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# Foamy virus vectors for gene transfer

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## Abstract

Foamy virus (FV) vectors are efficient gene delivery vehicles that have shown great promise for gene therapy in preclinical animal models. FVs or spumaretroviruses are not endemic in humans, but are prevalent in nonhuman primates and in other mammals. They have evolved means for efficient horizontal transmission in their host species without pathology. FV vectors have several unique properties that make them well-suited for therapeutic gene transfer including a desirable safety profile, a broad tropism, a large transgene capacity, and the ability to persist in quiescent cells. They mediate efficient and stable gene transfer to hematopoietic stem cells (HSCs) in mouse models, and in the canine large animal model. Analysis of FV vector integration sites in vitro and in hematopoietic repopulating cells shows they have a unique integration profile, and suggests they may be safer than gammaretroviruses or lentiviral vectors. Here properties of FVs relevant to the safety and efficacy of FV vectors are discussed. The development of FV vector systems is described, and studies evaluating their potential in vitro, and in small and large animal models is reviewed.

## 1. Introduction: Foamy virus (FV) vectors for therapeutic gene transfer

Foamy viruses (FVs) are unique retroviruses that have evolved means for efficient transmission and infection of their hosts without pathology. This lack of pathogenicity is one reason why FV vectors were developed, but there are other properties of FVs that contribute to their efficacy. FVs have a broad tropism and are the largest of the retroviruses, so FV vectors can transduce many therapeutic targets and carry large transgene cassettes. FVs also have a unique replication strategy and share some characteristics with pararetroviruses (such as hepatitis B virus) including reverse transcription of the genome in virus-producing cells. Here, the development of FV vectors as efficient and safe tools for stable gene transfer will be reviewed. First, it is useful to discuss some aspects of FVs relevant to the utility and safety of FV vectors.

### 1.1 FVs as a safe platform to develop FV vectors

FVs infect many mammals including cats, rabbits, cows, horses and nonhuman primates, but they are not endemic in human populations. In nonhuman primates FVs have adapted to their hosts over an estimated 30 million years [1], and have developed a replication strategy that permits efficient horizontal transmission without pathology. There have been reports of diseases associated with FV infection in humans [2], but a comprehensive study of the prevalence of FV infection using multiple detection techniques showed that FVs are not endemic in human populations [3]. The exception is zoonotic infection of people that interact with nonhuman primates [4, 5]. There has been no pathology directly linked to FV infection in any species [6, 7]. This lack of pathogenicity of FVs contrasts with other retroviruses developed as gene transfer vectors including HIV-1 which causes AIDS, and murine leukemia viruses (MLVs) which cause neoplasms in mice and can also cause lymphoma in nonhuman primates [8]. In nonhuman primates FVs replicate in differentiated cells in oral tissues to high titer [9] which facilitates

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efficient horizontal transmission via biting or other intimate contact (reviewed in [10]). In FV-infected humans, no pathology has been observed despite monitoring over several years [4, 11]. FVs do not replicate efficiently in humans, and horizontal transmission to additional humans has not been observed, even when there was intimate contact or blood transfusion [11, 12].

### 1.2 Aspects of FV replication relevant to the development of FV vectors

Like all retroviruses FVs (spumaretroviruses) have the canonical *gag, pol* and *env*, have an RNA intermediate, and integrate in host cell chromosomes forming a provirus. They are complex retroviruses containing the accessory genes *tas* (formerly called *bel-1*) and *bet* (Figure 1). Foamy virus replication is regulated by Tas (transactivator of spumavirus) in a unique way. Tas and Bet are expressed at a basal level from an internal promoter within the *env* gene and when Tas accumulates it efficiently transactivates transcription of the FV long terminal repeat (LTR) to produce viral genomes and Gag, Pol and Env RNAs [13, 14]. In the absence of Tas there is essentially no transcription from the FV LTR [15, 16]. Tas is thus not required for FV vectors, since transgene transcription can be driven by an internal promoter, and Tas can simply be eliminated from the vector design [17, 18]. Bet allows FVs to overcome host cell restriction mediated by some APOBEC3s ([19], see section 2.4) and is also involved in limiting superinfection [20]. Bet is also not required for FV vectors.

