

## RNA VIRUS VECTORS: GENE TRANSFER AND GENE EXPRESSION

### 1. Persistent Gene Expression in Mouse Nasal Epithelia Following Baculovirus GP64 Pseudotyped FIV-Based Gene Transfer

Patrick L. Sinn,<sup>1</sup> Erin R. Burnight,<sup>1</sup> Melissa A. Hickey,<sup>1</sup> Gary W. Blissard,<sup>2</sup> Paul B. McCray, Jr.<sup>1</sup>

<sup>1</sup>*Pediatrics, Program in Gene Therapy, The University of Iowa, Iowa City, IA;* <sup>2</sup>*Boyce Thompson Institute, Cornell University, Ithaca, NY.*

The use of gene transfer to treat or prevent cystic fibrosis lung disease has been limited in part by the inability of vectors to efficiently and persistently transduce airway epithelia. Influenza A is an enveloped virus with natural lung tropism; however, pseudotyping feline immunodeficiency virus (FIV)-based lentiviral vector with the HA and HEF envelope proteins proved unsuccessful. Conversely, influenza D has a single envelope protein (GP64) and pseudotyping FIV with influenza D GP64 (from Thogotovirus) resulted in titers of 10<sup>6</sup> TU/ml. Further, influenza D GP64 conferred FIV apical entry into well-differentiated human airway epithelial cells. Baculovirus GP64 envelope glycoproteins share sequence identity with influenza D envelope glycoproteins, the result of a postulated lateral transfer of genetic material during the evolution of the two viral families. Pseudotyping FIV with GP64 from 3 species of baculovirus resulted in titers ranging from 10<sup>7</sup> – 10<sup>9</sup> TU/ml. Of note, GP64 from *Autographa californica* multinuclear polyhedrosis virus resulted in high titer FIV preparations (upwards of 10<sup>9</sup> TU/ml) and also conferred apical entry into polarized primary cultures of human airway epithelia. These titers equal those obtained with VSV-G pseudotyping. Using a luciferase reporter gene and bioluminescent imaging, we observed persistent *in vivo* gene transfer in the mouse nose with *A. californica* GP64 pseudotyped FIV (AcGP64-FIV). Longitudinal bioluminescence analysis has documented the persistence of expression in the nasal epithelia for >7 months without significant decline. We used a nuclear targeted beta-galactosidase reporter and histological analysis to demonstrate that surface epithelial cells were transduced in the mouse sinuses. In addition, AcGP64-FIV transduced mouse nasal epithelia with much greater efficiency than VSV-G pseudotyped FIV, as determined by both bioluminescence and histological analysis. These data suggest that AcGP64-FIV efficiently and persistently transduces nasal epithelia in the absence of agents that disrupt the cellular tight junction integrity and have important implications for CF gene therapy.

### 2. Cellular Mechanisms Involved with Lentiviral Gene Transfer

Shangming Zhang,<sup>1</sup> Karen Pollok,<sup>2</sup> Lakshmi Sastry,<sup>1</sup> Lionel Berthoux,<sup>3</sup> Jeremy Luban,<sup>3</sup> Kenneth Cornetta.<sup>1</sup>

<sup>1</sup>*Medical and Molecular Genetics, Indiana University School of Medicine, Indianapolis, IN;* <sup>2</sup>*The Herman B Wells Center for Pediatrics and Research, Indiana University School of Medicine, Indianapolis, IN;* <sup>3</sup>*Microbiology and Medicine, Columbia University, College of Physicians and Surgeons, New York, NY.*

Lentiviral vectors derived from the human immunodeficiency virus-1 (HIV-1) have potential for gene therapy applications. We demonstrate that Genistein (GEN), a previously known protein tyrosine kinase (PTK) inhibitor which also induces G2 cell cycle arrest, enhances lentiviral gene transduction by 2-5 folds in a variety of human cell lines, primary human CD34+ peripheral blood progenitor cells and murine primary lymphocytes. In contrast to lentiviral vectors, transduction with a retroviral vector, MFG-eGFP,

is inhibited by GEN. Enhanced lentiviral gene transfer is seen in vectors pseudotyped with RRV, VSVG or ecotropic envelopes, indicating that the effect is vector-dependent and envelope-independent. The effect is dose dependent as GEN at 15 mM or 60 mM increased vector copy number by 2- or 6-fold, respectively. The effect was confirmed by expression of GFP in transduced cells and by vector copy number using real-time PCR. The GEN effect on lentiviral transduction positively correlates with the percentage of cells in G2 arrest. A similar increase in lentiviral gene transfer was noted with another G2 arresting agent, Nocodazole. Arrest in G1 with dexamethasone or G0 using serum deprivation failed to increase or significantly reduced gene transfer. However, Pentoxifylline, which is known to reverse G2 arrest, only partially antagonized the effect of GEN on gene transfer while it completely reversed GEN-mediated G2 arrest. This suggests that G2 cell cycle arrest contributes to but is not the only factor mediating increased gene transfer.

