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Introduction of shRNAs into human NK-like cell lines with retrovirus

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Abstract

Natural killer (NK) cell lines are difficult to transfect using standard techniques, which limits the ability to establish long-term knockdown of proteins with short hairpin (sh)RNAs. We have developed a method to stably knockdown protein expression in human NK-like lines by introducing shRNAs in retroviral vectors. After a single transduction with retrovirus, shRNA-containing cells can be selected with drug treatment or sorted for enhanced green fluorescent protein (EGFP) expression. With this method, protein expression can be stably decreased to less than 10% of wildtype levels.

Keywords

retroviral transduction; shRNAs; NK-like cell lines

1. Introduction

Primary NK cells are difficult to transfect with standard vectors under a variety of conditions proven successful in other cell types (2). These cells are also not generally amenable to viral or retroviral infection (1), although recent successes with lentivirus (see protocols by Kung and Savan/Young in this volume) are overcoming this technical hurdle. In contrast, NK-like cell lines are more permissive to gene transfer by transfection (3,4), and especially by retroviral transduction (1), thereby allowing for overexpression and knockdown of genes of interest.

The technique of RNA interference (RNAi) has revolutionized modern cell biology by enabling researchers to selectively eliminate the expression of specific mRNAs. To achieve short-term knockdown of the target mRNA expression (and subsequent protein expression), double-stranded short interfering (si)RNAs of 21–23 nucleotides (nt) matching specific sequences in the target mRNA are introduced into cells by electroporation or lipofection. Alternatively, short hairpin (sh)RNAs can be expressed in cells (see below) and processed by the Dicer endonuclease complex to generate sustained expression of double-stranded 21–23 nt siRNAs, achieving long-term, stable knockdown of target mRNAs (5). The siRNAs hybridize with target mRNAs to tag them for degradation by the RNAi-induced silencing complex (RISC), which contains an endoribonuclease (6).

The stable expression of shRNAs in cell lines can be achieved by transfection with certain vectors or transduction with retroviral or lentiviral vectors. Constructs cloned into these vectors

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¹⁶Determine the optimal concentration of drug needed to kill each NK-like cell line being used. For transduction of KHYG-1, NK-92 and NKL cells, puromycin was used at 2.5 µg/ml and G418 at 1.25 mg/ml.

generally consist of a 60 nt oligo that, when expressed in cells, is processed to generate a 19-nt siRNA with uridine overhangs (Figure 1). Expression of the shRNA construct is driven by the polymerase III HI promoter, which produces a small RNA transcript, lacking a poly-A tail, and can be processed into a standard siRNA molecule.

Here, we describe a method for knocking down proteins of interest in NK-like cell lines by the co-expression of two different retroviral vector-based shRNAs. This protocol can be modified to include co-expression of up to three distinct shRNAs simultaneously in the same NK cell line.

2. Materials

2.1 Generating shRNA containing retroviral vectors

1. Buffered saline: 100mM NaCl and 50mM HEPES, pH 7.4, in water
2. shRNA oligos: design an oligo targeting a specific sequence in the mRNA of interest. We have successfully designed shRNAs that knocked down the expression of several proteins using the online software provided by Oligoengine (www.oligoengine.com; Seattle, WA). The software scans an input mRNA sequence to predict the optimal target siRNAs. Upon choosing a particular target sequence, the software designs a double-stranded oligo consisting of a sense and an anti-sense strand with an intervening hairpin sequence. Each oligo also contains appropriate restriction site overhangs (BglII and HindIII) for cloning. These oligos can be purchased directly from Oligoengine.
3. pSuperior.retro.puromycin, -.neomycin and -.neomycin.gfp vectors (Oligoengine) (see Note 1)
4. Agarose (Invitrogen, Carlsbad, CA)
5. SV Gel and PCR Clean Up System (Promega, Madison, WI)
6. Restriction enzymes: BglII, HindIII, EcoRI and associated buffers (Invitrogen)
7. T4 ligase and 5x ligase buffer (Invitrogen)
8. Stbl2 competent bacterial cells (Invitrogen)
9. Liquid LB supplemented with 0.4% glucose and 50 µg/ml ampicillin
10. Wizard Plus SV Miniprep kit (Promega)
11. High purity plasmid purification system (Marlingen, Ijamsville, MD)