FVs are unique among the retroviruses in that reverse transcription is largely completed in the cell producing virions, rather than in the target cell [21, 22]. Consequently the functional genome for FV vectors is dsDNA, although some reverse transcription may also occur in target cells [23]. In quiescent target cells, low nucleotide levels can limit reverse transcription [24], so completing reverse transcription prior to entering a quiescent target cell is an advantage that FV vectors have over both gammaretroviral and lentiviral vectors. This may allow FV vectors to persist in quiescent cells until cell division, which is required for FV vector transduction (see section 2.5). Another unique feature of FVs is that Gag which forms the viral capsid, and Pol which encodes the protease, reverse transcriptase, RNaseH, and integrase are translated from different viral RNAs [22, 25], and their relative expression is regulated at the transcriptional level rather than by a ribosomal frameshift or suppression of stop codons. This is relevant to the production of FV vectors since Gag and Pol can be provided from separate plasmids if provided in appropriate ratios [26]. This differs from gammaretroviral and lentiviral vectors where Gag and Pol are typically expressed from the same helper plasmid in order to achieve high titers. The ability to express Gag and Pol from separate plasmids when producing FV vectors is a safety feature, since an additional recombination between helper and vector plasmids would be required to generate a contaminating novel replication competent virus (RCR).

The ability of FVs to integrate allows for stable transgene expression in actively dividing cells, because following mitosis each daughter cell receives a copy of the vector provirus. This is critical for stem cell gene therapy approaches where the target cells expand extensively following transduction. The FVs are the largest of the retroviruses with a proviral genome of over 13 kb and FV vectors can deliver large transgene cassettes. It is unclear what the upper packaging limit is for FV vectors, since the composition of the transgene cassette can influence the efficiency of reverse transcription and packaging. But at a minimum FV vectors are capable of packaging 9.2 kb of foreign DNA [26]. However, the ability of FV vectors to efficiently deliver any specific transgene cassette must be determined experimentally.

## 2. Development of FV vectors

The above attributes led to the development of vectors derived from several FVs including the prototypic foamy virus (PFV) [17, 18, 26-29], simian foamy virus type 1 (SFV-1, macaque) [30, 31], and feline foamy virus (FFV)[32, 33]. PFV was originally named human foamy virus (HFV)

since it was isolated from a patient with nasopharyngeal carcinoma [34], and was originally thought to be a human virus. However, it is now known that FVs are not endemic in human populations, and sequence analysis of PFV indicates that it is a chimpanzee isolate [35, 36]. Like PFV-derived vectors, SFV-1 vectors have shown promise for gene transfer to hematopoietic repopulating cells [37]. More recently FFV vectors have been described. Although less is known about their efficacy and safety, they transduce human cells efficiently, suggesting they may be useful for gene therapy. Here we focus on PFV-derived vectors, which have been the most extensively studied. For retroviral vectors, components of the viral genome that are not necessary for efficient gene transfer should be removed, and only essential *cis*-acting regions should be retained in the vector genomes. The required viral proteins Gag, Pol, and Env can be produced in *trans* to package the vector genome.

### 2.1 Early replication-competent and second generation Tas-independent FV vectors

The first described FV vectors were replication-competent [27]. These vectors were derived from an infectious plasmid clone of PFV with reporter genes inserted into *bet*. They could be generated at relatively high titers (over $10^5$ transducing units/ml) and could deliver a chloramphenicol acetyltransferase (CAT) transgene to human fibroblasts, demonstrating the potential of FV vectors. Non-replicating vectors were then developed, but these early vectors contained contaminating wild-type FV in vector preparations [38, 39]. Despite this limitation the potential for FV vector transduction of hematopoietic cells was established [39]. However, higher titers were needed to improve the transduction efficiency into a range where experiments to assay gene transfer in animal models using cell-free vector containing supernatants could be attempted.

High titer helper-free vector preparations were attained by developing second-generation FV vectors that expressed the vector genomes, and the helper functions Gag, Pol and Env from two separate plasmids using strong cytomegalovirus (CMV) immediate early promoters in transfected human embryonic kidney (HEK293) cells [17, 18]. To produce the vector transcripts the U3 region of the LTR was replaced with a constitutive CMV promoter (CMV-R-U5) such that transcription of FV vector genomes initiates at the start of the R region in the 5′ LTR. In the context of a FV vector, this modification results in a system where vector transcription is independent of Tas (Bel-1), so it can be eliminated from the system. Removal of *tas* also makes FV vectors self-inactivating (SIN), since following reverse transcription there is no expression from the FV LTR. Unconcentrated titers in excess of $10^5$ transducing units/ml were obtained from these vectors and removal of *tas* from the system resulted in the absence of detectable replication-competent virus [17]. The resulting high titer helper-free FV vector preparations led to experiments to evaluate transduction of hematopoietic stem cells (HSCs) in mouse models (see 3.1 below).

