Published in final edited form as:

Curr Opin Virol. 2016 December ; 21: 41–46. doi:10.1016/j.coviro.2016.07.010.

Retroviral vector interactions with hematopoietic cells

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Abstract

Hematopoietic stem cell (HSC) gene therapy using retroviral vectors has immense potential. Increasing the transduction of quiescent HSCs and reducing genotoxicity are major challenges in the field. Retroviral vectors, including lentiviral and foamy vectors, have been extensively modified resulting in improved safety and efficacy. This review will focus on recent advances to improve vector entry, transduction efficiency, control of transgene expression and approaches to improve safety by modifying the retroviral integration profile.

Introduction

Hematopoietic stem cell (HSC) gene therapy using retroviral vectors has the potential to cure many diseases including primary immunodeficiencies, enzyme deficiencies and even acquired diseases like HIV. In this approach, gene modified HSCs self-renew and differentiate providing a life-long supply of hematopoietic cells with a therapeutic transgene. Although HSC gene therapy is promising, the appearance of serious adverse events in early HSC gene therapy clinical trials as a result of vector-mediated genotoxicity has limited its use clinically. It has also resulted in significant efforts to better understand the interactions of retroviral vectors with hematopoietic cells. This review will focus on recent advances made in improving vector entry, regulating expression of the therapeutic transgene, and reducing the genotoxicity of retroviral vectors for HSC gene therapy.

Vector entry

Retroviruses have an envelope protein on their outer surface that binds to host cell surface molecules and mediates viral entry. After binding, the retroviral and cellular membranes fuse, allowing the retroviral core to gain entry into the host cell cytoplasm. The retroviral envelope protein is a primary determinant of viral tropism. Envelope pseudotyping,

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replacing the native envelope protein with a heterologous envelope protein from a different enveloped virus, can expand, limit, or alter vector tropism. Pseudotyping can also increase the efficiency of gene transfer and the stability of vector particles. Various pseudotypes have been incorporated into retroviral vectors including vesicular stomatitis virus glycoprotein G (VSV-G), gibbon ape leukemia virus, feline endogenous RD114 virus and the cocal pseudotype [1,2,3,4,5••]. Of these, VSV-G is the most popular and well-studied due in part to its broad tropism. The VSV-G pseudotype mediates efficient gene transfer into large animal long term repopulating cells [6,7] and allows the vector to be efficiently concentrated via centrifugation [1]. However, VSV-G pseudotyped vectors are toxic, which has led to difficulty in developing stable vector-producing cell lines [8]. Also, VSV-G can be inactivated by human sera, limiting its efficacy for *in vivo* delivery [9].

The cocal vesiculovirus envelope glycoprotein is an intriguing alternative to VSV-G. The cocal virus is a member of the genus *Vesiculovirus* and causes vesicular stomatitis primarily in cattle and horses [4]. The cocal virus glycoprotein is 71.5% identical at the nucleic acid level to VSV-G Indiana and is thought to use the same receptor as VSV-G for cell entry [4]. Similar to VSV-G, the cocal envelope has a broad tropism and mediates efficient gene transfer to human primary cells derived from blood, lung fibroblasts, retinal epithelia, bone epithelia, skin fibroblasts and stroma [4]. Cocal pseudotyped vector virions are more resistant to inactivation by human sera than VSV-G pseudotyped vector virions and may be more effective for gene delivery *in vivo*. Recently, a high titer 3rd generation self-inactivating lentiviral producer cell line was generated based on the cocal envelope [5••]. In a direct comparison, cocal pseudotyped lentiviral vectors were produced in packaging cells at higher titers than with the VSV-G pseudotype [5••]. Cocal pseudotyped lentiviral vectors also transduce human and non-human primate CD34⁺ and CD4⁺ cells more efficiently than VSV-G [4,5••].

