NON-REPLICATING, NON-INTEGRATING VECTOR FOR IMMUNOTHERAPY AND VACCINE APPLICATIONS

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ABSTRACT

Non-replicating, non-integrating vectors can provide transient expression in proliferating cells and stable transgene expression in quiescent cells. We explored further engineering of our highly modified non-replicating Moloney Murine Leukemia Viral (MoMLV) vector to become non-integrating using our optimized Herpes Simplex Virus Thymidine Kinase (HSV-TK) gene construct as a model system.

The optimal vectors for gene delivery should exhibit large payload capacity, high transduction efficiency, target cell selectivity, and no genotoxicity. For cancer therapy and vaccine use, an acceptable immune response is also necessary. Non-integrating vectors will dilute progressively in proliferating cells. However, for certain gene products, transient expression may be sufficient. Additionally, non-integrating vectors in particular offer a reduced risk of genotoxicity. For vaccine applications, transient expression may also be useful. If stable expression in non-dividing cells is required, repeated administration of non-integrating vectors may be considered.

In order to generate a non-integrating vector, seven integrase mutants were constructed at the Mg^{2+} binding motif of the catalytic core domain of the integrase in the vector *gagpol* gene: single mutations, dual combinations, and a triple mutant. The constructs were tested by triple transfection of 293T cells with *env*, *gagpol*, and *payload*. None of these mutations affected the physical titer of vector. Using our optimized HSV-TK suicide gene construct as the payload, the level of protein expression was examined in melanoma A375 cells transduced with vectors made with *gagpol* containing the integrase mutants and compared with payload expression from vectors made with wild-type *gagpol*. Data from FACS and Western blots for HSV-TK protein from the various mutants indicated that early protein expression is strong and nearly comparable to that observed with a functional integrase. This expression correlates well with the cell kill activity in the presence of ganciclovir (GCV). As expected, expression decreased over time to undetectable levels as the cells divided. Lack of integration in the host genome was confirmed using relative integration qPCR. All mutations and their combinations were able to knock out host genome integration. A range of cancer cell lines were tested with the non-integrating vectors. When the cells were successfully transduced with the vectors, HSV-TK expression became undetectable between 7 and 14 days for most of the cell lines tested. This correlated with the loss of cell kill activity in the presence of GCV.

To examine the functional effect of the mutated integrase with the reverse transcription deficiency, the reverse transcriptase (RT) gene was also mutated in the *gagpol.* When the mutated RT construct was used in the triple transfection of 293T, the vector particle was produced at a high titer, but transduced A375 cells failed to express the HSV-TK protein. No GCV induced cell killing was observed as well. Not surprisingly, when a combination of integrase mutated and RT mutated vector was tested, expression of the payload was also not present. This implies that RT activity is required even in the non-integrating vector, and that payload RNA alone is insufficient in transduced A375 cells for detectable expression of HSV-TK protein.

With non-replicating, non-integrating vectors, there is the opportunity to explore many additional indications in the immunotherapy and vaccine areas. An example is incorporating both an antigen gene and a second gene, e.g., a viral thymidine kinase. Incorporation of both of these genes within a non-replicating, non-integrating vector would permit the combination of antigen production with subsequent GCV-mediated cell killing, with the potential to augment the immune response against the co-delivered antigen.

INTRODUCTION

Non-replicating Moloney (MLV) vectors deliver transgenes that integrate into a host cell genome when the cell nucleus is broken down during cell division (1). These vectors are particularly efficient in actively dividing cancer cells. The integrase, located in the MLV *gagpol* sequence, is the essential enzyme that catalyzes the integration process (2). Once integrated, the transgenic protein (or vector payload) can be expressed constitutively. Non-replicating vectors that do not integrate into the host cell DNA present a reduced risk of insertional mutagenesis; non-integrating lentivectors are being investigated for use in many applications (3, 4). We explored here the potential of non-replicating, non-integrating MLV vectors to express an active protein, using our optimized Herpes Simplex Virus Thymidine Kinase (HSV-TK) gene construct (5). With protein expression at sufficient levels and for several days, non-replicating non-integrating MLV vectors could provide a new approach for many applications in gene therapy, immunotherapies and vaccines.

The vector integrase is a highly conserved protein (6), and mutant defective integrase *gagpol* sequences were engineered based on homology to lentivectors (7) and available data for MLV (8, 9). To inactivate the integrase, the triad of residues D125, D184, E220, that form the Mg^{2+} binding motif within the catalytic core domain (Fig. 1), were targeted and mutated to A by single base-point mutations. Furthermore, we also investigated whether the payload RNA itself could contribute to payload protein expression by knocking-out the function of the reverse transcriptase (RT) (10) with and without a defective integrase.

