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Expression of cDNAs in Human Natural Killer Cell Lines by Retroviral Transduction

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Abstract

Human NK-like cell lines are difficult to transfect using standard mammalian expression vectors and conventional transfection protocols, but they are susceptible to retroviral transduction as a means to introduce cDNAs. Our lab has exploited this technique to study a number of receptors in human NK cell lines. The method utilizes a bicistronic retroviral vector that co-expresses either drug resistance or enhanced green fluorescent protein (EGFP) in parallel with the gene of interest. After a single infection with recombinant retrovirus, transduced NK cells can be sorted for expression of EGFP or the transduced cell surface marker. Alternatively, cells expressing the transduced cDNAs can be selected for by treatment with neomycin, puromycin or hygromycin. Using this method, the sorted/selected cells uniformly express the gene of interest and the expression is stable for many weeks of culture.

Keywords

retroviral transduction; NK cell lines; EGFP

1. Introduction

A number of transformed human natural killer (NK)-like cell lines have been adapted to culture and provide valuable models for studying NK cell function and signal transduction. Most of these cell lines lack expression of many normal NK cell surface receptors, especially killer cell Ig-like receptors (KIR), CD94/NKG2 heterodimers, and CD16. Therefore, it is very attractive to express these receptors in the transformed NK cell lines to examine molecular functions. Unfortunately, the available NK cell lines are highly resistant to transfection with standard mammalian expression vectors.

In the late 1990's, several groups successfully expressed cDNAs in NK-like cell lines by retroviral transduction. Amphotropic retroviral transduction was first successfully used to introduce the IL-2 cDNA into the human NK-92 cell line (1). Cohen et al. subsequently introduced the ecotropic receptor into the human YTS cell line, which permitted susceptibility to transduction with mouse ecotropic retrovirus ((2) and further described in the chapter by Mandelboim in this volume). Amphotropic retroviruses can infect most mammalian cells, including human, and can therefore be biohazardous to laboratory personnel. On the other hand, ecotropic virus can only infect mouse and rat cells, and hence, working with ecotropic-sensitive YTS cells has the advantage of avoiding some biosafety issues. Nonetheless, many versions of replication-incompetent amphotropic retrovirus have been engineered, and these strains are not particularly dangerous if carefully handled under BSL2 biosafety conditions,

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which are achievable in most modern biology laboratories (*see* Note 1). Our lab has exploited amphotropic retroviral transduction to introduce a number of cDNAs into a variety of NK-like cell lines, including NK-92, NKL, NK3.3, and KHYG-1 ((3⁷) and our optimized transduction protocol is detailed in this chapter (*see* Note 2).

Retroviral vectors derived from Moloney murine leukemia virus (MMLV) are the most widely used and allow the delivery of genes to dividing mammalian cells. The expression of a cloned gene of interest is strongly promoted by the flanking long-terminal repeat (LTR) elements within these vectors, and the vectors integrate into the cell's chromosomes, thereby establishing long-term, stable protein expression after a single transduction procedure. Retroviral infection generates a polyclonal transduced population, since the distinct random chromosomal integration events occur in multiple clones. The polyclonal nature of the transduced population thereby dilutes potential bias that may be introduced by influences an integrated vector's promoter on genes adjacent to the integration site when studying monoclonal transfected populations.

To allow purification of transduced cells that express the gene of interest, retroviral vectors may also encode selectable markers, such as neomycin-, puromycin- or hygromycin-resistance genes or a fluorescent marker, especially enhanced green fluorescence protein (EGFP). Standard mammalian expression vectors contain independent transcription units for the selectable marker and the gene of interest. A number of bicistronic retroviral vectors have been developed, however, that contain an internal ribosome entry site (IRES), which allows both the marker and the gene of interest to be expressed independently from a single transcript.

Our retroviral transduction of NK cell lines has utilized a system developed and made readily available by Dr. Garry Nolan (Stanford University, Stanford, CA). Detailed information about this system can be found at: www.stanford.edu/group/nolan/. This system utilizes the retroviral vector pBMN-IRES-EGFP, which co-expresses EGFP and the Phoenix-amphotropic packaging cell line. The Phoenix packaging lines encode three major retroviral elements: 1) pol, which functions as a reverse transcriptase, RNase H, and integrase, 2) gag, which is a large protein that is processed into viral matrix and core structures, and 3) the envelope (env) protein, which exists in the lipid layer and determines viral tropism. When Phoenix cells are transfected with the pBMN plasmid, these elements package a replication-incompetent retrovirus that is secreted into the culture medium and used to infect the NK cell lines.

