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Vector design for expression of O⁶-methylguanine-DNA methyltransferase in hematopoietic cells

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Abstract

Enhancing DNA repair activity of hematopoietic cells by stably integrating gene vectors that express O(6)-methylguanine-DNA-methyltransferase (MGMT) is of major interest for innovative approaches in tumor chemotherapy and for the control of hematopoietic chimerism in the treatment of multiple other acquired or inherited disorders. Crucial determinants of this selection principle are the stringency of treatment with O(6)-alkylating agents and the level of transgenic MGMT expression. Attempts to generate clinically useful MGMT vectors focus on the design of potent expression cassettes, an important component of which is formed by enhancer sequences that are active in primitive as well as more differentiated hematopoietic cells. However, recent studies have revealed that vectors harboring strong enhancer sequences are more likely to induce adverse events related to insertional mutagenesis. Safety-improved vectors that maintain high levels of MGMT expression may be constructed based on the following principles: choice of enhancer-promoter sequences with relatively mild long-distance effects despite a high transcription rate, improved RNA processing (export, stability and translation), and protein design. The need for optimizing MGMT protein design is supported by recent observations suggesting that the P140K mutant of MGMT, developed to be resistant to inhibitors such as O6-benzylguanine, may confer a selective disadvantage when expressed at high levels. Here, we provide a review of the literature exploring MGMT expression vectors for bone marrow chemoprotection, and describe experimental evidence suggesting that high expression of MGMT P140K induces a selective disadvantage in the absence of alkylating agents. We conclude that the appropriate design of expression vectors and MGMT protein features will be crucial for the long-term prospects of this promising selection principle.

Keywords

gene therapy; lentiviral vector; retroviral vector

Introduction

The transfer and expression of genes mediating resistance against cytotoxic agents serves as a basic approach in genetic therapy to protect healthy cells and tissues from the adverse effects of tumor chemotherapy, and to establish a conditional selective advantage in gene-modified cells. The gene-modified, chemoprotected cell population may then gradually displace

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unmodified competitor cells. Given that chemoprotection occurs in a self-renewing stem cell and the selective pressure is applied repeatedly, the majority of cells present in a given organ or tissue will express the desired phenotype. Major determinants regulating the degree of chimerism are the efficiency of the initial gene transfer rate and the stringency of the cytotoxic regime (reviewed in [1-3]).

As cytotoxic therapy may have to be administered repeatedly, stably integrating gene vectors are considered particularly promising for this approach. While physicochemical modes of stable transgene delivery are emerging [4], retroviral (including lentiviral) vectors still represent the best characterized and most efficient tool for stable gene delivery [5]. It is important to consider that hematopoietic stem cells (HSCs) form a rare and somewhat hidden population that is difficult to target *in vivo*, even when using most advanced lentiviral technology [6]. Therefore, present clinical protocols prefer to enrich and transduce HSCs *ex vivo*. However, typically less than 10% of the endogenous pool of HSCs can be harvested for *ex vivo* gene transfer. Furthermore, to avoid the integration of multiple gene vectors into single cells, *ex vivo* gene transfer protocols may have to be designed to transduce less than 50% of the target cells [7,8]. Chemoprotection may thus be instrumental to increase the contribution of gene-modified cells to hematopoiesis [1-3,9-12], especially if the therapeutic gene does not confer a spontaneous selective advantage and if no or dose-reduced conditioning of endogenous hematopoiesis is administered before transplanting the gene-modified population. For most clinical applications including protection against side effects of cancer chemotherapy, much more than 10% of the cells within the hematopoietic system should express the therapeutic phenotype [1,13,14].