### 2.2 Third generation FV vectors and vector preparation methods

The *cis*-acting regions required for efficient gene transfer were then mapped and additional nonessential regions of *gag, pol* and *env* were removed from FV vectors [26, 29, 31]. Enhancer regions in the U3 region of the 3′ LTR were also removed resulting in FV vectors with deleted LTRs and a 2.2–2.5 kb *cis*-acting region that includes the region between the 5′ LTR and *gag*, a 5′ region in *gag* (*cis*-acting region I or CAS I) and a second *cis*-acting region, CAS II in the 3′ region of *pol* and the 5′ region of *env* (Figure 2A). CAS II contains 4 central purine rich sequences that fulfill different functions in viral replication, but only one of these probably serves as a central polypurine tract [29, 40, 41]. Mutations were introduced into CAS I to eliminate Gag expression from the vector plasmid, and further refinements have been made to the FV *cis*-acting regions [41]. To minimize the potential for recombination between the helper and vector plasmids that might lead to a replication-competent virus, the *gag, pol*, and *env* genes are separated onto separate plasmids, and each is expressed from a CMV promoter with an

intron to obtain high level expression. In the resulting third generation FV vector system, HEK293 cells are transfected with four plasmids to generate vector virions (Figure 2A,B). It should be noted that since *tas* and *bet* are deleted on both the helper and vector plasmids, it is not possible to generate a wild-type FV from recombination between the plasmids. When sensitive assays have been used to detect a potential novel recombined FV virus, none has been detected [26]. This is in contrast to the potential to generate a novel RCR when *tas* is present, even in SIN vectors [42]. A novel FV-derived replication-competent virus without *tas* was created experimentally, but this virus failed to replicate efficiently [43]. In summary, the current generation of FV vectors do not have detectable RCR, and coupled with the fact that FVs do not have any known pathology in humans, the FV vector system appears to be relatively safe. To date calcium phosphate transfection has been used for most studies where high titers were achieved, including studies in large animal models [44, 45], but polyethylenimine (PEI)-mediated transfection can also be used to generate high titer FV vector preparations [46].

### 2.3 The FV envelope and vector tropism

Like other retroviruses FVs are enveloped, and the FV *env* gene expresses a transmembrane glycoprotein that is required to bind to target cells and fuse the virion membrane with the host cell membrane. The FV receptor has not yet been identified but studies on PFV envelope-receptor interactions indicate that a bipartite sequence motif in the surface domain of the envelope is essential to form the receptor binding domain [47]. The envelope in part determines the target tropism of FV vectors which is extremely broad [38, 48]. FV vectors efficiently transduce cells from humans, all tested nonhuman primate species, and all large and small animal models that have been used for preclinical gene therapy studies. Although the transduction efficiency varies for different cell types, to date no mammalian cell that is completely refractory to FV vector transduction has been identified. Thus it is not necessary to pseudotype FV vectors with a heterologous envelope such as the vesicular stomatitis virus glycoprotein (VSV-G) to increase the tropism, as it is for MLV and HIV-1 vectors. This is fortunate since heterologous envelopes such as VSV-G do not pseudotype FVs, reducing the transduction efficiency to less than 0.5% of the FV Env [49]. The FV envelope is required for the egress of FV virions from infected cells, and there are specific interactions between the FV envelope and Gag that are unique among the retroviruses [50, 51]. This may provide an additional safety advantage, since FV vectors cannot be trans-complemented by other Env proteins to generate a novel RCR. FV vectors with the native FV envelope yield high titers, can be efficiently concentrated by centrifugation [52], and are resistant to human serum [38, 53]. The FV envelope is thus well suited for therapeutic applications, and chimeric FV envelopes have been developed to pseudotype MLV [54] or HIV vectors [55].

### 2.4 Restriction factors and FV vector transduction

The interaction between viruses and their hosts has led to a molecular arms race where host cells have evolved mechanisms to restrict viral infection, and viruses have evolved means to overcome host restriction [56, 57]. In some cases host restriction systems target steps of the viral replication cycle that are required for transduction by viral vectors. Understanding these systems is important from the perspective of developing more effective vector production protocols [58], and methods to increase transduction efficiency [59, 60]. The APOBEC3 family of cytidine deaminases inhibit viral replication by hypermutating viral genomes, and by inhibiting reverse transcription [56]. Several human APOBEC3s restrict FV infection, and the FV accessory protein Bet overcomes this restriction [19, 61, 62]. This mechanism was thought to be unimportant for Bet-deficient FV vectors since APOBEC3s are incorporated into virions from the virion-producing cell, and FV vectors are made in HEK293 cells that were characterized as APOBEC3-deficient based on Western blot analysis [63]. This explains why Bet, which counteracts APOBEC3 restriction, is not required for high titer FV vector preparations produced in HEK293 cells. However, recent evidence suggests there may be residual

