Chapter 3
Cell and Tissue Gene Targeting with Lentiviral Vectors

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Abstract One of the main advantages of using lentivectors is their capacity to transduce a wide range of cell types, independently from the cell cycle stage. However, transgene expression in certain cell types is sometimes not desirable, either because of toxicity, cell transformation, or induction of transgene-specific immune responses. In other cases, specific targeting of only cancerous cells within a tumor is sought after for the delivery of suicide genes. Consequently, great effort has been invested in developing strategies to control transgene delivery/expression in a cell/tissue-specific manner. These strategies can broadly be divided in three; particle pseudotyping (surface targeting), which entails modification of the envelope glycoprotein (ENV); transcriptional targeting, which utilizes cell-specific promoters and/or inducible promoters; and posttranscriptional targeting, recently applied in lentivectors by introducing sequence targets for cell-specific microRNAs. In this chapter we describe each of these strategies providing some illustrative examples.

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3.1 Introduction

Lentivectors can effectively transduce a wide range of cells [1, 2]. This property allows gene correction of potentially any cell type. On the other hand, in some circumstances transgene expression is desirable in only a limited number of specific cell targets. For example, intravenous lentivector administration results in gene transfer to hepatocytes in mouse models. However, this also leads to transgene expression in professional antigen presenting cells such as plasmacytoid dendritic cells (pDCs). These cells then trigger a transgene-specific immune response that will result in elimination of transgene-expressing hepatocytes [3]. This "collateral transduction" limits the therapeutic efficacy of some gene therapy protocols. Therefore, in this case DC transduction has to be avoided at all costs. In other circumstances, transgene expression in immune cells is therapeutic. For example, expression of particular mitogen activated protein kinase (MAPK) constitutive activators in myeloid DCs can either enhance antitumor immune responses or inhibit immune responses by modulating DC functions [4, 5]. On the other hand, some of these MAPK modulators may favor cell transformation if expressed in poorly differentiated cell types [6–8]. Therefore, restricted transgene delivery to immune cells would increase biosafety. Finally, a transgene may be toxic in a particular cell lineage but only at certain differentiation stages. This is exemplified in the correction by gene therapy of globoid cell leukodystrophy, a lysosomal storage disease caused by inactivating mutations in galactocerebrosidase (GALC) [9]. While GALC expression is highly toxic in early hematopoietic progenitors, it is therapeutic in mature cells from the hematopoietic lineage [9]. This is an interesting case in which specific transgene delivery was achieved according to the cellular differentiation stage.

Hence, there are many circumstances in which specific targeting to cell types and tissues has to be achieved. Therefore, the lentivector tropism has been modulated by many experimental approaches, and here we will focus on the best-known examples.

3.2 Modification of Lentivector Tropism by Pseudotyping (Surface Targeting)

Transgene delivery by lentivectors depends on the recognition of the target cell by ENV, which is followed by entry into the cell. Therefore, the lentivector tropism is first determined by specific binding to cell surface receptors. As discussed in Chap. 2, lentivectors can acquire a wide range of different envelope glycoproteins during budding at the plasma membrane from the producer cell. This process is called pseudotyping because the resulting virions (pseudovirions) exhibit the surface antigenicity provided by a heterologous ENV [10, 11].

HIV-1 ENV can be used for "pseudotyping" lentivectors, although it does not lead to high titer preparations. For this reason, one of the most widely used envelopes for lentivector pseudotyping is the vesicular stomatitis virus glycoprotein (VSV-G) [2, 12–14]. VSV-G pseudotyping exhibits many advantages; firstly, it stabilizes the vector particle, leading to high titer vector preparations, and allows vector concentration by ultracentrifugation due to its stability [15]. Secondly, VSV-G is a pantropic envelope, and confers a very broad host cell range [16]. In fact, it is unclear whether VSV-G binds a specific ubiquitous cell receptor, or binds to phospholipids in the plasma membrane [15, 17, 18].

However, in some cases restriction of lentivector tropism results in safer in vivo gene delivery, and can also enhance the therapeutic effects by reducing the lentivector dose. This is of interest since reaching high titer retrovirus vector preparations is a major difficulty. For this reason, several strategies have been applied to achieve specific transductional targeting by surface modification of ENV as explained below.