2. Materials

1. Phoenix-ampho retroviral packaging cell line: The cell line can be obtained from Dr. Garry Nolan. It is based on the 293T cell line, a human embryonic kidney fibroblast that is transformed with adenovirus E1a and carries a temperature sensitive T antigen co-selected with neomycin. The Phoenix-ampho cell line was created by introducing genes producing gag-pol, and env for infection of most mammalian cells, including human. Gag-pol and envelope use different promoters to minimize their inter-recombination potential (*see* Note 3).
2. NK-like cell lines: NK-92 (ATCC #CRL-2407), NKL (obtained from Dr. Marco Colonna, Washington University, St. Louis, MO), NK3.3 (obtained from Dr. Jacki

¹Importantly, constructs encoding potential oncogenes should be avoided when working with retroviral, adenoviral, or lentiviral expression systems.

²This is a general protocol for transducing human NK cell lines, but can be applied with little variation to other cell lines.

³An important feature of the Phoenix cell lines is high transfection efficiency using conventional transfection methods (e.g. including calcium phosphate or lipid-based techniques). In our hands, approximately 60–90% of Phoenix-ampho cells can be transiently transfected with Lipofectamine reagents, depending on the construct introduced.

Kornbluth, St. Louis University School of Medicine, St. Louis, MO), KHYG-1 (obtained from Health Science Research Resources Bank, Japan Health Sciences Foundation, Osaka, Japan, #JCRB0156)

3. pBMN-IRES-EGFP retroviral vector (also obtained from Dr. Garry Nolan) with cDNA of interest sub-cloned into appropriate restriction sites.
4. Stbl2 bacteria (Invitrogen) (*see* Note 4).
5. Complete RPMI medium: RPMI 1640 medium (Life Technologies, Rockville, MD) containing 10% heat inactivated fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT), 2 mM L-glutamine, 100 µg/ml penicillin, 100 µg/ml streptomycin, 10 mM HEPES (pH 7.4), 1 mM sodium pyruvate (all supplements from Life Technologies or Mediatech, Manassas, VA), and 50 µM 2-mercaptoethanol.
6. Folic acid stock solution (2 mM): Mix 0.221g folic acid (Gibco) in 250 ml ddH₂O. Autoclave, wrap in foil, and store at 4°C. The final preparation will be a suspension and needs to be warmed to 70°C for 10 minutes prior to addition to the complete α-MEM preparation.
7. Myo-inositol stock solution (20mM): Mix 0.901g myo-inositol (Sigma-Aldrich, St. Louis, MO) in 250 ml ddH₂O, sterile filter, and store at 4°C.
8. Complete α-MEM: α-minimal essential medium (MEM; Life Technologies, Rockville, MD) containing 10% heat inactivated FBS (HyClone Laboratories, Logan, UT), 10% heat inactivated horse serum (Life Technologies), 2 mM L-glutamate, 100 µg/ml penicillin, 100 µg/ml streptomycin, 1 mM sodium pyruvate, 100 µM 2-mercaptoethanol, 2 mM folic acid (Sigma-Aldrich), 20 mM myoinositol (Sigma-Aldrich), and 100–500 U/ml recombinant human IL-2 (Roche).
9. OPTI-MEM reduced serum medium (Gibco/Invitrogen)
10. Recombinant human IL-2, available from a variety of commercial sources, such as Roche (Basel, Switzerland) that was generously provide by the National Cancer Institute Biological Resources Branch (Frederick, MD).
11. Freezing Medium: 94% heat inactivated FBS supplemented with 6% DMSO.
12. Cryogenic freezing vials (Nalgene)
13. NALGENE™ cryo freezing container (Nalgene)
14. Lipofectamine™ reagent and Plus™ reagent (Invitrogen)
15. Polybrene (1,5-dimethyl-1,5-diazaundecamethylene polymethobromide, hexadimethrine bromide; Sigma): Polybrene is dissolved at a stock concentration is 5 mg/ml in PBS, subsequently filtered through a 0.2 µm filter, and stored for several weeks at +4°C or long term at –20°C. The working concentration of polybrene is 10 µg/ml.
16. Plasticware: T25 and T75 culture flasks (Nunc, Denmark), 15 ml conical centrifuge tubes (BD Falcon, USA), sterile disposable 5 ml and 10 ml pipettes (FisherBrand).
17. Neomycin (Fisher Scientific) was dissolved in HEPES buffer, pH 7.2. at a concentration of 50 mg/ml and used to select transduced cells at a final concentration of 1.25 mg/ml.

⁴The Stbl2 bacterial cells are suitable for the cloning of unstable inserts such as LTR-containing retroviral sequences or direct repeats and for optimal performance, bacteria should be grown at 30°C.

18. Puromycin (EMD/Calbiochem, San Diego, CA) dissolved in DMSO at 5 mg/ml stock concentration and was used to select transduced cells at a concentration of 2.5 µg/ml.

