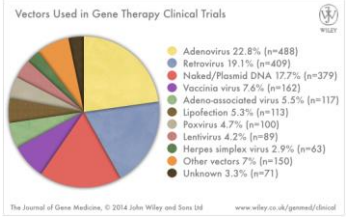
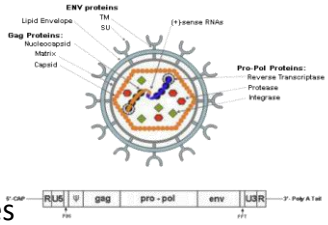
Retrovirus Vector

Vector	Gene Therapy Clinical Trials	
	Number	%
Adeno-associated virus	117	5.6
Adenovirus	463	22.3
Adenovirus + Modified vaccinia Ankara virus (MVA)	10	0.5
Adenovirus + Retrovirus	3	0.1
Adenovirus + Sendai virus	1	0
Adenovirus + Vaccinia virus	8	0.4
Alphavirus (VEE) Replicon Vaccine	1	0
Antisense oligonucleotide	6	0.3
Bifidobacterium longum	1	0
E. coli	2	0.1
Flavivirus	8	0.4
Gene gun	5	0.2
Herpes simplex virus	63	3
Lactococcus lactis	6	0.3
Lentivirus	89	4.3
Lipofection	113	5.4
Listeria monocytogenes	9	0.4
Measles virus	7	0.3
Minimalistic, immunologically defined gene expression (MIDGE)	1	0
Modified Vaccinia Ankara virus (MVA)	7	0.3
mRNA Electroporation	2	0.1
Naked/Plasmid DNA	369	17.8
Naked/Plasmid DNA + Adenovirus	3	0.1

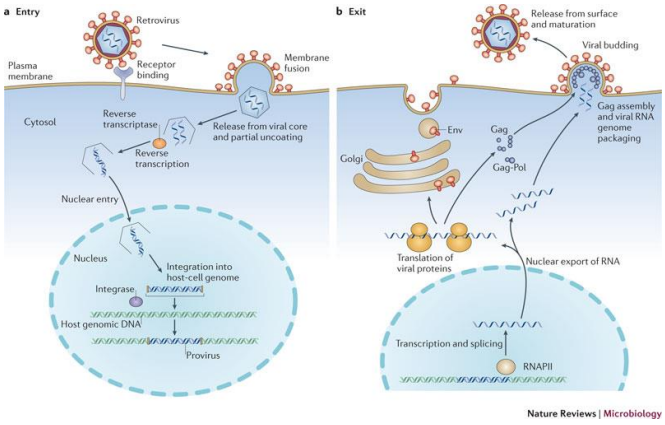


Vector	Gene Therapy Clinical Trials	
	Number	%
Naked/Plasmid DNA + Modified Vaccinia Ankara virus (MVA)	2	0.1
Naked/Plasmid DNA + Vaccinia virus	3	0.1
Naked/Plasmid DNA + Vesicular stomatitis virus	2	0.1
Newcastle disease virus	1	0
Poliovirus	1	0
Poxvirus	68	3.3
Poxvirus + Vaccinia virus	32	1.5
Retrovirus	406	19.6
RNA transfer	35	1.7
RNA virus	5	0.2
Saccharomyces cerevisiae	8	0.4
Salmonella typhimurium	4	0.2
Semliki forest virus	2	0.1
Sendai virus	2	0.1
Shigella dysenteriae	1	0
Simian Immunodeficiency Virus (SIVagm)	1	0
Simian virus 40	1	0
siRNA	2	0.1
Sleeping Beauty transposon	8	0.4
Streptococcus mutans	1	0
Vaccinia virus	119	5.7
Venezuelan equine encephalitis virus replicon	3	0.1
Vesicular stomatitis virus	3	0.1
Vibrio cholerae	1	0
Unknown	71	3.4

Retrovirus



- family of retroviridae :
  - simple α-, β-, γ-, and ε-retroviruses,
  - complex δ-retro-, lenti- and spumaviruses



