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Applications of Lentiviral Vectors for shRNA Delivery and Transgenesis

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Abstract

Lentiviral vectors are potent gene delivery vehicles that enable stable expression of transgenes in both dividing and post-mitotic cells. Development of lentiviral vectors expressing small hairpin RNAs generates a system that can be used to down regulate specific target genes *in vivo* and *in vitro*. In this review, we will discuss two examples of *in vivo* applications for the use of lentiviral vectors expressing shRNAs: Gene therapy of neurological disorders and generation of transgenic knockdown animals.

INTRODUCTION

A major challenge in modern biology is to determine the molecular function of over 30,000 human genes. The technique of gene knockout in mice by homologous recombination in ES cells has proven to be very powerful but is laborious and expensive. RNA interference (RNAi) has recently emerged as a novel pathway that allows modulation of gene expression. The pathway has been under intense study and details of its basic biological mechanism is described elsewhere [1,2]. Briefly, long dsRNA molecules are processed by the endonuclease Dicer into short 21–23 nucleotide small interfering RNAs (siRNAs) which are then incorporated into RISC (RNA induced silencing complex), a multi-component nuclease complex that selects and degrades mRNAs that are homologous to the dsRNA initially delivered. In mammalian systems, siRNAs can be delivered exogenously or expressed endogenously from pol III promoters (as short hairpin RNAs – shRNAs) or from pol II promoters (as a part of a miRNA molecule), resulting in sustained and specific downregulation of target mRNAs [3–6]. Most applications involving expression of siRNAs in mammalian systems *in vitro* take advantage of transient delivery systems. Thus, in order to apply this potent technique for *in vivo* studies and more specifically to obtain transgenic animals, efficient siRNA delivery methods must be developed [7]. In this review, we describe the design of lentiviral vectors expressing shRNA and their use in generation of local and whole animal gene knockdown.

OVERVIEW OF LENTIVIRAL VECTORS

During the past decade gene delivery vehicles based on HIV-1, the best characterized of the lentiviruses, have been developed. Lentiviral vectors are capable of infecting a wide variety of dividing and non-dividing cells, integrate stably into the host genome, and result in long term expression of the transgene. The HIV-1 genome contains nine open reading frames encoding at least 15 distinct proteins involved in the infectious cycle, including structural and regulatory proteins. In addition, there are a number of cis-acting elements required at various stages of

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the viral life cycle. These include the long terminal repeats (LTRs), the TAT activation region (TAR), primer binding site (PBS), splice donor and acceptor sites, packaging and dimerization signal, Rev-responsive element (RRE) and the central and terminal polypurine tracts (PPT) (for review see [8]). The general strategy used to produce vector particles has been to eliminate all dispensable genes from the HIV-1 genome and separate the cis-acting sequences from those trans-acting factors that are absolutely required for viral particle production, infection and integration. The widely used third generation of lentiviral vectors consists of four plasmids (Fig. 1): the transfer vector contains the transgene to be delivered in a lentiviral backbone containing all the cis-acting sequences required for genomic RNA production and packaging. The packaging system involves 3 additional plasmids, which provide the required trans-acting factors, namely Gag-pol, Rev and an envelope protein respectively. Gag-pol codes for integrase, reverse transcriptase and structural proteins. While the structural proteins are required for particle production, integrase and reverse transcriptase molecules are packaged in the viral particle and are required upon subsequent infection. Rev interacts with the Rev-responsive element (RRE), a sequence contained in the transfer vector, enhancing the nuclear export of unspliced viral genomic RNA and thus increases viral titer. Viral particles can be pseudotyped with a variety of envelope proteins. One commonly used envelope protein is the Vesicular Stomatitis Virus protein G (VSV-G), which is incorporated into the viral membrane and confers the ability to transduce a broad range of cell types, including stem cells and early embryos. The transfer vector also contains a woodchuck hepatitis virus regulatory element (WPRE) that enhances the expression of the transgene [9] and a central polypurine tract (cPPT) purported to be important for nuclear import of pre-integration complex [10]. In addition, an important safety feature is provided by a deletion in the 3'LTR that results in replication defective particles, as during reverse transcription the proviral 5'LTR is copied from the 3' LTR, thus transferring the deletion to the 5'LTR; the deleted 5'LTR is transcriptionally inactive, preventing viral genomic RNA production from the integrated provirus [11]. When these four plasmids are transfected into 293T human embryonic kidney cells, viral particles accumulate in the supernatant and high titer (typically between 10^8 to 10^{10} infectious units per ml) viral preparations can be prepared by ultracentrifugation.

