Chapter 8

Retroviral Transduction of T Cells and T Cell Precursors

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Abstract

Transduction of lymphoid progenitors with retroviral or lentiviral vectors is a powerful experimental strategy to tease out the role of a gene or pathway in T cell development via gain-of-function or loss-of-function strategies. Here we discuss different approaches to use this powerful technology, and present some protocols that we use to transduce murine HSCs, thymocytes, and lymphoid cell lines with these viral vectors.

Key words Retrovirus, Hematopoietic stem cell, Thymocytes, Bone-marrow chimera, Development

1 Introduction

Transduction of hematopoietic cells with retroviral vectors is an efficient way to manipulate gene expression during development of the immune system. Retroviral vectors are easy to manipulate in the laboratory and provide stable, long-term gene expression in the infected cells and their progeny because they stably integrate into the genome. Their major limitation is that they can only efficiently infect and integrate in cycling cells.

Most popular vectors are derived from the murine stem cell virus (MSCV) [1], a derivative of the Moloney murine leukemia virus (MoMLV) which can maintain long-term expression in infected cells and their progeny. While the genomes of these viruses are typically 7–12 kb in size and code for three structural genes, *gag*, *pol*, and *env*, the vectors consist only of the essential *cis*-acting elements of the viruses. *gag*, *pol*, and *env* are provided in *trans*, either in a stably transfected cell line (*see* ref. 2) or, as we will discuss in this review, cotransfected with the viral vector in independent plasmids. The separation of the structural elements in different plasmids increases the safety of the vectors, since the virus produced is replication defective, and provides space in the viral backbone to clone the gene of interest and/or markers to follow the infected cells.

The host range of a retrovirus is determined by its envelope glycoprotein (Env). The most widely used are the Env proteins of

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ecotropic MLVs. A major advantage (and a limitation) of ecotropic viruses is that they cannot infect human cells, and therefore can be used safely, with most inserts, under normal BSL2 laboratory conditions. For infection of human cells, the retrovirus can be packaged with an MLV amphotropic Env. Alternatively, Env proteins from a different virus can be used to package the retroviral vector. This process is called pseudotyping, and the most common heterologous Env used is the vesicular stomatitis virus glycoprotein G (VSV-G). Besides the extension in host range, VSV-G envelope protein forms very stable viral particles, which can be concentrated by ultracentrifugation [3]. The use of these modifications significantly increases the biosafety concerns (*see* **Note 1**).

An alternative to MSCV-based retroviruses is HIV-1- or FIV-based lentiviral vectors. Their major theoretical advantage over conventional MSCV-based retroviral vectors is their ability to transduce nondividing cells, which would make them better to infect HSCs. While this seems to be the case with human HSCs, the results with murine HSCs are not so clear [4–6] and it seems that murine HSCs need to be in G1 for an effective transduction to occur, and therefore they need to be stimulated in vitro with cytokines, as is the case for transduction with retroviruses. In our hands, lentiviral vectors have not been consistently superior to MSCV-based retroviral vectors for HSC transduction, and tend to be harder to manipulate in the laboratory.

One limitation of conventional retroviruses and lentiviruses in some experimental situations is that they express the transgene at high levels throughout development. If this early expression results in developmental phenotypes that predate one's stage of interest, the approach is not useful. There have been many different retroviral designs that try to overcome this limitation. These include tetracycline-inducible viruses [7], viruses engineered so that the transgene is turned on by Cre (either by inserting a stop sequence before the transgene [8, 9], or by inserting the floxed transgene in an antisense orientation that is irreversibly inverted [10]), or by the generation of self-inactivating viruses (SIN), where the endogenous LTR is disrupted upon integration, and the transgene is then expressed by a tissue-specific promoter (*see* refs. 11–14 for some examples relevant to T lymphocyte development). The SIN approach has been performed mostly with lentiviral backbones, because conventional retroviruses engineered this way suffered from very low titers. Recent modifications seem to have alleviated this problem [15, 16] (*see* **Note 2**).