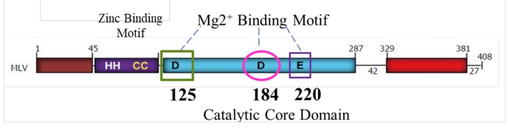


Figure 1. Schematic representation of MLV integrase protein, highlighting the highly conserved triad D125, D184, and E220 forming the Mg^{2+} binding motif in the catalytic core domain, and the HH-CC motif involved in Zinc binding motif. Figure adapted from ref. 6.

METHODS AND MATERIALS

Constructs. Single base mutations were introduced by site-directed mutagenesis at D125, D184, E220 of the integrase sequence in MLV *gagpol* gene using a Q5 Site-Directed Mutagenesis kit (New England Biolabs, MA). A second round was performed to yield double mutants, and a third round to generate a triple mutant integrase. Three mutations were introduced in the RT sequence at D150A/D224A/D225A. The mutant fragment was synthesized by GenScript Biotech, NJ and inserted into MLV wildtype *gagpol* gene or into the integrase-defective mutant D184A *gagpol* gene. **Vector**. Vectors were generated by transient triple transfection of 293T cells with control (wild-type) or mutant *gagpol* sequences, MLV amphotrophic envelope and payload plasmid. For the experiments shown here, the payload was an optimized HSV-TK sequence (see ASGCT Poster #1634). Titers were measured by RT-qPCR after isolation of the vector RNA. **Expression analysis.** Human melanoma cells A375, and other human cancer cell lines (see Fig. 6) were transduced with vector, and payload protein expression was examined by Western blot and FACS. Payload protein activity was determined by cell kill assays in presence of ganciclovir (GCV). **Relative integration analysis**. DNA was extracted from transduced cells using QIAcube (Qiagen, Germany) and tested for Psi signal copies using relative integration qPCR assay (relative to an internal control gene).

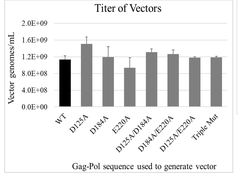
RESULTS

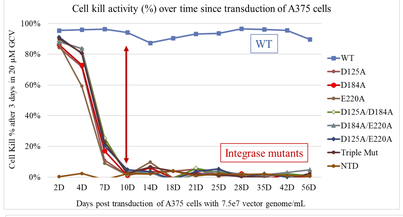
Defective MLV Integrase

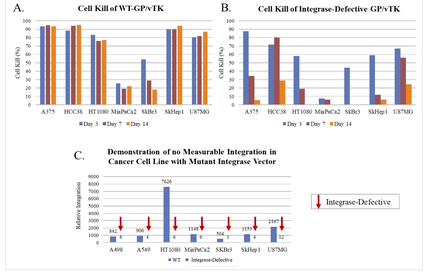
Figure 2. Seven different integrase defective mutants were produced by single point mutations within the catalytic core.

	Gagpol (GP) Sequences
Wild-type	WT
Single	D125A
	D184A
	E220A
Double	D125A/D184A
	D184A/E220A
	D125A/E220A
Triple	D125A/D184A/E220A

Figure 3. The physical titers of vectors generated with each integrase-defective *gagpol* were comparable to the titer obtained using wild-type (WT) gagpol. Titers were measured by RT-qPCR.







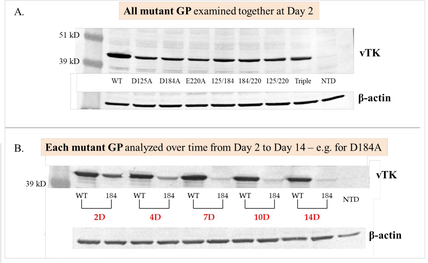


Figure 4. A375 cells were transduced with integrase-defective vectors and examined for HSV-TK (vTK) expression by Western Blotting. **A.** The 7 mutant GP and wild-type GP vectors were tested and compared at Day 2 (2D) post-transduction. **B.** For each mutant GP, vTK expression was followed up to Day 14 post-transduction. The blot shows a representative data of the expression over time for integrase mutant D184A. A similar pattern was observed for all mutants. NTD are non-transduced cells. β-actin was used as a loading control.

Figure 5. A375 cells transduced with integrase-defective HSV-TK vectors were analyzed for cell kill activity in the presence of GCV for 3 days. All 7 mutants vectors were examined in parallel and compared with the vector produced with wild-type GP (WT), and with non-transduced cells (NTD). The cell kill activity was gone by Day 10 (10D) post-transduction (as indicated by red arrow), with similar kinetics for all seven mutants.

Figure 6. Several cancer cell lines were transduced with vectors produced with WT-GP **(A)** or integrase-defective mutant GP D184A **(B)** containing a HSV-TK gene. Cells were collected at Day 3, 7, 14 post transduction and treated with 20 μM GCV for 3 days to induce cell killing activity. **C**. The DNA was extracted from the same cells, after at least 14 days, and examined for relative integration of the Psi packaging signal, using Relative Integration qPCR. The assay confirms that there was no significant detectable integration of the payload in host cell DNA when using the integrase-defective D184A vector.

