Title: Nipah virus envelope pseudotyped lentiviruses efficiently target ephrinB2+ stem cell populations in vitro and bypass the liver sink when administered in vivo.

Running title: NiV Pseudotypes Infect Stem Cell Populations

Karina Palomares, Frederic Vigant, Ben Van Handel, Olivier Pernet, Kelechi Chikere, Patrick Hong, Sean P. Sherman, Michaela Patterson, Dong Sung An, William E. Lowry, Hanna K.A. Mikkola, Kouki Morizono, April D. Pyle, Benhur Lee, William E. Lowry, Hanna K.A. Mikkola, Kouki Morizono, April D. Pyle, Benhur Lee, №

Department of Microbiology, Immunology and Molecular Genetics, Molecular Biology Institute, Department of Molecular, Cell, and Developmental Biology, School of Nursing, Department of Medicine, Division of Hematology and Oncology, Department of Pathology and Laboratory Medicine, Jonsson Comprehensive Cancer Center, Eli and Edythe Broad Center of Regenerative Medicine and Stem Cell Research, and AIDS Institute, David Geffen School of Medicine, University of California, Los Angeles, CA 90095, USA.

# Corresponding author. Mailing address: Department of Microbiology, Immunology and Molecular Genetics, University of California-Los Angeles, #257 BSRB, 615 Charles E. Young Dr. South, Los Angeles, CA, 90095. Phone: (310) 794-2132. Fax: (310) 267-2580. E-mail: bleebhl@ucla.edu.

Abstract word count: 249 words

Text word count (excluding references, table footnotes and figure legends): 7,277 words
Abstract

Sophisticated re-targeting systems for lentiviral vectors have been developed in recent years. Most seek to suppress the viral envelope’s natural tropism while modifying the receptor-binding domain such that its tropism is determined by the specificity of the engineered ligand-binding motif. Here, we take advantage of the natural tropism of Nipah virus (NiV), whose attachment envelope glycoprotein has picomolar affinity for ephrinB2, a molecule proposed as a molecular marker of “stemness” (present on embryonic, hematopoietic and neural stem cells) as well as being implicated in tumorigenesis of specific cancers. NiV entry requires both its fusion (F) and attachment glycoprotein (G). Truncation of the NiV-F cytoplasmic tail (T5F) alone, when combined with full-length NiV-G, resulted in optimal titers of NiV pseudotyped particles (NiVpp) (~10^6 IU/ml) even without ultracentrifugation. To further enhance the infectivity of NiVpp, we engineered a hyperfusogenic NiV-F lacking a N-linked glycosylation site (T5FΔN3). T5FΔN3/wt G exhibited enhanced infectivity on less permissive cell lines, and efficiently targeted ephrinB2+ cells even in a 1000-fold excess of ephrinB2-negative cells, all accomplished without any loss in specificity as entry was abrogated by soluble ephrinB2. NiVpp also transduced human embryonic, hematopoietic, and neural stem cell populations in an ephrinB2-dependent manner. Finally, intravenous administration of luciferase reporter NiVpp-T5FΔN3/G into mice resulted in signals detected in the spleen and lung, but not the liver. Bypassing the liver sink is a critical barrier for targeted gene therapy. The extraordinary specificity of NiV-G for ephrinB2 holds promise for targeting specific ephrinB2+ populations in vivo, or in vitro.
Introduction

Lentiviruses are common vectors used in gene therapy because they can transduce non-dividing cells and offer stable integration into a target cell’s genome. The host range can be altered by pseudotyping with glycoproteins derived from other enveloped viruses. The most commonly used is the glycoprotein (G) of vesicular stomatitis virus (VSV), which has great stability in the vector particle allowing concentration to high titers, and also has a ubiquitous host cell receptor allowing transduction of most cell types (45, 62). VSV-G pseudotyped particles (VSV-Gpp) have become the standard for evaluating the efficiency of transduction by other viral envelope pseudotypes. However, VSV-Gpp cannot be targeted to specific populations of cells, which is necessary for in vivo gene transfer applications.

More specific cell targeting can be achieved by pseudotyping with envelopes modified in various ways that allow for re-retargeting via some ligand specific domain (46, 48). Measles virus (MeV) glycoproteins (Edmonston strain) can also be efficiently pseudotyped onto a lentiviral vector, but only when the cytoplasmic tails of both envelope glycoproteins, the hemagglutinin (H) and fusion (F) proteins, were truncated. MeVEdm uses CD46 and/or SLAM as entry receptors. In humans, CD46 is expressed on all nucleated cells (53), thus its natural tropism does not offer MeVpp any specific targeting advantage in vivo. However, ex vivo, MeVpp can transduce unstimulated primary human B and T cells that are relatively resistant to even VSV-Gpp transduction, suggesting that MeVpp are at least useful as an experimental tool (20, 23).

More recently, the unique features of MeV entry have allowed for some innovations that have attracted considerable interest (7, 12, 21). Measles virus is a member of the morbillivirus genus in the Paramyxovirinae subfamily of paramyxoviruses. Paramyxovirus entry requires the
coordinated action of both the fusion (F) and attachment glycoproteins (designated HN, H, or G depending on its receptor binding properties); receptor binding to the viral attachment glycoprotein induces an allosteric change that triggers F to undergo a conformational cascade that results in virus-cell membrane fusion and entry (13, 36, 58). Morbillivirus is one of only two genera of paramyxoviruses that use protein-based receptors, the others use ubiquitous glycan-based receptors such as sialic acids. The aforementioned innovation takes advantage of the wealth of structure-function information that have not only mapped the receptor binding sites on MeV-H, but have also characterized key features of the ensuing receptor-binding triggered fusion cascade (50, 58). Thus, by mutating the native receptor-binding sites on MeV-H, and appending to the C-terminus of the mutated MeV-H (a type II transmembrane protein), the single-chain variable fragment (scFv) from a monoclonal antibody recognizing specific cell-surface antigens, MeVpp can be successfully re-targeted, at least in vitro, to neurons, endothelial cells, and hematopoietic progenitors (6). Nevertheless, the development as MeVpp as in vivo targeted human gene therapy vectors is limited by the wide-spread presence of pre-existing neutralizing antibodies in the vast majority of the human population that have received MeV vaccination.

Nipah (NiV) and Hendra (HeV) viruses belong to the only other genera (Henipavirus) of paramyxoviruses that use protein-based receptors. A recent study showed that the full-length Nipah virus envelope glycoproteins could be pseudotyped onto a lentiviral vector and mediate entry into various cell lines, although infectious titers were not determined (34). For NiV, the attachment glycoprotein, NiV-G, functions in recognition of the receptor. As for MeV, binding of the receptor to NiV-G triggers a series of conformational changes that eventually lead to NiV-F triggering and virus-cell membrane fusion (reviewed in (36)). Henipaviruses use ephrinB2 as
the primary receptor, and, somewhat less efficiently, ephrinB3 as an alternate receptor (11, 51, 52). The remarkably high affinity of NiV-G for ephrinB2 ($K_d = 0.06$ nM) (52) suggests that NiV pseudotyped particles (NiVpp) can be efficiently and specifically targeted to ephrinB2+ cells. Thus, instead of re-targeting strategies, we sought to exploit the natural tropism of NiV for specific targeting of primary ephrinB2-expressing cell types that are of significant biological and clinical interest to the gene targeting community.

Eph-ephrin receptor-ligand pairs are membrane-associated receptor tyrosine kinases (RTKs) with well-established roles in many developmental processes; they regulate cell boundaries during tissue and bone formation, as well as provide guidance cues during neurogenesis and angiogenesis (56). EphrinB2-ephB4 interactions have been strongly implicated in tumor angiogenesis, migration, and invasion (55). In addition, ephrinB2 has been proposed as a molecular marker of stemness, being expressed on murine embryonic stem cells, hematopoietic stem cells and neural stem cells (31). Thus, the ability to target lentiviral vectors specifically to ephrinB2+ cells may be of utility for studying specific stem cell populations, or be of use for disrupting tumorigenesis where the ephrinB2-ephB4 axis plays a critical role (56).