Approaches to increase transduction efficiency

Cell cycle requirements play a key role in determining how effective the retroviral vector will be for use in HSC gene therapy where the target cell is quiescent. Gammaretroviral vectors can only transduce actively dividing target cells [10]. Lentiviral vectors are able to transduce quiescent cells [11], however, they are much more efficient at transducing cells in the G_{1b} stage of the cell cycle [12]. Foamy viral vectors require mitosis for transduction, however, a major benefit of foamy viral vectors is that they can form a stable intermediate that can be maintained in quiescent cells [13]. Currently, cytokines are commonly used during *ex vivo* culture to stimulate cells to progress into the cell cycle. Commonly, fms-like tyrosine kinase 3, stem cell factor, granulocyte stimulating factor, thrombopoietin, interleukin-3 and interleukin-6 are used [14,15]. The use of CH-296 fibronectin, a recombinant fragment of human fibronectin, increases engraftment of gene-modified cells in patients by promoting co-localization of the retroviral vector with hematopoietic cells and also by maintaining HSC engraftment capacity [16,17]. An immobilized Notch ligand Delta-1 can also be used to accelerate hematopoietic engraftment in combination with cytokines [18].

Another approach to improving transduction is to overcome the restriction mechanisms that cells have evolved to inhibit viral infection. Inclusion of the drug rapamycin during *ex vivo* culture has shown promise in this regard. Rapamycin inhibits the mammalian target of rapamycin (mTOR) kinase and is commonly used as an immunosuppressant to prevent transplant rejection. The mTOR pathway regulates HSC quiescence. Rapamycin greatly increased lentiviral gene delivery without impairing engraftment of both primitive mouse HSCs and human primitive SCID-repopulating CD34⁺ cells [19••]. The mechanism appears to be enhanced cytoplasmic entry events after vector binding [19••].

Obtaining appropriate transgene expression

For gene therapy to be successful, sufficient transgene expression must occur to correct a disease phenotype. However, a strong promoter can dysregulate nearby genes leading to oncogenesis. House-keeping promoters with less oncogenic potential, such as phosphoglycerate kinase 1 (PGK) and elongation factor-1 α (EF1 α), are being proposed for clinical trials. A systematic comparison of the most commonly used constitutive promoters demonstrated that EF1 α promoters appear to be consistently strong in a variety of cell types [20]. A direct comparison in a competitive repopulation experiment in X-SCID canines showed that PGK was more effective than EF1 α for immune system reconstitution [21]. X-SCID canines were simultaneously injected with equal titers of foamy viral vectors carrying a therapeutic gene driven by a PGK or EF1 α promoter and a corresponding reporter gene. The PGK-driven vectors achieved lymphocyte marking of over 30% by 50 days post treatment and expanded to roughly 80% by 300 days post treatment. In comparison, EF1 α resulted in approximately 10% lymphocyte marking at all time points [21]. These studies suggest that disease-specific testing in vivo may be necessary to identify an ideal promoter.

Several approaches are being developed to reduce the expression of the therapeutic transgene in non-target cells. While off-target expression does not appear to be deleterious for many diseases, some diseases require very specific transgene expression. For example, in the case of Wiskott-Aldrich syndrome, expression of the transgene is highly toxic in non-hematopoietic cells. In a recent Phase I/II clinical trial for Wiskott-Aldrich syndrome, a lentiviral vector driven by the Wiskott-Aldrich syndrome protein gene-specific promoter resulted in robust and stable multi-lineage engraftment of gene-corrected hematopoietic cells in three patients [22]. Transcriptional targeting using microRNAs (miRNAs) can also be used to reduce off-target transgene expression. The combination of similar miRNA target sequences for a specific cell type has been shown to cooperatively reduce gene expression by 100-fold in human U937 monocytes and primary dendritic cells [23,24]. For more details Gentner et al. provide an excellent review on the various applications of miRNA manipulation [25]. For HSC gene therapy, retroviral vectors can be modified to contain sequences in the transgene cassette that are complementary to miRNAs specifically found in off-target cell types. Thus, if the transgene is expressed in the wrong cell type or blood lineage, the specific miRNAs will bind and inactivate the mRNA transcript (Figure 1).

Several miRNA targets have been discovered that are lineage and/or differentiation-stage specific to reduce off-target expression for HSC gene therapy. miR-223 can de-target transgene expression from myeloid cells [24] whereas miR-150 can de-target transgene

expression from mature T and B lymphocytes [26]. miR-144/451 is a positive regulator of late erythroid maturation and could have applications for hemoglobinopathies [26]. Examples of differentiation-stage specific miRNA targets include miR-181a, miR-155, miR-126 and miR-130a. miR-181a can be used to inhibit expression in immature T-cells while allowing for transgene expression in mature T-cells which may be beneficial in cancer immunotherapy [27]. miR-155 can be used to inhibit expression in immature dendritic cells while allowing for expression in mature dendritic cells [24], which might be helpful in correcting autoimmune diseases.

miR-126 and miR-130a are both selectively expressed in immature HSCs but not prevalently expressed in differentiated hematopoietic cells. Thus, adding a target sequence for miR-126 into a retroviral vector can allow for transgene expression in differentiated hematopoietic cells while inhibiting expression in primitive HSCs. The addition of four tandem miR-126 target sequences strictly targeted expression of the therapeutic transgene to differentiated hematopoietic cells, sparing HSCs and progenitors completely [28].

