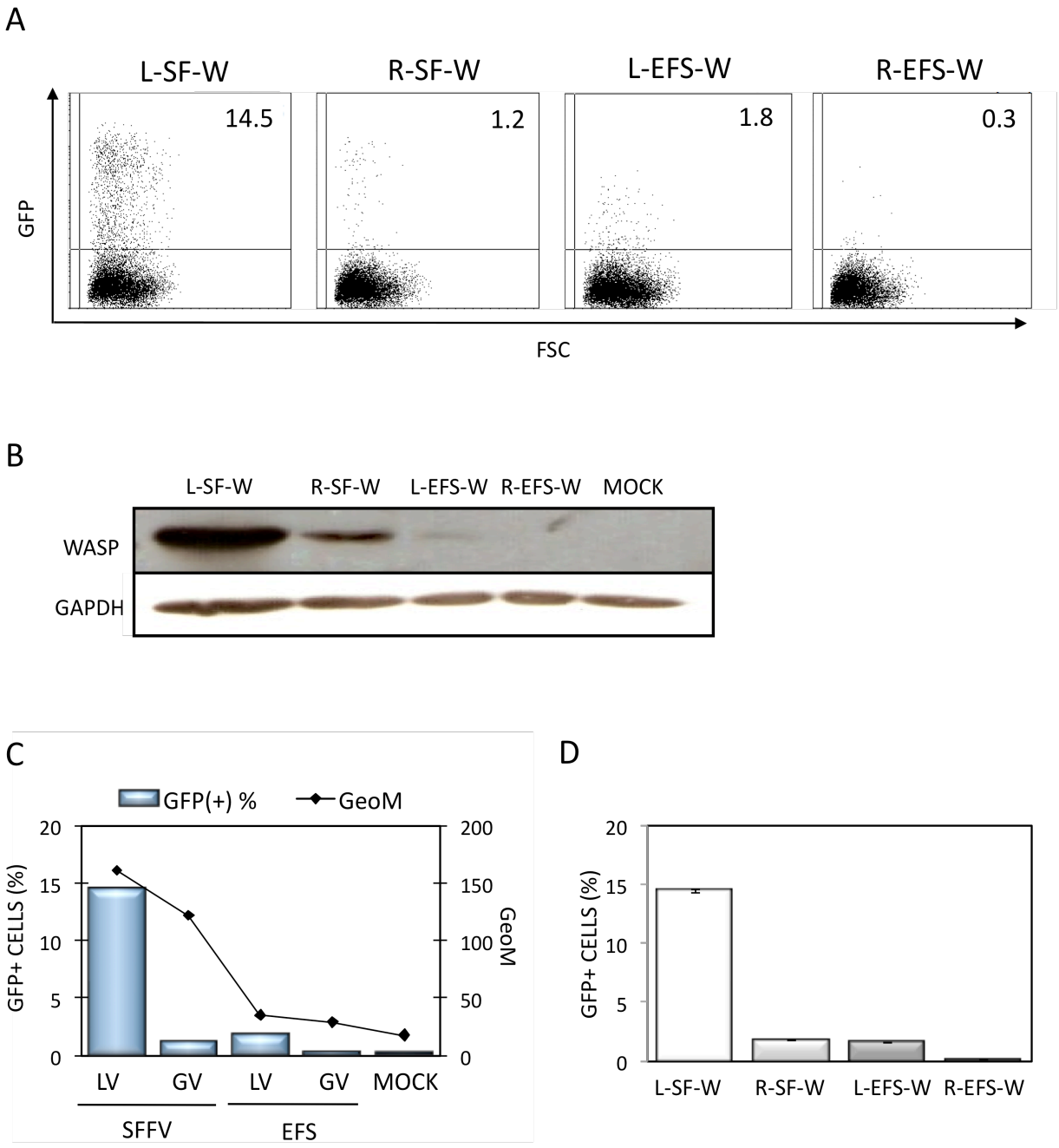

Figure 3.9. Comparative transgene expression of SIN-GV and SIN-LV in SC-1 cells. (A) FACS analysis 72h after transduction. Numbers in the upper right quadrant indicate the percentage of GFP+ cells. (B) Western-blot for detection of WASP and GAPDH. (C) Percentage of GFP+ cells (bars) and the corresponding GeoM value (bound points). (D) Mean percentage of GFP+ cells of two independent experiments measured by flow cytometry. Error bars indicate the standard deviation.

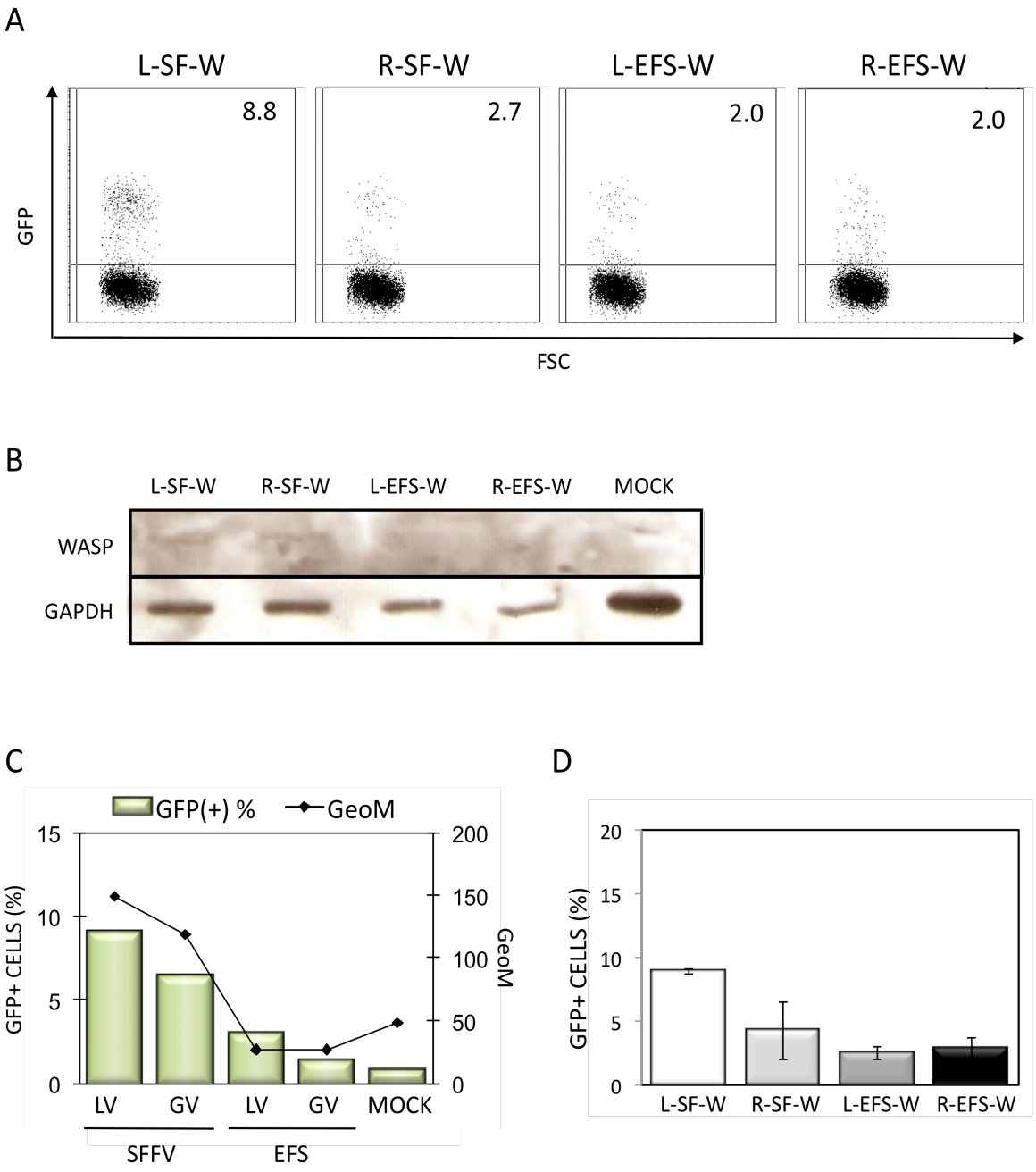

Figure 3.10. Comparative transgene expression of SIN-GV and SIN-LV in HT1080 cells. (A) FACS analysis 72h after transduction. Numbers in the upper right quadrant indicate the percentage of GFP+ cells. (B) Western-blot for detection of WASP and GAPDH. (C) Percentage of GFP+ cells (bars) and the corresponding GeoM value (bound points). (D) Average percentage of GFP+ cells of two independent experiments measured by flow cytometry. Error bars indicate the standard deviation.