3.2.1 Pseudotyping with Heterologous Viral Proteins

The availability of a broad range of existing viral ENVs combined with the capacity of retrovirus/lentivirus vectors to accommodate heterologous ENVs makes this strategy simple and straightforward. These lentivectors should exhibit the same cell/tissue tropism of the virus from which the ENVs originated. The list of available glycoproteins for lentivector pseudotyping is evergrowing [19]. Summarizing, viral glycoproteins from several viral families have been successfully used, including *Retroviridae*, *Baculoviridae*, *Filoviridae*, *Flaviviridae*, *Arenaviridae*, *Rhabdoviridae*, *Paramyxoviridae,* and *Coronaviridae* [19, 20] (Table 3.1). In this section we will provide key examples.

Lentivectors can be easily pseudotyped with γ-retroviral ENVs such as mouse leukemia virus amphotropic (MLV-A), gibbon ape leukemia virus (GALV), and feline endogenous retrovirus (RD114) envelopes [21–23]. These envelopes recognize cellular receptors expressed in a wide range of human cell types, such as phosphate cotransporters Pit2 for MLV A [24], Pit1 for GALV, and the neutral aminoacid transporter RDR for RD114 envelope [22, 25–27]. In particular cases, lentivector pseudotyping requires certain modifications in these ENVs. For GALV and RD114 ENVs, substitution of the cytoplasmic domain by that of the MLV enhances their incorporation [23, 28, 29]. The substitution of RD114 cleavage site with the site specific for HIV protease increases its activity [30]. Lentivectors pseudotyped with γ-retroviral envelopes effectively transduce CD34+ hematopoietic precursor cells, a requirement for the treatment of several human genetic pathologies [31]. In fact, correction of severe combined immunodeficiency (SCID) was achieved with GALV [32] and MLV-A [33] pseudotyped retrovirus vectors. GALV ENV was used again for the correction of X-linked chronic granulomatous disease (CGD) and Wiskott–Aldrich Syndrome [34, 35]. In contrast, correction of

Table 3.1 Some selected examples of virus envelope glycoproteins commonly used for lentivector and retrovirus vector pseudotyping

Family	Glycoprotein (species)	References
Retroviridae	Human T lymphotropic virus (HTLV)-1, maedi-visna virus, gammaretroviruses	[21, 50–53]
Togaviridae	Semliki forest virus (SFV), venezuelan equine encephalitis virus (VEEV), ross river virus (RRV), and sindbis virus	[38, 54–58]
Rhabdoviridae	Vesicular stomatitis virurs, rabies virus, and mokola lyssavirus	[59–61]
Filoviridae	Ebola, marburg virus	[62, 63]
Orthomyxoviridae	Influenza hemagglutinin	[64]
Coronaviridae	Severe acute respiratory syndrome (SARS) coronavirus	[20, 65]
Baculoviridae	Baculovirus	[40, 41]
Paramyxoviridae	Measles virus	[46]
Arenaviridae	Lymphotropic choriomeningitis virus (LCMV)	[42, 43, 66]

X-linked adrenoleukodystrophy and β-thalassaemia was achieved with VSV-G pseudotyped lentivectors because of their superior transduction efficiency [36, 37].

Lentivectors can also be effectively pseudotyped with envelope proteins from more distant virus families (Table 3.1). These include alphavirus envelopes (Ross River virus and Semliki Forest virus) which exhibit specific tropism towards mouse and human dendritic cells [38, 39]; baculovirus gp64, an insect virus envelope which confers high particle stability and transduction efficiency. Lentivectors pseudotyped with gp64 effectively transduce hepatocytes in vivo, but not cells from the hematopoietic lineage (or very poorly) including DCs [40, 41]. This property can be exploited to prevent transgene-specific immune responses. Lymphocytic choriomeningitis (L-CMV) virus ENV pseudotypes transduce cells from the central nervous system (neurons, neuroblasts, and astrocytes), glioma cells, and also insulin secreting β cells [42, 43].

