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(54) **SAFE LENTIVIRAL VECTORS FOR TARGETED DELIVERY OF MULTIPLE THERAPEUTIC MOLECULES**

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(58) **Field of Classification Search**

None

See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

5,674,703	A	10/1997	Woo et al.
6,156,514	A	12/2000	Acevedo et al.
6,635,472	B1	10/2003	Lauermann
8,124,752	B2	2/2012	Bumcrot et al.
8,993,532	B2	3/2015	Hannon et al.
9,834,790	B1	12/2017	Pauza et al.
9,914,938	B2	3/2018	Pauza et al.
10,023,880	B2	7/2018	Pauza et al.
10,036,038	B2	7/2018	Pauza et al.
10,036,040	B2	7/2018	Pauza et al.
10,137,144	B2	11/2018	Pauza et al.
10,233,464	B2	3/2019	Pauza et al.
2002/0168345	A1	11/2002	Doug et al.
2003/0013196	A1	1/2003	Engelman et al.
2003/0096787	A1	5/2003	Perridcaudet et al.
2003/0119770	A1*	6/2003	Lai ..... C12N 15/86 514/44 R
2003/0138444	A1	7/2003	Zavitz et al.
2004/0142416	A1	7/2004	Laipis et al.
2004/0161412	A1	8/2004	Penn et al.
2004/0192629	A1	9/2004	Xu et al.
2004/0214158	A1	10/2004	Sethi et al.
2004/0248296	A1	12/2004	Beresford et al.
2005/0019927	A1	1/2005	Markus et al.
2005/0138677	A1	6/2005	Pfister et al.
2006/0183230	A1	8/2006	Silla et al.
2006/0246520	A1	11/2006	Champagne et al.
2007/0026521	A1	2/2007	Colosi
2007/0141679	A1	6/2007	Sodroski
2007/0203333	A1*	8/2007	McSwiggen C12N 15/1138 536/24.5
2008/0003225	A1	1/2008	Vie et al.
2008/0003682	A1	1/2008	Lois-Caballe et al.
2008/0039413	A1	2/2008	Morris et al.
2008/0131940	A1*	6/2008	Chiu C12N 15/111 435/91.3
2008/0153737	A1	6/2008	Lieberman et al.
2008/0199961	A1	8/2008	Rasko et al.

(Continued)

FOREIGN PATENT DOCUMENTS

BR	2515	3/2019
CN	101805750	8/2010

(Continued)

OTHER PUBLICATIONS

Zufferey et al. (J. Virology (1998) vol. 72(12):9873-9880) (Year: 1998).*

Lin et al. (Cell Res. (2007) 17:531-536). (Year: 2007).*

Oh, et al. Retrovirology. 4(38), 10 pages. (Year: 2007).*

Vargas, J. Jr. et al., "Conditionally replicating lentiviral-hybrid episomal vectors for suicide gene therapy," Antiviral Res., vol. 80 No. 3, pp. 288-294, (Dec. 2008).

Thompson et al., "Alkylamines cause Vγ9Vδ2 T-cell activation and proliferation by inhibiting the mevalonate pathway," Blood, vol. 107, pp. 651-654, (Jan. 2006).

Gober et al., "Human T Cell Receptor γδ Cells Recognize Endogenous Mevalonate Metabolites in Tumor Cells," J. of Experimental Med., vol. 197, pp. 163-168, (Jan. 2003).

(Continued)

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(57) **ABSTRACT**

The present application discloses a lentiviral transfer system which includes: (i) a self-inactivating transfer vector comprising: multiple gene units, wherein each gene unit includes a heterologous nucleic acid sequence operably linked to a regulatory nucleic acid sequence; and (ii) a helper construct which lacks a 5' LTR, wherein the 5' LTR has been replaced with a heterologous promoter, in which the helper construct further comprises: a lentiviral env nucleic acid sequence containing a deletion, wherein the deleted env nucleic acid sequence does not produce functional env protein; and a packaging signal contains a deletion, wherein the deleted packaging signal is nonfunctional.

14 Claims, 50 Drawing Sheets

Specification includes a Sequence Listing.

(56) **References Cited**

U.S. PATENT DOCUMENTS

2008/0227736	A1	9/2008	Chen et al.
2008/0293142	A1	11/2008	Liu et al.
2009/0148936	A1	6/2009	Stout et al.
2009/0304688	A1	12/2009	Fournie et al.
2010/0017911	A1	1/2010	Dawson et al.
2010/0069372	A1	3/2010	Kazantsev
2010/0119511	A1	5/2010	Wang et al.
2010/0120155	A1	5/2010	Brennan et al.
2010/0286166	A1	11/2010	Pey Rodriguez et al.
2010/0316676	A1	12/2010	Sanders
2011/0008803	A1	1/2011	Stockwell et al.
2011/0207226	A1	8/2011	Ni et al.
2012/0053223	A1	1/2012	Benkirane et al.
2012/0027725	A1	2/2012	Galvin et al.
2012/0114607	A1	5/2012	Lai et al.
2012/0034197	A1	8/2012	Young et al.
2012/0201794	A1	8/2012	Chen et al.
2013/0078276	A1	3/2013	Robinson et al.
2013/0090371	A1	4/2013	Lu et al.
2013/0142766	A1	6/2013	Dodo et al.
2013/0211380	A1	8/2013	Aquino et al.
2014/0155468	A1	6/2014	Gregory et al.
2014/0162894	A1	6/2014	Hatchwell et al.
2014/0178340	A1	6/2014	Robbins et al.
2014/0234958	A1	8/2014	Kashara et al.
2014/0248277	A1	9/2014	Hoffman et al.
2014/0336245	A1	11/2014	Mingozzi et al.
2015/0010578	A1	1/2015	Balazs et al.
2015/0018539	A1	1/2015	Fellmann
2015/0126580	A1	5/2015	DePinho et al.
2015/0132255	A1	5/2015	Sorensen et al.
2015/0176006	A1	6/2015	Krause et al.
2016/0060707	A1	3/2016	Goldenberg et al.
2016/0243169	A1	8/2016	Chen et al.
2017/0028036	A1	2/2017	Mingozzi et al.
2017/0335344	A1	11/2017	Pauza et al.
2018/0010147	A1	1/2018	Pauza
2018/0142257	A1	5/2018	Pauza
2018/0142258	A1	5/2018	Pauza
2018/0161455	A1	6/2018	Pauza
2018/0177866	A1	6/2018	Pauza
2018/0256624	A1	9/2018	Pauza
2018/0305716	A1	10/2018	Pauza
2019/0046633	A1	2/2019	Pauza
2019/0062786	A1	2/2019	Pauza et al.
2019/0078096	A1	3/2019	Lahusen et al.
2019/0083523	A1	3/2019	Pauza

FOREIGN PATENT DOCUMENTS

CN	108883100	11/2018
EP	3402483	11/2018
EP	3413926	12/2018
EP	3426777	1/2019
EP	3468617	4/2019
EP	3468618	4/2019
EP	3481418	5/2019
EP	3481435	5/2019
IN	201947000153	2/2019
JP	2018-541270	4/2019
WO	2002020554	3/2002
WO	2004053137	6/2004
WO	2005033282	4/2005
WO	2006048215	5/2006
WO	2007000668	1/2007
WO	2007133674	11/2007
WO	WO2008/025025	2/2008
WO	2009100928	8/2009
WO	2009147445	12/2009
WO	2010051521	5/2010
WO	2010117974	10/2010
WO	2010127166	11/2010
WO	2011008348	1/2011
WO	2011071476	6/2011
WO	2012048303	4/2012
WO	2012061075	5/2012
WO	WO2012145624	10/2012
WO	2013096455	6/2013
WO	2014117050	7/2014
WO	2014187881	11/2014
WO	2015017755	2/2015
WO	2015042308	3/2015
WO	2015078999	6/2015
WO	2016046234	3/2016
WO	2016061232	4/2016
WO	WO2016061232	4/2016
WO	2016200997	7/2016
WO	2017007994	1/2017
WO	2017100551	6/2017
WO	2017123918	7/2017
WO	2017139065	8/2017
WO	2017156311	9/2017
WO	2017213697	12/2017
WO	2017214327	12/2017
WO	2018009246	1/2018
WO	2018009847	1/2018
WO	2018017882	1/2018
WO	2018126112	7/2018
WO	2018129540	7/2018
WO	2018187231	10/2018
WO	2018232359	12/2018
WO	WO2019070674	4/2019

OTHER PUBLICATIONS

Goepfert, et al., "Specificity and 6-Month Durability of Immune Responses Induced by DNA and Recombinant Modified Vaccinia Ankara Vaccines Expressing HIV-2 Virus-Like Particles," J. Infectious Diseases, vol. 210, pp. 99-110, (Jul. 2014).

Human papillomavirus type 16 (HPV16), complete genome; GenBank: K02718.1; Publication [online], https://www.ncbi.nlm.nih.gov/nucleotide/333031?report=genbank&log$=nucltop&blast_rank=22&RID=H3E1THFU014; pp. 1-4, (Mar. 1994).

{Long control region} [Human papillomavirus, type 16, Genomic, 860 nt]; Accession S60559. Publication [online], https://www.ncbi.nlm.nih.gov/nucleotide/237343?report=genbank&log$=nucltop&blast_rank=1&RID=H3FCKA00014; pp. 1, (May 1993).

Tebas, P. et al, "Antiviral effects of autologous CD4 T cells genetically modified with a conditionally replicating lentiviral vector expressing long antisense to HIV," Blood, vol. 121, No. 9, pp. 1524-1533, (2013).

Tebas, p. et al., "Gene Editing of CCR5 in Autologous CD4 T Cells of Persons Infected with HIV," The New England Journal of Medicine, vol. 370 (10), pp. 901-910, (Mar. 2014).

Li et al., "Reduced Expression of the Mevalonate Pathway Enzyme Farnesyl Pyrophosphate Synthase Unveils Recognition of Tumor Cells by Vγ9V82 T Cells," J. of Immunology, vol. 182, pp. 8118-8124, (2009).

Wang et al., "Indirect Stimulation of Human Vγ9V82 T Cells through Alterations in Isoprenoid Metabolism," J. of Immunology, vol. 187 pp. 5099-5113, (2011).

Stunkel et al., "The Chromatin Structure of the Long Control Region of Human Papillomavirus Type 16 Repress Viral Oncoprotein Expression," Journal of Virology, vol. 73, No. 3, pp. 1918-1930, (Mar. 1999).

Lu et al., "Anti-sense-Mediated Inhibition of Human Immunodeficiency Virus (HIV) Replication by Use of an HIV Type 1-Based Vector Results in Severely Attenuated Mutants Incapable of Developing Resistance," Journal of Virology, vol. 79, No. 13, pp. 7079-7088, (Jul. 2004).

Dieli et al., "Targeting Human yõ T Cells with Zoledronate and Interleukin-2 for Immunotherapy of Hormone-Refractory Prostate Cancer," Europe PMC Funders Group, Cancer Res. vol. 67(15), pp. 7450-1451, (Aug. 2007).

GenBank Accession No. S60559 "(long control region) [human papillomavirus, type 16, Genomic, 860 nt]," [located online Nov. 21, 2017 at https://ncbi.nlm.nih.gov/nuccore/S60559] entire DNA sequence, (May 1993).

GenBank Accession No. JG619773, MNESC1NG-T3-001_L15_6FEB2009_054 MNESC1NG cell culture from Mahonia nervosa

(56) **References Cited**

OTHER PUBLICATIONS

Berberis nervosa cDNA, mRNA sequence, (online). [Retrieved on Dec. 5, 2017]. Retrieved from the internet :< URL: https://www.ncbi.nlm.nih.gov/nucest/JG619773 > entire document, (Feb. 2014).
Moser et al., "yõ T cells: novel initiators of adaptive immunity," Immunological Reviews, vol. 215, pp. 89-102, (Feb. 2007).
PCT: International Search Report dated Nov. 7, 2016 in Application No. PCT/US2016/036519.
PCT: Written Opinion dated Nov. 7, 2016 in Application No. PCT/US2016/036519.
PCT: International Search Report dated Oct. 19, 2016 in Application No. PCT/US2016/041456.
PCT: Written Opinion dated Oct. 19, 2016 in Application No. PCT/US2016/041456.
PCT: International Search Report dated Jul. 20, 2017 in Application No. PCT/US2017/043157.
PCT: Written Opinion dated Jul. 20, 2017 in application No. PCT/US2017/043157.
PCT: International Search Report dated Jun. 9, 2017 in Application No. PCT/US2016/066185.
PCT: Written Opinion dated Jun. 9, 2017 in Application No. PCT/US2016/066185.
PCT: International Search Report dated Jul. 17, 2017 in Application No. PCT/US2017/013019.
PCT: Written Opinion dated Jul. 17, 2017 in Application No. PCT/US2017/013019.
PCT: International Search Report dated May 26, 2017 in Application No. PCT/US2017/013399.
PCT: Written Opinion dated May 26, 2017 in Application No. PCT/US2017/013399.
PCT: International Search report dated Aug. 25, 2017 in Application No. PCT/US2017/021639.
PCT: Written Opinion dated Aug. 25, 2017 Application No. PCT/US2017/021639.
PCT: International Search Report dated Nov. 8, 2017 Application No. PCT/US2017/041168.
PCT: Written Opinion dated Nov. 8, 2017 in Application No. PCT/US2017/041168.
PCT: International Search Report dated Dec. 15, 2017 in Application No. PCT/US2017/36433.
PCT: Written Opinion dated Dec. 15, 2017 in Application No. PCT/US2017/36433.
PCT: International Search Report date Jul. 14, 2017 in Application No. PCT/US2017/013024.
PCT: Written Opinion dated Jul. 14, 2017 in application No. PCT/US2017/013024.
USPTO; Notice of Allowance dated Oct. 13, 2017 in U.S. Appl. No. 14/706,481.
USPTO; Requirement for Restriction dated Oct. 23, 2017 in Application No. 15668223.
USPTO; Notice of Allowance dated Nov. 2, 2017 in Application No. 15652080.
Charron et al., "Dominant-Negative Interference in the Pah_{enu2} Mouse of PKU: Effectiveness of Vectors Expressing Modified Forms of Phenylalanine Hydroxylase (PAH) or Ribozymes Plus a Hardened PAH mRNA," Molecular Therapy, vol. 11: pp. S163-S164, (2005).
USPTO; Non-Final Office Action dated Jun. 15, 2018 in U.S. Appl. No. 15/904,131.
USPTO; Requirement for Restriction dated Jul. 12, 2018 in U.S. Appl. No. 15/736,284.
Capietto, A. H., et al., "Stimulated gammadelta T cells increase the in vivo efficacy of trastuzumab in HER-2+ breast cancer." J Immunol 187(2): 1031-1038, (2011).
Chen, Z. and M. S. Freedman, "CD16+ in gammadelta T cells mediate antibody dependent cellular cytotoxicity: potential mechanism in the pathogenesis of multiple sclerosis." Clin Immunol 128(2): 219-227, (2008).
Couzi, L., et al., "Antibody-dependent anti-cytomegalovirus activity of human gammadelta T cells expressing CD16 (FcgammaRIIIa)." Blood 119(6): 1418-1427, (2012).
Fisher, J. P., et al., "Effective combination treatment of GD2-expressing neuroblastoma and Ewing's sarcoma using anti-GD2 ch14.18/CHO antibody with Vgamma9Vdelta2+ gammadeltaT cells." Oncoimmunology 5(1): e1025194, (2016).
Gertner-Dardenne, J., et al., "Bromohydrin pyrophosphate enhances antibody-dependent cell-mediated cytotoxicity induced by therapeutic antibodies." Blood 113(20): 4875-4884, (2009).
Poonia, B. and C. D. Pauza, "Gamma delta T cells from HIV+ donors can be expanded in vitro by zoledronate/interleukin-2 to become cytotoxic effectors for antibody-dependent cellular cytotoxicity." Cytotherapy 14(2): 173-181, (2012).
Schiller, C. B., et al., "CD19-specific triple body SPM-1 engages NK and gamma delta T cells for rapid and efficient lysis of malignant B-lymphoid cells." Oncotarget 7(50): 83392-83408, (2016).
Tokuyama, H., et al., "V gamma 9 V delta 2 T cell cytotoxicity against tumor cells is enhanced by monoclonal antibody drugs—rituximab and trastuzumab." Int. J Cancer 122(11): 2526-2534, (2008).
USPTO; Non-Final Office Action dated Feb. 22, 2018 in U.S. Appl. No. 15/850,937.
USPTO; Non-Final Office Action dated Feb. 22, 2018 in U.S. Appl. No. 15/849,062.
PCT: International Search Report dated May 29, 2018 in Application No. PCT/US2018/012998.
PCT: Written Opinion dated May 29, 2018 in Application No. PCT/US2018/012998.
USPTO; Notice of Allowance dated Apr. 23, 2018 in U.S. Appl. No. 15/850,93.
USPTO; Notice Allowance dated Apr. 26, 2018 in U.S. Appl. No. 15/849,062.
USPTO; Notice of Allowance dated Mar. 26, 2018 in U.S. Appl. No. 15/668,223.
Ostertag et al., Brain Tumor Eradication and Prolonged Survival from Intratumoral Conversion of 5-Fluorocytosine to 5-fluorouracil Using a Nonlytic Retroviral Replicating Vector, Neoro-Oncology 14(2), pp. 145-159, Feb. 2012.
Twitty et al., Retroviral Replicating Vectors Deliver Cytosine Deaminase Leading to Targeted 5-Fluorouracil-Mediated Cytotoxicity in Multiple Human Cancer Types, Human Gene Therapy Methods, 27(1), pp. 17-31, Feb. 1, 2016.
Wang et al., "Butyrophilin Human 3A1 Plays an Essential Role in Prenyl Pyrophosphate Stimulation of Human Vg2Vd2 T Cells," Journal of Immunology, vol. 191(3), pp. 1029-1042, (Jul. 5, 2013).
Jiang et al., "A Novel EST-Derived RNAi Screen Reveals a Critical Role for Farnesyl Diphosphate Synthase in Beta2-Adrenergic Receptor Internalization and Down-Regulation," FASEB Journal, vol. 26(5), pp. 1-13, (Jan. 25, 2012).
Miettinen et al., "Mevalonate Pathway Regulates Cell Size Homeostasis and Proteostasis Through Autophagy," Cell Reports, vol. 13(11), pp. 2610-2620, (Dec. 2015).
Tolmachov, "Designing Lentiviral Gene Vectors," Viral Gene Therapy, Chapter 13, pp. 263-284, (2011).
Tracey, "Human DNA Sequence from Clone RP1-288M22 on Chromosome 6q 12-13," Complete Sequence, National Center for Biotechnology. GenBank Entry. Retrieved from the internet: <https://www.ncbi.nlm.nih.gov/nucleotide/AL035467.23?report=genbank&log$=nucltop&blast_rank=1&RID=UUD4GX2D014>; pp. 1-34, (Jan. 24, 2013).
PCT; International Search Report dated Nov. 9, 2018 in Application No. PCT/US2018/037924.
PCT; Written Opinion dated Nov. 9, 2018 in Application No. PCT/US2018/037924.
Mason et al., "Inactivated Simian Immunodeficiency Virus-Pulsed Autologous Fresh Blood Cells as an Immunotherapy Strategy," Journal of Virology, vol. 83(3), pp. 1501-1510, (2009).
Blick et al., "Cyclophosphamide Enhances SB-728-T Engraftment to Levels Associated with HIV-RNA Control," CROI Conference on Retroviruses and Opportunistic Infections, Boston, Massachusetts, p. 141, (2014), (Abstract Only).

(56) **References Cited**

OTHER PUBLICATIONS

De Rose et al., "Safety, Immunogenicity and Efficacy of Peptide-Pulsed Cellular Immunotherapy in Macaques," Journal of Medical Primatology, vol. 27(2), pp. 69-78, (2008).
Smith et al., "Developments in HIV-1 Immunotherapy and therapeutic Vaccination," F1000Prime Reports, vol. 6, p. 42, (2014).
Charron, "Gene Therapy for Phenylketonuria: Dominant-Negative Interference in a Recessive Disease," Dissertation, University of Florida 2005, http://etd.fcla.edu/UF/UFE0011392/charron_c.pdf>, (retrieved Jul. 26, 2018) (2005).
Ding et al., "Administration-Route and Gender-Independent Longterm Therapeutic Correction of Phenylketonuria (PKU) in a Mouse Model by Recombinant Adeno-Associated Virus 8 Pseudotyped Vector-Mediated Gene Transfer," Gene Therapy, vol. 13, pp. 583-587, (Dec. 1, 2005).
Nowacki et al., "The PAH Mutation Analysis Consortium Database: Update 1996," Nucleic Acid Research, vol. 25(1), pp. 139-142, (Jan. 1, 1997).
Condiotti et al., "Prolonged Liver-Specific Transgene Expression by a Non-Primate Lentiviral Vector," Biochemical and Biophysical Research Communications, vol. 320(3), pp. 998-1006, (Jul. 30, 2004).
PCT; International Search Report dated Sep. 24, 2018 in Application No. PCT/US2018/025733.
PCT; Written Opinion dated Sep. 24, 2018 in Application No. PCT/US2018/025733.
USPTO; Non-Final Office Action dated Oct. 19, 2018 in U.S. Appl. No. 15/736,284.
USPTO; Notice of Allowance dated Oct. 31, 2018 in U.S. Appl. No. 16/011,550.
Hafid et al., "Phenylketonuria: A Review of Current and Future Treatments," Translational Pediatrics, vol. 4(4), pp. 304-317, (2015).
Blau et al., "Phenylketonuria," The Lancet, vol. 376(9750), pp. 1417-1427, (2010).
Chandler et al., "Vector Design Influences Hepatic Genotoxicity After Adeno-Associated Virus Gene Therapy," Journal of Clinical Investigation, vol. 125(2), pp. 870-880, (2015).
Christophersen et al., "A Technique of Transumbilical Portal Vein Catheterization in Adults," The Archives of Surgery, vol. 95(6), pp. 960-963, (1967). (Abstract Only).
Bartholome, "Genetics and Biochemistry of the Phenylketonuria-Present State," Human Genetics, vol. 51(3), pp. 241-245, (1979).
Donsante et al., "AAV Vector Integration Sites in Mouse Hepatocellular Carcinoma," Science, vol. 317, p. 477, (2007).
Eisensmith et al., "Multiple Origins for Phenylketonuria in Europe," American Journal of Human Genetics, vol. 51(6), pp. 1355-1365, (1992).
Fisher et al., "The Inhibition of Phenylalanine and Tyrosine Hydroxylases by High Oxygen Levels," Journal of Neurochemistry, vol. 19(5), pp. 1359-1365, (1972). (Abstract Only).
Grisch-Chan et al., "Low-Dose Gene Therapy for Murine PKU Using Episomal Naked DNA Vectors Expressing PAH from Its Endogenous Liver Promoter," Molecular Therapy Nucleic Acids, vol. 7, pp. 339-349, (2017).
Guldberg et al., "Aberrant Phenylalanine Metabolism in Phenylketonuria Heterozygotes," Journal of Inherited Metabolic Disease, vol. 21(4), pp. 365-372, (1998).
Kaufman et al., "A Model of Human Phenylalanine Metabolism in Normal Subjects and in Phenylketonuric Patients," Proceedings of the National Academy of Sciences USA, vol. 96(6), pp. 3160-3164, (1999).
Kaufman et al., "Phenylalanine Hydroxylase Activity in Liver Biopsies from Hyperphenylalaninemia Heterozygotes: Deviation from Proportionality with Gene Dosage," Pediatric Research, vol. 9(8), pp. 632-634, (1975).
Longo et al., "Single-Dose, Subcutaneous Recombinant Phenylalanine Ammonia Lyase Conjugated with Polyethylene Glycol in Adult Patients with Phenylketonuria: An Open-Label, Multicentre, Phase 1 Dose-Escalation Trial," The Lancet, vol. 384(9937), pp. 37-44, (2014).
Mochizuki et al., "Long-Term Correction of Hyperphenylalaninemia by AAV-Mediated Gene Transfer Leads to Behavioral Recovery in Phenylketonuria Mice," Gene Therapy, vol. 11(13), pp. 1081-1086, (2004).
Nault et al., "Adeno-Associated Virus Type 2 as an Oncogenic Virus in Human Hepatocellular Carcinoma," Molecular & Cellular Oncology, vol. 3(2), p. e1095271, 3 pages, (2016).
Oh et al., "Reversal of Gene Expression Profile in the Phenylketonuria Mouse Model After Adeno-Associated Virus Vector-Mediated Gene Therapy," Molecular Genetics and Metabolism, vol. 86(Supp. 1), pp. S124-S132, (2005).
Oh et al., "Long-Term Enzymatic and Phenotypic Correction in the Phenylketonuria Mouse Model by Adeno-Associated Virus Vector-Mediated Gene Transfer," Pediatric Research, vol. 56(2), pp. 278-284, (2004).
Pan et al., "Biodistribution and Toxicity Studies of VSVG-Pseudotyped Lentiviral Vector After Intravenous Administration in Mice with the Observation of in Vivo Transduction of Bone Marrow," Molecular Therapy, vol. 6(1), pp. 19-29, (2002).
Shedlovsky et al., "Mouse Models of Human Phenylketonuria," Genetics, vol. 134(4), pp. 1205-1210, (1993).
Yagi et al., "Complete Restoration of Phenylalanine Oxidation in Phenylketonuria Mouse by a Self-Complementary Adeno-Associated Virus Vector," Journal of Gene Medicine, vol. 13(2), pp. 114-122, (2011).
Yano et al., "Evaluation of Tetrahydrobiopterin Therapy with Large Neutral Amino Acid Supplementation in Phenylketonuria: Effects on Potential Peripheral Biomarkers, Melatonin and Dopamine, for Brain Monoamine Neurotransmitters," PLoS One, vol. 11(8), p. e0160892, 14 pages, (2016).
USPTO; Non-Final Office Action dated Sep. 19, 2018 in U.S. Appl. No. 16/011,550.
USPTO; Invitation to Pay Additional Fees and, where Applicable, Protest Fee dated Sep. 11, 2018 in Application No. PCT/US2018/37924.
Gorziglia et al "Elimination of Both E1 and E2A from Adenovirus Vectors Further Improves Prospects for in Vivo Human gene Therapy," Journal of Virology, vol. 70(6), pp. 4173-4178, (1996).
Vargas et al., "Novel Integrase-Defective Lentiviral Episomal Vectors for Gene Transfer," Human Gene Therapy, vol. 15(4), pp. 361-372, (Apr. 2004).
Wendelburg et al., "An Enhanced EBNA1 Variant with reduced IR3 Domain for Long-Term Episomal Maintenance and Transgene Expression of ORIP-Based Plasmids in Human Cells," Gene Therapy, vol. 5, pp. 1389-1399, (Oct. 1998).
Westerhout et al., "A Conditionally Replicating HIV-Based Vector that Stably Expresses an Antiviral shRNA Against HIV-1 Replication," Molecular Therapy: The Journal of the American Society of Gene Therapy, vol. 14(2), pp. 268-275, (May 2006).
Lam et al., "T-Cell Therapies for HIV," Immunotherapy, Future Medicine, vol. 5(4), pp. 407-414, (Apr. 2013).
Munoz et al., "Ex Vivo Expansion and Lentiviral Transduction of *Macaca Nemestrina* CD4 + T Cells," Journal of Medical Primatology, vol. 38(6), pp. 438-443, (Dec. 2009).
Porichis et al., "HIV-Specific CD4 T Cells and Immune Control of Viral Replication," Current Opinion in HIV and Aids, vol. 6(3), pp. 174-180, (May 2011).
Kavanagh et al., "Expansion of HIV-Specific CD4+ and CD8+ T Cells by Dendritic Cells Transfected with mRNA Encoding Cytoplasm—or Lysosome—Targeted Nef," Blood, American Society of Hematology, vol. 107(5), pp. 1963-1969, (Mar. 2006).
Akinsheye et al., "Fetal Hemoglobin in Sickle Cell Anemia," Blood, vol. 118(1), pp. 19-27, (2011).
USPTO; Non-Final Office Action dated Dec. 31, 2018 in U.S. Appl. No. 16/182,443.
EPO; Extended Search Report dated Dec. 12, 2018 in EP Application No. 16808223.8.
EPO; Extended Search Report dated Dec. 11, 2018 in EP Application No. 16822021.8.
PCT; Invitation to Pay Additional Fees in Application No. PCT/US2018/053919 dated Feb. 22, 2019.