2.2 Generating retrovirus and transduction of NK-like cell lines

1. Phoenix-amphotropic retroviral packaging cell line (a gift from Dr. Garry Nolan, Stanford University, Stanford, CA)
2. Complete RPMI medium: RPMI-1640 medium (Mediatech, Herndon, VA) containing 10% heat inactivated fetal bovine serum (FBS; HyClone, Logan, UT), 2 mM L-glutamate, 100 I.U./ml penicillin (Mediatech), 100 µg/ml streptomycin (Mediatech), 50 mM Hepes and 50 µM 2-mercaptoethanol (ME)

¹Depending upon your selection scheme (selection with antibiotics versus GFP expression), it is important to choose the appropriate retroviral vector(s). With this system, one can express and select for up to three separate shRNAs at the same time (one shRNA in puromycin, neomycin and gfp vectors). We have had success using each shRNA-containing vector singly or all together.

3. Complete α -MEM: α -minimum essential medium (MEM; Life Technologies, Rockville, MD) containing 10% heat inactivated FBS, 10% heat inactivated horse serum (Invitrogen), 2 mM L-glutamate, 100 I.U./ml penicillin, 100 μ g/ml streptomycin, 1 mM sodium pyruvate (Sigma-Aldrich, St. Louis, MO), 20 mM myoinositol (Sigma-Aldrich), 2 mM folic acid (Sigma-Aldrich), 1X non-essential amino acids (Mediatech), and 100 μ M 2-ME
4. OPTI-MEM reduced serum medium (Invitrogen)
5. Recombinant IL-2, available from a variety of commercial sources, such as Roche (Basel, Switzerland) that was generously provide by the NCI Biological Resources Branch (Frederick, MD).
6. 6- and 12-well culture plates (Fisher Scientific)
7. Plus Reagent and Lipofectamine (Invitrogen)
8. 15 ml conical tubes (BD Falcon, USA)
9. Puromycin (Calbiochem, San Diego, CA) dissolved in DMSO at 5 mg/ml stock concentration
10. Neomycin dissolved in HEPES buffer, pH 7.2, at a stock concentration of 50 mg/ml (Fisher Scientific)

3. Methods

3.1 Designing shRNAs

There are numerous free programs available for designing shRNAs (Oligoengine, Dharmacon, etc). For this protocol, Oligoengine software was used, since the interface was user-friendly, GeneBank sequences could be uploaded directly and both the secondary structure and nucleotide usage were considered in the selection algorithm. We suggest designing a minimum of four shRNAs for each gene of interest, since we encountered an approximate 50% success rate. For knocking down SHP-2 phosphatase in the human NK-like cell line, KHYG-1, the expression of a single shRNA resulted in ~50% decrease in wildtype protein levels, while co-expression of two shRNAs decreased levels by >90% (7). Avoid designing shRNAs that target common domains within gene families (e.g. phosphatase domain), as these will be less specific for the gene of interest and could non-specifically suppress other members of the gene family. The final sequence should be tested for homology with other mRNAs with the BLAST program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>), and sequences with high homology to other known sequences should be abandoned. In addition, do not limit shRNAs to just one region of the mRNA. Finally, generate scrambled versions of each shRNA (having the same nucleotides as a shRNA targeting the gene of interest, in a randomly scrambled order) for use as controls. Scrambled control sequences should also not cross-react with known mRNAs in the targeted species, as assessed by a BLAST search. Alternatively, predesigned/pretested shRNAs of many genes cloned into retroviral vectors can be purchased from a commercial provider (e.g. Santa Cruz Biotechnology Inc, Santa Cruz, CA; Sigma-Aldrich, St. Louis, MO; Invitrogen, etc.).

3.2 Generating shRNA containing vectors

The procedure for generating shRNA constructs was adapted from the Oligoengine pSuperior protocol. The protocol can be adapted for other specific vector and restriction enzyme combinations.

1. To generate double stranded shRNAs, combine 1 μ l of each single stranded oligo (both sense and anti-sense strands with compatible restriction site overhangs at 3 mg/ml) in 48 μ l of Buffered Saline. Heat the oligo mix to 95°C for 10 min, and then slowly

cool to RT (see Note 2). Once RT is reached, shRNAs can be immediately used or stored at 4°C for future use.