Gene delivery of MGMT for selection of hematopoietic stem cells

O(6)-methylguanine-DNA-methyltransferase (MGMT) repairs DNA damage induced by agents considered to exert the majority of their toxic effects via alkylation at the O6-position of guanine in DNA (referred to as O(6)-alkylating agents), thus preventing cells from entering apoptosis [15]. This selection principle is particularly effective in the hematopoietic system, because HSCs and their progeny express relatively low amounts of endogenous MGMT and are thus highly susceptible to the toxic effects of alkylating agents. Evidence for the feasibility of genetic chemoprotection mediated by MGMT has been obtained using bone marrow transplantation (BMT) in mice, immunodeficient mice engrafted with human HSCs, and large animal (dog) models [1-3,8,9,11,12,16-39] (Table 1).

Particularly effective for chemoselection of HSCs is the use of MGMT mutants such as MGMT P140K which are resistant to O6-benzylguanine (O6BG) and related small compounds that inhibit the wild-type protein [40,41]. Human hematopoietic cells retrovirally engineered to express MGMT P140K and engrafted in immunodeficient NOD/SCID mice could be selected *in vivo* by combined treatment with O6BG and alkylating agents, following sublethal pretransplant conditioning by irradiation [17] or alkylating agents [37].

Unfortunately, a clinical study using retroviral vectors to over-express MGMT P140K in hematopoietic cells had too low gene transfer rates to establish a clinically useful selective advantage [19]. In contrast, studies in dogs have provided clear evidence of polyclonal chemoprotection of HSCs that were retrovirally engineered to express MGMT P140K [1,24, 27,42], strongly suggesting that similar effects can be achieved in humans given that technical hurdles are overcome.

Principles of retroviral vector design and expression

One of the major hurdles to implement bone marrow chemoprotection in humans relates to gene transfer and expression. Since the first demonstration of retroviral vector-mediated gene

transfer into repopulating hematopoietic cells of the mouse [43,44], numerous types of gene vectors and experimental protocols have been proposed to increase the efficiency and biosafety of somatic gene therapy in this cell population [5,45-48]. In general, gene transfer into hematopoietic cells in non-human primates has been found to be more cumbersome than predicted by small animal studies, owing to differences in cell cycle control, receptor status and other antiviral restriction factors [48]. Nevertheless, promising high gene transfer levels have been achieved by gammaretroviral and more recently also by lentiviral vectors. In one clinical trial and in a few non-human primates, gammaretroviral gene transfer attained more than 10% of the long-term repopulating cell population [48-50]. Lentiviral vectors such as those based on the human immunodeficiency virus (HIV) or the simian immunodeficiency virus (SIV) are expected to achieve even higher marking levels, as they can be actively transported into the nucleus of a non-dividing, partially activated cell [51,52]. Clinical data with lentiviral gene transfer into human HSCs may not be available before the end of this year. Meanwhile, many non-human primate studies have been hampered by the occurrence of species-specific antiretroviral restriction factors active against HIV-derived viral particles [53]. Improvements in the design of SIV-based vectors or the use of non-human primates lacking HIV-restriction factors may overcome these limitations [54-56].

In contrast to the difficulties encountered in animal models, recent improvements in lentiviral and gammaretroviral transduction protocols for human hematopoietic cells suggest that vector efficiency may be increased to a level that potentially results in the introduction of several transgene copies into single HSCs [7,57]. Besides the definition of suitable conditions for HSC mobilization, enrichment and short-term culture, the introduction of improved envelope proteins for particle “pseudotyping” is responsible for the increased gene transfer rates [51, 58-62]. Recent advances in clinical trials are partly based on such technical improvements [13,14,50,63,64].

Transgene expression increases as a function of gene copy number [7,65]. While appreciated from a standpoint of efficiency, the co-existence of several independent insertion events in single cells may cause major problems related to combinatorial insertional mutagenesis of cellular proto-oncogenes. In both mice and primates, insertional mutagenesis by replication-deficient vectors has now been documented as a potential cause of tumor induction [66-69]. To avoid side effects caused by insertional mutagenesis, the number of vector insertions in cells with self-renewal potential should be low and vectors need to be designed to avoid strong long-distance interactions with neighboring cellular genes [70].