Here, we systematically investigated what modifications to the cytoplasmic tails of the NiV glycoproteins could best enhance the efficiency of pseudotyping onto lentiviral particles. We found that efficient functional pseudotyping with NiV envelope requires only truncation of the F protein cytoplasmic tail, while full-length NiV-G can be used. Unlike MeVpp, full-length and truncated F were equally incorporated into NiVpp, indicating that the requirements for functional lentiviral pseudotyping differ between MeV and NiV. NiVpp can specifically target ephrinB2+ cells in a 1000-fold excess of ephrinB2-negative cells, and NiVpp transduced human embryonic, hematopoietic and neural stem cell populations in an ephrinB2-specific manner.
Intravenous administration of the luciferase reporter NiVpp resulted in signals detected in the spleen and lung, but not the liver. Biodistribution studies quantifying genome integrated vector copy numbers in various tissues confirm these observations. Bypassing the liver sink is a critical barrier for targeted gene therapy (17, 26), suggesting that the extraordinary specificity of NiV-G for ephrinB2 may allow for targeting of specific ephrinB2+ populations in vivo, or in vitro without the need for prior cell purification.
Materials and Methods

Plasmid construction. The codon-optimized NiV-F and NiV-G genes were tagged at their C termini with an AU1 (DTYRYI) or HA (YPYDVPDYA) tag, respectively, as previously described (37). The β-lactamase (β-la) gene was fused to NiV-M as previously described (72). NiV-G cytoplasmic truncation mutants were generated using the QuikChange site-directed mutagenesis kit (Stratagene, Cedar Creek, TX) with primers designed corresponding to the deletions. NiV T234F and T5FΔN3 mutants were previously made (2, 3). FUhLucW and FG12 were constructed from FUGW, as previously described (41, 60). pNL4-3.Luc.R−E was obtained through the NIH AIDS Research and Reference Reagent Program. VSV-ΔG-Luc has the G protein envelope replaced with Renilla Luc, as previously described (52).

Cells and culture conditions. 293T cells were cultured in IMDM with 10% FBS, 1% NEAA, 1% Glutamax, and antibiotics. CHO-pgsA745 is a mutant cell line derived from Chinese hamster ovary (CHO) cells that lack the endogenous expression of heparin sulfate proteoglycans (18), and was maintained in DMEM-F12 medium supplemented with 10% FBS. CHO cells expressing either ephrin-B2 (CHO-B2) or ephrin-B3 (CHO-B3) were made as previously described (52), and maintained in DMEM-F12 medium supplemented with 10% FBS and 1 mg/ml of G418 to drive plasmid expression through neomycin resistance. Vero (African green monkey kidney fibroblast) cells were maintained in α-MEM with 10% FBS. U87 cells were maintained in DMEM with 10% FBS. Human embryonic stem cells (hESCs) (H1, H9, and UCLA1 lines) were cultured on gelatin-coated plates on a feeder layer of mitotically-inactivated murine embryonic fibroblasts (MEFs). hESC medium is composed of DMEM/F:12 supplemented with 20% KnockOut Serum Replacement (KOSR, Life Technologies), 0.1 mM...
NEAA (Life Technologies), 1 mM L-Glutamine (Life Technologies), 0.1 mM 2-mercaptoethanol (Sigma-Aldrich), and 4 ng/ml basic fibroblast growth factor (bFGF, R&D Systems – obtained via National Cancer Institute Biological Resources Branch). hESCs were passaged in small clumps every 5-7 days using collagenase (Life Technologies). For viral transductions, hESCs were transferred to feeder-free conditions. hESCs were dissociated to single cells using trypsin (Life Technologies) for counting and then plated on Matrigel- (BD Biosciences) coated plates in hESC medium that was conditioned overnight on MEFs and supplemented with 10 µM HA-1077 (ROCK inhibitor, Sigma-Aldrich) to promote hESC survival (16). CD34+ cells were isolated from human fetal liver as previously described (68). For viral transductions, the cells were seeded onto Retronectin (Takara)-coated plates with 2% bovine serum albumin in Yssel’s medium (Gemini). As previously described (32), neuralization of undifferentiated HSF1 hESC colonies was induced in situ by switching to rosette media containing DMEM:F12, N-2 supplement (Gibco), B27 supplement (Gibco), 1µM retinoic acid (Sigma), 1µM Smoothened Agonist (Calbiochem), and 20 ng/mL FGF2. After 10-14 days, rosettes were mechanically isolated and passaged onto poly-ornithine- (Sigma) and laminin- (Sigma) coated plates. Once picked, rosettes were placed into neural progenitor cell (NPC) media containing DMEM:F12, N-2, B27, 50 ng/mL EGF (Peprotech), and 20 ng/mL FGF2 allowing for expansion and maintenance. 100,000 NPCs (passage #2) were plated onto 24-well plates using TrypLE (Gibco) for viral infection.

Virus production. Lentiviral vectors were produced by calcium phosphate-mediated transient transfection of 293T cells. 24 hours prior to transfection, 1.6 x 10^7 293T cells were seeded into a T175 flask. On the day of transfection, the medium was replaced with 25ml fresh medium
containing 10mM chloroquine. 7 μg of NiV-F variant, 7 μg of NiV-G variant, 12.5 μg of the packaging plasmid pCMVΔR8.9, and 12.5 μg of the lentiviral transfer vector plasmid (FG12 or FUHLucW) were mixed with 133 μl of 2M CaCl₂ and brought up to a final volume of 980 μl with ddH₂O. Next, 1,110 μl of 2X HEPES buffer saline buffer was added dropwise. Following a 20-minute incubation on ice, the precipitate was added to the cells. After 8 hours, the medium was replaced with 30 ml of AIM-V serum-free medium. 48 hours post-transfection, the cell supernatant containing the pseudotyped lentiviral vector particles was layered over a 20% sucrose cushion and concentrated by centrifugation at 28,000 rpm at 4°C for 2 hours (Beckman SW-32 rotor). The viral pellet was resuspended in 300 μl of PBS and filtered (0.45 μm filter).

To determine viral titer, serial dilutions of unconcentrated and concentrated stocks were added to 2 x 10⁵ 293T cells and incubated for 2 hours at 37°C. The medium was replaced with fresh medium. 72 hours post-infection, the cells were collected and analyzed by flow cytometry for eGFP expression. The titers are expressed as infectious units per ml (IU/ml). Typical unconcentrated titers for NiVpp are 10⁶ IU/ml, and can be concentrated to 10⁸-10⁹ IU/ml upon ultracentrifugation.

Western blot analysis. Concentrated viral stocks were normalized to HIV p24 (50 ng of p24 per lane). The samples were boiled in 6X sodium dodecyl sulfate (SDS) loading buffer containing 2-mercaptoethanol for 10 minutes, subjected to electrophoresis through a 10% SDS polyacrylamide gel, and transferred onto a PVDF (Millipore) membrane. Mouse anti-HA-tag (Covance), mouse anti-AU1-tag (Covance), and mouse anti-p24 (NIH AIDS Research and Reference Reagent Program) monoclonal antibodies were used to detect NiV-G, NiV-F, and p24 proteins, respectively. A goat anti-mouse IRDye 800CW (LI-COR Biosciences) was used as a
secondary antibody. Signals were detected using the Odyssey infrared imaging system (LI-COR Biosciences).

**β-lactamase-matrix entry assay.** NiV and VSV-G β-lactamase-matrix virus-like particles (β-la-M VLPs) were produced as previously described (72). 293T cells were seeded in a 24-well dish at 7.5 x 10^4 per well. 24 hours after seeding, concentrated NiV wt F/wt G, NiV T5F/wt G or VSV-G pseudotyped βla-M VLPs were added to the cells and spin-inoculated for 1 hour at 2,000 rpm at 4°C. After spin-inoculation, cells were gently washed and CCF2-AM (Invitrogen) was added according to manufacturer recommendations. Cells were then transferred to a pre-warmed 37°C micro-plate fluorometer (TECAN Infinite® M1000). Green (uncleaved CCF2-AM) and blue (cleaved CCF2-AM) fluorescence was monitored at 530 nm and 460 nm, respectively. Kinetic readings were taken every 10 min for up to 5 hours.

**In vitro infection of cells.** Increasing amounts of virus (based on MOI or p24) were added to 1 x 10^5 cells of each cell type and centrifuged at 2,000 rpm at 37°C for 2 hours. As a specificity control, 10 nM of soluble ephrinB2 (R&D Systems) was added to the infection medium. To exclude pseudotransduction, 5μM of nevirapine was added. For stem cell transductions, 4 ng/ml of polybrene (Sigma) was added. Following an overnight incubation with virus, the infection medium was removed and replaced with fresh medium. 72 hours post-infection, the cells were harvested and analyzed by flow cytometry for eGFP expression. For transduction of a mixed population of cells, ephrinB2+ human U87 cells were mixed with ephrinB2- non-human Chinese hamster ovary (CHO) cells at different ratios (U87:CHO ratios = 1:1, 1:10, 1:100, and 1:1000), and seeded at a density of 50,000 cells per well in 24-well plates. The next day, cells were...
infected with 1 or 10 ng of NiV T5F/wt G, T5FΔN3/wt G, and VSV-G pseudotypes. 72h post-infection, the cells were harvested, stained with the mouse W6/32 anti-human HLA-ABC monoclonal antibody (eBioscience), followed by Alexa 647-conjugated goat anti-mouse secondary antibodies. Samples were fixed and then analyzed by dual-color flow cytometry for human HLA and eGFP expression.