For the study of T cell development, the cells that are normally targeted are hematopoietic stem cells, derived from either bone marrow or fetal liver, immature thymocytes, and even early DP thymocytes. In all these cases, retroviral infection provides a fast and efficient method to induce or inhibit (via shRNA or dominant negative approaches) the function of a gene of interest in vivo

(via bone marrow chimeras, or intrathymic injection of infected cells) or in vitro (via rFTOCs, or using the OP9Δ system [17]).

Infection of hematopoietic stem cells, and adoptive transfer into irradiated hosts, is a very powerful approach to rapidly test the function of a gene of interest in development, to perform in vivo structure-function analysis, or genetic rescue experiments. The main limitation of this approach is the ability to infect a sufficient number of long-term reconstituting HSCs (since a very small proportion of them are normally in cycle). Therefore most approaches involve enrichment of LT-HSCs, and treatments to make them enter cell cycle, so that they can be infected by the retroviral vector while not losing their functionality. One approach is to treat the mice with fluorouracil, which depletes fast-replicating cells in the bone marrow. This has two effects; it increases the percentage of LT-HSC in the marrow, and promotes cell cycle entry, as these cells try to replenish the multipotent progenitors [18, 19]. Then cells are cultured in media with cytokines and infected (*see* **Note 3**). Another approach is to enrich for HSCs in vitro, using magnetic depletion with commercially available lineage marker cocktails (*see* **Note 4**), and then stimulate them with the same cytokine cocktail in vitro before infection. Below we detail our current protocols to infect HSCs and thymocytes.

2 Materials

2.1 Production of Retrovirus: Transient Transfection

1. 1.5 ml microfuge tubes.
2. 5 and 14 ml polypropylene tubes.
3. *293T growth media*: Dulbecco's modified Eagle's medium (DMEM), 10 % fetal bovine serum, 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin.
4. *HEK 293T cells* (ATCC, CRL-11268) (*see* **Note 5**).
5. *2× HBS*: 50 mM Hepes, 10 mM KCl, 12 mM dextrose, 1.5 mM NaH_2PO_4, 280 mM NaCl, pH 7.05 ± 0.05.

   Prepare three or four independent 100 ml batches, filter sterilize with a 0.2 μm filter, and aliquot in 10 ml/15 ml conical tubes. Test the four batches with viral vector and packaging stocks that have worked well previously, and discard those 2× HBS batches that do not work well (*see* **Note 6**).
6. *2 M CaCl2*: Prepare a 2 M solution (29.4 g in 100 ml), filter through a 0.2 μm filter, and store aliquots at –20 °C.
7. *Packaging plasmid(s)* containing retroviral gag, pol, and env genes: We routinely use pCL-Eco [20] for retroviral constructs (e.g., Addgene #12371). Alternatively, gag/pol, and another envelope such as VSV-G (e.g., Addgene #8454 or #12259), can be used. For lentivirus we use psPAX2 (e.g., Addgene #12260) to provide the missing structural elements.

8. *Vector plasmid*: There are many different vectors that can be used. As discussed above, for lymphoid cells, and long-term stable expression, backbones derived from MSCV are preferred. We use MIGR1, originally described in [21] for overexpression, and pBanshee [22] for shRNA expression [23]. For lentivirus, we normally use FUGW [13] or derivatives.

Prepare plasmid stocks using standard molecular biology techniques (*see* **Note 7**), and determine plasmid DNA concentration. Our laboratory routinely uses PEG purification [24], but other methods, or commercial kits, also produce good DNA.

2.2 Retroviral Transduction

1. *Bone marrow growth media* (BMGM): RPMI, 10 % fetal bovine serum, 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 ng/ml IL-3, 10 ng/ml IL-6, 50 ng/ml SCF (*see* **Note 8**).
2. 2 % BSA in PBS (sterile).
3. *Retronectin-coated plates*: Retronectin (*see* **Note 9**). 1 mg/ml in sterile PBS.