APOBEC3 activity in HEK293 cells that can lead to hypermutation of FV vector genomes, reducing their transduction efficiency [64]. FVs are also sensitive to TRIM5alpha which maps to the FV Gag [65], and is thus relevant to FV vectors. TRIM5alpha restriction of FV infection is species-specific so transduction of PFV (chimpanzee-derived) and SFV-1 (macaque-derived) FV vectors was inhibited by New World monkey, but not Old World monkey or ape TRIM5alpha. Feline FV vectors are potently inhibited by gorilla and orangutan Trim5alpha but not by human TRIM5alpha. The interferon (IFN) system is an ancient part of the innate immune system that restricts viral replication via many interferon-induced proteins [66] that act at several stages of viral replication and target many viruses including FVs [67-69]. It is currently unknown but possible that IFN-induced systems may also limit transduction by FV vectors, as they do for lentiviral vectors [60]. Of particular relevance for gene therapy, FV vectors are not sensitive to a form of restriction that inhibits lentiviral vector transduction in human hematopoietic progenitor cells, targeting lentiviral vectors for proteasomal degredation [70, 71].

### 2.5 Cell cycle requirements for FV transduction

The cell cycle requirements for retroviral transduction are important because some therapeutic target cells are quiescent. Early studies showed that transduction with the gammaretrovirus MLV was more efficient in dividing cultures than non-dividing cultures [72]. It was found that for MLV, transduction coincides with mitosis when the nuclear membrane breaks down, and that HIV-1 derived vectors do not require mitosis for efficient transduction [73]. HIV-1 can enter the nucleus independently of mitosis by active nuclear import, but MLV does not have means to enter the nucleus independently of mitosis. This difference is generally considered to explain the inability of MLV vectors to transduce non-dividing cells. However, it should be noted that although HIV-1 derived vectors efficiently transduce $G_1$-arrested cells, they do not efficiently transduce cells in $G_0$ [74, 75], and they do not efficiently transduce some non-dividing therapeutic targets in vivo [76]. There are additional factors that can limit the transduction of quiescent cells including the availability of nucleotides for reverse transcription [24], and less well-defined factors [77].

A study comparing the cell cycle requirements for infection and production of infectious FV, HIV-1 or MLV showed that PFV was unable to productively infect $G_1$/S or $G_2$ arrested cells and that the block to FV infection in $G_1$/S arrested cells extends to macaque SFV-1 and chimpanzee SFV-6 strains [78]. However, the cell cycle requirements for a full infectious cycle which includes post integration steps of viral replication might differ from vector transduction. After integration of a vector provirus, only transgene expression is required. The ability of FV and MLV vectors to transduce quiescent fibroblasts was compared and although FV vectors transduced stationary cultures less efficiently than dividing cultures, they transduced these cultures more efficiently than an MLV vector [38]. In this study the transduction rate in stationary cultures was higher when these cells were allowed to divide one day after vector exposure, suggesting that FV vectors could enter non-dividing cells and functional vector genomes could survive until these cultures were stimulated to divide. The cell cycle requirements of MLV, PFV and HIV-1 vectors were directly compared [75]. HIV-1 but not FV or MLV vectors efficiently transduced aphidicolin-treated $G_1$/S arrested cells. In quiescent $G_0$ serum-starved fibroblasts, FV vectors transduced cells at a similar efficiency to HIV-1 vectors and more efficiently than MLV vectors. However, in these cultures only cells that had gone through S phase as indicated by bromodeoxyuridine (BrdU) incorporation were transduced by FV vectors. FV vectors require mitosis, but are able to form a stable transduction intermediate in quiescent $G_0$ cells that persists until these cells are allowed to divide, even after up to 10 days of serum deprivation [75]. This dependence of FV vectors on cell cycle has been independently confirmed [46]. In quiescent cells incoming FV capsids track to the centrosome and appear to wait there until the cells undergo mitosis before uncoating occurs, suggesting a mechanism for

the observed dependence on mitosis [79]. A study investigating FV vector transduction after in vivo administration showed that only proliferating cells that had undergone DNA synthesis expressed transgene at late timepoints, consistent with the in vitro findings that FV vectors require cell division [80]. In summary the cell cycle requirements for FV vectors are complex and differ from both MLV and HIV-1 vectors.