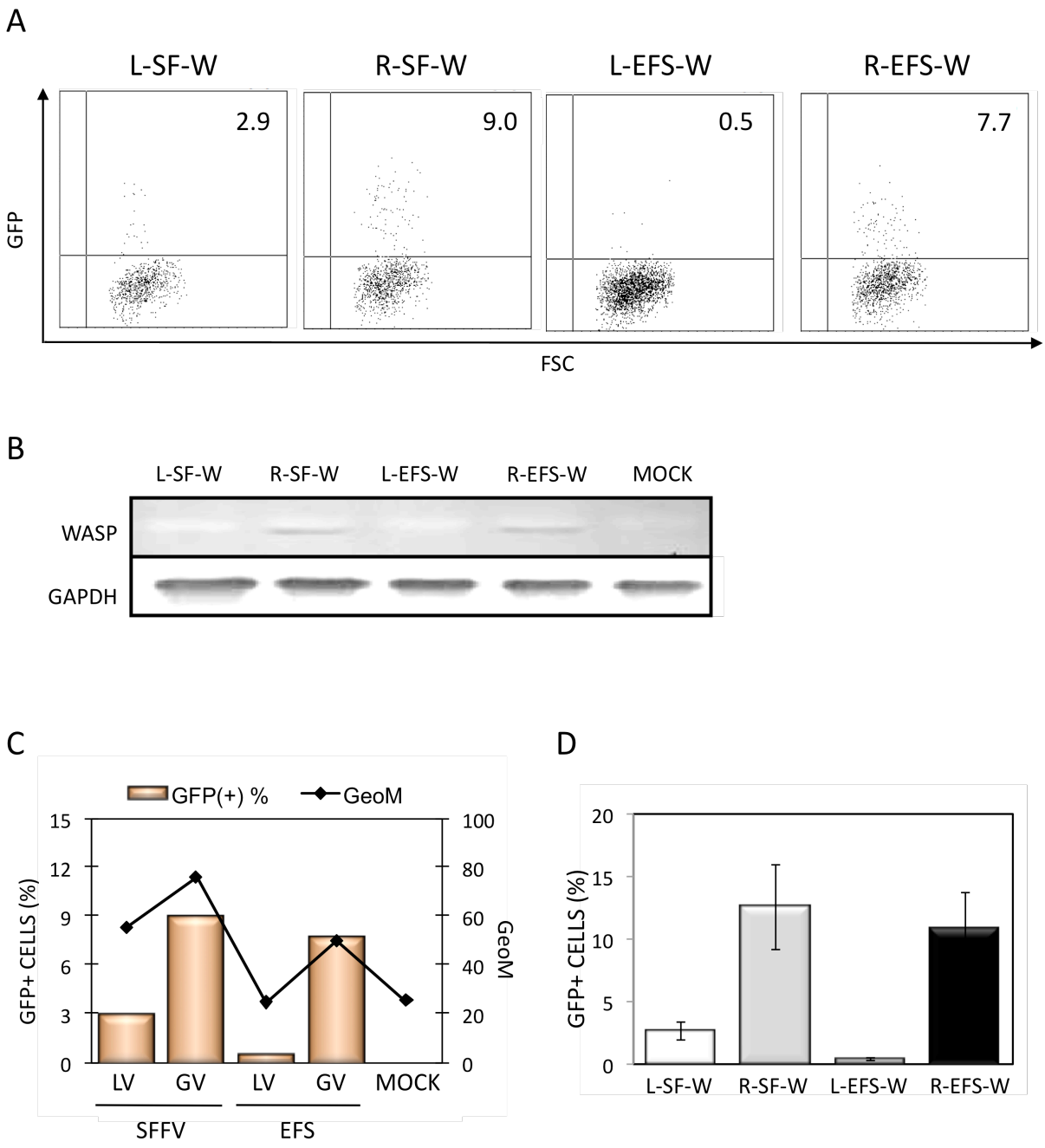

Figure 3.11. Comparative transgene expression of SIN-GV and SIN-LV in WAS-/- Lin- cells. (A) FACS analysis 72h after transduction. Numbers in the upper right quadrant indicate the percentage of GFP+ cells. (B) Western-blot for detection of WASP and GAPDH. (C) Percentage of GFP+ cells (bars) and the corresponding GeoM value (bound points). (D) Mean percentage of GFP+ cells of two independent experiments measured by flow cytometry. Error bars indicate the standard deviation.

3.5. Expression of WASP using a novel codon optimized version of *WASP* cDNA

To specifically improve the expression of the therapeutic transgene, a series of novel vectors was generated in which the sequence of the wild type *WASP* cDNA was substituted by a codon optimized-*WASP* (*coWASP*) sequence (see description in Section 4 and details in Section 6). By these means, two GV named R-SF-coW and R-EFS-coW, and two LV named L-SF-coW and R-EFS-coW were obtained (Fig. 3.1.B and 3.3.B respectively). To study the expression of this novel transgene, replicating HT1080, SC-1 and WAS-/- Lin- cells were transduced with VSV-G pseudotyped viruses at a MOI=20, reproducing exactly the same conditions described in Section 3.4. Three days after transduction, the expression of GFP was measured by flow cytometry and the expression of WASP was assessed by Western-blot. The complete set of experiments was performed twice.

In SC-1 cells, the percentages of GFP+ cells achieved in one representative experiment were 3.0% with L-SF-W, 12.4% with R-SF-W, 1.5% with L-EFS-W and 11.1% with R-EFS-W (Fig. 3.12. A, C and D). The protein analysis revealed a better correlation between the proportion of GFP+ cells and the expression of WASP in each sample (Fig. 3.12.B).

In HT1080 cells, the percentages of GFP+ cells observed in one representative experiment were 5.8% with L-SF-W, 13.7% with R-SF-W, 7.5% with L-EFS-W and 12.7% with R-EFS-W (Fig. 3.13. A, C and D). (Fig. 3.13. B).

Transduction of WASP-/- Lin- cells yielded the following percentages of GFP+ cells: 3.6% with L-SF-W, 7.8% with R-SF-W, 3.4% with L-EFS-W and 10.0% with R-EFS-W (Fig. 3.14. A, C and D). Again, a better correlation between the expression of WASP and the proportion of GFP+ cells could be observed in each sample (Fig. 3.14.B).

These results show that GV are more efficient than LV to transduce murine HSPC, when using the same viral envelope and MOI. Moreover, the expression of the therapeutical transgene could be improved by using an optimized alternative sequence. The expression of this new transgene provides a better correlation between proportion of GFP+ cells and expression of WASP. As a result, expression driven by EFS did not significantly differ from expression driven by SFFV, thus overcoming the “weakness” of the promoter of physiological origin (see *Discussion*).

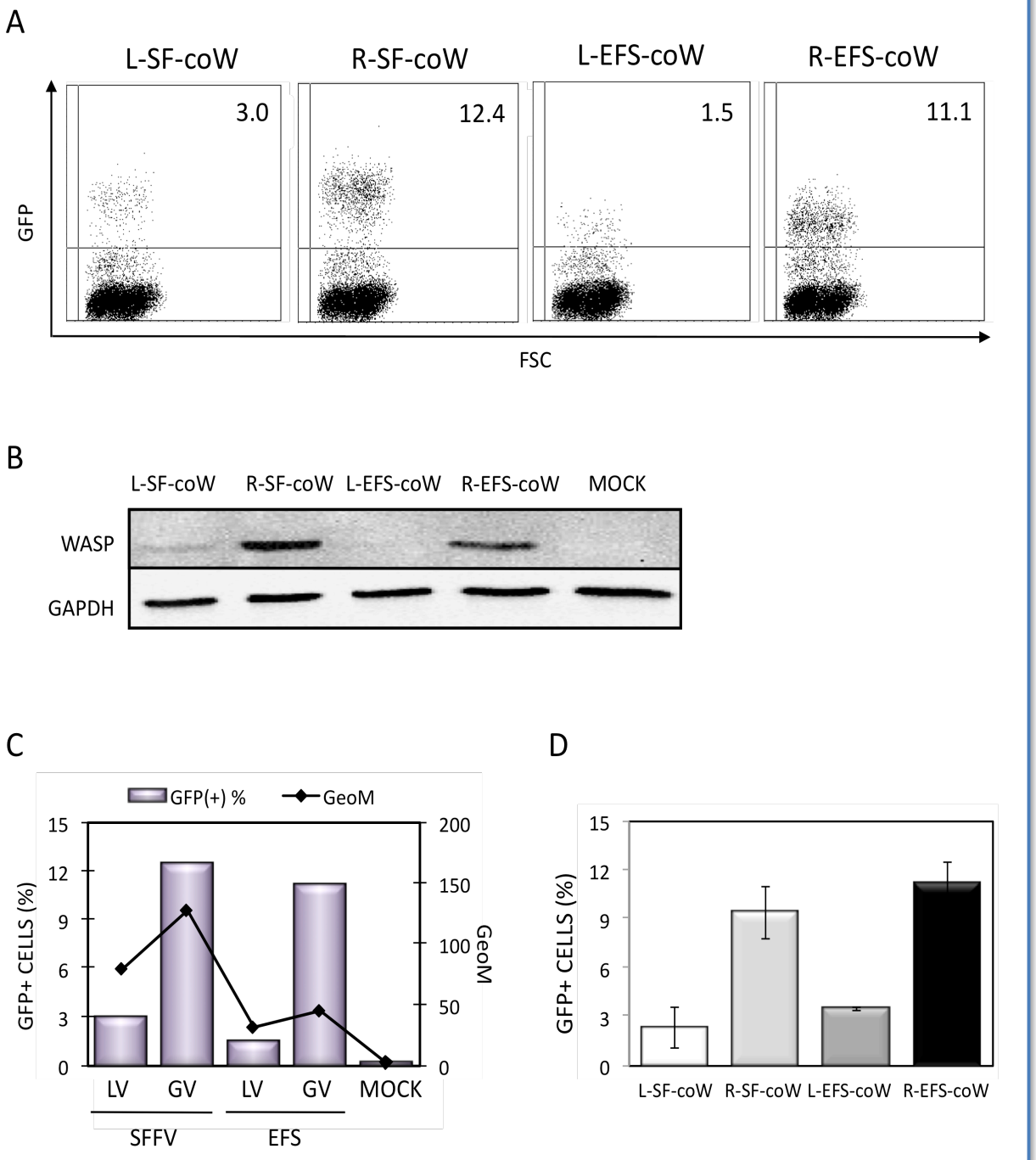

Figure 3.12. Comparative transgene expression in SC-1 cells upon transduction with SIN-GV and SIN-LV harbouring *coWASP*. (A) FACS analysis 72h after transduction. Numbers in the upper right quadrant indicate the percentage of GFP+ cells. (B) Western-blot for detection of WASP and GAPDH. (C) Percentage of GFP+ cells (bars) and the corresponding GeoM value (bound points). (D) Mean percentage of GFP positive cells of two independent experiments measured by flow cytometry. Error bars indicate the standard deviation.