**FACS-sorting of CD34+ fetal liver cells.** CD34+ cells were isolated from human fetal liver as previously described (68). The cell suspensions were stained with DAPI, CD34-APC, CD38-PE-Cy7, and CD90-FITC (BD-Biosciences) antibodies for 20 minutes on ice in the dark. Following washing, the cells were sorted on a BD FACS Aria into 4 populations: CD90+CD34+CD38-, CD90-CD34+CD38-, CD90-CD34+CD38+, and CD90-CD34-CD38+. Immediately following sorting, RNA was extracted from each population using a RNeasy Micro kit (Qiagen) and used for preparation of cDNA using a QuantiTect Reverse Transcription kit (Qiagen). Specific primers for ephrinB2 (Forward- TCCCGATTGAGCCTTACGACACTT, Reverse- TTCACCTTGACACAGAGCACC) and GAPDH (Forward- ATCAAGAAGGTGGTGAAGCAGG, Reverse- TCAAAGGTGGAGGAGTGGGTGT) were used for real-time PCR analysis of gene expression.

**Immunostaining.** Coverslips were fixed in 4% PFA at room temperature for 15 minutes, permeabilized in 0.5% Triton-X-100 at room temperature for 10 minutes, and blocked in 5% bovine serum, 1% BSA, and 0.2% Triton-X-100 at room temperature for 30 minutes. They were then incubated overnight at 4°C with a mouse anti-Nestin antibody (Neuromics). Next, the coverslips were incubated with goat anti-mouse 594 (Molecular Probes) at room temperature for
1 hour and mounted in Prolong Gold with DAPI (Invitrogen). Imaging was performed using the Zeiss Axio Imager A1.

**In vivo analysis of infection.** 5-week-old female C57/BL6 (The Jackson Laboratory) were maintained in the animal facilities at UCLA in accordance with the University of California Animal Research Committee guidelines. The FUhLucW vector was pseudotyped with VSV-G, T5F/wt G, or T5F ΔN3/wt G envelopes. 5 or 10 μg of p24 of each virus stock was injected into the tail vein. 5 days post-injection, the mice were anesthetized and injected intraperitoneally with 3 mg of D-luciferin (Xenogen). A cooled IVIS CCD camera (Xenogen) was used to obtain whole-body images. Organs (brain, lung, heart, spleen, liver) were excised from sacrificed mice for imaging. For biodistribution studies, which required the quantitation of integrated vector copy number in various tissues, the FG12 vector was pseudotyped with VSV-G or NiV envelopes. 5 ug of p24 of each virus stock was administered as above. 4 days post-injection, the mice were sacrificed and organs (liver, spleen, lung, brain, heart, kidney, bone marrow) were harvested. Organs were minced and cells dissociated as previously described (71). We modified the protocol to extend the digestion time to one hour. Genomic DNA was harvested using an Allprep kit (Qiagen). Quantitation of the vector copy number and cell number in the DNA isolate was performed with LightCycler 480 SYBR Green I Master (Roche) using the LightCycler 480 real-time PCR system (Roche). The FG12 plasmid was used as the standard for quantitation of vector copy number. The primers for the analysis of vector copy number were GFP-For (5’ GCAGAAGAAGGCATCACAGTG3’) and GFP-Rev (5’ TGGGTGCTCAGGATGTGTG3’). The primers for the analysis of cell number were HPRT-For (5’ GCAGCGTTTCTGAGCCATT3’) and HPRT-Rev (5’ AAAGCGGTCTGAGGAGGA3’).
Gene Expression Analysis. The gene expression profiles of various pluripotent stem cell (PSC)-derived and primary (fetal and adult) tissues were determined by the human U133plus2.0 array (Affymetrix) at the UCLA Clinical Microarray Core. Multiple independent arrays (>3) were performed on each cell type shown in Table I.
Results

Efficient pseudotyping of a lentiviral vector with the Nipah virus envelope glycoproteins only requires truncations in the cytoplasmic tail of the F protein. Previous studies have shown that pseudotyping of lentiviral vectors with unmodified paramyxoviral glycoproteins is highly inefficient (35). However, recent studies with the measles virus envelope showed that when the cytoplasmic tails of both the fusion (F) and attachment (H) glycoproteins are truncated, infectious particles are produced (20, 23). The highest titers were obtained when only 3 residues were left in the F protein cytoplasmic tail and 15 residues in the H protein cytoplasmic tail. In contrast, a study with the Nipah virus (NiV) envelope showed that the full-length glycoproteins (F and G) could be used to pseudotype a lentiviral luciferase reporter vector, although a truncated F mutant with only 4 residues left in its cytoplasmic tail did result in a ten-fold increase in luciferase expression compared to wild-type F (34). However, no data regarding the infectious titers produced were given, and neither the transduction efficiency of their NiVpp on relevant primary cells, nor the potential of NiVpp for targeted transduction in vivo was examined.

To confirm and extend these findings, we took advantage of a previously characterized truncated variant of NiV-F, T5F (2), with 5 residues left in the cytoplasmic tail (Fig. 1A, top panel), but which was otherwise expressed and processed at wild-type levels (2). When used in combination with wild-type (wt) NiV-G to pseudotype the FG12 lentiviral vector containing a GFP reporter gene, T5F/wtG NiVpp gave a titer of ~10^6 I.U./ml on 293T cells, a 100-fold increase in titer compared to wtF/wtG pseudotypes (Fig. 1B). Pseudotyping of NiV T5F/wtG onto the pNL4-3-Luc-E’R vector that was used in the above mentioned study (34) also resulted in a 100-fold increase in luciferase expression compared to wtF/wtG NiVpp across a three-log dilution of the virus stock (Fig. 1C). However, pNL4-3-Luc-E’R is obviously not suitable as a
gene therapy vector as it expresses the entire set of HIV genes except for Env, and lacks the cardinal safety features of lentiviral-based gene therapy vectors. Thus, in our subsequent studies, we will focus on using the FG12 vector, a HIV derived self-inactivating lentiviral vector designed for gene therapy purposes (60, 68).

In an effort to further increase viral titers, we generated variants with stepwise truncations in the NiV-G cytoplasmic tail (Fig. 1A, bottom panel) and screened them in combination with T5F. Although the T5F/Δ10G and T5F/Δ25G variants demonstrated similar titers to T5F/wtG, none of the NiV-G variants produced greater titers than wtG (Fig. 1B). Moreover, all combinations of wt F with the different NiV-G truncation variants produced extremely low titers (data not shown). Collectively, these results indicate that only truncations in NiV-F are critical for producing high titer functional pseudotypes when combined with wt NiV-G. Thus, all subsequent experiments were performed with the T5F/wtG variant as a starting point. As for VSV-Gpp, NiVpp could be concentrated by ultracentrifugation without loss of infectivity to produce titers of ~10^8-10^9 IU/ml compared to 10^10 for VSV-G (data not shown).

To determine whether the difference in titer between the T5F/wtG and wtF/wtG pseudotypes was due to the efficiency of envelope incorporation onto the lentiviral particle, we purified NiVpp by ultracentrifugation and determined the amount of F and G on an equivalent amount of virions (normalized by the amount of HIV capsid p24) by western blot. Unlike that for MeVpp, where full-length wtF/wtH were not detectably incorporated into pseudotyped particles, there was no difference in levels of NiV F and G incorporated between wtF/wtG and T5F/wtG pseudotypes (Fig. 1D). In addition, both wtF and T5F were equivalently processed (F0/F1). Thus, truncation of the NiV-F cytoplasmic tail did not necessarily enhance incorporation onto virus particles, as was demonstrated by studies with the measles virus envelope (20, 22, 23).
This suggests that there may be some incompatibility of the cytoplasmic tail of wild-type NiV-F with the matrix (gag) protein of HIV that compromises the fusogenicity of NiV-F, and hence the infectivity of NiVpp.

