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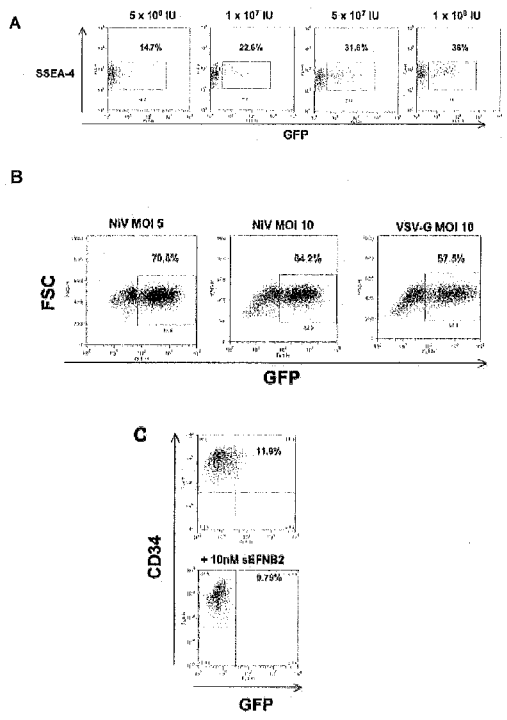
(54) Title: NIPAH VIRUS ENVELOPE PSUEDOTYPED LENTIVIRUSES AND METHODS OF THEIR USE



(57) Abstract: The present invention realtes to lentiviral particles which have been pseudotyped with Nipah virus (NiV) fusion (F) and attachment (G) glycoproteins (NiVpp-F/G). Additionally, the present invention relates to truncated NiV-F glyocproteins useful in producing such NiVpp lentiviral particles, as well as to additional variant peptides which enhance activity. Further, the present invention relates to methods of using such lentiviral particles or sequences, for example in the treatment of cancer or CNS disorders.

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NIPAH VIRUS ENVELOPE PSUEDOTYPED LENTIVIRUSES AND METHODS OF THEIR USE

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

This invention was made with Government support of Grant No. AIO69317, awarded by the National Institutes of Health, and Grant No. RB-01571, awarded by CIRM. The Government has certain rights in this invention.

CROSS-REFERENCE TO RELATED APPLICATIONS:

This application claims priority to U.S. Provisional Patent Application No. 61/615,534, filed March 26, 2012, which is incorporated by reference herein in its entirety for all purposes.

BACKGROUND OF THE INVENTION

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 of lentivirus vectors can be altered by pseudotyping with glycoproteins derived from enveloped viruses. Current gene therapy typically employs lentiviral vectors pseudotyped with the VSV-G envelope proetin (VSV-Gpp), which has a ubiquitous host cell receptor, thereby allowing transduction of most cell types. However, VSV-G itself is known to be cytotoxic and the envelope cytotoxicity limits the amount of VSV-Gpp that can be concentrated and used for cell transduction. That is, while VSV-G envelope has great stability in the vector particle, and can be concentrated to high titers via ultracentrifugation, the toxicity of VSV-G itself limits the viral titer that can be used as too high a concentration of VSV-Gpp applied to the target cell population results in apoptotic cell death. In addition, because it has a ubiquitous host cell receptor, VSV-Gpp canot be targeted to specific

populations of cells. Additionally, when VSV-Gpp is administered intravenously to mice, the majority is trapped in the liver, sometimes termed the "liver sink" effect, which is detrimental to the gene therapy unless the desired target cells reside in the liver.

To overcome these shortcomings of VSV-Gpp, other strategies have been devised for targeted lentiviral gene therapy. One common strategy involves pseudotyping lentiviral vectors with a modified Sindbis virus envelope that has been mutated to remove its own receptor binding site and engineered to dsiplay a "ZZ" motif from proteinA – a motif that binds to the Fc region of most antibodies. Incubation of the Sindbis-ZZ pseudotyped vectors with a specific monoclonal antibody theoretically should target the lentiviral particles to the cell-type in question. (*See* Morizono K *et al.*, 2005, Nat Med Vol 11(3):346-52). However, while the technique works well *in vitro*, *in vivo* the majority of the intravenously administered Sindbis-ZZ pseudoptyped vector is still trapped in the liver, regardless of the antibody used. As such, improved methods of overcoming the shortcomings of VSV-Gpp are still needed.

Nipah virus (NiV) is an emerging paramyxovirus that causes acute fatal encephalitis. Two envelope glycoproteins (the fusion and attachment glycoproteins) mediate cellular entry of Nipah virus. The attachment protein, NiV-G, functions in recognition of the receptor (EphrinB2 and EphrinB3). Binding of the receptor to NiV-G triggers a series of conformational changes that eventually lead to the triggering of NiV-F, which exposes the fusion peptide of NiV-F, allowing another series of conformational changes that lead to virus-cell membrane fusion. EphrinB2 was previously identified as the primary NiV receptor (Negrete *et al.*, 2005), as well as ephrinB3 as an alternate receptor (Negrete *et al.*, 2006). In fact, NiV-G has an

extremely high affinity for ephrinB2 and B3, with affinity binding constants (Kd) in the picomolar range (Negrete *et al.*, 2006) (Kd=0.06 nM and 0.58 nM for cell surface expressed ephrinB2 and B3, respectively). Significantly, residues important for ephrinB2/B3 interactions with their endogenous ephB receptors are also critical for their activity as NiV receptors, indicating that the NiV attachment glycoprotein (NiV-G) can block endogenous ephrinB2-ephB4 receptor interactions.

Ephrin receptor-ligand pairs (Eph-ephrin) are membrane associated receptor tyrosine kinases (RTKs) with well-established roles in development; they regulate cell boundaries during tissue formation, and provide guidance cues during neurogenesis and angiogenesis. (*See* Pasquale EB. Eph-ephrin bidirectional signaling in physiology and disease. *Cell.* 2008;133:38-52.) Cognate interactions activate both the Eph receptor (forward signaling) and ephrin ligand (reverse signaling) on opposing cells. These bi-directional signaling cascades result in cell-cell repulsion or attraction, depending on cell type or other microenvironmental cues.

EphrinB-ephB receptor-ligand interactions are a common regulator of multiple somatic stem cells, *e.g.*, intestinal crypt stem cells and hematopoietic stem cells (Pasquale (2008) Cell 133:38-52; Poliakov et al. (2004) Dev. Cell. 7:465-480), where differentiation is a carefully choreographed molecular and cellular response to local environmental determinants. EphrinB2, in particular, has been identified as a molecular stem cell signature common to human embryonic, neural, and hematopoietic stem cells (hESC, hNSC and hHSC) (Ivanova et al. (2002) Science 298:601-604). Its cognate receptor, EphB4, has also been shown to affect mouse ESC fate. Despite much evidence from model systems that ephrinB2/ephB4 axis may be intimately involved in ESC fate (survival, self-renewal, and pluripotency), this particular axis has not been carefully studied in human ESC.