Traditionally, the strongest enhancer-promoter elements active in mammalian cells were derived from viruses. For HSCs and their progeny, various enhancer-promoter sequences of murine leukemia viruses (MLV), the cytomegalovirus, or hybrids of these elements with cellular promoters have been proposed to maximize gene expression [71-76]. These enhancer-promoter elements were typically placed into the long terminal repeats (LTRs) of simple gammaretroviral vectors, from where they may direct high levels of transcription (Figure 1A). However, insights from preclinical models suggest that the LTR architecture plays a major role in the induction of transformation by insertional mutagenesis [77,78]. To reduce the frequency and severity of insertional effects on neighboring genes, improved constructs are designed as so-called self-inactivating (SIN) vectors which lack the enhancer-promoter elements in the LTRs and rather place the gene(s) of interest under control of an internal enhancer-promoter [79]. Initially, retroviral SIN vectors suffered from low titers [79]. The choice of more powerful promoters that drive the genomic RNA of the vector and the introduction of vector production technology based on transient transfection now allow the production of SIN vectors (either lentiviral or gammaretroviral) at titers that are sufficient for clinical trials [80,81]. Recently, an elegant system allowing high titer of gammaretroviral SIN vectors from stable producer cells based on a cassette exchange technology has been introduced [82]. Progress with stable

producer cells for lentiviral vectors has also been reported [83,84], suggesting that SIN vectors of either origin (gammaretroviral or lentiviral, Figure 1B-E) will be available for future clinical studies.

Compared with a conventional LTR-controlled expression cassette, a SIN vector harboring an internal promoter already reduces the insertional risk, even if a strong enhancer is connected to the promoter [78]. However, enhancer-mediated alteration of cellular proto-oncogene expression may still occur following integration of such SIN vectors [78], similar to observations made with transposons carrying an internal single LTR sequence [85]. It would thus be naïve to assume that SIN vector design will completely solve the problem of insertional genotoxicity.

Lentiviral vectors as opposed to gammaretroviral vectors are less likely to insert their genetic cargo in the vicinity of cellular promoter sequences [86]. It was therefore encouraging to note that lentiviral SIN vectors in contrast to gammaretroviral LTR vectors failed to promote tumors when investigated in a murine model with predisposition to lymphoma [77]. However, in this study the lentiviral SIN vectors used different enhancer-promoter sequences than the gammaretroviral LTR vector, leaving open whether the observed differences were related to the insertion pattern or enhancer configurations. In contrast, lentiviral vectors harboring stronger viral enhancers were associated with tumor induction in other murine models, although the potential role of insertional mutagenesis remained unclear [87,88].

Insulators are *cis*-acting DNA elements that separate transcriptional domains in the chromosomal context, thus preventing “regulatory conflicts”. Incorporating insulator sequences into semi-randomly integrating gene vectors may shield their expression cassette against disturbing influences of the cellular genome and, vice versa, the cellular genome against side effects that may be triggered by the insertion of the transgene cassette [89,90]. To achieve this, the insulator should be placed, to fill the “gap” left by the deleted enhancer-promoter sequences, within the Δ U3 region of SIN vectors (Figure 1C). Whether the insulator sequences may cause unexpected epigenetic effects or operate in a cell-type specific manner will have to be evaluated in suitable experimental models before recommending their use to increase vector biosafety [91].

Using the newly introduced models of insertional mutagenesis mediated by retroviral (including lentiviral) vectors [77,78,92], it may be possible to identify the enhancer sequences and vector configurations that are least prone to cause insertional upregulation of neighboring proto-oncogenes. At present, the strongest cellular enhancer-promoter sequence identified for gene expression in hematopoietic cells is derived from the human elongation factor 1 alpha (EF1a) gene [21,74,93-95]. The promoter of the human phosphoglycerate kinase gene (PGK) is also popular, although weaker. Compared to a powerful MLV-derived enhancer-promoter such as that from the polycythemic strain of the spleen-focus-forming virus (SFFVp), the expression levels of EF1a and PGK promoters are 3-10 fold lower (per transgene copy) [21].