We hypothesized that if the incompatibility is specific to the HIV gag protein, then wtF/wtG “pseudotyped” onto NiV matrix (NiV-M), to make infectious virus-like particles (VLPs), should not show a significant difference in infectivity compared to VLPs produced with T5F/wtG. To test this, we used an established β-lactamase-NiV matrix (βla-M) based assay to compare entry of wild-type NiV-F and T5F VLPs (72). Entry of VLPs is detected by cytosolic delivery of βla-M to target 293T cells preloaded with the fluorescent CCF2-AM substrate. βla-mediated cleavage of CCF2-AM results in a shift of green to blue fluorescence. Thus, the blue to green fluorescence ratio can be monitored in real-time to compare the relative differences in entry efficiency due to virus-cell fusion. Fig. 1E shows that the rate and extent of virus-cell fusion between wt F and T5F VLPs were very similar, plateauing at 2.38 and 2.65, respectively.

To further confirm this finding, we pseudotyped wild-type NiV-G with either NiV-F or T5F onto a VSVΔG-rLuc core, and infected 293T cells with 10-fold dilutions of each virus stock. T5F pseudotypes demonstrated only up to a 2-fold difference in R.L.U. compared to wt F pseudotypes (Fig. 1F). Thus, the 100-fold difference in titers of wt F and T5F lentiviral pseudotypes is most likely due to a specific incompatibility of the long cytoplasmic tail of NiV-F with HIV gag that compromises the fusogenic activity of NiV-F but not its ability to be incorporated into lentiviral particles.

A hypoglycosylated hyperfusogenic NiV-F mutant demonstrates increased infectivity in vitro. EphrinB2 is likely the primary entry receptor for NiV (11, 51), while ephrinB3 may serve
an alternate receptor on some cell types (52, 57). Chinese hamster ovary (CHO) cells do not express endogenous ephrinB2 and B3, and are therefore refractory to NiV envelope-mediated infection. However, stable CHO cell lines expressing ephrinB2 (CHO-B2) or ephrinB3 (CHO-B3) can readily support NiV infection (52). To compare the relative entry efficiency of NiVpp via the ephrinB2 and ephrinB3 receptors, we first infected CHO-B2 or CHO-B3 cells with 0.01, 0.1, and 1 ng p24 equivalents of NiVpp bearing T5F/wtG (Fig. 2A-C, grey bars), and normalized the infectivity observed with that obtained with 1 ng of VSV-Gpp (Fig. 2D). Since VSV-Gpp infection should not depend on the presence of ephrinB2 or B3, this normalization allows for comparison across multiple independent experiments. Fig. 2A-C shows that T5F/wtG pseudotypes infected a similar percentage of CHO-B2 and CHO-B3 cells in a dose-dependent manner such that at the maximal viral input (1 ng p24), 38% of CHO-B2 and 39% of CHO-B3 cells were infected relative to an equivalent amount of VSV-Gpp (compare Fig. 2C and 2D, grey bars at 1 ng).

Since we found that only modifications to NiV-F were critical for pseudotyping (Fig. 1), we sought to further improve transduction efficiency by pseudotyping lentiviral particles with a hyperfusogenic NiV-F variant in which an N-linked glycosylation site has been removed (T5FΔN3) (3). The titer of T5FΔN3/wtG pseudotypes on highly permissive 293T cells was similar to that of T5F/wtG pseudotypes (data not shown). However, on CHO-B2 cells, the hyperfusogenic T5FΔN3/wtG pseudotypes consistently exhibited a two-fold increase in infectivity relative to the T5F/wtG pseudotypes (Fig. 2A-C). This held true across a 100-fold difference in the amount of viral inoculum used. Although receptor specificity is determined by the attachment protein, there are examples of hyperfusogenic mutations in paramyxoviral F proteins that enable fusion triggering in the absence of their homotypic attachment proteins (8,
61, 65, 66). This does not appear to be true for the hyperfusogenic NiV-F as the increased infectivity of T5FΔN3/wtG pseudotypes was abrogated by soluble ephrinB2. Similar results were observed on CHO-B3 cells at moderate (0.1 ng) and high (1 ng) viral input levels (Fig. 2B-C, compare black and grey bars); however, no specific infectivity was detected at the lowest viral inoculum level on CHO-B3 cells (Fig. 2A). At the highest viral inoculum used, T5FΔN3/wtG NiVpp infection approached the transduction efficiency of VSV-Gpp, infecting 80% and 97% of CHO-B2 and CHO-B3 cells, respectively (Fig. 2D).

Lastly, since ephrinB2 is endogenously expressed at high levels on endothelial cells and cells of the central nervous system, we compared the transduction efficiencies of T5F/wtG and T5FΔN3/wtG pseudotypes on human microvascular endothelial cells (HMVECs) and the U87 glioblastoma cell line (Fig. 2E-F). The two-fold increase in infection efficiency of T5FΔN3/wtG over T5F/wtG pseudotypes was observed for U87 (59.8% versus 32.3% at 1 ng) cells, but not for HMVECs (96.6% versus 87.6%), which were already highly permissive to NiVpp infection. In summary, although both pseudotypes had similar infectivity on highly permissive cells such as 293Ts and HMVECs, the hyperfusogenic T5FΔN3/wtG pseudotypes nevertheless exhibited increased infectivity on some cell lines.

**NiV pseudotypes specifically target ephrinB2-positive cells in a vast excess of receptor-negative cells.** Entry of T5F/wtG and T5FΔN3/wtG pseudotypes into multiple cell types was inhibited by soluble ephrinB2 (Fig. 2), confirming the specificity of receptor-mediated entry. Next, we investigated whether the NiV pseudotypes could specifically target ephrinB2-positive cells in a mixture of ephrinB2-positive and -negative cells. U87 (ephrinB2-positive) cells were mixed with CHO (ephrinB2-negative) cells in 1:1, 1:10, 1:100, and 1:1000 ratios, and transduced
with 1 or 10 ng p24 equivalents of NiV or VSV-G pseudotypes (Fig. 3). To distinguish infection of the human U87 cells from non-human CHO cells, the infected cells were stained with an anti-HLA (anti-MHC class I) monoclonal antibody specific for human HLAs. At a 1:1 ratio and 1 ng of virus, the GFP+ cells transduced by both the T5F/wtG and T5FΔN3/wtG pseudotypes remained entirely within the HLA+ population (Fig. 3A, top panel). Increasing the amount of virus inoculum by 10-fold (10 ng) did not affect the specificity, since the NiVpp transduced GFP+ cells remained within the HLA+ population (Fig. 3A, bottom panel). Furthermore, across all cell ratios, the NiV pseudotypes selectively transduced the ephrinB2+ U87 cells even after accounting for the differential permissivity of CHO cells versus U87 cells for HIV-1 based lentiviral transduction (Fig. 3B-C). VSV-G pseudotypes, on the other hand, transduced both HLA+ and HLA- cells, indicating its relative lack of specificity. In sum, our data suggest that NiV pseudotypes can selectively target ephrinB2-positive U87 cells even in a 1000-fold excess of ephrinB2-negative CHO cells (Fig. 3C). Our data also demonstrates that increasing the fusogenicity of NiVpp did not necessarily compromise its specificity.

NiV pseudotypes mediate entry into human embryonic, neural and hematopoietic stem cells. On the basis of microarray and bioinformatics analysis, ephrinB2 has been identified as a molecular stem cell signature common to mouse embryonic (ESCs), hematopoietic (HSCs) stem cells, and neural (NSCs) (31). To determine if ephrinB2 also marks for their human stem cell counterparts, we determined if our NiVpp could mediate gene transfer into human ESCs, HSCs, and NSCs (Fig. 4). Indeed, adding increasing amounts of T5F/wtG pseudotypes to H9 hESCs resulted in a dose-dependent increase (14-36%) in the amount of cells positive for GFP and SSEA-4, a cell-surface human pluripotency marker (Fig. 4A). This infection was specific since
it was blocked by soluble ephrinB2 (Fig. 4B). To ensure that this ephrinB2-mediated transduction was not specific to the H9 hESC line, we infected two other hESC lines (H1 and UCLA1) and obtained similar results (Fig. 4B). Since stem cells are more difficult to transduce than standard cell lines, we expected that the hyperfusogenic T5FΔN3/wt G variant would mediate entry more efficiently than T5F/wtG NiVpp. However, we did not see an increase in infection using the T5FΔN3/wt G pseudotypes (Fig. 4C).