DESIGN OF LENTIVECTORS EXPRESSING shRNAs

A crucial breakthrough occurred with the report that siRNAs could be expressed as shRNA from pol III promoters cloned into plasmids [4,5]. The two pol III promoters most commonly used are H1 and U6 (both human and mouse). These promoters are characterized by their compact size (less than 400 bp), and by the fact that all sequences required for promoter function are upstream of the +1 transcriptional start site. Pol III promoters have ubiquitous expression and efficiently express short RNAs. Thus, they are ideally suited to express short hairpins RNAs. ShRNAs to be driven by the H1 promoter can begin with any base, but the stronger U6 promoter requires a G as its first base [12]. In our experience the U6 promoters (especially mU6) generates slightly better knockdown effect than H1 promoter. Nevertheless, there are some indications that U6 promoters might be more toxic in some tissues (e.g. bone marrow) probably due to their robust transcription capacity. Our preferred shRNA design consists of a 19–23 nucleotide sense sequence that is identical to the target sequence in the mRNA to be downregulated, followed by a 9 bp loop [4] and an antisense 19–23 nucleotide sequence. A stretch of 5 T's provides a pol III transcriptional termination signal. The total length of the silencing cassette is ~350 bp. Thus, when this construct is expressed, a short 21–23 base pair hairpin is formed; the loop is digested by Dicer and the resulting siRNA triggers degradation of the mRNA target (Fig. 2).

Ideally, a silencing lentiviral vector would contain both a marker gene such as EGFP or an antibiotic resistance gene and the shRNA silencing cassette. Previous experience in our lab indicates that the position and precise combination of elements in a lentiviral vector construct

can have unforeseen effects both on the viral titer and the efficiency of expression of both the marker and the silencing cassette. We have designed two different versions of lentiviral silencing vectors that differ both in the position of the silencing cassette and in the cloning strategy required to construct them (Fig. 3).

The first design involved a lentiviral vector carrying GFP cassette as a marker gene and an additional silencing cassette inserted into a unique restriction site in the 3'LTR [13,14]. Upon transduction of target cells by the lentiviral particles, reverse transcriptase generates a viral cDNA, which then stably integrates into the host genome. During this process, the 5'LTR is generated from the 3'LTR. Thus, cloning of the silencing cassette into the 3'LTR results in duplication of the cassette and doubling of the shRNA expression levels in every transduced cell (Fig. 3a). This feature is important since following transduction of primary cells and especially live animals, single integrations can be common and might not be sufficient for robust silencing.

In a second design the silencing cassette is inserted in the middle of the viral vector genome using a gateway cloning strategy (Fig. 3b). While this design does not result in duplication of silencing cassette it can be used to generate a CRE recombinase induced knockdown phenotype [15].

The effectiveness of a particular siRNA is largely unpredictable and presumably reflects both mechanistic constraints of the RNAi pathway and accessibility of the target sequence within the tertiary structure of the target mRNA. A number of algorithms have been developed to predict effective siRNA sequences [16] and many of them are available [17,18].

In general, the target sequence should be 19 to 23 bases long, but lengths of up to 29 bases have been reported [19]. Longer targets should be avoided, as longer dsRNA molecules can trigger a PKR response [20]. Targets can be directed to 5'UTR, ORF or 3'UTR of the target mRNA as desired.