The survey provided in Table 1 (not considered to be exhaustive) of various gammaretroviral and lentiviral vectors used to express MGMT reveals that most constructs used viral enhancer-promoter sequences, although more recently, vectors employing cellular promoter sequences have become more popular. We and others have observed that the promoter of EF1a, but probably not that of PGK, is sufficiently strong to express MGMT P140K in hematopoietic cells to a level that allows polyclonal chemoprotection from a single vector copy [12,21,96]. The presence of the first intron of EF1a enhances gene expression mediated by this promoter by a factor of 2 to 3. It is likely, although not formally shown, that this effect is primarily caused by improved RNA processing (as opposed to the presence of additional enhancer sequences within the EF1a intron).

The introduction of an intron and other RNA enhancer motifs represents a promising avenue of vector design (Figure 1), as improvements of RNA processing are not expected to result in an increased risk of insertional mutagenesis [70]. The importance of introns to elevate transgene expression from retroviral vectors is clearly demonstrated [97-105]. However, intron sequences are not reliably transmitted to target cells by retroviral vectors, because these use cytoplasmic mRNA as the mobile form of their genome. While several approaches to maintain introns in retroviral vectors have been described, these have to be further improved to increase titers or avoid the potential recombination with other viral elements [104,106,107]. Most lentiviral vectors rely on the Rev-responsive element (RRE) to interact with lentiviral Rev protein in packaging cells, thus increasing the efficiency of mRNA export [52]. Rev-mediated mRNA export may suppress splicing, thus allowing the nuclear export of vector mRNA that has preserved an intron located shortly downstream of the RRE (Figure 1D). Unfortunately, Rev/RRE-mediated escape from splicing is not complete [108,109], resulting in the coexistence of transduced cells that contain vector copies with and without the intron.

Improved processing of the mRNA 3' end may also significantly improve protein expression. The most widely used element improving 3' RNA processing is the post-transcriptional regulatory element (PRE) derived from the woodchuck hepatitis virus (WHV). Introducing this element into the 3' untranslated region (Figure 1A) typically improves both vector titer and expression [98, 99, 102]. It was suggested that this element may cause side effects because it overlaps with a potential tumorigenic sequence of WHV [110]. However, this hypothesis still lacks experimental validation. PRE sequences lacking major WHV reading frames and cryptic promoter elements have been designed that are expected to reduce concerns related to the introduction of this relatively large viral module (>600 bp) [102].

As indicated above, the EF1a promoter [93] is the most active cellular promoter known to date for constitutive transgene expression in hematopoietic cells. We recently found that gammaretroviral and lentiviral SIN vectors designed to express MGMT from an internal EF1a promoter are similarly efficient to protect hematopoietic cells against alkylating agents [21]. However, additional studies are required to reveal whether human CD34+ cells can be chemoselected in vivo (NOD/SCID model), as previously demonstrated using gammaretroviral or lentiviral vectors harboring viral enhancer-promoter sequences to express MGMT (mostly the P140K variant, Table 1). It also remains to be determined whether or not inclusion of the PRE of WHV or alternative RNA enhancer motifs further increases MGMT expression. Of note, such RNA processing motifs act in a very context-dependent manner such that lessons learned in the context of other cDNAs are not necessarily predictive [99].

Various approaches have been explored to achieve reliable co-expression of two or more cDNAs from a single vector. The use of internal ribosomal entry sequences (IRES) or the self-cleaving 2A proteinase of the foot-and-mouth disease virus may lead to coordinated protein expression under control of a single promoter. However, both strategies typically leave one of the two proteins expressed at relatively low levels. Recently, bidirectional promoters have been shown to result in consistent co-expression of two different cDNAs (Figure 1E) [111]. This strategy could be combined with IRES or the 2A-proteinase, potentially allowing the constructions of complex vectors expressing 3 or more different proteins. Unfortunately, the freedom of thought is greater than the flexibility of the viral life cycle. Complex transgene cassettes may be unstable during reverse transcription (due to the potential formation of RNA hairpins, cryptic splice sites or transcriptional termination signals), resulting in a low titer and a major genetic variability of the transduced cell population. The design of optimal co-expression vector may thus demand the screening of several alternative designs and strategies.