In mouse ESC, ephB4 inactivation results in bias against differentiation: ephB4-deficient mouse ESCs appear to remain in a more primitive state and are impaired in embryoid body (EB) formation in general and mesodermal differentiation in particular. (Wang et al. (2004) Blood 103:100-109)). Conversely, over expression of ephB4 in umbilical cord blood CD34+ cells results in a loss of the most primitive progenitors (LTC-ICs and CD34+/CD38- cells) likely due to differentiation into more committed precursors. (Wang et al. (2002) Blood 99:2740-2747)). EphrinB-ephB ligand-receptor interactions are promiscuous, and the lack of highly specific yet versatile reagents to interrogate this axis has hampered the understanding of ephrinB2/ephB4's role in hESC fate (pluirpotency, survival and self-renewal) and HSC lineage commitment. Understanding the regulation of this signaling axis could improve the culture of hESCs and the efficiency of HSC lineage differentiation, both previously key barriers in the field.

EphB4 and ephrinB2 are both expressed in ESC and likely contribute to some aspect of stem cell fate. However, while ephrinB2 is clearly also involved in ectoderm and endoderm differentiation, ephB4 is unique amongst ephB receptors for not being expressed in the central nervous system. Thus, ephrinB2 "reverse" signaling and ephB4 "forward" signaling likely play overlapping but distinct roles in germ layer commitment and differentiation. Understanding the relative contribution of each signaling pathway may result in more optimal conditions for directing the differentiation of specific cell types.

Finally, ephrinB-ephB usually follows a gradient of ligand-receptor interactions, and expression of ephrinB2 is indeed heterogeneous within an ESC colony. Understanding the basis for the heterogeneity seen in human ES cell

cultures will lead to more robust culture conditions that give rise to more homogenous population of cells suitable for regenerative medicine.

Eph-ephrin RTK expression is dysregulated in multiple cancers, and various members of this RTK family have been implicated in cancer development, progression, and subsequent metastases (*See* Pasquale EB. Eph receptors and ephrins in cancer: bidirectional signaling and beyond. *Nat Rev Cancer*. 2010;10:165-180).

Deciphering the role of Eph signaling activities in cancer is confounded by the promiscuity of interactions between Eph-ephrin receptor-ligand pairs, and the complexity of the resultant signaling cascades. Nevertheless, the centrality of ephrinB2 in facilitating tumor angiogenesis and promoting invasion and metastasis is supported by a slew of studies that provide a sound mechanistic basis for its action (*See* Pasquale EB. Eph receptors and ephrins in cancer: bidirectional signaling and beyond. *Nat Rev Cancer*. 2010;10:165-180). As such, soluble EphB4 inhibits tumor growth in multiple xenograft models (*see* Kertesz N, Krasnoperov V, Reddy R, *et al.* The soluble extracellular domain of EphB4(sEphB4) antagonizes EphB4-EphrinB2 interaction, modulates angiogenesis, and inhibits tumor growth. *Blood*. 2006;107:2330-2338; Kumar SR, Scehnet JS, Ley EJ, et al. Preferential induction of EphB4 over EphB2 and its implication in colorectal cancer progression. *Cancer Res.* 2009;69:3736-3745; Spannuth WA, Mangala LS, Stone RL, et al. Converging evidence for efficacy from parallel EphB4-targeted approaches in ovarian carcinoma. *Mol Cancer Ther*. 2010;9:2377-2388), while molecular genetic evidence implicates ephrinB2 reverse signaling in the activation of VEGFR2 that leads to vessel sprouting (*See* Branco-Price C, Johnson RS. Tumor vessels are Eph-ing complicated. *Cancer Cell*. 2010;17:533-534; Sawamiphak S, Seidel S, Essmann CL,

et al. Ephrin-B2 regulates VEGFR2 function in developmental and tumor angiogenesis. *Nature.* 2010;465:487-491). The latter point suggests the exciting possibility that blocking ephrinB2 signaling may synergize with anti-VEGF therapies. Furthermore, amongst all the ephrins examined, only ephrinB2 on stromal cells (fibroblast, endothelial cells, or pericytes) activates ephB3/ephB4 on invasive prostate cancer cells leading to loss of contact inhibition of locomotion (CIL), the tumor invasive phenotype responsible for cancer metastases (*See* Astin JW, Batson J, Kadir S, *et al.* Competition amongst Eph receptors regulates contact inhibition of locomotion and invasiveness in prostate cancer cells. *Nat Cell Biol.* 2010;12:1194-1204; Wang B. Cancer cells exploit the eph-ephrin system to promote invasion and metastasis: tales of unwitting partners. *Sci Signal.* 2011;4:pe28).

Use of Nipah virus in conjunction with a lentivirus vector has heretofor been hampered by the fact that paramyxoviral envelopes are known not to pseudotype functionally onto lentiviral particles, presumbly due to some incompatibility of the cytoplasmic tail of the fusion and attachment glycoproteins with the matrix (gag) protein of HIV.

There remains a need for improved gene therapy compositions and methods that allow for enhanced delivery of the gene product to the target cells or tissues.

BRIEF SUMMARY OF THE INVENTION

The present inventors have successfully pseudotyped NiV glycoproteins onto lentiviral particles (NiVpp) by using appropriate cytoplasmic tail truncations. The inventors found that efficient functional pseudotyping requires only truncation of the F protein cytoplasmic tail, while full-length NiV-G can be used. Additional variations can also be introduced into the NiV-F or NiV-G peptide sequence to impact the

properties of the resulting NiVpp lentivirus, e.g., increasing or decreasing infectivity of the NiVpp lentivirus. Codon-optimization of the NiV-F and G genes also allows for high-level expression of F and G, which enables efficient pseudotyping of NiV-F/G onto lentiviral particles (NiVpp).

NiVpp can be specifically targeted to various ephrinB2 expressing primary cells. The normal biology of ephrinB2, which undergoes rapid endocytosis upon interactions with its cognate receptor (*e.g.*, EphB4, another membrane assocaited receptor-tyrosine kinase), can also be exploited. Thus, NiVpp targeted to endothelial cells may also be transcytosed across the blood-brain barrier to deliver gene-therapeutic payloads globally across the CNS. This could be useful, for example, in the treatment of Hungtington's disease, which requires global correction of the gene at issue.

Additionally, the ephrinB2-ephB4 axis is dysregulated in many cancers. In some breast cancers, tumor angiogenic vessels that supply the breast cancer stroma over express ephrinB2, while in other cancers (*e.g.*, prostate), over expression of ephrinB2 has been implicated in the loss of contact inhibition of locomotion and thus may be responsible for metastasis. As such, NiVpp could be used to target cancer cells or angiogenic vessels, for example, to treat or otherwise impact various tunors or cancers.

Finally, EphrinB2 has been implicated as a molecular signature of stemness (Ivanova, NB *et al.*, 2002, Science, 298, 601), and the inventors have confirmed that NiVpp can specifically target subpopulations of human embryonic stem cells (SSEA4+), human neuroprogenitor stem cells (nestin+), and human hematopoietic stem cells (CD34+).

Thus, NiVpp pseudotyped lentivirus has many potential uses, including but not limited to: (1) To deliver any gene to neurons or endothelial cells, which over-express ephrinB2; (2) To deliver any gene to ephrinB2+ embryonic, neural, and hematopoietic stem cell populations; (3) To target tumors over-expressing ephrinB2; (4) To target ephrinB2+ cell populations *in vivo* or *in vitro*, *e.g.*, for better transduction of neural stem cells for eventual transplantation; (5) To deliver therapeutic genes across the blood-brain barrier to the CNS.