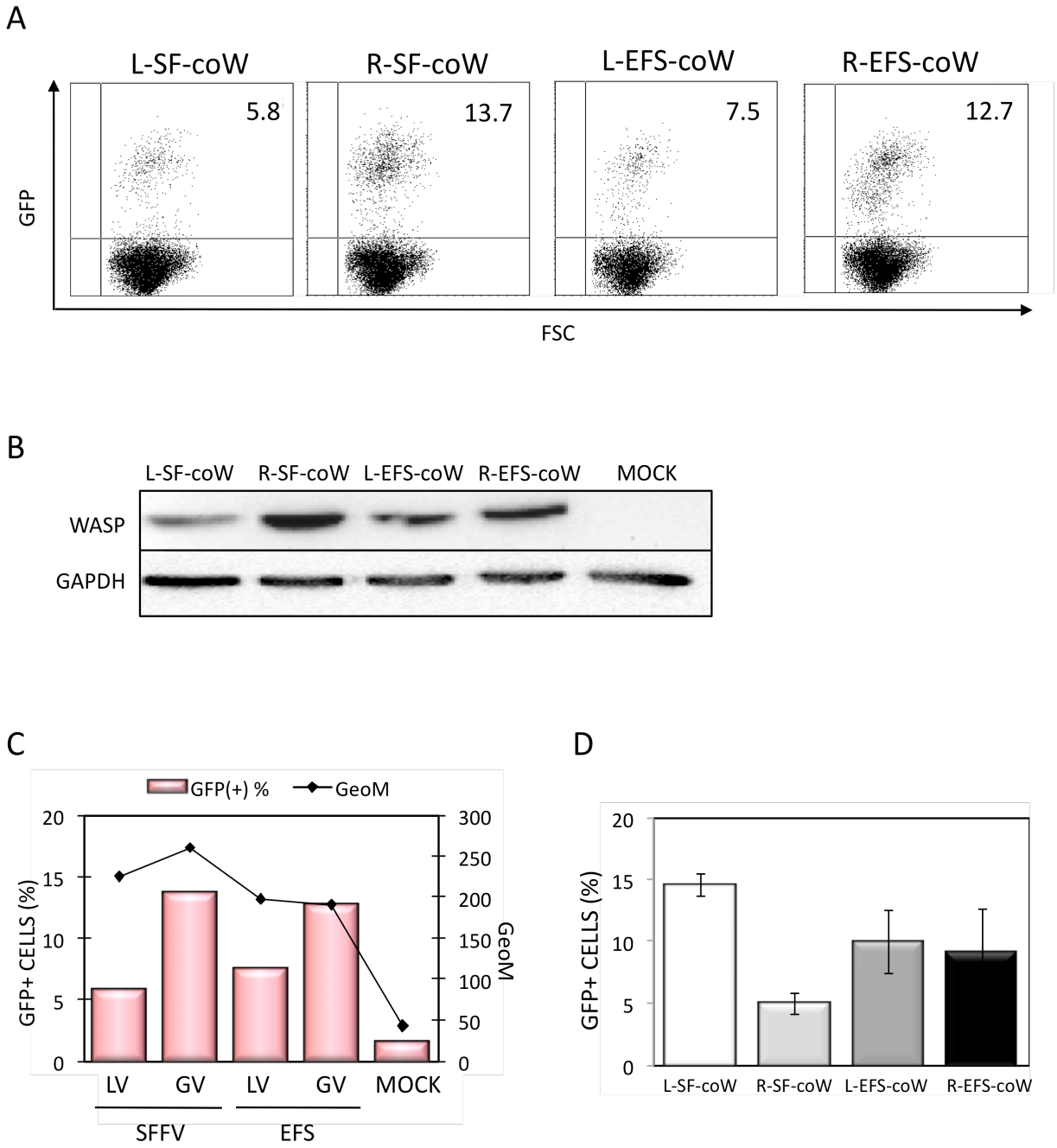

Figure 3.13. Comparative transgene expression in HT1080 cells upon transduction with SIN-GV and SIN-LV harbouring *coWASP*. (A) FACS analysis 72h after transduction. Numbers in the upper right quadrant indicate the percentage of GFP+ cells. (B) Western-blot for detection of WASP and GAPDH. (C) Percentage of GFP+ cells (bars) and the corresponding GeoM value (bound points). (D) Mean percentage of GFP positive cells of two independent experiments measured by flow cytometry. Error bars indicate the standard deviation.

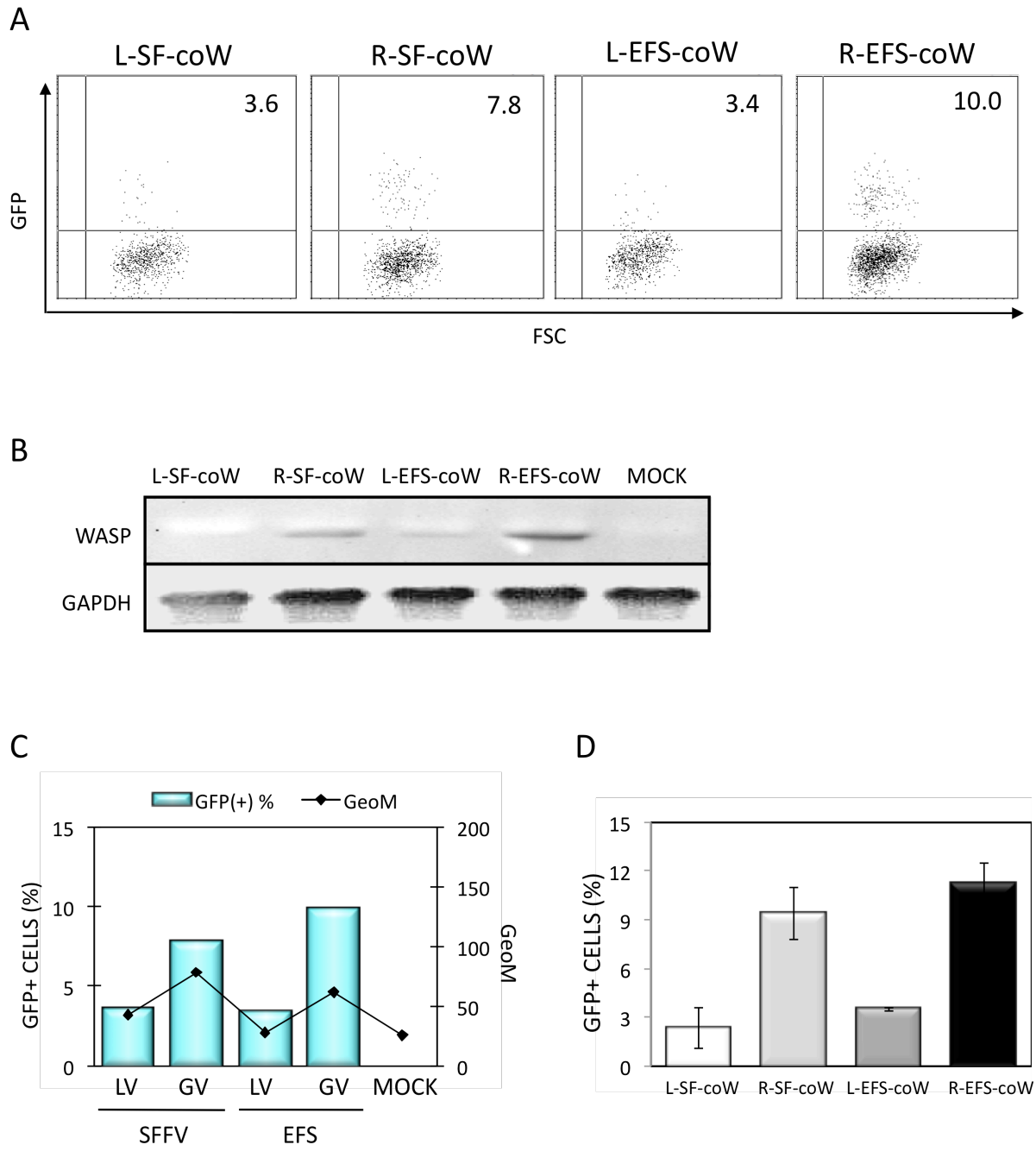

Figure 3.14. Comparative transgene expression in WAS-/- Lin- cells upon transduction with SIN-GV and SIN-LV harbouring *coWASP*. (A) FACS analysis 72h after transduction. Numbers in the upper right quadrant indicate the percentage of GFP expressing cells. (B) Western-blot for detection of WASP and GAPDH. (C) Percentage of GFP+ cells (bars) and the corresponding GeoM value (bound points). (D) Mean percentage of GFP+ cells of two independent experiments measured by flow cytometry. Error bars indicate the standard deviation.

3.6. Codon optimization of *WASP* increases the level of mRNA

To analyze the stability of the mRNA corresponding to the transcription of *coWASP*, a northern-blot analysis was performed using mRNA harvested from HT1080 cells transduced with R-EFS-W, R-EFS-coW, R-SF-W and R-SF-coW at the same MOI (Fig. 3.15).

Our results show that when the wild-type cDNA is used as transgene, the level of mRNA clearly depends on the strength of the promoter. However, mRNA level upon transduction with vectors harbouring *coWASP* did no longer differ from one vector to the other, thus neutralizing the impact of the internal promoter. This experiment shows that by incorporating the novel optimized sequence of *WASP* as transgene, it is possible to overcome the "weakness" of EFS, a promoter of cellular origin, thus obtaining similar mRNA level as with SFFV, a stronger promoter of viral origin.

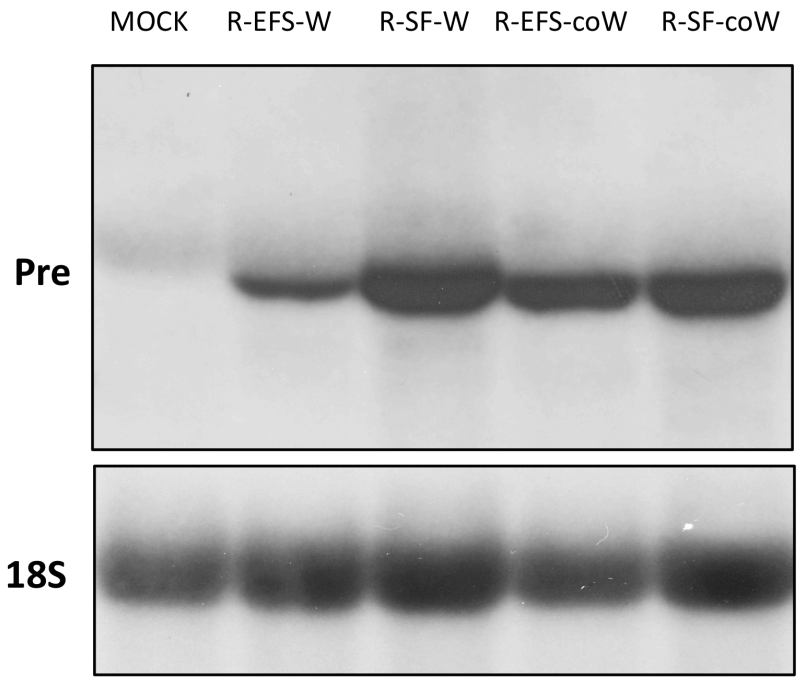

Figure 3.15. Analysis of mRNA by northern-blot. Data kindly provided by Daniela Zychlinski.

3.7. Novel codon optimized *WASP* transgene efficiently reconstitutes aberrant actin cytoskeleton in myeloid progeny of WASP deficient CD34+ cells upon gene transfer

After demonstrating that the novel vector, R-EFS-coW, allows for higer mRNA level and improved transgene expression, the next goal of this study was to check whether it also provides, in a clinically relevant scenario, the same efficiency as the earlier vector used in the clinical trial in Hannover. To this aim, we tested whether this vector could reconstitute WAS phenotype in CD34+ derived monocytes. WASP-deficient macrophages and dendritic cells are characterized by the absence of adhesion structures called podosomes (171, 172). Therefore, to assess the functionality of the protein expressed by *coWASP*, we investigated whether retroviral gene transfer into WASP-deficient CD34+ cells could reconstitute the formation of podosomes in myeloid cells differentiated *in vitro*. CD34+ cells were isolated from two independent WAS patients (named WAS3 and WAS4), transduced at MOI=5 with GALV-pseudotyped CMMP or R-EFS-coW respectively. Three days later samples were differentiated *in vitro* into myeloid progeny. After differentiation, CD14+ cells were magnetically separated and vinculin and F-actin were intracellularly stained. The specific co-localization of vinculin and F-actin corresponds to the organized structure of podosomes (173).

In patient WAS3, the FACS analysis of the sample transduced with R-EFS-coW showed 18.1% GFP+ cells on day three after transduction and 21.7% GFP+ cells after differentiation. Samples transduced with CMMP showed 14.3 and 26.3% GFP+ cells before and after transduction, respectively. The percentage of CD14+ cells with podosomes was 23% with the novel construct R-EFS-coW and 28% with CMMP (Figure 3.16).

Figure 3.16. (*See next page*) Functional restoration of podosome formation upon gene transfer in patient WAS3. (A) FACS analysis for detection of GFP in transduced CD34+ cells (upper panel) and differentiated CD14+ cells (bottom panel). Numbers in the upper right quadrant indicate the percentage of GFP positive cells. (B) Percentage of CD14+ cells with podosomes in an untransduced healthy donor (HD) and transduced and untransduced WAS3 patient. (C) Representative stainings of: untransduced healthy donor cells (top panel), mock and reconstituted cells from WAS3 (middle panel) and mock and transduced cells from WAS3 (bottom panel). Podosomes correspond to a specific co-localisation of vinculin (red) and F-actin (green). Cell nuclei are recognized by DAPI stain (blue).