The list of lentivector pseudotypes and their application is long. However, there is one more case worth explaining in detail due to its relevance for T and B cell human gene therapy. Gene modification of naïve, nonactivated B, and T lymphocytes has always been a scientific challenge. Their efficient transduction requires their activation usually with antiCD3/antiCD28 agonistic antibodies, or by pretreatment with cytokines [44]. This activation alters their phenotype and effector functions before they can be transduced. Even VSV-G lentivector pseudotypes transduce nonactivated T cells inefficiently [44]. Interestingly, efficient transduction of naïve, nonactivated human lymphocytes is achieved with measles virus H and F ENV (H/F) pseudotypes [45, 46]. Measles virus H/F binds to SLAM and CD46 leading to efficient virus entry, nuclear transport, and integration [47]. These lentivectors can also transduce some B cell lymphomas particularly resistant to lentivector transduction [48, 49].

All these examples, especially the last one, demonstrate that it is possible to find an adequate ENV pseudotype for any target cell type.

3.2.2 Pseudotyping with Modified Viral Glycoproteins

The binding function and tropism of ENV pseudotypes can also be altered by modification of ENV residues involved in receptor binding. In some cases, their original tropism can be completely abrogated without affecting their fusion activities. Then, other molecules such as antibodies, cytokines, or receptor ligands can provide an alternative binding method.

An example of altering the natural tropism of ENV to achieve specific DC tropism is the introduction of selected mutations in the Sindbis virus envelope proteins E1/E2. E1/E2 binds to heparan sulfate, present in most cell types, and also to DC-SIGN, a DC-specific molecule. While E2 binds to the cell receptor, E1 mediates membrane fusion. Interestingly, E1 fusion activity is independent of E2 binding to the cell receptor [67]. Specific E2 mutations abolished binding to heparan sulfate but not to DC-SIGN. This modification allowed specific lentivector gene transfer to DCs in vivo [58]. The Sindbis E1/E2 envelope system is also susceptible to other targeting strategies. In some cases, E2 binding capacities have been completely abrogated, while providing alternative binding methods alongside E1/E2 pseudotyping. For example, cell-specific antibodies conjugated to E2 conferred specific tropism towards P-glycoprotein-expressing melanoma cells [68], prostate cancer [69], endothelial cells [70], and CD34+ hematopoietic progenitor cells [71]. Strong antibody conjugation was achieved by introducing the ZZ domain of protein A in E2. Incorporation of antibodies or any other surface molecule alongside modified Sindbis ENVs can effectively target lentivectors to specific cell types [72].

A major setback from the Sindbis-based modification strategies is the dependence on endocytosis for pH-dependent fusion to occur. Physical retargeting of lentivectors does not guarantee their endocytosis. Fortunately, pseudotyping with measles virus F/H envelope glycoproteins circumvents this hurdle. While the H subunit mediates cell binding, the F protein triggers pH-independent fusion [73]. Therefore, F/H lentivector pseudotypes can gain access by direct fusion with the plasma membrane [74]. Similarly to the Sindbis virus E1/E2 system, the measles virus H subunit binding residues can be mutated, and bound to different molecules targeting specific ligands. For example, fusion with either the epidermal growth factor (EGF) or with a CD20-specific single-chain antibody resulted in specific lentivector transduction of EGF receptor expressing cells and CD20+ B lymphocytes, respectively [75]. Of note, the authors of this study remark the high B cell transduction efficiency. However, it is possible that the measles virus H/F envelope system itself is the main determinant for B cell modification [46, 47].

Engineering of retargeted envelope proteins by covalent fusion to natural ligands such as cytokines has proved to be a challenge [76]. These strategies have limited success as the inclusion of a ligand usually inhibits viral entry, with some limited exceptions such as the Sindbis and Measles virus envelope systems [76, 77]. One of these examples is the fusion of influenza heamaglutinin with EGF to target retroviral transduction to EGF receptor-expressing cells [78]. To overcome the inhibition of vector entry, sequence targets for cellular proteases such as metalloproteases (MMP) were introduced to release ENV from the fused ligand/antibody. This strategy has also been applied for the targeting of MMP-expressing tumors using retrovirus and lentivirus vectors [79–83].

3.3 Transcriptional Targeting

Selective targeting of transgene expression to specific cell types can be effectively achieved with cell and tissue-specific promoters. In this situation lentivector transduction is not prevented at cell entry, but rather transgene expression is restricted to specific cell types. The large number of endogenous cellular promoters potentially allows targeted expression to any cell type or tissue. In addition, inducible promoters can also be incorporated in lentivector systems, leading to controlled transgene expression by administration of a given drug. These strategies add an additional control point for the development of cell-specific lentivectors.