Given all of this, in one embodiment, the invention is directed to a Nipah virus envelope pseudotyped lentivirus. In another embodiment, the invention is directed to Nipah virus (NiV) glycoproteins pseudotyped onto lentiviral particles (NiVpp).

In another embodiment, the invention is directed to a method for specifically targeting or delivering a gene or peptide product to ephrinB2+ cells or cell populations using the foregoing pseudotyped lentivirus. Such methods may be used, for example, to treat cancer or to combat angiogenic vessels. In certain examples, the ephrinB2+ cells comprise embryonic, neural, or hematopoietic stem cells.

In another embodiment, the invention is directed to a method for delivering or transporting a gene or peptide product across the blood-brain barrier using the foregoing pseudotyped lentivirus.

In another embodiment, the invention is directed to a method for altering brain function in a subject comprising injection of NiVpp into specific areas of said subject's brain.

In another embodiment, the invention is directed to a method for delivering any gene or peptide product to neurons or endothelial cells which overexpress ephrinB2 using the foregoing pseudotyped lentivirus.

In another embodiment, the invention is directed to a method for targeting tumors using the foregoing pseudotyped lentivirus.

BRIEF DESCRIPTION OF THE FIGURES

This application file contains at least one drawing executed in color. Copies of this application with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

Figure 1 shows relevant portions of the NiV-F and NiV-G glycoproteins and the mutations made thereto.

Figure 2 shows the titer obtained from NiVpp pseudotyped lentivirus produced using various NiV-F and NiV-G truncated glycoproteins.

Figure 3 shows the relative infection of various cell types by various forms of NiVpp pseudotyped lentivirus, in some cases in the presence of soluble ephrinB2. In some panels, infectivity of VSV-G is also shown.

Figure 4 shows the ability of NiVpp pseudotyped lentivirus to infect various cell types at various MOIs., in some cases in the presence of soluble ephrinB2.

Figure 5 shows the selectivity index of various pseudotypes of lentivirus for ephrinB2+ cells, when those cells are co-cultured with ephrinB2- cells at different ratios (1:1, 1:10, 1:100, and 1:1000).

Figures 6 and 7 show the localization of various lentivirus pseudotypes when they are injected into the animal for an *in vivo* examination of infectivity.

DETAILED DESCRIPTION

Before describing the present invention in detail, it is to be understood that this invention is not limited to particular embodiments, which can, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting. Unless defined otherwise, all technical and scientific terms used herein generally have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Generally, the nomenclature used herein and the laboratory procedures in cell culture, molecular genetics, organic chemistry, and nucleic acid chemistry and hybridization are those well-known and commonly employed in the art. Standard techniques are used for nucleic acid and polypeptide synthesis. Procedures used for genetic engineering are well known and can be found, for example, in Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, N.Y.).

As used in this specification and the appended claims, terms in the singular and the singular forms "a," "an," and "the," for example, include plural referents unless the content clearly dictates otherwise. Thus, for example, reference to "polypeptide," "the polypeptide" or "a polypeptide" also includes a plurality of polypeptides. Additionally, as used herein, the term "comprises" is intended to indicate a non-exhaustive list of components or steps, thus indicating that the given composition or method includes the listed components or steps and may also include additional components or steps not specifically listed. As an example, a composition "comprising a polypeptide" may also include additional components or polypeptides. The term "comprising" is also intended to encompass embodiments "consisting essentially of" and "consisting of" the listed components or steps. Similarly, the term

"consisting essentially of" is also intended to encompass embodiments "consisting of" the listed components or steps.

Numeric ranges recited within the specification are inclusive of the numbers defining the range (the end point numbers) and also are intended to include each integer or any non-integer fraction within the defined range.

In describing and claiming the present invention, the following terminology will be used in accordance with the definitions set out below.

The terms "polypeptide," "peptide," and "protein" are generally used interchangeably herein and they refer to a polymer in which the monomers are amino acids that are joined together through amide bonds. Additionally, unnatural amino acids, for example, β-alanine, phenylglycine, and homoarginine are also included. Amino acids that are not gene-encoded can also be used with the technology disclosed herein. Furthermore, amino acids that have been modified to include reactive groups, glycosylation sites, polymers, therapeutic moieties, biomolecules, and the like can also be used. All of the amino acids used herein can be either the D- or L- isomer. The L-isomer is generally preferred. As used herein, "polypeptide," "peptide," and "protein" refer to both glycosylated and unglycosylated forms.

The term "amino acid" refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, e.g., hydroxyproline, γ-carboxyglutamate, and O-phosphoserine. "Amino acid analogs" refers to compounds that have the same basic chemical structure as a naturally occurring amino acid, i.e., an α carbon that is bound to a hydrogen, a

carboxyl group, an amino group, and an R group, e.g. homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs have modified R groups (e.g., norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. "Amino acid mimetics" refers to chemical compounds that have a structure that is different from the general chemical structure of an amino acid, but that function in a manner similar to a naturally occurring amino acid.

As used herein, "NiVpp," "NiVpp lentivirus," "NiVpp pseudotyped lentivirus," NiV pseudotyped lentivirus," or the like refers to a lentivirus particle which has been pseudotyped using Nipah virus envelope glycoproteins NiV-F and NiV-G. The NiV-F glycoprotein on such NiVpp lentivirus particles is a variant form which has been modified such that it possesses a cytoplasmic tail truncation. In certain examples, the truncation will be a deletion of amino acid residues 525-544 of the NiV-F peptide, which will be referred to herein as the "T5F" or "T234F" form of the NiV-F glycoprotein (see **FIG. 1**). In other examples, the NiV-F glycoprotein will further include a mutation to an N-linked glycosylation site, more specifically a substitution of glutamine (Q) for asparagine (N) at amino acid position 99 of the NiV-F peptide, which will be referred to herein as the "DeltaN3" or "ΔN3" form of the NiV-F glycoprotein. The NiV-G glycoprotein can be either a wild-type form or a modified or variant form of the protein, such as a truncated NiV-G. Deletions of 5, 10, 15, 20, 25, and 30 amino acids at or near the N-terminus of the NiV-G peptide were constructed, which are referred to herein as "Δ5G," "Δ10G," "Δ15G," "Δ20G," "Δ25G," and "Δ30G," respectively. A partial amino acid sequence of the NiV-F and NiV-G peptides showing each one of these variations is shown in **FIG. 1**.

The present inventors engineered the Nipah virus envelope glycoproteins to be efficiently pseudotyped onto lentiviruses, and such NiV pseudotyped lentiviruses can efficiently target ephrinB2 expressing cells *in vitro* and *in vivo*. In certain examples, the NiVpp can be used to target a subpopulation of ephrinB2+/SSEA-4+ human embryonic stem cells (hESC). In other examples, NiVpp can be used to deliver agents that antagonize EphB-ephrinB2 mediated signaling specifically to ephrinB2-expressing target cells.