3. Methods

3.1 Cell culture

3.1.1 Culture of Phoenix-ampho cells (see Note 5)

1. Phoenix cells are cultured in complete RPMI medium in 25 mm flasks (set horizontally) maintained at 37°C in humidified 7% CO₂ atmosphere.
2. When cells reach about 80% confluence, they should be split 1:10 to 1:20 every 3–4 days with fresh culture medium.

3.1.2 Culture of NK-like cell lines—Several different NK cell lines, including KHYG-1, NK-92, NK3.3 and NKL, can be cultured under the following conditions. Cultures should be replaced with freshly thawed stocks every 4–6 weeks to maintain biological uniformity that can drift upon long-term culture.

1. NK cell lines are cultured in 50 ml of fresh complete α -MEM medium in T75 flasks (standing vertically) at 37°C in a humidified 7% CO₂ atmosphere.
2. Cells are passaged between 1:5 to 1:10 into fresh medium with human IL-2 every 4 days. Optimal growth is achieved by seeding new cultures with 4 million cells/50 ml.

3.1.3 Freezing cell lines—To assure the optimal viability of cell lines, they should be harvested from log phase cultures prior to freezing. NK cell lines grow on suspension, whereas Phoenix cells are adherent and easily detach from the tissue culture flask by gentle shaking.

1. Collect cells in a centrifuge tube and centrifuge the cells at 500 ×g for 5 min.
2. Remove the medium, resuspend in fresh medium, and count the cells
3. Centrifuge again at 500 ×g for 5 min.
4. Remove supernatant and resuspend in freezing medium at 2–3×10⁶ cells per ml.
5. Transfer 1 ml to a 2 ml cryogenic freezing vial, put the vial in a NALGENETM cryo freezing container at room temperature, and transfer to –70°C overnight.
6. Transfer vials to liquid nitrogen on the following day for long-term storage.

3.1.4 Thawing cells

1. Remove vial from liquid nitrogen and thaw rapidly in a 37°C water bath.
2. Immediately after complete thaw, add 1 ml of warm culture medium (37°C) to the freezing vial and transfer this solution to 15 ml sterile conical screw cap tube containing 13 ml of warm culture medium.
3. Centrifuge tube at 500 ×g for 5 min
4. Remove the supernatant and resuspend the cell pellet by flicking the tube. Add warm culture medium and transfer to a culture flask. Expand the culture at 37°C in humidified 7% CO₂ atmosphere.

⁵The transfection efficiency of Phoenix-ampho retroviral packaging cells depends on their health and growth status, which must be maintained by regular passage. If the cells are 100% confluent, transfection is very inefficient, so never let the cells reach confluence.

3.2 Retroviral transduction of human NK-like cell lines (see Note 2)

3.2.1 Transfection of Phoenix-ampho cells—The first step of retroviral transduction is to clone your gene of interest into the pBMN-IRES-EGFP vector (*see* Note 4) and use this engineered vector to prepare recombinant retrovirus by transfecting into the packaging cell line, Phoenix-ampho. The Phoenix-ampho cell line should be maintained at less than 80% confluence, and cultures should be replaced with freshly thawed stocks every 6–8 weeks (*see* Note 3).

1. Plate $0.1\text{--}0.2 \times 10^6$ Phoenix-ampho cells/6 ml complete RPMI medium per well in a 6-well plate 24 hours prior to transfection. Phoenix cells should be about 70–80% confluent on the day of transfection (*see* Note 5).
1. In a 1.5 ml microfuge tube, mix at least 4 μg pBMN-IRES-EGFP vector containing cDNA of interest with 10 μL Plus Reagent, and bring total volume to 100 μL with the addition of pre-warmed reduced serum OPTI-MEM.
2. In a separate microfuge tube, add 8 μL Lipofectamine to 92 μL pre-warmed reduced serum OPTI-MEM.
3. Incubate both samples at RT for 15 min
4. After incubation, mix the contents of both tubes together for a total volume of 200 μL and incubate at RT for another 15 min
5. Wash Phoenix-amphotropic cells once by aspirating the culture medium and gently adding 6 ml pre-warmed reduced serum OPTI-MEM along the side of the well. Add the medium slowly, because Phoenix cells do not adhere tightly and the added medium should not detach the cells
6. After the DNA and transfection reagents have finished incubating, add 800 μL pre-warmed reduced serum OPTI-MEM for a total volume of 1 ml
7. Gently aspirate the wash medium from the culture well, and gently add the 1 ml transfection reaction slowly to the Phoenix cells by releasing along the side of the well with a pipette
8. Incubate the plate at 37°C in 7% CO_2 atmosphere for at least 3 hrs
9. After 3 hrs of incubation add 4 mL pre-warmed complete RPMI medium to the transfected well and incubate plate at 37°C , 7% CO_2 overnight
1. Remove medium by aspiration and wash cells once with 5 mL pre-warmed serum free OPTI-MEM.
2. Add 2 mL serum free OPTI-MEM and incubate at 37°C , 7% CO_2 for 24 hrs (add 1.3 mL OPTI-MEM if use 2 wells for single transfection).