   Non-tissue culture-treated plates (*see* **Note 10**). Dilute retronectin stock to 12 μg/ml and add 250 μl/well for 24-well plates, or 1 ml/well for 6-well plates. Incubate for 2 h at room temperature, or wrap the plates and incubate overnight at 4 °C. Remove the retronectin solution and block the plates with 2 % BSA (in sterile PBS) for 30 min at room temperature. Wash twice with PBS. Plates can be stored with PBS at 4 °C.
4. Polybrene.
5. Lipofectamine.

3 Procedures

3.1 Transient Transfection

1. Twenty-four hours before transfection plate $0.4–0.8 \times 10^6$ exponentially growing 293T cells per 6 cm Petri dish in a 4 ml volume of 293T growth media; or $1–2 \times 10^6$ cells per 10 cm dish in 8 ml (*see* **Note 11**).
2. On the day of the transfection: have 2× HBS, 2 M $CaCl_2$ solution, and sterile water at room temperature. Use polypropylene tubes (microfuge, 4 or 15 ml):

   For 10 cm dish:

   (A) Microfuge tube: In a final volume of 500 μl H_2O, mix, 7 μg retroviral construct (*see* **Note 12**), 7 μg pCL/Eco (*see* **Note 13**), and 62 μl 2 M $CaCl_2$.

   (B) 4 or 15 ml tube: 500 μl 2× HBS.

   For 6 cm dish:

   (A) Microfuge tube: In a final volume of 300 μl H_2O, mix 4 μg retroviral construct, 4 μg pCL/Eco, and 37 μl 2 M $CaCl_2$.

(B) 4 or 15 ml tube: 300 µl 2× HBS.

Take the Petri dish with the 293T cells to be transfected out of the incubator into the tissue culture hood. Take one plate out at a time. It is very important to keep the pH of the media buffered.

Mix (A) and add dropwise to (B) while vortexing (B). Alternatively, instead of vortexing (B), blow air into (B) using a Pasteur pipette while adding (A) dropwise. Keep vortexing or blowing air into (B) for 30 s or so. Immediately add the mix to the 293Ts distributing it dropwise throughout the plate, and then swirl dish gently; do not eject liquid into dish because 293T cells will be stripped off. Put dish back into incubator. Transfect next dish.

3. Incubate cells with transfection mix for 16–20 h, after which the plates are rinsed once with PBS or media and fed with 3 ml of fresh media. At this stage the cells are slightly detached and rinses should be done gently to avoid stripping them off.
4. Supernatant is collected 24 and 48 h after the rinse. Filter through a 0.45 µm syringe filter to remove cells. It is ideal to prepare fresh supernatant for every experiment, but we routinely snap freeze the supernatant in aliquots (keep at least at −70 ° C). The freezing causes a 50 % reduction in viral titer.

   GFP expression can be seen 16 h after transfection and is maximal at 24–48 h. Check transfection efficiency of the 293T cells by flow cytometry. If the transfection efficiency is not high (>60 %), you can assume that the virus titer will probably not be good (*see* **Note 14**).

3.2 Transduction

In general, the method involves plating cells in 24-well plates with 2 ml of fresh warm viral supernatant and 4 µg/ml lipofectamine or 5 µg/ml polybrene, centrifuging cells at 20 °C for 1–1.5 h at 460×*g*, culturing cells for 1 h at 37 °C, and then replacing the viral supernatant with media (*see* **Note 15**); additional details are provided below. These approaches yield good efficiency of infection when coupled with a good virus, e.g., >90 % with lymphoid lines, up to 60 % with fetal liver and early thymocytes, 5–20 % with DP thymocytes, and 15–30 % with enriched bone marrow cells.