(56) **References Cited**

OTHER PUBLICATIONS

Hee Yeon Kim., "Farnesyl diphosphate synthase is important for the maintenance of glioblastoma stemness," Experimental & Molecular Medicine, (2018).
Hong Wang., "Indirect Stimulation of Human V2V2 Cells Through Alterations in Isoprenoid Metabolism," The Journal of Immunology, (2011).
Z. Li, "Inhibition of farnesyl pyrophosphate synthase prevents angiotensin II-induced cardiac fibrosis in vitro," Clinical & Experimental Immunology, (2014).
Xiaofeng Jiang, "A novel EST-derived RNAi screen reveals a critical role for famesyl diphosphate in B2-adrenerigic receptor internalization and down-regulation," (1995).
Jian Yang, "Lentiviral-Mediated Silencing of Farnesyl Pyrophosphate Synthase through RNA Interference in Mice," Hindawi Publishing Corporation, (2015).
Yang Ye, "Knockdown of farnesyl pyrophosphate synthase prevents angiotensin II-medicated cardiac hypertrophy, The International Journal of Biochemistry & Cell Biology," (2010).
Jianqiang Li, "Reduced Expression of Mevalonate Pathway Enzyme Farnesyl Pyrophosphate Synthase Unveils Recognition of Tumor Cells by V9V2 Cells," (2019).
Daryl S. Schiller, "Parameters Influencing Measurement of the Gag Antigen-Specific T-Proliferative Response to HIV Type 1 Infection," (2000).
PCT; International Search Report dated Jul. 22, 2019 in the Application No. PCT/US2019/24410.
PCT; Written Opinion of the International Search Report dated Jul. 22, 2019 in the Application No. PCT/US2019/24410.
PCT; International Preliminary Report on Patentability dated Jul. 9, 2019 in the Application No. PCT/US2018/012998.
USPTO; Restriction Requirement dated Jul. 12, 2019 in the U.S. Appl. No. 15/736,284.
USPTO; Advisory Action dated Jul. 23, 2019 in the U.S. Appl. No. 15/736,284.
USPTO; Notice of Allowance dated Aug. 14, 2019 in the U.S. Appl. No. 16/008,991.
USPTO; Notice of Allowance dated Sep. 25, 2019 in the U.S. Appl. No. 16/218,010.
USPTO; Notice of Allowance dated Jul. 19, 2019 in the U.S. Appl. No. 16/132,247.
EPO; European Search Report dated Aug. 12, 2019 in the EP Application No. 17764128.9.
EPO; Supplementary European Search Report dated Sep. 6, 2019 in the Application No. 17750547.6.
USPTO; Notice of Allowance dated Jun. 18, 2019 in the U.S. Appl. No. 16/182,443.
USPTO; Notice of Allowance dated Jul. 3, 2019 in U.S. Appl. No. 16/182,443.
USPTO; Final Office Action dated Jul. 1, 2019 in the U.S. Appl. No. 16/132,247.
USPTO; Final Office Action dated May 2, 2019 in U.S. Appl. No. 15/736,284.
USPTO; Final Office Action dated May 2, 2019 in U.S. Appl. No. 16/182,443.
USPTO; Non-Final Office Action dated May 7, 2019 in U.S. Appl. No. 16/008,991.
USPTO; Non-Final Office Action dated May 16, 2019 in U.S. Appl. No. 16/132,247.
USPTO; Non-Final Office Action dated May 24, 2019 in U.S. Appl. No. 16/218,010.
EPO; Extended Search Report dated Jun. 6, 2019 in EP Application No. 17739028.3.
GenBank Sequence M65141.1 Retrieved from the Internet <URL: https://www.ncbi.ntm.nih.gov/nuccore/M65141.1. Especially Sequence, nt 301-420, (Retrieved Mar. 31, 2019).
PCT; Written Opinion dated Apr. 12, 2019 in Application No. PCT/US2018/053919.
PCT; International Search Report dated Apr. 12, 2019 in Application No. PCT/ US2018/053919.
Fusetti, et al., "Structure of Tetrameric Human Phenylalanine Hydroxylase and Its Implications for Phenylketonuria," J. Bio. Chem., vol. 273, No. 27, pp. 16962-16958 (1998).
USPTO; Invitation to Pay Additional Fees and, Where Applicable, Protest Fee dated Jul. 17, 2018 in Application No. PCT/US2018/25733.
USPTO; Requirement for Restriction dated Aug. 3, 2018 in U.S. Appl. No. 16/011,550.
USPTO; Notice of Allowance dated Aug. 10, 2018 in U.S. Appl. No. 15/904,131.
Hassan et al., "Isolation of umbilical cord mesenchymal stem cells using human blood derivative accompanied with explant method," Stem Cell Investigation, pp. 1-8, (2019).
Huang et al., "An Efficient protocol to generate placental chorionic plate-derived mesenchymal stem cells with superior proliferative and immunomodulatory properties," Stem Cell Research & Therapy, pp. 1-15, (2019).
USPTO; Restriction Requirement dated Oct. 22, 2019 in the U.S. Appl. No. 15/580,661.
USPTO; Restriction Requirement dated Nov. 4, 2019 in the U.S. Appl. No. 16/076,655.
USPTO; Restriction Requirement dated Nov. 7, 2019 in the U.S. Appl. No. 16/083,384.

* cited by examiner

FIGURE 1A

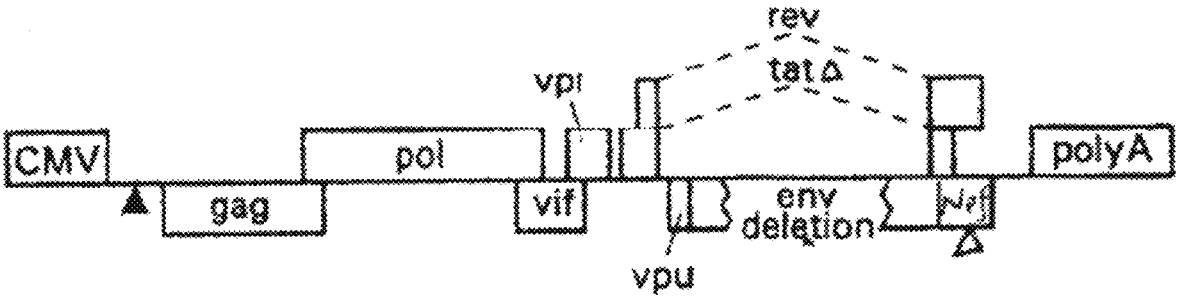

FIGURE 1B

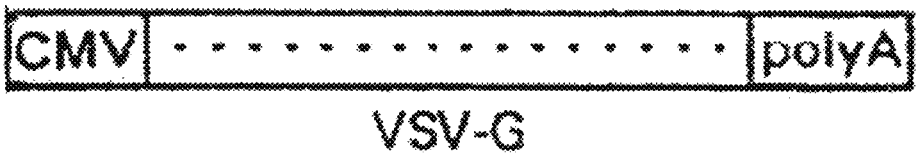

FIGURE 1C

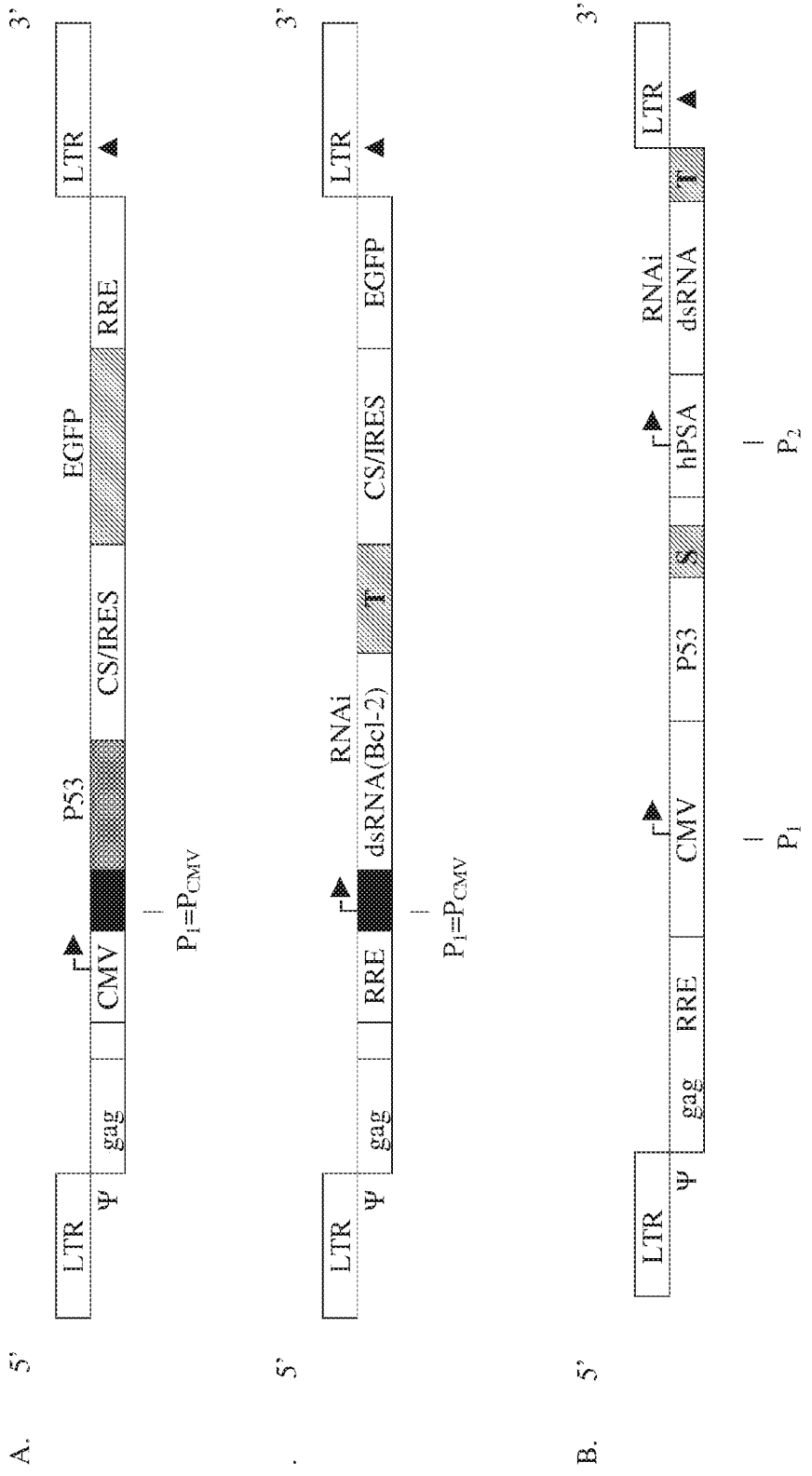
A.
5'
3'
LTR
Ψ
gag
CMV
P53
CS/IRES
EGFP
RRE
LTR
P1=PCMV
RNAi
dsRNA(Bcl-2)
T
EGFP
P1=PCMV
B.
P53
S
hPSA
dsRNA
P1
P2

FIGURE 1C (cont.)

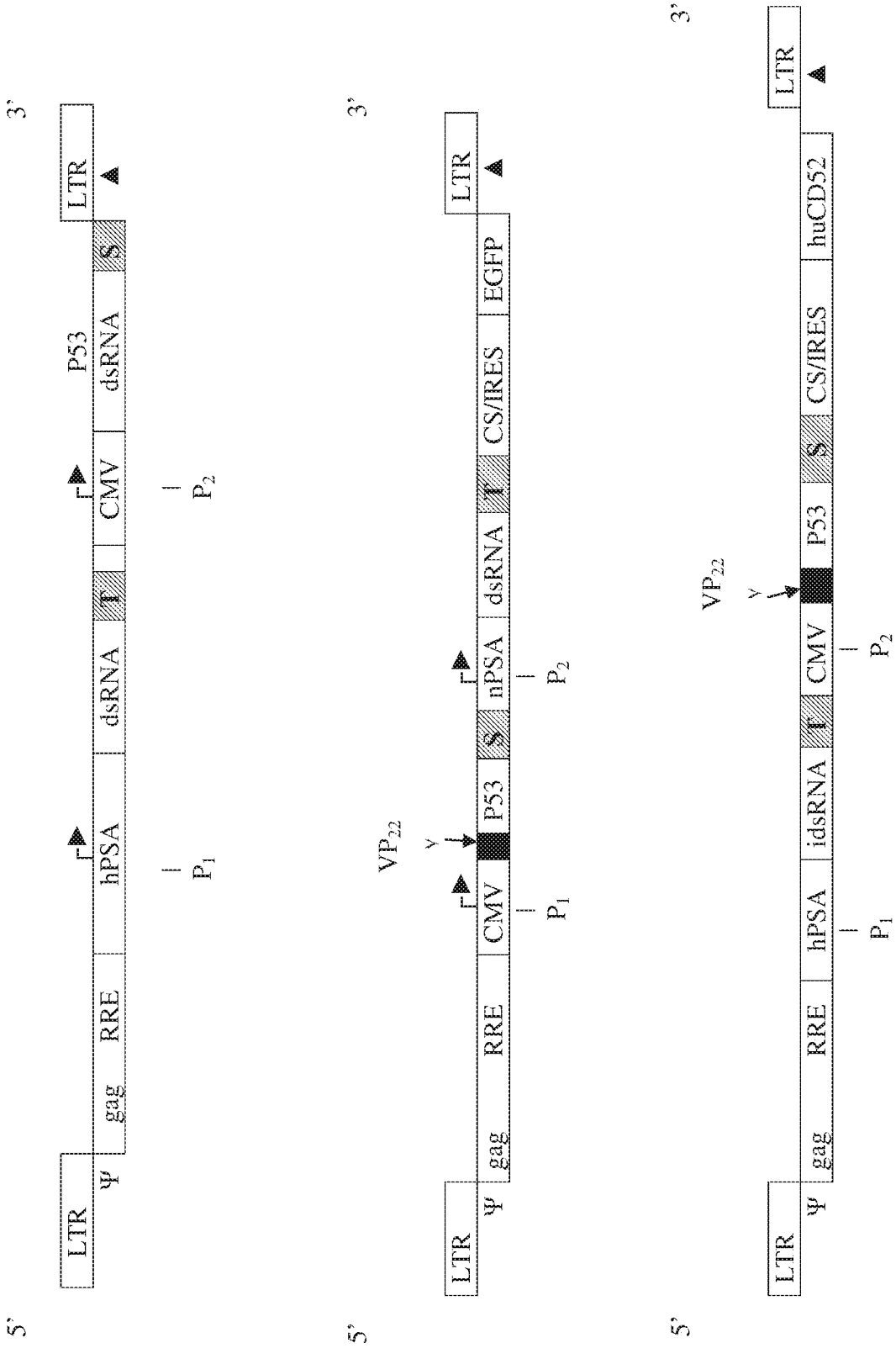

FIGURE 1C (cont.)

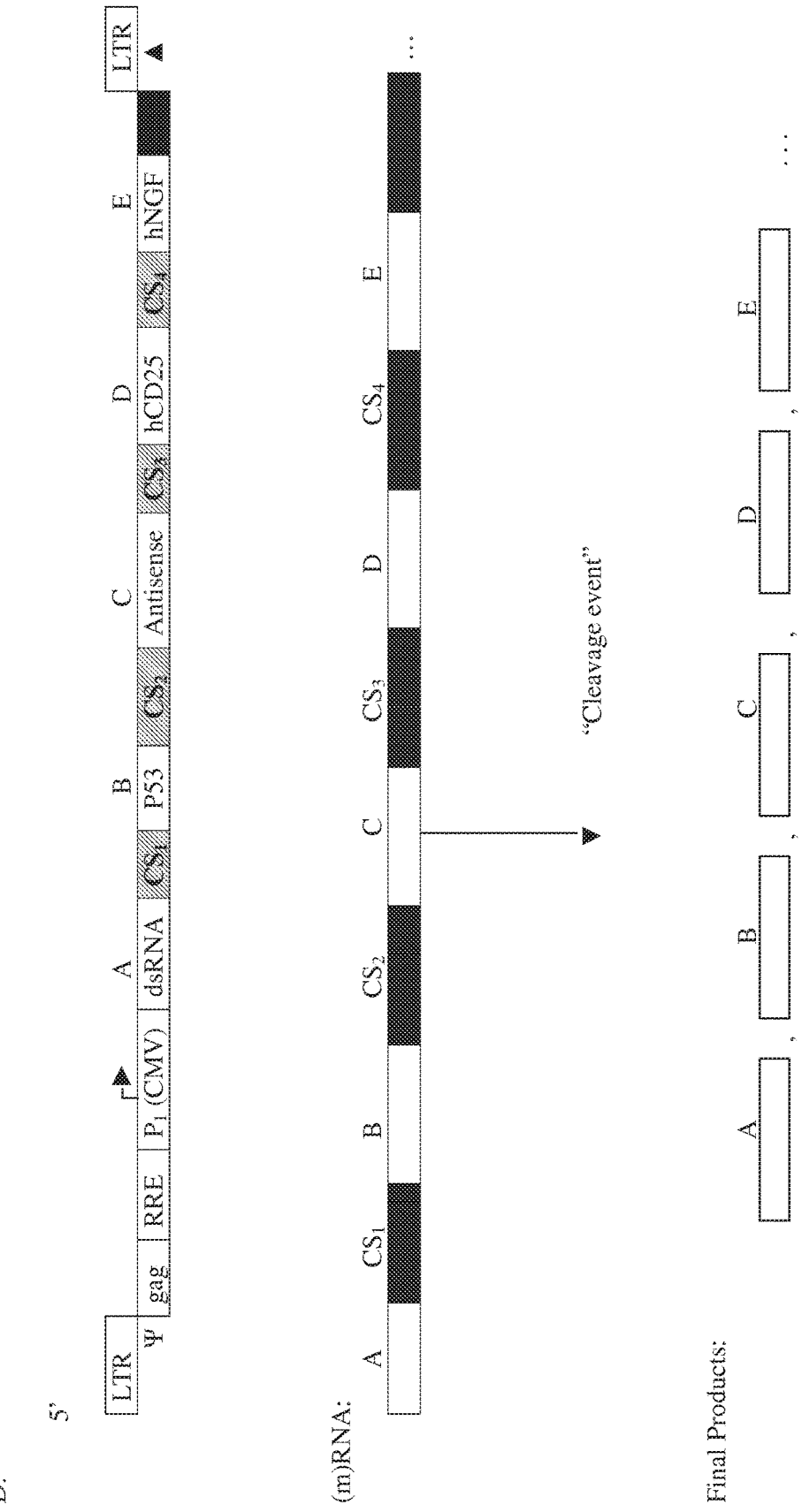

FIGURE 2

Heterologous Proteins for Use in the Present Invention	
Heterologous Protein	Reference
Human:	
2AP-70 protein-tyrosine kinase	Isakov et al. 1996
ABC transporter tap1 processing (TAP)	Meyer et al. 1994
ABC transporter tap2 processing (TAP)	Meyer et al. 1994
α_2 C_2 adrenoceptor	Marjamaki et al. 1994
α-galactosidase A	Coppola et al. 1994
α and β globins	Groebe et al. 1992
α_1 glycine receptor	Cascio et al. 1993
α-macroglobulins (αM)	Rompaey & Marynen 1992
α and β platelet-derived growth factor receptors	Jeusen et al. 1992
Adenosine deaminase	Medin et al. 1990
aldase reductase	Nishimura et al. 1991

FIGURE 2 (cont.)

Heterologous Proteins for Use in the Present Invention	
Heterologous Protein	Reference
α-interferon	Maeda et al. 1985
5-a reductase (type 1)	Delos et al. 1994
Ah receptor and Ah receptor nuclear translocater	Chan et al. 1994
Alzheimer amyloid precursor protein	Ramakrishna et al. 1991
Alzheimer β-amyloid peptide precursor	Currie et al. 1991
Amyloid peptide precursor	Essalmani et al. 1996
Amyloid precursor protein	Bhasin et al. 1991
Amyloid b protein precursor	Bhasin et al. 1991
Amyloid precursor protein	Lowery et al. 1991
Androgen receptor	Beitel et al. 1995
Angiotensin	Williams et al. 1994
Androgen receptor	Chang et al. 1992
Antithrombin III	Gillespie et al. 1991
Apolipoprotein E	Gretch et al. 1991
Aromatase P450	Amarneh & Simpson 1995
Autoantigen of Wegener's granulomatosis (PR3)	Szymkowiak et al. 1996
b1,2-N-acetylglucosaminyl-transferase I (hGNT-I)	Wagner et al. 1996
β1 γ2 dimers of G-protein	Dietrich et al. 1992
β1,β2,γ2 subunits of heterotrimeric guanine nucleotide binding protein	Graber et al. 1992
β1 –adrenergic receptor	Ravet et al. 1992
β2 –adrenergic receptor	Kleymann et al. 1993
β-adrenergic receptor kinase	Sohlemann et al. 1993
β galactosidase	Itoh et al. 1990
β interferon	Smith et al. 1983
β2 glycoprotein I	Igarashi et al. 1996
BCl2	Alnemri et al. 1992
BCl-2 oncoprotein	Reid et al. 1992

FIGURE 2 (cont.)

Heterologous Proteins for Use in the Presint Invention	
Heterologous Protein	Reference
bone morphogenetic protein-2	Maruoka et al. 1995
Cc gene	Poul et al. 1995
Cg1 sequence	Poul et al. 1995
C-reactive protein	Mamell et al. 1995
cAMP-specific phosphodiesterase	Amegadzie et al. 1995
CD95/APO 1/Fas ligand	Mariani et al. 1996
CD4	Murphy et al. 1990; Lazarte et al. 1992
Cdc42 GTP-binding protein	Cerione et al. 1995
c-fos protein	Tratner et al. 1990
CYP2A6	Nanji et al. 1994
calpain I	Meyer et al. 1996
Carcinoembryonic antigen	Bei et al. 1994
Carcinoembryonic antigen CD 66b	Yamamaka et al. 1996
Carcinoembryonic antigen CD66c	Yamamaka et al. 1996
Cholecystokinin B (CCK_B)	Gimpl et al. 1996
Choriogonadotropin α subunit	Nakhai et al. 1991
Choriogonadotropin β-subunit	Chen et al. 1991
Choriogonadotropin β-subunit descarboxyl-terminal peptide	Chen and Rahl 1991
Chorionic gonadotropin hormone precursor	Nakhai et al. 1991
Chorionic gonadotropin hormone (β-subunit)	Hasnain et al. 1994
Chorionic gonadotropin hormone β subunit	Nakhai et al. 1992
Complement C1r	Sass et al. 1977
Complement C1r proenzyme	Gal et al. 1989
Complement protein C9	Tomlinson et al. 1993
Corticosteroid binding globulin	Ghose Dastidar et al. 1991
c-myc protein	Miyamoto et al. 1985

FIGURE 2 (cont.)

Heterologous Proteins for Use in the Presint Invention.	
Heterologous Protein	Reference
Complement protein C9	Tomlinson et al. 1993
Corticosteroid binding globulin (hCBG)	Ghose-Dastidar et al. 1991
Creatine kinase B (B-CK)	de Kok et al. 1995
Cyclooxygenase-2	Cromlish et al. 1994
Cytochrome b5	Patten & Koch 1995
Cytochrome B558	Katkin et al. 1992
Cytochrome CYP3A4	Lee et al. 1995
Cytochrome P450 CYP3A4	Buters et al. 1994
Cytochrome P-450 isoform(s)	Clair et al. 1994
Cytomegalovirus 65K tegument phosphoprotein	La Fauci et al. 1994
Cytomegalovirus IE1, IE1 exon 4	Davrinche et al. 1993
Cytosolic phospholipase A2	Abdullah et al. 1995
D4 dopamine receptor	Mills et al. 1993
DNA ligase I	Gallina et al. 1995
DNA polymerase α subunit	Copeland and Wang 1991
DNA polymerase d catalytic subunit	Zhou et al. 1996
DNA topoisomerase I	Zhelkovsky & Moore 1994
Dopamine D2 receptor	Javitch et al. 1994
EGF receptor	Greenfield et al. 1988
EGF receptor-tyrosine kinase domain	Wedegaertner et al. 1989
Endothelial nitric oxide synthase	Chen et al. 1996
Epidermal growth factor receptor	Waterfield & Greenfield 1991
Epidermal-growth-factor receptor protein-tyrosine kinase	McGlynn et al. 1992
Epidermal growth factors IX and XIIa	Astermark et al. 1994
Erythrocyte anion exchanger	Dalo et al. 1996
Erythropoietin	Quelle et al. 1992
Estrogen receptor	Beekman et al. 1994

FIGURE 2 (cont.)

Heterologous Proteins for Use in the Present Invention	
Heterologous Protein	Reference
factor VIII - B domain deleted	Webb et al. 1993
Fibroblast growth factor receptor subtype ligand binding domain	Sisk et al. 1992
follicle-stimulating hormone receptor	Christophe et al. 1993
furin	Bravo et al. 1994
$GABA_A$ receptor α1 subunits	Birnir et al. 1995
$GABA_A$ receptor β1 subunits	Birnir et al. 1995
ga773 - 2 antigen	Strassburg et al. 1992
GMP synthetase	Lou et al. 1995
Glucocerebrosidase	Martin et al. 1988
Glucocorticoid receptor	Srinivasan et al. 1990
Glutamic acid decarboxylase	Mauch et al. 1993
glycine receptor α1	Morr et al. 1995
group b rotavirus ADRV, VP4	Mackow et al. 1993
group II Phospholipase A2	Tremblay et al. 1993
growth hormone	Sumathy et al. 1996
growth hormone receptor - extracellular domain	Ota et al. 1991
$5\text{-}HT_{1A}$ receptor	Mulheron et al. 1994
hst-1 transforming protein	Miyagawa et al. 1988
heart (R)-3-hydroxybutyrate dehydrogenase	Green et al. 1996
Hematopoietic glycopeptide erythropoietin	Quelle et al. 1992
Hemopexin	Satoh et al. 1994
Heparin cofactor II	Ciaccia et al. 1995
Hepatitis b virus X protein	Klein et al. 1992
Hepatocyte growth factor	Yee et al. 1993
Hepatocyte growth factor	Lee et al. 1993
high-affinity IgE receptor-a chain	Yagi et al. 1994
17b-hydroxysteroid dehydrogenase	Breton et al. 1994

FIGURE 2 (cont.)

Heterologous Proteins for Use in the Present Invention	
Heterologous Protein	Reference
5-hydroxytryptamine$_{1A}$	Butkerait et al. 1995
5-hydroxytryptamine receptors (5-HT$_{1A}$, 5-HT$_{1Dα}$, 5-HT$_{1Dβ}$, 5-HT$_{1E}$)	Parker et al. 1994
IgA	Carayannopoulos et al. 1994
IL2 receptor α & β chains	Lindqvist et al. 1993
Immunodeficiency virus-type 1 *gag* precursor	Chazal et al. 1994
Immunodeficiency virus-1 gp41	Lu et al. 1993
Immunodeficiency virus-1 gp120	Yeh et al. 1993
insulin holoreceptor	Paul et al. 1990
insulin receptor substrate-1	Siemeister et al. 1995
insulin receptor β-subunit	Herrera et al. 1988
insulin receptor β subunit transmembrane/cytoplasmic domain	Li et al. 1992
insulin receptor ectodomain	Sissom et al. 1989; 1991
insulin receptor protein-tyrosine kinase domain	Ellis et al. 1988
insulin receptor cytoplasmic domain of β subunit	Herrera et al. 1988
insulin receptor protein tyrosine-kinase-cytoplasmic domain	Ellis and Levine 1991
insulin-like growth factor II	Congote and Li, 1994
insulin-like growth factor II	Marumoto et al. 1992
Intercellular adhesion molecule 1 (ICAM-1)	Cobb et al. 1992
Interferon-g glycoforms	Ogonah et al. 1995
Interleukin 2	Smith et al. 1985
Interleukin 2 glycoprotein variants	Grabenhorst et al. 1993
Interleukin-2 receptor gamma chain	Raivio et al. 1995
Interleukin 5	Brown et al. 1995
Interleukin 6	Matsuura et al. 1991
Interleukin-6 receptor	Weiergraber et al. 1995

FIGURE 2 (cont.)

Heterologous Proteins for Use in the Present Invention	
Heterologous Protein	Reference
Intrinsic factor	Gordon et al. 1992
iron regulatory factor	Emery-Goodman et al. 1993
Isoforms (neuronal, inducible, endothelial) nitric oxide synthase	Nakane et al. 1995
Ku autoantigen	Allaway et al. 1990
Lecithin-cholesterol acyltransferase	Chawla & Owen 1995
Leukotriene A_4 hydrolase	Gierse et al. 1993
link protein	Grover & Roughley 1994
liver carboxylesterase	Kroetz et al. 1993
Lymphocytic activation gene (LAG-1)	Baizleras et al. 1990
lysyl hydroxylase	Krol et al. 1996; Pirskanen et al. 1996
Lysosomal β-galactosidase	Itoh et al. 1991
5'lipoxygenase	Dunk et al. 1989
m1 muscarinic acetylcholine receptors	Haga et al. 1996
μ2 muscarinic cholinergic receptor	Debburman et al. 1995
μ3 (hμ3) muscarinic cholinergic receptors	Debburman et al. 1995
MHC class I HLA-b27 antigen	Levy and Kvist 1990
MHC class II DR4a, DR4b, extracellular domain	Scheerle et al. 1992
Macrophage colony stimulating factor	Qiu et al. 1995
Matrilysin	Lopez de Turiso et al. 1996
Metallothionein-II	Schmiel et al. 1985
Mineralocorticosteriod receptor	Binart et al. 1991
Monocyte chemoattractant protein-1	Ueda et al. 1994; Ishii et al. 1995
Multidrug resistance 1	Germann et al. 1990
Multidrug resistance P-glycoprotein	Rao et al. 1994
Muscarine receptor μ2	Kameyama et al. 1994

FIGURE 2 (cont.)

Heterologous Proteins for Use in the Present Invention	
Heterologous Protein	Reference
Myeloperoxidase	Taylor et al. 1992
Myogenic factors myf4, myf5	Braun et al. 1991
N-formyl peptide receptor	Quehenberger et al. 1992
Na^+/H^+ antiporter	Fafournoux et al. 1991
NADPH-P450 oxidoreductase	Tamura et al. 1992
nerve growth factor	Buxser et al. 1991
nerve growth factor receptor	Vissavajjhala et al. 1990
Neutrophil NADPH oxidase factors p47 [phox], p67[phox]	Leto et al. 1991
Nuclear hormone receptor H-2RIIBP	Marks et al. 1992
Nucleolar protein p120	Ren et al. 1996
Oxytocin receptor	Gimpl et al. 1995
p53	Patterson et al. 1996
P450 2E1	Patten & Koch 1995
Pancreatic lipase	Thirstrup et al. 1993
Pancreatic procolipase	Lowe 1994
Papillomavirus type 11 E1, E2	Bream et al. 1993
Papillomavirus type 11 L1 protein	Rose et al. 1993
Papillomavirus type 16 E2	Sanders et al. 1993
Papillomovirus type 16 L1 protein	Kirnbauer et al. 1993
Papillomavirus type 45 L1 protein	Touze et al. 1996
Parainfluenza virus type 3, 7, HN, 7HN	Lehman et al. 1993
Parathyroid hormone	Mathavan et al. 1995
Parvovirus B19 vp1, vp2	Cubie et al. 1993
Phospholipase A_2	Abdullah et al. 1995
Placental aromatase (CYP19A1)	Sigle et al. 1994
plasma plasminogen	Whitefleet-Smith et al. 1989
Plasminogen	Davidson et al. 1991

FIGURE 2 (cont.)

Heterologous Proteins for Use in the Present Invention	
Heterologous Protein	Reference
Plasminogen (HPg)	Castellino et al. 1993
Plasminogen activator inhibitor-2	Pei et al. 1995
Platelet glycoprotein IBb	Finch et al. 1996
platelet 12-lipoxygenase	Chen et al. 1993
poly(ADP-ribose) polymerase	Giner et al. 1992
pre-pro endothelin-1	Benatti et al. 1992
pre-pro gastrin releasing peptide	Lebacq-verheyden et al. 1988
pro-al(III) chains	Tomita et al. 1995
ProapoA-I	Sorci-Thomas et al. 1996
Progesterone receptor (A form)	Elliston et al. 1992
Progesterone receptors A&B forms	Christensen et al. 1991
prolyl 4-hydroxylase a, b subunits	Vuori et al. 1992
prolyl 4-hydroxylase a subunit with BiP polypeptide	Veijola et al. 1996
prosaposin	Leonova et al. 1996
prostoglandin G/H synthase	George et al. 1996
prostaglandin G/H synthase 1	Barnett et al. 1994
prostaglandin G/H synthase 2	Barnett et al. 1994
protein disulphide isomerase	Vuori et al. 1992
protein kinase c-d	Rankl et al. 1994
protein kinase Cm	Dieterich et al. 1996
pro-urokinase	Gao and Hu 1994
rab 6	Yang et al. 1992
rap1A	Quilliam et al. 1990
recombinant IL-8	Kang et al. 1992
recombinant p56^{lck}	Fiotow et al. 1996
renin	Mathews et al. 1996
respiratory syncytial virus F and G glycoproteins	Wathen et al. 1989

FIGURE 2 (cont.)