Next, we infected purified CD34+ cells isolated from human fetal liver with NiV pseudotypes. CD34 is expressed on human hematopoietic stem and progenitor cells (HSPCs), although only a small fraction of CD34+ cells are true hematopoietic stem cells (HSCs) that have extensive self-renewal capacity in vitro and can engraft immunodeficient mice (27, 60). At a multiplicity of infection (MOI) of 1000, both T5F/wtG and T5FΔN3/wt G pseudotypes reproducibly transduced 3.6% and 3.5% of CD34+ cells, respectively. The specificity of this low-level infection was confirmed by blocking with soluble ephrinB2 (Fig. 4D). True human HSCs have two cardinal properties: multipotency, defined as the ability to differentiate into all blood cell lineages, and long-term self-renewal, defined by the inexhaustible ability to produce progeny functionally identical to the parent upon cell division (27). Human HSCs with these properties are enriched in the Lin-CD90+CD34+CD38- fraction of cord blood (44). To determine whether ephrinB2 is expressed in this fraction, we FACS-sorted 4 populations from CD34+ cells isolated from human fetal liver: CD90+CD34+CD38-, CD90-CD34+CD38-, CD90-CD34+CD38+, and CD90-CD34-CD38+ (Fig. 4E). RNA was extracted from each sorted population and ephrinB2 expression quantified using real-time PCR analysis. Our results indicate that ephrinB2 is expressed the highest in the CD90+CD34+CD38- fraction (Fig. 4F).
Thus, the NiV pseudotypes may be targeting the cognate population of CD34+ cells enriched for true HSC activity.

Lastly, we assessed the transduction efficiencies of the NiV pseudotypes on nestin+ NSCs derived from hESCs (Fig. 4G). Unlike hESCs and CD34+ HSPCs, increasing the MOI resulted in a dose-dependent increase in the percent of NSCs transduced such that the percent of GFP+ cells approached 100% at a MOI of 100 (Fig. 4H). However, similar to the hESC and hHSC transductions, the T5FΔN3/wt G pseudotypes did not demonstrate increased infectivity compared to the T5F/wtG pseudotypes. Nevertheless, we confirmed that ephrinB2 is functionally expressed on human ESCs, HSCs, and NSCs, at least at levels that can mediate NiVpp infection.

**NiV pseudotypes bypass the liver sink in vivo.** EphrinB2 is expressed on endothelial cells, smooth muscle cells, and neurons (25, 69). In contrast, ephrinB3 is not expressed in the endothelium, and demonstrates overlapping but also distinct expression patterns in the central nervous system (19). This is consistent with our own microarray expression studies in a variety of human- and fetal tissue-derived as well as pluripotent stem cell (PSC) derived cell types (Table 1). The ephrinB2/ephrinB3 expression patterns are in concordance with NiV infection in vivo, since histopathological studies on human patients detected the highest levels of viral antigens in neurons and endothelial cells of small blood vessels in the brain, but some was also observed in the vasculature of the lung and spleen. (43, 73). Importantly, no viral antigens were detected in the liver (73), also consistent with our data in Table I, which shows the lack of ephrinB2 and B3 expression in adult tissue derived hepatocytes.
The inability to detect viral antigens in liver autopsy tissues from NiV infected patients, and the lack of viral receptor expression in the liver prompted us to examine whether intravenous administration of NiVpp might bypass the liver sink and target accessible ephrinB2+ cell types in vivo. We pseudotyped the FvcFlw lentiviral vector containing a firefly luciferase reporter gene with the NiV and VSV-G envelopes, and administered the viruses intravenously through the tail vein of C57/BL6 mice (Fig. 5). A CCD camera was used to quantify the level of luciferase expression in the mice after injection of the D-luciferin substrate. Consistent with previous studies, VSV-Gpp-mediated transgene expression was detected primarily in the liver and spleen (Fig. 5B, top panel). For the T5F/wtG pseudotypes, a slight signal was detected in the spleen in one case (Fig. 5B, middle panel). Strikingly, the T5FΔN3/wt G pseudotypes showed a substantially enhanced signal in the spleen in all cases and lung in one case (Fig. 5B, bottom panel). Thus, the T5FΔN3/wt G hyperfusogenic mutant demonstrates increased infectivity in vitro and in vivo. Significantly, neither the T5F/wtG or T5FΔN3/wtG pseudotypes exhibited any signal in the liver in all cases.

To complement and confirm these results, we also examined genomic vector integration in various tissues in an independent set of mice. The FG12 vector was pseudotyped with VSV-G and NiV envelopes, and viruses were administered as above. Four days post-injection, whole organs (liver, spleen, and lung) were harvested and cells dissociated. Genomic DNA was extracted and quantitation of vector integration was performed using real-time PCR analysis. This PCR based assay was more sensitive (limit of sensitivity ~0.01 vector integrants/10,000 cells) and confirmed key aspects of our luciferase imaging results: while VSV-Gpp and NiVpp transduced the spleen with high efficiencies, only the VSV-Gpp transduced the liver, confirming that the NiVpp clearly bypassed the liver sink (Fig. 5C). Interestingly, using the FG12 vector,
VSV-G also transduced the lung as well as the NiVpp, although it is unclear whether the same cell types were transduced. The significance of these findings will be discussed.

Discussion

Measles virus and Nipah virus belong to the only two paramyxovirus genera that use protein-based receptors for entry. Studies with the measles virus envelope have shown that complex modifications to its attachment protein, including disruption of its binding site to its natural receptors and appending scFv or other targeting domains, result in specific re-targeting to desired cell populations in vitro and in vivo (12, 24). Some of these modifications have been successfully adapted to make scFV-directed targeting lentiviral MeVpp (23, 49). However, pre-existing neutralizing antibodies due to wide-spread MeV vaccination may compromise the transduction efficiency of MeVpp when administered in vivo, although deletion of immunodominant epitopes on MeV-H and other modifications have reduced the sensitivity of MeVpp to serum neutralization in vitro (38). In contrast, NiV is an emerging and lethal pathogen thus far confined to Southeast Asia (42). Thus, it is unlikely that pre-existing antibodies will pose a barrier to the development of NiVpp as a vehicle for targeted gene therapy. However, in the case of NiV, we sought to take advantage of the physiologically restricted and pathologically relevant expression patterns of ephrinB2, the primary high affinity receptor for NiV. Thus, instead of mimicking the re-targeting strategies used for MeV, we investigated the prospects of generating high-titer NiV pseudotypes that allow for specific targeting of biologically significant ephrinB2+ populations in vitro and in vivo.

The picomolar affinity of NiV-G for ephrinB2 is amongst the strongest viral envelope-receptor interactions known (36). This likely accounts for the extraordinary specificity of NiVpp
for ephrinB2-expressing cells. We examined whether further increasing the efficiency of transduction on a per virion basis without compromising the specificity of NiV-G-mediated infection may facilitate the development of NiVpp for targeted gene therapy to ephrinB2 expressing cells. To that end, we generated NiVpp with a hyperfusogenic F and wt G. Our hyper-fusogenic T5FΔN3/wt G NiVpp appeared to infect some cell types (CHO-B2, CHO-B3, and U87 cells) twice as well as T5F/wt G pseudotypes, but not in other highly permissive cells such as 293T cells and HMVECs, where transduction efficiencies of the normo-fusogenic T5F/wt G NiVpp already approached that of VSV-Gpp.

Unexpectedly, in hard-to-transduce stem cell populations such as human ESCs and HSCs, the hyper-fusogenic T5FΔN3/wt G pseudotypes also did not show an increase in transduction efficiency over T5F/wt G NiVpp. In addition, both demonstrated similar dose-dependent transduction efficiencies that plateaued at a relatively low percentage of the putative stem cell population. Thus, even at a saturating MOI of 1,000, both T5F- and T5FΔN3-based NiVpp transduced only ~36% and ~3.5% of SSEA4+ hESCs and CD34+ hHSCs, respectively. The limited transduction efficiency seen in human ESCs and CD34+ HSCs may be due to ephrinB2 expression only on a subset of these stem cell populations.

In hESCs, which are optimally passaged as colonies of cells, each colony contains heterogeneous subpopulations of cells that interact as an “ecosystem” to maintain the cardinal properties of hESCs: self-renewal and pluripotency (28, 30). For example, Stella and Nanog expressing subpopulations are biased towards self-renewal, whereas GATA-6 expressing subpopulations are more poised towards differentiation, and in between is a continuum of cells that contributes to the unique phenotypic properties of each hESC line (29). The ephrinB2+ subpopulation, or rather the fraction of ephrinB2+ cells that is maintained in hESC colonies, may
be regulated to provide the optimal milieu for maintaining the cardinal properties of “stemness”. This speculation is consistent with the known properties of ephrin-eph ligand-receptor interactions (both are receptor tyrosine kinases) for maintaining or enforcing tissue and cell type boundaries (59, 64). Thus, the ability to mark a subpopulation of hESCs with NiVpp-mediated transduction provides an experimentally tractable tool to examine the role of these ephrinB2+ subsets in hESC fate: survival, self-renew and pluripotency.