The discovery that miRNAs can act as siRNAs and vice versa [21,22] had led to development of a new type of shRNAs – miRsiRNAs. These molecules are generated by changing the stem sequence of an endogenous miRNA into a given siRNA while maintaining the extra stem sequences, loop and 5' and 3' sequences. Processing of miRNAs require two maturation steps, nuclear processing of pri-miRNA into pre-miRNA require recognition and cleavage of 5' and 3' extra stem sequences by Drosha/DGCR8 complex and cytoplasmic processing of pre-miRNA into mature miRNA require recognition and cleavage of loop sequences by Dicer (reviewed by [23]). Since mammalian miRNAs are naturally transcribed by RNA pol II promoters, any tissue specific and drug-regulated pol II promoter can transcribe miRsiRNAs [24]. Many miRNAs are initially expressed as a polycistronic transcript containing several individual miRNAs and in some cases an additional protein-coding region. This finding enables the expression of several miRsiRNAs and a marker gene under one pol II promoter [25]. This is an extremely desirable attribute since it allows the combination of regulated expression with complex silencing of several genes driven by one promoter. Nevertheless, RNA pol II promoters are significantly weaker than pol III promoters and achieving sufficient knockdown is more demanding in comparison to the robust transcription of shRNA by pol III promoter.

VALIDATION OF shRNAs

Typically, as only a fraction of selected target siRNA sequences will be functional with varying degrees of efficiency, several siRNAs need to be generated and tested for every target gene. Depending on the quality of the algorithms used, 3–7 different siRNA target sequences are normally screened for most effective siRNA. In order to ensure potent knockdown in animals, only siRNA sequences showing highest efficiencies should be selected. A validation assay for

most effective shRNA should include a control shRNA. Over expression of shRNAs can result in an unexpected non-specific effect, which can affect the expression of the targeted gene. Therefore it is always recommended to use a proper control for validation assays. A proper control should contain the same lentiviral backbone expressing the same marker gene (e.g. GFP) and an irrelevant shRNA cassette driven by the same pol III promoter as the gene specific shRNA. Several control shRNAs are commonly used: A scrambled control (sequence of α -sense do not have a target in genebank), a no-target control (sequence of α -sense do not have a target in assayed cell, e.g. shLuciferase) or mutated control (sequence of α -sense has 3 or more mismatches to target gene). Two or more different control shRNAs should be used in each validation assay. It is recommended to select siRNA targets showing 80% or higher silencing levels for stable knockdown studies. Sub-optimal siRNAs will require an increase in shRNA expression levels, which can result in lethality due to non-specific toxicity [26]. It is highly recommended that at least one of the irrelevant shRNA be used as control for the actual *in vivo* study.

IN VIVO DELIVERY OF shRNA EXPRESSION LENTIVIRAL VECTORS

One of the very desirable features of lentiviral vectors is their ability to transduce non-dividing cells such as neurons. Through the use of stereotactic injection device, concentrated lentiviral particles can be injected into defined sites of the brain [27]. This kind of localized delivery technique can be used as a mean of gene therapy for many types of brain specific diseases through expression of therapeutic proteins [28] or expression of shRNAs targeting disease associated genes. Many human brain disease models have been generated by over expressing a mutated human gene in transgenic animals. These models can be used as a proof of principle in therapy experiment since shRNAs can be directed to target only the transgenic human allele carrying the mutation without targeting the wild type mouse allele, thus avoiding any deleterious effects. This was shown with the use of AAV vectors for targeting the human allele carrying polyglutamine expansion in a spinocerebellar ataxia mouse model [29] and lentiviral vectors for targeting the human allele carrying SOD1 mutant in a mouse model of ALS [30, 31]. Applying such a technique for human trials is likely to require generation of siRNAs that can target specifically and exclusively the mutated sequence, a complex task, since many of the human diseases are generated by a single point mutation. Many neurodegenerative diseases, such as Alzheimer's disease (AD), progress as a consequence of interaction between several genetic components. This characteristic provides several possible targets for shRNAs – e.g. amyloid precursor protein (APP) and the γ -secretase presenilin 1 (PS1). One of the most studied targets is the β -secretase BACE1, required for processing and generating the toxic amyloid - β peptide. BACE1, initially thought to be a redundant protein, is known to be elevated in affected brain area of AD patients as in AD animal models. BACE1 is an appealing target since it is considered to be an important component of the feedback loop that accelerates the production of amyloid - β peptide. Indeed, reduction of endogenous levels of BACE1 by a lentiviral vector expressing shRNA in an AD mouse model was shown to be therapeutic without obvious deleterious effects [32].