Heterologous Proteins for Use in the Present Invention	
Heterologous Protein	Reference
retinoblastoma pp110[RB]	Wang et al. 1990
retinoic acid receptor a1	Quick et al. 1994
retinoic acid receptor - g1	Reddy et al. 1992
ssDNA-binding protein	Stigger et al. 1994
sex steroid-binding protein (hSBP/hABP, hSHBG)	Sui et al. 1995
soluble human insulin receptor - ectodomain	Sissom and Ellis 1992
soluble human insulin receptor tyrosine kinase	Ahn et al. 1993
Sos1 protein	Frech et al. 1995
steroid 5a-reductase	Ichiki et al. 1993
synthetic basic fibroblast growth factor	Hills & Crane-Robinson 1995
TII (CD2) t-lymphocyte surface glycoprotein	Richardson et al. 1988
TII (CD2)	Alcover et al. 1988
T-cell leukemia virus type I p40	Nyunoya et al. 1988
T-cell protein tyrosine kinase	Lehr et al. 1996
T-cell protein-tyrosine-phosphatase	Zander et al. 1991
T-lymphotropic virus type I envelope protein	Yamashita et al. 1992
terminal transferase	Chang et al. 1988
terminal deoxynucleotidyl transferase	di Primio et al. 1992
thrombomodulin	Marumoto et al. 1993
thromboxane synthase	Yokoyama et al. 1993
thyroid hormone B1 receptor	Putlitz et al. 1991
thyroid peroxidase	Kendler et al. 1993
thyrotropin receptor extracellular domain	Seetharamaiah et al. 1993
thyrotropin hormone receptor- extracellular domain	Huang et al. 1993
tissue inhibitor of metalloproteinases-1	Gomez et al. 1994
tissue plasminogen activator	Jarvis et al. 1993
tissue-type plasminogen activator	Steiner et al. 1988

FIGURE 3

Transport genes	References
VP22 in lentivirus	Lai and Brady, 2002
Pancreatic presecretory proteins	Thomas, 1982
Resistance -cell (cancer) division proteins (Transporter AcrB)	Krishan et al., 2000 Gary et al., 2006
HIV-1-Tat	Schwarze et al., 1999
Antennapedia homeobox domain	Derossi et al., 1996
Mitochondrial carrier proteins	Walker et al., 1992
Amino acid: cation symporters (Proton glutamate symporter)	Koch et al., 2007
Drug/Metabolite transporter (Multidrug resistance transporter EmrE)	Livshits, et al., 2003
Light absorption-driven transporter (Bacteriorhodopsin-like proteins)	Waschuk et al., 2005
Transmembrane cytochrome b-like proteins (Coenzyme Q-cytochrome reductase)	Dudkina et al., 2005
Electrochemical potential-driven transporters (ATPases)	Ishmukhametov et al., 2007
Mitochondrial membrane transport protein	Nicholls et al., 2005
Glucose transporters	Hediger et al., 1994
P-glycoprotein	Michael et al., 2002
Glutamate transporter	Shigeri et al., 2004
Norepinephrine transporter	Shannon et al., 2000

FIGURE 3 (cont.)

Transport genes	References
Vesicular monoamine transporter	Rang et al., 2003
The H+/peptide co-transporters (PEPT1/2)	Kenichi et al., 2000
Renal peptide transporters	Daniel et al., 1997
Apolipoprotein E	Gretch et al., 1991

Fig. 3 - 2

FIGURE 4

Promoter Element	Inducer	References
MT II Regulation of human metallothionein – IIA gene	Phorbol Ester (TFA) Heavy metals	Palmiter et al., 1982; Haslinger and Karin, 1985; Searle et al., 1985; Stuart et al., 1985; Imagawa et al., 1987, Karin et al., 1987; Angel et al., 1987b; McNeall et al., 1989
MMTV (mouse mammary tumor virus) promoter involved in regulation by Glucocorticoids involved Pathway in Chronic Obstructive Pulmonary disease	Glucocorticoics	Huang et al., 1981; Lee et al., 1981; Majors and Varmus, 1983; Chandler et al., 1983; Lee et al., 1984; Ponta et al., 1985; Sakai et al., 1988
β-Interferon regulation of infection disease	Poly(rI)x poly(rc)	Tavernier et al., 1983
Adenovirus 5 E2 Gene therapy promoter in anti-cancer infection disease	Ela	Imperiale and Nevins, 1984
Collagenase anti-wrinkle application	Phorbol Ester (TPA)	Angel et al., 1987a

FIGURE 4 (cont.)

Promoter Element	Inducer	References
Stromelysin application on cardiovascular disease	Phorbol Ester (TPA)	Angel et al., 1987b
SV40 Gene Vector selection	Phorbol Ester (TPA)	Angel et al., 1987b
Murine MX Gene for Newcastle disease in bird industry	Interferon, Newcastle Disease Virus	
GRP78 Gene regulation of Hepatitis C virus	A23187	Resendez et al., 1988
α-2-Macroglobulin Sickle cell disease	IL-6	Kunz et al, 1989
Vimentin Serum inductibility of the human vimentin promoter	Serum	Rittling et al., 1989
MHC Class I Gene H-2Kb regulation for cancer/gene therapy	Interferon	Blanar et al., 1989
HSP70 Cell-specific activation of Glial-cell	Ela, SV40 Large T Antigen	Taylor et al., 1989; Taylor and Kingston, 1990a, b
Proliferin Chronic myelogenous leukemic cell	Phorbol Ester-TPA	Mordacq and Linzer, 1989
Tumor Necrosis Factor cancer cell gene therapy, immune disease	FMA	Hensel et al., 1989

Fig 4 - 2

FIGURE 4 (cont.)

Promoter Element	Inducer	References
Thyroid Stimulating Hormone a Gene Thyroid cell specific promoter activator	Thyroid Hormone	Chatterjee et al., 1989
CMV promoter general mammalian promoter		
hSyn-1 human neuron-specific promoter		
hPSA human Prostate Specific Antigen Promoter		
hCCKAR Human Cholecystorinin (CCK) promoter for gene drug specific expression in pancreatic cancer cells, but not in normal cells		
hAFP human Alpha-Fetoprotein promoter Specificity: Hepatocellular carcinoma (liver cancer cells)		
Neu-Oncoven/c-erbB2 promoter human breast pancreatic cancer (promoter)		

Fig. 4 - 3

FIGURE 4 (cont.)

Promoter Element	Inducer	References
CEA promoter (Carcinoembryonic Antigen) human epithelial cancer cells (lung cancer, gastric cancer cells)		

Fig. 4 - 4

FIGURE 5

ENHANCER	REFERENCES
Immunoglobulin Heavy Chain	Banerji et al., 1983; Gilles et al., 1983; Grosschedl and Baltimore, 1985; Atchinson and Perry, 1986, 1987, Imler et al., 1987, Weinberger et al., 1984; Kiledjian et al., 1988; Porton et al., 1990
Immunoglobulin Light Chain	Queen and Baltimore, 1983; Picard and Schaffner, 1984
T-Cell Receptor	Luria et al., 1987; Winoto and Baltimore, 1989; Redondo et al., 1990
HLA DQα and DQβ β-Interferon	Sullivan and Peterlin, 1987; Goodbourn et al., 1986; Fujita et al., 1987; Goodbourn and Maniatis, 1988
Interleukin-2	Greene et al., 1989
Interleukin-2 Receptor	Greene el al., 1989; Lin et al., 1990
MHC Class II 5	Koch et al., 1989
MHC Class II HLA-DRa	Sherman et al., 1989
β-Actin	Kawamoto et al., 1988; Ng et al.; 1989
Muscle Creatine Kinase	Jaynes et al., 1988; Horlick and Benfield, 1989; Johnson et al., 1989a
Prealbumin (Transthyretin)	Costa et al., 1988
Elastase I	Ornitz et al., 1987
Metallothionein	Karin et al., 1987; Culotta and Hamer, 1989
Collagenase	Pinkert et al., 1987; Angel et al., 1987
Albumin Gene	Pinkert et al., 1987; Tronche et al., 1989, 1990
α-Fetoprotein	Godbout et al, 1988; Campere and Tilghman, 1989

Fig. 5 - 1

FIGURE 5 (cont.)

ENHANCER	REFERENCES
t-Globin	Bodine and Ley, 1987; Perez-Stable and Constantini, 1990
β-Globin	Trudel and Constantini, 1987
c-fos	Cohen et al., 1987
c-HA-ras	Triesman, 1986; Deschamps et al., 1985
Insulin	Edlund et al., 1985
Neural Cell Adhesion Molecule (NCAM)	Hirsh et al., 1990
α_1-Antitrypain	Latimer et al., 1990
H2B (TH2B) Histone	Hwang et al., 1990
Mouse or Type I Collagen	Rpe et al., 1989
Glucose-Regulated Proteins (GRP94 and GRP78)	Chang et al., 1989
Rat Growth Hormone	Larsen et al., 1986
Human Serum Amyloid A (SAA)	Edbrooke et al., 1989
Troponin I (TN I)	Yutzey et al., 1989
Platelet-Derived Growth Factor	Pech et al., 1989
Duchenne Muscular Dystrophy	Klamut et al., 1990
SV40	Banerji et al., 1981; Moreau et al., 1981; Sleigh ard Lockett, 1985; Firak and Subramanian, 1986; Herr and Clarke, 1986; Imbra and Karin, 1986; Kadesch and Berg, 1986; Wang and Calame, 1986; Ondek et al., 1987; Kuhl et al., 1987; Schaffner et al., 1988
Polyoma	Swartzendruber and Lehman, 1975; Vasseur et al, 1980; Katinka et al., 1980, 1981; Tyndell et al, 1981; Dandolo et al., 1983; de Villiers et al., 1984; Hen et al., 1986; Satake et al., 1988; Campbell and Villarreal, 1988

FIGURE 5 (cont.)

ENHANCER	REFERENCES
Retroviruses	Kriegler and Botchan, 1982, 1983; Levinson et al., 1982; Kriegler et al., 1983, 1984a, b, 1988; Bosze et al., 1986; Miksicek et al., 1986; Celander and Haseltine, 1987; Thiesen et al., 1988; Celander et al., 1988; Choi et al., 1988; Reisman and Rotter. 1989
Papilloma Virus	Campo et al., 1983; Lusky et al., 1983; Spandidos and Wilkie, 1983; Spalholz et al., 1985; Lusky and Botchan, 1986; Cripe et al., 1987; Gloss et al., 1987; Hirochika et al, 1987; Stephens and Hentschel, 1987; Glue et al., 1988
Hepatitis B Virus	Bulla and Siddiqui, 1986; Jameel and Siddiqui, 1986; Shaul and Ben-Levy, 1987; Spandau and Lee, 1988; Vannice and Levinson, 1988
Human Immuno- deficiency Virus	Maesing et al., 1987; Hauber and Cullan, 1988; Jakobovits et al., 1988; Feng and Holland, 1988; Takebe et al., 1988; Rosen et al., 1988; Berkhout et al., 1989; Laspia et al., 1989, Sharp and Marciniak, 1989; Braddock et al., 1989
Cytomegalovirus	Weber et al., 1984; Boshart et al., 1985; Foecking and Hofstetter, 1986
Gibbon Ape Leukemia Virus	Holbrook et al., 1987; Quinn et al., 1989

FIGURE 6

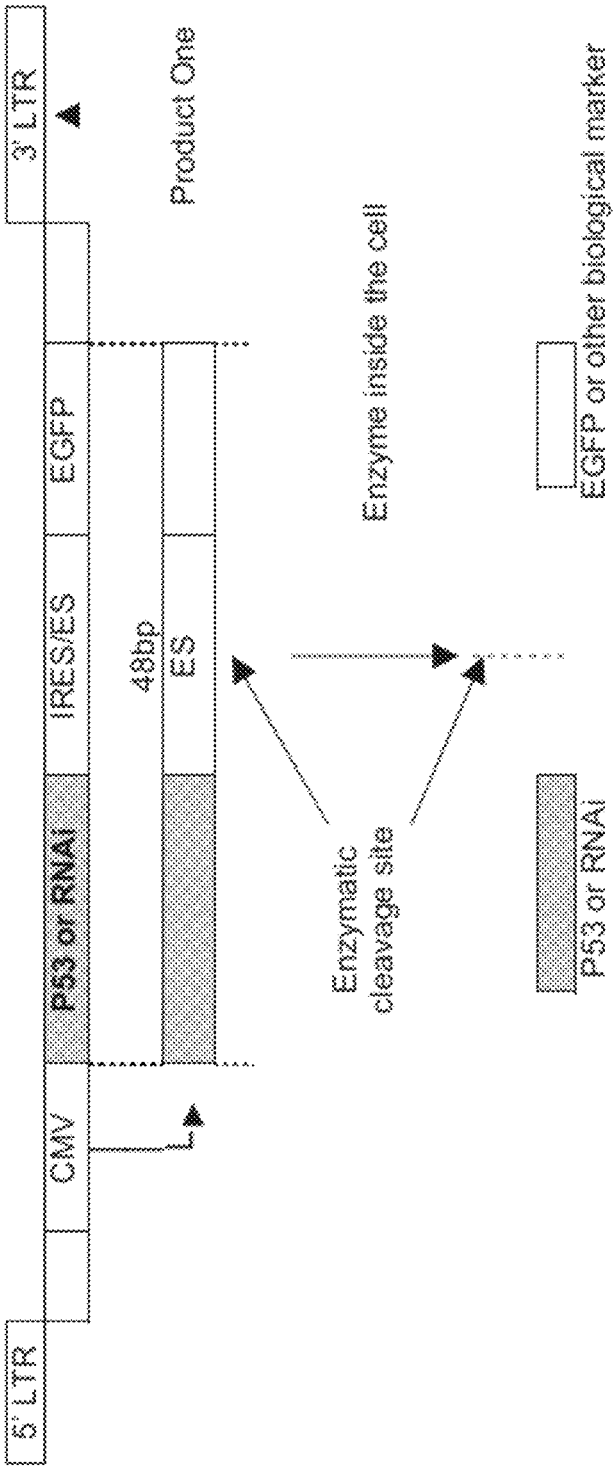
5' LTR
CMV
P53 or RNAi
IRES/ES
EGFP
3' LTR
48bp
ES
Product One
Enzymatic cleavage site
Enzyme inside the cell
P53 or RNAi
EGFP or other biological marker

FIGURE 7

ELEMENT / INDICATION	First Regulatory Nucleic Acid Sequence	First Heterologous Nucleic Acid Sequence	Second Regulatory Nucleic Acid Sequence	Second Heterologous Nucleic Acid Sequence	Reference
PROSTATE CANCER	CMV	P53	hPSA None HIV-1at VP22	Bcl-2 RNAi	
PANCREATIC CANCER	CMV	P53	hCCKAR None HIV-Tas VP22	K-ras RNAi	
LIVER CANCER	CMV	P23	hAFP None HIV-Tas VP22	Bcl-2 RNAi	
GAUCHER'S DISEASE	CMV None VP22	Glucocerebrosidase	IRES/Cleavage site None	Human intrinsic selectable marker, e.g.: IL-2Ra (huCD25) huNGF	
FABRY'S DISEASE	CMV None VP22	Alpha Galactosidase A	IRES/Cleavage site None	IL-2Ra (huCD25)	
ALZHEIMER'S DISEASE	CMV None VP22	hNGF	Cleavage site/hNSE	AAP (beta-amyloid precursor protein) RNAi	
PARKINSON'S DISEASE	CMV None VP22	hNGF	Cleavage site/hNSE	AAP (beta-amyloid precursor protein) RNAi + third gene CS-hBDNF (brain-derived neurotropic factor	
PARKINSON'S DISEASE	CMV None	hBDNF (brain-derived neurotropic factor)	Cleavage site/hNSE	hGAD (human glutamic acid decarboxylase)	
PARKINSON'S DISEASE	CMV VP22	hBDNF (brain-derived neurotropic factor)	Cleavage site/hNSE	hGAD (human glutamic acid decarboxylase) + third gene CS-hNGF	
LEBER CONGENITAL AMAUROSIS (blindness and optic nerve disease)	CMV	RPE65 (retinal pigment epithelium)	Cleavage site/hNSE	hBDNF (brain-derived neurotropic factor)	

FIGURE 7 (cont.)

ELEMENT / INDICATION	First Regulatory Nucleic Acid Sequence	First Heterologous Nucleic Acid Sequence	Second Regulatory Nucleic Acid Sequence	Second Heterologous Nucleic Acid Sequence	Reference
LEBER CONGENITAL AMAUROSIS (blindness and optic nerve disease)	CMV VP22	RPE65 (retinal pigment epithelium)	Cleavage site/hNSE	hBDNF (brain-derived neurotropic factor) + third gene CS-hNGF	
COSMETIC Anti-Wrinkle Gene Reagent Formulate 1	CMV	MMP (which one?) RNAi	Cleavage site	TL (tropoelastin) RNAi	
COSMETIC Anti-Wrinkle Gene Reagent Formulate 2	CMV	Collagen type I alpha 1	Cleavage site	FE (fibroblast elastases)	
COSMETIC Anti-Wrinkle Gene Reagent Formulate 3	CMV	SOD (superoxide dismutase)	Cleavage site	??	
COSMETIC Hair-enhancement Reagent	CMV	SH (sonic hedgehog)	Cleavage site	DSG4 (desmoglein 4)	

Fig. 7 - 2

FIGURE 7 (cont.)

ELEMENT / INDICATION	Cloned Structural Gene	Clone Type	Exemplary Construct	Reference
Adrenal Gland Disease	Activin	Porcine-cDNA	LV - CMV - p-cDNA - (CS) - EGFP	Mason AJ, Nat, 318:659 1985
Coronary Artery Disease	Adenosine Deaminase	h-cDNA	LV - CMV - h-cDNA - (CS) - RNAi (fatty acid)	Wiginton DA, PNAS, 80:7481, 1983
Cardiovascular Disease	Angiotensinogen I	r-cDNA r-gDNA	LV - CMV - (r)cDNA - (CS) - r-gDNA	Ohkubo H, PNAS, 80:2196, 1983; Tanaka T, JBC, 259:8063, 1984
Congenital Antithrombin IV Deficiency Liver Disease	Antithrombin III	h-cDNA h-cDNA and gDNA	LV - CMV - h-cDNA - (CS) - AFP-gDNA	Bock SC, NAR 10:8113, 1982, Prochownik EV, JBC, 258:8389, 1983
Alpha-1 Antitrypsin Deficiency Lung Disease	Antitrypsin, alpha 1	h-cDNA h-gDNA RFLP	LV - CMV - h-cDNA - (CS) - h-gDNA (RFLP)	Kurachi K, PNAS, 78:6826, 1981; Leicht M, Nat, 297:655, 1982; Cox DW, AJHG, 36:1348, 1984
Tangier Disease with Coronary Artery Disease	Apolipoprotein A-I	h-cDNA, l-gDNA RFLP h-gDNA	LV - CMV - h-cDNA - (CS) - h-gDNA - (CS) - RFLP	Shoulders CC, NAR, 10:4873, 1982; Karathanasis SK, Nat, 301:718, 1983; Karathanasis SK, PNAS, 80:6147, 1983
Coronary Artery Disease	Apolipoprotein A-II	h-cDNA Chr h-cDNA	LV - CMV - h-cDNA - (CS) - Chr	Sharpe CR, NAR, 12:3917, 1984; Sakaguchi, AY, AJHB, 36:2073, 1984, Knott TJ, BBRC, 120:734, 1984
Risk Factor for Alzheimer's Disease	Apolipoprotein C-I	h-cDNA	LV - CMV - h-cDNA - (CS) - RNAi (B-amyloid)	Knott TJ, NAR, 12:3909, 1984
Premature Vascular Disease	Apolipoprotein C-II	h-cDNA h-cDNA h-cDNA RFLP	LV - CMV - h-cDNA - (CS) RFLP	Jackson CL, PNAS, 81:2945, 1984, Mykelbost O, JBC, 249:4401, 1984; Fojo SS, PNAS, 81:6354, 1984; Humphries SE, CGen, 26:389, 1984
Cardiovascular Disease with Metabolic Syndrome (HLTriacylglycero-rich Lipoprotein	Apolipoprotein C-III	h-cDNA and gDNA h-cDNA	LV - CMV - h-cDNA - (CS) - h-cDNA	Karanthanasis SK, Nat, 304:371, 1983; Sharpe CR, NAR, 12:3917, 1984
Cerebral Palsy Alzheimer's Disease	Apolipoprotein E	h-cDNA	LV - CMV - h-cDNA - (CS) - RNAi (B-amyloid)	Brewslow JL, JBC, 257:14639, 1982

Fig. 7 - 3

FIGURE 7 (cont.)

ELEMENT / INDICATION	Cloned Structural Gene	CloneType	Exemplary Construct	Reference
Congenital Heart Disease	Atrial Natriuretic Factor	h-cDNA h-cDNA h-cDNA h-gDNA h-gDNA h-gDNA	LV - CMV - h-gDNA - (CS) - h-cDNA	Oikawa S, Nat, 309:724, 1984; Nakayama K, Nat, 310:699, 1984; Zivin RA, PNAS, 81:6325, 1984; Seidman CE, Sci, 226:1206, 1984; Nemer M, Nat, 312:654, 1984; Greenberg BI, Nat, 312:665, 1984
Crohn's Disease Inflammatory Bowel Disease	Chorionic Gonadotropin, Alpha Chain	h-cDNA RFLP	LV - CMV - h-cDNA - (CS) - RFLP	Fiddes JC, Nat, 281:351, 1981; Boothby M, JBC, 256:5121, 1981
Gestational Trophoblastic Disease	Chorionic Gonadotropin Beta Chain	h-cDNA h-gDNA h-gDNA	LV - CMV - h-cDNA - (CS) - h-gDNA	Fiddes JC, Nat, 286:684, 1980; Boorstein WR, Nat, 300:419, 1982; Talmadge K, Nat, 307:37, 1984
Infection Disease	Chymosin, pre (rennin)	bovine-cDNA	LV - CMV - h-cDNA - (CS) - EGFP	Harris TJR, NAR, 10:2177, 1982
Cardiac Disease	Complement, Factor B	h-cDNA h-cDNA and gDNA	LV - CMV - h-cDNA - (CS) - gDNA	Woods DE, PNBAS, 79:5661, 1982; Duncan R, PNAS, 80:4464, 1983
Grave's Disease	Complement C2	h-cDNA h-gDNA (C2, C4, and B)	LV - CMV - h-cDNA - (CS) - h-gDNA	Bentley DR, PNAS, 81:1212, 1984; Carroll MC, Nat, 307:237, 1984
Renal Disease	Complement C3	m-cDNA h-gDNA	LV - CMV - h-gDNA- (CS) - m-cDNA	Dunkley H, PNAS, 79:7619, 1983; Whitehead AS, PNAS, 79:5021, 1982
Lupus Disease	Complement C4	h-cDNA and gDNA h-cDNA	LV - CMV - h-cDNA - (CS) - gDNA	Carroll MC, PNAS, 80:264, 1983; Whitehead AS, PNAS, 80:5387, 1983
Autoimmune Disease	Complement C9	h-cDNA	LV - CMV - h-cDNA - (CS) - EGFP	DiScipio RC, PNAS, 81:7298, 1984
Alzheimer's Disease A Stress Factor	Corticotropin releasing factor	sheep-cDNA h-gDNA	LV - CMV - h-gDNA - (CS) - RNAi (B-amyloid)	Furutani Y, Nat, 301:537, 1983; Shibahara S, EMBO J, 2:775, 1983
Salivary Disease Prostate Disease/Cancer	Epidermal growth factor	m-cDNA m-cDNA h-gDNA	LV - CMV - h-gDNA - (CS) - m-cDNA	Gray A, Nat, 303:722, 1983; Scott J, Sci, 21:236, 1983; Brissenden JE, Nat, 310:781, 1984
Tongue (oral) Cancer	Epidermal growth factor receptor, oncogene c-erb B	h-cDNA and Chr	LV - CMV - h-cDNA - (CS) - Chr	Lan CR, Sci, 224:843, 1984
Immune Disorder	Epoxide dehydratase	r-cDNA	LV - CMV - r-cDNA - (CS) - EGFP	Gonzalez FJ, JBC, 256:4697, 1981

Fig. 7 - 4

FIGURE 7 (cont.)

ELEMENT / INDICATION	Cloned Structural Gene	CloneType	Exemplary Construct	Reference
Congenital Heart Disease	Erythropoietin	h-cDNA	LV - CMV - h-cDNA - (CS) - RNAi (fatty acids)	Lee-Huang S, PNAS, 81:2708, 1984
Inflammation Disease	Esterase inhibitor, dehydratase	h-cDNA	LV - h-cDNA - (CS) - RNAi (IL-2)	Stanley KK, EMBO J, 3:1429, 1984
N/K	Factor VIII	h-cDNA and gDNA h-cDNA		Gitschier J, Nat, 312:326, 1984;
N/K	Factor IX, Christmas factor	h-cDNA h-cDNA RFLP h-gDNA		Kurachi K, PNAS, 79:6461, 1982; Choo KH, Nat, 299:178, 1982; Camerino G, PNAS, 81:498, 1984; Anson DS, EMBO J, 3:1053, 1984
N/K	Factor X	h-cDNA		Leytus SP, PNAS, 81:3699, 1984
Dysfibrinogenemia Disorder	Fibrinogen A, alpha	h-cDNA	LV - CMV - hc-DNA - (CS) - hNGF	Kant JA, PNAS, 80:3953, 1983
Cardiovascular Disease	B beta, gamma	h-gDNA gamma) h-cDNA alpha gamma) h-gDNA gamma)	LV - CMV - h-gDNA - (CS) - h-cDNA	Fornace AJ, Sci, 224:161, 1984; Imam AMA, NAR, 11:7427, 1983; Fornace AJ, JBC, 259:12826, 1984
Respiratory Disease	Gastrin releasing peptide	h-cDNA	LV - CMV - h-cDNA - (CS) - RNAi (IL-2)	Spindel ER, PNAS, 81:5699, 1984
Human Diabetes	Glucagon, prepro	hamster-cDNA h-gDNA	LV - CMV - h-gDNA - (CS) - h-gDNA	Bell GI, Nat, 302:716, 1983; Bell GI, Nat, 304:368, 1983
Growth Hormone Deficiency and Other Growth Disorders i.e., Creutzfeldt-Jakob Disease	Growth hormone	h-cDNA h-gDNA GH-like gene	LV - CMV - h-cDNA - (CS) - h-gDNA	Martial JA, Sci, 205:602, 1979; DeNoto FM, NAR, 9:3719, 1981; Owerbach, D, Sci, 209:289, 1980
Growth Hormone Deficiency and Other Growth Disorders i.e., Creutzfeldt-Jakob Disease	Growth hormone, RF	h-cDNA	LV - CMV - h-cDNA - (CS) - IGF-I	Gubler V, PNAS, 80:3411, 1983
Acromegaly	Somatocrinin	h-cDNA	LV - CMV - h-cDNA - (CS) - RNAi (GHRH)	Mayo KE, Nat, 306:86:1983
Retina Disease	Hemopexin	h-cDNA	LV - CMV - h-cDNA - (CS) - RNAi (IL-2)	Stanley KK, EMBO J, 3:1429, 1984
Trophoblastic Disease	Inhibin	porcine-cDNA	LV - CMV - p-cDNA - (CS) - EGFP	Mason AJ, Nat, 318:659, 1985

Fig. 7 - 5

FIGURE 7 (cont.)

ELEMENT / INDICATION	Cloned Structural Gene	Clone Type	Exemplary Construct	Reference
Obesity Syndrome	Insulin, prepro	h-gDNA	LV - CMV - h-gDNA - (CS) - RNAi (fatty acid)	Ullrich A, Sci, 209:612, 1980
Ischemic Heart Disease Amyotrophic Lateral Sclerosis	Insulin-like growth factor I	h-cDNA h-cDNA Chr	LV - CMV - h-cDNA - (CS) - Chr	Jansen M, Nat, 306:609, 1983; Bell GI, Nat, 3 10:775, 1984; Brissenden JE, Nat, 310:781, 1984
Chronic Liver Disease	Insulin-like growth factor II	h-cDNA h-gDNA Chr	LV - CMV - h-cDNA - (CS) - h-gDNA - (CS) - Chr.	Bell GI, Nat, 310:775, 1984; Dull TJ, Nat, 310:777, 1984; Brissenden JE, Nat, 310:781, 1984
Hemophilia/Von-Willebrand's Disease Hepatitis Disease	Interferon, alpha (leukocyte), multiple	h-cDNA h-cDNA h-gDNA h-gDNA h-gDNA	LV - CMV - h-cDNA - (CS) - h-gDNA -	Maeda S, PNAS, 77:7010, 1980; Goeddel DV, NAT, 290:20, 1981; Lawn RM, PNAS, 78:5435, 1981; Tadokoro K, EMBO J, 3:1909, 1984; Tonczynski RM, PNAS, 81:6451, 1984
Regulation of Immune Response to Viral Infection Rheumatic/Inflammation Diseases	Interferon, beta (fibroblast)	h-cDNA h-gDNA h-gDNA (related) h-gDNA (related) h-cDNA	LV - CMV - h-gDNA - (CS) - h-cDNA	Taniguchi T, Gene; 10:11, 1980; Lawn RM, NAR, 9:1045, 1981; Sehgal P, PNAS, 80:3632, 1983; Sagar AD, Sci, 223:1312, 1984; Gray PW, Nat, 295:503, 1982
Rheumatoid Arthritis Disease	Interferon, gamma (immune)	h-cDNA h-gDNA	LV - CMV - h-cDNA - (CS) - h-gDNA	Gray PW, Nat, 298:859, 1982
Rheumatoid Arthritis	Interleukin-1	m-cDNA	LV - CMV - m-cDNA - (CS) - EGFP	Lomedico PT, Nat, 312:458, 1984
HIV-Therapy	Interleukin-2	h-cDNA	LV - CMV - h-cDNA - (CS) - RNAi (Pol)	Devos R, NAR, 11:4307, 1983
Immunoregulatory = IL-2	T-cell Growth factor	h-cDNA h-gDNA Chr	LV - CMV - h-cDNA - (CS) - h-gDNA - (CS) - Chr	Taniguchi T, Nat, 302:305, 1983; Hulbrook NJ, PNAS, 81:1634, 1984; Siegg LF, Sci, 223:175, 1984
Autoimmune Disease Myeloproliferative Disease	Interleukin-3	m-cDNA	LV - CMV - m-cDNA - (CS) - EGFP	Fung MC, Nat, 307:233, 1984
Rheumatoid Disease	Kininogen, two forms	bovine-cDNA bovine,-cDNA and gDNA	LV - CMV - h-cDNA - (CS) - gDNA	Nawa H, PNAS, 80:90, 1983; Kitamura N, Nat, 305:542, 1983
Gynecologic Disease	Leutenizing hormone, beta subunit	h-gDNA and Chr	LV - CMV - h-gDNA - (CS) - Chr	Talmadge K, Nat, 307:37, 1984

Fig. 7 - 6

FIGURE 7 (cont.)