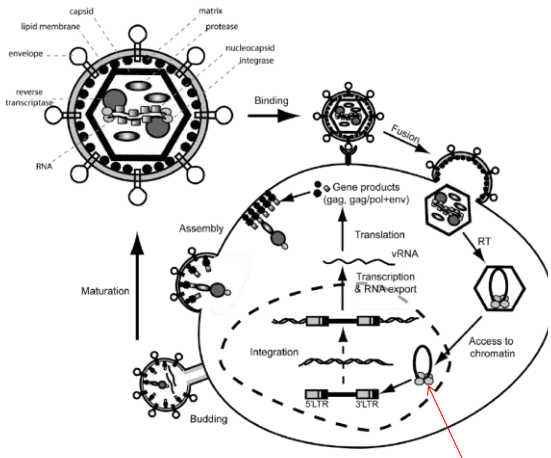
Stoye. 2012. *Nat. Rev.Microbiol.***10**, 395-406

Retroviral life cycle. Different events in the life cycle of retroviruses are illustrated. **a** | Viral entry into cells involves the following steps: binding to a specific receptor on the cell surface; membrane fusion either at the plasma membrane or from endosomes (not shown); release of the viral core and partial uncoating; reverse transcription; transit through the cytoplasm and nuclear entry; and integration into cellular DNA to give a provirus. **b** | Viral exit involves the following steps: transcription by RNA polymerase II (RNAPII); splicing and nuclear export of viral RNA; translation of viral proteins, Gag assembly and RNA packaging; budding through the cell membrane; and release from the cell surface and virus maturation.

Retrovirus

- Can only enter dividing cells

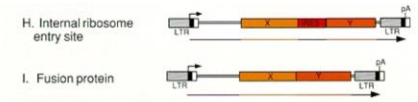
→ viral preintegration complex (PIC), a large nucleoprotein complex responsible for viral cDNA integration, cannot enter the cell nucleus and requires disassembly of the nuclear envelope during mitosis

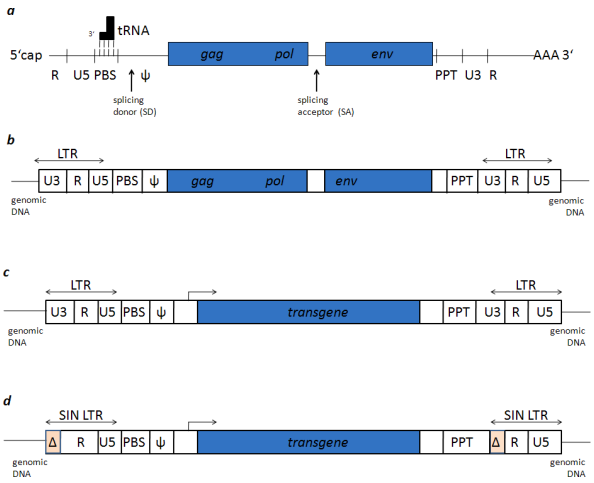


viral preintegration complex (PIC),

Three general strategies to design retroviral vectors

1. expression of different proteins from alternatively spliced messenger RNAs transcribed from one promoter,
2. use of the promoter in the LTR and internal promoters to drive transcription of different cDNAs and
3. use of IRES elements to allow translation of multiple coding regions from a single mRNA .





Sequence elements present in the RNA genome of all infectious virus particles

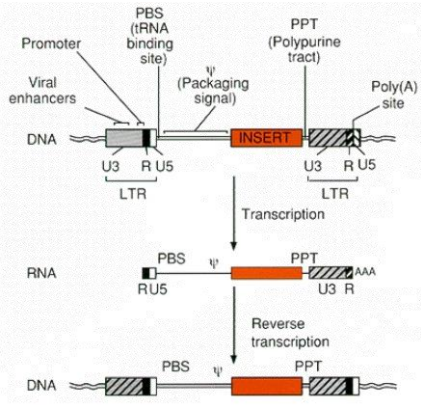
Sequence elements present in the RNA genome of all infectious virus particles and in the proviral genome after integration into the host genome

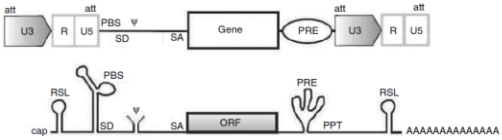
Sequence elements of a retroviral vector with complete LTR

composition of a safety-optimized vector with deletions in the U3 region of LTRs

The arrow indicates the internal promoter that controls transgene expression.
R: redundant region; U3 and U5: unique regions at 3t- or 5t- end, respectively;
PPT: polypurine tract;
LTR, "*Long Terminal Repeat*";
SIN: self inactivating,
PBS, primer binding site;
ψ, packaging signal.