3.3.1 Cell and Tissue Specific Promoters

Specific cell type expression can be achieved by incorporating promoters active in these specific cells into the lentivectors. Endogenous cellular promoters are in addition less sensitive to promoter silencing [84, 85]. This is key in human gene therapy; silencing of the γ-retroviral promoter and loss of transgene expression could have contributed to patient death in the CGD clinical trial [86]. Using endogenous cellular promoters results in improved stability and longevity of transgene expression in the target cells. Consequently, a wide range of endogenous promoters has been introduced in retrovirus and lentivirus transfer vectors.

Using this approach and sometimes by combining viral enhancers with endogenous promoters, specific gene expression was achieved in a number of cell types and tissues such as erythroid cells [87–89], endothelial cells [90], retinal cells [91, 92], neurons [93, 94], glial cells [89, 95, 96], and several cell types in the hippocampus [97]. Cells of the liver have also been targeted after intravenous lentivector administration with the use of specific promoters which effectively restricted expression to hepatocytes [84, 98]. In this particular case, the benefits of cell-specific gene expression were clearly shown using the albumin promoter, which resulted in long-term transgene expression in rat liver. In contrast, transgene

expression with the cytomegalovirus promoter (CMV) was rapidly silenced [84, 98]. Importantly, hepatocyte-specific promoters prevent transgene expression in professional antigen presenting cells, which could raise transgene-specific immune responses. This is exemplified in the correction of mucopolysaccharidosis type I in a mouse model with lentivector gene therapy. This disease is caused by α-L-iduronidase (IDUA) deficiency, which leads to toxic glycosaminoglycan accumulation in a wide range of cells [99]. Its correction relies on expression of IDUA in the liver by intravenous administration of a therapeutic lentivector. However, IDUA is also expressed in antigen presenting cells, limiting the efficacy, and durability of the correction. To prevent this, IDUA expression was controlled by the albumin promoter, resulting in long-term expression in the liver, and minimal transgene-specific immune responses [99].

Cancer cells have also been specifically targeted using "tumour cell-specific promoters". A lentivector containing a metalloprotease-specific promoter was used to express proapoptotic genes Bax and tBID in MMP2-expressing cancer cell lines [100]. The α-fetoprotein promoter was used to deliver suicide genes to hepatocarcinoma cells [101, 102], and the prostate specific antigen (PSA) promoter for targeting prostate cancer cells. In fact, a lentivector delivering the diphtheria toxin A gene under the control of the PSA promoter has been used to eradicate prostate cancer cells in culture and in a mouse tumor model [103].

In other experimental settings, transgene expression is required in immune cells, particularly DCs. DCs comprise a group of specialized professional antigen presenting cells, which regulate, and control immune responses [4, 104, 105]. DC-specific expression has been achieved to induce antitumor immunity using HLA DRα [106] and Dectin-2 promoters [107]. On the other hand, transcriptional targeting to DCs has been applied to achieve immune suppression. For example, transgene-specific tolerance was achieved by lentivector-mediated CD11c promoter-controlled expression in transgenic mice [108]. Specific DC targeting to achieve immunological tolerance widens the application of gene therapy approaches for the treatment of autoimmune diseases and prevention of graft-versus-host disease.

As mentioned above, possibly one of the most complex tissues/organs to target is the central nervous system, exhibiting a high cellular diversity [109]. In this instance, transcriptional targeting has proved to be a reliable technique. Many cellular promoters are effective for expression in neurons, glial, and hippocampus cells, such as the synapsin and synapsin-1 promoters [89, 93, 97], enolase promoter [94], CD44 promoter, glial fibrillary acidic protein, and vimentin promoters [89, 95]. In some of these cases, high and longlasting transgene expression has been achieved [94, 97], while other promoters have been less efficient [95]. In fact, it is often the case that endogenous promoters are not as strong as those of viral origin. To boost endogenous promoters while retaining their cell specificity, researchers have modified particular cell-specific promoters by combination with other promoters or adding enhancers, and artificial transcriptional activators. This is the case for bidirectional promoters in which a minimal CMV promoter is positioned next to the cell-specific promoter leading to
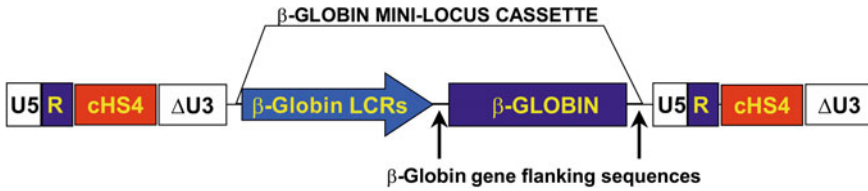
Fig. 3.1 Lentivector design for correction of human β-thalassaemia. The design of the therapeutic lentivector used for the correction of β-thalassaemia is shown. The lentivector contains an expression cassette resembling the endogenous β-globin gene. This includes, apart from the endogenous promoter, the 5′ and 3′ locus control regions (LCRs) placed upstream the β-globin gene [110]. In addition, 5′ and 3′ immediate flanking regions from the endogenous β-globin gene are included and indicated with arrows. Additionally, chicken hypersensitive site 4 (cHS4) β-globin insulator sequences [113] were placed within the LTRs, which prevents silencing of the expression cassette and transcriptional transactivation of adjacent host genes. Please note that this lentivector is a self-inactivating construct (Chap. 2) and the minilocus is placed within the lentivector construct in reverse orientation