On the other hand, only a small subpopulation of CD34+ cells harbors true multipotent HSCs capable of long-term (LT) self-renewal, operationally defined by multi-lineage reconstitution in immunodeficient NOD-SCID mice. These LT-SCID repopulating cells can be found at least within the CD34+/CD38-/CD90+ subset (9, 15, 44). Intriguingly, we found that ephrinB2 is expressed highest in this subset (Fig. 4F). This is also consistent with the finding that ephrinB2 is found in the functional murine equivalent of LT-HSC (31). Moreover, data from Fig. 4E-F indicate that the CD38-/CD90+ subset comprises less than 8% of total CD34+ cells from human fetal liver, which is close to but more than the ~3.5% of CD34+ cells infected by NiVpp at maximal MOI. Since LT-SCID repopulating cells are highly enriched in, but do not comprise the totality of CD34+/CD38-/CD90+ cells, this raises the possibility that NiVpp may indeed be targeting the elusive "true" HSC population within the CD34+/CD38-/CD90+ subset. Functional confirmation will require limiting dilution in LT-SCID repopulating assays, which is a focus for future studies.

We are cognizant that cell-type dependent post-entry restriction factors may limit the efficiency of NiVpp transduction no matter how fusogenic we make the F protein to be. However, the extraordinary specificity of NiVpp exhibited by its ability to selectively target ephrinB2+ cells even in a 1000-fold excess of ephrinB2-negative cells (Fig. 3), prompted us to
examine the transduction efficiency of NiVpp \textit{in vivo}, especially when the NiV pseudotypes are administered intravenously, and therefore subjected not only to dilution into the blood and tissue volume, but also to the problem of hepatic clearance, a critical barrier for \textit{in vivo} virus-based gene therapy (17, 26, 77). Interestingly, when VSVpp and NiVpp carrying a luciferase reporter gene were injected into mice intravenously, and subsequently subjected to whole animal and organ imaging for D-luciferin-induced bioluminescent signals, T5FΔN3/wt G pseudotypes demonstrated an enhanced signal in the spleen and lungs compared to T5F/wt G (Fig. 5B). Significantly, we did \textit{not} detect a signal in the liver with either NiVpp as was observed with the VSV-G pseudotypes, suggesting that NiVpp could bypass the liver sink. However, \textit{in vivo} bioluminescent imaging has limited sensitivity as signals are generally detected only when high local concentrations of cells are transduced (typically $>10^3$ to $10^4$) (14, 67). Thus, we performed a sensitive PCR based biodistribution study to quantify the copy number of genomic vector integrants in these tissues (Fig. 5C). This PCR assay confirmed that NiVpp did not transduce the liver to any significant level above background, while VSV-Gpp clearly could.

Rapid clearance and degradation of intravenously administered viral vectors by the liver has long been noted as an important obstacle in virus-based gene therapy (17, 26, 77). Our data suggests that the NiVpp can effectively bypass the liver sink and target ephrinB2+ cells in selected organs \textit{in vivo} without the need for modifying the intrinsic specificity of the receptor binding attachment protein (NiV-G). Interestingly, our PCR based biodistribution studies (Fig. 5C) showed that both normo- (T5F/wt G) and hyper-fusogenic (T5FΔ3)/wt G) NiVpp could transduce the spleen and lung with equivalent efficiencies, which is in contrast to the bioluminescent results (Fig. 5B). Additionally, our biodistribution assay also showed that VSV-Gpp (FG12 vector based) could transduce the lung at the same levels as NiVpp, but
bioluminescent signals was clearly lacking in the lung of VSV-Gpp (FvCF1w vector based) transduced animals. VSV-Gpp can transduce a wide array of tissues, and thus the lack of detectable bioluminescent signals in the lung is likely a reflection of the limits of the detection methodology as discussed above. However, identification of the specific cell populations infected in the spleen and lungs is necessary for future optimization of the NiVpp platform for targeted gene therapy. EphrinB2 is expressed highly on endothelial cells, smooth muscle cells surrounding some arterioles, and neurons (25, 69). Unlike ephrinB2, ephrinB3 is mostly expressed in the CNS (19). Our own expression studies on multiple cell types and tissues confirm and extend these findings (Table I). As mentioned, these expression patterns are in concordance with the tissues that are targeted in the context of a natural human Nipah virus infection (74). Importantly, Table I also shows that endoderm tissues do not express ephrinB2 or B3. Indeed, of the major organs examined in a large autopsy series, the liver is one of the few organs that exhibited no pathology or presence of any detectable viral antigens (73). The latter observations are consistent with the lack of liver transduction seen with our NiVpp. In spleen, viral antigen staining can be seen in macrophages and multinucleated giant cells. In the lung, viral antigen is most commonly seen in small blood vessels, and less often, in bronchial epithelial cells and alveolar macrophages. Since intravenous administration of NiVpp does not reflect the natural mode of NiV infection, determining the cell types transduced by NiVpp in the spleen and lung will be an important focus of future studies.

Altogether, our data demonstrates that we can generate high-titer, concentrated stocks (10⁸-10⁹ IU/ml) of NiVpp that can specifically target ephrinB2- and ephrinB3-positive cells in vitro and in vivo. Although NiV uses ephrinB3 as an alternate receptor, the affinity of NiV-G for ephrinB3 is less than that for ephrinB2 (52). Nevertheless, ephrinB3 is expressed in regions in
the CNS where ephrinB2 is lacking, including the corpus callosum and spinal cord (39). Thus, NiV pseudotypes can potentially be used to also target these ephrinB3-positive regions. EphrinB2 has been shown to be upregulated in many types of cancer, including ovarian (4), uterine (5), and colon (40). In the appropriate context, inhibition of ephrinB2-ephB4 interactions has resulted in inhibition of tumor growth and angiogenesis (33). In some cases, breast cancer stromal cells over express ephB4 to attract tumor angiogenic vessels that overexpress ephrinB2 (54). Thus, NiV pseudotypes can be potentially used to either target appropriate tumors where overexpression of ephrinB2 has been linked to poorer prognosis, or antagonize ephrinB2 interactions with ephB4 to inhibit tumor angiogenesis (reviewed in (55, 56)).

Many other viral envelopes have been modified for targeted gene therapy. For many of them, the receptor-binding domain and the fusion domain of the envelope are produced from a single viral env gene. Thus, manipulation to enhance receptor-targeting specificity is more likely to adversely affect the fusion domain of the envelope protein, resulting in low viral titers. For paramyxoviruses, the receptor-binding attachment protein and the fusion protein are produced from two independent viral genes. Mechanistic studies as to how receptor binding to the attachment protein leads to allosteric triggering of the fusion protein is an area of intense study by many labs (reviewed in (10, 12, 24, 36, 49, 63)). For the henipaviruses, a large body of work has accumulated regarding the independent determinants of fusogenicity in F and G. Thus, F can be made even more fusogenic by incorporating other mutations that are already well characterized in the literature (1-3, 46, 47, 75). Indeed, even the specificity and fusogenicity of G itself can be optimized based on published structural and functional data (8, 36, 38, 42, 53, 61, 76). Altogether, this confluence of properties makes NiV pseudotypes highly attractive for further development as a targeted gene therapy vector for in vitro and in vivo applications.
Acknowledgements

The stem cell work was supported by a California Institute for Regenerative Medicine grant RB2-01571 and the non-stem cell work by NIH grant AI069317-06. We acknowledge support from the UCLA CFAR Virology Core (p24 determinations) and the Gene and Cellular Therapy Core (human CD34+ cells) (NIH AI028697), the UCLA AIDS Institute, and the UCLA Council of Bioscience Resources. We also acknowledge support from Eli and Edythe Broad Center of Regenerative Medicine and the Stem Cell Research Center FACS Core. WEL was supported by the Eli and Edythe Broad Center of Regenerative Medicine and Stem Cell Research at UCLA Innovation Award.
References