Further, NiVpp is the first demonstration of any lentiviral vector administered intravenously that can bypass the liver sink, which allows for targeting of specific ephrinB2+ populations *in vivo*. In addition, the natural tropism of NiVpp can be altered by mutating the natural receptor binding site to make it more ephrinB2 or B3 specific, depending on the clinical context of its use. NiVpp opens up the possibility for therapeutic targeting of ephrinB2-overexpressing cells common in various solid cancers or their tumor angiogenic vessels (*see* E.Pasquale, 2011).

EphrinB2 and its endogenous receptor, EphB4, are both receptor tyrosine kinases that undergo bi-directional signaling as well as bidirectional endocytosis upon interaction with each other. NiVpp can take advantage of this biological property for transcytosis across the blood brain barrier. This is a critical barrier in CNS targeted gene therapy (by systemic administration). NiVpp can transcytose across functional microvascular endothelial cell layers to infect target cells at the bottom of the transwell chamber. Further, considering that NiVpp can transduce Nestin+ neural stem cells even more efficiently than VSV-Gpp, direct stereotatic injection of NiVpp into specific CNS areas where neurogenesis (proliferation of neurons from stem cell progenitors) is known to occur in the adult brain, such as the hippocampus and the subventricular zone, is possible.

The efficiency of NiVpp transduction can be improved by engineering hyperfusogenic mutations in one or both of NiV-F and NiV-G. Several such mutations have been previously described (see, e.g., Lee at al, 2011, Trends in Microbiology). This could be useul, for example, for maintaining the specificity and picomolar affinity of NiV-G for ephrinB2 and/or B3 while independently enhancing the entry efficiency of NiVpp. Additionally, mutations in NiV-G that completely abrogate ephrinB2 and B3 binding, but that do not impact the association of this NiV-G with NiV-F, have been identified. This could allow for specific targeting of other desired cell types that are not ephrinB2+ through the addition of a single chain variable fragment (scFV) directed against a different cell surface molecule

The inventors have generated several mutants of the NiV fusion protein (NiV-F), and have also generated stepwise truncations in the cytoplasmic tail of the attachment protein (NiV-G), and screened each in combination with the NiV-F variant(s) for the ability to pseudotype lentivirus. Infectivity has been examined using a variety of cell types, including 293T and CHO-B2 cells, both of which express the NiV primary receptor, ephrinB2. While many of the G-truncations were expressed and could be pseudotyped onto lentiviruses, the highest increase in viral transduction titers (~100-fold) was obtained with the NiV-F variant and wild-type NiV-G, indicating that only truncations in the cytoplasmic tail of NiV-F are critical for efficient pseudotyping. Infection was blocked using soluble ephrinB2, confirming specificity of NiV pseudotyped lentivirus for ephrinB2+ cells. Moreover, NiV pseudotyped lentiviruses can suitably transduce primary human neurons and microvascular endothelial cells. Thus, lentivirus pseudotyped with NiV envelope may be used for targeted gene therapy in situations where ephrinB2/B3 is upregulated in the diseased tissue, thereby overcoming limitations of current gene therapy.

The NiVpp psuedotyped lentivirus vectors disclosed herein could be used to deliver any desired nucleic acid encoding for any desired peptide to any cell that expresses an appropriate receptor for NiV. In certain examples, these nucleic acid "payloads" will be delivered to cells expressing ephrin, for example ephrinB2 or ephrin B3. In other examples, the payload may be a nucleic acid encoding for a peptide product that is absent from the gene, such as is commonly done in gene therapy. This could be useful, for example, for targeting a genetic payload to neural stem cells. In other examples, the payload may be a nucleic acid or peptide that is toxic to the cell, for example to combat cancer cells. In other examples, the payload may be an ephrin antagonist, such as a soluble ephrinB2 or a nucleic acid capable of silencing or downregulating ephrinB2, such as an siRNA. Delivery of such ephrinB2 antagonists may be useful, for example, for impacting cell pluripotency or development, or for decreasing metastasis of certain cancer cells.

The following examples are offered to illustrate, but not to limit, the claimed embodiments. It is to be understood that the examples and embodiments described herein are for illustrative purposes only, and persons skilled in the art will recognize various parameters that can be altered without departing from the spirit of the disclosure or the scope of the appended claims.

EXAMPLES

EXAMPLE 1 – Generation of truncated glycoproteins and NiVpp pseudotyped lentivirus

Previous studies have shown that pseudotyping of lentiviral vectors with unmodified paramyxoviral glycoproteins is highly inefficient. In the present study, we obtained chemically-synthesized, codon-optimized wild-type NiV-F and NiV-G

nucleotides. These codon-optimized NiV-F and NiV-G sequences included a tag at the 3' end encoding an AU1 peptide tag (DTYRYI) or a hemaglutinin peptide tag (YPYDVPDYA), respectively. These were subcloned into pcDNA3.1 vectors for mutagenesis. Variants of NiV-F and NiV-G were produced using a QuickChange site directed mutagenesis kit (Stratagene, Cedar Creek, TX) with primers designed to correspond to the desired deletions. A NiV-F variant, termed T5F or T234F, with a truncation of the cytoplasmic tail, as discussed above, was produced (see, e.g., Aguilar et al. (2007) J. Virol. 81:4520-4532). NiV-G variants were produced by making stepwise truncations of the cytoplasmic tail of NiV-G. **FIG. 1** shows the variant forms of NiV-F and NiV-G that were produced.

NiVpp lentiviral vectors were created using various combinations of these variant NiV-F and NiV-G glycoproteins. All lentiviral vectors were produced by calcium phosphate-mediated transient transfection of 293T cells. One day prior to transfection, 1.6 x 10^7 293T cells were seeded in a T175 flask. 7μg of NiV-F (wild-type of variant), 7μg of NiV-G (wild-type or variant), 12.5μg of the packaging plasmid pCMVΔR8.9, and 12.5μg of the lentiviral transfer vector plasmid FG12-GFP or FUhLucW were transfected into cells. After 8h, the transfection medium was removed and fresh medium was added. 48h post-transfection, the viral supernatant was harvested and concentrated by centrifugation at 28,000rpm at 4°C for 2h over a 20% sucrose cushion. To determine viral titer, serial dilutions of concentrated viral stocks were added to 293T cells and incubated at 37°C for 2h. 3 days post-infection, the cells were analyzed by flow cytometry for eGFP expression. Titers are expressed as infectious units per mL (IU/mL).

Truncation of the NiV-F cytoplasmic tail alone resulted in a titer of ~10^6 IU/mL on 293T cells, a 100-fold increase in titer compared to wtF/wtG pseudotypes (**FIG. 2**).

With regard to the NiV-G variants, although the T234F/Δ10G and T234F/ Δ25G variants demonstrated similar titers to T234F/wtG, none of the NiV-G variants produced greater titers than T234F/wtG (**FIG. 2**). Moreover, combinations of wt F with the NiV-G truncation variants produced extremely low titers (data not shown), indicating that truncations in NiV-F are critical for efficient pseudotyping. Following concentration, titers of ~10^8-10^9 were obtained, compared to 10^{10} for VSV-G (data not shown). These high titer NiV pseudotyped lentiviruses can be used for efficient infection of ephrinB2+ cells, including for infection of hESCs, to deliver marker genes to tag ephrinB2+ hESCs, or to deliver siRNAs or other genes to antagonize the ephrinB2-ephB4 axis on hESCs.