Exploring side effects of continued transgene expression

Having defined a vector configuration that can be produced at high titers and mediates sufficient levels of transgene expression from a single vector copy, extended studies need to be conducted in preclinical models to test the safety and efficiency of genetic chemoprotection. To date, no standards have been appointed for such studies. Most researchers conduct BMT in mice of the strains C57Bl6/J, BALB/c and C3H (Table 1). Human CD34+ cells are examined in vitro or after transplantation into immunodeficient mice such as NOD/SCID mice (Table 1). Finally, large animal studies may be performed [24,27]. Given the overwhelming evidence for the feasibility of bone marrow chemoprotection mediated by MGMT over-expression, experiments addressing potential limitations of this approach are relatively sparse.

Before conducting BMT experiments in mice, we typically evaluate retroviral vector expression in 32D cells, a murine cell line representing non-tumorigenic myeloid progenitor cells [21,112]. When examining various retroviral and lentiviral vectors expressing MGMT P140K in these cells, we made a surprising observation. As expected, all vectors expressing MGMT P140K mediated a clear survival advantage when cells were treated with O6-alkylating agents (BCNU) and O6BG (data not shown). However, if transduced 32D cells were kept for prolonged periods of time in the absence of a selective pressure, we noted a steady decline of cells expressing MGMT P140K in polyclonal cultures. This phenomenon appeared to be specific for vectors containing strong enhancer-promoter sequences derived from the spleen focus-forming virus (SFFVp). The decline of MGMT-expressing cells was observed following transduction with either gammaretroviral LTR vectors or lentiviral SIN vectors, both expressing similar high levels of the transgene [21]. In contrast, we noted constant levels of cells expressing MGMT P140K when using SIN vectors employing the relatively weak internal PGK promoter (Figure 2).

We next addressed whether this selective disadvantage was also mediated by the wild-type form of MGMT. Surprisingly, our data (Figure 2) suggested that the P140K mutation was required for the selective disadvantage caused by high expression of MGMT. The frequency of cells expressing the wild-type form of MGMT remained constant. Treatment of cells with O6BG, which inactivates MGMT without inducing DNA damage, also resulted in a stable proportion of cells over-expressing MGMT (Figure 2).

The decline of cells expressing high levels of MGMT P140K was reproduced in a second experiment (Figure 3), using vectors containing the SFFVp-driven LTR with or without the PRE of WHV. Cells transduced with SFFVp-driven LTR vectors expressing the enhanced green fluorescent protein (EGFP) instead of MGMT P140K showed no substantial loss of EGFP+ cells over time (Figure 3). Together with the observation that the population of cells expressing the wild-type form of MGMT remained stable, this strongly argued that neither the vector backbone nor the MGMT P140K cDNA are potent triggers of epigenetic silencing in 32D cells. The most likely explanation remained that high expression of MGMT P140K mediated a selective disadvantage, irrespective of the vector backbone used (gammaretroviral or lentiviral). Interestingly, high expression of MGMT G156A, another O6BG-resistant mutant used in preclinical studies (Table 1), did not give rise to a selective disadvantage in the absence of chemoselection (Figure 3). Intriguingly, when the P140K and G156A mutations were combined, the selective disadvantage reappeared, although with some delay (Figure 3). It remains to be addressed whether the selective disadvantage observed dependent on the mutation introduced into MGMT can be explained by changes in protein stability or altered interactions with cellular proteins or DNA. In our experiments, protein levels in transduced cells (determined by FACS) were consistently highest when using the P140K mutant (~2-fold elevated compared to G156A and wt MGMT).