There have been recent successes in the use of dual regulated retroviral vectors [29,30••] that employ a gene or tissue-specific promoter to target the correct tissue and miRNA to eliminate expression in off-target cells. A hepatocyte-specific promoter along with target sequences for a hematopoietic lineage-specific miRNA, miR-142-3p, resulted in stable disease correction with reduced off-target transgene expression in antigen presenting cells [29]. Poor engraftment has limited the efficacy of X-linked chronic granulomatous disease (X-CGD) gene therapy [30••]. A dual-regulated vector employing a myeloid specific promoter and two tandem target sequences for miR-126 was designed to treat X-CGD. This vector provided therapeutic benefit, restoring NADPH activity to 50% in both X-CGD mice and primary X-CGD patient phagocytes, while targeting expression to specifically myeloid cells and sparing expression in HSCs [30••].

Genotoxicity

Genotoxicity occurs as the result of the retroviral vector provirus dysregulating the expression of nearby genes and remains a major challenge for HSC gene therapy. Vector-mediated genotoxicity has led to serious adverse events such as vector-mediated leukemias [31-34]. The appearance of serious adverse events in early clinical trials [34-37] demonstrated the need for a better understanding of retroviral interactions with hematopoietic cells. Thus, major efforts have been made to develop retroviral vectors that reduce the risk for genotoxicity.

Different retroviral vectors have unique integration preferences that greatly affect the genotoxic potential of the vector. Gammaretroviral vectors tend to integrate near proto-oncogenes and have a strong preference for integrating near transcription start sites and within CpG islands [38-41] and lentiviral vectors preferentially integrate within actively transcribed regions of RefSeq genes [39-41]. Foamy viral vectors have a promising integration profile, displaying only a weak preference for integrating within CpG islands and near transcription start sites and no preference for integrating within actively transcribed regions of genes [39-41]. Foamy viral vectors also integrate less often near proto-oncogenes

than lentiviral vectors [41,42]. While it is generally accepted that lentiviral vectors are safer than gammaretroviral vectors [40,41], more recent studies are starting to suggest that foamy viral vectors may be a potentially safer vector type [42,43••]. Beard et al. showed that foamy viral vectors integrate less frequently near proto-oncogenes in canine long-term repopulating cells than lentiviral vectors [42]. Additionally, in a side-by-side comparison of lentiviral and foamy viral vectors in human CD34⁺ SCID repopulating cells, foamy viral vectors were shown to have less clonal dominance and fewer integrations near proto-oncogenes [43••].

Integration profiles are determined by interactions between the retroviral pre-integration complex (PIC) and host cell proteins. Once in the host cell nucleus, the PIC interacts with host proteins which facilitates chromosomal tethering [44]. One promising approach to reduce retroviral genotoxicity involves retargeting vectors to integrate in safer locations by modifying PIC-host protein interactions. Gijsbers et al. successfully retargeted lentiviral integration into gene-poor regions of the genome by modifying a host cell line to express a chimeric lens epithelium-derived growth factor-chromobox protein homolog 1 (LEDGF-CBX1) protein. To accomplish this, LEDGF, a host protein known to interact with the viral integrase, was fused to chromobox protein homolog 1, a protein that interacts with trimethylated lysine 9 of histone H3 and is associated with heterochromatin. In this approach, the CBX1 portion of the chimeric protein retargets LEDGF to gene sparse regions of the human genome. LEDGF binds the viral integrase, tethering the PIC to the heterochromatin. It is important to note that this approach is not clinically relevant as it requires a modified cell line [45].

Lentiviral vector integration profiles have been weakly retargeted by fusing zinc finger endonucleases to the viral integrase [46,47]. Recently, gammaretroviral vectors have been successfully retargeted away from actively transcribed regions of genes. Gammaretroviral integrase interacts with host bromodomain and extra-terminal (BET) proteins to direct the PIC near transcription start sites of genes. By introducing specific mutations into the Gammaretroviral integrase that inhibit interactions with BET proteins, gammaretroviral vector integration profiles are retargeted away from gene transcription start sites [48•]. However, the vector titers in this study were not reported as functional transducing units/ml, and this approach has not yet been used for hematopoietic repopulating cells.