transcription in the opposite direction. In this way, transgene expression in the target cells was enhanced [89]. The combination of several promoters within the same construct also allows cell-specific expression of more than one transgene. For example, the interphotoreceptor retinoid binding protein promoter and the guanylate cyclase activating protein promoters were evaluated together with the rhodopsin promoter. These combinations were aimed to achieve specific expression of two trangenes in retinal cells [92].

There is a specific case in which the promoter design has been critical to achieve therapeutic activities in a human gene therapy [37]. Patients suffering from β-thalassaemia contain a nonfunctional allele of β-globin, which results in a marked reduction of its expression. These patients rely on life-long blood transfusions. An obvious approach to correct the disease is to drive β-globin expression in erythroid cells. Although straightforward from a theoretical point of view, the accomplishment of relevant functional β-globin expression has been a challenge. This has been achieved after carefully engineering a lentiviral vector to include the β-globin gene under the transcriptional control of its endogenous promoter, introns, and locus control regions [37, 110–112] (Fig. 3.1).

Summarizing, there is a long list of cell-specific promoters that have been successfully applied in lentiviral vectors, which will surely improve their performance and safety in gene therapy.

3.3.2 Regulatable Promoters

Transgene expression can also be controlled using regulatable promoters. The capacity to regulate transgene expression is crucial for the treatment of genetic diseases for which the timing or levels of expression is critical. A typical example

of this is diabetes, in which high blood glucose levels trigger insulin secretion. Many research groups have developed inducible promoters, and many of these systems can be incorporated in lentivectors. Probably, one of the first and most widely used systems utilize tetracycline induction [93, 114–116]. Briefly, there are two main variations of the tetracycline system; tet-on, leading to inducible transgene expression after tetracycline (doxycycline) delivery, and tet-off, which needs constant antibiotic administration to prevent transgene expression [117]. For obvious reasons, the tet-on system is preferred for gene therapy, and most published lentivector systems belong to this category [100, 114, 115, 118, 119], with a few exceptions [93, 120]. In any case, tetracycline-inducible systems are also prone to inactivation and leaky transcription, and their in vivo application is not straightforward [121].

To overcome the disadvantages of tetracycline-dependent inducible systems, other systems have also been adapted to lentivectors, such as the *Drosophila* ecdysone receptor system [122, 123]. This is based on the binding of either ecdysone or synthetic analogs to a heterodimeric protein made of the herpex simplex virus protein VP16 activation domain fused to the ecdysone receptor (VgEcR) and the retinoid X receptor (RXR). VgEcR-RXR then binds to the inducible promoter driving gene transcription [122]. However, this system depends on the administration of multiple lentivector backbones [123]. More recently, it has been successfully reduced to a single lentivector backbone by fusing the tetracycline repressor with the Kruppel-associated Box (KRAB) domain repressor. This novel fusion protein acts as the regulator. This system has achieved tightly regulated conditional transgene expression in the brain, for a drug-inducible transgenic mouse model, or gene silencing in hematopoietic cells [124, 125].

There are quite a number of other inducible systems also adapted to the lentivector system such as the glucocorticoid inducible promoters and mifepristone-inducible systems [126, 127].