These data suggest that the therapeutic index of MGMT P140K over-expression might be narrower than previously anticipated. Relatively high levels of this protein are required to ensure sufficient DNA repair following treatment with O6-alkylating agents. However, too high expression may impede cell growth. The exact mechanism underlying the selective disadvantage (cell cycle control, metabolic exhaustion etc.) and its potential relevance for primary hematopoietic cells in vivo remain to be determined. If subsequent studies underline the relevance of this observation for human cells, the use of other vector backbones (avoiding very high levels of expression) or the choice of MGMT mutants other than P140K may offer solutions.

Future directions and summary

Based on the above, we conclude that vectors mediating permanent ectopic expression of MGMT in hematopoietic cells can be identified, although the therapeutic index might be relatively narrow. Inducible expression of the drug resistance phenotype may offer an even better solution. Fusing MGMT with the estrogen-receptor domain that mediates conditional nuclear translocation in the presence of Tamoxifen may offer regulation of intercellular MGMT protein trafficking, as previously shown with the Cre recombinase [113]. Another option is inducible transcription, which could be provided by vectors that combine an expression unit for the tetracycline-dependent transactivator and the corresponding regulated expression cassette [114-116]. Sufficient levels of MGMT expression may thus be achieved in the induced state and cells may be allowed to switch off MGMT expression when the serum levels of the alkylating agents have declined. Potential challenges related to the potential immunogenicity and toxicity of the artificial transactivator remain to be addressed. Alternatively, episomal vectors might be useful [117]. To advance this technology, lentiviral delivery of episomal DNA [118,119] and the incorporation of scaffold attachment region to improve episome retention represent promising developments [120-122].

In summary, chemoprotection mediated by transfer and expression of MGMT or variants thereof is a promising approach in the treatment of cancer and for conditional enrichment of gene-modified cells. Proof of principle has been obtained in preclinical studies and large animal models. The design of improved transgene vectors and MGMT mutants go hand in hand to advance the clinical prospects of this approach. Potential side effects of insertional mutagenesis, protein over-expression, residual DNA damage caused by cytotoxic drugs in insufficiently protected cells, and leukemia-promoting co-factors such as replication stress, require careful attention. Further clinical studies should be conducted, if possible using state-of-the-art technology for MGMT gene transfer and expression.

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Figure 1.

Principles of gammaretroviral and lentiviral vector design for MGMT expression. The modular composition of the vectors is shown with the respective LTRs (enhancer-promoter (EP), R, U5 regions), splice sites (SD, SA) forming an intron within the expression cassette, rev-responsive element (RRE) and packaging signal (ψ) in the 5' UTR, internal enhancer-promoter (EP) and MGMT transgene. Optionally a posttranscriptional regulatory element (PRE) is introduced into the 3' UTR for improved mRNA processing. (A) shows a gammaretroviral LTR-driven vector, (B) - (E) display self-inactivating vectors (SIN, which harbor a 3' U3 deletion) either of the gammaretroviral (B) or lentiviral origin (C-E). Optionally, additional sequences (e.g. χ HS4 insulator) can be incorporated into the 3' U3 deletion and are present also in the 5' LTR after reverse transcription. In (D) an intron is installed 3' of the internal promoter. In (E) two expression cassettes are driven by an internal, bi-directional promoter. For further details see text.

Figure 2.

Selective disadvantage of transduced 32D cells with high expression of P140K MGMT. We examined the percentage of MGMT-positive cells in 32D cells, a murine myeloid precursor cell line, for up to 65 days without any exposure of alkylating agents (e.g. BCNU, temozolomide). Analysis was performed using a previously described protocol for intracellular MGMT-staining and FACS detection [21]. A set of 5 gammaretroviral (SF91 derivatives) and lentiviral (RRL derivatives) vectors (see Figure 1 for comparison) was used for transduction of 32D cells. Time post transduction is given on the X-axis, percentage of MGMT-positive cells on the Y-axis. Polyclonal populations with high-expressing P140K vectors experience a loss in MGMT marking (SF91.P140K.p and pRRL.PPT.SF91.P140K.p), whereas cells with control constructs with weaker promoters (RRL.PPT.PGK.P140K.p) or the MGMTwt variant do not show this effect.