3.2.2 Collection of Virus and transduction of NK cell line—This section describes the generation and manipulation of biohazardous retrovirus. Therefore, BSL2 biosafety procedures should be followed throughout the following steps. Upon finishing this section, incubate all disposed plasticware (including pipettes) under the UV light of a biohazard hood for at least one hour to destroy the retroviral contamination.

1. On day 4, the retroviral supernatant is ready for harvesting. Collect supernatant (containing virus) into a 15mL centrifuge tube.
2. Centrifuge the tube at $500 \times g$ for 5 min to remove any remaining cells or filtering through a $45 \mu\text{m}$ filter (*see* Note 6).

3. Transfer virus into a new 15mL centrifuge tube
4. Add 20 μ L Plus Reagent to virus suspension and incubate at RT for 15 min
5. After incubation add 8 μ L Lipofectamine to virus suspension and incubate at RT for another 15 min
6. During the virus/Plus/Lipofectamine incubation, wash NK cells once with OPTI-MEM by spinning at 500 $\times$ g for 5 minutes. Count the cells and transfer 0.5×10^6 cells to a 15 ml tube.
7. Resuspend NK cells to be transduced with 2 mL virus-containing supernatant and transfer to a single well of a 12-well plate. Centrifuge the plate at 700 $\times$ g for 30 min (*see* Note 7).
8. Incubate the plate at 37°C in a humidified 7% CO₂ atmosphere for at least 3 hrs
9. Centrifuge the plate again at 700 $\times$ g for 30 min, incubate again for 3 hours at 37°C, and then proceed to step 10 or 11.
10. Transfer cells from each well of the 12-well plate to a separate T25 flask, and add 8mL of fresh IL-2-containing α -MEM culture medium to each flask. Incubate the culture at 37°C in a humidified 7% CO₂ atmosphere until cells are confluent and proceed to step 12.
11. Alternatively after step 9, to maximize transduction efficiency, add 2 mL of fresh IL-2-containing α -MEM culture medium to the virus-infected NK cells in well(s) of the 12-well plate (total 4 ml of medium), and incubate overnight at 37°C in a humidified 7% CO₂ atmosphere. Then transfer cells from the plate to the T25 culture flask (most of the cells will detach from plastic, and can be easily transferred), and add another 6 ml of fresh complete α -MEM containing IL-2. Incubate the culture at 37°C in a humidified 7% CO₂ atmosphere until cells are confluent and proceed to step 12.
12. When cells are ready (~6 days after transduction, *see* Note 8) sort the transduced population for EGFP or for the expression of the transduced surface marker by flow cytometry (*see* Note 9). Alternatively, select transduced cells by treatment with antibiotics (if using a vector containing an antibiotic resistance gene; *see* Note 10). The fraction of transduced cells depends on the cell line used. The resulting cell population will retain expression for many weeks of culture.
13. Expand the transduced NK cell population and freeze several vials as described in section 3.1.3. Discard growing transduced NK cell populations after 4–6 weeks of culture and replace with a newly thawed aliquot of frozen stock (*see* Note 11).

⁶The retroviral supernatant can be used immediately for transduction of target cells or kept on ice if used within several hours. Otherwise, retroviral supernatant may be frozen at -80°C, resulting in a minimal loss of viral titer.

⁷Our usual centrifugation period is 30 min, but increasing the centrifugation time up to 90 minutes can increase transduction efficiency in some cell lines.

⁸Depending on growth rate, cells are generally sorted 6–8 days after transduction. Transduction efficiency can depend upon the number of cells infected, the construct used and the NK cell line to be transduced. Starting with a higher number of cells usually requires relatively shorter times to be ready for sorting.

⁹We have successfully used this transduction protocol to express cDNAs in the following human NK-like cell lines at the indicated efficiency of transduction: KHYG-1 (20–50%), NK-92 (5–15%), NKL (15–30%), and NK3.3 (5–15%). KHYG-1 cells are highly susceptible to retroviral transduction and can be successfully transduced by adding polybrene (10 μ g/ml) instead of using Lipofectamine reagents. To improve transduction efficiencies, more NK-92 or NK3.3 cells can be infected in steps 6–7 of section 3.2.2.

¹⁰Starting on day 2 after transduction with retrovirus containing an antibiotic-resistance gene, the transduced cells should be selected with antibiotics for 5 days.

¹¹For testing any biological effect in transduced cells, the results should always be compared in cells from separate transduction procedures using the same construct. This will assure that the impact is not unique to the cells derived from a specific transduced population.

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