### 2.6 Integration profile of FV vectors and safety for gene therapy

The ability of retroviral vectors to integrate into the genome is a desirable property from the perspective of long-term, stable therapeutic transgene expression. But this also means that retroviral vectors including FV vectors are mutagens. The oncogenic properties of gammaretroviruses are well established. Acute-transforming gammaretroviruses that carry a viral counterpart of a cellular gene led to the discovery of proto-oncogenes [81], but retroviruses can also transform cells by integration near proto-oncogenes in a process known as proviral insertional mutagenesis. When a provirus integrates near a gene controlling growth and alters its expression, the host cell may gain a selective growth advantage, and after a latent period become transformed. This process can also be caused by retroviral vectors, but it was commonly thought that the probability of oncogenic transformation by replication-incompetent vectors in a gene therapy setting was low. Supporting this, in early T cell and HSC gene therapy trials leukemias were not observed. This viewpoint changed when the first reports came out that gammaretroviral vector-mediated insertional mutagenesis had caused leukemia in children treated for X-linked severe combined immunodeficiency (SCID-X1) (reviewed in [82]). Studies have suggested that contributing factors included the vector design, the biology of the target cell, vector type, and the contribution of the specific therapeutic transgene [82]. Thus, genotoxicity has become an important consideration in the development and evaluation of gene therapy vectors, and FV vectors have unique properties that suggest they may be relatively safe in this regard.

The integration profile of vectors contributes to their potential to cause cancer in a gene therapy setting. The sequencing of the human genome and animal model genomes including the mouse, dog and rhesus macaque has allowed large-scale analysis of the location of different retroviral vector integration sites relative to genomic features. Retroviral vectors differ in their integration pattern relative to genes and proto-oncogenes. Small numbers of FV vector integration sites were identified in mouse and human cells using polymerase chain reaction (PCR) amplification [83, 84], but the first large scale analysis of FV integration sites was performed in normal human fibroblasts and human peripheral blood $CD34^+$ cells which are enriched for HSCs. In this study 2,829 unique FV vector provirus integration sites were identified by plasmid rescue [85]. The FV vector integration profile differs from both MLV which integrates preferentially near transcription start sites[86], and HIV-1 vectors which integrate preferentially in transcribed genes [87]. FV vectors have a modest preference for transcription start sites but do not integrate preferentially within genes. Transcriptional profiling showed that the activity of genes has little influence on FV vector integration [85]. A study where 628 FV integration sites were identified using a polymerase chain reaction PCR-based technique confirmed this integration pattern [88]. Thus FV vectors integrate less frequently within genes than HIV-1 vectors, and less frequently near transcription start sites than MLV vectors. How these differences translate to their relative safety in a clinical setting remains to be determined.

Another factor that contributes to safety is the relative ability of vector proviruses to activate nearby genes. Transactivation can occur by enhancer effects, and by transcription from vector LTRs or internal promoters. Third generation HIV-1 and FV vectors are SIN unlike the gammaretroviral vectors used in the SCID-X1 study that can activate downstream genes via transcription from an active 3′ LTR. A direct comparison of gammaretroviral vectors, FV vectors and HIV-1 vectors using a plasmid-based transactivation assay showed that a FV vector

provirus had a lower propensity than an HIV-1 or gammaretrovirus vector provirus to transactivate a reporter gene [89]. This suggests that FV vectors may also be safer than MLV or HIV-1 vectors from the perspective of the potential to transactivate nearby proto-oncogenes or growth-promoting genes.

## 3. FV vector applications

The broad tropism and relative safety of FV vectors suggests they will be useful for many scientific and therapeutic applications where stable gene transfer is desired. This includes expression of short hairpin RNAs to knockdown expression of target genes [90, 91]. FV vectors have been extensively evaluated for HSC gene therapy applications where they have shown great promise. However, their ability to efficiently transduce embryonic stem cells [92], and neural progenitors [93] suggests they may be useful for other stem cell-based therapies. Their resistance to human serum [38, 53], suggests they may be useful for some in vivo delivery applications.