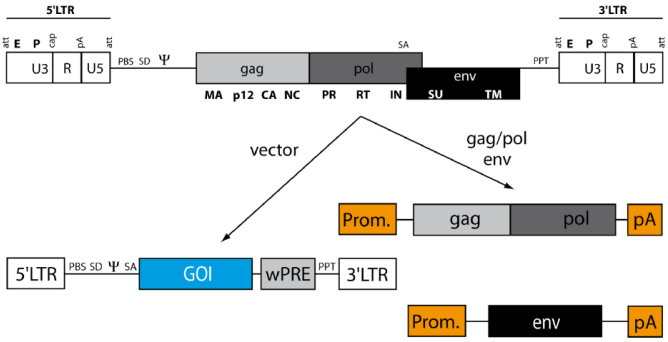
- Efficient gene transduction and integration depend on the inclusion in the retroviral vector of a number of *cis*-acting viral elements:
  - (1) a promoter and poly adenylation signal in the viral genome;
  - (2) a viral packaging signal (ψ or E) to direct incorporation of vector RNA into virions;
  - (3) signals required for reverse transcription, including a transfer RNA-binding site (PBS) and poly purine tract (PPT) for initiation of first- and second-strand DNA synthesis, and a repeated (R) region at both ends of the viral RNA required for transfer of DNA synthesis between templates; and
  - (4) short partially inverted repeats located at the termini of the viral LTRs required for integration.
- Vectors derived from simple retroviruses:
  - a transgene capacity of up to 9kb
  - only enter the nuclei of dividing cells





Gene therapy strategy using retroviral vector

- SPLIT PACKAGING DESIGN
- SELF-INACTIVATING VECTOR DESIGN
- Termination enhancers and insulators
- Coexpression
- Targeting integration
- pseudotyping

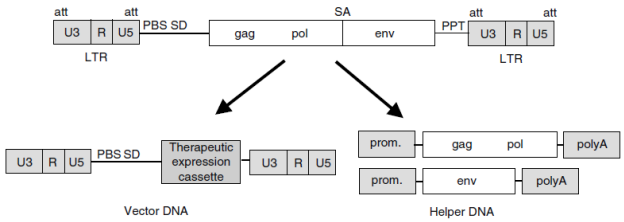


general genome structure

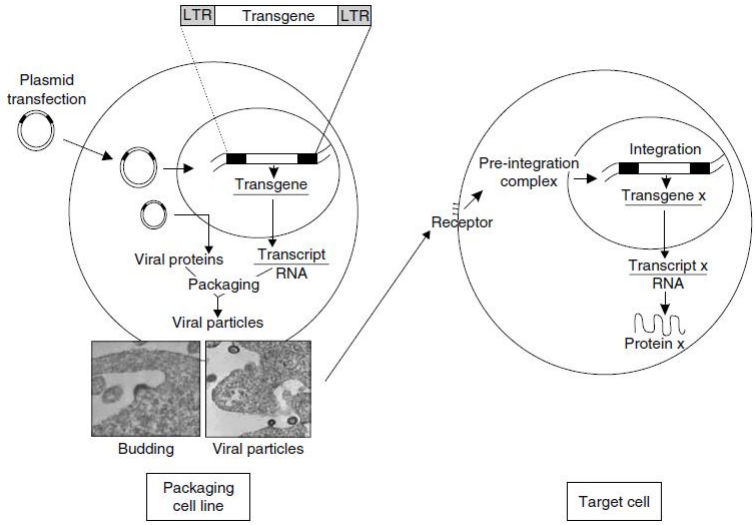
For construction of a gene transfer tool, the *cis*-acting elements (LTRs and leader region with Ψ) and the open reading frames for gag/pol and env are divided onto separate plasmids.
Gag/pol and env are placed in a heterologous DNA context (Prom: promoter; pA: polyA signal) that can be delivered as a plasmid transiently or stably inserted into the host cell DNA of the packaging cell. The gag/pol and env plasmids lack Ψ, so that the encoded RNA cannot be packaged into retroviral particles.
the vector DNA containing gene of interest (GOI, e.g., a therapeutic transgene cassette), flanked by the LTRs, harbors Ψ for efficient packaging into the viral particle.

Maetzig, 2011

SPLIT PACKAGING DESIGN



- The general genome organization of a simple retrovirus :
  - LTRs (LTR, with U3, R, and U5 regions),
  - PBS, splice signals (SD, SA),
  - packaging signal ψ, the
  - PPT,
  - attachment signals of the IN (att).
- To construct a (replication-deficient) gene transfer tool, the cis-acting elements (LTR and leader region) and the ORFs for gag, pol, and env are divided on separate plasmids (vector DNA and helper DNA).
  - Gag, pol, and env are encoded by helper plasmids that can be delivered as a single molecule or split into different DNA molecules for safety reasons.
  - The helper DNA lacks the packaging signal (ψ) and thus cannot be packaged into a viral particle.
  - The vector DNA contains the therapeutic expression cassette, flanked by the LTR. It harbors a packaging signal (ψ) for efficient packaging into the viral particle.