3.2.1 Retroviral Transduction Hematopoietic Cells

1. Day 0: Cells are plated in BMGM, and cultured for 24–36 h at 2×10^6 cells/ml (the yield is approximately $2–5 \times 10^6$ cells per 5-FU treated mouse).
2. Day 1: Use freshly produced virus, or thaw on ice an aliquot of previously frozen virus. Calculate how much virus you will need, depending on how many cells are going to be infected. For a 6-well plate we use 2 ml viral supernatant and 2.5×10^6 cells/well. For a 24-well plate, we use 0.5 ml virus and 0.5×10^6 cells/well.

3. Add virus to previously prepared retronectin-coated plates. Incubate at 37 °C for 2 h or centrifuge at 2000 × *g* for 2 h at 22 °C and then wash with PBS (*see* **Note 16**).
4. Add the cells in BMGM and centrifuge at 2000 × *g* for 5 min, to facilitate adhesion to the fibronectin. Incubate at 37 °C overnight.
5. Day 2: Collect the cells (*see* **Note 17**). Wash, resuspend in fresh BMGM, either culture for 48 h or repeat the infection on fresh virus-loaded retronectin plates (*see* **Note 18**), and then culture for 24–48 h.
6. Day 3: If your virus has a fluorescent marker, infection efficiency can be checked by flow cytometry.
7. Day 4: Adoptively transfer the infected cells into irradiated hosts following standard bone marrow chimera protocols (*see* **Note 19**).
8. Analyze the bone marrow chimeras 6–12 weeks after transfer for your phenotype.

3.2.2 Retroviral Transduction of Lymphoid Cell Lines

1. Resuspend cells from a culture in exponential growth at 5×10^6/ml of fresh media. Mix 100 μl of the cell suspension with 2 ml of fresh viral S/N, and polybrene at 5 μg/ml, and dispense in one well, in 24-well plates.
2. Centrifuge plate for 1 h at RT, 450 × *g*.
3. Incubate cells for 1 h in the incubator (37 °C 5 % CO_2). Replace 1.5 ml of media in each well with fresh media. Afterwards, cells are fed and split as necessary.

3.2.3 Retroviral Transduction of Primary Thymocytes

1. Plate $1.5–2 \times 10^6$ cells/well in 24-well plates, in 2 ml of retroviral supernatant, plus Lipofectamine at 4 μg/ml. Spin for 1–1.5 h, at 450 × *g*, at RT (*see* **Note 20**).
2. Wash cells by aspirating most of the supernatant without disturbing the layer of cells and replace with fresh media.
3. Expression of the transgene (at least when monitored by GFP expression can be detected after 18–24 h). The thymocytes can be now used in your model (rFTOC, OP9Δ differentiation, etc.) (*see* **Note 21**).

4 Notes

1. Using oncogenic inserts increases the biosafety requirements. In any case, approval from the Institutional Biosafety Committee is required for any work with these vectors, and different institutions have slightly different requirements.
2. When considering all these alternatives it is important to keep in mind that retroviral and lentiviral vectors have a limited cargo

capacity, and that the bigger the final construct is, the lower the titer of the virus we will be able to produce [25]. Retroviruses bigger than 8 kb, or lentiviruses bigger than 10 kb, are very hard to work with. Keep this in mind when thinking about SIN lentiviruses with a tissue-specific promoter, your gene of interest, and IRES-GFP to follow infection. Also, systems that work well with one transgene may not work with other transgenes.