### 3.1 Efficient FV-mediated transduction of HSCs

HSCs are a promising target for gene therapy since they can be easily obtained by bone marrow (BM) aspirate or leukapheresis, cultured ex vivo to allow efficient transduction, and re-infused into a patient where they will repopulate the entire hematopoietic system for the life of an individual. Early FV vectors showed promise for this application in studies where hematopoietic progenitors were transduced in vitro at similar efficiency to MLV vectors [39]. However, transduction of HSCs must be evaluated in vivo, since HSCs are defined in part by their ability to repopulate a lethally irradiated host. The development of helper-virus free vector preparations that could be concentrated to high titer by centrifugation, facilitated studies in mice [17, 52]. FV vectors expressing either green fluorescent protein or alkaline phosphatase reporter genes efficiently transduced mouse hematopoietic repopulating cells with a mean of 24% in white blood cells and 36% in red blood cells at four to seven weeks after transplantation [52]. FV vectors transduced mouse long-term HSCs as evidenced by the ability of repopulating cells in primary BM transplant recipients to engraft and repopulate secondary transplant recipients. In this study these second generation FV vectors also efficiently transduced human progenitor cells. The ability of FV vectors to transduce human hematopoietic repopulating cells has been demonstrated in the xenogeneic NOD/SCID mouse model [37, 83, 94, 95]. Using cord blood $CD34^+$ cells, very high transduction efficiency was achieved with over 50% of repopulating cells expressing a second generation FV green fluorescent protein (GFP) vector at four to seven weeks after transplant with a low multiplicity of infection (MOI) [83]. Similar marking rates were observed in lymphoid and myeloid lineages and there was no evidence of significant silencing. Immortalized B cell clones were derived from engrafted lymphocytes and analysis of integration sites showed myeloid and lymphoid repopulating cells derived from the same transplanted NOD/SCID repopulating cell (SRC)[83], establishing the ability of FVs to transduce pluripotent SRCs. In a direct comparison FV vectors transduced cord blood-derived SRCs at least as efficiently as HIV vectors, and more efficiently than MLV vectors [95]. FV vectors can also efficiently transduce SRCs derived from mobilized peripheral blood, which is more commonly used for transplantation [94]. In this study, third generation FV vectors transduced 34% of engrafted mobilized peripheral blood-derived SRCs and long-term SRCs (18 weeks) were efficiently transduced. Importantly, relatively short ex vivo transduction protocols were employed which is important to maintain the engraftment potential of HSCs. In summary, FV vectors efficiently transduce mouse HSCs and human SRCs using clinically relevant sources of enriched HSCs, and short ex vivo transduction protocols designed to maintain HSC engraftment.

### 3.2 HSC transduction by FV vectors in the dog large animal model

The ability of FV vectors to efficiently transduce long-term repopulating cells has also been demonstrated in the dog large animal model. The dog allows evaluation of long-term efficacy and safety in a setting that more closely predicts the efficacy of stem cell gene transfer in patients. In this setting third generation FV vectors mediated efficient long-term gene transfer (<450 days in one dog and <650 days in another)[45]. Marking with an EGFP vector was polyclonal and observed in all hematopoietic lineages examined, with stable long-term transgene expression in approximately 15% of repopulating granulocytes and lymphocytes in both animals. This marking level would be sufficient to cure many hematopoietic diseases. In these studies a low MOI (8.0–9.8) was used, yet the marking was similar to that observed in previous studies with lentiviral vectors used at a much higher MOI (100) [96]. The ability of FV and lentiviral vectors to transduce canine repopulating cells was directly compared at the same MOI (5) using a competitive repopulation assay [44]. Remarkably similar long-term marking efficiency was observed for FV and lentiviral vectors at late timepoints in both dogs. FV vector integration sites were analyzed in canine repopulating cells and the integration profile was consistent with in vitro studies [97]. In these canine repopulating cells FV vectors had a slight preference to integrate near transcription start sites, but the percentage of FV integrants within proto-oncogenes or within 10 kb of a proto-oncogene transcription start site was lower than for either lentiviral or gammaretroviral vectors. There were no adverse events in these studies from FV-mediated gene transfer.

### 3.3 Demonstration of the therapeutic potential of FV vectors

The above studies strongly support the use of FV vectors for HSC gene therapy. One advantage of the dog model is that dogs are available that accurately model human genetic diseases, so gene therapy can be tested in a clinically relevant setting. FV vectors were evaluated for HSC gene therapy in the dogs with canine leukocyte deficiency. In this model a mutation in the leukocyte integrin *ITGB2* gene (CD18) predisposes dogs to infection, and is life-threatening. Five dogs were transplanted with BM $CD34^+$ cells transduced with a third generation FV vector expressing canine CD18 [98]. One dog died from a transplant-related cause, but the four dogs that survived long-term had stable multilineage marking, and complete reversal of the disease phenotype. This was stable for at least two years after transplantation. Comparison of integration sites in this disease model with dogs that were treated with a gammaretroviral vector showed that FV vectors were found less often within or near proto-oncogenes [98]. In mouse models FV vectors that express the Fanconi C gene were able to restore the repopulating activity of HSCs [99], and FV vectors that express gamma-aminobutyric acid (GABA) reduced pain after subcutaneous administration [100].

## 4. Expert opinion (conclusions and perspective)

The unique properties of FVs have been exploited in FV vectors resulting in a platform that allows efficient gene transfer. Their broad tropism, large therapeutic transgene capacity, and demonstrated ability to mediate long-term, stable gene transfer suggest they will be useful for numerous therapeutic applications. To date many different reporter and therapeutic transgenes have been efficiently delivered by FV vectors demonstrating their versatility.