The differential effects of GEN on lentiviral and retroviral gene transfer led us to hypothesize that Cyclophilin A (CypA) is involved in the GEN's action since CypA is known to be specifically incorporated into HIV-1 virions but not into virions of other retroviruses. GEN significantly increased CypA protein in a dose-dependent manner, and the percentage of eGFP positive cells increased from 19% in untreated 293 cells to 56% in 60 mM GEN-treated cells. Furthermore, the improved vector copy number and percentage of eGFP positive cells by GEN were completely abolished by 2.5 mM Cyclosporine A (CSA). These results indicate that CypA is involved in the improved gene transfer by GEN. CypA-deficient Jurkat cells have much lower rates of lentiviral gene transfer compared to the parent cell line but GEN increases gene transfer to a similar degree in both CypA +/+ and -/- Jurkat cells. Interestingly, CSA reversed GEN-mediated G2 cell cycle arrest, a property of CSA not previously reported. Taken together, we conclude that Cyclophilin A and G2 cell cycle arrest are two mechanisms important for lentiviral gene transfer.

### 3. Effects of the cHS4 Insulator Elements on Transgene Expression and Biological Titer of Lentiviral Vector

Hideki Hanawa,<sup>1</sup> Takashi Shimada.<sup>1</sup>

<sup>1</sup>*Biochemistry and Molecular Biology, Nippon Medical School, Tokyo, Japan.*

Modification of retroviral vector to minimize the chance of insertional mutagenesis is the urgent require for stem cell gene therapy. A possible approach is the use of an insulator element identified in the 1.2 Kb fragment from chicken  $\beta$ -globin hypersensitivity site 4 (cHS4). When the gene is flanked by the element, both enhancer blocking effect and stable gene expression independent of chromosomal positional effect are observed. One of the problems of the insulator element in the case of HIV-1 vector is the decrease in biological titer, which is thought to be attributed to the increase in the vector RNA size (Blood 101: 4717-4724, 2003). The other possible reason is the characteristic of HIV-1 reverse transcriptase (RT). To form DNA flap during reverse transcription, HIV-1 RT aborts at central termination sequence when it is already double stranded. Retroviral RT have to go through already double stranded LTR for successful reverse transcription, therefore HIV-1 LTR can be a weak point when it is modified. To test the hypothesis, we constructed a series of HIV-1 based vectors. The parent vector carries the EGFP marker gene under the control of U3 from MSCV oncoretroviral vector. The 1.2 Kb cHS4 element or the 0.5 Kb core insulator element was inserted into the parent vector at various positions with either orientation. The relative orientation of cHS4 element against  $\beta$ -globin genes and INS IR vector genome is identical. The values shown are the relative biological titer measured on HeLa cells (TU/ml) and the relative biological titer normalized using p24

level measured by ELISA (TU/ng) against those of parental vector, and these are average of four independent experiments (Exp.1) or three independent experiments (Exp.2). The actual titers of parental vector are  $2.7 \times 10^7$  TU/ml and  $7.6 \times 10^4$  TU/ng in Exp.1, and  $1.9 \times 10^7$  TU/ml and  $4.0 \times 10^4$  TU/ng in Exp.2.

	Exp.1		Exp.2	
	relative TU / ml	relative TU / ng	relative TU / ml	relative TU / ng
Parent	1.00	1.00	1.00	1.00
INS1R	0.18	0.18	0.12	0.17
INS1L	0.25	0.21		
INS2R	0.85	0.88		
INS2L	0.55	0.87		
C1R			0.58	0.83
C1L			0.79	0.93
C4L-C2R			< 0.001	-

The result of Exp.1 indicates that the decrease of vector titer is related to position of cHS4 but not the size of the vector RNA, and that the decrease is due to reduced transducibility of the vector. Interestingly, MFI of INS1L is higher than that of parental vector, while that of INS1R is lower (Parent, 5%, 329; INS1R, 4%, 132; and INS1L, 4%, 514; %GFP+ and MFI respectively). The shorter insulator improved normalized biological titer as well as absolute biological titer. As it is observed with INS1L, C1L has higher EGFP expression than parental vector (Parent, 12%, 107; C1L, 11%, 149). The vector production of C4L-C2R is completely impaired, but the cause is unclear at this point. In conclusion, utilization of the 0.5Kb core cHS4 element in reverse orientation improves insulated vector in terms of both titer and gene expression. Further investigations of the constructs are on going.

#### 4. Optimization of Globin Lentiviral Vector Design for the Treatment of $\beta$ -Thalassemia

Leszek Lisowski,<sup>1</sup> Stefano Rivella,<sup>1</sup> Michel Sadelain.<sup>1</sup>  
<sup>1</sup>Laboratory of Gene Transfer and Gene Expression, Memorial Sloan-Kettering Cancer Center, New York, NY.