3. Mice are injected I.P. with 250 μg/g body weight of 5-fluorouracil dissolved in PBS (10 mg/ml), and the bone marrow cells are harvested 4–5 days later.
4. We use the BD Biosciences Mouse Hematopoietic Progenitor (Stem) Cell Enrichment Set and magnetic depletion, but there are other equally good commercial alternatives.
5. 293T cells are grown in a 37 °C degree incubator containing 5 % CO_2. The cells should not be allowed to become overconfluent, and should not be split more than 1:5. 293T cells easily detach from the tissue culture dishes after approximately 30 s of treatment with trypsin at room temperature (0.05 % trypsin/0.53 mM EDTA). It is important to freeze multiple (50–100) vials of the 293T cells after first receiving and expanding them. This will ensure a ready supply of backup vials to allow for uniform virus production over several years. Go back to an early freeze and expand again when running low on backup vials.
6. This is the most finicky reagent in the process. It normally takes two or three batches of 2× HBS before finding one that works well for transfections. The ability of the 2× HBS solution to produce working $CaPO_4$ precipitates deteriorates after 6 months to 1 year, even when the 2× HBS solution is stored at –20 °C. Prepare and test a fresh batch before the old one is finished or too old! Despite this, in our experience no commercial reagent works as well as a good homemade batch.
7. It is recommended to grow the viral backbone in RecA-negative strains, and at 30 °C, to minimize recombination.
8. Other cytokines may also be added to this cocktail such as Flt3 (50 ng/ml) and TPO (25 ng/ml).
9. Matrix proteins such as fibronectin mediate colocalization of target cells and vector [26]. The highest gene transfer is obtained with the recombinant protein CH-296 (RetroNectin).
10. This is important. Retronectin binds much better to non-tissue culture-treated plates [27].
11. Be careful with your 293T cells. Do not use cells that have been kept in culture for a long time, or your transfection efficiency will be reduced. It is extremely important that the cells are not overly clumped and are at the correct density. It is essential that the cells are extremely healthy prior to

plating. It is recommended to count the cells rather than estimating the split.

12. The amount of DNA may be increased up to certain limits, when it starts to be toxic. This is our default start amount.
13. We normally use pCL/eco as the packaging vector. This can be substituted by other combinations. Please note that if you pseudotype the retrovirus with VSV-G, as discussed, it is important to titer the amount of VSV-G env plasmid in the transfection. Too much results in toxicity, cell fusion, and death, and low virus titers.
14. For most applications that require infection of primary cells, it is important to obtain a high titer virus. As a general rule, the bigger the final vector, the lower the titer, but there are other factors that affect this process that are not well defined. If your viral backbone expresses GFP or another surface marker, virus titer is easy to estimate by infecting a receptive, exponentially growing cell line. In our laboratory we routinely use 16610D9, a murine thymoma cell line [28], to test our viral preps.
15. Most primary hematopoietic and lymphoid cells show reduced viability when cultured for long periods of time with retroviral supernatant and other infection reagents such as lipids or polybrene. Therefore, we use either spin infection or incubation on virus-loaded retronectin-coated plates as our preferred method for retroviral transduction because it minimizes the time cells are exposed to these reagents.
16. Alternatively, cells can be mixed with the viral supernatant, incubated in the retronectin plates for 4 h, and then washed and replated with fresh media. Some viral supernatants can affect HSC survival and function, so we prefer to preabsorb the virus to retronectin.
17. They will be weakly adherent, so forceful pipeting or gentle scraping with a rubber policeman is recommended.
18. The second infection increases the infection efficiency, but also prolongs the amount of time the cells spend in culture, which may decrease their ability to reconstitute irradiated hosts. We tend not to do a second infection, unless the virus we are using is not very good.
19. If the infection efficiency is very low, infected cells can be enriched by sorting, and then injected alone (or with rescue bone marrow if their numbers are low). This approach can salvage some experiments. We prefer to inject both infected and uninfected cells, because the uninfected cells provide a good internal control in each reconstituted animal.
20. Use freshly isolated cells and never put on ice for best results.

21. We have used this approach to infect DP thymocytes, and analyze the role of different signal transduction pathways on GATA-3 induction [29]. In this case it is useful to culture the infected thymocytes on a OP9Δ layer, because it seems to improve their viability. For these experiments we use them in experiments 24–36 h after infection.

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