35 Virus gp Overcome Pre-existing Immunity in In Vivo-like Transduction of Human T and B Cells. Mol Ther.


Figure 1. Characterization of NiV-F and -G protein variants used for pseudotyping of an HIV-1-derived lentiviral vector. (A) A schematic of the amino acid sequences of the cytoplasmic tails of the NiV F (top) and G (bottom) protein variants is shown. T5F is a cytoplasmic tail truncation of NiV-F previously described (2). The 6 amino acids to the right is the AU1 tag at the cytoplasmic C-terminus of the F protein. Stepwise truncations in the cytoplasmic tail of NiV-G were generated and screened in combination with T5F for the ability to form functional lentivirus pseudotypes. (B) Pseudotyped lentiviruses were made with the FG12 vector where the UbiC promoter drives eGFP expression. Codon-optimized NiV-F and NiV-G genes were transfected at a 1:1 ratio and supernatants harvested at 48h after transfection. Serial dilutions of unconcentrated viral supernatants were titered on 293T cells. Cells were examined for GFP expression 72h post-transduction by FACS analysis. Titers are expressed as IU/ml. Data shown are averages ± standard deviations from three independent experiments. (C) pNL4-3.Luc.R’E’ was pseudotyped with VSV-G or NiV envelopes. 293T cells were infected with 0.01 ng, 0.1 ng, 1 ng, and 10 ng (p24 equivalents) of VSV-G or NiV pseudotypes. 72h post-infection, the cells were lysed and analyzed for luciferase activity. Data shown are averages of three replicates ± standard deviations. (D) Western blot analysis of HIV-1 pseudotyped particles, pseudotyped with VSV-G and NiV wild-type or variant proteins. NiV-F was detected using an anti-AU1 antibody and NiV-G with an anti-HA antibody. One out of three representative experiments is shown. (E) Virus-like particles (VLPs) were produced with NiV β-lactamase-matrix (β-lac-M) and VSV-G, NiV wt F/wt G or NiV T5F/wt G envelopes. Based on densitometry of βla-M blots, equivalent amounts of concentrated VLPs were added to 293T cells for 1 hour at 4°C and then incubated with CCF2-AM substrate at 37°C. The blue and green...
fluorescence ratios were monitored as a measure of virus-cell fusion as described in the text and Methods. Data is presented as blue:green ratios every 10 minutes. Kinetic readings up to the 50 minutes time point are shown. Duplicate readings are taken at each time point. Data shown are averages ± standard deviations from three independent experiments. (F) A VSV-ΔG-Luc core was pseudotyped with NiV wt F/wt G or NiV T5F/wt G envelopes as previously described (52). Serial dilutions of unconcentrated viral supernatants were titered on Vero cells. 24 hours post-infection, the infected cells were lysed and analyzed for luciferase activity. Data shown are averages of four replicates ± standard deviations.

Figure 2. NiV T5FAN3/ wt G hyperfusogenic mutant demonstrates increased infectivity in vitro. (A-C) CHO, CHO-B2, and CHO-B3 cells were infected with 0.01 ng, 0.1 ng, and 1 ng (p24 equivalents) of NiV envelope or (D) VSV-G lentiviral pseudotypes carrying the GFP reporter gene (CHO cells not shown for NiVpp infection). Infectivity was determined by the percent of GFP+ cells at 48h post-infection via FACS analysis. The % GFP+ cells in each of the CHO cell lines infected by VSV-Gpp at maximal viral input (1 ng) was set at 100%, and all other infections in that cell line were normalized to this value. For reference, at 1 ng, VSV-G infected 20.2% of CHO, 22.7% of CHO-B2, and 21.6% of CHO-B3 cells. For clarity of comparisons, the relative infectivity of T5F/wtG NiVpp versus the hyperfusogenic T5FΔN3/wtG variant on CHO-B2 and CHO-B3 cells using low (0.01 ng), medium (0.1 ng), or high (1 ng) amounts of viral inoculum are shown separately in (A), (B), and (C), respectively. (E) U87 cells and (F) HMVECs were infected with T5F/wt G and T5FΔN3/wt G pseudotypes as described for (A-C) but normalized to VSV-Gpp infection of the same cell line (U87 or HMVECs) at maximal viral input (1 ng). For reference, at 1 ng, VSV-G infected 36.5% of U87 cells and 14.4% of
HMVECs. Inhibition by 10 nM of soluble ephrinB2 (sEFNB2) was used to demonstrate specificity of NiV receptor-mediated entry. All pseudotyped particle infections, regardless of envelope used, were also abrogated by 5 μM niverapine (NVP), a reverse transcriptase inhibitor (data not shown). Data shown in (A-F) are averages ± standard deviations for three independent experiments. Statistical analyses were performed using a two-way ANOVA with Bonferroni post-test comparison using GraphPad PRISM™. *: p < 0.05, **: p < 0.01, ****: p < 0.0001.

Figure 3. NiV pseudotypes can specifically target ephrinB2-positive cells in up to a 1000-fold excess of ephrinB2-negative cells. (A) U87 (ephrinB2+) cells were mixed with CHO (ephrinB2-) cells at different ratios (U87:CHO ratios = 1:1, 1:10, 1:100, and 1:1000) and seeded at a density of 50,000 cells per well in 24-well plates. The next day, cells were infected with 1 or 10 ng of NiV T5F/wt G, T5FΔN3/wt G, and VSV-G pseudotypes. 72h post-infection, the cells were harvested and stained with the W6/32 anti-human HLA-ABC monoclonal antibody and the infection rate (GFP-positive cells) was determined by FACS analysis. Representative FACS plots are shown for data acquired on infection of the 1:1 mixture of U87:CHO cells. Although the cells were seeded and infected at the indicated ratio, the CHO cells divided faster and outgrew the U87 cells by about ten-fold in each sample. Each FACS plot is representative of one of the triplicates at 1 ng and one of the duplicates at 10 ng. Data from 300,000 cells were acquired for every condition used for analysis in part B of this figure. (B) To take into account the differential permissivity of U87 and CHO cells to lentiviral transduction, we first calculated the “cell-specific selectivity index” for U87 cells, the U87 SI as \( \frac{\{B/(A+B)\}}{\{D/(C+D)\}} \) where B and D represents the % of infected (GFP+) U87 and CHO cells, respectively, and A and C represents their uninfected counterparts, such that the total fraction of U87 (A+B) and CHO
(C+D) cells in any given mixture upon analysis must equal 100%. A U87 SI of >1 indicates a selective preference for infecting U87 over CHO cells. For VSV-Gpp, the U87 SI at 1 and 10 ng is 5.14 and 1.93, respectively. This likely reflects the receptor-independent preference for U87 over CHO cells due to the HIV-1 based vector backbone alone. The reduction in U87 SI at a higher inoculum of VSV-Gpp is also consistent with the known ability of VSV-G-delivered gag to saturate non-human post-entry restriction factors such TRIM5α. Since VSV-G is not known to have a cell-type specific receptor, we calculated the “NiV receptor-specific selectivity index”, or the “EphrinB2 SI” as the VSV-G or NiV Env specific U87 SI divided by the U87 SI for VSV-G. This normalizes for differences in the intrinsic permissiveness of U87 over CHO cells for lentiviral transduction. This formulation now allows one to evaluate the selectivity of NiVpp for infecting ephrinB2-expressing cells relative to VSV-Gpp under all conditions analyzed. The values of the U87 SI and EphrinB2 SI for the data shown in (A) are indicated here as an example of our analysis. (C) The EphrinB2 Selectivity Index for VSV-Gpp, and NiVpp bearing T5F or T5F-ΔN3 was calculated for all the indicated conditions. Data shown are averages ± standard deviations for triplicates done at 1 ng, and average ± range for duplicates done at 10 ng.

Figure 4. NiV pseudotyped lentiviruses infect human embryonic, neural and hematopoietic stem cells. (A) Increasing amounts of NiV T5F/wt G pseudotypes were added to H9 hESCs. Cells were stained for the cell-surface pluripotency marker, SSEA-4, and examined for GFP expression 72h post-transduction by FACS analysis. (B) H1 and UCLA1 hESC lines were infected with NiV T5F/wt G pseudotypes as in part (A). Infection was blocked with 10 nM soluble ephrinB2 or 5 μM nevirapine. Data shown are averages ± standard deviations from three independent experiments. (C) H9 hESCs were infected with NiV T5F/wt G and T5FΔN3/wt G...
pseudotypes as in part (A). One out of two representative experiments is shown. (D) Purified CD34+ cells from human fetal liver were infected with the indicated NiVpp in the presence or absence of 10 nM sEFNB2 and 5 μM NVP. 72h post-transduction, cells were stained for the cell-surface marker, CD34, and analyzed for GFP expression by FACS analysis. One representative donor out of three is shown. (E) CD34+ cells isolated from human fetal liver were stained with CD90-FITC, CD34-APC, CD38-PE-Cy7 antibodies and DAPI, and FACS-sorted into the 4 populations as indicated. Data shown are averages ± standard deviations from 3 donors. (F) RNA was extracted from each cell population indicated in (E) and ephrinB2 expression was examined by real-time PCR analysis and normalized against GAPDH as indicted in methods. Data shown are averages ± standard deviations from 3 donors. (G) Neural progenitors were derived from HSF1 hESCs and infected with NiVpp. 72h post-transduction, cells were stained for nestin and examined by microscopy. (H) hNSC GFP expression at each M.O.I. (0.01, 0.05, 0.1, 0.5, 1, 5, 10, and 50) was quantified by FACS analysis. Data shown in (G) and (H) are from one representative experiment out of four.