ELEMENT / INDICATION	Cloned Structural Genes	CloneType	Exemplary Construct	References
Disorders of Reproductive Endocrine System	Leutinizing hormone releasing hormone	h-cDNA and gDNA	LV - CMV - h-cDNA - (CS) - gDNA	Seeburg PH, Nat, 311:666, 1984
Liver Related Disease	Lymphotoxin	h-cDNA and gDNA	LV - CMV - h-cDNA - (CS) - gDNA	Gray PW, Nat, 312:721, 1984
Cutaneous Mastocytoma Atherosclerosis	Mast cell growth factor	m-cDNA	LV - CMV - m-cDNA - CS) - RNAi	Yokoya T, PNAS, 81:1070, 1984
Neurological Disorders	Nerve growth factor, beta subunit	m-cDNA h-gDNA Chr	LV - CMV - h-gDNA - (CS) - Chr - (CS) - NGF	Scott J, Nat, 302:538, 1983; Ullrich A, Nat, 303:821, 1983; Franke C, Sci, 222:1248, 1983
Autoimmune Disease	Oncogene, c-sis, PGDF	h-gDNA	LV - CMV - h-gDNA - CS) - P53	Dalla-Favera R, Nat, 295:31, 1981
Pancreatic Disease	Pancreatic polypeptide and fragments	h-cDNA h-cDNA	LV - CMV - h-cDNA - (CS) - h-gDNA	Clarke MF, Nat, 308:464, 1984; Boel E, EMBO J, 3:909, 1984
Pseudo Hypoparathyroidism	Parathyroid hormone, prepro	h-cDNA h-gDNA	LV - CMV - h-cDNA - (CS) - h-gDNA	Hendy GN, PNAS, 78:7365, 1981; Vasicek TJ, PNAS, 80:2127, 1983
Liver Disease	Plasminogen	h-cDNA and gDNA	LV - CMV - h-cDNA - (CS) - gDNA	Malinowski DP, Fed P, 42:1761, 1983
Cardiometabolic Disorder	Plasminogen activator	h-cDNA h-cDNA h-gDNA h-cDNA t-gDNA	LV - CMV - h-cDNA - (CS) - h-gDNA - (CS) - t-gDNA	Edlund T, PNAS, 80:349, 1983, Pennica D, Nat, 301:214, 1983, Ny T, PNAS, 81:5355, 1984; Cook NE, JBC, 256:4007, 1981, Cooke NE, Nat, 297:603, 1982
Cushing's Disease	Proopiomelanocortin	h-cDNA h-gDNA	LV - CMV - h-cDNA - (CS) - h-gDNA	Herbert ER, Sci, 220:721, 1983, Cochet M, Nat, 297:335, 1982
Congenital Thrombotic Disease	Protein C	h-cDNA	LV - CMV - h-cDNA - (CS) - h-cDNA	Foster D, PNAS, 81:4766, 1984
Coronary Heart Disease	Prothrombin	bovine-cDNA	LV - CMV - h-cDNA - (CS) - GFP	MacGillivray RTA, PNAS, 77:5153, 19850
Vasoconstriction Disease	Relaxin	h-gDNA h-cDNA (2 genes) Chr	LV - CMV - h-gDNA - (CS) - h-cDNA - (CS) - Chr.	Hudson P, Nat, 301:628, 1983; Hudson P, EMBO J, 3:2333, 1984, Crawford, RJ, EMBO J, 3:2341, 1984

Fig. 7 - 7

FIGURE 7 (cont.)

ELEMENT / INDICATION	Cloned Structural Gene	CloneType	Exemplary Construct	Reference
Diabetic Renal Disease	Renin, prepro	h-cDNA h-gDNA h-gDNA Chr	OV - CMV - h-cDNA - (CS) - h-gDNA - (CS) - Chr	Imai T, PNAS, 80:7405, 1983; Hobart PM, PNAS 81:5026, 1984; Miyazaki H, PNAS, 81:5999, 1984; Chirgwin JM, SCMG, 10:415, 1984
Pituitary Disease Huntington's Disease	Somatostatin	h-cDNA h-gDNA and Ri-IP	LV - CMV - h-cDNA - (CS) - h-gDNA - (CS) - Ri-IP	Shen IF, PNAS, 79:4575, 1982; Naylor SI, PNAS, 80:2088, 1983
Inflammatory Disease	Substances P & K	bovine-gDNA	LV - CMV - h-gDNA - (CS) - opioid analogous gene	Nawa H, Nat, 312:729, 1984
Gastroenterological Therapy	Tachykinin, prepro,	bovine-cDNA	LV - CMV - h-cDNA - (CS) - EGFP	Nawa H, Nat, 306:32, 1983
Thromboembolic Disease	Urokinase	h-cDNA	LV - CMV - h-cDNA - (CS) - NGF	Verde P, PNAS, 81:4727, 1984

Fig. 7 - 8

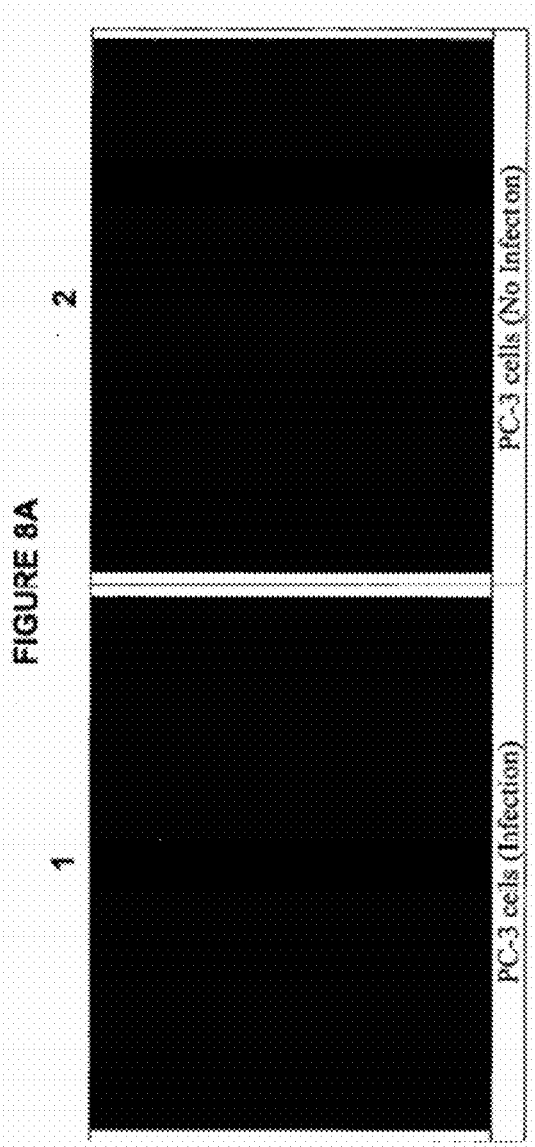

FIGURE 8A

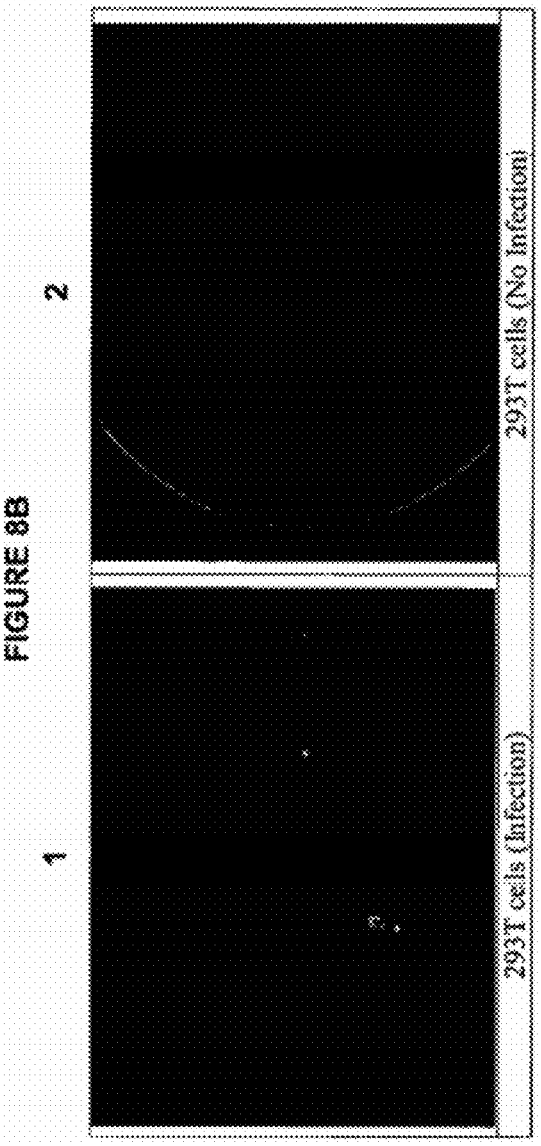

FIGURE 8B

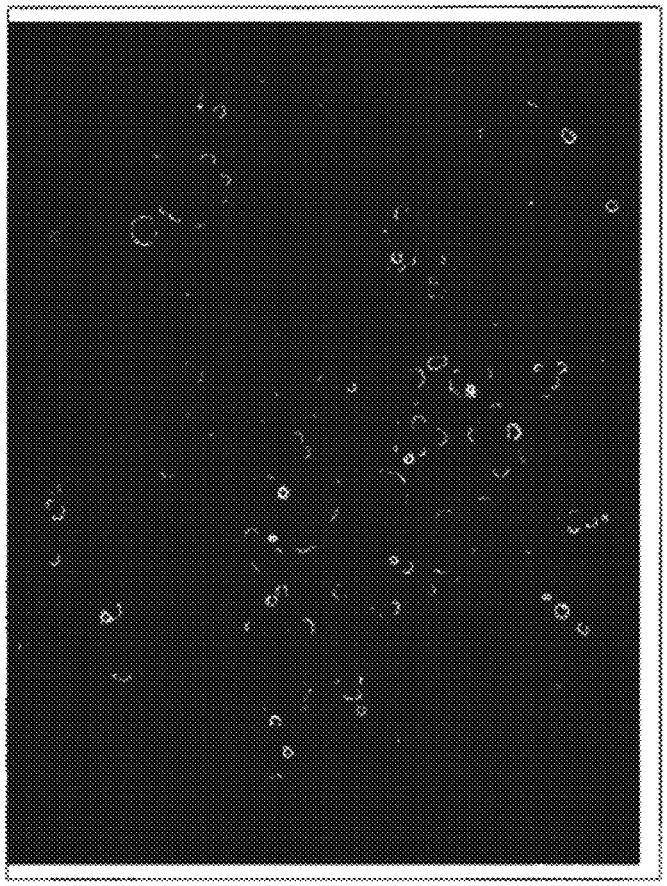

FIGURE 8C

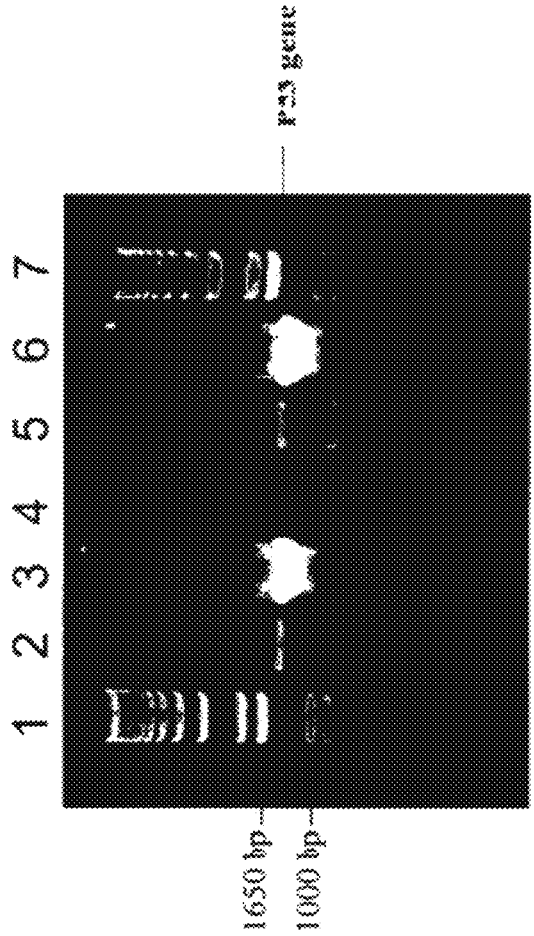

FIGURE 8D

Figure 9

Selected Cloned Structural Genes

		Gene	Clone Type	Reference
1	LV-CMV-pcDNA-(cs)-EGFP	Activin	Porcine-cDNA Adrenal gland disease	Mason AJ, Nat., 318:659, 1985
2	LV-CMV-hcDNA-(cs)-RNAi (fatty acid)	Adenosine deaminase (Coronary Artery disease)	h-cDNA	Wigmon DA, PNAS, 80:7481, 1983
3	LV-CMV-(r)cDNA-(cs)-r-gDNA	Angiotensinogen1 (Cardiovascular disease)	r-cDNA r-gDNA	Ohkubo H, PNAS, 80:2196, 1983; Tanaka T, JBC, 259: 8063, 1984
4	LV-CMV-hcDNA-(cs)-AFP-gDNA Liver disease	Antithrombin III (Congenital antithrombin IV deficiency)	h-cDNA h-cDNA and gDNA	Bock SC, NAR 10:8113, 1982; Prochownik EV, JBC, 258:8389, 1983

Figure 9 (cont.)

Selected Cloned Structural Genes

		Gene	Clone Type	Reference
5	LV-CMV-hcDNA-(cs)-h-gDNA(RTLP)	Antitrypsin, alpha1 (α-antitrysin deficiency Lung Disease)	h-cDNA h-gDNA RFLP	Kurachi K, PNAS, 78:6826, 1981; Leicht M, Nat, 297:655, 1982; Cox DW, AJHG, 36:134S, 1984
6	LV-CMV-hcDNA-(cs)-h-gDNA-(cs)-RFLP	Apolipoprotein A-I Tangier disease with coronary artery disease	h-cDNA, h-gDNA RFLP h-gDNA	Shoulders CC, NAR, 10:4873, 1982; Karathanasis SK, Nat, 301: 718, 1983; Karathanasis SK, PNAS, 80:6147, 1983
7	LV-CMV-h-cDNA-(cs)-Chr	Apolipoprotein A-II Coronary artery disease	h-cDNA Chr h-cDNA	Sharpe CR, NAR, 12:3917, 1984; Sakaguchi, AY, AJHB, 36:207S, 1984; Knott TJ, BBRC, 120:734, 1984
8	LV-CMV-hcDNA-(cs)-RNAi(β-amyloid)	Apolipoprotein C-I Risk factor for Alzheimer's disease	h-cDNA	Knott TJ, NAR, 12:3909, 1984
9	LV-CMV-hcDNA-(cs)-RFLP	Apolipoprotein C-II Premature vascular disease	h-cDNA h-cDNA h-cDNA RFLP	Jackson CL, PNAS, 81:2945, 1985; Mykelbost O, JBC, 249:4401, 1984; Fojo SS, PNAS, 81:6354, 1984; Humphries SE, C Gen, 26:389, 1984
10	LV-CMV-hcDNA-(cs)-h-cDNA	Apolipoprotein C-III Cardiovascular disease with metabolic syndrome (TRL = triacylglycerol rich lipoprotein)	h-cDNA and gDNA h-cDNA	Karathanasis SK, Nat, 304:371, 1983; Sharpe CR, NAR, 12:3917, 1984
11	LV-CMV-hcDNA-(cs)-RNAi(β-amyloid)	Apolipoprotein E Cerebral palsy Alzheimer's disease	h-cDNA	Breslow JL, JBC, 257: 14639, 1982

Figure 9 (cont.)

Selected Cloned Structural Genes

		Gene	Clone Type	Reference
12	LV-CMV-h-gDNA-(cs)-hcDNA	Atrial natriuretic factor Congenital heart disease	h-cDNA h-cDNA h-cDNA h-gDNA h-gDNA h-gDNA	Oikawa S, Nat. 309:724, 1984; Nakayama K, Nat, 310:699, 1984; Zivin RA, PNAS, 81: 6328, 1984; Seidman CE, Sci, 226: 1206, 1984; Nemer M, Nat, 312:654, 1984; Greenberg BI, Nat, 312:665, 1984
13	LV-CMV-hcDNA-(cs)-RFLP	Chorionic gonadotropin, alpha chain Crohn's disease Inflammatory bowel disease	h-cDNA RFLP	Fiddes JC, Nat, 281:351, 1981; Boothby M, JBC, 256:5121, 1981
14	LV-CMV-hcDNA-(cs)-h-gDNA	Chorionic gonadotropin, beta chain Gestational trophoblastic disease	h-cDNA h-gDNA h-gDNA	Fiddes JC, Nat. 286:684, 1980; Boorstein WR, Nat, 300:419, 1982; Talmadge K, Nat, 307:37, 1984
15	LV-CMV-h-cDNA-(cs)-EGFP	Chymosin, pro (renain) Infectious disease	Bovine-cDNA	Harris TJR, NAR, 10:2177, 1982.
16	LV-CMV-hcDNA-(cs)-gDNA	Complement, factor B Coelinedisease	h-cDNA h-cDNA and gDNA	Woods DE, PNAS, 79:5661, 1982; Duncan R, PNAS, 80:4464, 1983
17	LV-CMV-hcDNA-(cs)-hgDNA	Complement C2 Grave's Disease	h-cDNA h-Gdna (C2, C4, and B)	Bentley DR, PNAS, 81:1212, 1984; Carroll MC, Nat, 307: 237, 1984
18	LV-CMV-hcDNA-(cs)-m-Cdna	Complement C3 Renal disease	m-cDNA h-gDNA	Domsley IL PNAS, 79:7619, 1983; Whitehead AS, PNAS, 79:8021, 1982
19	LV-CMV-h-cDNA-(cs)-gDNA	Complement C4 Lupus disease	h-cDNA and gDNA	Carroll MC, PNAS, 80:264

Figure 9 (cont.)

Selected Cloned Structural Genes

		Gene	Clone Type	Reference
20	LV-CMV-hcDNA-(cs)-EGFP	Complement C9	h-cDNA h-c-DNA	1983; Whitehead AS, PNAS, 80:5387, 1983 DiScipio RC, PNAS, 81:7298, 1984
21	LV-CMV-h-gDNA-(cs)RNAi (β-amyloid)	Corticotropin releasing factor Alzheimer's disease A stress factor	Sheep-cDNA h-gDNA	Furutani Y, Nat, 301:537, 1983; Shibahara S, EMBO J, 2:775, 1983
22	LV-CMV-h-gDNA-(cs)-m-cDNA	Epidermal growth factor Salivary disease Prostate disease/cancer	m-cDNA m-cDNA h-gDNA	Gray A, Nat, 303:722, 1983; Shibahara S, EMBO J, 2: 775, 1983
23	LV-CMV-h-cDNA-(cs)-Chr	Epidermal growth factor receptor, oncogene c-erb B Tongue (oral) cancer	h-cDNA and Chr	Lax CR, Sci, 224:843, 1984
24	LV-CMV-r-cDNA-(cs)-EGFP	Epoxide dehydratase Immuno disorder	r-cDNA	Gonzalez FI, JBC, 256:4697, 1981
25	LV-CMV-h-cDNA-(cs)-RNAi (fatty acid)	Erythropoietin Congenital heart disease	h-cDNA	Lee-Huang S, PNAS, 81:2708, 1984
26	LV- hcDNA-(cs)-RNAi (IL-2)	Esterase inhibitor, dehydratase Inflammation disease	h-cDNA	Stanley KK, EMBO J,3:1429, 1984
27		Factor VIII	h-cDNA and gDNA h-cDNA	Gitschier J, Nat, 312:326, 1984; Toole JJ, Nat, 312:342, 1984
28		Factor IX, Christmas factor	h-cDNA h-cDNA RFLP h-gDNA	Kurachi K, PNAS, 79:6461, 1982; Cisso KH, Nat, 299:178, 1982; Camerino G, PNAS, 81:498, 1984; Anson DS, EMBO J, 3: 1053, 1984
29		Factor X	h-cDNA	Leytus SP, PNAS, 81:3699, 1984

Figure 9 (cont.)

Selected Cloned Structural Genes

		Gene	Clone Type	Reference
29	LV-CMV-hcDNA-(cs)-hNGF	Fibrinogen A, alpha Dysfibrinogenemia disorder	h-cDNA	Kant JA, PNAS, 80:3953, 1983
30	LV-CMV-h-gDNA-(cs)-h-cDNA	B beta, gamma Cardiovascular disease	h-gDNA (gamma) h-cDNA (alpha gamma) h-gDNA(gamma)	Fornace AH, Sci, 224:161, 1984; Inami AMA, NAR, 11:7427, 1983; Fornace AJ, JBC, 259:12826, 1984
31	LV-CMV-h-cDNA-(cs)-RNAi(IL-2)	Gastrin releasing peptide Respiratory disease	h-cDNA	Spindel ER, PNAS, 81:5699, 1984
32	LV-CMV-h-Gdna-(cs)-h-insulin	Glucagon, prepro Human diabetes	hamster-cDNA h-gDNA	Bell GI, Nat, 302:716, 1983; Bell GI, Nat, 304:368, 1983
33	LV-CMV-h-cDNA-(cs)-h-gDNA	Growth horomone Growth horomone deficiency and other growth disorders i.e. Creutzfeldt-Jakob disease	h-cDNA h-gDNA GH-like gene	Martial JA, Sci, 205:602, 1979; DeNoto FM, NAR, 9:3719, 1981; Owerbach, D, Sci, 209:289, 1980
34	LV-CMV-h-cDNA-(cs)-IGF-1	Growth horomone, RF Same as above	h-cDNA	Gubler V, PNAS, 80:3411, 1983
35	LV-CMV-h-cDNA-(cs)-RNAi (EHRH)	Somatocrinin Acromegaly	h-cDNA	Mayo KR, Nat, 306:86:1983
36	LV-CMV-h-cDNA-(cs)-BCL-xP	Hemopexin Retina disease	h-cDNA	Stanley KK, BMBO J, 3: 1429, 1984
37	LV-CMV-p-cDNA-(cs)-EGFP	Inhibin Trophoblastic disease	Porcine-cDNA	Mason AJ, Nat, 318:659,1985
38	LV-CMV-h-gDNA-(cs)-RNAi (fatty acid)	Insulin, prepro Obesity syndrome	h-gDNA	Ullrich A, Sci, 209:612, 1980
39	LV-CMV-hcDNA-(cs)-Chr	Insulin-like growth factor I Ischemic heart disease amyotrophic lateral sclerosis	h-cDNA h-cDNA Chr	Jansen M, Nat, 306:609, 1983; Bell GI, Nat, 3 10:775, 1984; Brissenden JE, Nat, 310:781, 1984

Figure 9 (cont.)

Selected Cloned Structural Genes

		Gene	Clone Type	References
40	LV-CMV-h-cDNA-(cs)-Chr	Insulin-like growth factor II Chronic liver disease	h-cDNA h-gDNA Chr	Bell GI, Nat, 310:775, 1984; Dull TJ, Nat, 310:777, 1984; Brissenden JE, Nat, 310:781, 1984
41	LV-CMV-h-cDNA-(cs)-h-gDNA	Interferon, alpha (leukocyte), multiple Hemophilia/Von-Willebrand's disease Hepatitis disease	h-cDNA h-cDNA h-gDNA h-gDNA h-gDNA	Maeda S, PNAS, 77:7010, 1980; Goeddel DV, NAT, 290:20, 1981; Lawn RM, PNAS, 78:5435, 1981; Todokoro K, EMBO J, 3:1809, 1984; Torczynski RM, PNAS, 81:6451, 1984
42	LV-CMV-h-gDNA-(cs)-h-cDNA	Interferon, beta (fibroblast) Regulation of immune response in viral infections Rheumatic/inflammation disease	h-cDNA h-gDNA h-gDNA (related) h-gDNA (related) h-cDNA	Taniguchi T, Gene, 10:11, 1980; Lawn RM, NAR, 9:1045, 1981; Sehgal P, PNAS, 80:3632, 1983; Sager AD, Sci, 223:1312, 1984; Gray PW, Nat, 295:503, 1982;
43	LV-CMV-h-cDNA-(cs)-h-gDNA	Interferon, gamma (immune) rheumatoid arthritis disease	h-cDNA h-gDNA	Gray PW, Nat, 298:859, 1982
44	LV-CMV-h-cDNA-(cs)-EGFP	Interleukin-1 Rheumatoid arthritis	m-cDNA	Lomedico PT, Nat, 312:458, 1984
45	LV-CMV-h-cDNA-(cs)-RNAi(pol)	Interleukin-2 HIV-Therapy	h-cDNA	Devos R, NAR, 11:4307, 1983
46	LV-CMV-hcDNA-(cs)-h-gDNA-(cs)-Chr	T-cell Growth factor Immunoregulatory IL-2	h-cDNA h-gDNA Chr	Taniguchi T, Nat, 302:305, 1983; Hollbrook NJ, PNAS, 81:1634, 1984; Siegel LF, Sci, 223: 175, 1984

FIGURE 9 (cont.)

		Selected Cloned Structural Genes		
		Gene	Clone Type*	Reference
(47)	LC-CMV-m-cDNA-(CS)-EGFP	Interleukin-3 Autoimmune disease myeloproliferating disease	m-cDNA	Fung MC, Nat, 307:233, 1984
(48)	LV-CMV-bcDNA-(CS)-gDNA	Kininogen, two forms Reheumatoid disease	bovine-cDNA bovine, -cDNA and gDNA	Nawa H, PNAS, 80:90,1983; Kitamura N, Nat, 305:545,1983
(49)	LV-CMV-b-gDNA-(CS)-chr	Leutenizing hormone, beta subunit Gynecologic disease	b-gDNA and Chr	Talmadge K, Nat, 307:37, 1984
(50)	LV-CMV-bcDNA(CS)-gDNA	Leutenizing hormone disorders of reproductive endocrine system releasing hormone	b-cDNA and gDNA	Seeburg PH, Nat, 311:666, 1984
(51)	LV-CMV-bcDNA-(CS)-gDNA	Lymphotoxin Liver related disease	b-cDNA and gDNA	Gray PW, Nat, 312:721, 1984
(52)	LV-CMV-m-cDNA-(CS)-RNAi	mast cell growth factor Cutaneus mastocytoma atherosclerosis	m-cDNA	Yokoya T, PNAS, 81:1070, 1984
(53)	LV-CMV-b-gDNA-(CS)-Chr-(CS)-NGF	nerve growth factor, beta subunit Neurological disorders	m-cDNA b-gDNA Chr	Scott J, Nat, 302:538,1983; Ullrich A, Nat, 303:821, 1983; Franke C, Sci, 222:1248, 1983
(54)	LV-CMV-b-gDNA-(CS)-P53	oncogene, c-sis, PGDF autoimmune disease	b-gDNA	Dalla-Favera R, Nat, 295:31, 1981
(55)	LV-CMV-b-cDNA-(CS)-RNAi	Pancreatic polypeptide and icosapeptide pancreatic disease	b-cDNA b-cDNA	Clarke MF, Nat, 308:464, 1984 Boel E, EMBO J, 3:909, 1984
(56)	LV-CMV-bcDNA-(CS)-bgDNA	Parathyroid hormone, prepro Pseudohypopara thyroidi-ism	b-cDNA b-gDNA	Hendy GN, PNAS, 78:7365, 1981; Vasicek TJ, PNAS, 80:2127, 1983
(57)	LV-CMV-bcDNA-(CS)-gDNA	Plasminogen Liver disease	b-cDNA and gDNA	Malinowski DP, Fed P, 42:1761, 1983
(58)	LV-CMV-bcDNA-(CS)-bgDNA-(CS)-r-g-DNA	Plasminogen activator Cardiometabolic disorder	b-cDNA b-cDNA b-gDNA	Edlund T, PNAS, 80:349, 1983; Pennica D, Nat, 301:214, 1983; Ny T, PNAS

FIGURE 9 (cont.)

