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***In vivo* manipulation of gene expression in non-human primates using lentiviral vectors as delivery vehicles**

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Abstract

Nonhuman primates (NHPs) are an invaluable resource for the study of genetic regulation of disease mechanisms. The main disadvantage of using NHPs as a preclinical model of human disease is the difficulty of manipulating the monkey genome using conventional gene modifying strategies. Lentiviruses offer the possibility of circumventing this difficulty because they can infect and transduce either dividing or nondividing cells, without producing an immune response. In addition, lentiviruses can permanently integrate into the genome of host cells, and are able to maintain long-term expression. In this article we describe the lentiviral vectors that we use to both express transgenes and suppress expression of endogenous genes via RNA interference (RNAi) in NHPs. We also discuss the safety features of currently available vectors that are especially important when lentiviral vectors are used in a species as closely related to humans as NHPs. Finally, we describe in detail the lentiviral vector production protocol we use and provide examples of how the vector can be employed to target peripheral tissues and the brain.

Keywords

Lentiviral Vectors; Gene Regulation; Non-Human Primates

1. Introduction

Non-human primates (NHPs), as discussed in other articles of this issue, are close phylogenetic relatives of humans and thus represent a valuable animal model for the study of the relationship that exists between genetics and disease in higher primates. The difficulty with using NHPs is that conventional genetic tools, such as the production of transgenic animals or the use of gene targeting techniques, are impractical because of the long time required for these gene

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manipulations to generate expected phenotypes and the large number of animals required to achieve the desired gene modification. Given these major obstacles, somatic cell gene transfer emerges as a viable approach to study the role that genetic modifications have in the genesis of various pathologies, as well as a tool to investigate potential therapeutic intervention in NHPs. An ideal gene therapy tool would allow investigators to enhance or suppress the expression of specific genes in a cell- or tissue-specific, and temporally defined, manner. Lentiviruses, or slow retroviruses naturally perform some of these activities as they infect cells as directed by the glycoprotein specificity of their envelope, permanently integrate into the host cell genome, and upon integration express viral proteins [1,2]. An important characteristic that sets lentiviruses apart from other viral vectors and enhances their value as agents for gene therapy is their ability to transduce nondividing cells, as well as dividing cells in contrast to non-lenti retroviruses that transduce only dividing cells [1–4]. Although the adeno-associated virus (AAV) system also shares this capability, lentiviral vectors hold two key advantages over AAV vectors: a) lentiviruses allow for a larger packaging capacity (8–10 kb) [3,5] compared to less than 5 kb for AAV [3] and b) the majority of self-inactivating (SIN) HIV-based infective lentiviral particles become integrated into the genome 3 days after infection [6] as compared to less than 10% for AAV [3]. Because of these advantages, lentiviruses appear ideal for studies aimed at manipulating gene expression in NHPs.

2. Lentiviral-derived vector systems

2.1 General description

The first lentiviral vectors described were based on HIV-1 [2] and although other vector systems have been developed using lentiviruses that are specific for other species [7] the HIV-1 vector system remains the vector of choice. The life cycle of HIV-1 lends itself well to the purpose of viral-mediated gene transfer, such as host cell attachment, receptor-mediated entry into host cells, viral mediated reverse transcription, and integration of the viral genome into the host-cell chromatin [2,8]. Another feature of HIV-1 that makes it an ideal gene therapy vector and that is its ability to escape from cellular immune responses [9]. Following HIV-1 infection, neutralizing antibodies are rarely generated *in vivo* [10] and lentiviral vectors similarly integrate their genome into that of target cells without an inflammatory response [2]. This is not to imply that the current versions of lentiviral vectors are ready for all uses and methods of administration. For example, lentiviral vectors that have been pseudotyped with the most commonly used envelope protein are inactivated by human serum complement preventing the use of these vectors in protocols involving systemic administration [11]. Thus while lentiviral vectors might not be ready for use in all situations, HIV-1 based lentiviral vectors are promising gene therapy tools [1–4,7,12].

2.2 Vector Components

An HIV-1 viral vector system consists of a replication-incompetent, non-pathogenic version of the virus where the *cis*- and *trans*-acting components required to generate a viral particle are separated on different plasmids. The viral sequences required *in cis* are included in the vector genome plasmid (see below; Fig 1A, B), which contains the transgene (TG) or RNA interference (RNAi) (TG-RNAi) cassette (Fig 1A, B) which, once transcribed into RNA, is packaged into the vector particles and ultimately transferred into the target cells. The *trans*-acting sequences, typically encoding proteins required to assemble functional viral particles, are located on separate plasmids (Fig 1C). These plasmids are used during the vector production phase (called “packaging”), but the genes for the *trans*-elements are not included in the viral particle and therefore, not transduced into target cells. The result is a viral particle that upon infecting a cell does not propagate the infection further.

2.3 Cis-elements

The vector genome plasmid is the structure that will provide the *cis* elements required for efficient packaging, reverse transcription, and integration [2]. Importantly, the vector carrying the TG-RNAi cassette also contains the regulatory components necessary for their expression. The vector cassette is a stripped version of the HIV genome; it contains less than 5% of the parental genome [3]. Although the 5' end of the HIV-1Gag (including the ATG) is included in the vector, the coding sequence for Gag has been interrupted with stop codons (the first of which occurs 21 codons after the ATG). The rest of the vector contains no other HIV-1 open reading frames. It is bounded by long terminal repeats (LTRs; Fig 1A) and includes the packaging signal (ψ) which is required for incorporating the RNA in the virion [13]. It also contains the primer binding site, the HIV-1 splice donor site, the splice-acceptor site (all located in the same vicinity as the ψ signal), the *rev* response element (RRE), the central polypurine tract (cPPT), and a foreign *cis* element known as the woodchuck hepatitis posttranscriptional regulatory element (wPRE; Fig 1A, B; [2,14]). Each LTR is divided into the U3 - retroviral promoter; R - repeat region/transcription start site; and U5 - polyadenylation region; the LTRs are responsible for controlling viral replication and integration of the viral genome into the host genome [13,14].