EXAMPLE 2 – *In vitro* and *in vivo* infection using NiVpp pseudotyped lentivirus

Increasing amounts of virus (based on MOI or p24 equivalent) 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 in some studies. To exclude pseudotransduction, 5μM of nevirapine (NVP; a reverse transcriptase inhibitor) was added in some studies. 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.

CHO, CHO-B2, and CHO-B3 cells were infected with 0.01 ng, 0.1 ng, and 1 ng (p24 equivalents) of NiVpp or VSV-Gpp lentiviral pseudotypes carrying the GFP reporter gene (**FIG. 3**, panels A-D). 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. U87 cells and HMVECs were infected with T5F/wt G and T5FΔN3/wt G pseudotypes as described for panels A-C but normalized to VSV-Gpp infection of the same cell line (U87 or HMVECs) at maximal viral input (1 ng) (**FIG. 3**, panels E & F). 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 **FIG. 3** 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™. *: $p < 0.05$, **: $p < 0.01$, ****: $p < 0.0001$. As this figure demonstrates, NiVpp pseudotyped lentiviurus is able to effectively infect all cell types tested in an ephrinB2 dependent manner. Moreover, T5FΔN3 showed an improved infectivity versus T5F.

EphrinB2 is a functional marker of human embryonic, neural, and hematopoietic stem cells (hESC, hNSC and hHSC). To confirm that ephrinB2 is functionally expressed on hESC, hNSC and hHSC, and to confirm that T234F/wtG pseudotype can mediate transfer into these ephrinB2+ cells, we transduced human ESCs, HSCs, and NSCs with NiVpp pseudotyped lentiviruses carrying a marker gene for EGFP. **FIG. 4** shows that NiV pseudotypes infected SSEA-4+ hESC (H1 line) (panel A), hNSC (Nestin+) (panel B), and a subpopulation of purified CD34+ cells from human fetal liver (panel C). More specifically, in panel A of **FIG. 4**, increasing amounts of NiVpp 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. $1x10^8$ IU of NiVpp produced an infection rate of approximately 36% of SSEA-4+ hESC (**FIG. 4**, panel A). For panel B of **FIG. 4**, progenitor cells derived from the medial temporal lobe of a 17-week human fetus were infected with NiVpp. 72h post-transduction, cells were stained for nestin and GFP expression was quantified by FACS analysis. The results of this analysis suggest that the NiV pseudotypes may infect NSC more efficiently than VSV-G pseudotypes. In panel C of **FIG. 4**, purified $CD34^+$ cells from human fetal liver were infected with NiVpp. 72h post-transduction, cells were stained for the cell-surface marker CD34 and analyzed for GFP expression by FACS analysis. At a multiplicity of infection (MOI) of 10, T234F/wtG pseudotypes specifically transduced ~12% of purified CD34+ cells from human fetal liver. Moreover, this infection was inhibited with soluble ephrinB2 (10nM) in all cases (data only shown for fetal liver CD34+ cells, which shows a reduction from ~12% to <1% infection in the presence of soluble ephrinB2).

In the ehprinB2+/B2- ratio study, 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. 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. Data from 300,000 cells were acquired for every condition used for analysis. 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 {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 admixture 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. 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 analysed. The values of the U87 SI and EphrinB2 SI for VSV-G, T5F, and T5FΔN3 pseudotypes are provided in Table 1:

Table 1

Specificity Index	Infection rate	VSV-G	T5F	T5FΔN3
U87 SI	1 ng	5.14	258.7	292.5
U87 SI	10 ng	1.93	362.8	342.9
EphrinB2 SI	1 ng	1.00	50.3	56.9
Ephrin B2 SI	10 ng	1.00	188.0	177.7

The EphrinB2 Selectivity Index calculated for VSV-Gpp, and NiVpp bearing T5F or T5F-ΔN3 for all the indicated conditions is shown in **FIG. 5**. Data shown are averages ± standard deviations for triplicates done at 1 ng, and average ± range for duplicates done at 10 ng. As these results demonstrate, the NiVpp pseudotyped lentivirus vectors have a greatly increased specificity for EphrinB2 bearing cells as compared to VSV-Gpp pseudotyped lentivirus.

For *in vivo* analysis, the FvcFlw (firefly luciferase) vector was pseudotyped with VSV-G and two variant NiV pseudotypes, T234F and T234FΔN3 , as discussed above, as discussed above. 10ng of p24 equivalents of each pseudotyped lentivirus was injected into C57/BL6 mice through the tail vein. At 5 days post-injection, luciferase expression was imaged. Following whole-body imaging (see **FIG. 6**), each organ was isolated to image luciferase expression (see **FIG. 7**). As the images demonstrate, the NiVpp pseudotyped lentivirus is able to avoid the liver sink and to effectively infect cells and deliver genetic payloads in other tissues.

In addition to the other publications cited throughout this application, the following references are incorporated herein in their entireties for all purposes:

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WHAT IS CLAIMED IS:

1. A Nipah virus envelope pseudotyped lentivirus.
2. Nipah virus (NiV) glycoproteins pseudotyped onto lentiviral particles (NiVpp).
3. A method for specifically targeting ephrinB2 expressing primary cells using the pseudotyped lentivirus of Claim 1 or Claim 2.
4. The method according to Claim 3 used for treating cancer.
5. The method according to Claim 3 for use against agiogenic vessels.
6. A method for crossing the blood brain barrier using the pseudotyped lentivirus of Claim 1 or Claim 2.
7. A method for altering brain function in a subject comprising injection of NiVpp into specific areas of said subject's brain.
8. A method for delivering any gene to neurons or endothelial cells which overexpress ephrinB2 comprising using the pseudotyped lentivirus of Claim 1 or Claim 2.
9. A method for delivering any gene to enphrinB2+ embryonic, neural, and/or hematopoietic cell populations comprising using the pseudotyped lentivirus of Claim 1 or Claim 2.
10. A method for targeting tumors comprising using the pseudotyped lentivirus of Claim 1 or Claim 2.
11. A method for delivering one or more therapeutic genes across the blood-brain barrier comprising using the pseudotyped lentivirus of Claim 1 or Claim 2.

```
NiV-F (SEQ ID NO: 1 or 2):        EKKRNTYSRLEDRRVRPTSSGDLYYIGTDTYRYI
NiV-F T234 (SEQ ID NO: 4 or 6):   EKKRNT..................GTDTYRYI

NiV-G (SEQ ID NO: 10 OR 12):   MGPAENKKVRFENTTSDKGKIPSKVIKSYYGTMDIKKINEGLLDSK
NiV-G Stop:                    S............................MDIKKINEGLLDSK
NiV-G Δ5 (SEQ ID NO: 13):      MG.....KVRFENTTSDKGKIPSKVIKSYYGTMDIKKINEGLLDSK
NiV-G Δ10 (SEQ ID NO: 15):     MG.........NTTSDKGKIPSKVIKSYYGTMDIKKINEGLLDSK
NiV-G Δ15 (SEQ ID NO: 17):     MG.............KGKIPSKVIKSYYGTMDIKKINEGLLDSK
NiV-G Δ20 (SEQ ID NO: 19):     MG.................SKVIKSYYGTMDIKKINEGLLDSK
NiV-G Δ25 (SEQ ID NO: 21):     MG.....................SYYGTMDIKKINEGLLDSK
NiV-G Δ30 (SEQ ID NO: 23):     ............................GTMDIKKINEGLLDSK
```