SELF-INACTIVATING VECTOR DESIGN

- lack enhancer–promoter sequences in the U3 region of their LTRs and rather make use of an internal promoter to initiate transcription of the transgene cassette
- SIN:
  - reduces the risk of insertional upregulation of neighboring genes by decreasing long-distance enhancer interactions or promoter activity from the 3'LTR
  - lowers the risk of recombination to create a replication-competent retrovirus;
  - if a cell expressing the SIN transgene is superinfected by a replicating retrovirus, the SIN-vector RNA (due to the lack of genomic packageable RNA) is unlikely to be mobilized;
  - avoids potential interference with more cell-type specific or inducible transcriptional control systems that are introduced to express the gene(s) of interest.

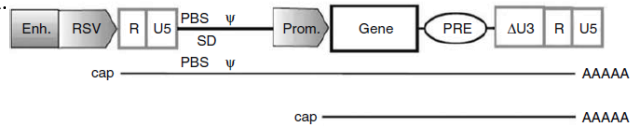


FIGURE 1.5 SIN retroviral vectors. SIN vectors are devoid of promoter/enhancer elements in the 3′U3 region and are therefore dependent on an internal promoter. Moreover, this internal promoter can be chosen according to the tissue specificity and expression strength needed for the vector application. To overcome transcriptional interference and to produce sufficient amounts of packageable genomic RNA (upper), a strong promoter should be taken to drive the genomic message (e.g., Rous sarcoma virus [RSV] promoter plus SV40 enhancer).

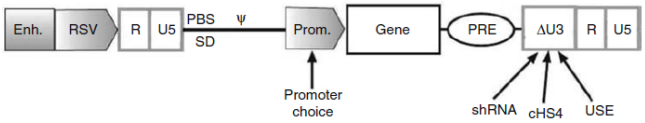
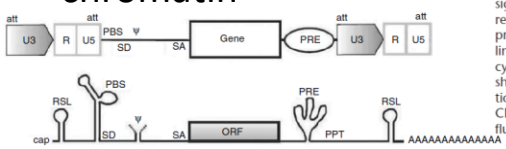


FIGURE 1.6 SIN vectors within the 3′UTR and the 3′ΔU3 region. The deletion of promoter/elements of the 3′U3 (ΔU3) creates space for the incorporation of useful features. Interestingly, these elements will be present (due to the mode of reverse transcription) in its integrated form. For RNAi applications short hairpin RNA (shRNA) expression cassettes, consisting of a Pol. I promoter and a shRNA RNA, can be introduced. In addition, chromatin insulators (e.g., cHS4) and polyadenylation enhancers (upstream polyadenylation enhancers, USE) can be incorporated here (for details see text).

Termination enhancers and insulators

- Insulators are cis-elements that inhibit enhancer or silencer interactions between the integrated transgene and the neighboring chromatin



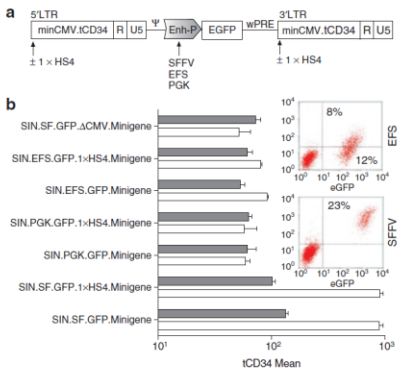
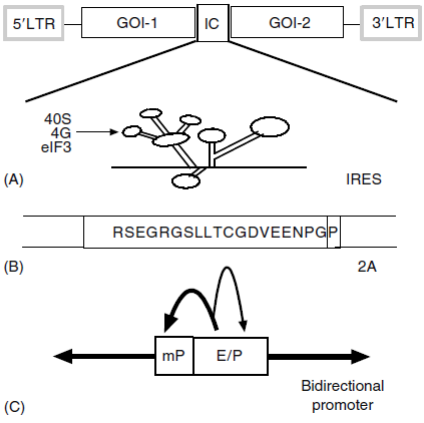