2. Digest pSuperior.retro vectors with BglII and HindIII restriction enzymes for 1 hr at 37°C.
3. Separate the digested vector on a 1% agarose gel and purify with Promega Gel Extraction kit according to the manufacturer's instructions.
4. Ligate shRNAs (1 µl of 1:100 dilution) into the purified linearized vector at 14°C overnight (see Note 3 and Note 4).
5. Transform Stb12 recombination deficient competent cells (see Note 5) with 8 µl of the ligation reaction.
6. Plate the bacteria on LB culture plates containing 50 µg/ml ampicillin and grow at 30°C overnight.
7. Pick at least 6 colonies and culture each in 5 ml liquid LB with 0.4% glucose and 100 µg/ml ampicillin at 30°C overnight (see Note 6).
8. Isolate bacterial DNA with the Promega Miniprep kit, digest with EcoRI and HindIII for 1 hr at 37°C and separate digests on 1% agarose gels. Positive colonies are identified by the presence of a 300 bp band upon digestion (see Note 7).
9. Confirm the orientation of the shRNA insert by sequencing.

3.3 Transfection of Phoenix-ampho cells

It is important for both the Phoenix-amphotropic and NK-like cell lines to be freshly passed a day prior to transfection and transduction, respectively. This protocol works well for KHYG-1, NKL, NK3.3 and NK-92 cell lines (7).

DAY 0

- 1 One day prior to transfection, plate 5 mls Phoenix-ampho cells into a 6-well culture plate (9 mls of a confluent Phoenix culture + 21 ml of complete RPMI medium) and culture overnight in a 37°C incubator with 7% CO₂ atmosphere (see Note 8 and Note 9).

DAY 1

- 2 For each transfection reaction, combine 4 µg of each shRNA-containing plasmid, 10 µl Plus Reagent and OPTI-MEM to a final volume of 100 µl and incubate for 15 min at RT.

²It is easiest to use a heat block for this step, turning it off after the 95°C incubation and allowing the block to cool to RT.

³It is recommended to have a 1:3 ratio of vector:insert for the ligation reaction. Some PCR and gel extraction kit elution buffers disrupt accurate determinations of the DNA concentration by absorbance spectroscopy. To avoid this issue, measure the concentration of the insert and digested vector on an agarose gel using a DNA ladder for the concentration control.

⁴To decrease the number of false positive colonies, digest the ligation reaction with BglII for 1 hr at 37°C. The BglII site is destroyed upon successful cloning of the shRNA pair, therefore vectors containing the shRNA insert will not be cut.

⁵To prevent recombination at retroviral LTRs, always use recombination-defective bacteria (e.g. Stb12) when working with retroviral vectors, and always culture bacteria at 30°C.

⁶Grow colonies for longer times to compensate for the reduced growth temperature.

⁷False positives that stem from re-ligation of empty vector will have a ~1 kb band in the Oligoengine system.

⁸Phoenix cells should be ~80% confluent for optimal transfection. Never allow the cells to reach full confluence or transduction efficiency will suffer.

⁹Several varieties of the Phoenix packaging cells are available (e.g. amphotropic, ecotropic, polytropic), which differ primarily in the expression of the viral envelope proteins that mediate viral entry into target cells (8). Use Phoenix-amphotropic cells when generating virus for transduction of human cells, Phoenix-ecotropic for murine cells.

- 3 In a separate tube, combine 8 μ l Lipofectamine with 92 μ l OPTI-MEM for each reaction, and incubate for 15 min at RT.
- 4 After 15 min, combine the plasmid and Lipofectamine solutions, and incubate for 15 min more.
- 5 During the incubation, wash Phoenix-ampho cells once with 6 ml OPTI-MEM (see Note 10).
- 6 Add 800 μ l of OPTI-MEM to the plasmid/lipofectamine solution (total volume is now ~1 ml). Remove the wash solution from Phoenix-ampho cells and cover the cells with the plasmid/lipofectamine solution.
- 7 Transfect cells for at least 3 hrs at 37°C, and then cover with 4 ml complete RPMI medium and incubate overnight.

DAY 2

- 8 On the next day (afternoon), remove the medium, wash once with 6 ml OPTI-MEM and cover cells with 2 ml OPTI-MEM.