The mechanism by which the virus genome is replicated allows the production of the SIN viral vector [15,16], resulting from a modification made to the 3' LTR. Self-inactivation is achieved by deleting a 400 bp segment of the U3 retroviral promoter region in the 3' LTR of the DNA used to produce the vector RNA [15,17]. During reverse transcription of either the wild-type HIV-1 or the SIN attenuated viral vector, the 3' LTR is duplicated and replaces the 5' LTR. As a consequence of this duplication, the deletion in the U3 region of the 3' LTR results in a vector that loses the ability to replicate once integrated into the host genome [17]. Although the native HIV-1 LTR promoter is not active in the absence of Tat, the developers of these vectors have used the virus's own mechanism of replication to completely eliminate the LTR promoter in the SIN vectors. Advantages of using a SIN vector include the reduced probability that target cell coding sequences adjacent to the vector integration site will be aberrantly expressed either due to the promoter activity of the 3' LTR or through an enhancer effect [17], and the prevention of any potential transcriptional interference between the LTR and the internal TG-RNAi promoter [17].

The RRE is the binding site for Rev which allows for the nuclear export of full-length RNA vector genomes [8,14]. The remaining two *cis* elements known to enhance the effectiveness of the vector are the cPPT [18] and the wPRE [19]; together they synergize to provide enhanced transduction and transgene expression [20].

2.4 Trans-elements

HIV-1 contains 9 genes that play a role in the life cycle of the virus and determine its pathogenic properties [1,2]. Six of the genes (*vif*, *vpr*, *vpu*, *nef*, *env*, *tat*), known as accessory genes, are either directly related to pathogenesis or not necessary for vector production and functionality. Consequently, these genes have been deleted from the vector system, or replaced with another gene as in the case of *env* [2,5,8]. Because the remaining 3 genes, *gag*, *pol*, and *rev* (Fig 1C), are necessary for viral packaging, they have been retained in the vector system [2,5,21,22]. The *gag* and *pol* genes code for the p55 Gag and p160 Gag-Pol polypeptide precursors which are cleaved into mature products by viral proteases and are required for packaging of the viral vector. The p55 Gag precursor is cleaved into p17 matrix, p24 capsid, p9 nucleocapsid, p6 proteins, and two spacer peptides [14]. The p160 Gag-Pol protein is the precursor of mature Gag protein, p12 viral protease, p51/66 reverse transcriptase, and p31 integrase [14]. Some of the proteins serve a structural function in the virus capsule. More importantly, the Gag-Pol cleavage products have several functions in the viral life cycle including: 1) reverse

transcriptase which is responsible for reverse transcription of viral DNA from the RNA genome, and 2) integrase, required for nuclear import and integration of the viral DNA into the host genome [14,23]. The *rev* (Fig 1C) is a single 21 kDa protein that serves a single, but important role. It binds mRNAs, removing them from the spliceosome complex, and links the rescued mRNA to a nuclear pore such that full length and partially spliced mRNAs are transported out of the nucleus and into the cytoplasm [8,14]. This transfer is required for some of the transcripts from *gag* and *pol*, but more importantly it is absolutely critical for the production of the full-length viral RNA genome [8].

There is an additional *trans* element required to generate active packaged particles surrounded by a protein envelope. The *env* gene from HIV-1 provides host cell specificity such that HIV-1 only infects CD4 producing cells [1]. By replacing the HIV-1 *env* gene, with the vesicular stomatitis virus glycoprotein (VSV-G; Fig 1C; [24,25]), a process known as pseudotyping, three important objectives are achieved: 1) the tropism of the vector is greatly broadened, 2) the vector particles are stabilized such that they can withstand ultracentrifugation to allow for vector concentration, and 3) the VSV-G directs the vector to an endocytic pathway, reducing the requirement for HIV-1 accessory proteins for infectivity [26]. The tropism is determined by the target molecule on the surface of the host cell that the VSV-G protein attaches to; in this case the target molecules are phosphatidylserine phosphatidylinositol and GM3 ganglioside [25]. While the VSV-G envelope is the most commonly used envelope, there are potential advantages to pseudotyping with other envelope proteins. Modifying the envelope protein is one possible strategy for targeting the vector specifically to cells of a given phenotype. Among several examples [4], Ross River virus pseudotyped viruses stand out because of their selectivity for glial cells. The proteins described above all act *in trans* to achieve packaging of the lentiviral vector; the protein products are coded by genes present on the HIV genome, but can be separated from the RNA genome backbone (see below) creating room for the insertion of additional genetic material, such as the TG-RNAi cassettes we describe below.

2.5 Packaging cells

Packaging cells are used as the viral factory where the *trans* and *cis* elements are brought together to produce the vector particles. The packaging cells used to produce lentiviral vectors are 293T/17 cells (ATCC#CRL-11268), a highly transfectable derivative of the 293 human fetal kidney cell line, into which the temperature sensitive gene for simian virus 40 (SV40) large T antigen has been inserted [27]. There are several versions of the HIV-1 based lentiviral packaging systems.; they are referred to as first [28], second [29], and third [30] generation. The differences between these versions reside in the number of plasmids used for packaging, allowing the *cis* and *trans* elements to be split on different plasmids and thereby improving the biosafety profile of the vector preparations. The first and second generations consist of three plasmids; a vector plasmid, a packaging plasmid and an envelope plasmid. The number of accessory genes present on the packaging plasmid varies depending on the system [28,29]. The third generation system includes four expression cassettes; a vector plasmid, a *gag/pol* plasmid, a *rev* plasmid and an *env* plasmid [30]. There is an additional system that comprises 4 or more plasmids; the key feature to this system is that the *gag/pol* cassette has been split onto two plasmids [21,22,26]. This system also includes *tat* and a *vpr* fusion protein, and it is being marketed as a fourth generation system (Clontech, La Jolla, CA). We have primarily used the third generation system, which provides the *gag* and *pol* genes on one plasmid and the *rev* on a separate plasmid (Fig 1C). In our work with rhesus monkeys we have utilized a packaging plasmid in which the *gag* gene has been altered such that the HIV-1 Gag protein has been mutated in one site to be more similar to the Gag of simian immunodeficiency virus (SIV) [31]. In simian cells, HIV-1 fails to replicate because of an early post-entry block, so alteration of the *gag* gene significantly increases the ability of HIV-based lentiviral vectors to transduce simian cell lines [31].