FIG. 1

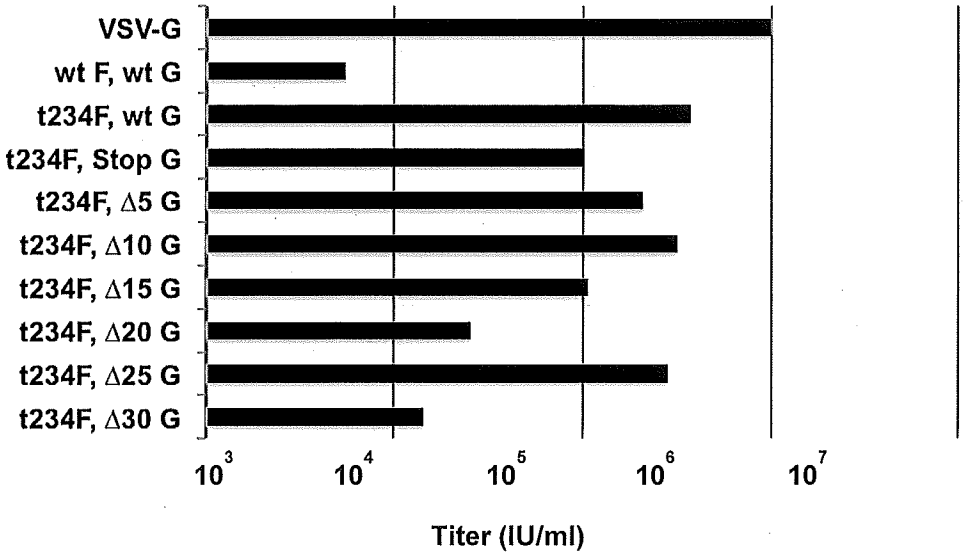
VSV-G
wt F, wt G
t234F, wt G
t234F, Stop G
t234F, Δ5 G
t234F, Δ10 G
t234F, Δ15 G
t234F, Δ20 G
t234F, Δ25 G
t234F, Δ30 G
10^3
10^4
10^5
10^6
10^7
Titer (IU/ml)

FIG. 2

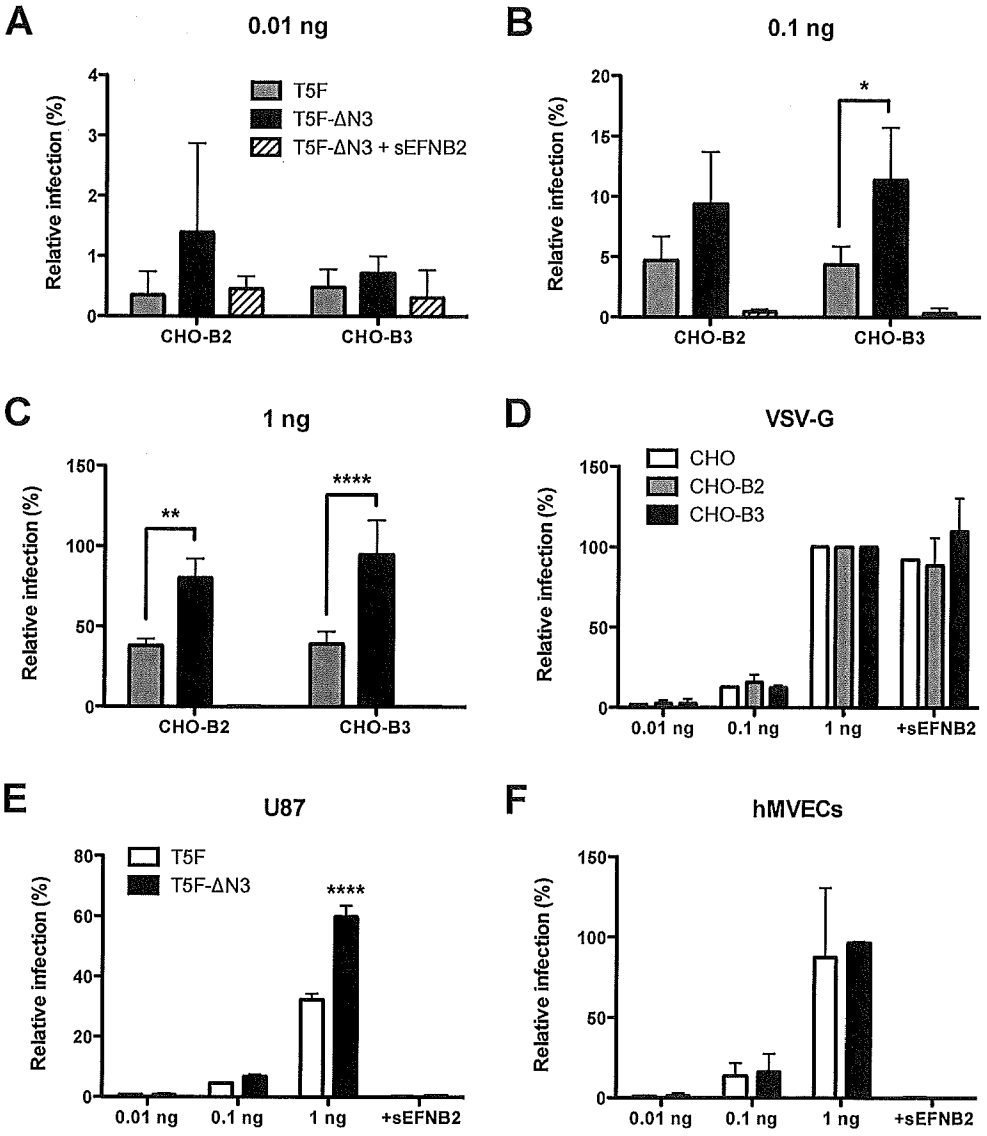
A
0.01 ng
T5F
T5F-ΔN3
T5F-ΔN3 + sEFNB2
Relative infection (%)
CHO-B2
CHO-B3
B
0.1 ng
*
C
1 ng
**
****
D
VSV-G
CHO
CHO-B2
CHO-B3
0.01 ng
0.1 ng
1 ng
+sEFNB2
E
U87
F
hMVECs