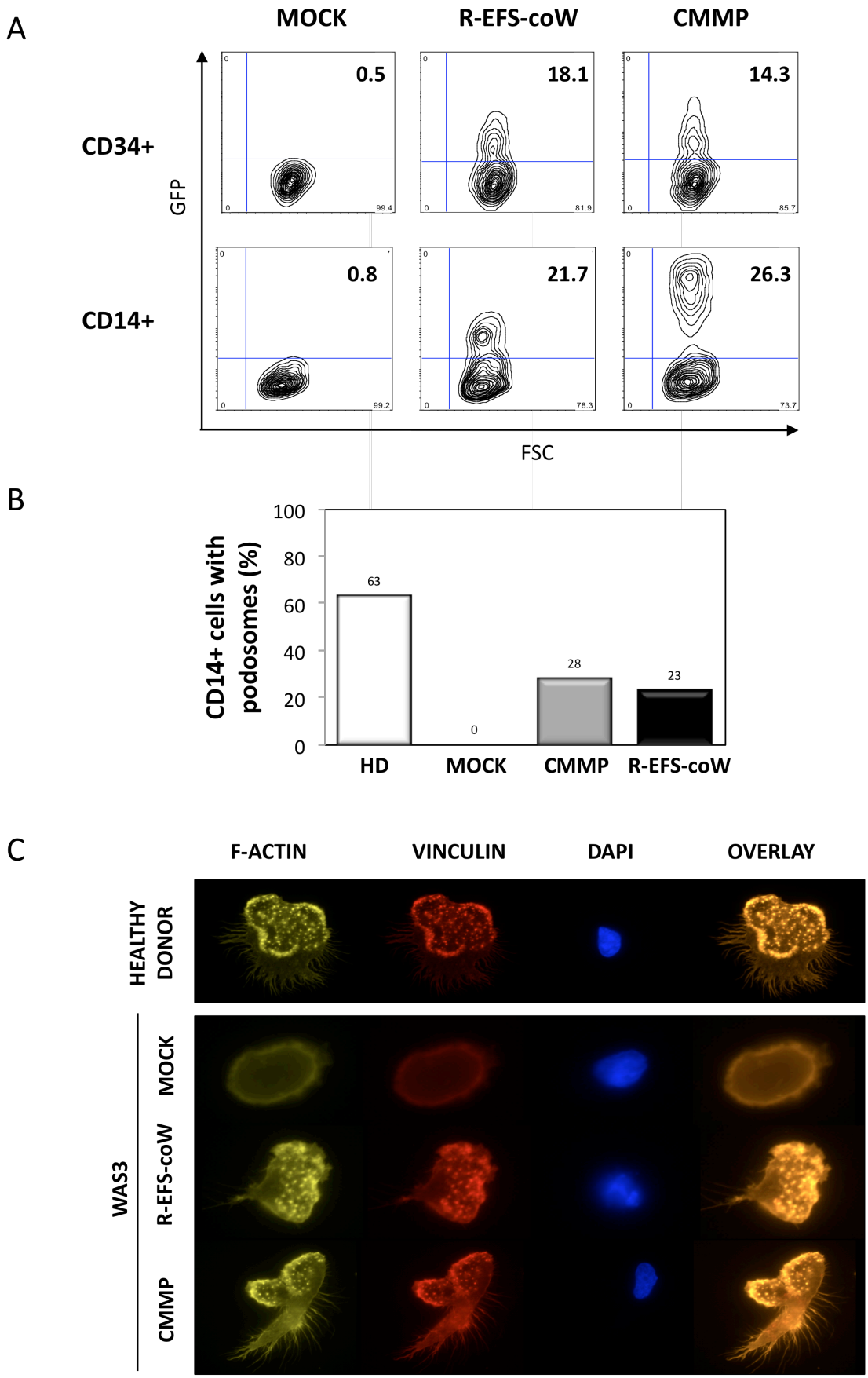

Figure 3.16. Functional restoration of podosome formation upon gene transfer in patient WAS3. (*See previous page).*

In patient WAS4, flow cytometry showed 16.1 and 8.2% GFP+ CD34+ cells transduced with R-EFS-coW and CMMP respectively. After subsequent myeloid differentiation, the percentage of GFP+ cells was 13.0 with R-EFS-coW and 15.2 with CMMP. Podosomes were detected in 30% and 37% cells differentiaten from CD34+ cells transduced with R-EFS-coW and CMMP, respectively (Figure 3.17).

As expected, *in vitro* differentiated WASP-deficient CD14+ cells completely failed to assemble vinculin and F-actin to form podosomes in both patients, whereas the percentage of CD14+ cells with podosomes ranged were 63 adn 77% in two independent healthy individual donors, thus reflecting the interindividual variability (Figures 3.16 and 3.17).

In summary, this data indicate that the novel SIN-retrovirus presented here in which a novel codon optimized version of *WASP* cDNA is driven by an internal promoter of cellular origin, EFS, could offer an efficacious and potentially less genotoxic tool for WAS hematopoietic stem and progenitor cell-based gene therapy.

Figure 3.17. (*See next page*) Functional restoration of podosome formation upon gene transfer in patient WAS4. (A) FACS analysis for detection of GFP in transduced CD34+ cells (upper panel) and differentiated CD14+ cells (bottom panel). Numbers in the upper right quadrant indicate the percentage of GFP positive cells. (B) Percentage of CD14+ cells with podosomes in an untransduced healthy donor (HD) and transduced and untransduced WAS4 patient. (C) Representative staininings of: untransduced healthy donor cells (top panel), mock and reconstituted cells from WAS3 (middle panel) and mock and transduced cells from WAS4 (bottom panel). Podosomes correspond to a specific co-localisation of vinculin (red) and F-actin (green). Cell nuclei are recognized by DAPI stain (blue).

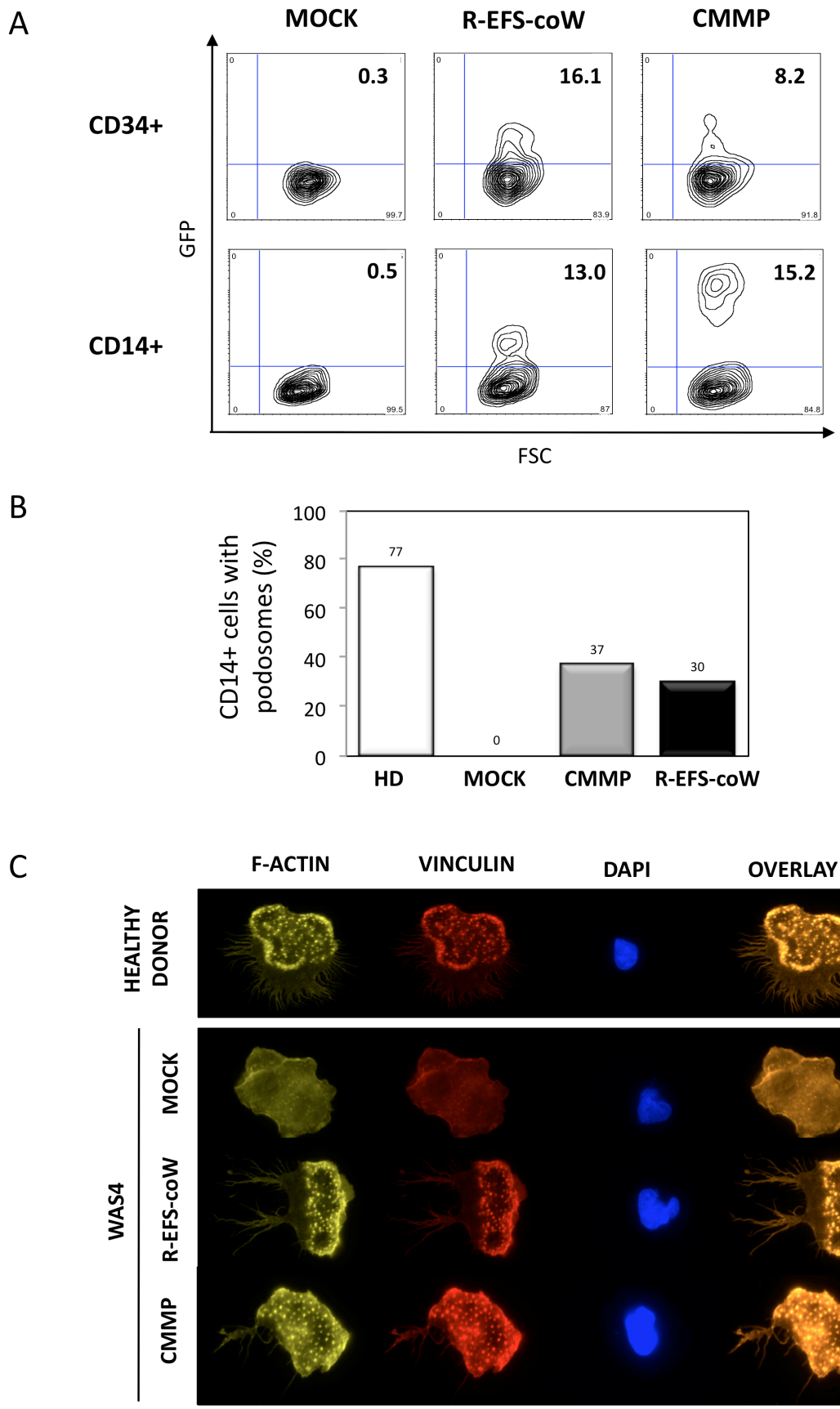

Figure 3.17. Functional restoration of podosome formation upon gene transfer in patient WAS4. (*See previous page*).

4. DISCUSSION

Gene therapy has proven as a very successful therapeutic approach to treat primary immunodeficiencies. Indeed, previous clinical hematopoietic gene therapy studies have reported sustained or temporary correction of lymphocytes (X-SCID, ALD) (57, 86, 174), myeloid cells (CGD, ALD) (97, 174) and red blood cells (thalassemia) (175). Also the clinical trial for WAS that is currently ongoing at the Hannover Medical School (Germany) has recently reported the successful correction of all cell lineages of blood in the first two treated patients, including for the first time correction of platelets upon transplantation of retrovirus-transduced HSC in humans (103). Noteworthy, the fractions of corrected lymphocytes increased over time, in line with the proliferative advantage of WASP-positive cells (102). Clinically, both patients have markedly improved their susceptibility to infection, autoimmunity, and bleeding. This clinical trial provides first proof-of-principle that gene therapy for WAS is feasible and safe up to three years after gene therapy, indicating that early hematopoietic progenitor/stem cells contributing quantitatively to all cell lineages of blood, were successfully corrected (103). However, an extensive study of the clonal inventory of both patients reveals that *LMO2* and *CCND2* were targeted preferentially in lymphoid cells, and *MDS1/EVI1* was almost exclusively hit in myeloid cells. Activation of *LMO2* upon viral integration has already triggered a leukemia-like T lymphoproliferative disorder in four XSCID patients treated with gene therapy in the past (see Section 1.2.2.3.), and integrations in *MDS-1*, *PRDM16* and *SETBP1* have caused the development of myelodysplasia in two CGD patients treated with gene therapy (see Section 1.2.2.4.). Although no persistent clonal imbalance has been observed so far in the WAS clinical trial, the risk of insertional mutagenesis remains a major drawback of this strategy (156, 176), thus reinforcing the need of developing safer vectors for gene transfer into HSC. Therefore, the aim of the present study was to design a novel vector for the gene therapy of WAS as efficient as the vector currently used in the Hannover clinical trial but with reduced risk of genotoxicity.