Figure 5. NiV pseudotypes bypass the liver sink in vivo. (A) The FvcFlw (firefly luciferase) vector was pseudotyped with VSV-G and the indicated NiV envelopes. 5-10 ng of p24 equivalent of each pseudotyped lentivirus was injected into C57/BL6 mice through the tail vein. 5 days post-injection, luciferase expression was monitored by CCD imaging of the whole animal after injection of the D-luciferin substrate as described in methods. Three mice from three independent experiments are shown per virus. (B) Following whole-body imaging, each organ was isolated and luciferase activity was imaged and quantified as in (A). Three organs from three different mice are shown per virus. (C) The FG12 (GFP) vector was pseudotyped with
VSV-G and the NiV envelopes. 5 μg of p24 equivalent of each pseudotyped lentivirus was administered to mice as above. 4 days post-injection, the mice were sacrificed and the indicated organs harvested. Genomic DNA was extracted and quantitation of the vector copy number was determined using real-time PCR analysis for GFP vector sequences. GFP copy numbers were normalized to HPRT copy numbers. Normalized GFP copy numbers are presented as fold-increase over background numbers obtained from matched organs in an uninfected mouse. 3 mice were used for each indicated pseudotyped vector (VSV-G, NiV-T5F, and NiV-T5FΔ3). The median and range are shown as box plots, using the average of quadruplicates for each PCR reaction. Statistical significance was accessed by t tests corrected for multiple comparisons by the Holm-Sidak method in GraphPad PRISM™ 6. ***, p<0.001.

Table 1. Tissue and cell type expression of EphrinB2 and EphrinB3. A human U133plus2.0 array (Affymetrix) was performed on various pluripotent stem cell (PSC)-derived and primary (fetal and adult) tissues to examine whole genome expression. Shown here are the normalized mean expression values from multiple biological repeats (≥ 3). EphrinB2/B3 (yellow for significant positives), housekeeping genes (grey), and cell-specific genes (red) are color coded as indicated.

Footnote:

PSC, Pluripotent Stem Cell
DE, Definitive Endoderm derived from PSC cultured in chemically defined medium (CDM-ABFLY) supplemented with Activin, BMP4, FGF2, and the PI3K inhibitor LY294002. DE gives rise to other endoderm progenitors (pancreatic, endocrine etc.), and can retain expression of the POU5F1 pluripotency marker (also called Oct4) during early stages of PSC→DE differentiation (70).
<table>
<thead>
<tr>
<th>Gene</th>
<th>Specificity Marker</th>
<th>Source: Cell Type</th>
<th>PSC</th>
<th>PSC DE</th>
<th>Adult Tissue Hepatocytes</th>
<th>PSC Neural Progenitor</th>
<th>PSC Neurons</th>
<th>Fetal Tissue Neural Progenitor</th>
<th>Fetal Tissue Keratinocytes</th>
<th>Adult Tissue Mesothelial</th>
<th>Adult Tissue Kidney Epithelial</th>
<th>Adult Tissue Blood Vessels</th>
</tr>
</thead>
<tbody>
<tr>
<td>EFNB2</td>
<td></td>
<td></td>
<td>1186</td>
<td>716</td>
<td>85</td>
<td>2131</td>
<td>1794</td>
<td>3961</td>
<td>2244</td>
<td>1935</td>
<td>3008</td>
<td>5415</td>
</tr>
<tr>
<td>EFNB3</td>
<td></td>
<td></td>
<td>349</td>
<td>525</td>
<td>53</td>
<td>1622</td>
<td>7789</td>
<td>2275</td>
<td>275</td>
<td>190</td>
<td>219</td>
<td>184</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Housekeeping</td>
<td></td>
<td>19143</td>
<td>17814</td>
<td>13047</td>
<td>17359</td>
<td>17637</td>
<td>17303</td>
<td>19032</td>
<td>22349</td>
<td>21258</td>
<td>19544</td>
</tr>
<tr>
<td>ACTB</td>
<td>Genes</td>
<td></td>
<td>18106</td>
<td>18917</td>
<td>17098</td>
<td>18191</td>
<td>17055</td>
<td>19256</td>
<td>17449</td>
<td>20505</td>
<td>19893</td>
<td>18052</td>
</tr>
<tr>
<td>FABP7</td>
<td>NPCs</td>
<td></td>
<td>281</td>
<td>64</td>
<td>22</td>
<td>4351</td>
<td>4640</td>
<td>15172</td>
<td>31</td>
<td>17</td>
<td>30</td>
<td>25</td>
</tr>
<tr>
<td>ALB</td>
<td>hepatocytes</td>
<td></td>
<td>13</td>
<td>11</td>
<td>22065</td>
<td>11</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>9</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>VWF</td>
<td>endothelium</td>
<td></td>
<td>65</td>
<td>105</td>
<td>67</td>
<td>63</td>
<td>49</td>
<td>40</td>
<td>37</td>
<td>46</td>
<td>31</td>
<td>12953</td>
</tr>
<tr>
<td>KRT14</td>
<td>keratinocytes</td>
<td></td>
<td>13</td>
<td>11</td>
<td>13</td>
<td>12</td>
<td>12</td>
<td>11</td>
<td>11</td>
<td>16</td>
<td>11</td>
<td>12</td>
</tr>
<tr>
<td>POU5F1</td>
<td>NPCs</td>
<td></td>
<td>332</td>
<td>11792</td>
<td>229</td>
<td>508</td>
<td>133</td>
<td>123</td>
<td>188</td>
<td>144</td>
<td>169</td>
<td>146</td>
</tr>
</tbody>
</table>

| Pluripotent | Endoderm | Endoderm | Ectoderm | Ectoderm | Ectoderm | Ectoderm | Endoderm | Endoderm | Endoderm | Endoderm | Endoderm |
|-------------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|
| Pluripotent |          | Endoderm | Endoderm | Ectoderm | Ectoderm | Ectoderm | Endoderm | Endoderm | Endoderm | Endoderm | Endoderm | Endoderm |
**A**

![Image of a diagram showing cell lines and mock, VSV-G, TSF, and TSF-ΔN3 under different conditions.]  

**B**

<table>
<thead>
<tr>
<th>Cell-specific Selectivity Index</th>
<th>N/V receptor-specific Selectivity Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>U87 SI = (B/(A+B))</td>
<td>EphrinB2 SI = (VSV or N/V Env) U87 SI</td>
</tr>
<tr>
<td></td>
<td>VSV-G U87 SI</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>VSV-G</th>
<th>TSF</th>
<th>TSF-ΔN3</th>
</tr>
</thead>
<tbody>
<tr>
<td>U87 SI</td>
<td>1 ng</td>
<td>5.14</td>
<td>258.7</td>
</tr>
<tr>
<td>U87 SI</td>
<td>10 ng</td>
<td>1.93</td>
<td>362.8</td>
</tr>
<tr>
<td>EphrinB2 SI</td>
<td>1 ng</td>
<td>1.00</td>
<td>50.3</td>
</tr>
<tr>
<td>EphrinB2 SI</td>
<td>10 ng</td>
<td>1.00</td>
<td>188.0</td>
</tr>
</tbody>
</table>

**C**

![Image of bar graphs showing cell-specific selectivity index changes with different concentrations of VSV-G, TSF, and TSF-ΔN3.]
Nipah Virus Envelope-Pseudotyped Lentiviruses Efficiently Target ephrinB2-Positive Stem Cell Populations \textit{In Vitro} and Bypass the Liver Sink When Administered \textit{In Vivo}

Karina Palomares, Frederic Vigant, Ben Van Handel, Olivier Pernet, Kelechi Chikere, Patrick Hong, Sean P. Sherman, Michaela Patterson, Dong Sung An, William E. Lowry, Hanna K. A. Mikkola, Kouki Morizono, April D. Pyle, Benhur Lee

Department of Microbiology, Immunology and Molecular Genetics, Molecular Biology Institute, Department of Molecular, Cell, and Developmental Biology, School of Nursing, Department of Medicine, Division of Hematology and Oncology, Department of Pathology and Laboratory Medicine, Jonsson Comprehensive Cancer Center, Eli and Edythe Broad Center of Regenerative Medicine and Stem Cell Research, and AIDS Institute, David Geffen School of Medicine, University of California, Los Angeles, California, USA

Volume 87, no. 4, p. 2094–2108, 2013. Page 2100, column 2, paragraph 2, line 6: “(30, 44)” should read “(78, 79).”

Page 2100, column 2, paragraph 2, line 14: “(44)” should read “(79).”

Page 2108, References: Add the following references.