Chapter 8

Studying Autoimmunity by In Vivo RNA Interference

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Abstract

The occurrence of autoimmunity is strongly associated with multiple gene variants that predispose individuals to disease. The identification of the gene polymorphisms that modulate disease susceptibility is key to our understanding of disease etiology and pathogenesis. While genetic studies in humans have uncovered several associations and have provided possible candidate genes for further study, the use of animal models is indispensable for detailed functional studies. In order to facilitate the genetic manipulation of experimental models of autoimmunity, we employ lentiviral transgenesis in combination with RNA interference (RNAi). This approach bypasses the need for targeted mutagenesis of embryonic stem cells and/or backcrossing of genetically modified animals onto the relevant genetic background. Lentiviral RNAi offers several advantages compared to conventional transgenesis or knockout technology, and these, as well as the technique's weaknesses, are discussed herein.

Key words: Lentivirus, RNAi, autoimmunity, transgenesis, mouse, embryo.

1. Introduction

RNAi has found widespread use in many laboratories, and has rapidly become a method of choice for genetic manipulation of transformed cell lines as well as whole organisms such as *Caenorhabditis elegans* and even *Drosophila melanogaster*. Its use in mammalian organisms is still limited by one main hurdle: specific and long-lasting delivery of oligonucleotides to cells within a living animal. Just seven years ago, Baltimore and colleagues demonstrated that lentivirus could be employed to deliver a transgene to single-cell mouse embryos that subsequently developed into transgenic adult animals *(1)*. That same year, Brummelkamp et al. demonstrated that small interfering RNAs (siRNAs) could

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be expressed from a short-hairpin motif (shRNA) incorporated within a retroviral vector *(2)*. The following year, several groups had combined these two technologies to generate transgenic mice in which a particular gene was silenced by RNAi *(3, 4)*. Since then, lentiviral vectors have been refined and modified to accommodate the inducible expression of shRNA sequences within transgenic animals *(5, 6)*.

The study of autoimmunity, and particularly of type 1 diabetes (T1D), requires the use of very specific mouse strains. In the case of T1D, the best and most widely studied model is the nonobese diabetic (NOD) mouse. This mouse model closely resembles human T1D, and shares not only pathophysiological aspects, but also genetic susceptibility loci with human disease. The availability of the NOD mouse has allowed several disease-associated human genes to be confirmed and studied in more detail in the NOD model. However, the NOD mouse is notoriously refractory to genetic manipulation. Despite prolonged efforts, no NOD-derived embryonic stem cell line is yet available that would allow direct manipulation of the NOD genome by targeted mutation. Even conventional transgenesis by pronuclear injection is more challenging in this strain, such that the generation of mutant NOD mice often requires lengthy backcrossing of mutant animals from a different strain onto the NOD background. We have now used lentiviral transgenesis to silence genes directly in the NOD embryo *(7)*, thereby greatly reducing the time required to generate new experimental models and to test the involvement of candidate genes in the disease process.

2. Materials

2.1. Cloning of Oligonucleotides into the Lentiviral Vector

1. Synthetic oligonucleotides (ordered commercially).
2. Lentiviral vector (e.g. pLB, available from Addgene).
3. Restriction enzyme (for pLB: *Hpa*I, *Xho*I, *Xba*I).
4. T4 Polynucleotide kinase with T4 PNK buffer.
5. T4 ligase with ligase buffer.
6. Competent bacteria (e.g. Novablue (Novagen)).
7. LB medium, supplemented with 50 μg/ml ampicillin.
8. LB-agar plates, supplemented with 50 μg/ml ampicillin.

2.2. Generation of Lentivirus

1. DMEM supplemented with penicillin (100 Units/ml) and streptomycin (100 μg/ml), with and without 5 or 10% fetal bovine serum (FBS).
2. HEK 293 cells.
3. Transfection reagent (e.g. FuGene6 (Roche) or Polyfect (Qiagen)).

4. Lentiviral packaging plasmids (pMDLg/pRRE and pRSV-Rev, available from Addgene).
5. Envelope plasmid (pCMV-VSVg, available from Addgene).
6. Ultracentrifuge tubes (40 ml capacity).
7. Sterile PBS.

2.3. Embryo Generation, Manipulation and Injection

1. Embryo donor mice (females from strain of choice).
2. Male stud mice (from strain of choice).
3. Pregnant Mare Serum (PMS) gonadotropin (Sigma-Aldrich) and human Chorionic Gonadotropin (hCG) (Sigma-Aldrich).
4. M16 medium, M2 medium, mineral oil, hyaluronidase (all from Sigma-Aldrich).
5. Microinjection set-up.