Figure 3.

MGMT G156A does not induce a selective disadvantage. Using the SF91 vector with or without the PRE (p), we examined MGMT or GFP marking in transduced 32D cells for up to 65 days. Again, populations transduced with vectors expressing high levels of MGMT P140K (SF91.P140K with or without PRE) show a significant drop in marking while the control with EGFP (SF91.GFP.p) or the G156A variant does not. A double mutant (P140K/G156A) shows a prolonged drop in MGMT marking over time. Individual time points were monitored in triplicate.

Table 1

Overview of MGMT vector studies (chosen from 78 papers listed in PubMed)

Vector type	Ref.	Promoter	MGMT variant	SIN / LTR	PRE	cell line	primary cells	in vivo
gammaretroviral	[19]	MSCV	P140K	LTR	-	K562	CD34+	brain tumor patients
	[18]	SFFV	P140K	LTR	-	K562	lin-, CD34+	C57BL/6
	[20]	MoMLV	P140K, G156A	LTR	-	K562	lin-, CD34+	NOD/SCID
	[21]	PGK, EFS, SFFV, MPSV	P140K	LTR, SIN	+	32D, K562	lin-, CD34+	C57BL/6
	[22]	SFFV	P140K	LTR	-	K562	CD34+	dog model
	[23]	SFFV	P140K	LTR	-		canine CD34+	C57BL/6
	[24]	MSCV	P140K	LTR	-		canine CD34+	C57BL/6
	[25]	SFFV	P140K	LTR	-		lin-, CD34+	dog model
	[26]	MoMLV	P140K-GFP	LTR	-		canine CD34+	C57BL/6
	[27]	MSCV	P140K	LTR	-		canine CD34+	C57BL/6
	[17]	MSCV, SFFV	P140K	LTR	-	K562	CD34+	NOD/SCID
	[28]	MoMLV, MPSV, SFFV	G156A	LTR	-	K562	5-FU	C3H
	[30]	MoMLV	P140K, G156A	LTR	-	K562	5FU	C57BL/6
	[9]	MSCV, MoMLV	P140K, G156A	LTR	-		MSC	NOD/SCID
	[31]	MLV	wt	LTR	-		CD34+	C57BL/6
	[32]	MoLV	G156A	LTR	-		MSC	NOD/SCID
	[33]	MSCV, SFFV, PGK, MoMLV	P140K	LTR	-		mCFU, hCFU	C57BL/6
	[34]	MSCV	P140K	LTR	-		5-FU	C3H
	[29]	MoLV	G156A	LTR	-		5-FU	C57BL/6
	[35]	PGK	wt	LTR	-			
lentiviral	[36]	PGK	P140K	SIN	+	K562, HeLa	lin-, CD34+	BALB/c
	[21]	PGK, EFS, SFFV	P140K	LTR, SIN	+	32D, K562	5-FU/sca+	
	[12]	MND, PGK	G156A	SIN	-	K562	5-FU, CD4+, CD34+	NOD/SCID
	[8]	CMV	P140K	SIN	-		canine CD34+	dog model
	[11]	HIV	P140K	LTR	-		CD34+	NOD/SCID
	[27]	SFFV	P140K	SIN	-		CD34+	
	[37]	CMV	P140K	SIN	-		CD34+	
	[38]	MSCV	G156A	SIN	+	K562	5FU	C57BL/6
	[9]	MSCV	P140K	SIN	+			
	[39]	CMV	wt	SIN	-	K562, HeLa		