Figure 2 Stably integrated "minigene" reporter constructs reveal the low enhancer activity of the cellular promoters tested. (**a**) The retroviral self inactivating (SIN) vector contains a minigene cassette in the U3 region of the long-terminal repeat (LTR), consisting of a minimal promoter followed by a truncated CD34 surface marker gene (*tCD34*). Termination is mediated by signals in the R region of the LTR. The same vectors were also tested with 1×HS4 insulator element inserted upstream of the minigene cassette. Enh-P sequences were introduced as indicated. A control vector contained no minimal promoter (ΔCMV). (**b**) In SC1 fibroblasts and lineage-negative primary murine hematopoietic cells, only the internal spleen focus–forming virus (SFFV) promoter led to a significant activation of the minigene cassette. Background levels are reflected in the tCD34 expression of the construct that lacks a minimal promoter. Data obtained from SC1 cells are shown in white columns and lineage-negative cells in grey ones. The insets show representative flow cytometry data (SC1 cells) for illustration of the assay, CD34 expression shown on the y-axis. Potential activation of the minigene by transcriptional readthrough could be ruled out (**Supplementary Figure S2**). CMV, cytomegalovirus; EFS, short form of EF1α EGFP, enhanced green fluorescent protein; PGK, phosphoglycerate kinase.

Coexpression



Bicistronic expression can be achieved via several strategies. For efficient coexpression the internal cassette (IC) can be formed by internal ribosome entry sites, 2A "self-cleavage" sites and single or bidirectional promoters. (A) Internal ribosome entry sites recruit parts of the translation machinery (e.g., eukaryotic initiation factor 4G and eIF3 plus 40S ribosome). (B) Self-cleavage indicated for the *Thosea asigna* virus 2A-cleavage site (T2A). The peptide consists of 20 amino acids. Cleavage occurs via a co-translational ribosome skipping mechanism between the C-terminal Glycin and Prolin residues, leaving the indicated amino acids attached to the end of the 5′ protein and the start of the 3′protein. (C) Bidirectional promoters consist of two antisense oriented (minimal = m) promoters (P) sharing the same enhancer (E) but driving transgene expression in opposite directions.

Targeting Integration

Table 2. Chromosomal features associated with preferential retroviral integration sites.

Retroviridae genera	in Transcription Units[a]	± 2kb Transcription Start Sites[a]	± 2 kb CpG Islands[a]
Lentiviruses	+/++	0	-/0
Alpharetroviruses	+	0	+
Betaretroviruses	0	0	0
Gammaretroviruses	+	++	++
Deltaretroviruses	+	+	+
Epsilonretroviruses	NA	NA	NA
Spumaviruses	0	++	++
HERV-class II	+	++	++

[a] ratio between the proportion of the chromosomal feature over the random proportion in the human genome, according to RefSeq databases and with values from [69,87,88,90,94,95].
0: no statistical difference over random;
+/++: statistically favored feature over random with ++ for ratio >2 and + for a ratio <2;
-: statistically disfavored feature over random
NA : not available

Pseudotyping of Retroviral Vectors for Targeting Approaches

- retroviruses enter cells by a defined envelope/host cell receptor interaction

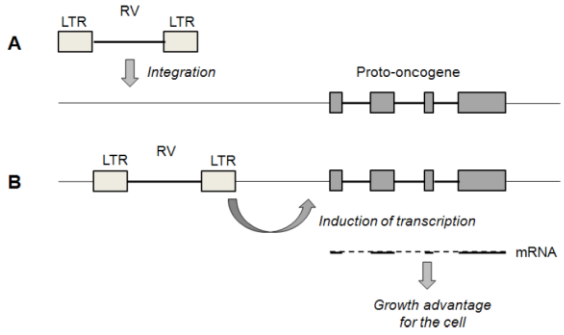
Table 1. List of most important pseudotypes, their receptors and envelope modifications required for pseudotyping of MLV to transduce certain target cells / species.