The risk of activating cellular proto-oncogenes as a result of insertional mutagenesis can be significantly reduced by using a self-inactivating vector (see Section 1.4.1.9.), and a more physiological internal promoter to drive the expression of the gene of interest (126). Cell-based assays especifically designed to detect the clonal dominance of insertional mutants which expand in initially polyclonal cultures of primary murine HSPC revealed that γ-retroviral vectors (GV) with a SIN design are weaker insertional mutagens than their LTR-driven counterparts (125). Nevertheless, MLV-derived SIN vectors with an internal enhancer-promoter derived from SFFV were able to transform cells by insertional activation of proto-oncogenes such as *EVI1* (125). In the same line of investigation, further studies have shown that in hematopoietic cells, the EFS promoter is much less likely to activate neighbouring genes than are viral promoters. Actually, SIN vectors carrying the EFS enhancer-promoter were unable to immortalize primary hematopoietic cells even in the absence of insulators, despite rather drastic dose escalation and accordingly, they did not lead to detectable levels of *EVI1* upregulation in cultured cells (126). Despite its reduced intrinsic potential of cell transformation by activation of neighbouring proto-oncogenes, EFS is a relatively potent cellular promoter which, in hematopoietic cells, is only threefold less active than MLV enhancer-promoters (169, 197). Following this line of thought, our first goal was to comparatively analyze *in vitro* the transgene expression driven by different physiological internal promoters in the context of a SIN-γ-retroviral vector and select the most suitable one. Among all physiological promoters tested (EFS, PGK, and WASP), EFS is the one whose transduction rates and GeoM value of GFP+ cells were closest to those corresponding to the SFFV promoter and to the CMMP vector with intact LTR, thus being the most efficient physiological promoter. We therefore selected it to further develop the novel vector presented in this study.

Another important side that must be carefully considered when designing a new transfer vector with potential clinical application is the selection of the type of vector. Retroviruses (mainly GV) and lentiviruses are the preferred vehicle to perform gene transfer into HSC because the viral vector integrates into the cell genome, thus allowing for long-term transgene expression (188-190). Nevertheless, they differ in their ability to transduce non-replicating cells: unlike lentivirues, retroviruses require the breakdown of the nuclear membrane to access the cellular chromosomal DNA, therefore, they can only transduce

actively dividing cells (187, 188). Retroviruses may be considered safer, because they show no homology with HIV-1, a potent human pathogen. However, retroviral-mediated transduction of HSC, the target cell population in gene therapy, requires at least 24h of *in vitro* stimulation with cytokines to induce the cells to actively divide before undergoing transduction (201). Therefore, the use of lentiviruses may be considered a better choice, because they can integrate into non-dividing HSPC under conditions of limited preactivation with cytokines which are more likely to preserve long-term multilineage hematopoietic reconstitution (202, 203). Specifically, the possible employment of a lentiviral vector system based on the human immunodeficiency virus type 1 (HIV-1) has attracted much attention in the field of gene therapy (177-179). However, its application is a very controversial issue, mainly because both ethical and practical issues oppose the application of HIV-based vectors in clinical trials. The first concern is that a gene delivery system cannot be based on an infectious agent that is pathogenic for humans or might trigger AIDS after chemotherapy, especially if the pathogenesis of the disease is still not completely clear and is not curable. Another concern in the matter of HIV-based vectors is the seroconversion of the subject to certain components of HIV-1 (180). In addition, if the vector will be administered in patients with AIDS, or if the patient developes AIDS after chemotherapy, the risk of a recombination event between the vector and HIV-1 should be considered (180). The resulting infectious agent might even be more pathogenic than HIV-1 itself. At this stage, it is not possible to assess the likelihood of such a risk (181).

The production and manipulation of HIV-based vectors must be carried out in biosafety laboratory level two or even three facilities, depending on the country. These manipulations are very cumbersome for producing large quantities of clinical-grade lentiviral vectors stocks. In addition, scaling up the production of HIV-based vectors may increase the possibility of generating replication-competent viruses by homologous recombination (180).

Other lentiviral vector systems have emerged, which are based on lentiviruses that are not pathogenic in humans, such as feline immunodeficiency virus (FIV) (182, 183), equine infectious anemia virus (EIAV) (184), bovine immunodeficiency virus (BIV) (185) and sheep Visna virus (186). The eventual employment of these lentiviral vectors in gene therapy clinical trials would circumvent the critical issue of HIV-seroconversion of the subject. However, these vectors may still generate replication competent lentiviruses by homologous recombination events and transduction efficiency, levels of transgene expression and

duration of transgene expression are still rather sub-optimal, if compared to the latest generation of HIV-1-derived vector systems (180).

The usage of a MLV-derived vector overcomes these drawbacks of HIV-1-derived vectors. Murine (such as MLV) retroviruses are distantly related to primate retroviruses, which contributes to minimize possible interactions with human endogenous retroviruses. Moreover, retroviruses have a more simple genome than lentiviruses, allowing the generation of vectors in which gag/pol can be completely deleted. Production of retroviral particles can be accomplished by cotransfection of the canonical gag/pol, envelope and transfer vector, and does not require the addition of any accesory genes (e.g., rev), as it is mandatory in the case of lentiviruses. Noteworthy, in retroviruses developed using the split-packaging design, viral particles can carry up to 9 kb of chimeric viral genome.

The viral integration pattern is also a matter of controversy. We know now that neither GV or LV integrate randomly in the genome, as initially thought (194, 195). LV with their tendency to insert themselves into transcribed genes have been considered less genotoxic than GV with their preference for integration next to transcriptional start sites and regulatory gene regions. However, recent published data reveals that mutants induced by LV also show robust replating in an *in vitro* transformation assay (196). Moreover, in spite of the differences in their integration pattern, the potent *EVI1* proto-oncogene represents a common insertion site for both GV and LV (196). The latest advances in the field indicate that altering the vectors´ enhancer-promoter elements has a greater effect on safety than the retroviral insertion pattern (196) and that the insertional gene activation is determined by the characteristics of the transcriptional regulatory elements carried by the vector, thus it is largely independent from the vector type (193).

To assess whether transgene expression could be further improved using SIN-LV, R-EFS-W and R-SF-W were comparatively analyzed *in vitro* to their respectives lentiviral counterparts. In non-hematopoietic cells, all vectors yielded very low (<3% GFP+cells) transduction rates except L-SF-W, whereas in WASP-/- Lin- cells, both GV were clearly more efficient than LV. These preliminary results suggested that R-EFS-W was the vector that combined the safest configuration with a relatively good transduction rate in murine HSPC. However, we also observed that the expression of WASP did not correlate to the percentage of GFP+ cells in

any of the cell types tested. Instead, it depended on the strenght of the internal promoter rather than the transduction rate or the type of the vector.

To circumvent or decrease at least the need of a strong enhancer/promoter to achieve high expression of WASP in the newly developed R-EFS-W, the sequence of the transgene itself, i.e. the wild-type *WASP* cDNA, was optimized, without altering the aminoacid sequence (see details in Section 6). Indeed, the analysis of the *WASP* cDNA revealed the presence of rare codons with a high frequency, together with several negatively *cis*-acting motifs (seven prokaryotic inhibitory motifs and one cryptic splice donor site), and a quite low GC content, that might hamper expression in mammals and facilitates quick mRNA turnover, respectively. These features explain why the transgene displays a relatively low codon adaptation index (CAI=0,79). The parameter CAI describes how well the codons match the codon usage preference of the target organism. Thus, a CAI of 1,0 would be perfect, however, a CAI of >0,9 is considered as very good, i.e., allowing high expression. To adapt the codon usage to the codon bias of *Homo sapiens* genes for increased mammalian expression, an optimization process was carried out in which regions of very high (>80%) or very low (<30%) GC content were avoided where possible. The following *cis*-acting sequence motifs were also avoided: internal TATA-boxes, chi-sites and ribosomal entry sites; AT-rich or GC-rich sequence stretches; repeat sequences and RNA secondary structures; cryptic splice donor and acceptor sites, branch points; AgeI, SalI and XbaI, except as indicated for cloning purposes; and two STOP codons were added to ensure efficient termination. The optimization process resulted in a sequence with no negative *cis*-acting sites which may negatively influence expression and increased GC-content to prolong mRNA half-life (from 62% in *WASP* cDNA to 65% in *coWASP*). Codon usage was adapted to the bias of *Homo sapiens* resulting in a high CAI value of 0,91. The optimized gene should therefore allow for high and stable expression rates in human or other mammalian cells.

Our *in vitro* comparative study of transgene expression shows that the codon optimization procedure results in higher percentages of GFP+ cells with all vectors in all cell types tested, probably reflecting the improved procesivity of *coWASP*. Importantly, there is a better correlation between the percentage of GFP+ cells and the expression of coWASP, indicating that the transgene expression now depends on the transduction rate, rather than on the streght of the internal promoter. The northern-blot analysis of transduced cells reveals that

the optimization of the codon allows to overcome the weakness of the physiological promoter already at mRNA level. Indeed, the levels of *coWASP* mRNA displayed upon transduction with R-EFS-coW or R-SF-coW are very similar. These results provide solid evidence, that the SIN-GV containing the EFS promoter is the most effective vector in murine HSPC, the target cell population for BMT in preclinical models.

At that stage, it still remained unknown whether R-EFS-coW, our newly desingned transfer vector containing the novel sequence of *WASP*, could correct the WAS phenotype, as efficiently as the CMMP vector containing an intact LTR and the wild-type *WASP* cDNA does. To address this question, we tested our new vector in CD34+ cells from WAS patients. Transduction rates obtained with R-EFS-coW did not signigficantly differ from those obtained with CMMP, whereas the percentage of GFP+ cells as well as the intensity of the fluorescence remained more stable along the *in vitro* differentiation with the former vector. The myeloid progeny of CD34+ cells treated with R-EFS-coW displayed the same morphology and phenotypical correction than those treated with the CMMP vector and were indistinguishable from the healthy donor´s. These results clearly show, in a clinically relevant scenario, that our novel vector can correct the aberrant cytoskeleton and rescue the formation of podosomes in the myeloid progeny as effciently as the vector currently used in the clinical trial, despite the low multiplicity of infection (MOI=5). Additionally, the present data validate the ability to restore WASP expression in patients´ CD34+ cells while preserving their *in vitro* differentiation potential, two important requisites for clinical use.