			Selected Cloned Structural Genes	
		Gene	Clone Type[a]	Reference
			h-cDNA r-gDNA	81:5355, 1984; Cook NE, JBC, 256:4007, 1981; Cooke NE, Nat. 297:603, 1982
(59)	LV-CMV-h-cDNA-(CS)-h-gDNA	Proopiomelanocortin Cushing's disease	h-cDNA h-gDNA	Debold CR, Sci, 220:721, 1983; Cochet M, Nat, 297:335, 1982
(60)	LV-CMV-h-cDNA-(CS)-bcDNA	Protein C Congenital thrombotic disease	h-cDNA	Foster D, PNAS, 81:4766, 1984
(61)	LV-CMV-bcDNA-(CS)-GFP	Prothrombin Coronary heart disease	bovine-cDNA	MacGillivray RTA, PNAS, 77:5153, 1980
(62)	LV-CMV-h-gDNA-(CS)-h-cDNA-(CS)-chr	Relaxin Vasoconstriction disease	h-gDNA h-cDNA (2 genes) Chr	Hudson P, Nat, 301:628, 1983; Hudson P, EMBO J, 3:2333, 1984; Crawford RJ, EMBO J, 3:2341, 1984
(63)	LV-CMV-h-cDNA-(CS)-h-gDNA-(CS)-Chr	renin, prepro diabetic renal disease	h-cDNA h-gDNA h-gDNA Chr	Imai T, PNAS, 80:7405, 1983; Hoban PM, PNAS, 81:5026, 1984; Miyazaki H, PNAS, 81:5999, 1984; Chirgwin JM, SCMG, 10:415, 1984
(64)	LV-CMV-bcDNA-(CS)-h-gDNA-(CS)-Ri-IP	Somatostatin Pituitary disease huntington's disease	h-cDNA h-gDNA and Ri-IP	Shen IP, PNAS, 79:4575, 1982, Naylor SI, PNAS, 80:2686, 1983
(65)	LV-CMV-h-gDNA-(CS)-opioid analogous gene	Substances P & K inflammatory disease	bovine-gDNA	Nawa H, Nat, 312:729, 1984
(66)	LV-CMV-h-cDNA-(CS)-EGFP	Tachykinin, prepro. Gastroenterological therapy	bovine-cDNA	Nawa, Nat, 306:32, 1983
(67)	LV-CMV-bcDNA-(CS)-NGF	Urokinase Thromboembolic disease	h-cDNA	Verde P, PNAS, 81:4727, 1984

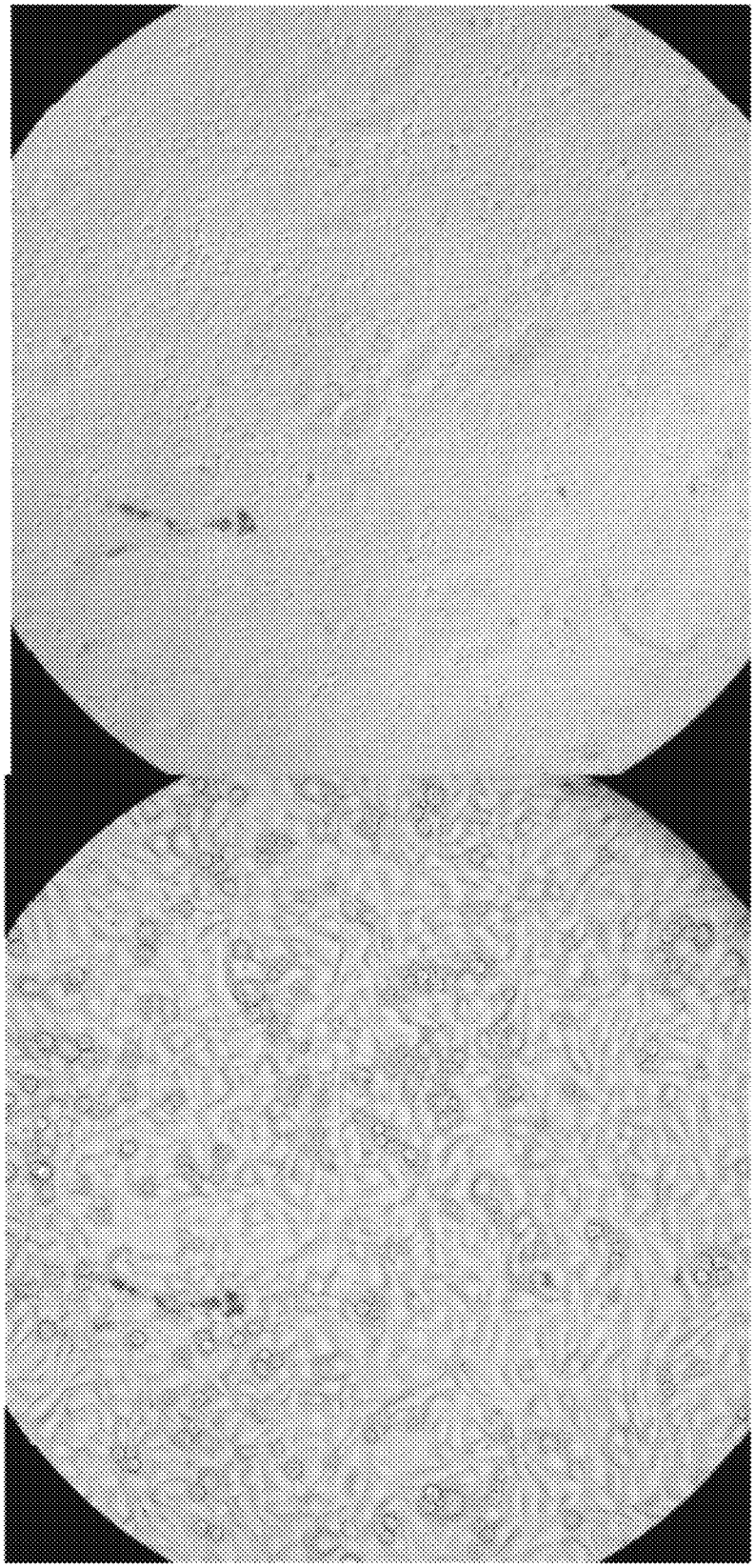

FIG. 10

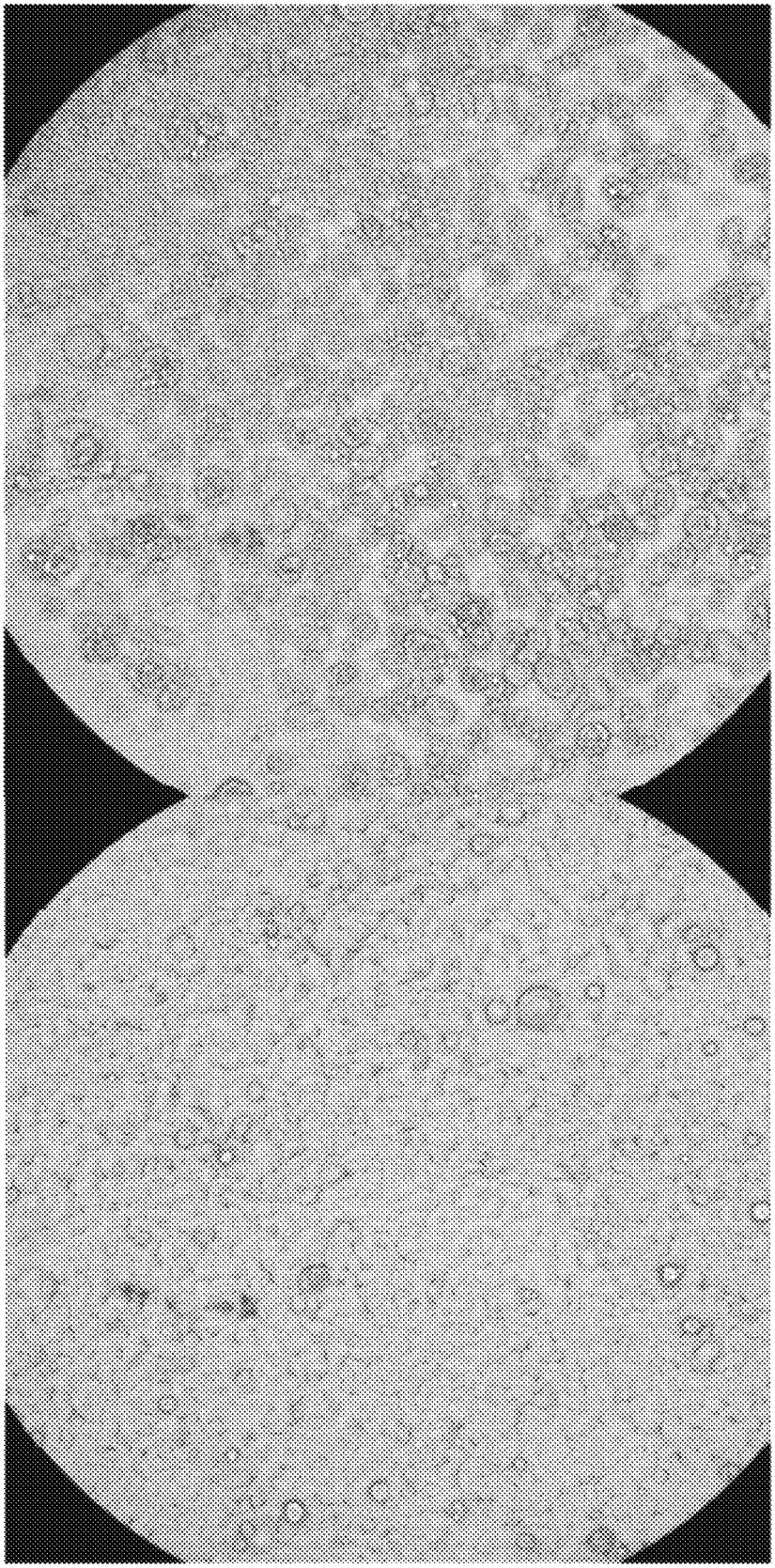

FIG. 11

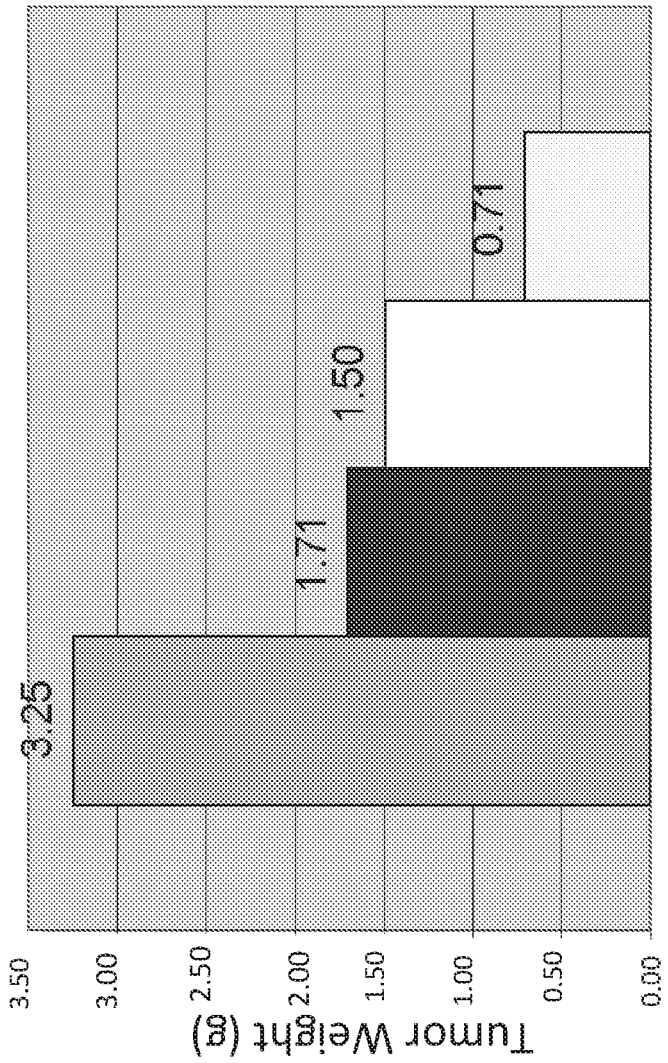

FIG. 12

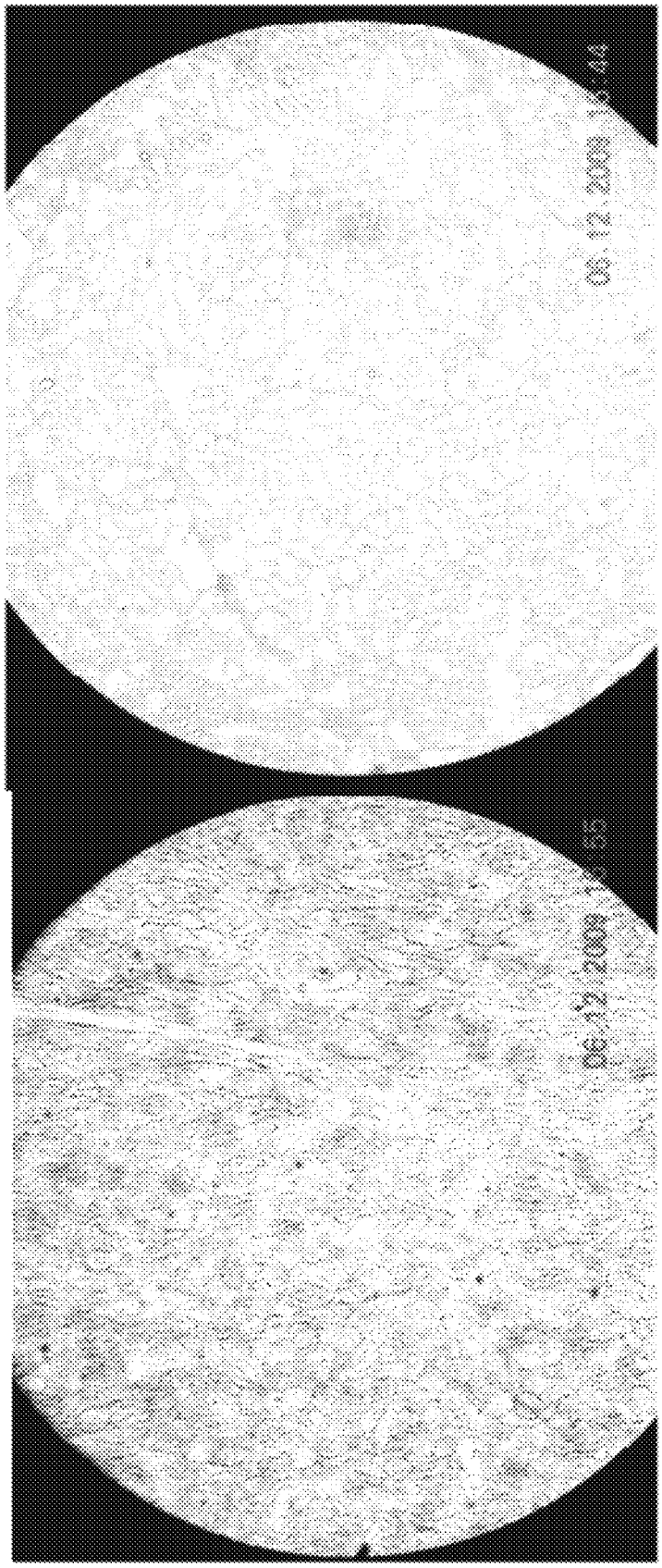

FIG. 13

FIG. 14A

Group Name	Day 7		Day 12		
	Tumor Size L x W x H = Vol. (mm^3)		Tumor Size L x W x H = Vol. (mm^3)		Tumor Growth Rate**
	Individual	Average	Individual	Average	
V1	180.00	309.6	31.50	61.3	-80.2%
	224.00		90.00		
	360.00		64.00		
	300.00		24.00		
	432.00		96.00		
V2	540.00	346.8	72.00	72.6	-79.1%
	240.00		90.00		
	440.00		63.00		
	264.00		50.00		
	252.00		70.00		
V3	450.00	393.1	126.00	99.4	-74.7%
	360.00		140.00		
	260.00		100.00		
	420.00		64.00		
	405.00		50.00		
PC	64.00	34.6	90.00	109.8	217.8%
	6.00		48.00		
	50.00		162.00		
	75.00		120.00		
	12.00		150.00		
Remark	1st time drug injection		2nd time drug injection		

FIG. 14B

Group Name	Day 16			Day 21		
	Tumor Size L x W x H = Vol. (mm^3)		Tumor Growth Rate	Tumor Size L x W x H = Vol. (mm^3)		Tumor Growth Rate
	Individual	Average		Individual	Average	
V1	8.00	41.8	-86.5%	0.50	10.2	-96.7%
	62.50			8.00		
	45.00			30.00		
	6.00			0.02		
	147.00			60.00		
V2	31.50	45.4	-86.9%	15.00	23.0	-93.4%
	60.00			40.00		
	70.00			40.00		
	13.50			1.00		
	62.50			48.00		
V3	90.00	44.8	-88.6%	30.00	17.1	-95.7%
	84.00			48.00		
	67.50			18.00		
	22.50			18.00		
	4.00			0.20		
PC	150.00	143.5	315.3%	225.00	246.2	612.5%
	70.00			165.00		
	192.00			360.00		
	100.00			234.00		
	220.00			200.00		
Remark	3rd time drug injection			4th time drug injection		

FIG. 14C

Group Name	Day 28					
	Tumor Size L x W x H = Vol. (mm^3)		Tumor Growth Rate	Tumor Weight (mg)		Ratio of Tumor Weight/Starting Tumor Size** (mg/mm^3)
	Individual	Average		Individual	Average	
V1	180.00	20.5	-93.4%	280.0	132.0	0.43
	24.00			220.0		
	20.00			90.0		
	0.00			30.0		
	6.00			40.0		
V2	25.00	33.0	-90.5%	270.0	250.0	0.72
	250.00			350.0		
	12.00			200.0		
	10.00			400.0		
	6.00			30.0		
V3	160.00	24.9	-93.7%	220.0	176.0	0.45
	40.00			290.0		
	1.20			190.0		
	24.00			140.0		
	0.10			40.0		
PC	1050.00	1026.4	28/0.0%	1350.0	1303.0	37.70
	1260.00			1170.0		
	960.00			1210.0		
	1600.00			1580.0		
	450.00			1205.0		
Remark	3th time injection					

SAFE LENTIVIRAL VECTORS FOR TARGETED DELIVERY OF MULTIPLE THERAPEUTIC MOLECULES

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application is a continuation application of U.S. application Ser. No. 12/581,871 filed Oct. 19, 2009, which claims the benefit of priority to U.S. Provisional Application No. 61/243,121, filed Sep. 16, 2009; U.S. Provisional Application No. 61/116,138, filed Nov. 19, 2008; and U.S. Provisional Application No. 61/196,457, filed Oct. 17, 2008, the contents of which are incorporated by reference herein in their entirety.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to the field of medicine, specifically to delivery of multiple therapeutic molecules strategically combined with regulatory elements, using a safe for human use, highly and long-term expressing in human subject lentiviral gene transfer vector for the treatment of a condition, disease or disorder.

2. General Background and State of the Art

The present application relates to a gene transfer vector that provides versatility, control, high expression, stable multiple gene expression, tolerability, and safety, for the design of effective gene therapies for disease. Virus vectors in development each have limitations for researchers to address.

Identification of target genes involved in neoplastic transformation and tumor progression has encouraged the idea that nucleotide sequences of cancer-relevant genes could lead to the development of tailored anticancer agents that lack many of the toxic side effects of traditional cytotoxic drugs. Mutations of the p53 gene are associated with transformation to a malignant phenotype. Transfer of wild-type P53, which plays a critical role in the regulation of cell growth and downregulation of genes that contribute to cancer progression, is hoped to result in selective and specific inhibition of tumor growth while minimizing undesirable side effects on normal cells. Delivery of the p53 gene has been reported in a replication-deficient adenoviral vector containing the wild-type p53 gene sequence. Functional activity and expression of the transgene product in tumor cells treated with the adenoviral vector has been reported. (Baker, et al., 1990, Suppression of human colorectal carcinoma cell growth by wild-type p53, Science 249: 912-915.) Two adenovirus-based gene therapeutics for the treatment of cancer were recently commercialized in China. These two agents, combined with chemotherapy, have been used there as an alternative treatment for some types of refractory cancer. (Peng, 2005, Current Status of Gendicine in China: Recombinant Human Ad-p53 Agent for Treatment of Cancers, Hum. Gene Ther. 16, 1016-1027; Yu, W., and Fang, H., 2007, Clinical Trials with Oncolytic Adenovirus in China. Curr. Cancer Drug Targets 7, 141-148). Although adenovirus vectors can efficiently deliver therapeutic genes in both dividing and non-dividing cells, and can be manufactured at high viral titers, adenovirus vectors are highly immunogenic (Shirakawa, et al., 2008, The Current Status of Adenovirus-based Cancer Gene Therapy, Mol. Cells 25(4): 462-466). Furthermore, long-term expression in target tissues is not observed.

Lentiviruses, such as HIV, are "slow viruses." Vectors derived from lentiviruses can be expressed long-term in the host cells after a few administrations to the patients, e.g., via ex vivo transduced bone marrow stem cells. For most diseases and disorders, including genetic diseases, cancer, and neurological disease, long-term expression is crucial to successful treatment. Safety has been a concern with lentiviral vectors, but a number of strategies for eliminating the ability of lentiviral vectors to replicate have now been described. For example, the deletion of promoter and enhancer elements from the U3 region of the long terminal repeat (LTR) are thought to have no LTR-directed transcription. The resulting vectors are called "self-inactivating" (SIN). However, it has been reported that HIV-1-derived vectors containing the SIN deletion in the U3 region of the LTR are capable of expressing full-length genomic transcripts (Logan, et al., 2004, Integrated Self-Inactivating Lentiviral Vectors Produce Full-Length Genomic Transcripts Competent for Encapsidation and Integration, J. Virology 78(16): 8421-8436). Therefore, combination of this deletion with other safety measures must be considered.

The last few years have seen immense excitement regarding the use of RNAi agents for disease therapies. Investigators reported that they were able to specifically silence mutant oncogenic ras without affecting wild-type ras in vitro (Zhang, et al., 1995, Safety evaluation of Ad5CMV-p53 in vitro and in vivo, Human Gene Therapy 6:155-164; Ishii, et al., 2001, Potential cancer therapy with the fragile histidine triad gene review of the preclinical studies, JAMA 286: 2441-2449). It is believed that treatment costs for siRNA would be similar to most protein-based therapies, e.g., antibody therapies. Preclinical cancer studies have shown inhibition of growth and survival of tumor cells by RNAi-mediated downregulation of several key oncogenes or tumor-promoting genes, including growth and angiogenic factors or their receptors (vascular endothelial growth factor, epidermal growth factor receptor), human telomerase (hTR, hTERT), viral oncogenes (papillomavirus E6 and E7) or translocated oncogenes (BCR-abl).

Various studies report on the in vivo activity and the potential of RNAi agents to suppress tumor growth. These include an intratumoral injection of an shRNA-adenoviral vector construct targeting a cell-cycle regulator causing inhibition of subcutaneous small cell lung tumor in mice, and systemic administration of an siRNA targeting a carcinoembryonic antigen-related cell adhesion molecule (CEACAM6) in mice with subcutaneously xenografted pancreatic adenocarcinoma cells. In another report, direct injection of a plasmid vector expressing shRNAs to matrix metalloproteinase MMP-9 and a cathepsin showed efficacy in established glioblastoma (Chen, et al., 2005, Reversal of the phenotype by K-rasval12 silencing mediated by adenovirus-delivered siRNA in human pancreatic cancer cell line Panc-1, World J. Gastroenterol. 11(6): 831-838). However, delivery of siRNA for long-term expression in target cells and tissues has been particularly difficult in vivo.

Another problem in gene transfer is the delivery of therapeutic molecules to a sufficient number of target cells to elicit a therapeutic response. Recently, a series of virus-encoded and other regulatory proteins were found to possess the ability to cross biological membranes. These proteins include HIV-Tat and the herpes simplex virus type 1 tegument protein VP22. VP22 was also reported to exhibit a unique property of effecting intercellular spread. VP22 is a basic, 38-kDa phosphorylated protein (Knopf, et al., 1980, J. Gen. Virol. 46:405-414) encoded by the viral UL49 gene (Elliott, et al., 1992, J. Gen. Virol. 73:723-726).

Specific and controlled delivery of therapeutic molecules to an affected cell population, e.g., to tumor cells and even circulating cancer cells, can potentially be achieved by strategically positioning nucleic acid and protein regulatory elements, e.g., cell and tissue-specific promoters and enzyme cleavage sites. These elements are recognized by the production machinery that is present only in certain cell types. The ability to easily combine and regulate the expression and delivery of multiple therapeutic molecules, while taking effective safety measures without compromising expression levels, in methods for using a lentiviral gene transfer vector or lentiviral transfer system, would provide a researcher with a critical tool for treating a broad range of diseases and disorders.

SUMMARY OF THE INVENTION

The present invention is related to lentiviral transfer systems including safe, self-inactivating, recombinant lentiviral vectors with the capacity to accommodate strategic combinations of genes for therapeutic molecules and novel regulatory sequences, in methods for treating a broad range of diseases and disorders.

In one aspect, the invention relates to a lentiviral gene transfer system comprising: a self-inactivating transfer vector comprising: a first gene unit with a first heterologous nucleic acid sequence, operably linked to a first regulatory nucleic acid sequence; and a second gene unit with a second heterologous nucleic acid sequence, operably linked to a second regulatory nucleic acid sequence; and a helper construct which lacks a 5' LTR, wherein said 5' LTR has been replaced with a heterologous promoter, said helper construct further comprising: a lentiviral env nucleic acid sequence containing a deletion, wherein said deleted env nucleic acid sequence does not produce a functional env protein; a packaging signal containing a deletion, wherein said deleted packaging signal is nonfunctional. The transfer vector may be preferably derived from HIV-1. Preferably, RNAi or a polypeptide may be encoded by the heterologous nucleic acid sequence. In addition, expression of the first and second heterologous nucleic acid sequences may have a synergistic effect in inhibiting progression of a disease or disorder.

The transfer vector may include mammalian insulator sequence and splice acceptor and splice donor sites, and may be free of wPRE "wood-chuck" hepatitis virus post-transcriptional element downstream of a cloning site (Gao et al., J. Virol., Mar. 2008; p. 2938-2951).

The RNAi may inhibit expression of a gene that contributes to progression of a disease or disorder. In addition, the first or second heterologous nucleic acid sequence may include a sequence encoding a trafficking signal, and the trafficking signal may be expressed as a fusion with a protein expressed from the second heterologus nucleic acid sequence. The intercellular trafficking signal may be a membrane-penetrating protein or a fragment thereof, such as a plant or bacterial protein toxin, or viral protein, any other sequence domain with transporting function between cells, in particular, cancer cells. The trafficking signal may be derived from herpesvirus VP22 or HIV-Tat, or may be a HIV-Tat eleven amino acid transduction sequence. The herpesvirus may be HSV1, and the trafficking signal may further be a VP22 protein homologue of HSV1 VP22. The VP22 transport signal may include a C-terminal 34 amino acid sequence of VP22 of HSV1, or a fragment having 80% or greater identity to the terminal 34 amino acid sequence of VP22 of HSV1. Further, the VP22 transport signal may include one or more of RSASR, RTASR, RSRAR, RTRAR, ATATR, or RSAASR.

The transfer vector may utilize general or cell or tissue specific promoters such as TSTA promoter, mesothelin promoter, hPSA promoter, hCCKAR promoter, hAFP promoter, and hNSE promoter.

Tissue-specific enzyme cleavage sites may be included in the transfer vector, wherein cleavage at the site occurs within a polypeptide that is encoded by the first and second heterologous nucleic acid sequences. The regulatory nucleic acid sequence may include a sequence encoding a cell or tissue-specific enzyme cleavage site, wherein cleavage at the site occurs within at least one polypeptide that is encoded by two or more of the first, second and third heterologous nucleic acid sequences. The cell or tissue-specific enzyme cleavage site may be a protease 2A cleavage site, a presecretory protein signal peptidase cleavage site, or a pancreatic prechymotrypsinogen cleavage site.

The transfer vector may also include a nucleic acid sequence encoding translation initiation site. Such a sequence may be positioned between the gene units, and in certain aspects may be considered to belong to a "regulatory sequence" of a gene unit. Although a variety of sequences may be used, an internal ribosome entry site (IRES) is preferred.

The inventive lentiviral transfer system may be used to prepare a treatment for a disease or disorder. The disease or disorder may be cancer. In a preferred embodiment, a heterologous nucleic acid sequence may encode the P53 protein.

The antisense RNA, RNAi, or any polypeptide expressed from the heterologous nucleic acid sequence may be expressed consistently and for long period of time to inhibit expression of a gene or the activity of a gene product that contributes to progression of the cancer. The antisense RNA, RNAi and the polypeptide may inhibit expression and activity of a tumor promoting gene or gene product. The RNAi and the expressed polypeptide may inhibit expression of or activity of a growth factor, growth factor receptor, angiogenic factor, angiogenic factor receptor, cell cycle regulator, apoptosis-inducing molecule, or cell adhesion molecule. The RNAi or the expressed polypeptide may inhibit the expression or activity of a vascular endothelial growth factor, Bcl-2, K-ras, AEC-1, Myc, including c-Myc, a vascular endothelial growth factor receptor, epidermal growth factor receptor, hTR, hTERT, papillomavirus E6, papillomavirus E7, BCR-abl, CEACAM6, MMP9, or a cathepsin.

The cancer to be treated may be prostate cancer in which a regulatory nucleic acid sequence may include hPSA. If the cancer is liver cancer then a regulatory nucleic acid sequence may include hAFP. If the cancer is pancreatic cancer then a regulatory nucleic acid sequence may include hCCKAR to control the expression of RNAi or a polypeptide.

The inventive lentiviral transfer system may be used to prepare a treatment for a genetic disorder, such as a metabolic disorder, including Gaucher's Disease or Fabry's Disease. In the case of Gaucher's Disease, a first heterologous nucleic acid sequence may encode glucocerebrosidase and a second heterologous nucleic acid sequence may encode a human intrinsic selectable marker such as huCD25 protein or huNGF protein.

The lentiviral transfer system may include a regulatory nucleic acid sequence that includes a sequence encoding a trafficking signal that is expressed as a fusion with a glu-

cocerebrosidase protein expressed from an adjacent heterologous nucleic acid sequence. A second regulatory nucleic acid sequence may include translation initiation sequence as well. A cell or tissue-specific enzyme cleavage site may also be included.

In the case of Fabry's Disease, said first heterologous nucleic acid sequence may encode an alpha-galactosidase-A protein, and a second heterologous nucleic acid sequence may encode the huCD25 protein.

In another aspect, the inventive lentiviral transfer system may include a first heterologous nucleic acid sequence comprising a sequence encoding a trafficking signal that is expressed as a fusion with the alpha-galactosidase-A protein expressed from the first heterologous nucleic acid sequence. The trafficking signal may be a VP22 trafficking signal or an HIV-Tat trafficking signal.

In yet another aspect, if the genetic disorder is Leber Congenital Amaurosis, the first heterologous nucleic acid sequence may encode the RPE65 protein, and a second heterologous nucleic acid sequence may encode the hBDNF protein, and a second regulatory nucleic acid sequence may include hNSE, and further the vector may include a third heterologous nucleic acid sequence encoding the hNGF protein, and also a third regulatory nucleic acid sequence including a sequence encoding a cell or tissue-specific enzyme cleavage site.

In another aspect, the disease or disorder to be treated may be a neurological disorder, such as Alzheimer's Disease, in which case, a first heterologous nucleic acid may encode the hNGF protein, a second heterologous nucleic acid may encode an RNAi targeted to beta-amyloid precursor protein, and wherein the second regulatory nucleic acid sequence may include a sequence encoding a cell or tissue-specific enzyme cleavage site. A third third heterologous nucleic acid that encodes the hBDNF protein may also be included, in which the first regulatory nucleic acid sequence may include a sequence encoding a trafficking signal that is expressed as a fusion with the hNGF protein expressed from the first heterologous nucleic acid sequence.