3.3.3 Promoters Controlled by Activation State

There are also many promoters upregulated depending on the activation state of different cell types. In most cases, these promoters have been utilized as reporter constructs [4, 128]. An example of these, an NF-κB transactivatable promoter was engineered by fusing NF-κB binding sites upstream of the minimal CMV promoter, driving expression of reporter fluorescent proteins. Addition of toll-like receptor (TLR) agonists such as LPS to DCs modified with these lentivectors resulted in strong transcriptional upregulation of the fluorescent proteins. The interferon β promoter also achieved similar results. These promoters could be useful to express transgenes following DC activation, although they have not been applied in a therapeutic setting yet [4, 128].

3.3.4 Posttranscriptional Targeting

Without any doubt, the discovery of a regulatory system of gene expression based on small noncoding RNAs (microRNAs or miRNAs) has revolutionized biomedical research. These small noncoding 20–24 nt RNAs, termed siRNAs, are partially complementary to a wide range of mRNAs. They can posttranscriptionally inhibit gene expression by either leading to mRNA degradation, translational repression, or mRNA destabilization.

The miRNAs and their activities are regulated by complex mechanisms with many variations depending on the species. Therefore, briefly and oversimplifying, we will describe the main steps controlling miRNA regulation in animal cells. Firstly, active siRNAs are encoded within large precursor RNA molecules called miRNAs (Fig. 3.2) which are transcribed by the RNA polymerase II. These long precursor miRNAs are recognized by a specialized enzymatic pathway (Pasha/ Drosha), which will release the siRNAs in the form of short hairpins (shRNA). The siRNA refers to the hairpin stem together its complementary strand (in some particular cases, the complementary strand can also play regulatory roles). This shRNA is actively exported out of the nucleus to the cytoplasm where it will be recognized by the enzyme complex Dicer (DCR), which will degrade most of the shRNA leaving the stem containing the siRNA target and its complementary sequence (miRNA–miRNA* duplex). This duplex is loaded in the AGO complex (Argonaut), forming the preRISC (RNA Interference Silencing Complex). Subsequently, the miRNA strand is degraded, leaving its complementary miRNA* intact within the RISC complex. The RISC complex will scan mRNAs and when "sufficient" complementation is found between the target mRNA and the miRNA* strand, the mRNA will be degraded. In some cases, the poly-A tail is removed, leading to mRNA destabilization. Alternatively, mRNA translation may be stalled (Fig. 3.2).

So, how has this mechanism been exploited for cell specific targeting of transgene expression? In fact, it is strikingly simple. Different cell types express different patterns of miRNAs, because they are intimately involved in regulation of cell differentiation. Therefore, if a transgene delivered by the lentivector contains a target that is complementary to an endogenously expressed miRNA in cell type A but not cell type B, transgene expression will take place only in cell type B. The transgene mRNA will be degraded in type A alone (Fig. 3.3). This system is called miRNA tagging. However, this is a saturable system. High mRNA levels can saturate RISC complexes, and the mRNA excess will be translated (although resulting in reduced expression levels).