2.6 Constructs

The TG-RNAi vector plasmid contains all the *cis* elements necessary for production of the viral RNA genome, infection of target cells, integration into the host genome, and expression of TG-RNAi cassettes. The transcription of the RNA genome is directed by the 5' LTR (Fig 1A, B). In the 3rd generation system, the promoter sequences of the 5' LTR have been replaced by a constitutively active promoter, such as the cytomegalovirus (CMV) or the Rous sarcoma virus (RSV) promoter creating a heterologous U3 (htU3) promoter [30,32]. This replacement eliminates the need for *tat* [30]. Because the 2nd generation and 4th generation vector plasmids have retained the HIV 5' LTR U3 promoter, both systems require a packaging plasmid that supplies *tat* [21,22,29].

The TG-RNAi cassette in the transfer vector is the region in which the gene or RNAi molecule of interest can be inserted (Fig 1A). Lentiviruses are limited to an overall TG insert size of 8–10 kb [3,5]. Primarily two types of constructs are used; they either express a transgene of interest or produce RNAi molecules to suppress expression of a target gene. In either case a promoter must be provided, as the upstream LTR promoter activity has been eliminated once the viral genome is reverse transcribed and integrated into the host genome. The transgene promoter sequence can be a constitutively active promoter, a tissue specific promoter, or a regulated promoter. For our work we have used the constitutively active CMV promoter (Fig 1A). Because of space limitations, we have found it is best to use just the coding region, including the Kozak sequence [33] of the cDNA encoding the transgene of interest. In one of our studies we used the coding region of the human nerve growth factor (hNGF) gene. By inserting an internal ribosome entry site (IRES), after the transgene we produced a lentiviral construct that is capable of producing a bicistronic mRNA (Fig 1A). The IRES is from the encephalomyocarditis virus and was generated by PCR from the pERV3 plasmid (Stratagene, La Jolla, CA). The PCR primers used to generate the IRES were as follows: upstream 5'-ACGCGTCCCCCTCTCCCT-3' and downstream 5'-ACGCGTGATCGTGTTTTTCAAAGG-3'. An Mlu I site was inserted at the 5' end of both primers to facilitate cloning. Downstream of the IRES a cell marker can be used to identify infected cells. We commonly use enhanced green fluorescent protein (eGFP), but other cell markers such as different color fluorescent proteins, can replace the eGFP. Alternatively, an antibiotic resistance gene can be inserted in place of the eGFP (Fig 1A). There is one more DNA fragment incorporated into the transgene construct and that is a heterologous intron (Fig 1A). It is well known that heterologous introns enhance expression of transgenes in mice [34]. In order to determine if the heterologous intron has the same effect in the context of a lentiviral vector, we inserted the rat insulin II intron A sequence [35] used in transgenic mice [34,36] between the CMV promoter and the cDNA for hNGF (Fig 1A) and compared NGF production from infected 293T/17 cells. NGF released into the culture medium was determined with the NGF Emax immunoassay system (Promega Co. Madison WI). It was undetectable in culture medium from 293T/17 cells infected with virus lacking the NGF cDNA, clearly measurable in the medium of cells transduced with a viral construct lacking the heterologous intron, and greatly enhanced (>3-fold) when the intron was present (Fig 2).

The second type of construct we use is intended to suppress expression of target genes. The system called pPRIME (potent RNAi using miR expression) was developed in the Elledge lab [37] and has been made available through Addgene (www.addgene.org). Artificial microRNAs (miRNAs) have improved host safety over short hairpin constructs [38] thus avoiding potentially lethal oversaturation of cellular pathways [38,39]. The pPRIME vectors use an RNA polymerase II to direct the expression of a marker gene (eGFP, RFP, or an antibiotic resistance gene such as neomycin) followed by an artificial miRNA precursor (Fig 1B). The artificial miRNA is derived from miR-30 [40] and is inserted downstream from the marker coding sequence; in our studies the marker sequence is eGFP. The miRNA sequence is engineered to

allow replacement of the hairpin sequence so that any mRNA sequence can be targeted with this system [37,41]. We improved the pPRIME system by placing multiple hairpins in the same miRNA, a modification shown to increase the suppressive effectiveness of the RNAi cassette [42].

3. Application

3.1 Screening of NHPs for studies involving lentivirus administration

The major safety concern with using HIV-1 lentivirus is that a recombination event could occur resulting in the production of replication competent lentivirus (RCL). While no such event has been reported to occur, vigilance in this area during production is warranted. A brief discussion of a screening method to detect RCLs is presented below. A heightened concern regarding recombination events arises when the host animals are NHPs, because of the presence of retroviruses native to NHPs. Prescreening of candidate animals for retroviruses that have the potential for recombination is a prudent preventative measure. All animals used for our studies are screened for antibodies specific for SIV, simian retrovirus (SRV; types 1, 2, 3 and 5), and simian T-cell leukemia virus (STLV; types 1 and 2). The presence of antibodies for any of these viruses prevents an animal from being included in any study involving administration of lentiviruses.