The stable introduction of a functional globin gene into human hematopoietic stem cells (HSC) is a potentially curative treatment for the  $\beta$ -thalassemias and sickle-cell diseases. To this end, we are investigating the transcriptional requirements for expressing the highest possible levels of the human  $\beta$ -globin gene in murine and primate erythroid cells.

The endogenous  $\beta$ -globin gene is controlled by two proximal enhancers and distal regulatory elements known as the  $\beta$ -globin locus control region (LCR), which spans 7 DNase hypersensitive sites (HS) located 50-80kb upstream of the human  $\beta$ -globin gene. We have previously shown that the juxtaposition of HS 2, 3 and 4 with the  $\beta$ -globin gene promoter from position -615 and the 3'  $\beta$ -globin enhancer yield tissue specific and therapeutic  $\beta$ -globin expression in thalassemic mice. Furthermore, addition of the LCR reduces the position effect and transcriptional inactivation.

To identify the best possible vector design before investigating globin gene transfer in phase I clinical studies, we are further investigating the role of these elements as well as insulators and lentiviral vector elements.

To investigate the role of HS1/HS4 in LCR function as well as the activity of different length  $\beta$ -globin promoters on globin transgene

expression we have generated a panel of constructs with specific modifications in the  $\beta$ -globin promoter, HS1 and/or HS4 regions of the LCR. These constructs were transduced into murine erythroleukemia (MEL) cells to assess tissue specificity, stage specificity and average expression level of the vector-encoded human  $\beta$ -globin gene.

In contrast to one previously published report, the extended promoter (1.6kb) proved to be less active in MEL cells. Conversely, the shortening the promoter from -615 to -265, and removal of the putative silencer element believed to be present in that region, might have enhanced  $\beta$ -globin expression.

The HS4 region of the LCR, containing GATA-1 and AP-1 binding sites, significantly contributes to expression of the human  $\beta$ -globin gene in transgenic mice. AT rich flanking regions of HS4 might encompass scaffold/matrix-associated regions, which may play an important role in LCR controlled  $\beta$ -globin gene expression. To test this hypothesis we deleted either the 3' or 5' AT rich flanking regions of HS4. Deletion of the 3' flanking region did not affect the expression level, however truncation of the 5' AT rich region decreased  $\beta$ -globin gene expression in MEL cells.

Furthermore we have assessed the role of HS1 on  $\beta$ -globin expression. HS1 has been reported to contain a GATA-1 binding site and provides position-independent expression on a linked human  $\beta$ -globin gene in transgenic mice, without affecting the level of expression. Addition of HS1 did not affect the average globin gene expression in MEL cells, and we are further investigating the role of this element on  $\beta$ -globin gene expression *in vivo*.

In light of our data we have selected four lentiviral vectors containing the human  $\beta$ -globin gene under the control of  $\beta$ -globin promoter with or without the silencer element as well as full HS4 elements with or without the HS1 element. We are currently studying these vectors in an animal model of  $\beta$ -Thalassemia.

#### 5. Differential Expression of PIT1 and PIT2 after G-CSF Mobilization Is Associated with Efficient Gene Transfer Using GALV-Pseudotyped Gammaretroviral Vectors

Brian C. Beard,<sup>1</sup> Pau Mezquita,<sup>1</sup> Julia C. Morris,<sup>1</sup> Hans-Peter Kiem.<sup>1,2</sup>

<sup>1</sup>Clinical Research, Fred Hutchinson Cancer Research Center, Seattle, WA; <sup>2</sup>Department of Medicine, University of Washington, Seattle, WA.

The optimal stem cell source for stem cell gene therapy has yet to be determined. Most large animal studies have utilized peripheral blood or marrow-derived cells collected after administration of granulocyte colony-stimulating factor (G-CSF) and stem cell factor (SCF), however, SCF is unavailable for clinical use. A recent study of a competitive repopulation assay in the rhesus macaque model showed very inefficient engraftment of transduced G-CSF-mobilized peripheral blood (G-PBSC) CD34+ cells relative to G-CSF- and SCF-mobilized cells using amphotropic pseudotypes (Hematti P et al Blood 101, 2003, 2199-2205). Because G-PBSC would be the preferred target cell population for most clinical stem cell gene therapy applications, we asked whether we could achieve efficient engraftment of transduced G-PBSC in our baboon model using Phoenix-GALV pseudotype vectors. In order to better compare these results to those from previous experiments utilizing G-CSF- and SCF-primed bone marrow (G&S-BM) as a stem cell source, the first two baboons also received G-CSF-primed BM (G-BM) in a competitive repopulation. We transplanted three baboons with G-CSF-mobilized CD34+ cells transduced with GALV pseudotyped retroviral vectors. We observed high-level, persistent engraftment of transduced G-PBSC in all three animals with gene marking in granulocytes in one animal up to 60%. To determine retrovirus integration pattern of clones that contributed to long-term