2.4. Genotyping of Transgenic Animals

1. (Optional) Multi-LED Flashlight with blue filter (e.g. Lee Filters #47B Tricolor Blue) and goggles with yellow filter (e.g. Lee Filters #12).
2. Fluorescent microscope with appropriate filters (for GFP visualization).
3. Flow cytometer.
4. PBS supplemented with 5 mM EDTA.
5. Red blood cell lysis buffer (ACK: 0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.2–7.4).
6. PBS supplemented with 1% FBS.

3. Methods

The principle of the procedure consists in designing a lentiviral vector that contains an shRNA-encoding construct directed against the gene of interest. The lentiviral vector is packaged in VSVg-pseudotyped viral particles that are microinjected into the perivitelline space of single-cell embryos. The embryos are reimplanted within 24 h of injection, and develop into fully transgenic animals in which the gene of interest is silenced by RNAi.

3.1. Cloning of Oligonucleotides into the Lentiviral Vector

All procedures described below apply to cloning into the pLB vector. Using a different vector will require adaptation of the procedure to incorporate the relevant design and restriction sites.

1. Identify suitable 19-nt target sequences within the gene of interest, using one of the freely available algorithms.
2. The 19-nt target sequence is incorportated into an shRNA design *(3)* as follows:
   Forward strand: $T(N+)_{19}TTCAAGAGA(N-)_{19}TTTTTC$

Reverse strand: TCGAGAAAAA$(N+)_{19}$TCTCTTGAA $(N-)_{19}$A

where $(N+)_{19}$ denotes the target sequence and $(N-)_{19}$ denotes the antisense complementary sequence to the target sequence.

3. Order the forward and reverse strands as unmodified, desalted oligonucleotides at a small (10 or 25 nmol) scale. Resuspend oligonucleotides at 100 pmol/μl (*see* **Note 1**).
4. Phosphorylate 5 μl of each oligonucleotide in a total volume of 10 μl with T4 polynucleotide kinase in the appropriate buffer for 1 h at 37°C.
5. Combine the two phosphorylation reactions into one PCR tube (total 20 μl) and anneal the two strands by heating to 94°C for 2 min, followed by 10 min at 70°C, and cooling down to 4°C slowly (1°C/s). Dilute the annealed dsDNA in a total of 500 μl.
6. Digest 3 μg of the lentiviral vector (pLB) using *Hpa*I and *Xho*I. Dephosphorylate the vector (optional) and purify the digested vector (using a commercially available PCR-purification kit, for example) into 30 μl.
7. Set up a ligation with 5 μl digested vector together with 1 μl annealed oligonucleotide and 1 μl T4 ligase in 20 μl using the appropriate ligase buffer. Leave at room temperature for 2–3 h, and transform chemically competent bacteria with 0.5–1 μl of the ligation reaction.
8. Colonies are screened after selection on LB-agar/ampicillin plates and growth in LB/ampicillin medium by restriction digest of the purified plasmid DNA using *Xba*I and *Xho*I. Visualize the digest on a 1.5% agarose gel: the empty vector should yield a band at approximately 330 bp, while a vector with the correct insert should have a band at 385 bp. Positive colonies should then be verified by sequencing, as mutations in the shRNA-encoding sequence do occasionally occur during the cloning process.

3.2. Generation of Lentivirus

1. Prepare HEK 293 cells the day prior to transfection: plate out approximately 8–10 million cells in a 15-cm tissue culture plate or, alternatively, in a 175 cm^2 flask in 20 ml DMEM with 10% FBS.
2. The next day, prepare the transfection mixture as follows: 20 μg lentiviral vector, 4 μg pMDL-g/p RRE, 3 μg pRSV-Rev, 3 μg pCMV-VSVg, together with 60 μl transfection reagent in serum-free DMEM. Incubate at room temperature for at least 20 min, and pipette onto the cells (*see* **Note 2**).
3. The day following transfection, replace the medium with DMEM 5% FBS (*see* **Note 3**).