DAY 3

- 9 Collect the Phoenix cell supernatant containing the retrovirus and transfer into a 15 ml conical tube (see Note 11). Remove any cells by centrifugation at 500 xg for 3min. Transfer the viral supernatant to a new tube containing 20 μ l of Plus Reagent.
- 10 Incubate the viral supernatant for 15 min at RT.
- 11 After 15 min, add 8 μ l of Lipofectamine and incubate 15 min longer (see Note 12).
- 12 During incubation, prepare NK cells for transduction by washing once with OPTI-MEM.
- 13 Transfer 5x10⁵ NK cells to a 15 ml conical tube and pellet cells by centrifugation 500 xg to remove the medium.
- 14 Resuspend the NK cells in the viral supernatant (about 2mls), transfer to a 12-well cell culture plate and spin at 700 xg for 30 min at RT.
- 15 Incubate cells for at least 6 hrs at 37°C.
- 16 After incubation, cover cells with ~2 ml complete α -MEM with 50 U/ml recombinant IL-2 and incubate cells overnight at 37°C (see Note 13).

DAY 4

- 17 Remove supernatant, and continue to culture in fresh complete α -MEM with 50 U/ml recombinant IL-2 (see Note 14).

¹⁰Phoenix cells are semi-adherent and can easily detach from the plate. Exercise caution when manipulating the cells or when changing the medium. Add medium very slowly to the side of the 6-well plate, keeping the pipet tip horizontal to the plane of the cells. Never add medium directly on top of the cells.

¹¹Although the retrovirus is replication defective and should be non-infectious, be sure to UV-treat all contaminated glass and plasticware for at least 1 hr before discarding. The use of retroviral technology requires standard class BSL2 biohazard safety precautions and approval by the local biohazard safety committee in your institution.

¹²Retrovirus can be used immediately or can be stored at -80°C for up to 4 months with an estimated potency loss of ~50%. We have had success using virus that was frozen to co-transduce KHYG-1 cells with two vectors simultaneously when subsequently selected in medium containing the appropriate combination of antibiotics.

¹³To increase viability, let cells detach from the 12-well plate overnight instead of forcing cell detachment with pipeting.

¹⁴Transduction efficiency varies widely among known NK-like cells lines. For example, NK-92 cells had ~4% transduction efficiency with this protocol, while KHYG-1 had almost 30% efficiency for single vector transductions.

3.4 shRNA selection

Selection scheme will depend upon which vectors (puromycin, neomycin or gfp) were used.

DAY 6

- 1 After resting cells for 2 days following transduction, add fresh complete α -MEM with 50 U/ml recombinant IL-2, and/or 2.5 μ g/ml puromycin, 1.25 mg/ml neomycin (see Note 15–Note 17).

DAY 7

- 2 Remove the old medium and add fresh complete α -MEM with 50 U/ml recombinant IL-2, and/or 2.5 μ g/ml puromycin, 1.25 mg/ml neomycin (see Notes 18).

DAY 9

- 3 Remove the old medium and add fresh, drug-free complete α -MEM with 50 U/ml recombinant IL-2.
- 4 Check knockdown at the protein level by Western blotting or at the mRNA level by RT-PCR. For SHP-2 and SHP-1, protein knockdown was observed after 3 days of drug treatment (earliest timepoint analyzed), but knockdown did not become stable and consistent until 7 days after drug treatment was concluded.
- 5 Compare cellular protein or mRNA levels in cells transduced to express single shRNAs versus cells expressing the scrambled shRNAs or empty pSuperior vector.

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¹⁵To improve drug selection, expand cells to multiple wells if overgrown.

¹⁷To ensure proper drug selection, include non-transduced cells during selection.

¹⁸Puromycin-induced death was observed by days 1–2 of drug treatment and days 3–5 for neomycin.

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Figure 1.

Schematic of a 60-nt siRNA-generating oligo. The ds oligo is flanked by restriction sites for cloning at the 5' and 3' ends, a 19 nucleotide sense sequence of the siRNA, a 9 nucleotide hairpin spacer sequence followed by the complementary 19 nucleotide antisense sequence of the siRNA. This figure was reproduced with permission from Oligoengine.