3.2 Packaging

The lentivirus is prepared by transient transfection of the 293T/17 packaging cells. The cells are grown, maintained, and transfected in antibiotic-free, high glucose Dulbecco's modified eagle medium (D-MEM) (Invitrogen, Carlsbad, CA) plus 10% defined fetal bovine serum (Hyclone-ThermoFisher, Waltham, MA). The day before transfection the cells are plated at 70–80% confluency (six million cells per dish) in 10 cm tissue culture dishes (BD-Falcon, Franklin Lakes, NJ) that have been pre-coated with 20 µg/ml poly-L-lysine (Sigma-Aldrich, St Louis, MO) in phosphate buffered saline. The next day each dish is transfected with a mixture of the TG-RNAi expression plasmid (10 µg) plus the packaging plasmids (6.5 µg gB gag LV; 2.5 µg pLP2; 3.5 µg pLP/VSVG; Fig 1A, B, C). The gB gag LV was kindly provided by Dr. I. Verma [31]; the pLP2 and pLP/VSVG were acquired as part of the ViraPower Lentivirus Expression System (Invitrogen). The transfection is carried out using a calcium-phosphate solution consisting of a 1:1 mixture of 0.25 M CaCl₂ (J.T. Baker, Phillipsburg, NJ); 2XBBS [0.28 M NaCl, (Sigma-Aldrich); 0.05 M N,N-bis-(2-Hydroxyethyl)-2-aminoethanesulfonic Acid (BES), (Calbiochem, La Jolla, CA); 1.5 mM Na₂HPO₄ (Sigma-Aldrich)]. The calcium-phosphate-DNA mixture is added drop-wise to each dish and the cells are incubated at 3% CO₂/37C. After 18 hours, the transfection medium is replaced with fresh D-MEM and the cells are incubated overnight in an atmosphere of 10% CO₂/37C. The virus is then harvested by collection and filtration of the medium through 0.22 µm steriflip filters (Millipore, Billerica, MA) followed by ultracentrifugation at 20,000 rpm using a swinging-bucket rotor (Beckman SW28, Beckman Coulter Inc., Fullerton, CA). The viral pellets are re-dissolved in Hank's balanced salt solution (HBSS; Invitrogen), shaken at room temperature for 45 minutes and stored overnight at 4C. Fresh D-MEM is added to the dishes and cells are again incubated overnight at 10% CO₂/37C. The virus is harvested as mentioned above and viral pellets are combined with the re-suspended virus from the previous day and shaken again to mix. The virus is concentrated by an additional ultracentrifugation spin at 21,000 rpm, in an SW60 rotor (Beckman Coulter Inc.) over 20% sucrose (Sigma-Aldrich) in phosphate buffered saline. The viral pellet is re-suspended in HBSS, shaken for 45 minutes at room temperature, aliquoted, and stored at –85C.

Titration of the virus is performed using flow cytometry to analyze infected cells for GFP expression. 293T/17 cells are plated at 400,000 cells per well in 6-well plates (Costar, Corning,

NY) and various volumes of viral supernatant or dilutions of concentrated virus are added to each well, along with 0.16 µg/ml polybrene (hexadimethrine bromide, Sigma-Aldrich) to enhance infection efficiency. The plates are incubated at 10% CO₂/37°C for approximately 72 hours and the cells are lifted with trypsin (TrypLE, Invitrogen), fixed with 0.5% paraformaldehyde in phosphate buffered saline (to inactivate remaining virus), transferred to FACS cell-strainer cap tubes (BD-Falcon, Franklin Lakes, NJ), and analyzed by flow cytometry (FACScalibur, BD Biosciences, San Jose CA) for GFP fluorescence. Calculations are made using the percentage of GFP-positive cells to yield titer values that represent the number of transforming units (TU) per ml. For example if a 2 µl aliquot of conditioned medium resulted in 10% of the cells being counted as positive for GFP the calculation would be as follows: 400,000 cells × 0.1 (percent GFP positive cells) × 1000/2 µl (dilution factor correction) = 2 × 10⁶ TU/ml. Concentrated viral preparations routinely have titer values of at least 1 million viral particles (TU) per µl. If it is not possible to include the reporter GFP protein to facilitate titration of the viral preps, real-time PCR or p24 ELISA (see below) can be used to titer viral preparations [43,44].

To ensure that RCL is not produced by recombination events in the host cell, a suspension of SUP-T1 (ATCC#CRL-1942) lymphoblast cells, seeded at 1 million cells per 25 cm² flask (Corning, Lowell, MA), are infected with 10 million virus particles, and cells are propagated for two weeks in antibiotic free RPMI-1640 medium (Sigma-Aldrich) plus 10% defined fetal bovine serum (Hyclone-ThermoFisher). The medium is collected from the flasks containing the infected cells and assayed for the HIV-1 p24 antigen [28,45] using a commercial ELISA kit (Zeptometrix Corp., Buffalo, N.Y.). The sensitivity of the ELISA kit is approximately 7.8 pg/ml. However because the cells are propagated for two weeks prior to assay, a RCL would be amplified during this propagation step, making it unlikely that the lack of RCL detection is due to low sensitivity of the assay. It is important to note that should a RCL develop, the most likely env protein present would be VSV-G, which would result in the virus having a very broad tropism.

We have used the above described lentiviral vector system for both gene expression and gene suppression in the ovary and in the brain of NHPs. We will use these studies as examples of the two major applications, one involving a peripheral tissue and the other, involving the brain.