FIG. 3

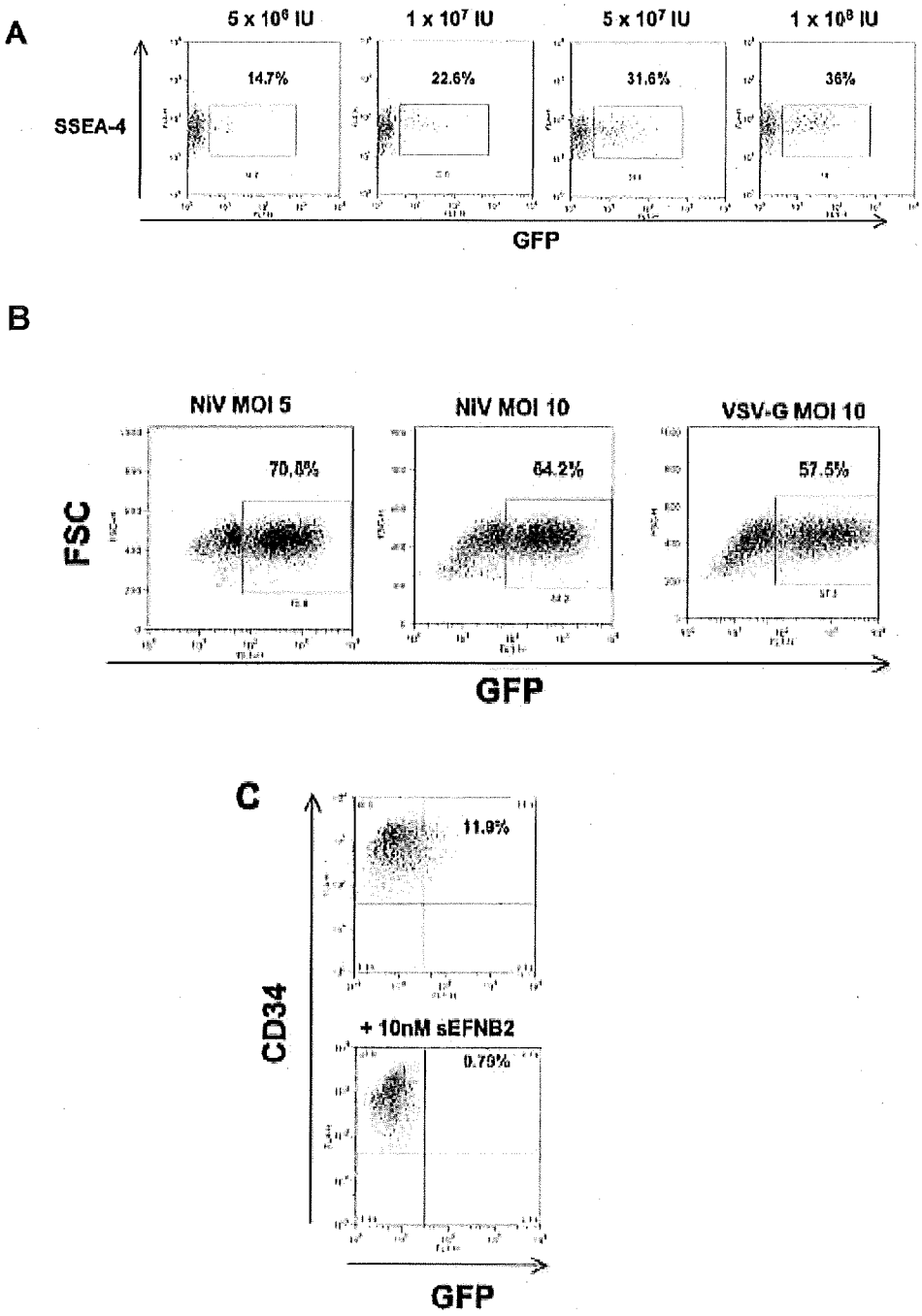
A
5 x 10^6 IU
1 x 10^7 IU
5 x 10^7 IU
1 x 10^8 IU
14.7%
22.6%
31.6%
36%
SSEA-4
GFP
B
NiV MOI 5
NiV MOI 10
VSV-G MOI 10
70.8%
64.2%
57.5%
FSC
GFP
C
11.9%
+ 10nM sEFNB2
0.79%
CD34
GFP

FIG. 4

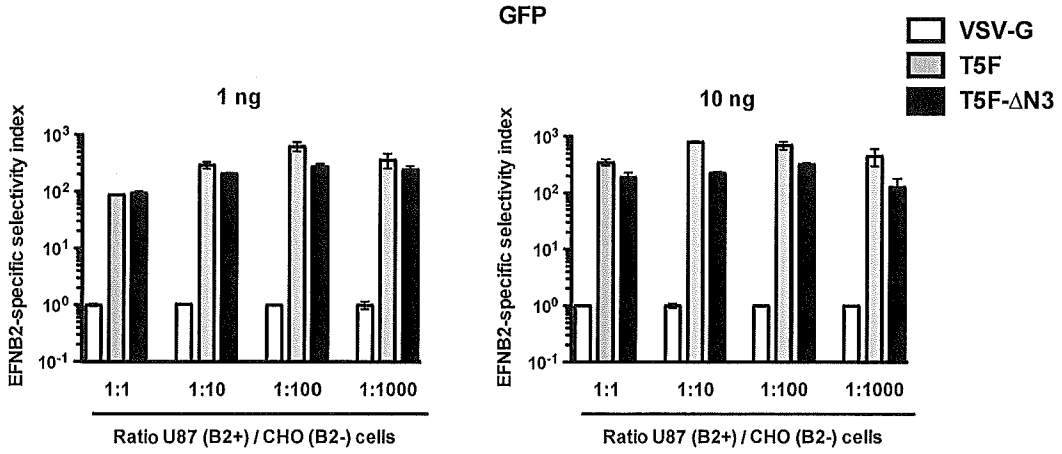
GFP
VSV-G
T5F
T5F-ΔN3
1 ng
10 ng
EFNB2-specific selectivity index
10^3
10^2
10^1
10^0
10^{-1}
1:1
1:10
1:100
1:1000
Ratio U87 (B2+) / CHO (B2-) cells

FIG. 5

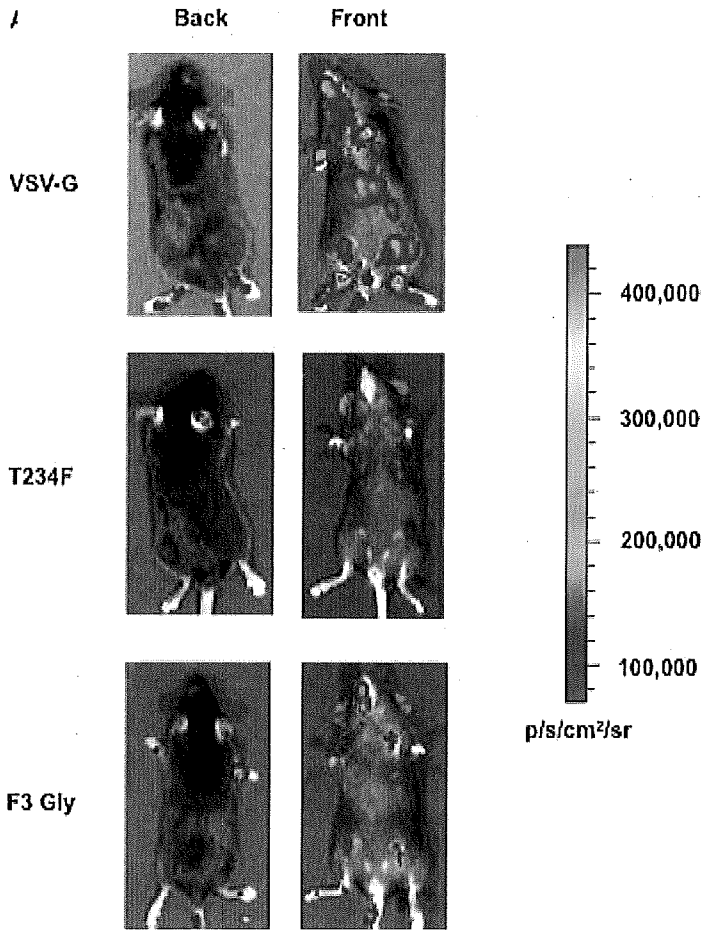
Back
Front
VSV-G
T234F
F3 Gly
400,000
300,000
200,000
100,000
p/s/cm²/sr