If the neurological disorder is Parkinson's Disease, a first heterologous nucleic acid may encode the hBDNF protein or the hGDNF protein, and the second heterologous nucleic acid encodes hGAD. The second regulatory nucleic acid sequence may include a sequence encoding a cell or tissue-specific enzyme cleavage site. Further, the first regulatory nucleic acid may include a sequence encoding a trafficking signal that is expressed as a fusion with the hBDNF protein or hGDNF protein expressed from the first heterologous nucleic acid sequence, wherein the vector may further include a third heterologous nucleic acid sequence encoding hNGF, in which the vector may further include a third regulatory nucleic acid sequence encoding a cell or tissue-specific enzyme cleavage site.

In another aspect, the invention is directed to a method for treating a condition, comprising administering to a patient a lentiviral particle for gene transfer, said lentiviral particle produced using a lentiviral transfer system comprising: a self-inactivating transfer vector comprising: a first gene unit with a first heterologous nucleic acid sequence, operably linked to a first regulatory nucleic acid sequence; and a second gene unit with a second heterologous nucleic acid sequence, operably linked to a second regulatory nucleic acid sequence; and a helper construct which lacks a 5' LTR, wherein said 5' LTR has been replaced with a heterologous promoter, said helper construct further comprising: a lentiviral env nucleic acid sequence containing a deletion, wherein said deleted env nucleic acid sequence does not produce functional env protein; a packaging signal containing a deletion, wherein said deleted packaging signal is nonfunctional. The condition may be cancer, such as liver cancer, pancreatic cancer, or prostate cancer. The condition may also be a genetic disorder, such as Gaucher's Disease or Fabry's Disease. The condition may also be a neurological disorder, such as Parkinson's Disease or Alzheimer's Disease. The condition may also be a need for cosmetic enhancement.

A pharmaceutical composition comprising a lentiviral particle for gene transfer, said lentiviral particle produced using a lentiviral transfer system comprising: a self-inactivating transfer vector comprising: a first gene unit with a first heterologous nucleic acid sequence, operably linked to a first regulatory nucleic acid sequence; and a second gene unit with a second heterologous nucleic acid sequence, operably linked to a second regulatory nucleic acid sequence; and a helper construct which lacks a 5' LTR, wherein said 5' LTR has been replaced with a heterologous promoter, said helper construct further comprising: a lentiviral env nucleic acid sequence containing a deletion, wherein said deleted env nucleic acid sequence does not produce functional env protein; a packaging signal containing a deletion, wherein said deleted packaging signal is nonfunctional.

In a further aspect, the invention is directed to a pharmaceutical composition that includes a lentiviral transfer vector, said lentiviral transfer vector comprising a first heterologous nucleic acid sequence, operably linked to a first regulatory nucleic acid sequence; and a second heterologous nucleic acid sequence, operably linked to a second regulatory nucleic acid sequence, wherein said transfer vector is self-inactivating.

The pharmaceutical compositions as described above may further include a chemotherapeutic agent, a steroid agent such as prednisolone, cortisone, corticosterone, or dexamethasone.

In one aspect of the invention, SIN element is incorporated into the recombinant lentivirus; the tat region has been modified so as to optionally allow for infection efficiency without allowing the replication functions and uncontrolled infection of the recombinant lentivirus beyond the intended target; and the rev protein has been inactivated so as to prevent further unwanted infectivity while preserving the basic function of the rev to support the expression efficiency of the therapeutic gene(s). Tat and rev proteins may be inactivated of their original replication activity without necessarily removing them from the lentivirus and thereby preserve their desirable attributes while keeping the resulting vector bio-safe.

These and other objects of the invention will be more fully understood from the following description of the invention, the referenced drawings attached hereto and the claims appended hereto.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention will become more fully understood from the detailed description given herein below, and the accompanying drawings which are given by way of illustration only, and thus are not limitative of the present invention, and wherein;

FIG. **1** Lentiviral transfer system. The drawing shows an HIV-1-based gene transfer system carrying multiple functional (therapeutic) genes. A. Helper (packaging) construct. The 5' LTR of the helper construct is replaced with the CMV promoter, to avoid integration of the viral elements pre-

sented in that construct. This is an important biosafety feature of the lentiviral transfer system of the invention. The triangles represent deletions: one represents a 36-bp deletion harboring the putative packaging signal from nucleotides 753 to 789 between the 5' major splice donor site and the beginning of the gag ATG coding region; one represents a deletion in the tat gene, and a third represents a deletion in nef. The packaging signal is functionally absent from this construct, to avoid production of an active gag-pol precursor. The poly (A) site was derived from the bovine growth hormone gene. The helper construct provides a nucleic acid sequence encoding lentiviral gag and pol, operably linked to a heterologous regulatory nucleic acid sequence. The construct further contains a deleted, nonfunctional env protein and is devoid of lentiviral sequences both upstream and downstream from a splice donor site to a lentiviral gag initation site. B. Envelope expression construct. An envelope construct encoding vesicular stomatitis virus G glycoprotein (VSV-G) is shown, though other non-lentiviral envelope proteins can be used instead. Expression is driven by the HIV-1 LTR. The poly(A) site was derived from the simian virus 40 late region. C. Transfer vector constructs. In these constructs, Tat, Vpr, and Nef are inactivated. Boxes interrupted by jagged lines contain partial deletions. RRE=Rev-response element; ψ=cis-acting packaging signal; IRES=internal ribosome entry site; huCD25=human IL-2Ra chain gene; GFP=Green Fluorescent Protein coding sequence; RNAi=interfering RNA coding sequence; S=stop codon; T=termination signal (e.g., SV40 polyA or BGH polyA); CS=cleavage site (e.g., viral 2A-like peptide cleaved by the 2A protease, presecretory protein cleavage site, pancreatic prechymotrypsinogen cleavage site); hPSA=human prostate specific antigen promoter; P1=promoter 1; CMV, Human CMV-IE promoter; P2=promoter-2; P53=tumor suppressor gene; dsRNA=Bcl-2 RNAi (human).

FIG. **2** Heterologous Proteins. This table provides examples of heterologous proteins contemplated for use in the vectors and methods of the present invention.

FIG. **3** Transport Genes. This table provides examples of transport genes contemplated for use in developing transport sequences for the vectors and methods of the present invention.

FIG. **4** Promoter Elements. This table provides examples of promoter elements contemplated for use in the vectors and methods of the present invention.

FIG. **5** Enhancer Elements. This table provides examples of enhancer elements contemplated for use in the vectors and methods of the present invention.

FIG. **6** SIN-LV-P53-EGFP and SIN-LV-BCL2 RNAi-EGFP. The drawing shows the vector constructs used as described in the Examples.

FIG. **7** Therapeutic Constructs. This table provides examples of constructs for use in the vectors and methods of the invention for certain therapeutic applications.

FIG. **8** Infection of Prostate Cancer Cells. A. EGFP expression in PC-3 cells infected with SIN-HIV-p53-IRES-EGFP (Panel 1) and PC-3 cells not infected with virus (2). B. EGFP expression in 293T cells infected with SIN-HIV-p53-IRES-EGFP (Panel 1) and 293T cells not infected with virus (2). C. Viral packaging of Vector. D. P53 expression. Lane 1: Size standards 1 kb plus DNA Ladder. Lane 2: PC-3 cells (no infection). Lane 3: PC-3 cells (infection). Lane 4: Blank. Lane 5: 293T cells (no infection). Lane 6: 293T cells (infection).

FIG. **9** Therapeutic Constructs 2. This table provides examples of constructs for use in the vectors and methods of the invention for certain therapeutic applications.

FIGS. **10**A-**10**B Experimental evidence of efficacy of the viral vector construct P53-Bcl-2 RNAi, expressing P53 and an RNAi agent targeting human Bcl-2. FIGS. **10**A and **10**B show phenotype of in vitro cell culture under phase-contrast microscope indicating that the P53-Bcl-2 RNAi construct induces cell necrosis in PC3 prostate cancer cells. A. untreated living cells. B. treated with viral vector construct P53-Bcl-2 RNAi, expressing P53 and an RNAi agent targeting human Bcl-2.

FIGS. **11**A-**11**B Experimental evidence of efficacy of the viral vector construct P53-Bcl-2 RNAi, expressing P53 and an RNAi agent targeting human Bcl-2. FIGS. **11**A and **11**B show phenotype of in vitro cell culture under phase-contrast microscope indicating that the P53-Bcl-2 RNAi construct does not cause necrosis in 293T cells. A. untreated cells. B. treated with viral vector construct P53-Bcl-2 RNAi, expressing P53 and an RNAi agent targeting human Bcl-2.

FIG. **12** In vivo test demonstrates tumor reduction efficacy of viral vector construct P53-Bcl-2 RNAi, expressing P53 and an RNAi agent targeting human Bcl-2. In order from left to right, far left bar is weight of the control tumor group after three weeks; tumor treated with P53 alone expressed through viral vector construct (repair genetic defect); tumor treated with BCL2 SiRNA alone expressed through viral vector construct (down regulation of BCL2 gene) only; and on the far right tumor treated with viral vector construct P53-Bcl-2 RNAi, expressing P53 and an RNAi agent targeting human Bcl-2 for simultaneous P53 (repair) and BCL2 siRNA.

FIGS. **13**A-**13**B FIGS. **13**A and **13**B show in vivo tumor staining of control (untreated tumor) versus treatment groups. Immunohistochemical staining by specific antibodies show that tumor cells treated with viral vector construct P53-Bcl-2 RNAi, expressing P53 and an RNAi agent targeting human Bcl-2 no longer produce hPSA (human prostate specific antigen), which means that the cells are no longer active tumor cells or are no longer actively cancerous. A. control tumor expresses hPSA. B. treatment tumor shows cell necrosis and no expreession of hPSA.

FIGS. **14**A-**14**C FIGS. **14**A-**14**C show a table that shows results from another mouse (in vivo) study confirming tumor reduction findings. Group V1 is prostrate tumor mice treated with P53 expressed alone in a viral vector; Group V2 is tumor mice treated with BCL2 siRNA alone expressed through viral vector; V3 is tumor mice treated with the dual viral construct expressing P53 and BCL2 siRNA. Group PC shows untreated tumor mice control. "**" in the tables indicates tumor sizes on Day 7 as the starting sizes for calculating the tumor growth rate and the ratio of Tumor Weigh/Starting Tumor Size for groups of V1, V2, V3, and PC.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

In the present application, "a" and "an" are used to refer to both single and a plurality of objects.

Unless otherwise indicated, all terms used herein have the same ordinary meaning as they would to one skilled in the art of the present invention.

Citation of documents herein is not intended as an admission that any of the documents cited herein is pertinent prior art, or an admission that the cited documents are considered material to the patentability of the claims of the present

application. All statements as to the date or representations as to the contents of these documents are based on the information available to the applicant and do not constitute any admission as to the correctness of the dates or contents of these documents.

As used herein, reference to "upstream", "downstream", "first gene", "second gene", "last gene", "before", "after" and so forth in relation to the spatial positioning of the various DNA sequences in a vector, is meant to be with respect to the 5' to 3' orientation of the vector sequence. For example "before" will have the same meaning as "upstream of" or 5' of a particular reference position, and "after" will have the same meaning of "downstream of" or 3' with respect to a particular reference point on the vector.

As used herein, "gene unit" includes a regulatory region that may include a promoter and a heterologous nucleic acid sequence encoding either an antisense RNA, RNAi or polypeptide of interest that is controlled by the regulatory sequence, which is typically referred to in the context of a multigene transfer vector. However, in situations where a fused polypeptide is desirous of being generated from the multigene vector of the encoded polypeptides of adjacent gene units, the regulatory region of the downstream gene units may include a nucleic acid sequence encoding a cleavage site instead of a separate promoter.

Modes of Carrying out the Invention

It is to be understood that this invention is not limited to particular formulations or process parameters, as these may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments of the invention only, and is not intended to be limiting. Further, it is understood that a number of methods and materials similar or equivalent to those described herein can be used in the practice of the present invention.

I. Lentiviral Transfer System

The present invention provides a recombinant lentivirus capable of infecting dividing and non-dividing cells. The virus is useful for the in vivo and ex vivo transfer and expression of nucleic acid sequences. Lentiviral vectors of the invention may be lentiviral transfer plasmids or infectious lentiviral particles. Construction of lentiviral vectors, helper constructs, envelope constructs, etc., for use in lentiviral transfer systems has been described, e.g., in U.S. Patent App. Pub. No. 2003/0119770, "Intercellular delivery of a herpes simplex virus VP22 fusion protein from cells infected with lentiviral vectors," incorporated herein by reference in its entirety.

Lentiviruses

Lentiviruses are RNA viruses wherein the viral genome is RNA. When a host cell is infected with a lentivirus, the genomic RNA is reverse transcribed into a DNA intermediate which is integrated very efficiently into the chromosomal DNA of infected cells. This integrated DNA intermediate is referred to as a provirus. Transcription of the provirus and assembly into infectious virus occurs in the presence of an appropriate helper virus or in a cell line containing appropriate sequences enabling encapsidation without coincident production of a contaminating helper virus. As described below, a helper virus is not required for the production of the recombinant lentivirus of the present invention, since the sequences for encapsidation are provided by co-transfection with appropriate vectors.

The lentiviral genome and the proviral DNA have three genes: the gag, the pol, and the env, which are flanked by two long terminal repeat (LTR) sequences. The gag gene encodes the internal structural (matrix, capsid, and nucleocapsid) proteins; the pol gene encodes the RNA-directed DNA polymerase (reverse transcriptase) and the env gene encodes viral envelope glycoproteins. The 5' and 3' LTRs serve to promote transcription and polyadenylation of the virion RNAs. The LTR contains all other cis-acting sequences necessary for viral replication. Lentiviruses have additional genes including vit, vpr, tat, rev, vpu, nef, and vpx (in HIV-1, HIV-2 and/or SIV).

Adjacent to the 5' LTR are sequences necessary for reverse transcription of the genome (the tRNA primer binding site) and for efficient encapsidation of viral RNA into particles (the Psi site, ψ). If the sequences necessary for encapsidation (or packaging of lentiviral RNA into infectious virions) are missing from the viral genome, the result is a cis defect which prevents encapsidation of genomic RNA. The resulting mutant is still capable of directing the synthesis of all virion proteins, but lacks function of replication.

In a first embodiment, the invention provides a recombinant lentivirus capable of infecting a dividing or non-dividing cell. The recombinant lentivirus comprises a nucleic acid sequence containing a lentiviral packaging signal flanked by lentiviral cis-acting nucleic acid sequences necessary for reverse transcription and integration, a heterologous nucleic acid sequence operably linked to a regulatory nucleic acid sequence, and a nucleic acid sequence encoding an intercellular trafficking signal, where the nucleic acid sequence encoding the intercellular trafficking signal is fused in-frame with the heterologous nucleic acid sequence, where the lentivirus does not contain either a complete gag, pol, tat, rev, or env gene.

The recombinant lentivirus of the invention is therefore genetically modified in such a way that some of the structural, infectious genes of the native virus have been removed, and some removed sequences replaced with a nucleic acid sequence to be delivered to a target non-dividing cell. After infection of a cell by the virus, the virus releases its nucleic acid into the cell and the lentivirus genetic material can integrate into the host cell genome. The transferred lentivirus genetic material is then transcribed and translated, e.g., as dictated by the regulatory sequences, into proteins within the host cell.

Lentiviral Vector Systems

The invention provides a method of producing a recombinant lentivirus capable of infecting a dividing or non-dividing cell comprising transfecting a suitable host cell with the following: a transfer vector providing a nucleic acid encoding a lentiviral gag and a lentiviral pol, where the gag and pol nucleic acid sequences are operably linked to a heterologous regulatory nucleic acid sequence and where the transfer vector is defective for nucleic acid sequence encoding functional env protein and devoid of lentiviral sequences both upstream and downstream from a splice donor site to a gag initiation site of a lentiviral genome; an envelope construct providing a nucleic acid encoding a non-lentiviral env protein; and a helper construct providing a nucleic acid sequence containing a lentiviral packaging signal flanked by lentiviral cis-acting nucleic acid sequences for reverse transcription and integration, and providing a cloning site for introduction of a heterologous nucleic acid sequence operably linked to a regulatory nucleic acid sequence and optionally to a nucleic acid sequence encoding an intercellular trafficking signal, where the nucleic acid sequence encoding the intercellular trafficking signal is fused in-frame with the heterologous nucleic acid sequence, where the helper construct does not contain either a complete gag, pol, or env gene, and recovering the recombinant lentivirus. An

illustration of the individual vectors used in the method of the invention is shown in FIG. **1**.

The method of the invention includes the combination of a minimum of three vectors in order to produce a recombinant virion or recombinant lentivirus. For example, a vector of the invention can include (a) the p53 gene product, expressed and driven by a regulatory nucleic acid sequence to treat tumor cells or migrating cells having a p53 gene mutation; (b) a specific siRNA driven by second regulatory nucleic acid sequence to down-regulate tumor activity of the tumor cells in target tissue or organs; wherein the double or multiple gene system is able to enhance delivery efficacy and therapeutic response. It is understood that in the vectors and methods of the present invention, the relative positions in the transfer vector of the therapeutic molecules—be they proteins, RNAi or other types of antisense agents—can vary as needed. Therefore, for example, any of the first, second, or third heterologous nucleic acid sequences can encode an RNAi. Furthermore, multiple heterologous nucleic acid sequences (e.g., two or three) can encode an RNAi or other antisense agent.

A first vector is a helper construct, which provides a nucleic acid encoding a lentiviral gag and a lentiviral pol (FIG. **1**A).

A second vector is an envelope construct, which provides a nucleic acid encoding a non-lentiviral env protein (FIG. **1**B). The env gene can be derived from any virus excluding lentiviruses. The env gene is ideally derived from a virus other than HIV. The env gene may be amphotropic envelope protein which allows transduction of cells of human and other species, or may be ecotropic envelope protein, which is able to transduce only mouse and rat cells. Further, it may be desirable to target the recombinant virus by linkage of the envelope protein with an antibody or a particular ligand for targeting to a receptor of a particular cell-type. By inserting a sequence (including regulatory region) of interest into the viral vector, along with another gene which encodes the ligand for a receptor on a specific target cell, for example, the vector is now target specific. Lentiviral vectors can be made target specific by inserting, for example, a protein. Targeting is often accomplished by using an antibody to target the lentiviral vector. Those of skill in the art will know of, or can readily ascertain without undue experimentation, specific methods to achieve delivery of a lentiviral vector to a specific target.

Examples of retroviral-derived env genes include, but are not limited to: Moloney murine leukemia virus (MoMuLV), Harvey murine sarcoma virus (HaMuSV), murine mammary tumor virus (MuMTV), gibbon ape leukemia virus (GaLV), and Rous Sarcoma Virus (RSV). Other env genes such as Vesicular stomatitis virus (VSV) (Protein G) can also be used.

The construct providing the viral env nucleic acid sequence is operably associated with regulatory sequence, e.g., a promoter or enhancer. Preferably, the regulatory sequence is a viral promoter. The regulatory sequence can be any eukaryotic promoter or enhancer, including for example, the Moloney murine leukemia virus promoter-enhancer element, the human cytomegalovirus enhancer, or the vaccinia P7.5 promoter. In some cases, such as the HIV-1 promoter-enhancer element, these promoter-enhancer elements are located within or adjacent to the LTR sequences.

A third vector, the transfer vector, provides a nucleic acid sequence, which contains the cis-acting viral sequences necessary for the lentiviral life cycle. Such sequences include the lentiviral psi packaging sequence, reverse transcription signals, integration signals, viral promoter, enhancer, and polyadenylation sequences. The transfer vector also contains a cloning site for a heterologous nucleic acid sequence to be transferred to a dividing or non-dividing cell, and optionally a nucleic acid sequence encoding an intercellular trafficking signal, where the nucleic acid sequence encoding the intercellular trafficking signal is fused in-frame with the heterologous nucleic acid sequence (FIG. **1**C).

Since recombinant lentiviruses produced by standard methods in the art are defective, they require assistance in order to produce infectious vector particles. Typically, this assistance is provided, for example, by using a helper cell line that provides the missing viral functions. These plasmids are missing a nucleotide sequence which enables the packaging mechanism to recognize an RNA transcript for encapsidation. Suitable cell lines produce empty virions, since no genome is packaged. If a lentiviral vector is introduced into such cells in which the packaging signal is intact, but the structural genes are replaced by other genes of interest, the vector can be packaged and vector virion produced.

The method of producing the recombinant lentivirus of the invention is different than the standard helper virus/packaging cell line method described above. The three or more individual vectors used to co-transfect a suitable packaging cell line collectively contain all of the required genes for production of a recombinant virus for infection and transfer of nucleic acid to a non-dividing cell. Consequently, there is no need for a helper virus.

Conveniently during the cloning stage, the nucleic acid construct referred to as the transfer vector, having the packaging signal and the heterologous cloning site, also contains a selectable marker gene. Marker genes are utilized to assay for the presence of the vector, and thus, to confirm infection and integration. Typical selection genes encode proteins that confer resistance to antibiotics and other toxic substances, e.g. histidinol, puromycin, hygromycin, neomycin, methotrexate, etc.

"Non-dividing" cell refers to a cell that does not go through mitosis. Non-dividing cells may be blocked at any point in the cell cycle, (e.g., G_0/G_1, G_1/S, G_2/M), as long as the cell is not actively dividing. For ex vivo infection, a dividing cell can be treated to block cell division by standard techniques used by those of skill in the art, including, irradiation, aphidocolin treatment, serum starvation, and contact inhibition. However, it should be understood that ex vivo infection is often performed without blocking the cells since many cells are already arrested (e.g., stem cells). The recombinant lentivirus vector of the invention is capable of infecting any non-dividing cell, regardless of the mechanism used to block cell division or the point in the cell cycle at which the cell is blocked. Examples of pre-existing non-dividing cells in the body include neuronal, muscle, liver, skin, heart, lung, and bone marrow cells, and their derivatives.

The method of the invention provides at least three vectors which provide all of the functions required for packaging of recombinant virions as discussed above. The method also envisions transfection of vectors including viral genes such as vpr, vif, nef, vpx, tat, rev, and vpu. Some or all of these genes can be included, for example, on the packaging construct vector, or, alternatively, they may reside on individual vectors. There is no limitation to the number of vectors which are utilized, as long as they are co-transfected to the packaging cell line in order to produce a

single recombinant lentivirus. For example, one could put the env nucleic acid sequence on the same construct as the gag and pol.

The vectors are introduced via transfection or infection into the packaging cell line. The packaging cell line produces viral particles that contain the vector genome. Methods for transfection or infection are well known by those of skill in the art. After co-transfection of the at least three vectors to the packaging cell line, the recombinant virus is recovered from the culture media and titered by standard methods used by those of skill in the art.

In another embodiment, the invention provides a recombinant lentivirus produced by the method of the invention as described above.

The invention also provides a method of nucleic acid transfer to a non-dividing cell to provide expression of a particular nucleic acid sequence. Therefore, in another embodiment, the invention provides a method for introduction and expression of a heterologous nucleic acid sequence in a non-dividing cell comprising infecting the non-dividing cell with the recombinant virus of the invention and expressing the heterologous nucleic acid sequence in the non-dividing cell.

It may be desirable to modulate the expression of a gene regulating molecule in a cell by the introduction of a molecule by the method of the invention. The term "modulate" envisions the suppression of expression of a gene when it is over-expressed, or augmentation of expression when it is under-expressed. Where a cell proliferative disorder is associated with the expression of a gene, nucleic acid sequences that interfere with the gene's expression at the translational level can be used. This approach utilizes, for example, antisense nucleic acid, ribozymes, or triplex agents, siRNA to block transcription or translation of a specific mRNA, either by masking that mRNA with an antisense nucleic acid or triplex agent, or by cleaving it with a ribozyme.

The method of the invention may also be useful for neuronal or glial cell transplantation, or "grafting," which involves transplantation of cells infected with the recombinant lentivirus of the invention ex vivo, or infection in vivo into the central nervous system or into the ventricular cavities or subdurally onto the surface of a host brain. Such methods for grafting will be known to those skilled in the art and are described in Neural Grafting in the Mammalian CNS, Bjorklund and Stenevi, eds. (1985). Procedures include intraparenchymal transplantation, (i.e., within the host brain) achieved by injection or deposition of tissue within the host brain so as to be apposed to the brain parenchyma at the time of transplantation.

Self-Inactivating Lentiviral Vectors

Self-inactivating (SIN) lentiviral vectors have a deletion in the U3 region of the 3' LTR that eliminates regulatory sequences, including the TATA box. The deletion has been reported to result in transcriptional inactivation of the LTR in proviruses without affecting vector titers or transgene expression in vitro. SIN vectors are described, e.g., by Zufferey, et al., 1998, J. Virology 72(12):9873-9880, who made a 400 by deletion, and Miyoshi, et al., 1998, J. Virology 72(10):8150-8157, who made a 133 bp deletion.

It has been reported that a certain U3 deletion actually results in increased expression from the vector in vivo (Bayer, et al., 2008, A Large U3 Deletion Causes Increased In Vivo Expression from a Nonintegrating Lentiviral Vector, Molecular Therapy doi:10.1038/mt.2008.199). This finding suggests that additional alterations to the lentivirus sequences are needed to ensure safety of gene transfer systems.

II. Heterologous Nucleic Acid Sequences

A heterologous nucleic acid sequence is operably linked to a regulatory nucleic acid sequence. As used herein, the term "heterologous" nucleic acid sequence refers to a sequence that originates from a foreign species, or, if from the same species, it may be substantially modified from its original form. Alternatively, an unchanged nucleic acid sequence that is not normally expressed in a cell is a heterologous nucleic acid sequence. The term "operably linked" refers to functional linkage between the regulatory sequence and the heterologous nucleic acid sequence. The heterologous sequence can be linked to a promoter. The heterologous nucleic acid sequence can be under control of either the viral LTR promoter-enhancer signals or of an internal promoter, and retained signals within the lentiviral LTR can still bring about efficient integration of the vector into the host cell genome. The use of nonintegrating vectors for certain purposes, e.g., where transient expression is sufficient, is also contemplated.

The recombinant virus of the invention is capable of transferring nucleic acid sequences into a non-dividing cell. The term "nucleic acid sequence" refers to any nucleic acid molecule, preferably DNA. The nucleic acid molecule may be derived from a variety of sources, including DNA, cDNA, synthetic DNA, RNA, or combinations thereof. Such nucleic acid sequences may comprise genomic DNA which may or may not include naturally occurring introns. Moreover, such genomic DNA may be obtained in association with promoter regions, introns, or poly(A) sequences. Genomic DNA may be extracted and purified from suitable cells by means well known in the art. Alternatively, messenger RNA (mRNA) can be isolated from cells and used to produce cDNA by reverse transcription or other means.

FIG. **2** shows examples of heterologous proteins that can be expressed from their genes using the vectors and methods of the present invention. FIGS. **7** and **8** further list examples of cloned structural genes that can serve as, e.g., a first, second, or third heterologous nucleic acid sequence of the invention.

A preferred protein for expression using the vectors and methods of the present invention is tumor antigen P53. Expression of P53 is defective in most cancers, e.g., due to mutation of the gene or lowered expression. Delivery of the wild-type gene encoding the 53-kilodalton protein is therefore a goal of gene therapy for many cancers.

Nucleic acids encoding the same proteins or targeting the same RNAs can be used in a single transfer vector, for example, two genes for the same protein can be cloned from different sources and used as the first and second heterologous nucleic acid sequences. Similarly, RNAi sequences that are specific for different parts of the same target RNA, or that differ in their percent homology to the target RNA, can be used together.

It may be desirable to transfer a nucleic acid encoding a biological response modifier. Included in this category are immunopotentiating agents including nucleic acids encoding a number of the cytokines classified as "interleukins." These include, for example, interleukins 1 through 12. Also included in this category, although not necessarily working according to the same mechanisms, are interferons, and in particular gamma interferon (γ-IFN), tumor necrosis factor (TNF) and granulocyte-macrophage-colony stimulating factor (GM-CSF). It may be desirable to deliver such nucleic acids to bone marrow cells or macrophages to treat enzy-

matic deficiencies or immune defects, or cancer disease. Nucleic acids encoding growth factors, toxic peptides, ligands, receptors, or other physiologically important proteins can also be introduced into specific non-dividing cells.

Selection of RNAi Agents and Other Antisense Nucleic Acid Sequences

An RNAi agent used in the vectors and methods of the present invention can be targeted to any RNA molecule. Besides messenger RNA (mRNA), RNAi agents can target, e.g., various species of microRNA. The use of RNAi in gene therapy and RNAi selection and sequence design, are described, e.g., in WO 2007/109131, "Lentiviral Vectors That Provide Improved Expression and Reduced Variegation after Transgenesis," and WO 2007/087113, "Natural Antisense and Non-Coding RNA Transcripts as Drug Targets," both of which are incorporated herein by reference.

It may be desirable to modulate the expression of a gene regulating molecule in a cell by the introduction of a molecule by the method of the invention. The term "modulate" envisions the suppression of expression of a gene when it is over-expressed, or augmentation of expression when it is under-expressed. Where a cell proliferative disorder is associated with the expression of a gene, nucleic acid sequences that interfere with the gene's expression at the translational level can be used. This approach utilizes, for example, antisense nucleic acid, ribozymes, or triplex agents, siRNA to block transcription or translation of a specific mRNA, either by masking that mRNA with an antisense nucleic acid or triplex agent, or by cleaving it with a ribozyme.

Antisense nucleic acids are DNA or RNA molecules that are complementary to at least a portion of a specific mRNA molecule (Weintraub, 1990 Scientific American 262:40). In the cell, the antisense nucleic acids hybridize to the corresponding mRNA, forming a double-stranded molecule. The antisense nucleic acids interfere with the translation of the mRNA, since the cell will not translate an mRNA that is double-stranded. Antisense oligomers of about 15 nucleotides are preferred, since they are easily synthesized and are less likely to cause problems than larger molecules when introduced into the target cell. The use of antisense methods to inhibit the in vitro translation of genes is well known in the art (Marcus-Sakura, 1988 Anal Biochem 172:289).

The antisense nucleic acid can be used to block expression of a mutant protein or a dominantly active gene product, such as amyloid precursor protein that accumulates in Alzheimer's disease. Such methods are also useful for the treatment of Huntington's disease, hereditary Parkinsonism, and other diseases. Antisense nucleic acids are also useful for the inhibition of expression of proteins associated with toxicity.