More recently, foamy viral vectors have been retargeted into H3K9me3 regions and away from proto-oncogenes using an approach that is practical for clinical translation. Hocum et al. modified two of the foamy viral helper plasmids by modifying the chromatin binding site in the foamy viral Gag protein and fusing CBX1 to the foamy viral integrase (Figure 2). This approach effectively retargeted the foamy viral vectors into the gene-sparse heterochromatin and away from proto-oncogenes in human cord blood CD34⁺ cells. These retargeted vectors were produced at clinically relevant titers and do not require engineered target cells, making this approach clinically feasible. An additional benefit to this retargeting approach is that any foamy viral vector can be retargeted with these modified helper plasmids (Hocum et al. abstract in the 19th annual meeting of the *American Society of Gene and Cell Therapy*, May 2016). Studies are underway in the authors' lab (GDT) in human CD34⁺ SRCs transduced with retargeted foamy viral vectors to test the efficacy and safety of these vectors in vivo.

The vector-mediated leukemias previously observed in HSC gene therapy clinical trials can primarily be attributed to enhancer-mediated activation and transactivation of nearby genes. Chromatin insulators are a class of DNA regulatory elements that can be added to retroviral vectors to control gene expression and reduce genotoxicity. Insulators recruit proteins that promote changes in the chromatin structure and prevent genes from being inappropriately expressed. There are two classes of insulators: barrier insulators and enhancer-blocking insulators. Barrier insulators prevent gene silencing by physically blocking heterochromatin from moving into transcriptionally active regions of open chromatin and consequently inactivating the promoter [49]. Enhancer-blocking insulators block enhancer elements, such as the retroviral long terminal repeat, from acting on promoters and thereby causing the transactivation of nearby genes [50]. Clinically relevant insulated vectors have been developed for gammaretroviral vectors, lentiviral vectors, and foamy viral vectors [49-54] and has resulted in reduced genotoxicity in pre-clinical studies [52-54].

Conclusions

Many different approaches have been taken to enhance the efficacy and safety of HSC gene therapy using retroviral vectors. In each case a detailed understanding of the biology of gene transfer with retroviruses has led to improved efficacy or safety. A future challenge will be to combine these approaches and to develop sensitive clinically relevant models to compare next generation vectors.

Acknowledgments

This article was supported by grants from the National Institute of Health Grants AI097100 and AI102672 (GDT).

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Highlights

- Cocal envelope pseudotype has low toxicity, broad tropism and resists human sera
- The small molecule drug rapamycin can be used to increase transduction efficiency
- Novel promoters or miRNAs can target transgene expression
- Foamy vector integration can be retargeted into heterochromatin to improve safety

Figure 1.

Detargeting transgene expression from off-target cells using microRNAs (miRNAs). A hematopoietic pluripotent stem/progenitor cell (HSPC) has been transduced with a retroviral vector containing a therapeutic gene (green rectangle) and an artificial target sequence specific for miR-126 (red rectangle). miR-126 is expressed in HSPC but not in differentiated hematopoietic cells. In the HSPC, the transgene is transcribed into mRNA. The miR-126 binds to the artificial target sequence which blocks translation of the transgene and directs the degradation of the transgene mRNA. In the differentiated hematopoietic cell the transgene is transcribed into mRNA and translated into proteins, as there is no miR-126 to bind the target sequence.

Figure 2.

Retargeting foamy viral vector integration using modified helper plasmids. **a)** A foamy vector provirus with modified group-specific antigen (Gag) and polymerase (Pol) helper plasmids is retargeted to integrate into satellite regions of the genome. A triple alanine substitution of RTY has been introduced into the chromatin binding site domain of the Gag protein. This mutation decreases the binding affinity of the chromatin binding site domain with host cell chromatin, retargeting the provirus away from genes. The Pol is modified to express chromobox protein homolog 1 (CBX1) fused to the integrase protein. CBX1 is a protein that interacts with tri-methylated lysine 9 of histone H3 and is associated with heterochromatin. Together, the modified Gag and CBX1 fused to IN targets the foamy vector provirus into satellite regions of heterochromatin.