Pseudotype	Abbreviation	Receptor	Modification	Target Species	References
Ecotropic MLV env	Eco	mCAT	not required	mouse and rat	[110]
Amphotropic MLV env	Ampho	PiT2	not required	multiple	[110]
Xenotropic MLV env	Xeno	XPR1	not required	human and others	[98]
Vesicular Stomatitis Virus glycoprotein	VSVg	not determined	not required	multiple	[111]
Simian Endogenous Retrovirus env	RD114	RDR/ASCT2	not required	human and others	[112]
Gibbon Ape Leukemia Virus env	GALV	PiT1	not required	human and ape	[98]
Measles Virus (vaccine strain) H and F proteins	MV	CD150, CD46	not determined	human	[113]
Human Immunodeficiency Virus gp120 env	HIV	CD4 and co-receptor	C-terminal truncation	human	[114]

- Vector retrovirus has been used for:
  - X-chromosomal inherited severe combined immunodeficiency disorder (X-SCID).
  - adenosine deaminase (ADA) SCID and
  - chronic granulomatous disease (CGD)

Side Effects in Retroviral Gene Therapy

- integration of retroviruses in the cellular genome →disruption of the gene coding regions
  - replication-deficient oncoviral vectors → the risk of insertional mutagenesis leading to cell transformation for a long time: low
- Insertion-mediated mutagenesis can lead to a wide spectrum of phenomena, ranging from *in vitro* immortalization to clonal dominance and oncogenesis *in vivo*



Mechanism of proto-oncogene activation by insertional mutagenis. (A) Integration of viral genomic DNA into somatic cellular DNA in the proximity of a nontranscribed oncogene. (B) Integrated proviral DNA induces the transcription of the oncogene. RV, retroviral vector. LTR, "*Long Terminal Repeat*".

Distribution of Retroviral Integration Sites in the Cellular Genome

- Using LAM-PCR →
  - integration of MLV-derived vectors preferentially occurs close to transcription start sites and in proximity to CpG islands.
  - HIV-1-derived vectors
    - 2/3 cases : integrate into gene coding regions over the complete length of the gene, with a preference for active genes
    - revealed a relationship between the expression of the transcription coactivator lens epithelium-derived growth factor (LEDGF) and the integration of the vector in transcribed regions in Jurkat cells

Preclinical and Clinical side effect

- Serial transplantations of gamma retroviral gene-marked CD34+ cells derived from murine bone marrow
  - tumor development in secondary and tertiary recipients
  - alterations of their hematopoiesis
  - both proviral LTRs caused an overexpression of *Evi1*
- 2006, retroviral gene transfer in hematopoietic precursor cells non-human primate model→ died five years after gene therapy due to a myeloid sarcoma the first acute myeloid leukemia
  - Two vector integrants : *Bcl2-A1*, *Cdw91*

- Two to six years after successful gene therapy for X-SCID, 5 of 19 patients developed T-cell leukemia
  - vector integrations in the proto-oncogene *LMO2*
- Gene therapy for correction of X-CGD in two adult patients
  - Integration analyses : insertion sites in the genes *MDS1-EVI1*, *PRDM16* or *SETBP1* ➔ resulting in an expansion of gene-corrected myelopoesis.
  - A myelodysplastic syndrome

Table 2. Selected gene therapy trials targeting the hematopoietic system or skin disorders.

Disease	Phenotype	Affected Gene(s)	Conventional Therapy	Target Cells	Vector	Vector Related Side Effects	Reference
Epidermolysis bullosa	Detaching skin and mucosal tissues	Keratin or collagen	Skin graft transplantation	MSC	GV-LTR	n.a.	[89]
Wiskott- Aldrich Syndrome	Immuno deficiency, thombocytopenia and blood cancer	WAS	Drug therapy and / or BM transplantation	HSC	GV-LTR	Leukemia	[162]
Chronic granulomatous disease	Lack of phagocytic lymphocytes	gp91-phox	BM transplantation	HSC	GV-LTR	Leukemia	[161]
SCID-X1	Lack of T and NK cells, and of mature B cells	IL2γc	BM transplantation	HSC	GV-LTR	Leukemia	[86,159,174,180]
SCID-ADA	Immunodeficiency, skeletal and neurologic abnormalities	Adenosine deaminase	Drug therapy and / or BM transplantation	HSC	GV-LTR	n.a.	[160,181]
Beta thalassemia	Anemia	Beta-globin	Blood transfusion	HSC	LV-SIN	Clonal dominance	[182]
Adrenoleuko-dystrophy	Cerebral demyelination	ALD	BM transplantation	HSC	LV-SIN	n.a.	[177]
Melanoma	Skin cancer	various	Dissection of the tumor, chemo- or radiotherapy	T-cells	GV-LTR	n.a.	[183,184]
Graft *versus* host disease	Various	n.a.	Drug therapy	T-cells	GV-LTR	n.a.	[185]
HIV-Aids	Immunodeficiency	n.a.	HAART	HSC, T-cells	LV-LTR	n.a.	[186]