Use of an oligonucleotide to stall transcription is known as the triplex strategy since the oligomer winds around double-helical DNA, forming a three-strand helix. Therefore, these triplex compounds can be designed to recognize a unique site on a chosen gene (Maher, et al. 1991 Antisense Res and Dev 1:227; Helene, C. 1991 Anticancer Drug Design 6:569).

Ribozymes are RNA molecules possessing the ability to specifically cleave other single-stranded RNA in a manner analogous to DNA restriction endonucleases. Through the modification of nucleotide sequences which encode these RNAs, it is possible to engineer molecules that recognize specific nucleotide sequences in an RNA molecule and cleave it (Cech, 1988 J Amer Med Assn 260:3030). A major advantage of this approach is that, because they are sequence-specific, only mRNAs with particular sequences are inactivated.

RNA interference (RNAi) is mediated by double stranded RNA (dsRNA) molecules that have sequence-specific homology to their target nucleic acid sequences (Caplen, N. J., et al, Proc. Natl. Acad. ScL USA 98:9742-9747 (2001)). Biochemical studies in Drosophila cell-free lysates indicate that, in certain embodiments of the present invention, the mediators of RNA-dependent gene silencing are 21-25 nucleotide "small interfering" RNA duplexes (siRNAs). The siRNAs are derived from the processing of dsRNA by an RNase enzyme known as Dicer (Bernstein, E., et al, Nature 409:363-366 (2001)). siRNA duplex products are recruited into a multi-protein siRNA complex termed RISC (RNA Induced Silencing Complex). Without wishing to be bound by any particular theory, a RISC is then believed to be guided to a target nucleic acid (suitably mRNA), where the siRNA duplex interacts in a sequence-specific way to mediate cleavage in a catalytic fashion (Bernstein, E., et al, Nature 409:363-366 (2001); Boutla, A., et al, Curr. Biol. 11:1776-1780 (2001)). Small interfering RNAs that can be used in accordance with the present invention can be synthesized and used according to procedures that are well known in the art and that will be familiar to the ordinarily skilled artisan. Small interfering RNAs for use in the methods of the present invention suitably comprise between about 0 to about 50 nucleotides (nt). In examples of non-limiting embodiments, siRNAs can comprise about 5 to about 40 nt, about 5 to about 30 nt, about 10 to about 30 nt, about 15 to about 25 nt, or about 20-25 nucleotides.

"RNAi" or "RNAi agent" refers to an at least partly double-stranded RNA having a structure characteristic of molecules that are known in the art to mediate inhibition of gene expression through an RNAi mechanism or an RNA strand comprising at least partially complementary portions that hybridize to one another to form such a structure. When an RNA comprises complementary regions that hybridize with each other, the RNA will be said to self-hybridize. An RNAi agent includes a portion that is substantially complementary to a target gene. An RNAi agent, optionally includes one or more nucleotide analogs or modifications. One of ordinary skill in the art will recognize that RNAi agents that are synthesized in vitro can include ribonucleotides, deoxyribonucleotides, nucleotide analogs, modified nucleotides or backbones, etc., whereas RNAi agents synthesized intracellularly, e.g., encoded by DNA templates, typically consist of RNA, which may be modified following transcription. Of particular interest herein are short RNAi agents, i.e., RNAi agents consisting of one or more strands that hybridize or self-hybridize to form a structure that comprises a duplex portion between about 15-29 nucleotides in length, optionally having one or more mismatched or unpaired nucleotides within the duplex. RNAi agents include short interfering RNAs (siRNAs), short hairpin RNAs (shRNAs), and other RNA species that can be processed intracellularly to produce shRNAs including, but not limited to, RNA species identical to a naturally occurring miRNA precursor or a designed precursor of an miRNA-like RNA.

The term "short, interfering RNA" (siRNA) refers to a nucleic acid that includes a double-stranded portion between about 15-29 nucleotides in length and optionally further comprises a single-stranded overhang {e.g., 1-6 nucleotides in length) on either or both strands. The double-stranded portion is typically between 17-21 nucleotides in length, e.g., 19 nucleotides in length. The overhangs are typically

present on the 3' end of each strand, are usually 2 nucleotides long, and are composed of DNA or nucleotide analogs. An siRNA may be formed from two RNA strands that hybridize together, or may alternatively be generated from a longer double-stranded RNA or from a single RNA strand that includes a self-hybridizing portion, such as a short hairpin RNA. One of ordinary skill in the art will appreciate that one or more, mismatches or unpaired nucleotides can be present in the duplex formed by the two siRNA strands. One strand of an siRNA (the "antisense" or "guide" strand) includes a portion that hybridizes with a target nucleic acid, e.g., an mRNA transcript. Typically the antisense strand is perfectly complementary to the target over about 15-29 nucleotides, typically between 17-21 nucleotides, e.g., 19 nucleotides, meaning that the siRNA hybridizes to the target transcript without a single mismatch over this length. However, one of ordinary skill in the art will appreciate that one or more mismatches or unpaired nucleotides may be present in a duplex formed between the siRNA strand and the target transcript.

"Short hairpin RNA" refers to a nucleic acid molecule comprising at least two complementary portions hybridized or capable of hybridizing to form a duplex structure sufficiently long to mediate RNAi (typically between 15-29 nucleotides in length), and at least one single-stranded portion, typically between approximately 1 and 10 nucleotides in length that forms a loop connecting the ends of the two sequences that form the duplex. The structure may further comprise an overhang. The duplex formed by hybridization of self-complementary portions of the shRNA has similar properties to those of siRNAs and, as described below, shRNAs are processed into siRNAs by the conserved cellular RNAi machinery. Thus shRNAs are precursors of siRNAs and are similarly capable of inhibiting expression of a target transcript. As is the case for siRNA, an shRNA includes a portion that hybridizes with a target nucleic acid, e.g., an mRNA transcript and is usually the perfectly complementary to the target over about 15-29 nucleotides, typically between 17-21 nucleotides, e.g., 19 nucleotides. However, one of ordinary skill in the art will appreciate that one or more mismatches or unpaired nucleotides may be present in a duplex formed between the shRNA strand and the target transcript.

An RNAi agent is considered to be "targeted" to a transcript and to the gene that encodes the transcript if (1) the RNAi agent comprises a portion, e.g., a strand, that is at least approximately 80%, approximately 85%, approximately 90%, approximately 91%, approximately 92%, approximately 93%, approximately 94%, approximately 95%, approximately 96%, approximately 97%, approximately 98%, approximately 99%, or approximately 100% complementary to the transcript over a region about 15-29 nucleotides in length, e.g., a region at least approximately 15, approximately 17, approximately 18, or approximately 19 nucleotides in length; and/or (2) the Tm of a duplex formed by a stretch of 15 nucleotides of one strand of the RNAi agent and a 15 nucleotide portion of the transcript, under conditions (excluding temperature) typically found within the cytoplasm or nucleus of mammalian cells and/or in a Drosophila lysate as described, e.g., in U.S. Patent App. Pubs. 2002/0086356 and 2004/0229266, is no more than approximately 15° C. lower or no more than approximately 10° C. lower, than the Tm of a duplex that would be formed by the same 15 nucleotides of the RNAi agent and its exact complement; and/or (3) the stability of the transcript is reduced in the presence of the RNAi agent as compared with its absence. An RNAi agent targeted to a transcript is also considered targeted to the gene that encodes and directs synthesis of the transcript. A "target region" is a region of a target transcript that hybridizes with an antisense strand of an RNAi agent. A "target transcript" is any RNA that is a target for inhibition by RNA interference. The terms "target RNA" and "target transcript" are used interchangeably herein.

Selection of appropriate RNAi agents is facilitated by using computer programs that automatically align nucleic acid sequences and indicate regions of identity or homology. Such programs are used to compare nucleic acid sequences obtained, for example, by searching databases such as GenBank or by sequencing PCR products. Comparison of nucleic acid sequences from a range of species allows the selection of nucleic acid sequences that display an appropriate degree of identity between species. In the case of genes that have not been sequenced, Southern blots are performed to allow a determination of the degree of identity between genes in target species and other species. By performing Southern blots at varying degrees of stringency, as is well known in the art, it is possible to obtain an approximate measure of identity. These procedures allow the selection of RNAi that exhibit a high degree of complementarity to target nucleic acid sequences in a subject to be controlled and a lower degree of complementarity to corresponding nucleic acid sequences in other species. One skilled in the art will realize that there is considerable latitude in selecting appropriate regions of genes for use in the present invention.

Selection of an appropriate antisense nucleic acid is facilitated by using computer programs that automatically align nucleic acid sequences and indicate regions of identity or homology. Such programs are used to compare nucleic acid sequences obtained, for example, by searching databases such as GenBank or by sequencing PCR products. Comparison of nucleic acid sequences from a range of species allows the selection of nucleic acid sequences that display an appropriate degree of identity between species. In the case of genes that have not been sequenced, Southern blots can be performed to allow a determination of the degree of identity between genes in target species and other species. By performing Southern blots at varying degrees of stringency, as is well known in the art, it is possible to obtain an approximate measure of identity. These procedures allow the selection of antisense nucleic acids that exhibit a high degree of complementarity to target nucleic acid sequences in a subject to be controlled and a lower degree of complementarity to corresponding nucleic acid sequences in other species. One skilled in the art will realize that there is considerable latitude in selecting appropriate regions of genes for use in the present invention.

Selection of hybridization sites for antisense nucleic acids can be made by one of skill in the art using methods described in the literature. For example, Ding, et al., report a method for defining mRNA hybridization sites based on determining RNA structures using algorithms and thermodynamic and structural properties of the RNA (Ding, et al., 2001, Statistical prediction of single-stranded regions in RNA secondary structure and application to predicting effective antisense target sites and beyond, Nucleic Acids Research 29(5):1034-1046; incorporated herein by reference in its entirety). Sczakiel, et al., also describe a method for computer-supported design of antisense oligonucleotides (Sczakiel, et al., 2000, Theoretical and experimental approaches to design effective antisense oligonucleotides, Frontiers in Bioscience 5: D194-201; Schen, et al., 2000, RNA accessibility prediction: a theoretical approach is con-

sistent with experimental studies in cell extracts, Nucleic Acids Research 28: 2455-2461; Patzel, et al., 1999, A theoretical approach to select effective antisense oligodeoxyribonucleotides at high statistical probability, J. Biol. Chem. 266:18162-18171; all incorporated herein by reference in their entirety).

Reports of other methods for identifying mRNA hybridization sites used include, e.g., Chiang, et al., who describe a method based on calculating melting temperatures (Chiang, et al., 1991, Antisense oligonucleotides inhibit intercellular adhesion molecule 1 expression by two distinct mechanisms, J. Biol. Chem. 266: 18162-18171, incorporated herein by reference in its entirety). Methods based on calculation of duplex formation free energies have been used (see, e.g., Stull, et al., 1992, Predicting antisense oligonucleotide inhibitory efficacy: a computational approach using histograms and thermodynamic indices, Nucleic Acids Research 20:3501-3508; Ding, et al., 1999, A bayesian statistical algorithm for RNA secondary structure prediction, Comput. Chem. 23:387-400; all incorporated herein by reference in their entirety). Still other methods rely on the use of combinatorial oligonucleotides to identify the hybridization sites within the target RNA. Identification of the hybridization sites is made using RNase H cleavage (Lloyd, et al., 2001, Determination of optimal sites of antisense oligonucleotide cleavage within TNFα mRNA, Nucleic Acids Research 29:3664-3673, incorporated herein by reference in its entirety), microarray analysis (Mir, et al., 1999, Determining the influence of structure on hybridization using oligonucleotide arrays, Nature Biotechnology 17:788-792; and Sohail, et al., 2001, Antisense oligonucleotides selected by hybridization to scanning arrays are effective reagents in vivo, Nucleic Acids Research 29:2041-2051, both incorporated herein by reference in their entirety) or MALDI-TOF mass spectrometry (Altman, et al., 1999, Selection of modified oligonucleotides with increased target affinity via MALDI-monitored nuclease survival assays, J. Comb. Chem. 1:493-508, incorporated herein by reference in its entirety).

The utility of an antisense nucleic acid molecule for modulation (including inhibition) of an mRNA can be readily determined by simple testing. Thus, an in vitro or in vivo expression system comprising the targeted mRNA, mutations or fragments thereof, can be contacted with a particular antisense nucleic acid molecule (modified or un modified) and levels of expression are compared to a control, that is, using the identical expression system which was not contacted with the antisense nucleic acid molecule. In vitro assays of oligonucleotide activity can also be useful for identifying antisense nucleic acids of the invention. For example, Lloyd, et al., report a direct inverse correlation between predicted chimeric antisense oligonucleotide activities, as determined using an in vitro RNase H assay, and the resultant levels of mRNA and protein expression (Lloyd, et al., 2001, Determination of optimal sites of antisense oligonucleotide cleavage within TNFα mRNA, Nucleic Acids Research 29(17): 3664-3673). According to Lloyd, et al., the ability of the in vitro assay to predict oligonucleotide efficacy was superior to other computationally based RNA structural predictions, ΔG calculations and in vivo trial and error methodologies.

Bcl-2 and molecules that work in conjunction with Bcl-2 are also targets for cancer therapy. B cell leukemia/lymphoma-2 (Bcl-2) is the prototype member of a family of cell death regulatory proteins. Bcl-2 is found mainly in the mitochondria and blocks apoptosis by interfering with the activation of caspases. Gene transfer of Bcl-2 into tumor cells has been shown to enhance their metastatic potential (Miyake et al., 1999). Bcl-2 gene transfer may be applied to bone marrow transplant since Bcl-2 enhances the survival of hematopoietic stem cells after reconstitution of irradiated recipient (Innes et al., 1999). Also, Bcl-2 gene transfer could be useful against neurodegenerating diseases since expression of Bcl-2 in neurons protects them from apoptosis (Saille et al., 1999). Bcl-XS (short isoform) is a dominant negative repressor of Bcl-2 and Bcl-XL. It has been used in gene therapy experiments to initiate apoptosis in tumors that express Bcl-2 and Bcl-XL. Expression of Bcl-XS reduces tumor size (Ealovega et al., 1996) and sensitizes tumor cells to chemotherapeutic agents (Sumatran et al., 1995), suggesting a role for Bcl-XS in initiating cell death in tumors that express Bcl-2 or Bcl-XL (Dole et al., 1996). Expression of these genes or RNAi agents targeting them can be selected as appropriate for the condition being treated.

Equivalent Molecules

The invention comprehends that the therapeutic molecules delivered using the vectors and methods of the present invention can be modified. The nucleic acid molecule encoding a given protein be modified, for instance, due to the degeneracy of codon usage, a coding sequence can be modified, and modified and truncated forms of a protein can be used, such as those which may be found in the literature or analogous to truncated or modified forms found in the literature.

Likewise, analogs, homologs, derivatives, and variants of the coding sequences can be used and analogs, homologs, derivatives and variants of proteins can be expressed; such expressed analogs, homologs, derivatives and variants of proteins can have activity analogous to that of the full-length protein, and the analogs, homologs, derivatives and variants of the protein coding sequence encode such active analogs, homologs, derivatives, and variants.

III. Heterologous Regulatory Sequences

A "first heterologous regulatory sequence" is positioned upstream (5' of) the first heterologous nucleic acid sequence, encoding, e.g., a therapeutic gene. Similarly, a "second heterologous regulatory sequence" can be positioned upstream (5' of) the second heterologous nucleic acid sequence and downstream of the first heterologous nucleic acid sequence, and a "third heterologous regulatory sequence" can be positioned upstream (5' of) the third heterologous nucleic acid sequence and downstream of the second heterologous nucleic acid sequence. A heterologous regulatory sequence can be a sequence that influences, e.g., expression or localization, of a therapeutic molecule encoded by a heterologous nucleic acid sequence. A heterologous regulatory sequence can comprise a promoter, enhancer, protease recognition (cleavage) sequence, internal ribosome binding site, intracellular or intercellular trafficking (transport) signal, etc. Certain heterologous regulatory sequences, e.g., cleavage sequences and trafficking sequences, can be expressed as part of a fusion with a therapeutic molecule.

In embodiments, a heterologous regulatory sequence affects an upstream heterologous nucleic acid sequence. Therefore, a fourth regulatory sequence, located downstream of a third heterologous nucleic acid sequence, can be included in the transfer vector. Also, for example, the second heterologous regulatory sequence can contain elements that affect expression or localization of the first heterologous nucleic acid and its corresponding therapeutic molecule, and the third heterologous regulatory sequence can contain ele-

ments that affect expression or localization of the second heterologous nucleic acid and its corresponding therapeutic molecule.

Promoters and Enhancers

The promoter sequence may be homologous or heterologous to the desired gene sequence. A wide range of promoters may be utilized, including viral or mammalian promoters. Cell or tissue specific promoters can be utilized to target expression of gene sequences in specific cell populations. Suitable mammalian and viral promoters for the present invention are available in the art.

Examples of promoters, cellular promoters/enhancers and inducible promoters/enhancers that can be used in combination with the present invention are listed in FIGS. **4** and **5**. Any suitable promoter/enhancer combination (as per the Eukaryotic Promoter Database, EPDB) can be used to drive expression of constructs of the invention.

Trafficking Signals

Trafficking signals can direct a molecule to different compartments within a cell, as well as outside the cell and into other cells. An "intercellular trafficking signal," or transport signal, is an amino acid sequence that imparts the property to a protein of being able to pass through membranes between cells. Examples of membrane-penetrating proteins include, but are not limited to, several plant and bacterial protein toxins, such as ricin, abrin, modeccin, diphtheria toxin, cholera toxin, anthrax toxin, heat labile toxins, and *Pseudomonas aeruginosa* exotoxin A. Examples of membrane-penetrating proteins that are not toxins include the TAT protein of human immunodeficiency virus and the protein VP22, the product of the UL49 gene of herpes simplex virus type 1. One line of research involves adapting such molecules from their naturally destructive role into therapeutic compositions.

The effectiveness of lentiviral vectors to deliver genes encoding proteins fused to herpes simplex virus type 1 tegument protein VP22 has been reported (see, e.g., U.S. Pat. App. Pub. No. 2003/0119770). "VP22" denotes: protein VP22 of HSV, e.g., of HSV1, and transport-active fragments and homologues thereof, including transport-active homologues from other herpesviruses including varicella zoster virus VZV, marek's disease virus MDV and bovine herpesvirus BHV.

Among sub-sequences of herpesviral VP22 protein with transport activity, investigators have found that, for example, transport activity is present in polypeptides corresponding to amino acids 60-301 and 159-301 of the full HSV1 VP22 sequence (1-301). A polypeptide consisting of aa 175-301 of the VP22 sequence has markedly less transport activity, and is less preferred in connection with the present invention. Accordingly, the present invention relates in one aspect to a sub-sequence of VP22 containing a sequence starting preferably from about aa 159 (or earlier, towards the N-terminal, in the native VP22 sequence), to about aa 301, and having (relative to the full VP22 sequence) at least one deletion of at least part of the VP22 sequence which can extend for example from the N-terminal to the cited starting point, e.g., a deletion of all or part of the sequence of about aa 1-158. (Less preferably, such a deletion can extend further in the C-terminal direction, e.g., to about aa 175.) For example, partial sequences in the range from about aa 60-301 to about aa 159-301 are provided.

VP22 sequences as contemplated herein extend to homologous proteins and fragments based on sequences of VP22 protein homologues from other herpesviruses, e.g., the invention provides corresponding derivatives and uses of the known VP22-homologue sequences from VZV (e.g., all or homologous parts of the sequence from aa 1-302), from MDV (e.g., all or homologous parts of the sequence from aa 1-249) and from BHV (e.g., all or homologous parts of the sequence from aa 1-258). The sequences of the corresponding proteins from HSV2, VZV, BHV and MDV are available in public protein/nucleic acid sequence databases. Thus, for example, within the EMBL/Genbank database, a VP22 sequence from HSV2 is available as gene item UL49 under accession no. Z86099 containing the complete genome of HSV2 strain HG52; the complete genome of VZV including the homologous gene/protein is available under accession numbers X04370, M14891, M16612; the corresponding protein sequence from BHV is available as "bovine herpesvirus 1 virion tegument protein" under accession number U21137; and the corresponding sequence from MDV is available as gene item UL49 under accession number L10283 for "gallid herpesvirus type 1 homologous sequence genes". In these proteins, especially those from HSV2 and VZV, corresponding deletions can be made, e.g., of sequences homologous to aa 1-159 of VP22 from HSV1. Homologies between these sequences are readily accessible by the use of standard algorithms, default parameters, and software.

Furthermore, chimeric VP22 proteins and protein sequences are also useful within the context of the present invention, e.g., a protein sequence from VP22 of HSV1 for part of which a homologous sequence from the corresponding VP22 homologue of another herpesvirus has been substituted. For example, into the sequence of polypeptide 159-301 from VP22 of HSV1, C-terminal sequences can be substituted from VP22 of HSV2 or from the VP22 homologue of BHV.

Investigators have found that deletion of the 34-amino acid C-terminal sequence from VP22 of HSV1 abolishes transport-activity, thus this sequence region contains essential elements for transport activity. According to a further aspect of the invention, there are provided in-frame fusions comprising a nucleic acid sequence encoding the 34-amino acid C-terminal sequence from VP22, or a variant thereof, together with a sequence for a heterologous nucleic acid sequence. In-frame fusions of nucleic acid sequences encoding modified terminal fragments having at least one mutation insertion or deletion relative to the C-terminal 34 amino acid sequence of HSV1 VP22 are also provided.

Investigators have also been found that sequences necessary for transport activity contain one or a plurality of amino acid sequence motifs or their homologues from the C-terminal sequence of VP22 of HSV1 or other herpesviruses, which can be selected from RSASR (SEQ ID NO: 1), RTASR (SEQ ID NO: 2), RSRAR (SEQ ID NO: 3), RTRAR (SEQ ID NO: 4), ATATR (SEQ ID NO 5), and wherein the third or fourth residue A can be duplicated, e.g., as in RSAASR (SEQ ID NO: 6). Corresponding in-frame fusions of nucleic acid sequences encoding these signals are also provided.

The HIV-1 Tat protein was also reported to enhance intercellular trafficking in vitro. It is composed of 86 amino acids and contains a highly basic region and a cysteine-rich region. It was found that Tat-derived peptides as short as eleven amino acids are sufficient for transduction of proteins (Fawell, et al., 1994, Proc Natl Acad Sci USA 91:664-668). However, the exact mechanism by which the 11-amino acid transduction domain crosses lipid bilayers is poorly understood. Schwarze et al. (Science, 385:1569-1572, 1999) reported generating a Tat-β-galactosidase fusion protein that was delivered efficiently into brain tissue and skeletal muscle in vivo.

In embodiments of the present invention, a Tat-derived trafficking protein of eleven amino acids Try-Gly-Arg-Lys-lys-Arg-Arg-Gln-Arg-Arg (SEQ ID NO: 7) is used to enhance intercellular trafficking of therapeutic molecules. Any appropriate nucleic acid sequence can be used to express this protein, e.g., tat ggc agg aag aag cgg aga cag cga cga aga (SEQ ID NO:8) with a start codon.

Control of intracellular as well as intercellular transport is contemplated for use in the methods of the invention. This level of control can be used to target therapeutic proteins to particular cellular compartments, e.g., to correct defects for proteins involved in specific disease processes.

Other potentially useful Tat-derived trafficking sequences have been described. For example Chauhan, et al., 2007, The Taming of the Cell Penetrating Domain of the HIV Tat: Myths and Realities, J. Control Release 117(2): 148-162, incorporated herein by reference in its entirety, disclose variants of the Tat protein transduction domain.

Following are Tat-derived cell penetrating peptides described by Chauhan, et al.:

PTD	YGRKKRRQRRR	(SEQ ID NO: 9)
PTD-4	YARAAARQARA	(SEQ ID NO: 10)
YM-3	THRLPRRRRRR	(SEQ ID NO: 11)
CTP	GGRRARRRRRR	(SEQ ID NO: 12)

As reported by the authors, depending on the nature of the protein being transported, these peptides effect transport, including transport among cellular compartments. For example, cytoplasmic proteins reportedly end up in the nucleus when PTD is used, nucleo-cytoplasmic proteins go to the nucleus when PTD-4 is used, secretory proteins are found in the nucleus and outside the cell when YM-3 is used, and membrane proteins go to the membrane and nucleus when CTP is used.

Other cell penetrating proteins useful for introducing recombinant proteins into cells are penetratin, polylysine, polyarginine, Kaposi FGF, Syn B1, FGF-4, nuclear localization signal, anthrax toxin derivative 254-amino acids peptide segment, diphtheria toxin "R" binding domain, MPG (described below), WR peptide, and exotoxin A. Penetratin peptide has also been used for siRNA delivery to cells. In embodiments, these proteins or their derivatives used in as trafficking signals in the vectors and methods of the invention.

A fusion peptide, "MPG," has been described for efficient transduction of nucleic acids. This peptide is a bipartite amphipathic peptide obtained by combining the fusion domain of HIV-gp41 protein and the NLS domain of SV40 large T antigen. This peptide is being used as a nanoparticle for transduction of siRNA in vitro and is also available commercially. (See, e.g., Chauhan, et al., 2007; Morris, et al., 1999, A novel potent strategy for gene delivery using a single peptide vector as a carrier, Nucleic Acids Research 27:3510-3517.)

In embodiments of the vectors and methods of the invention, an siRNA or other antisense agent is transported intercellularly or intracellularly.

The inventive transfer vector may include splice acceptor and splice donor sequences flanking the gene construct. In the case of multigene transfer vector, the splice acceptor sequence may be inserted upstream of the first promoter-structural gene unit and splice donor site may be located in the downstream of the last promoter-structural gene unit in the 5' to 3' order. A mammalian insulator sequence (MIS) may be inserted downstream of the gene units, or if specific promoters are used for the gene units, then the MIS may be placed before the first gene unit and after the last gene unit followed by splice donor site. A translation initiation sequence may also be included in the multigene gene transfer vector. The use of the translation initiation sequence causes the second and subsequent multigene units to be expressed evenly and stably compared with the first gene expression product.

Translation Initiation Sequence

The transfer vector may optionally comprise a sequence that allows for translation initiation in the middle of a messenger RNA (mRNA) sequence as part of the greater process of protein synthesis. While this sequence may be typically IRES other sequences may be used, which may have a similar sequence.

Splice Acceptor/Splice Donor Sequence

The transfer vector may optionally comprise a promoter-gene sequence flanked by a splice acceptor site 5' to a gene unit and a splice donor site 3' to the promoter-gene. For instance, the following elements may be present 5' to 3': a splice acceptor site, first promoter for the first gene unit, first heterologous nucleic acid sequence and splice donor site 3' to the first gene unit, then a splice acceptor site and a second promoter for the second gene, the second heterologous nucleic acid sequence, splice donor site, and then if there is a third gene, a splice acceptor site, third promoter for the third gene unit, the third heterologous nucleic acid sequence, and a splice donor site, and so forth. The splice acceptor or donor site typically may include about 5 to 10 bases.

Mammalian Insulator Sequence (MIS)

The transfer vector may optionally comprise an insulator sequence, in particular mammalian insulator sequence (MIS). Insulators are DNA sequence elements that can protect against the activation influence of distal enhancers associated with other genes, and also help to preserve the independent function of genes embedded in a genome in which they are surrounded by regulatory signals so that cross interaction is avoided. The insulators as used in the present application may not necessarily be limited to use in lentiviruses. The insulators may be used with other viral vectors.

To provide background on these insulator sequences, the zinc finger protein CCCTC-binding factor (CTCF) is a versatile transcription regulator that binds to insulators and shows enhancer-blocking activity for regulating gene expression control. Chicken (beta-globin) insulator with about 1.2 kb is widely used in vitro or in vivo animals, but generally does not have human or mammalian compatible factors to be entirely useful in treating humans or mammals.

Bovine or human growth hormone transcriptional stop sequences may also be used as insulators. A short-element of about 238 bp containing the "HS" DNA element core sequence, which is the binding site for CTCF is also effective as an "enhancer blocking" element. Template DNA for generation of such element, for example a pcDNA3, which contains bovine growth hormone transcriptional stop sequence may be as follows: PCR primers: 5'-agctagatagt-gtcacctaaatgc-3' (SEQ ID NO:13) and 5'-agcatgcctgctatt-3' (SEQ ID NO:14).

A binding site for the transcription factor CTCF may be responsible for enhancer-blocking activity in a variety of insulators, including the insulators at the 5' and 3' chromatin boundaries of the chicken and human beta-globin locus. The minimal element responsible for this activity may be a binding site for CTCF.

When several different sequences of insulators of 5' and 3' HS/CTCF are compared using "human insulator" as the template, there are at least two mutations in the mouse, at least 5 mutations in chicken at the 5' HS/CTCF. In addition, there are at least two mutations in mouse, at least 4 mutations at the 3' HS/CTCF.

The insulator sequence as used in the lentivirus transfer vector may be placed as follows. In a single gene vector where a general promoter is used to control gene expression, MIS may be placed 3' of the gene and upstream of the splicing donor site. However, if a specific promoter is used to control the expression of the gene, the MIS may be placed upstream of the specific promoter and downstream of the gene, wherein the MIS sequences are optionally flanked by the splice acceptor on the 5' side and splice donor on the 3' side of the gene. In a single gene construct, for RNAi expression where specific promoter is used, MIS may flank the specific promoter-structural gene construct, optionally with a splice donor site downstream of the 3' MIS. Two or more MIS may be used together to enhance the blocking effects.

For multiple gene vectors, if a general promoter is used, an MIS may be included downstream of the gene units with splice donor site 3' to the MIS. However, if a specific promoter is used MIS is place upstream of the first specific promoter for the first structural gene, and another MIS downstream of the last specific promoter-structural gene set. The MIS are optionally flanked by splice acceptor on the 5' side and splice donor on the 3' side.

IV. Therapeutic Applications

The invention includes a variety of therapeutic applications for the lentiviral vectors of the invention. In particular, lentiviral vectors are useful for gene therapy. Exemplary therapeutic applications are listed in FIG. **7**. The invention provides methods of treating and/or preventing infection by an infectious agent, the method comprising administering to a subject prior to, simultaneously with, or after exposure of the subject to the infectious agent a composition comprising an effective amount of a lentiviral vector, wherein the lentiviral vector directs transcription of at least one RNA that hybridizes to form an shRNA or siRNA that is targeted to a transcript produced during infection by the infectious agent, which transcript is characterized in that reduction in levels of the transcript delays, prevents, and/or inhibits one or more aspects of infection by and/or replication of the infectious agent.