FV vectors require mitosis for efficient transduction, and this dependence on cell division limits their utility for therapeutic applications where the target cell is post-mitotic. Despite this limitation stable FV-mediated gene transfer to dividing target cells has been obtained after in vivo delivery [80], and the therapeutic potential of FV vectors following in vivo delivery has been established [100]. Their ability to form a stable transduction intermediate in quiescent cells makes FV vectors ideal for ex vivo applications such as HSC gene therapy, where a quiescent target stem cell can be briefly exposed to FV vectors, then re-introduced into a patient. Current

generation FV vectors have several safety features and can be produced by transient transfection at high titer in the absence of contaminating replicating virus. Since no special equipment is required other than tissue culture facilities and a centrifuge for concentration, FV vector technology can be quickly established in most laboratories. Because of their demonstrated utility and ease of use, more widespread adoption of FV vectors and studies to evaluate their potential for additional therapeutic applications should be expected.

Further improvements to FV vectors and to techniques for FV vector production are likely. FV vectors are sensitive to host restriction factors including TRIM5alpha and APOBEC3 enzymes. As we better understand the interactions of FV vectors with host restriction systems, means to increase the transduction efficiency by interfering with these processes may further increase their efficacy. For example, it should be possible to generate HEK293 cells that do not have residual APOBEC3 activity to increase the titer of FV vectors produced in these cells [41]. It may also be possible to identify ways to transiently interfere with other factors that limit transduction in target cells, as has been achieved with lentiviral vectors [101]. Towards this goal, studies to better understand the interactions of FV vectors with restriction factors are needed.

The safety of FV vectors compares favorably to MLV and HIV-1 vectors from the perspective of lack of pathogenicity of the parent virus, integration profile, and lower potential to transactivate nearby genes. However, FV vectors do integrate so dysregulation of proto-oncogenes is a risk when using these and other integrating vectors in clinical studies. To date, adverse events resulting from FV vector integration have not been reported in either the mouse or dog HSC gene therapy models, and there have not been any reports of pre-leukemic clonal expansion in the canine model where long-term safety can be assessed. The available preclinical efficacy and safety data thus support evaluating FV vectors in clinical studies for severe hematopoietic diseases such as immunodeficiencies and hemoglobinopathies. In these severe life-threatening diseases, the risk of an adverse event is warranted by the potential for lifelong therapeutic benefit, and the lack of safer alternative treatment options. Clinical trials with FV vectors that analyze the clonality of engrafted FV-transduced repopulating cells, and vector integration sites are needed, along with a comparison to similar studies using gammaretrovirus and lentivirus vectors. Such studies will hopefully establish FV vectors as a safe and effective alternative to gammaretroviruses and lentiviruses.

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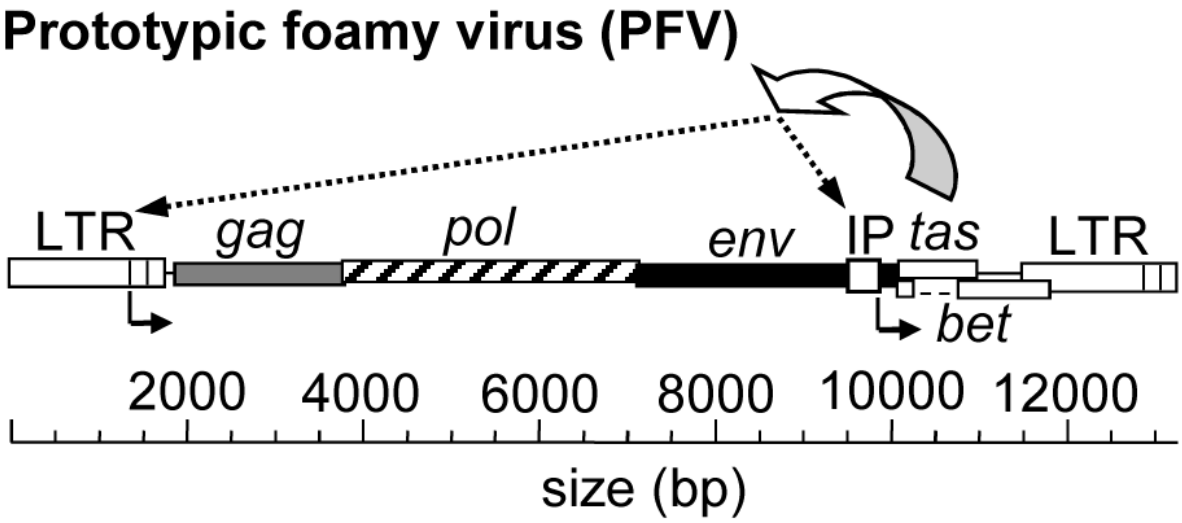


**Figure 1. Prototypic foamy virus (PFV)**
In addition to *gag, pol* and *env*, PFV also expresses two accessory genes *tas* and *bet*. Tas and Bet are expressed from the internal promoter (IP) in *env*. Tas transactivates transcription from both the IP and the long terminal repeat (LTR). Arrows indicate transcription.