4. Collect culture supernatant at 48 h and store at 4°C, replacing it with 20 ml fresh medium (5% FBS).
5. Collect culture supernatant at 72 h, and combine with the 48 h supernatant.
6. Spin the combined supernatants at 2500 rpm for 10 min to pellet cell debris. Filter the supernatant through a 0.45-μm sterile filter and ultracentrifuge at 25,000 rpm for 90 min.
7. Carefully remove the supernatant by aspiration. Add 100 μl sterile PBS, taking care not to disrupt the pellet, if one is visible (*see* **Note 4**). Cover tubes with parafilm and leave undisturbed at 4°C overnight.
8. The following day, hold the ultracentrifuge tube at an angle and pipette the PBS onto the opposite tube wall, so it flows over the pellet. Repeat 10–20 times, then transfer the virus solution into 1.5-ml tubes (usually as 10–20 μl aliquots) and freeze at –80°C (*see* **Note 5**).
9. To titer the virus, prepare HEK 293 cells (400,000/well) in a six-well plate with DMEM/10% FBS. The following day, dilute 1 μl virus in 1 ml DMEM/10% FBS, and transfer 100 μl of this mixture into 1 ml of DMEM/10% FBS to generate a 1:10 dilution. Repeat the dilution to obtain a 1:100 dilution. Replace the medium in three wells of the previously prepared HEK 293 cells with the three virus dilutions and in one well with virus-free DMEM/10% FBS. Assuming that approximately 800,000 cells were present (doubling of the initial cell number) at the time of infection, the proportion of infected cells can be measured 48 h post-infection by flow cytometry, and the virus titer extrapolated from the percentage infected (GFP-positive) cells (*see* **Note 6**).
10. The virus can be used to infect a variety of cells, and validation of the chosen shRNA sequences can be performed by measuring mRNA or protein level in an adequate cell type after infection (*see* **Note 7** for an alternative validation method).

3.3. Embryo Generation, Manipulation and Injection

1. Superovulate donor females of the chosen mouse strain by intraperitoneal injection of 5 I.U. PMS on day 2, and then with 5 I.U. hCG 47 h later, on day 0. The females are mated with stud males of the same strain immediately after hCG injection (*see* **Note 8**).
2. Collect embryos on day 1, shortly after fertilization: the oviduct is removed, placed into M2 medium supplemented with hyaluronidase (300 μg/ml), and embryos are freed by tearing of the oviduct. Hyaluronidase acts to free the embryos from surrounding cumulus cells.

3. Wash embryos in M2 medium, and transfer them into a drop of M16 medium to keep at 37°C in the incubator while preparing the injection needle.
4. Thaw a virus aliquot on ice, and spin at 2,000 rpm for 2 min to pellet any larger debris. Backload the microinjection needle with 3 μl of virus (*see* **Note 9**).
5. Transfer embryos into a drop of M2 medium overlayed with mineral oil for injection. Each embryo is injected with sufficient virus solution to achieve a visible expansion of the zona pellucida. Take care not to damage the actual zygote, as the virus is injected between the zona pellucida and the zygote, in the perivitelline space.
6. Transfer injected embryos into M16 medium, place in incubator at 37°C. Reimplant embryos either the same or following day into the oviduct of pseudopregnant recipients (*see* **Note 10**).

3.4. Genotyping of Transgenic Animals

Once potentially transgenic animals are born (after the usual 20–21 days gestation), three simple methods can be used to test animals for expression of the GFP marker gene.

1. The earliest possible genotyping can be performed using a flashlight and goggles with appropriate filters (see materials for filter details, and **Note 11** for further information on building your own set of flashlight and goggles). This method can be applied from the first day post-partum, and is particularly effective in albino or agouti mice with light skin. The animals are illuminated with the filtered light and observed through the filter-goggles within their cage.
2. Alternatively, a tissue biopsy (e.g. tail-tip) can be obtained and observed under a fluorescent microscope equipped with appropriate filters (*see* **Note 12**).
3. For quantitation of cells that express the marker gene, a blood sample can be collected for analysis by flow cytometry. For this purpose, a small amount of blood (50–100 μl) is collected in a microfuge tube filled with 200 μl PBS/EDTA buffer to prevent clotting. The red blood cells are lysed with ACK buffer (2–3 ml for 3 min), and then washed twice with PBS/1% FBS. Cells are analysed by flow cytometry to quantify the proportion of GFP-positive cells. Additionally, specific cell-surface markers can be labelled with the relevant fluorescent antibodies to distinguish individual cell populations, if required (*see* **Note 13**).

3.5. Summary of Advantages of Lentiviral RNAi in Mice

1. Using lentiviral transgenesis bypasses the limitations imposed by the availability of embryonic stem cells from a small number of mouse strains, or even species. Lentiviral transgenesis combined with RNAi allows the downregulation of genes directly in many specialized mouse models.

2. RNAi in itself has the advantage of allowing silencing of individual splice variants by targeting unique exon boundaries, for example. This was previously not possible in many cases using conventional knockout technology. In addition, RNAi can be used to downregulate, rather than completely eliminate, a particular gene product to mimic physiological polymorphisms encountered in human disease-associated genes.
3. The use of RNAi also enables simpler tissue-specific downregulation of genes, using appropriate promoters for the expression of shRNA constructs *(8)*.
4. Lentiviral embryo injection is technically easier and more efficient than the targeting of embryonic stem cells and their injection into blastocysts or pronuclear injection of DNA.