3.3 Anesthesia and analgesia in NHPs

Anesthesia and analgesia regimens were consistent among the three surgical procedures described below. Animal positioning and incision sites varied with procedures. These variations are noted in the corresponding text. Anesthesia was induced with a 10 mg/kg intramuscular injection of Ketamine HCl (Ketaved, Bioniche Teoranta, Inverin, Co. Galway, Ireland). After endotracheal intubation, anesthesia was maintained with Isoflurane (Hospira, Inc., Lake Forest, IL) vaporized in 100% oxygen delivered via a non-rebreathing circuit. Bupivacaine, HCl 0.25% (Hospira, Inc., Lake Forest, IL) and Lidocaine, HCl 1% (Xylocaine, AstraZeneca LP, Wilmington, DE) were injected intradermally along the intended incision sites to provide local anesthesia. Hydromorphone (0.2 mg/kg intravenous injection) was administered to provide intraoperative analgesia. Post-operative analgesia consisted of Hydromorphone (0.2 mg/kg intramuscular injections) administered every 4 hours for 48 hours (0800–1600 hrs). Buprenorphine (Ben Venue Laboratories, Inc., Bedford, OH) was administered (0.03 mg/kg intramuscular injections) to provide overnight analgesia (at 2000 hrs).

3.4 Lentivirus administration to a peripheral tissue

Our work with a peripheral tissue has been focused on the ovary. The ovary is a suitable example of a peripheral tissue amenable to lentiviral gene transfer by localized injection,

because it has clearly defined boundaries and is of a size that allows good penetration by injection. The goal of this study was to overexpress the gene encoding hNGF (Fig 1A). The delivery of the lentivirus to the ovary was via laparotomy. After ventral midline laparotomy, each ovary was surgically suspended with forceps at the utero-ovarian ligament and vascular pedicle. The lentivirus was delivered using a 100 μ l Hamilton syringe (Hamilton Company, Reno, NV) with a disposable 30 ga needle. The needle was inserted into the ovary adjacent to the utero-ovarian ligament and 12 μ l of lentivirus (200,000 TU/ μ l) were injected into each of four ovarian quadrants, for a total of 48 μ l/ovary. A cotton-tipped applicator was held against the injection site for 10–15 seconds site to prevent leakage. The ovary was then returned to its normal position. The abdominal incision was closed using 3-0 Vicryl (Ethicon, Inc. Somerville, NJ) suture in a simple continuous pattern to appose the rectus fascia and subcutaneous layers. 4-0 Monocryl (Ethicon, Inc.) was used to appose the skin layer in an intradermal pattern. After the monkeys have been injected with the lentivirus they are kept separate from other animals of the rhesus colony in standard housing in an animal biological safety level II facility. The monkeys were then observed for changes in the menstrual cycle, both by daily observation of menses and regular blood sampling. We found that collecting blood samples three times a week is sufficient to characterize the length of the monkey's cycle and assess its normality.

3.5 Administration to the brain

For our studies we were interested in the effects of knockdown of a gene known as enhanced at puberty 1 (EAP1). EAP1 mRNA expression was initially discovered to be upregulated in NHPs at puberty [46]. Further, *EAP1* was shown to be required for normal cyclicity and to be an important regulator of luteinizing hormone releasing hormone (LHRH) release in rodents [46]. Thus it was important to test the hypothesis that EAP1 is critical for cyclicity in NHPs. We targeted cells expressing EAP1 mRNA, which are located in a region of the hypothalamus known as the arcuate nucleus. The pPRIME lentivirus was constructed as described above with 3 copies of the hairpin specific for the target mRNA embedded into the body of miR30 (Fig 1B; [37,42]).

In adult rhesus monkeys the arcuate nucleus is an oval shaped nucleus at the base of the brain and lateral to the 3rd ventricle, and it is 3–4.5 $\times$ 1–2 $\times$ 2–3 mm (anterior-posterior $\times$ lateral $\times$ dorsal-ventral) in size [47]. An important challenge posed by this location is delivering with accuracy the lentivirus particles to cells included in this area. The Oregon National Primate Research Center pioneered the stereotaxic surgical and injection methods for rhesus macaques using x-ray images [48,49]. A lateral x-ray image (Fig 3A) shows the anterior and posterior clinoids of the sella turcica, bony structures that provide clear land marks in the anterior-posterior and dorsal-ventral orientations. However, there are no comparable landmarks in the x-rays for the medial-lateral orientation. In order to guide injections along this dimension we constructed a set of fiducial markers, which were visualized using the Siemens Magnetom Trio 3T magnetic resonance imaging (MRI) system (New York, NY), located on-campus at ONPRC. The fiducial markers were made from handmade glass spheres (approximately 1 mm id; 2.5 mm od; TRS Scientific Glass, Hillsboro, OR) filled with a 1:500 dilution of the gadolinium-based MRI contrast agent Prohance, (Bracco Diagnostics, Princeton, NJ). For ease of visualization throughout the surgical procedures, Prohance was diluted in trypan blue (0.4% in phosphate buffered saline, MP Biomedicals Inc., Solon, OH). The fill opening of the glass spheres was sealed with Loctite 3335, ultraviolet (UV)/cationic epoxy (Loctite Corporation, Rocky Hill, CT) which was cured using a handheld 366 nm UV light source (UVP Inc. San Gabriel, CA). The actual shape of the spheres was that of a flask complete with neck at the fill hole. The fiducial markers were sterilized in a solution of Cidex Plus (Advanced Sterilization Products, Irvine, CA) prior to surgical placement.