FIG. 6

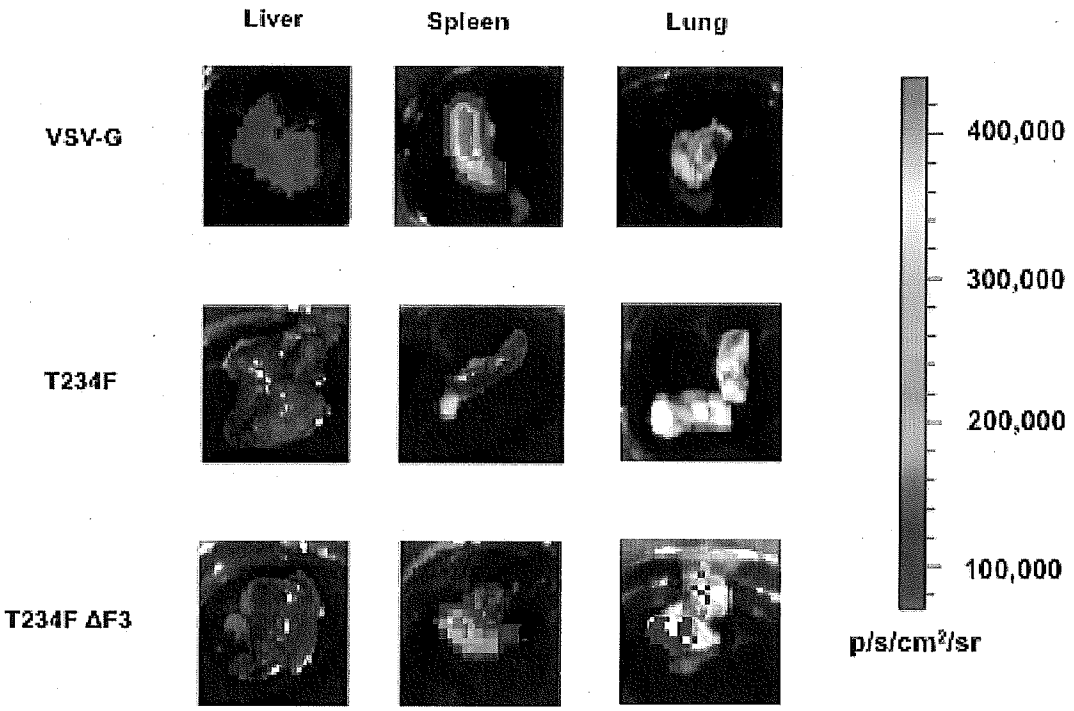
Liver
Spleen
Lung
VSV-G
T234F
T234F ΔF3
400,000
300,000
200,000
100,000
p/s/cm²/sr

FIG. 7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/032197

A. CLASSIFICATION OF SUBJECT MATTER

C12N 7/01(2006.01)i, A61K 39/12(2006.01)i, A61P 35/00(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N 7/01; A61K 39/285; A61K 39/275; A61K 39/12; A61P 35/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & Keywords:Nipah virus, glycoproteins, envelope, pseudotyped lentivirus, ephrinB2, tumor

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
PX	PALOMARES, K. et al., `Nipah virus envelope-pseudotyped lentiviruses efficiently target ephrinB2-positive stem cell populations in vitro and bypass the liver sink when administered in vivo`, Journal of Virology, 28 November 2012, Vol. 87, No. 4, pp. 2094-2108. See the whole document.	1,2
X	KHETAWAT, D. et al., `A functional henipavirus envelope glycoprotein pseudotyped lentivirus assay system`, Virology Journal, 12 November 2010, Vol. 7, No. 312, pp. 1-14. See abstract; page 2, right column, lines 41-50; figures 1-3 and 7.	1,2
A	NEGRETE, O. A. et al., `EphrinB2 is the entry receptor for Nipah virus, an emergent deadly paramyxovirus`, Nature, 6 July 2005, Vol. 436, No. 7049, pp. 401-405. See abstract; figures 4-5.	1,2
A	ZHANG, XIAN-YANG et al., `Cell-specific targeting of lentiviral vectors mediated by fusion proteins derived from Sindbis virus, vesicular stomatitis virus, or avian sarcoma/leukosis virus`, Retrovirology, 25 January 2010, Vol. 7, No. 3, pp. 1-15. See abstract; figures 1-6.	1,2

☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 June 2013 (26.06.2013)	**28 June 2013 (28.06.2013)**

Name and mailing address of the ISA/KR
Korean Intellectual Property Office
189 Cheongsa-ro, Seo-gu, Daejeon Metropolitan City, 302-701, Republic of Korea
Facsimile No. 82-42-472-7140

Authorized officer
HEO, Joo Hyung
Telephone No. 82-42-481-8150

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/032197

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2007-0031455 A1 (AUDONNET, JEAN C. F.) 8 February 2007 See abstract; claims 1-4 and 14-15.	1,2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/032197

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 3-11
because they relate to subject matter not required to be searched by this Authority, namely:
Claims 3-11 pertain to methods for treatment of the human body by therapy, and thus relate to a subject matter which this International Searching Authority is not required, under Article 17(2)(a)(i) of the PCT and Rule 39.1(iv) of the Regulations under the PCT, to search.

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required addtional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/US2013/032197

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2007-0031455 A1	08.02.2007	AT 461710 T	15.04.2010
		CA 2605629 A1	02.11.2006
		CA 2605629 C	26.06.2012
		CN 101208104 A0	25.06.2008
		CN 101208104 B	31.10.2012
		DE 602006013117 D1	06.05.2010
		DK 1881845 T3	07.06.2010
		EP 1881845 A2	30.01.2008
		EP 1881845 B1	24.03.2010
		EP 2198881 A1	23.06.2010
		ES 2343270 T3	27.07.2010
		HR 20100314 T1	31.08.2010
		PT 1881845 E	31.05.2010
		RS 51324 B	31.12.2010
		SI 1881845 T1	30.06.2010
		US 2010-0278862 A1	04.11.2010
		US 7803612 B2	28.09.2010
		US 8420389 B2	16.04.2013
		WO 2006-115843 A2	02.11.2006
		WO 2006-115843 A3	24.01.2008