The miRNA tagging technology was quickly applied to solve a major problem in lentivector gene therapy. Direct in vivo lentivector administration leads to rather efficient transgene-specific immune responses, and while this is a desirable characteristic to boost immunity [5, 107, 128–131], it is detrimental for gene therapy of genetic/metabolic disorders. Transgene-specific immune responses dramatically limit the therapeutic activity and survival of corrected cells [3, 99, 132]. To solve
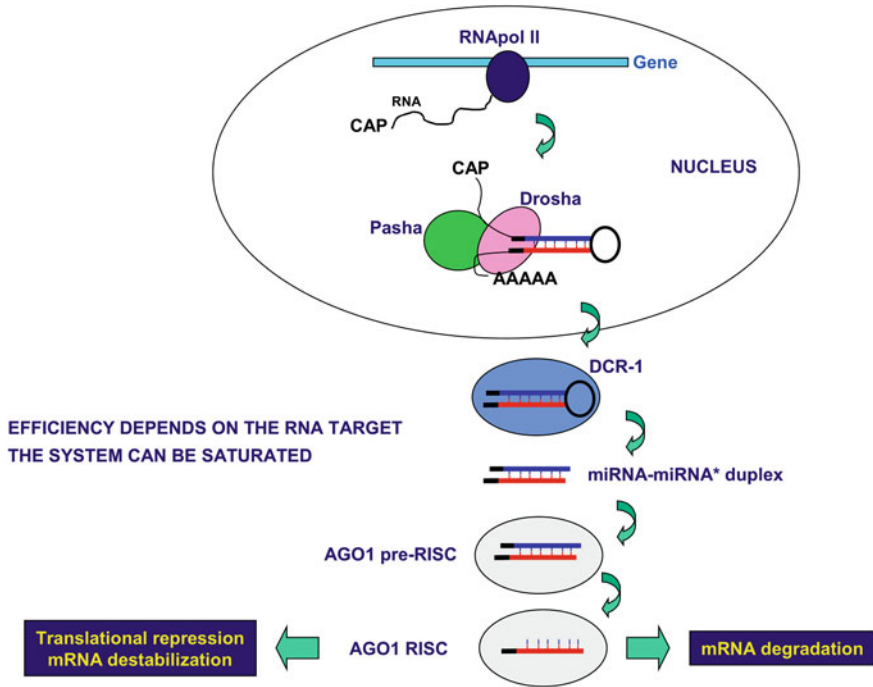
Fig. 3.2 Simplified mechanism of microRNA (mIR) pathways. A simple scheme for the production and function of miRNA-dependent control of gene expression is shown. In the cell nucleus (*upper part*), miRNAs are encoded in large capped RNA molecules transcribed by the cellular RNA polymerase II. These large precursors are recognized by a protein complex containing Drosha and Pasha that will remove the siRNA segment of the short RNA hairpin, which presents a specific stem-loop secondary structure. The shRNA is exported to the cytoplasm and it is bound by Dicer (DCR-1), which will degrade the shRNA leaving a miRNA–miRNA* duplex (miRNA* refers to the complementary sequence to the actual target sequence). This duplex is loaded in a protein complex containing Argonaute (AGO1 pre-RNA Interference Silencing Complex or pre-RISC), and the miRNA strand of the duplex is degraded. The AGO1 RISC contains the complementary strand to the target sequence, which is used to "scan" mRNA molecules exhibiting total and partial complementarity. When matching occurs, the mRNA is either degraded, destabilized or its translation is repressed

this problem, transgene expression was abrogated in cells of the hematopoietic lineage by including four copies of a sequence target for the hematopoietic-specific miRNA 142 3p, downstream of the transgene coding sequence [133]. This strategy ensured that the mRNA encoding the transgene would be degraded only in cells from the hematopoietic lineage, such as lymphocytes, granulocytes and more importantly, macrophages, and DCs. Consequently, intravenous administration of 142 3p-tagged lentivectors resulted in lack of transgene-specific immune responses and sustained, long-term transgene expression in hepatocytes [133]. Interestingly, this strategy resulted in transgene-specific tolerance, as shown by expansion of Foxp3+ regulatory CD4 T cells (Tregs) [134]. Curiously, detargeting antigen