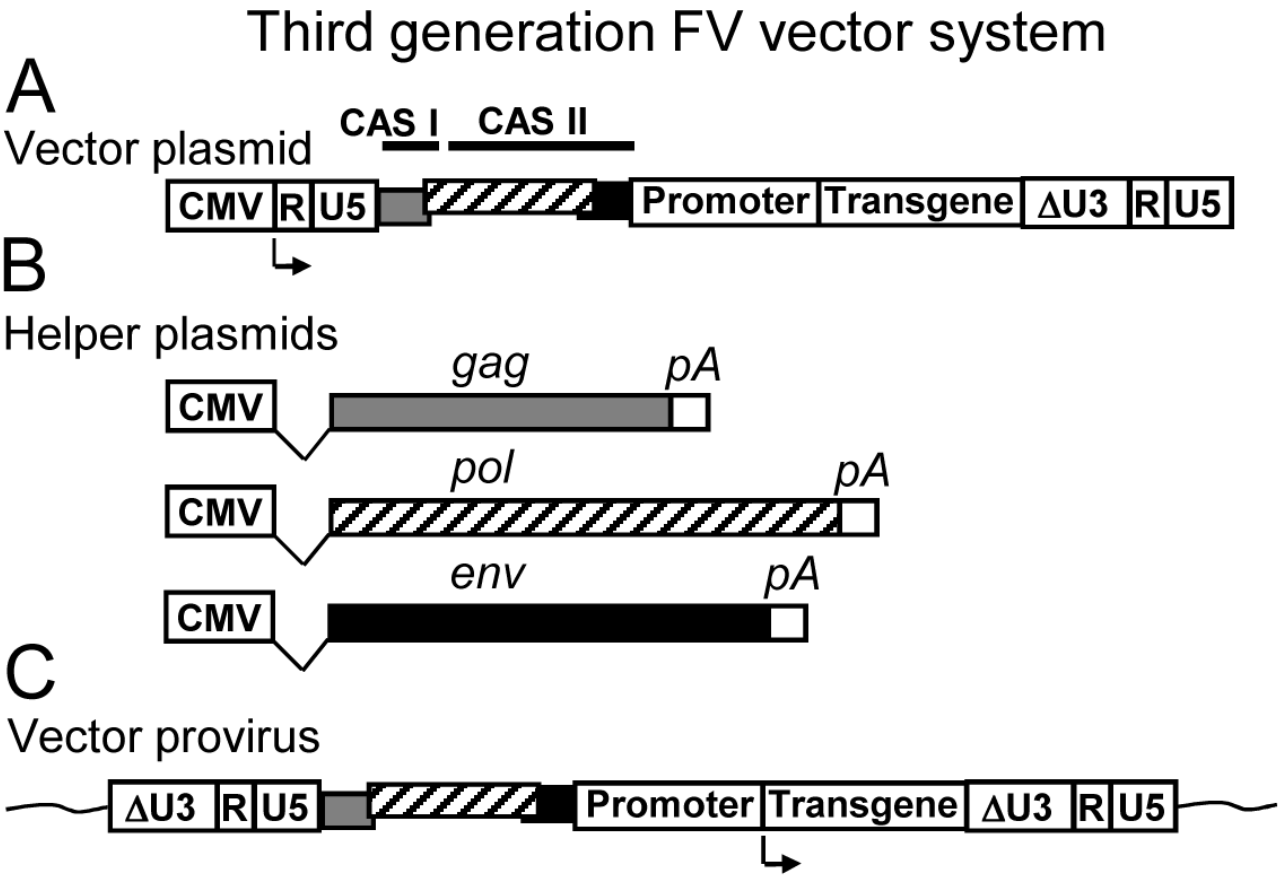


**Figure 2. Third generation FV vectors**

**A. FV vector plasmid.** Regions of *gag, pol* and *env* have been removed and *cis*-acting regions necessary for efficient transduction are retained. These include the long terminal repeats and *cis*-acting regions I and II (CAR I, II). Transcription is driven by a CMV-LTR fusion promoter. The 3′ LTR has deletions in the U3 region. **B. FV Helper plasmids.** *gag, pol* and *env* are each expressed from a CMV promoter with a heterologous intron and a heterologous polyadenylation (pA) site on three different plasmids. **C. Integrated vector provirus.** During reverse transcription the deletion in the vector plasmid 3′ LTR U3 region is copied to the vector provirus 5′ LTR. Both LTRs are transcriptionally inactive and an internal promoter is used to drive transcription of the desired transgene. Arrows indicate transcription.