Assay LOD = 4 Relative Psi Copies; LOQ = 8 Relative Psi Copies.

RESULTS CONTINUED

Defective MLV Reverse Transcriptase

Figure 7. Schematic diagram of the polymerase showing where the three RT mutations (D150A/D224A/D225A) were introduced into the Fingers/Palm region (10).

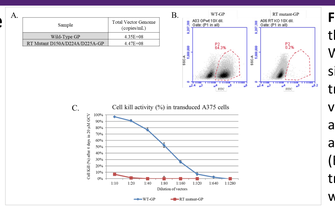


Figure 8. A. Physical titer of the vectors produced with WT-GP or RT mutant-GP are similar. **B.** A375 cells were transduced with these vectors, stained with FITC-anti-HSV-TK antibody and analyzed after 3 days by FACS (BD Accuri). **C.** A375 cells transduced with the vectors were examined for cell kill activity in 20 μM GCV for 4 days. The results demonstrate that there is no protein expression as well as no cell kill activity with the RT mutant although vector particles were made.

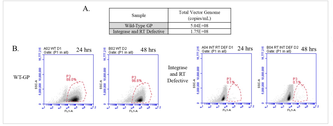


Figure 9. Combination of integrase-defective and RT mutant. **A.** Physical titers of vectors produced with either WT-GP or with integrase-defective mutant D184A/RT mutant. **B.** A375 cells were transduced with the vectors and analyzed for vTK expression after 24 and 48h by FACS. The results are similar to the RT mutant construct only (Fig. 8).

CONCLUSIONS

Seven mutant integrase MLV *gagpol* sequences were engineered. All seven mutants of defective integrase successfully generated vector particles at high titers similar to the wild-type *gagpol* sequence. All mutants also resulted in similar levels of expression of the payload protein HSV-TK in transduced target cells. The expression disappeared over time as would be expected for a payload gene that did not integrate into the host genome. The lack of integration was also confirmed by qPCR of DNA of host cells. In cancer cell lines that are most receptive to MLV vectors, expression and activity of the payload HSV-TK diminished over time but could still be detected up to 14 days after transduction, by Western blot and cell kill assays in the presence of GCV, despite the lack of integration.

The data obtained with the reverse transcriptase mutant *gagpol* sequence show that the mutations did not affect formation of vector particles and generated the same titers as with wild-type *gagpol.* However, when the RT function was knocked-out, HSV-TK protein could not be detected in target cells, indicating that payload RNA alone is insufficient for detectable protein expression in transduced cells and that reverse transcription is necessary.

Non-replicating non-integrating MLV vectors represent an attractive alternative for transgene therapy as the duration of payload expression outperforms current approaches, such as with formulated mRNA for example. These constructs provide a new platform with improved safety for testing a variety of therapeutic and prophylactic applications, ranging from cancer therapy to vaccine delivery.

REFERENCES

1. T. Maetzig, M. Galla, C. Baum, A. Schambach. Gammaretroviral Vectors: Biology, Technology and Application. Viruses (2011) 3:677-713.
2. P. Lesbats, A. N. Engelman, P. Cherepanov. Retroviral DNA Integration. Chem. Rev. (2016) 116:12730-12757.
3. M.B. Banasik and P. B. McCray, Jr. Integrase-Defective Lentiviral Vectors (IDLV): Progress and Applications. Gene Therapy (2010) 17:150-157.
4. A. Gallinaro *et al*. Integrase Defective Lentiviral Vector as a Vaccine Platform for Delivering Influenza Antigens. Front. Immunol. (2018) 9:171.
5. ASGCT Poster #1634: Optimization of a Suicide Gene Vector for the GEN2 Cancer Immunotherapy Clinical trial. C. Roh *et al.* (2023).
6. D. P. Grandgenett, K. K. Pandey, S. Bera, H. Aihara. Multifunctional facets of retrovirus integrase. WJBC (2015) 6:83-94.
7. R. Sengupta et al. An Optimized Protocol for Packaging Pseudotyped Integrase Defective Lentivirus. Biological Procedures Online (2016) 18:14.
8. L. Lai, H. Liu, X. Wu, J. C. Kappes. Moloney Murine Leukemia Virus Integrase Protein Augments Viral DNA Synthesis in Infected Cells. J. Virol. (2001) 75:11365-11372.
9. A. Steinrigl, D. Nosek, R. Ertl, W. H. Günzburg, B. Salmons, D. Klein. Mutations in the Catalytic Core or the C-terminus of Murine Leukemia Virus (MLV) Integrase Disrupts Virion Infectivity and Exerts Diverse Effects on Reverse Transcription. Virology (2007) 362:50-59.
10. D. Das and M. M. Georgiadis. The Crystal Structure of the Monomeric Reverse Transcriptase from Moloney Murine Leukemia Virus. Structure (2004) 12:819-829.

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