GENERATION OF TRANSGENIC KNOCKDOWN ANIMALS

We and others have previously shown that lentiviral vectors have the unique ability to generate transgenic rodents by *in vitro* transduction of fertilized eggs at different preimplantation stages [33,34]. Since lentiviral vectors integrate into the host chromosome, the progeny of a lentivirus mediated transgenic animal is likely to inherit the provirus and express the transgene or silencing cassette. Although retroviral vectors (e.g. MLV based vectors) have the ability to integrate their genomic DNA into chromosomes of dividing cells and achieve long-term expression, they cannot be used to make transgenic animals due to the fact that the proviral DNA is silenced during embryogenesis, probably as a result of methylation of LTR sequences

[35]. Other viral vectors, such as adenoviruses and adeno-associated viruses (AAV) remain as episomal DNA in transduced cells and will not result in germ line transmission of viral DNA.

While generation of transgenic animals had been performed by pronuclear injection of DNA to single cell embryos, generation of mouse knockouts is time consuming and laborious. An ES knockout line must be generated, characterized and injected into a blastocyst in order to obtain a chimeric founder that can be subsequently bred to homozygosity. As an alternative, taking advantage of the unique ability of lentiviral vectors to generate transgenic animals, lentiviral vectors were also used for generation of transgenic knockdown mice by expressing shRNAs from pol III promoters such as H1 and mU6 [13,36]. Two methods are available for delivering lentiviral particles to the embryo to obtain lentivirus transgenesis: zona pellucida removal and sub-zonal injection [37]. Both methods are designed to deliver virus particles through the zona pellucida protective layer and both results with high percent of transgenesis (typically 50% and higher). Although zona pellucida removal does not require special machinery, zona removal is toxic to the embryos and results in low survival of embryos. The sub-zonal injection method is associated with only minimal toxicity for the embryos; therefore, survival of embryos is much higher. Nevertheless, injection of viral particles through the zona layer requires special apparatus and manipulation of embryos using micromanipulator apparatus requires patience and practice.

As opposed to traditional transgenesis (achieved by pro-nuclear DNA injection), lentiviral vector transgenesis will cause the numbers of integration copies to vary between the progeny since each provirus integrates independently. This is the source of the major difference between traditional transgenesis and lentiviral vector transgenesis; traditional pronuclear DNA injection result in transgene insertion into a single locus, however the linearized DNA will form larger multimer comprised of many head-to-tail copies. The results will be a single transgenic locus that will be pass to next generation by simple Mendelian inheritance but will contains many (e.g. 50–100) copies of transgene. Lentiviral vector trans-genesis will generate several independent copies of integrated provirus that will segregate independently in the next generations. Since lentiviral vectors can integrate in a variety of genomic sites (as opposed to retroviral vectors that are more restricted to active transcription units – especially the first exon) some copies are expected to be in a non-active chromatin. This is probably the reason why some integrated copies do not express (silencing of integrated provirus). The majority of lentiviral vector transgenic animals result in mosaic founders (F_0) and levels of chimerism are directly correlated with the developmental stage of manipulated embryo. Zona pellucida removal technique is possible only in embryos that are at 2-cell stage or higher and therefore always results in some level of chimerism due to uneven transduction of the embryo's cells. Direct lentiviral vector perivitelline injection at a single cell stage embryo can minimize chimerism. In each case, the transgenic founders should be crossed to each other or to a non-transgenic mouse in order to ensure germ line transmission of transgene. In our experience, once transgenic F_1 mice are generated the transgene will be stably transmitted to next generations. Some founders will give poor germ line transmission for reasons that are not fully understood. The expected copy number for lentiviral vector transgenesis is normally between 1–5 copies per animal, although higher copy number can sometimes be noticed. Since the efficiency of shRNAs is influenced by amount of RNA production this can generate a variety of different hypomorphs within the same litter. In order to achieve a more homogeneous silencing phenotype it is possible to segregate the different copies by out breeding until a single copy line is generated [38]. Of course, it is essential that the selected shRNA sequence is sufficient to generate the desired silencing phenotype from a single integration. It is our experience that once germline transmission is achieved, the knockdown phenotype is generally not altered. Other factors that should be taken into account are position effect variegation of integration sites that results in altering expression levels of some of the integrated copies and the possibility of toxicity due to the gene specific silencing phenotype. Expression of shRNAs