General anesthesia was induced as described in section 3.3. The monkey was placed in the prone position and the head secured to a stereotaxic apparatus (Model 1404, Kopf Instruments, Tujunga, CA). After sterile surgical preparation, a linear sagittal skin incision over the dorsal scalp was made to expose the top of the skull. Three partial-thickness burr holes were created in the top of the skull (see orientation Fig 3B) using a Hall air drill and medium round bur (Conmed Linvatec Corp., Largo, FL). The fiducial markers were laid on their sides in these depressions and affixed in place with sterile dental cement (CO-ORAL-ITE Dental MFG. Co., Inc., Diamond Springs, CA), leaving the uppermost side wall of the sphere (approximately 1 mm diameter) exposed. Because the fiducial markers were filled with blue dye, they were clearly visible. With the injection syringe (used as a pointer) attached to the micromanipulator, the stereotaxic coordinates for each fiducial marker were recorded in each of the 3 planes. The incision site was closed using 4-0 Monocryl in a simple interrupted pattern to appose the subcutaneous layer in a simple interrupted pattern; followed by 4-0 Monocryl intradermal pattern to appose the skin layer. The monkey was then removed from the stereotaxic apparatus and placed on a transport anesthesia cart allowing for maintenance of the Isoflurane anesthesia while transporting the monkey to the MRI unit (housed in an adjacent building).

In the MRI unit the monkey was maintained on Isoflurane anesthesia, which was continually supplied throughout the MRI procedure through tubing extending from the control room into the scanner room. The animal was kept warm using an underpad with circulating warm water as well as overlying towels, and physiological monitoring was maintained in the form of pulse rate, oxygenation, end-tidal CO₂, as well as respiration rate. The animal was positioned head-first, supine in a radiofrequency coil suitable for a non-human primate head. Following a localizer scan, a 3-D T1-weighted magnetization-prepared rapid gradient-echo (MP-RAGE) scan was acquired, where TR=2500 ms, TE=4.38 ms, Ti=1100 ms, fov=120 mm, 176 slices with a base resolution of 256, thickness=0.5 mm, at an isotropic image resolution of 0.5 mm yielding an isovoxel size of 0.5 mm.

The injections were made 1–2 weeks after the implantation of the fiducial markers and the MRI procedure. Using the MRI image, the distance from the midline of the brain to the posterior fiducial marker (Fig 3C, D) was measured using the software Slicer3D (www.slicer.org). This measurement was used as a correction to establish the midline of the monkey once it was mounted in the stereotaxic apparatus on the day of injections. A lateral x-ray was then obtained to establish the target (Fig 3A, E); the MRI image in figure 3D shows the arcuate nucleus (arrow). The target is 1 mm dorsal of the line drawn between the points of the anterior and posterior clinoids of the sella turcica bone structure on the x-ray image (Fig 3E). To access the target site, the animal was anesthetized and positioned in the stereotaxic apparatus as previously described. A linear sagittal incision was then made over the dorsal scalp to expose the skull. A circular 1.5 cm diameter craniotomy was created using the Hall air drill and medium round bur. The dura mater was incised on either side of the sagittal sinus and the lateral branches of the sagittal sinus were cauterized as necessary using a bipolar coagulating current. The stereotaxic micromanipulator was then used to lower the injection syringe to the targets. We used a 10 µl Hamilton syringe (Hamilton Co.) with a blunt 26 ga., 52 mm needle. A 19 ga guide tube set in a nylon ring was fitted over the 26 ga. needle to prevent deviation of the needle as it was lowered into place. A total of six 1.5 µl injections containing 1×10^6 TU/µl, 3 per side, were placed in the target area. The first target injection site is 1 mm lateral from the midline of the brain (as defined by the MRI; Fig 3C), and 1 mm dorsal of the midpoint of the line drawn between the points of the sella turcica bone structure on the x-ray image (Fig 3E). From this point, 2 additional injections were placed 1 mm anterior and 1 mm posterior of the midpoint injection. The dorsal-ventral depth for the anterior and posterior injections was adjusted to maintain 1 mm dorsal of the line drawn between the points of the sella turcica (Fig 3E). The injection needle was slowly lowered into place, for each injection, and placement was confirmed on the left side by lateral x-ray. The needle placement can be seen in figure 3E,

which is an example of the midpoint injection. Once it was confirmed that the needle point was in the target, the 1.5 μ l of the viral construct solution was slowly injected (30 to 45 sec). The needle remained in place for an additional 30 sec before it was removed, to allow the solution time to absorb into the target. Because the injections on the right side (1 mm to the right of the midline of the brain) used the same anterior-posterior and dorsal-ventral coordinates, lateral x-rays were not repeated. During development of the injection procedure, 1.5 μ l of a 1 μ M solution of MnCl (Sigma-Aldrich) in trypan blue (0.4% in phosphate buffered saline, MP Biomedicals Inc.) was injected using these methods. The MnCl solution was detected by MRI in the arcuate nucleus (Fig 3F), and the brain was then collected and grossly sectioned to verify that the trypan blue was correctly localized to the arcuate nucleus (Fig 3G).

After the injections were complete, the craniotomy site was filled with Gelfoam (Pharmacia & Upjohn Co., Kalamazoo, MI), and the incision was closed using 4-0 Monocryl in a simple interrupted pattern to appose the subcutaneous tissue followed by 4-0 Monocryl intradermal pattern to appose the skin layer. Following treatment the monkeys are housed as described above, in a standard housing but segregated from the rest of the colony. The monkeys were then observed for changes in menstrual cycles, as described above, induced by the siRNA.

4. Concluding Remarks

Lentiviral vectors are powerful tools to manipulate gene expression *in vivo* in a tissue and cell-specific manner following local delivery via intra tissue injections.