The invention provides methods of treating a disease or clinical condition by, for example, removing a population of cells from a subject at risk of or suffering from the disease or clinical condition and engineering or manipulating the cells to comprise an effective amount of therapeutic agents by infecting or transfecting the cells with a lentiviral vector. At least a portion of the cells are returned to the subject.

Without limitation, therapeutic approaches may find particular use in diseases such as cancer, in which a mutation in a cellular gene is responsible for or contributes to the pathogenesis of the disease, and in which specific inhibition of the target transcript bearing the mutation may be achieved by expressing an RNAi agent targeted to the target transcript within the cells, without interfering with expression of the normal (i.e. non-mutated) allele. Furthermore, treatment of any cancer in which P53 expression is defective is contemplated using the vectors and methods of the invention.

The invention is also useful for the treatment of genetic diseases, for example, Gaucher's Disease and Fabry's Disease. Gaucher's disease is a lysosomal storage disease caused by a deficiency of the enzyme glucocerebrosidase. This deficiency leads to an accumulation of the enzyme substrate, the fatty substance glucocerebroside (also known as glucosylceramide). Fatty material can collect in the spleen, liver, kidneys, lungs, brain and bone marrow. It has been reported, using a mouse model for Gaucher's Disease, that a lentiviral vector can transduce HSCs that are capable of long-term gene expression in vivo (Kim, et al., 2005, "Long-term expression of the human glucocerebrosidase gene in vivo after transplantation of bone-marrow-derived cells transformed with a lentivirus vector, J. Gene Med. 7:878-887, incorporated herein by reference).

According to certain embodiments of the invention, rather than removing cells from the body of a subject, infecting or transfecting them in tissue culture, and then returning them to the subject, inventive lentiviral vectors or lentiviruses are delivered directly to the subject.

Neurological Disorders

Cells infected with a recombinant lentivirus of the invention, in vivo, or ex vivo, used for treatment of a neuronal disorder for example, may optionally contain an exogenous gene, for example, a gene which encodes a receptor or a gene which encodes a ligand. Such receptors include receptors which respond to dopamine, GABA, adrenaline, noradrenaline, serotonin, glutamate, acetylcholine and other neuropeptides, as described above. Examples of ligands which may provide a therapeutic effect in a neuronal disorder include dopamine, adrenaline, noradrenaline, acetylcholine, gamma-aminobutyric acid and serotonin. The diffusion and uptake of a required ligand after secretion by an infected donor cell would be beneficial in a disorder where the subject's neural cell is defective in the production of such a gene product. A cell genetically modified to secrete a neurotrophic factor, such as nerve growth factor (NGF), might be used to prevent degeneration of cholinergic neurons that might otherwise die without treatment. Alternatively, cells can be grafted into a subject with a disorder of the basal ganglia, such as Parkinson's disease, or can be modified to contain an exogenous gene encoding L-DOPA, the precursor to dopamine. Parkinson's disease is characterized by a loss of dopamine neurons in the substantia nigra of the midbrain, which have the basal ganglia as their major target organ.

U.S. Pat. No. 6,800,281, "Lentiviral-mediated growth factor gene therapy for neurodegenerative diseases," incorporated herein by reference in its entirety, describes methods for treating Parkinson's Disease using glial cell derived neurotrophic factor (GDNF), highly conserved neurotrophic factor that potently promotes the survival of many types of neurons.

Parkinson's disease (PD) is a neurodegenerative disorder characterized by the loss of the nigrostriatal pathway; a progressive disorder resulting from degeneration of dopaminergic neurons within the substantia nigra. Although the cause of Parkinson's disease is not known, it is associated with the progressive death of dopaminergic (tyrosine hydroxylase (TH) positive) mesencephalic neurons, inducing motor impairment. The characteristic symptoms of Parkinson's disease appear when up to 70% of TH-positive nigrostriatal neurons have degenerated. Surgical therapies aimed at replacing lost dopaminergic neurons or disrupting aberrant basal ganglia circuitry have recently been tested (C. Honey et al. 1999). However, these clinical trials have focused on patients with advanced disease, and the primary goal of forestalling disease progression in newly diagnosed patients has yet to be realized. The administration can be by stereotaxic injection. The administration can be intracranially, e.g., intracranially to stiatum or to substantia nigra. The administration can also be by retrograde transport.

In an embodiment, the administration site is the striatum of the brain, in particular the caudate putamen. Injection into the putamen can label target sites located in various distant regions of the brain, for example, the globus pallidus, amygdala, subthalamic nucleus or the substantia nigra. Transduction of cells in the pallidus commonly causes

retrograde labelling of cells in the thalamus. In a preferred embodiment the (or one of the) target site(s) is the substantia nigra.

In another embodiment the vector system is injected directly into the spinal cord. This administration site accesses distal connections in the brain stem and cortex. Within a given target site, the vector system may transduce a target cell. The target cell may be a cell found in nervous tissue, such as a neuron, astrocyte, oligodendrocyte, microglia or ependymal cell. In a preferred embodiment, the target cell is a neuron, in particular a TH positive neuron.

The vector system can be administered by direct injection. Methods for injection into the brain (in particular the striatum) are well known in the art (Bilang-Bleuel et al (1997) Proc. Acad. Natl. Sci. USA 94:8818-8823; Choi-Lundberg et al (1998) Exp. Neuro1.154:261-275; Choi-Lundberg et al (1997) Science 275:838-841; and Mandel et al (1997)) Proc. Acad. Natl. Sci. USA 94:14083-14088). Stereotaxic injections may be given.

Administration of the cells or virus into selected regions of the recipient subject's brain may be made by drilling a hole and piercing the dura to permit the needle of a microsyringe to be inserted. The cells or recombinant lentivirus can alternatively be injected intrathecally into the spinal cord region. A cell preparation infected ex vivo, or the recombinant lentivirus of the invention, permits grafting of neuronal cells to any predetermined site in the brain or spinal cord, and allows multiple grafting simultaneously in several different sites using the same cell suspension or viral suspension and permits mixtures of cells from different anatomical regions.

For transduction in tissues such as the brain, it is necessary to use very small volumes, so the viral preparation is concentrated by ultracentrifugation. The resulting preparation should have at least 10^8 pfu./ml, preferably from 10^8 to 10^{10} pfu./ml, more preferably at least 10^9 pfu./ml. (The titer is expressed in transducing units per ml (pfu./ml) as titred on a standard D17 cell line). It has been found that improved dispersion of transgene expression can be obtained by increasing the number of injection sites and decreasing the rate of injection (Horellou and Mallet (1997) as above). Usually between 1 and 10 injection sites are used, more commonly between 2 and 6. For a dose comprising $1\text{-}5\times10^9$ pfu./ml, the rate of injection is commonly between 0.1 and 10 μl/min, usually about 1 μl/min.

In another embodiment the vector system is administered to a peripheral administration site. The vector may be administered to any part of the body from which it can travel to the target site by retrograde transport. In other words the vector may be administered to any part of the body to which a neuron within the target site projects.

The "periphery" can be considered to be all part of the body other than the CNS (brain and spinal cord). In particular, peripheral sites are those which are distant to the CNS. Sensory neurons may be accessed by administration to any tissue which is innervated by the neuron. In particular this includes the skin, muscles and the sciatic nerve.

In another embodiment the vector system is administered intramuscularly. In this way, the system can access a distant target site via the neurons which innervate the innoculated muscle. The vector system may thus be used to access the CNS (in particular the spinal cord), obviating the need for direct injection into this tissue. There is thus provided a non-invasive method for transducing a neuron within the CNS. Muscular administration also enables multiple doses to be administered over a prolonged period.

Other neuronal disorders that can be treated similarly by the method of the invention include Alzheimer's disease, Huntington's disease, neuronal damage due to stroke, and damage in the spinal cord. Alzheimer's disease is characterized by degeneration of the cholinergic neurons of the basal forebrain. The neurotransmitter for these neurons is acetylcholine, which is necessary for their survival. Engraftment of cholinergic cells infected with a recombinant lentivirus of the invention containing an exogenous gene for a factor which would promote survival of these neurons can be accomplished by the method of the invention, as described. Following a stroke, there is selective loss of cells in the CA1 of the hippocampus as well as cortical cell loss which may underlie cognitive function and memory loss in these patients. Once identified, molecules responsible for CA1 cell death can be inhibited by the methods of this invention. For example, antisense sequences, or a gene encoding an antagonist can be transferred to a neuronal cell and implanted into the hippocampal region of the brain.

The method of transferring nucleic acid also contemplates the grafting of neuroblasts in combination with other therapeutic procedures useful in the treatment of disorders of the CNS. For example, the lentiviral infected cells can be co-administered with agents such as growth factors, gangliosides, antibiotics, neurotransmitters, neurohormones, toxins, neurite promoting molecules and antimetabolites and precursors of these molecules such as the precursor of dopamine, L-DOPA.

Further, there are a number of inherited neurologic diseases in which defective genes may be replaced including: lysosomal storage diseases such as those involving β-hexosamimidase or glucocerebrosidase; deficiencies in hypoxanthine phosphoribosyl transferase activity (the "Lesch-Nyhan" syndrome"); amyloid polyneuropathies (-prealbumin); Duchenne's muscular dystrophy, and retinoblastoma, for example.

For diseases due to deficiency of a protein product, gene transfer could introduce a normal gene into the affected tissues for replacement therapy, as well as to create animal models for the disease using antisense mutations. For example, it may be desirable to insert a Factor IX encoding nucleic acid into a lentivirus for infection of a muscle or liver cell.

Stem cell therapy contemplates injection of stem cells transduced by a lentiviral vector carrying a therapeutic gene of interest into a fetus central nervous system. The correction or rescue of a genetic defect is achieved during cell differentiation. Stem cells at a nondividing stage should be efficiently transduced by such a vector using a convenient infection technique.

V. Pharmaceutical Compositions

The invention further provides pharmaceutical compositions comprising lentiviral vectors of the invention and one or more pharmaceutically acceptable carriers.

The pharmacologically active compounds of this invention can be processed in accordance with conventional methods of galenic pharmacy to produce medicinal agents for administration to patients, e.g., mammals including humans.

The compounds of this invention can be employed in admixture with conventional excipients, i.e., pharmaceutically acceptable organic or inorganic carrier substances suitable for parenteral, enteral (e.g., oral) or topical application, which do not deleteriously react with the active compounds. Suitable pharmaceutically acceptable carriers include but are not limited to water, salt solutions, alcohols, gum arabic, vegetable oils, benzyl alcohols, polyethylene glycols, gelatin, carbohydrates such as lactose, amylose or starch, magnesium stearate, talc, silicic acid, viscous paraffin, perfume oil, fatty acid monoglycerides and diglycerides, pentaerythritol fatty acid esters, hydroxy methylcellulose, polyvinyl pyrrolidone, etc. The pharmaceutical preparations can be sterilized and if desired mixed with auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents,

emulsifiers, salts for influencing osmotic pressure, buffers, coloring, flavoring and/or aromatic substances and the like which do not deleteriously react with the active compounds. They can also be combined where desired with other active agents, e.g., vitamins.

For parenteral application, particularly suitable are injectable, sterile solutions, preferably oily or aqueous solutions, as well as suspensions, emulsions, or implants, including suppositories. Ampoules are convenient unit dosages.

For enteral application, particularly suitable are tablets, dragees, liquids, drops, suppositories, or capsules. A syrup, elixir, or the like can be used wherein a sweetened vehicle is employed.

Sustained or directed release compositions can be formulated, e.g., by inclusion in liposomes or those wherein the active compound is protected with differentially degradable coatings, e.g., by microencapsulation, multiple coatings, etc. It is also possible to freeze-dry these compounds and use the lyophilizates obtained, for example, for the preparation of products for injection.

For topical application, there are employed as non-sprayable forms, viscous to semi-solid or solid forms comprising a carrier compatible with topical application and having a dynamic viscosity preferably greater than water. Suitable formulations include but are not limited to solutions, suspensions, emulsions, creams, ointments, powders, liniments, salves, aerosols, etc., which are, if desired, sterilized or mixed with auxiliary agents, e.g., preservatives, stabilizers, wetting agents, buffers or salts for influencing osmotic pressure, etc. For topical application, also suitable are sprayable aerosol preparations wherein the active ingredient, preferably in combination with a solid or liquid inert carrier material, is packaged in a squeeze bottle or in admixture with a pressurized volatile, normally gaseous propellant, e.g., a freon.

It will be appreciated that the actual preferred amounts of active compound in a specific case will vary according to the specific compound being utilized, the compositions formulated, the mode of application, and the particular situs and organism being treated. Dosages for a given host can be determined using conventional considerations, e.g., by customary comparison of the differential activities of the subject compounds and of a known agent, e.g., by means of an appropriate, conventional pharmacological protocol.

The compositions may, if desired, be presented in a pack or dispenser device which may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration.

The lentiviral vectors of the invention can also be administered in combination with other agents, for example, chemotherapeutic agents, radiation treatment, or steroids, according to methods known and described in the art. PCT Publication WO 2008/08069942, "Novel Methods of Enhancing Delivery of a Gene Therapy Vector Using Steroids," describes methods for enhancing expression of a viral vector-encoded therapeutic gene product by delivering to a subject the viral vector in conjunction with a steroid, e.g., prednisolone, cortisone, corticosterone, or dexamethasone. In embodiments, the individual therapies being combined are not necessarily administered together, e.g., they can be administered separately via different modes of administration, alternately, etc.

VII. Patients

The invention contemplates treatment of patients including human patients. The term patient as used in the present application refers to all different types of mammals including humans and the present invention is effective with respect to all such mammals. The present invention is effective in treating any mammalian species which have a disease potentially remedied by delivery of a gene product or inhibition of expression of a gene.

The contents of all cited references, including literature references, issued patents, published patent applications, and co-pending patent applications, cited throughout this application are hereby expressly incorporated by reference in their entirety.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims. The following examples are offered by way of illustration of the present invention, and not by way of limitation.

EXAMPLES

Example 1—Vector Construction

The additional components of the gene transfer system include a packaging (helper) plasmid and an envelope (Env) plasmid encoding VSV-G driven by the HIV-1 LTR (Mochizuki, H. et al., 1998, J Virol 72:8873-8883; Reiser, J. et al., 1996, PNAS USA 93:15266-15271). These packaging and envelope constructs are described herein and by Lai et al., 2000, PNAS, 97: 11297-11302, and Lai, et al., 2002, Neurosci. Res. 67: 363-371, incorporated herein by reference. The helper construct has a deletion in the packaging signal rendering it inactive, and the 5' LTR is replaced with the CMV-IE promoter. The CMV promoter was derived from pcDNA 3.1, which was used as a template for a PCR reaction yielding 590 bp of the CMV promoter. The HIV-1 helper construct was digested by EcoRV (33) and Afl II (517) to have generate a deletion of 475 bp from the U3 region of the 5'LTR. Insertion of the CMV promoter PCR fragment was then ligated into the HIV-1 helper construct to create a safer Tat-independent construct without compromising viral titer. The viral genes tat and nef were also inactivated. Pseudotyped vectors were produced in human embryonic kidney 293T cells using a three-component transient packaging system (Mochizuki, H. et al., 1998).

The transfer vectors were based on HIV-1 lentivirus vectors, and were made using methods similar to those described by Kim, et al., J. Gene Med. (2005), referenced above. The transfer vectors of the present invention are self-inactivating, i.e., they have a deletion in the U3 region of the 5' LTR that was introduced as follows: (a) the fragment between Nef and the 3' LTR was isolated by digesting with Xho-I and Afl-II; (b) the isolated fragment of about 820 bp was subcloned into the PUC18 vector; (c) the U3 region (containing the TATA box, SP1) of the 3' LTR of about 337 bp was deleted by EcoR-V and Rsa-I; and (d) a PCR fragment from (c) containing the regions of Nef and the 3' LTR with the U3 deletion were ligated back into the lentiviral vector.

Example 2—In Vitro Infection of a Prostate Cancer Cell Line with SIN-HIV-P53-EGFP

PC-3 (prostate cancer) cells were infected with a lentivirus containing a gene transfer vector expressing wild-type

P53 and EGFP. Three groups were tested. Treatment group: PC-3 cells were treated with SIN-HIV-P53-EGFP (construct shown in FIG. **6**). Control (negative) group: PC-3 cells were untreated. Control (positive) group: Normal cells were treated or not treated with SIN-HIV-P53-EGFP.

Prostate cancer cell-lines (PC-3) and 293-T cells were placed into the 12 well-plate with 0.4×10^6 cells, and were cultured in the RPMI 1640 and DMEM medium containing antibiotics. 200 μl of the packaged lentiviral vector SIN-CMV-p53-IRES/CS-EGFP (see FIG. **1**C) was added to the cells for the infection study under 1 mg/ml. The cells were then cultured by incubating for 6-16 hours at 37° C. in a CO_2 incubator. After infection for 48-72 hours, we evaluated the expression of EGFP using a fluorescence microscope in both the transduced PC-3 and 239-T cells. We found that a high level of EGFP was expressed in both PC-3 and 293-T cells transduced by SIN-CMV-p53-IRES/CS-EGFP, but not in the control group of those cells that were not infected (FIGS. **8**A-C). The results indicated that our vector system worked well to express both transgenes simultaneously.

After treatment, RNA was isolated from the cells, and RT-PCR was used to determine the p53 expression level at different time points. Corresponding cell growth curves and survival cell numbers were determined at different time points, and the results for treated and untreated cells compared.

To evaluate confirm P53 expression, we extracted total RNA 96 hours after infection. 0.5 μg total RNA from each group (treated and untreated) were used as template for RT-PCR. P53 mRNA was amplified using sense primer (from the partial promoter of CMV: 5'-tacgtattagtcatcgctatt-3) and antisense primer (from the end of the P53 gene: 5'-aggcctcattcagctctcgga-3'). The results showed that the cell lines infected by the vector expressed a high level of P53, and the uninfected cell lines expressed only the basic level of endogenous p53 (see FIG. **8**D). Thus, our data indicated that our vector system is functional for highly expressing both the two transgene protein in the cells at the same time.

We also compared growth and survival of the prostate cancer cells transduced with the vector with untreated cells. Taking time points for up to one week, we observed the growth condition and cell numbers under the light and fluorescent microscopes. Expression of EGFP indicated transduction by the vector. We observed that PC-3 cells transduced by the vector died quickly compared with the untreated PC-3 cells, and that both treated and untreated 293-T cells showed growth and no significant cell death. The result can be confirmed using FACS analysis to quantitate cell numbers at the respective time points.

Example 3—In Vivo Transduction with SIN-HIV-P53-EGFP and SIN-HIV-P53-Bcl-2 RNAi

Transduction with the SIN-HIV-P53-EGFP vector described in Example II, expressing P53 and GFP, or SIN-HIV-P53-Bcl-2 RNAi, expressing P53 and an RNAi agent targeting human Bcl-2, is done in vivo. See FIG. **1**C, Panel B, upper construct (CMV-P53-hPSA-Bcl-2 RNAi). The NOD SCID mouse model, characterized by a major immunodeficiency, is used to study gene therapy for prostate cancer using lentiviral transfer systems of the invention.

NOD SCID mice are subcutaneously implanted with a PC-3 cell suspension in a thoracic postero-lateral wound. Tumor cell suspensions are injected using a 30-gauge needle and a 1-ml disposable syringe. The volume of inoculation is 100 μl (2×10^6 tumor cells suspended in 100 μl of PBS). After tumor appearance (1-2 weeks post-implantation), the virus injections are made.

Tumor progression is monitored by palpation twice a week by the investigator, and subcutaneous tumor size is measured using a caliper. Viral vector is administered at a titer of about 10^8 pfu/ml, by tail-vein injection. Animals are euthanized according to tumor size or clinical status during the observation period. Cervical dislocation is performed 2 to 4 weeks after injection.

Prostate cancer tumor sizes are measured, and for EGFP immunostaining to evaluate distribution of the vector, liver, lung, heart, and bone marrow cells are harvested. These tissue samples are collected for immunostaining does as described by Lai, et al., PNAS, 2002. The tissues are subjected to total RNA and protein extractions, to evaluate expression of the transgenes. The tissue slides are made and examined for EGFP fluorescence under the fluorescence microscope, to determine the distribution of the vector and thereby identify target tissues in the animal model. P53 expression is evaluated by RT-PCR of tissue observed to express EGFP under the fluorescent microscope. The function of the RNAi agent targeted to bcl-2 is be tested by both RT-PCR of RNA and Western-blot protein analysis in the same samples, to determine whether the level of Bcl-2 in tumor tissue is significantly downregulated in the same target cells that express EGFP.

Co-localization of the target cells expressing the P53 or RNAi and EGFP simultaneously is evaluated, particularly those cells in tumors that underwent size reduction as a result of vector treatment. The distribution, e.g., in bone marrow, of the vector after i. v. injection is also determined, to provide information useful for human clinical trials using the vector coexpressing P53 and a bcl-2 RNAi agent.

Example 4—In Vitro Synergistic Effects of SIN-HIV-P53-Bcl-2 RNAi

PC3 prostate cancer cells grown on a substrate were contacted with double gene viral vector construct that express P53 and Bcl-2 RNAi, which is discussed above. FIGS. **10**A and **10**B show in vitro test results that show that double gene (P53 and Bcl-2 RNAi) expression construct viral vector induces cell necrosis of PC3 prostate cancer cells. In living culture, FIG. **10**A shows the untreated cells, and the FIG. **10**B shows cells treated with the double gene vector.

However, when tested on human embryonic kidney cells (293T), the double gene construct did not cause necrosis. FIG. **11**A shows the untreated cells and FIG. **11**B shows cells treated with the viral vector construct P53-Bcl-2 RNAi, expressing P53 and an RNAi agent targeting human Bcl-2. No difference in necrosis is observed, indicating specificity of the double gene construct for prostate tumor. The cells were observed three days after infection.

Example 5—In Vivo Synergistic Effects of SIN-HIV-P53-Bcl-2 RNAi Over P53 and Bcl RNAi Alone—First Study

In vivo effects of the double gene construct and the individual single gene constructs were compared against prostate tumor. The results show that synergistic tumor reducing effects were seen for the viral vector construct P53-Bcl-2 RNAi, expressing P53 and an RNAi agent targeting human Bcl-2. FIG. **12** is a graph showing this effect. In order from left to right, far left bar is untreated control

group; P53 alone expressed through viral vector construct (repair); Bcl-2 siRNA alone expressed through viral vector construct (downregulation of BCL2 gene) only; and on the far right viral vector construct P53-Bcl-2 RNAi, expressing P53 and an RNAi agent targeting human Bcl-2 for simultaneous P53 (repair) and Bcl-2 siRNA. Whereas application of P53 gene construct alone reduced the tumor weight to 1.71 grams, and 1.5 grams when only Bcl-2 siRNA construct was applied, application of the combination gene construct resulted in the tumor weight of 0.71 grams, indicating a synergistic effect of the double gene construct.

In the in vivo studies, 100 μl of viruses (5.0×10^8 cells) were injected into the tail vein of mice for three weeks. After injection for three weeks, the mice were sacrificed and tumors were harvested and weighed.

Further, cells obtained from the untreated tumor and tumor treated with the double gene contruct were examined for human prostate-specific antigen (hPSA) expression. FIGS. **13**A and **13**B show staining of control (untreated tumor) versus the treatment group. The results show that tumor cells treated with viral vector construct P53-Bcl-2 RNAi, expressing P53 and an RNAi agent targeting human Bcl-2 no longer produce hPSA, which means that the cells are no longer active tumor cells or are no longer actively cancerous. After human prostate specific antibody staining, FIG. **13**A shows the control tumor, which expresses hPSA, and FIG. **13**B shows treatment tumor that shows cell necrosis and no expression of hPSA.

Example 6—In Vivo Synergistic Effects of SIN-HIV-P53-Bcl-2 RNAi Over P53 and Bcl RNAi Alone—Second Study

A second study was carried out using mouse models for prostate cancer using the individual gene constructs for P53 and Bcl-2 RNAi, and the double gene construct that carry both of these genes. Multiple mice were tested. V1 Group is mice treated with P53 gene construct alone, V2 Group is mice treated with Bcl-2 RNAi construct alone, and V3 Group is mice treated with a double gene construct expressing both P53 and Bcl-2. PC is the untreated control mice. As shown in the table in FIGS. **14**A-**14**C, the mice were injected with the constructs and their tumor size measured. Injection days were Days 0, 5, 9, 14 and 21. Tumor size measurement days were Days 7, 12, 16, 21 and 28. Comparison of the tumor size of the V1, V2, V3 mice show that the tumor size in these mice were much reduced compared with the tumor size in the control PC mice. Comparison of the tumor weight on Day 28 in FIG. **14**C indicates that V1, V2, V3 mice tumor weight were much reduced compared with the tumor weight in the control PC mice. The ratio of tumor weight/starting tumor size at day 7 shows a large difference in the V1, V2 and V3 Groups compared with that of the PC control mice. V3 Group was injected with half of the dosage concentration compared with V1 and V2 Groups. Yet its tumor size is smaller than V2 and close to V1, thus indicating the synergistic effect of V3.

Example 7—Treatment of Human Patients with SIN-HIV-CMV-p53-hPSA-RNAi-huCD25

To investigate the combination gene therapy effects of our gene vector for treatment of human metastatic prostate cancer, adult bone marrow cells (BMC) genetically modified by transduction with SIN-HIV-CMV-p53-hPSA-RNAi-huCD25 (FIG. **1**C, Panel C, lower construct) are administered to patients with metastasized prostate cancer by the following procedure: (1) blood is harvested from the patient to collect stem cells. Recent medical advances now make it possible to collect stem cells from circulating blood as well. The collection or harvesting of bone marrow is typically done in a hospital operating room under general anesthesia. The bone marrow is then frozen and stored until gene therapy is completed. (2) The bone marrow cells are transduced ex vivo by the gene transfer vector expressing P53, RNAi and huCD25. To select only those cells stems transduced by the transfer vector, cells expressing the selectable marker huCD25 are selected. Stem cell selection is one method used for purging tumor cells. Recent clinical studies have demonstrated that stem cell selection reduces the tumor contamination found in mobilized blood. This is possibly because stem cells have unique properties not shared by tumor cells. (3) After evaluation of the transduced stem cells, and marker selection, the stem cells are thawed and returned to the patient. This procedure is often referred to as the transplant. Within a few days after completing the gene therapy, the stored stem cells are transplanted, or re-infused into the patient's bloodstream. The re-infusion process is similar to a blood transfusion and takes place in the patient's room: it is not a surgical procedure. The frozen bags of bone marrow or blood cells are thawed in a warm water bath, and then injected into the bloodstream through the catheter. It usually takes 2-4 hours for the infusion. Infused stem cells travel through the bloodstream, and eventually, to the bone marrow where they begin to produce new white blood cells, red blood cells, and platelets. (3) The patients are evaluated by collection of their blood to determine the level of P53, Bcl-2, and the selectable marker (i.e., huCD25) at different time points following treatment using Real Time PCR. At the same time a routine diagnosis of index is done to see if any indexes of cancer markers disappear in the current clinical settings.

All of the references cited herein are incorporated by reference in their entirety.

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention specifically described herein. Such equivalents are intended to be encompassed in the scope of the claims.

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What is claimed is:

1. A lentiviral transfer system comprising:

(i) a self-inactivating transfer vector comprising:

(a) multiple gene units, wherein each gene unit comprises a heterologous nucleic acid sequence operably linked to a regulatory nucleic acid sequence;

and

(b) a mammalian insulator sequence and splice acceptor and donor sites,

wherein the self-inactivating transfer vector is free of wPRE (wood-chuck hepatitis virus post-transcriptional element);

and

(ii) a helper construct which lacks a 5' LTR, wherein said 5' LTR has been replaced with a heterologous promoter, said helper construct further comprising: a lentiviral env nucleic acid sequence containing a deletion, wherein said deleted env nucleic acid sequence does not produce functional env protein; a packaging signal containing a deletion, wherein said deleted packaging signal is nonfunctional.

2. A pharmaceutical composition comprising a lentiviral particle for gene transfer, said lentiviral particle produced using a lentiviral transfer system comprising:

(i) a self-inactivating transfer vector comprising:

(a) multiple gene units, wherein each gene unit comprises a heterologous nucleic acid sequence operably linked to a regulatory nucleic acid sequence;

and

(b) a mammalian insulator sequence and splice acceptor and donor sites,

wherein the self-inactivating vector is free of wPRE (wood-chuck hepatitis virus post-transcriptional element);

and

(ii) a helper construct which lacks a 5' LTR, wherein said 5' LTR has been replaced with a heterologous promoter, said helper construct further comprising: a lentiviral env nucleic acid sequence containing a deletion, wherein said deleted env nucleic acid sequence does not produce functional env protein; a packaging signal containing a deletion, wherein said deleted packaging signal is nonfunctional.

3. The lentiviral transfer system of claim **1**, further comprising an envelope construct for providing a functional env protein.

4. The lentiviral transfer system of claim **1**, wherein the multiple gene units comprise a first gene unit operably linked to a first regulatory nucleic acid sequence and a second gene unit operably linked to a second regulatory nucleic acid sequence.

5. The lentiviral transfer system of claim **4**, wherein at least one of the first gene unit or the second gene unit encodes a trafficking signal.

6. The lentiviral transfer system of claim **1**, wherein the regulatory nucleic acid sequence comprises a cell-specific or tissue-specific promoter.

7. The pharmaceutical composition of claim **2**, further comprising an envelope construct for providing a functional env protein.

8. The pharmaceutical composition of claim **2**, wherein the multiple gene units comprise a first gene unit operably linked to a first regulatory nucleic acid sequence and a second gene unit operably linked to a second regulatory nucleic acid sequence.

9. The pharmaceutical composition of claim **8**, wherein at least one of the first gene unit or the second gene unit encodes a trafficking signal.

10. The pharmaceutical composition of claim **2**, wherein the regulatory nucleic acid sequence comprises a cell-specific promoter or tissue-specific promoter.

11. The pharmaceutical composition of claim **2**, wherein the pharmaceutical composition further comprises a chemotherapeutic agent or a steroid agent.

12. The lentiviral transfer system of claim **6**, where the cell or tissue-specific promoter is selected from: TSTA promoter, mesothelin promoter, hPSA promoter, hCCKAR promoter, hAFP promoter, and hNSE promoter.

13. The lentiviral transfer system of claim **1**, wherein the heterologous nucleic acid sequence encodes at least one RNAi agent or at least one polypeptide that inhibits expression of Bcl-2.

14. The pharmaceutical composition of claim **2**, wherein the heterologous nucleic acid sequence encodes at least one RNAi agent or at least one polypeptide that inhibits expression of Bcl-2.

* * * * *