from pol III promoters is robust and well established, however, these promoters are ubiquitous and express the shRNA in every cell, which might result in embryonic lethality due to silencing of an essential gene. Since traditional transgenesis always results in high copy number of transgene integration in one locus it is more likely that shRNA transgenic made this way will suffer from general toxicity of over expressed shRNAs [26]. This could explain the low success rate of some traditional shRNA transgenic reported [39]. One can expect that the low copy number transgenesis by lentiviral vectors will be more suitable for generating transgenic shRNA animals. The ability to segregate the independent provirus copies and dilute out possible toxicity adds up to this advantage. Alternatively, a more controlled expression system can be used (e.g. Tetracycline regulatable or tissue specific promoters).

Lastly, one of the great advantages of using lentiviral vectors for transgenesis is the ability to generate efficient transgenesis in a variety of species that are incompatible with pronuclear DNA injection and/or knockout technology. Production of transgenic immuno-compromised mice has always been time consuming and requires generation of an immunocompetent transgenic mouse followed by backcrossing into a NOD-SCID line, however, direct lentiviral vector perivitelline injection has proven a much more rapid and cost effective method [40]. Traditional generation of transgenic rats that suffer from lack of visible pronuclei (make pronuclear injection challenging) can be greatly improved by direct lentiviral vector perivitelline injection [33,41,42]. Similarly, direct lentiviral vector perivitelline injection or co culture was also shown to be most efficient with generation of transgenic birds [43,44] and farm animals [45].

CONCLUSIONS

The rapid development of gene delivery approaches holds the promise for establishing novel treatments for neurological disorders and other disorders, like cancer and infectious diseases. In combination with RNAi technology it is possible to modulate expression levels of disease related genes or even to silence mutated genes specifically [25,46]. Due to the larger packaging capacity of lentiviral vectors it is possible to include in a single vector genome several siRNAs targeting different genes and even an entire regulation system (e.g. Tet-regulatable shRNA in addition to the TetR - tetracycline repressor protein). The same delivery approach can also be used to study gene function in defined areas of the brain by selectively silencing genes involved in cognitive and emotional processes [47]. Similarly, expression of different mix of oncogenes can be used to generate new brain cancer models [48]. In combination with specific shRNAs targeting different tumor suppressor genes this, methodology can be used to better understand basic cancer biology questions.

We have described a methodology that can be successfully employed to generate a large number of transgenic mice where expression of specific genes can be substantially down regulated. The technology builds on the use of lentiviral vectors for transgenesis, combined with the use of RNA-interference to silence gene expression. Since RNAi only reduce the levels of silenced gene it is conceivable that some transgenic knockdown phenotypes will be different than the knockout phenotype and generate a variety of hypomorphs. In addition, it is likely that such hypomorphs will be more viable compare to the knockout and in some cases may escape embryonic lethality. In combination with a reliable regulation system this technology is expected to greatly enhance the study of genome function in mammals.

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Fig. 1.

a) Scheme of the HIV-1 proviral DNA. b) Third generation lentiviral vector system. The transfer vector contains all cis-acting elements required for replication and packaging of transfer vector RNA into viral particles. Three helper plasmids provide all required transacting factors (see text for details).

Fig. 2.

Scheme depicting the RNA interference pathway. A pol III promoter drives expression of the silencing cassette and produces a hairpin, which is exported into the cytoplasm, processed by Dicer and assembled into the RISC complex. Recognition of the target mRNA requires unwinding of the siRNA duplex and the antisense strand mediates target recognition and degradation.

Fig. 3.

a) 3' LTR double shRNA cassette design. b) Gateway single shRNA cassette design.