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References

1. Verma IM, Somia N. Nature 1997;389:239–242. [PubMed: 9305836]
2. Kafri T. Methods Mol.Biol 2004;246:367–390. [PubMed: 14970605]
3. Thomas CE, Ehrhardt A, Kay MA. Nat.Rev.Genet 2003;4:346–358. [PubMed: 12728277]
4. Wong LF, Goodhead L, Prat C, Mitrophanous KA, Kingsman SM, Mazarakis ND. Hum.Gene Ther 2006;17:1–9. [PubMed: 16409120]
5. Salmon P, Trono D. Curr.Protoc.Hum.Genet 2007;Chapter 12:Unit 12.10.1–Unit 12.10.24.
6. Butler SL, Johnson EP, Bushman FD. J.Virol 2002;76:3739–3747. [PubMed: 11907213]
7. Watson DJ, Karolewski BA, Wolfe JH. Methods Mol.Biol 2004;246:413–428. [PubMed: 14970607]
8. Debyser Z. Curr.Gene Ther 2003;3:517–525. [PubMed: 14683448]
9. Wodarz D, Nowak MA. Immunol.Rev 1999;168:75–89. [PubMed: 10399066]
10. Stebbing J, Patterson S, Gotch F. Cell Res 2003;13:1–7. [PubMed: 12643344]
11. DePolo NJ, Reed JD, Sheridan PL, Townsend K, Sauter SL, Jolly DJ, Dubensky TW Jr. Mol.Ther 2000;2:218–222. [PubMed: 10985952]
12. Zhou HS, Liu DP, Liang CC. Med.Res.Rev 2004;24:748–761. [PubMed: 15250039]
13. Debyser Z. Curr.Gene Ther 2003;3:495–499. [PubMed: 14683447]
14. Federico, M. From lentiviruses to lentivirus vectors. In: Federico, M., editor. Methods in Molecular Biology, Vol 229: Lentivirus Gene Engineering Protocols. Totowa, New Jersey: Humana Press Inc; 2003. p. 3-15.
15. Yu S-F, von Rüden T, Kantoff PW, Garber C, Seiberg M, Rüther U, Anderson WF, Wagner EF, Gilboa E. Proc.Natl.Acad.Sci.USA 1986;83:3194–3198. [PubMed: 3458176]
16. Miyoshi H, Blomer U, Takahashi M, Gage FH, Verma IM. J.Virol 1998;72:8150–8157. [PubMed: 9733856]

17. Zufferey R, Dull T, Mandel RJ, Bukovsky A, Quiroz D, Naldini L, Trono D. *J. Virol* 1998;72:9873–9880. [PubMed: 9811723]
18. Zennou V, Petit C, Guetard D, Nerhbass U, Montagnier L, Charneau P. *Cell* 2000;101:173–185. [PubMed: 10786833]
19. Zufferey R, Donello JE, Trono D, Hope TJ. *J. Virol* 1999;73:2886–2892. [PubMed: 10074136]
20. Barry SC, Harder B, Brzezinski M, Flinit LY, Seppen J, Osborne WRA. *Hum. Gene Ther* 2001;12:1103–1108. [PubMed: 11399231]
21. Wu X, Wakefield JK, Liu H, Xiao H, Kralovics R, Prchal JT, Kappes JC. *Mol. Ther* 2000;2:47–55. [PubMed: 10899827]
22. Wu X, Liu H, Xiao H, Conway JA, Hehl E, Kalpana GV, Prasad V, Kappes JC. *J. Virol* 1999;73:2126–2135. [PubMed: 9971795]
23. Frankel AD, Young JA. *Annu. Rev. Biochem* 1998;67:1–25. [PubMed: 9759480]
24. Burns JC, Friedmann T, Driever W, Burrascano M, Yee JK. *Proc. Natl. Acad. Sci. U.S.A* 1993;90:8033–8037. [PubMed: 8396259]
25. Hanazono Y, Terao K, Ozawa K. *Stem Cells* 2001;19:12–23. [PubMed: 11209087]
26. Cockrell AS, Kafri T. *Mol. Biotechnol* 2007;36:184–204. [PubMed: 17873406]
27. Pear WS, Nolan GP, Scott ML, Baltimore D. *Proc. Natl. Acad. Sci. U.S.A* 1993;90:8392–8396. [PubMed: 7690960]
28. Naldini L, Blomer U, Gallay P, Ory D, Mulligan R, Gage FH, Verma IM, Trono D. *Science* 1996;272:263–267. [PubMed: 8602510]
29. Zufferey R, Nagy D, Mandel RJ, Naldini L, Trono D. *Nat. Biotechnol* 1997;15:871–875. [PubMed: 9306402]
30. Dull T, Zufferey R, Kelly M, Mandel RJ, Nguyen M, Trono D, Naldini L. *J. Virol* 1998;72:8463–8371. [PubMed: 9765382]
31. Kootstra NA, Munk C, Tonnu N, Landau NR, Verma IM. *Proc. Natl. Acad. Sci. U.S.A* 2003;100:1298–1303. [PubMed: 12547912]
32. Follenzi A, Ailles LE, Bakovic S, Geuna M, Naldini L. *Nat. Genet* 2000;25:217–222. [PubMed: 10835641]
33. Kozak M. *Nucleic Acids Res* 1987;15:8125–8148. [PubMed: 3313277]
34. Palmiter RD, Sandgren EP, Avarbock MR, Allen DD, Brinster RL. *Proc. Natl. Acad. Sci. USA* 1991;88:478–482. [PubMed: 1988947]
35. Lomedico P, Rosenthal N, Efstratidis A, Gilbert W, Kolodner R, Tizard R. *Cell* 1979;18:545–558. [PubMed: 498284]
36. Hoyle GW, Graham RM, Finkelstein JB, Nguyen K-PT, Gozal D, Friedman M. *Am. J. Respir. Cell Mol. Biol* 1998;18:149–157. [PubMed: 9476901]
37. Stegmeier F, Hu G, Rickles RJ, Hannon GJ, Elledge SJ. *Proc. Natl. Acad. Sci. U.S.A* 2005;102:13212–13217. [PubMed: 16141338]
38. Boudreau RL, Martins I, Davidson BL. *Mol. Ther* 2009;17:169–175. [PubMed: 19002161]
39. Grimm D, Streetz KL, Jopling CL, Storm TA, Pandey K, Davis CR, Marion P, Salazar F, Kay MA. *Nature* 2006;441:537–541. [PubMed: 16724069]
40. Lagos-Quintana M, Rauhut R, Lendeckel W, Tuschl T. *Science* 2001;294:853–858. [PubMed: 11679670]
41. Silva JM, Li MZ, Chang K, Ge W, Golding MC, Rickles RJ, Siolas D, Hu G, Paddison PJ, Schlabach MR, Sheth N, Bradshaw J, Burchard J, Kulkarni A, Cavet G, Sachidanandam R, McCombie WR, Cleary MA, Elledge SJ, Hannon GJ. *Nat. Genet* 2005;37:1281–1288. [PubMed: 16200065]
42. Sun D, Melegari M, Sridhar S, Rogler CE, Zhu L. *BioTechniques* 2006;41:59–63. [PubMed: 16869514]
43. Tiscornia G, Tergaonkar V, Galimi F, Verma IM. *Proc. Natl. Acad. Sci. U.S.A* 2004;101:7347–7351. [PubMed: 15123829]
44. Galimi F, Saez E, Gall J, Hoong N, Cho G, Evans RM, Verma IM. *Mol. Ther* 2005;11:142–148. [PubMed: 15585415]
45. Sinn PL, Sauter SL, McCray PB Jr. *Gene Ther* 2005;12:1089–1098. [PubMed: 16003340]