A main point of this study is that it also provides evidence of the feasibility of producing this novel vector at high titers in human 293T cells, hence obtaining titers within the range necessary for clinical application. The production of viral supernatants at high titers was partially achieved by incorporating the woodchuck hepatitis virus post-transcriptional regulatory element (PRE) downstream the expression cassette, which improves the 3´ RNA processing of retroviral transcripts (170) and reduces readthrough transcription from retroviral vectors (198) without increasing the potential transformation of hematopoietic cells (196). Noteworthy, the substitution of the wild-type cDNA by *coWASP* did not affect the production of viral vectors, resulting in obtention of similar titer values. Although SIN-vectors are considered to be replication-incompetent, the transfer of intact viral genomes is

unacceptable for clinical applications, due to the risk of vector mobilization and the potentially immunogenic expression of viral proteins. This study additionally shows the feasibility of minimizing this risk by setting up a split-packaging system expressing the necessary viral proteins in *trans*. In the split-packaging design used to develop the new γ-retroviral vector, the *cis*-acting sequences required for vector packaging, reverse transcription and integration can be entirely separated from the sequences encoding the structural and enzymatically active retroviral proteins. Hence, this system prevents the potential recombination with retroviral sequences of the target cells as well as the formation of RCR, an even greater threat to the therapeutic use of retrovirus-based gene vectors than the insertional mutagenesis (174) that is therefore unacceptable for clinical applications. Besides minimizing the risk of RCR formation, the split-packaging design also avoids the potentially immunogenic expression of viral proteins from transduced cells and increases the theoretical packaging capacity of the γ-retroviral vector. In addition, the present study demonstrates proof-of-principle for the versatility of this novel vector for genetic modification of murine and human hematopoietic cells at a low multiplicity of infection. Moreover, this new vector can be successfully pseudotyped with different envelope proteins (VSVg, GALV and ecotropic) without significant variations in the titers. Furthermore, the envelope, which is provided in *trans*, can be selected to modify the tropism of the virus, with potential species-dependent restrictions. In fact, this study shows efficient transduction using GALV pseudotyped viruses with a low multiplicity of infection in human hematopoietic stem/progenitor cells, the target cell population for many gene therapy applications in diseases of the blood and immune systems.

Alltogether, this study presents a novel vector that combines improved safety features and improved transgene expression. The SIN design is a key element that reduces the risk of insertional mutagenesis by providing the opportunity to use the physiological promoter EFS that have less long range enhancer activity than viral LTRs. The codon optimization of *WASP* allows the same transgene expression levels than the strong SFFV promoter and the MLV-derived vector with intact LTR used in the Hannover clinical trial. For these reasons and because the present study also provides evidence of the efficacy of this vector in different murine and human cell types, including patients´ B and CD34+ cells, this vector appears to be

a good candidate vector to pursue for the development of a safer second generation gene therapy protocol for WAS, with greatly decreased risk of insertional mutagenesis.

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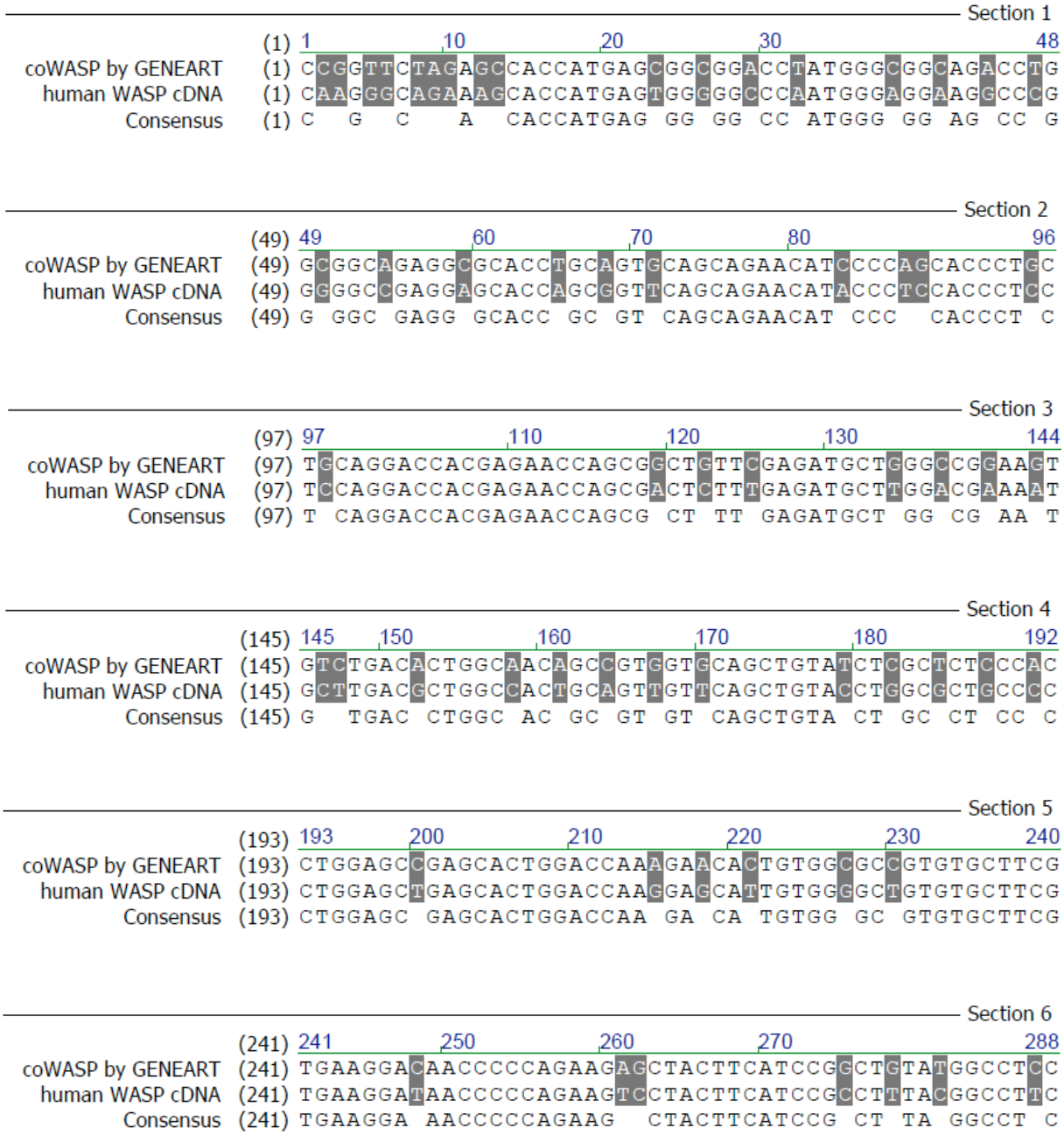
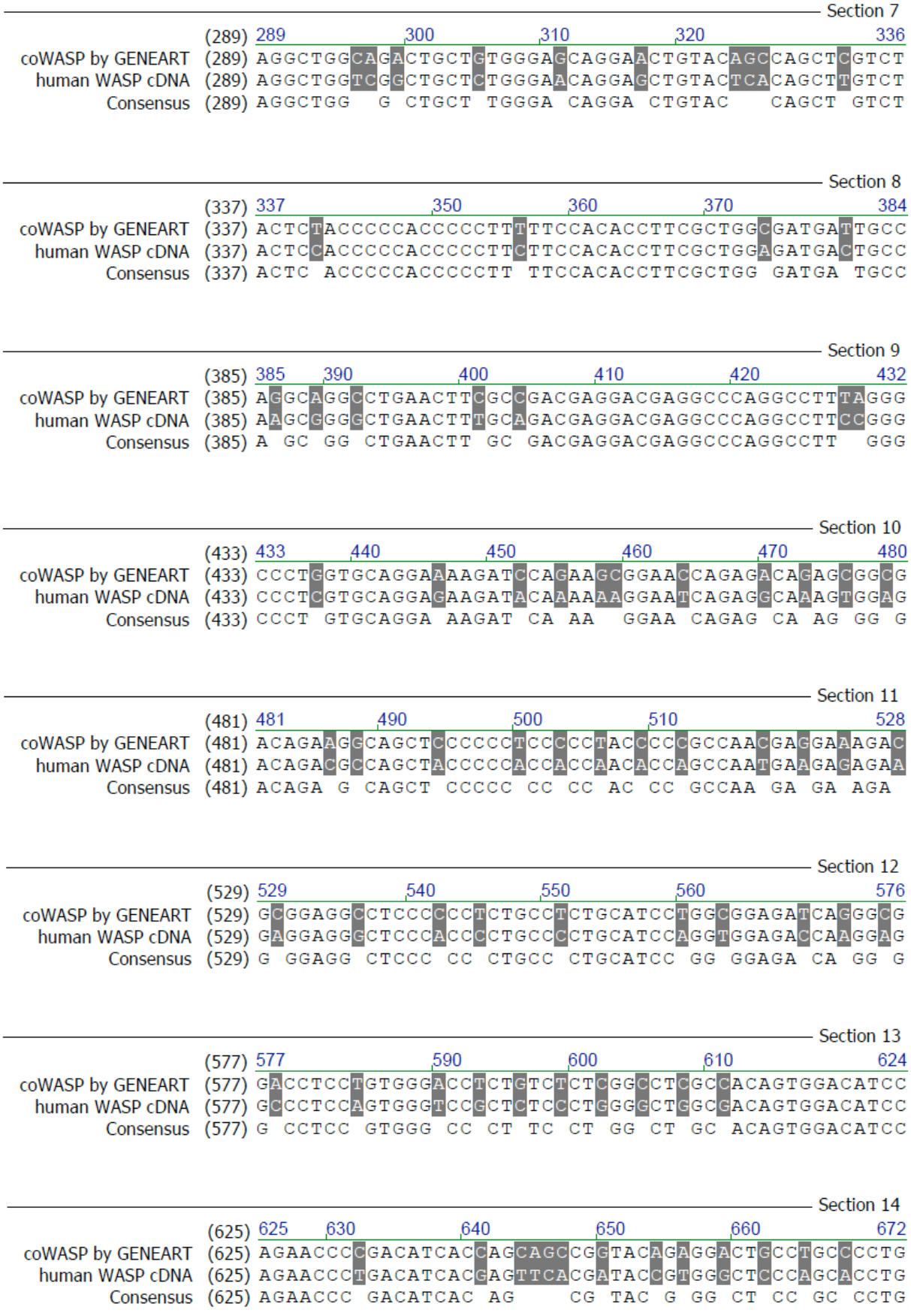
6. APPENDIX

Alignment of the codon-optimized *WASP* sequence against the wild-type human *WASP* cDNA