3.6. Summary of Disadvantages of Lentiviral RNAi in Mice

1. Expression of lentiviral transgenes is very often variegated, necessitating careful analysis of founders.
2. Lentiviral integration in the embryonic genome is random, and can result in multiple integrants spread throughout the genome. These are segregated during breeding, sometimes leading to changes in expression between founder and offsprings.
3. Downregulation of gene expression by RNAi is seldom complete. Conventional knockout technology is therefore still a more reliable approach in most instances.

RNAi in vivo by lentiviral transgenesis has opened new possibilities and should facilitate the study of specialized disease models such as the NOD model of T1D. But this method is unlikely to entirely replace conventional pronuclear transgenesis or knockout technology, as these approaches are, in many instances, still more reliable and appropriate. Nevertheless, lentiviral RNAi complements conventional methods and should, as it becomes more established, prove very useful in the genetic manipulation of mammalian disease models.

4. Notes

1. We initially ordered HPLC-purified oligonucleotides, but found desalted oligonucleotides to be of sufficient quality.
2. We have used both FuGene6 (Roche) and Polyfect (Qiagen) with success. At this large scale of transfection, we find that a twofold excess of transfection reagent over DNA amount is usually sufficient for very high transfection efficiency and production of high virus titers.
3. Replacing the medium with a lower percentage FBS improves the quality of the virus concentrate by reducing

debris and impurities that can be a major problem during microinjection.

4. The virus pellet itself is not visible, and if a light-brown pellet is visible, it is likely due to impurities and debris. As a general gauge of quality of the virus concentrate, the less you see, the cleaner the virus.
5. We usually flash-freeze virus aliquots in liquid nitrogen prior to transfer to –80°C, but it is debatable whether this is a necessary procedure to safeguard the virus against degradation during freezing.
6. This titration method yields an approximate measure of the minimum number of infectious particles present in the virus stock. We always aim to use virus preparations with no less than $2–3 \times 10^8$ infectious particles/ml for embryo injection. As a general guideline, good virus stock should infect >50% of the HEK 293 cells in the well containing 1 μl virus.
7. As an alternative to measuring endogenous gene expression in a cell line, we routinely use a luciferase reporter assay to validate shRNA sequences. The relevant cDNA (e.g. from the OpenBiosystems collection) is cloned into the 3′ UTR of the *Renilla* luciferase gene in the dual-luciferase reporter vector psiCheck2 (Promega), and knockdown efficiency is measured in a dual-luciferase assay after co-transfection of the lentiviral vector and reporter plasmid into HEK 293 cells.
8. While some laboratories use exclusively female donors aged 3–5 weeks, others use animals of 6–8 weeks. The success of the superovulation procedure is age-dependent as well as strain-dependent, and adjustments to hormone quantities and donor age have to be made on a case-by-case basis for optimal results.
9. The injection needles used for virus injection are considerably larger than needles used for pronuclear injection. The virus concentrate can be viscous and contain larger debris that will clog small openings, one of the main technical problems encountered during this procedure. At the same time, a larger opening inflicts more damage to the embryo, and it is a matter of experience to determine what size needle works best.
10. The typical survival rate of injected embryos, as measured by two-cell development the day following injection, is up to 90% for B6 embryos. However, survival is strain-dependent, and we found NOD embryos, for example, to be much less robust. We therefore reimplant NOD embryos on the day of injection, while B6 embryos can be reimplanted the following day.

11. While sets of flashlights and goggles equipped with appropriate filters for GFP visualization are available commercially, we find that "home-made" devices are entirely adequate and much cheaper. We use a 16-LED flashlight with blue LEDs, supplemented with a photographic filter to further narrow the excitation light wavelength range (the polyester filter is cut to size to fit the flashlight). We similarly use a yellow filter cut to fit onto laboratory safety goggles, through which we observe transgenic animals. This allows visualization of GFP in the skin of animals at ambient light, or for even better results, in a dark room.
12. The second method is particularly useful when animals are held off-site, such that tail-biopsies can be shipped between laboratories. The GFP is stable for several days, if not weeks, in the tissue sample, so these can be shipped at room temperature. The GFP can be visualized directly through an eppendorf tube wall, making it a very fast and easy procedure.
13. Depending on the integration site of the transgene, we have found widely variable expression levels. These can also change after breeding; for example, if the transgene is located in an imprinted locus or if it is present in multiple copies throughout the genome. Variegated expression is a very common occurrence in lentiviral transgenics, which makes it a less reliable method than conventional knockout technology or pronuclear transgenesis. However, the efficiency of transgenesis can be higher than 50% under optimal conditions, such that the number of positive founders compensates to some extent for the wide variability in transgene expression.

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