46. Heger S, Mastronardi C, Dissen GA, Lomniczi A, Cabrera R, Roth CL, Jung H, Galimi F, Sippell W, Ojeda SR. J.Clin.Invest 2007;117:2145–2154. [PubMed: 17627301]
47. Paxinos, G.; Huang, X-F.; Toga, AW. The Rhesus Monkey Brain in Stereotaxic Coordinates. San Diego, CA: Academic Press; 2000.
48. Clifton DK, Ochsner AJ, Uno H, Norman RL, Spies HG. Physiol.Behav 1975;14:103–107. [PubMed: 1098068]
49. Gliessman PM, Pau KY, Hill JD, Spies HG. J.Appl.Physiol 1986;61:2273–2279. [PubMed: 3804932]
50. Tavazoie SF, Alvarez VA, Ridenour DA, Kwiatkowski DJ, Sabatini BL. Nat.Neurosci 2005;8:1727–1734. [PubMed: 16286931]

Figure 1.

Maps of the 3rd generation lentiviral vector system containing either a TG or RNAi (TG-RNAi) cassette. The 5'LTR contains the heterologous U3 promoter (htU3), the repeat region/transcription initiation site (R), and the polyadenylation region (U5). The R and U5 regions are also present in the 3'LTR but importantly there is a 400 bp deletion in the 3'LTR U3 region (ΔU3) that results in the self-inactivation (SIN) of these vectors. The heterologous promoters are either the Rous sarcoma virus promoter (RSVp) or the cytomegalovirus promoter (CMVp); the CMVp also serves as the TG-RNAi cassette promoter. The other components include the packaging signal (Ψ), the Rev response element binding site (RRE), the central polypurine tract (cPPT), and the woodchuck-hepatitis-virus posttranslational regulatory element (wPRE). See

section 2.3 for descriptions of the vector components. A) Map of a lentivirus delivery vector [32] containing a bicistronic TG cassette composed of a human nerve growth factor (hNGF) cDNA linked to an enhanced green fluorescent protein (eGFP) cDNA via an internal ribosome entry site (IRES) and placed downstream of the rat insulin II intron A (Int). B) Map of the pPRIME lentivirus delivery vector [37] containing an artificial miR30 and replaceable hairpin structure able to accommodate 1 to 3 hairpins [42]; Xho I and Eco RI restriction enzyme sites are used as cloning sites to insert the hairpin(s). C) Maps of the packaging system containing the plasmids gB gag LV [50], pLP2, and pLP/VSVG (Invitrogen). The mutation noted in the *gag* gene increases the ability of the HIV-1 based lentiviral vector to transduce simian cells [31]. The other components in the packaging plasmids are the β -globin polyadenylation signal sequence (β -globin pA), HIV-1 polyadenylation signal sequence (HIV-1 pA), and the vesicular stomatitis virus glycoprotein (VSV-G). See section 2.4 for more information regarding the packaging plasmid components.

Figure 2.

A heterologous intron incorporated into a TG cassette of a lentiviral vector results in increased transgene expression, measured as an increase in NGF output. A) Maps of the lentiviruses carrying TG expression vector constructs. B) NGF release into the culture medium of 293T cells transduced with the viral vectors shown in A. Bars = mean ± SEM; numbers above bars are numbers of wells per group; * $P < 0.05$ (t-test) vs. no intron group. NGF was measured after 24 h of culture. See figure legend 1 and text for descriptions and definitions of vector components and abbreviations.

Figure 3.

X-ray and MRI guided stereotaxic injection of lentiviral particles into the arcuate nucleus of the hypothalamus in rhesus macaques. A) Initial x-ray showing the clinoids of the sella turcica (arrowheads). B) Drawing of a monkey skull showing the placement of fiducial markers. The most posterior marker (arrow) was placed on the midline and used to establish the true midline of the brain. C) MRI in which the posterior fiducial marker can be seen (arrow). D) MRI showing the arcuate nucleus of the hypothalamus (arrow). E) Final x-ray showing the injection needle (arrow); the tip of the needle shows the injection site, which is 1 mm dorsal to the line drawn between the clinoids (arrowhead). F) MRI following bilateral injection of $1.5 \mu\text{l}$ of a $1 \mu\text{M}$ solution of MnCl in 0.4% trypan blue (in phosphate buffered saline) into the arcuate nucleus

of the hypothalamus (arrow). G) Gross section of a monkey brain following bilateral injection of MnCl-trypan blue showing the sites of the injections (arrows).