Human Wiskott-Aldrich Syndrome Protein Accession Number: NM_000337

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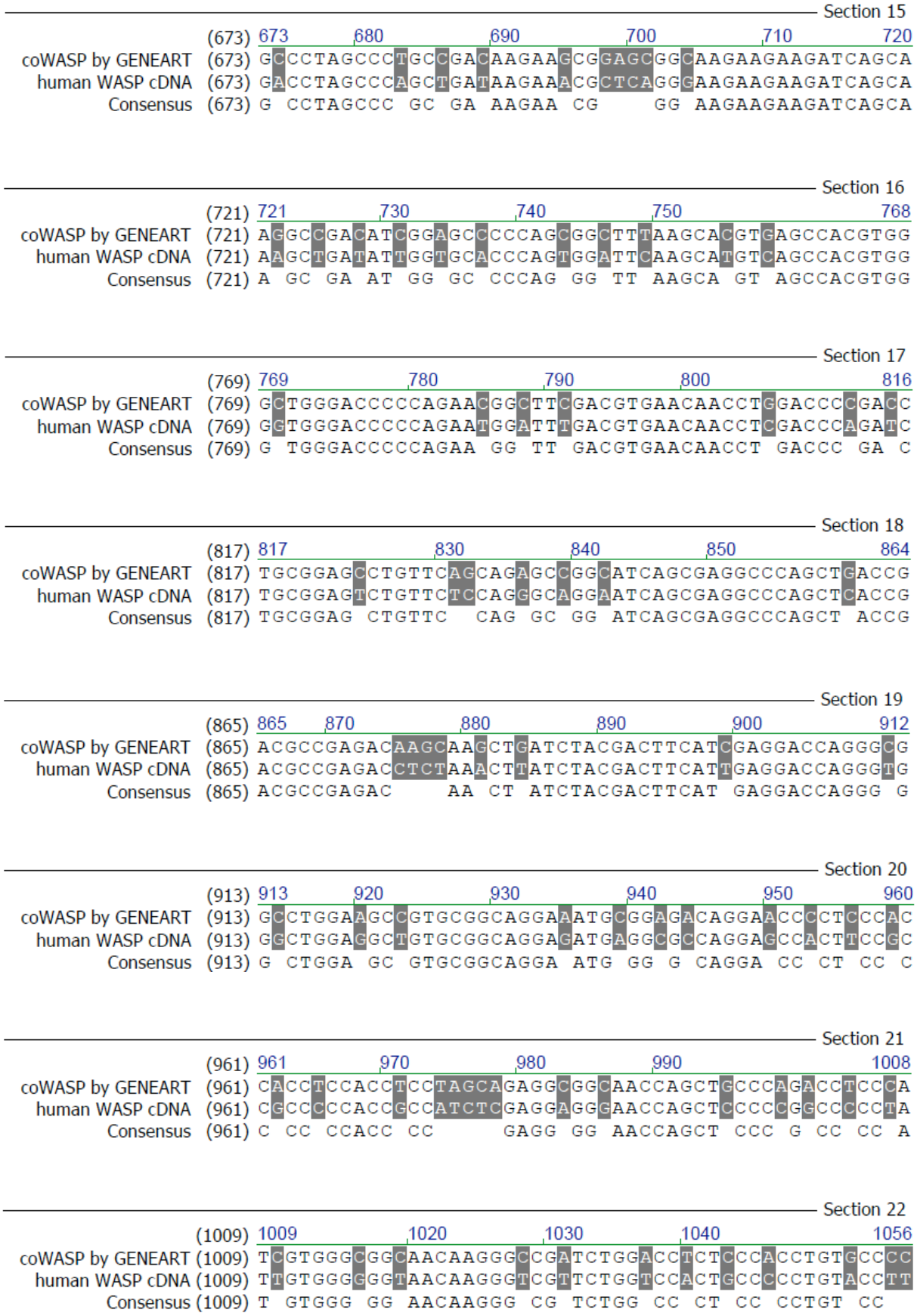
coWASP by GENEART (289) AGGCTGGCAGACTGCTGTGGGAGCAGGAACTGTACAGCCAGCTCGTCT
human WASP cDNA (289) AGGCTGGTCGGCTGCTCTGGGAACAGGAGCTGTACTCACAGCTTGTCT
Consensus (289) AGGCTGG G CTGCT TGGGA CAGGA CTGTAC CAGCT GTCT
Section 8
(337) 337 350 360 370 384
coWASP by GENEART (337) ACTCTACCCCCACCCCCTTTTTCCACACCTTCGCTGGCGATGATTGCC
human WASP cDNA (337) ACTCCACCCCCACCCCCTTCTTCCACACCTTCGCTGGAGATGACTGCC
Consensus (337) ACTC ACCCCCACCCCCTT TTCCACACCTTCGCTGG GATGA TGCC
Section 9
(385) 385 390 400 410 420 432
coWASP by GENEART (385) AGGCAGGCCTGAACTTCGCCGACGAGGACGAGGCCCAGGCCTTTAGGG
human WASP cDNA (385) AAGCGGGGCTGAACTTTGCAGACGAGGACGAGGCCCAGGCCTTCCGGG
Consensus (385) A GC GG CTGAACTT GC GACGAGGACGAGGCCCAGGCCTT GGG
Section 10
(433) 433 440 450 460 470 480
coWASP by GENEART (433) CCCTGGTGCAGGAAAAGATCCAGAAGCGGAACCAGAGACAGAGCGGCG
human WASP cDNA (433) CCCTCGTGCAGGAGAAGATACAAAAAAGGAATCAGAGGCAAAGTGGAG
Consensus (433) CCCT GTGCAGGA AAGAT CA AA GGAA CAGAG CA AG GG G
Section 11
(481) 481 490 500 510 528
coWASP by GENEART (481) ACAGAAGGCAGCTCCCCCCTCCCCCTACCCCCGCCAACGAGGAAAGAC
human WASP cDNA (481) ACAGACGCCAGCTACCCCCACCACCAACACCAGCCAATGAAGAGAGAA
Consensus (481) ACAGA G CAGCT CCCCC CC CC AC CC GCCAA GA GA AGA
Section 12
(529) 529 540 550 560 576
coWASP by GENEART (529) GCGGAGGCCTCCCCCCTCTGCCTCTGCATCCTGGCGGAGATCAGGGCG
human WASP cDNA (529) GAGGAGGGCTCCCACCCCTGCCCCTGCATCCAGGTGGAGACCAAGGAG
Consensus (529) G GGAGG CTCCC CC CTGCC CTGCATCC GG GGAGA CA GG G
Section 13
(577) 577 590 600 610 624
coWASP by GENEART (577) GACCTCCTGTGGGACCTCTGTCTCTCGGCCTCGCCACAGTGGACATCC
human WASP cDNA (577) GCCCTCCAGTGGGTCCGCTCTCCCTGGGGCTGGCGACAGTGGACATCC
Consensus (577) G CCTCC GTGGG CC CT TC CT GG CT GC ACAGTGGACATCC
Section 14
(625) 625 630 640 650 660 672
coWASP by GENEART (625) AGAACCCCGACATCACCAGCAGCCGGTACAGAGGACTGCCTGCCCCTG
human WASP cDNA (625) AGAACCCTGACATCACGAGTTCACGATACCGTGGGCTCCCAGCACCTG
Consensus (625) AGAACCC GACATCAC AG CG TAC G GG CT CC GC CCTG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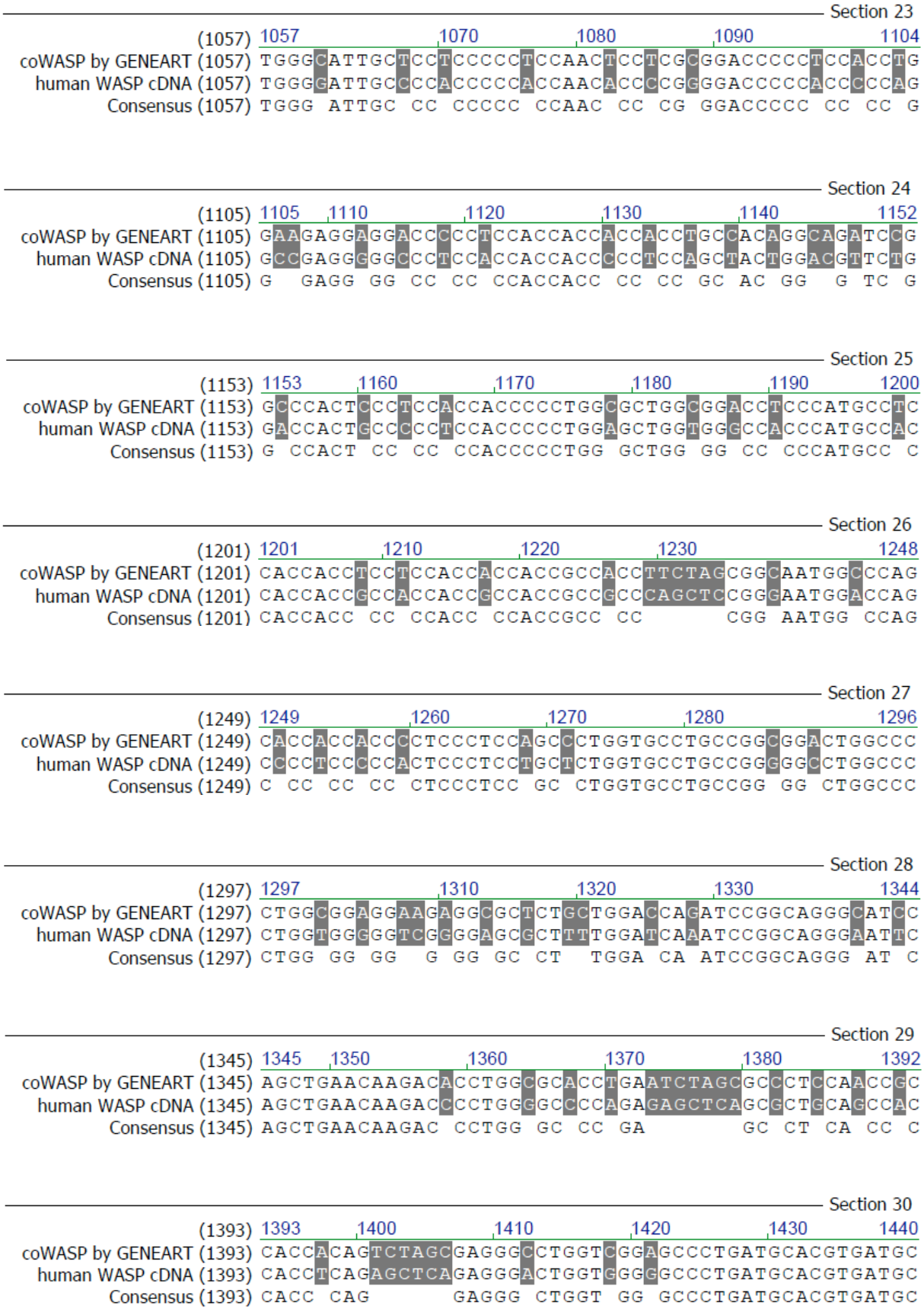
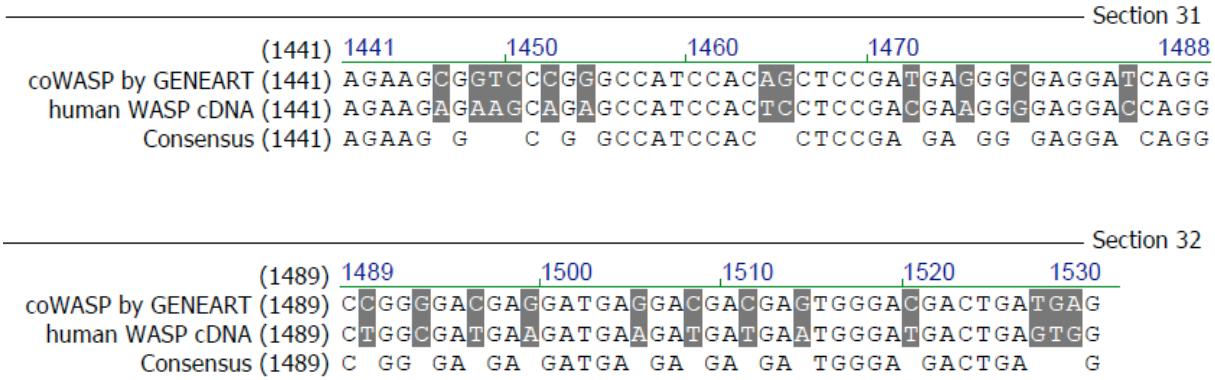
coWASP by GENEART (673) GCCCTAGCCCTGCCGACAAGAAGCGGAGCGGCAAGAAGAAGATCAGCA
human WASP cDNA (673) GACCTAGCCCAGCTGATAAGAAACGCTCAGGGAAGAAGAAGATCAGCA
Consensus (673) G CCTAGCCC GC GA AAGAA CG GG AAGAAGAAGATCAGCA
Section 16
(721) 721 730 740 750 768
coWASP by GENEART (721) AGGCCGACATCGGAGCCCCAGCGGCTTTAAGCACGTGAGCCACGTGG
human WASP cDNA (721) AAGCTGATATTGGTGCACCCAGTGGATTCAAGCATGTCAGCCACGTGG
Consensus (721) A GC GA AT GG GC CCCAG GG TT AAGCA GT AGCCACGTGG
Section 17
(769) 769 780 790 800 816
coWASP by GENEART (769) GCTGGGACCCCCAGAACGGCTTCGACGTGAACAACCTGGACCCCGACC
human WASP cDNA (769) GGTGGGACCCCCAGAATGGATTTGACGTGAACAACCTCGACCCAGATC
Consensus (769) G TGGGACCCCCAGAA GG TT GACGTGAACAACCT GACCC GA C
Section 18
(817) 817 830 840 850 864
coWASP by GENEART (817) TGCGGAGCCTGTTCAGCAGAGCCGGCATCAGCGAGGCCCAGCTGACCG
human WASP cDNA (817) TGCGGAGTCTGTTCTCCAGGGCAGGAATCAGCGAGGCCCAGCTCACCG
Consensus (817) TGCGGAG CTGTTC CAG GC GG ATCAGCGAGGCCCAGCT ACCG
Section 19
(865) 865 870 880 890 900 912
coWASP by GENEART (865) ACGCCGAGACAAGCAAGCTGATCTACGACTTCATCGAGGACCAGGGCG
human WASP cDNA (865) ACGCCGAGACCTCTAAACTTATCTACGACTTCATTGAGGACCAGGGTG
Consensus (865) ACGCCGAGAC AA CT ATCTACGACTTCAT GAGGACCAGGG G
Section 20
(913) 913 920 930 940 950 960
coWASP by GENEART (913) GCCTGGAAGCCGTGCGGCAGGAAATGCGGAGACAGGAACCCCTCCCAC
human WASP cDNA (913) GGCTGGAGGCTGTGCGGCAGGAGATGAGGCGCCAGGAGCCACTTCCGC
Consensus (913) G CTGGA GC GTGCGGCAGGA ATG GG G CAGGA CC CT CC C
Section 21
(961) 961 970 980 990 1008
coWASP by GENEART (961) CACCTCCACCTCCTAGCAGAGGCGGCAACCAGCTGCCCAGACCTCCCA
human WASP cDNA (961) CGCCCCCACCGCCATCTCGAGGAGGGAACCAGCTCCCCGGCCCCCTA
Consensus (961) C CC CCACC CC GAGG GG AACCAGCT CCC G CC CC A
Section 22
(1009) 1009 1020 1030 1040 1056
coWASP by GENEART (1009) TCGTGGGCGGCAACAAGGGCCGATCTGGACCTCTCCCACCTGTGCCCC
human WASP cDNA (1009) TTGTGGGGGGTAACAAGGGTCGTTCTGGTCCACTGCCCCCTGTACCTT
Consensus (1009) T GTGGG GG AACAAGGG CG TCTGG CC CT CC CCTGT CC