miRNA-TAGGED INTEGRATED PROVIRUS
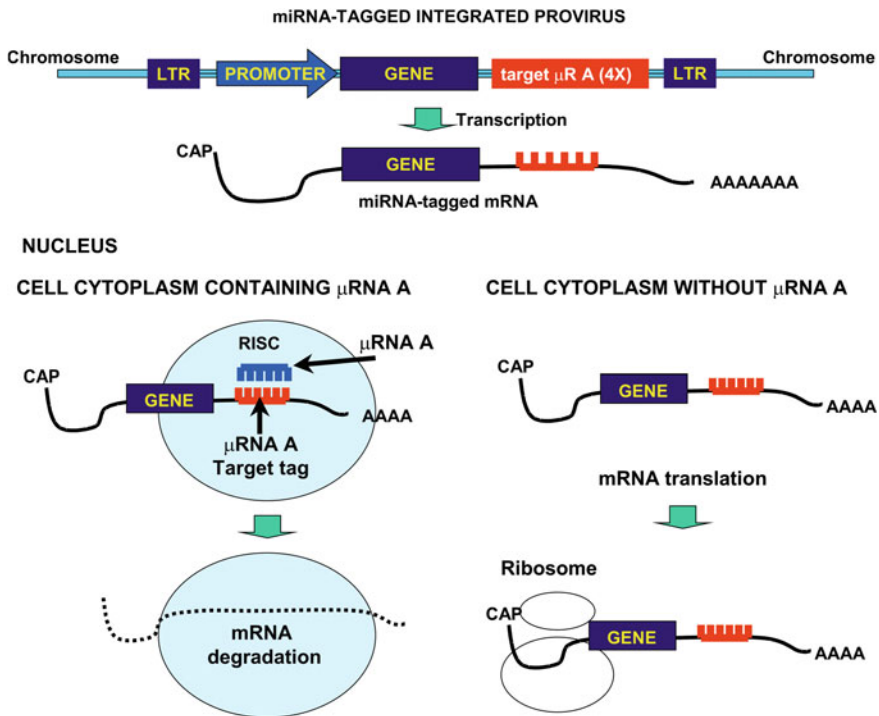
Fig. 3.3 Mechanism of action of miRNA tagging applied to lentivectors. The upper *panel* shows a simplified scheme of a chromosomal integrated lentivector in its "pro-virus" form. This lentivector contains four copies of the target sequence for an ideal miRNA (target μR A, shown as a *red caption*). After transcription, an mRNA is produced encoding the gene of interest followed by the miRNA target sequences. The mRNA is transported out of the nucleus to the cellular cytoplasm (*lower panels*). If the cell is expressing the miRNA A (*left panel*), an RNA silencing complex (RISC) containing the miRNA A (*blue comb*) will bind to its complementary target sequence present in the mRNA (*red comb*). This recognition will lead to disruption of gene translation from that particular mRNA, either by degradation (as shown) or inhibition of translation. If the cell does not express the miRNA A, gene expression will occur as normal by translation (*right panel*)

expression in APCs resulted in Treg expansion [134], when in other experimental settings antigen presentation plays a critical role for differentiation and expansion of antigen-specific Tregs [4, 5, 135, 136]. Interestingly, the same authors demonstrated by using the same miRNA-detargeting strategy that transgene expression in hepatocytes was required for immunological tolerance [134]. Detargeting transgene expression using miRNA 142 3p effectively allowed factor IX expression in liver without raising immune responses, leading to correction of hemophilia B in a mouse model [137].

Another application of miRNA tagging is transgene expression corresponding to specific differentiation or activation stages, by utilizing targets for miRNAs with

differentiation stage-dependent variable expression levels [138, 139]. For example, transgene expression can be achieved in only immature DCs, or a combination of different miRNA targets can achieve transgene expression in specific cell types within a given tissue [138]. Another example of targeting expression to cells at different differentiation stages is the introduction of the miRNA 126 target sequence. This particular miRNA is expressed in endothelial, and some epithelial cells, in addition to hematopoietic stem cells. This expression pattern was exploited to target expression of GALC to mature cells from the hematopoietic lineage for the correction of globoid cell leukodystrophy [9]. Curiously, GALC expression in hematopoietic stem cells and early progenitors is highly toxic. In contrast, it is therapeutic in mature cells from the hematopoietic lineage [9]. Therefore, to correct the disease, four copies of the miRNA 126 target sequence were placed downstream GALC gene. Consequently, GALC was only expressed in mature hematopoietic cells, leading to disease correction.

Finally, miRNA tagging can also be exploited to track differentiation pathways, utilizing to the expression pattern of reporter genes containing distinct miRNA target sequences [139].

3.4 Conclusions

The three main groups of lentivector targeting strategies have promising therapeutic applications. Surface targeting ensures the specific entry of the therapeutic vector to targeted cells, while the use of specific promoters can restrict transgene expression if transduction of nontarget cells occurs. Finally, miRNA tagging can add another level of control of transgene expression. In fact, the three strategies have been already applied for the treatment of hemophilia A. A baculovirus gp64-pseudotyped lentivector driving expression of factor VIII from the albumin promoter, in combination with miRNA tagging to avoid transgene expression in APCs, was applied in a mouse model of hemophilia A. Strikingly, in this particular case it was not sufficient to prevent factor VIII-specific immune responses even though liver-specific expression was achieved. Macrophage depletion before lentivector administration had to be performed to achieve therapeutic FVIII levels [40]. The results from this experiment are difficult to explain, as miRNA tagging alone was sufficient to correct hemophilia B without inducing FIX-specific immune responses [137]. This last case demonstrates that even though combining several targeting strategies to avoid transgene-specific immune responses looks promising, specific targeting of viral vectors to cells and tissues is still a challenge.

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