Section 23
(1057) 1057 1070 1080 1090 1104
coWASP by GENEART (1057) TGGGCATTGCTCCTCCCCCTCCAACTCCTCGCGGACCCCCTCCACCTG
human WASP cDNA (1057) TGGGGATTGCCCCACCCCCACCAACACCCCGGGGACCCCCACCCCCAG
Consensus (1057) TGGG ATTGC CC CCCCC CCAAC CC CG GGACCCCC CC CC G
Section 24
(1105) 1105 1110 1120 1130 1140 1152
coWASP by GENEART (1105) GAAGAGGAGGACCCCCTCCACCACCACCACCTGCCACAGGCAGATCCG
human WASP cDNA (1105) GCCGAGGGGGCCCTCCACCACCACCCCCTCCAGCTACTGGACGTTCTG
Consensus (1105) G GAGG GG CC CC CCACCACC CC CC GC AC GG G TC G
Section 25
(1153) 1153 1160 1170 1180 1190 1200
coWASP by GENEART (1153) GCCCACTCCCTCCACCACCCCCTGGCGCTGGCGGACCTCCCATGCCTC
human WASP cDNA (1153) GACCACTGCCCCCTCCACCCCCTGGAGCTGGTGGGCCACCCATGCCAC
Consensus (1153) G CCACT CC CC CCACCCCCTGG GCTGG GG CC CCCATGCC C
Section 26
(1201) 1201 1210 1220 1230 1248
coWASP by GENEART (1201) CACCACCTCCTCCACCACCACCGCCACCTTCTAGCGGCAATGGCCCAG
human WASP cDNA (1201) CACCACCGCCACCACCGCCACCGCCGCCCAGCTCCGGGAATGGACCAG
Consensus (1201) CACCACC CC CCACC CCACCGCC CC CGG AATGG CCAG
Section 27
(1249) 1249 1260 1270 1280 1296
coWASP by GENEART (1249) CACCACCACCCCTCCCTCCAGCCCTGGTGCCTGCCGGCGGACTGGCCC
human WASP cDNA (1249) CCCCTCCCCCCACTCCCTCCTGCTCTGGTGCCTGCCGGGGGCCTGGCCC
Consensus (1249) C CC CC CC CTCCCTCC GC CTGGTGCCTGCCGG GG CTGGCCC
Section 28
(1297) 1297 1310 1320 1330 1344
coWASP by GENEART (1297) CTGGCGGAGGAAGAGGCGCTCTGCTGGACCAGATCCGGCAGGGCATCC
human WASP cDNA (1297) CTGGTGGGGGTCGGGGAGCGCTTTGGATCAAATCCGGCAGGGAATTC
Consensus (1297) CTGG GG GG G GG GC CT TGGA CA ATCCGGCAGGG AT C
Section 29
(1345) 1345 1350 1360 1370 1380 1392
coWASP by GENEART (1345) AGCTGAACAAGACACCTGGCGCACCTGAATCTAGCGCCCTCCAACCGC
human WASP cDNA (1345) AGCTGAACAAGACCCCTGGGGCCCCAGAGAGCTCAGCGCTGCAGCCAC
Consensus (1345) AGCTGAACAAGAC CCTGG GC CC GA GC CT CA CC C
Section 30
(1393) 1393 1400 1410 1420 1430 1440
coWASP by GENEART (1393) CACCACAGTCTAGCGAGGGCCTGGTCGGAGCCCTGATGCACGTGATGC
human WASP cDNA (1393) CACCTCAGAGCTCAGAGGGACTGGTGGGGCCCTGATGCACGTGATGC
Consensus (1393) CACC CAG GAGGG CTGGT GG GCCCTGATGCACGTGATGC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(1441) 1441 1450 1460 1470 1488
coWASP by GENEART (1441) AGAAGCGGTCCGGGCCATCCACAGCTCCGATGAGGGCGAGGATCAGG
human WASP cDNA (1441) AGAAGAGAAGCAGAGCCATCCACTCCTCCGACGAAGGGGAGGACCAGG
Consensus (1441) AGAAG G C G GCCATCCAC CTCCGA GA GG GAGGA CAGG
Section 32
(1489) 1489 1500 1510 1520 1530
coWASP by GENEART (1489) CCGGGGACGAGGATGAGGACGACGAGTGGGACGACTGATGAG
human WASP cDNA (1489) CTGGCGATGAAGATGAAGATGATGAATGGGATGACTGAGTGG
Consensus (1489) C GG GA GA GATGA GA GA GA TGGGA GACTGA G

LIST OF OWN PUBLICATIONS

1. **Avedillo Díez I**., Dewey R. A., Zychlinski D., Modlich U., Baum C., Schambach A. and Klein C. Development of a novel SIN-gammaretroviral vector with improved efficacy and safety features for WAS stem cell based gene therapy. *In preparation.*
2. Boztug K., Schmidt M., Schwarzer A., Banerjee P. P., **Avedillo Díez I.**, Dewey R. A., Böhn M., Naundorf S., Kühlcke K., Blasczyk R., Maródi L., Kondratenko I., Orange J., von Kalle C. and Klein C. Successful hematopoietic stem cell gene therapy for Wiskott-Aldrich Syndrome. New England Journal of Medicine, 2010. *In press*.
3. Boztug K., Germeshausen M., Ballmaier M., Diestelhorst J., **Avedillo Díez I.**, Chernyshova L. I., Marodi L., Welte K. and Klein C. Multiple independent second-site mutations in two siblings with somatic mosaicism for Wiskott-Aldrich syndrome. Clinical Genetics 2008 Jul; 74(1): 68-74.
4. Dewey R. A., **Avedillo Díez I.**, Ballmaier M., Filipovich A., Greil J., Güngör T., Happel C., Maschan A., Noyan F., Pannicke U., Schwarz Snapper S., Welte K. and Klein C. Retroviral WASP gene transfer into human hematopoietic stem cells reconstitutes the actin cytoskeleton in myeloid progeny cells differentiated *In Vitro*. *Experimental Hematology* 2006 Sep; 34(9): 1161-9.

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DECLARATION / ERKLÄRUNG

Hiermit erkläre ich an Eides statt, dass die vorliegende Dissertation „A novel vector for the Gene Therapy for the Wiskott-Aldrich Syndrome“ selbständig verfasst und alle benutzten Hilfsmittel sowie evtl. zur Hilfeleistung herangezongenen Institutionen vollständig angegeben wurden.

Die Dissertation ist nicht schon als Diplomarbeit oder ähnliche Prüfungsarbeit verwendet worden.

Hannover, den

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Inés Avedillo Díez