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Intrabody Expression In Eukaryotic Cells

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i. Summary/Abstract

We describe procedures for intracellular expression of scFv in eukaryotic cells. Starting from a scFv gene cloned in a phage-display vector we describe the cloning step into a mammalian expression vector, the transient transfection of HeLa cell line and the monitoring of intrabody expression by immunofluorescence staining and FACS analysis.

ii. Key Words

Antibody fragment, scFv, intrabody, intracellular immunization, disulfide bond

1. Introduction

Single-chain Fv fragments (scFv) are 28 kDa immunoglobulin-derived molecules composed of the variable region of a heavy and a light chain, joined together by a flexible linker. ScFvs are expressed from a single recombinant gene and retain the specificity and the binding affinity of the parental antibodies.

Intracellular antibodies, or intrabodies, are scFvs that are ectopically expressed within the cell. Intrabodies are able to interact with their cognate antigen inside the cell. This interaction may result in the modulation or inhibition of the functions of the antigen, either by

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direct interference, or by diverting it from its normal intracellular location ***(1)***. In addition, by adding to the scFv a targeting signal, such as a nuclear localization signal (NLS) or a retention signal for the endoplasmic reticulum (ER), expression of the scFv can be restricted to a specific location within the cell.

One of the main problems associated with intrabodies is that most scFvs are not able to fold under the reducing conditions that pertain in the cell cytosol and nucleus. This is due to the limited stability of scFvs after the two conserved disulfide bonds are reduced. The main consequence is the formation of insoluble aggregates, rendering antibodies non-functional (**Fig. 1**). However, even when aggregated, some intrabodies may retain their binding affinity and can sequester the targeted antigen, preventing it to reach its physiological localization and therefore inhibiting its function ***(2-3)***. Even if the intrabody is expressed as soluble protein in the cell cytoplasm, its inhibitory potential may be limited by a low expression level and a limited half-life. This has promoted the development of stable frameworks ***(4)*** on which scFvs that fold correctly and in sufficient amount to be active as intracellular antibodies can be constructed.

In order to obtain efficient intrabodies, a panel of antibodies against an antigen must be first isolated. This step can be accomplished by different techniques, including phage display, ribosome display, yeast two-hybrid system, or even classical hybridoma technology. Then, the selected antibodies must be tested *in vivo* for their correct expression and ability to inhibit their target. Expressing scFvs that have been selected from an optimized library will greatly increase the success rate of this experiment ***(5-8)***.

In this chapter, we describe procedures for intracellular expression of scFv in eukaryotic cells. We also give details for the subcloning of a selected scFv sequence into a mammalian expression vector, the transient transfection of Hela cell line and the monitoring of intrabody expression by immunofluorescence staining and FACS analysis.

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2. Materials

1. pCMV/myc/cyto (Invitrogen) plasmid DNA (*see* **Note 1 & 2**).
2. Plasmid DNA of a selected scFv clone in pCANTAB6 or equivalent vector.
3. Restriction enzymes and buffers: *Nco*I, *Not*I.
4. T4 DNA ligase.
5. Competent *E.coli* cells.
6. Luria-Bertani (LB) liquid and solid media: 10 g tryptone (peptone), 5 g yeast extract, 10 g NaCl, make up to 1 liter with distilled water and adjust pH to 7.0 with 5M NaOH. For LB plates, add 15 g of agar. Autoclave. Allow the solution to cool to 60 °C or less before adding ampicillin at 100 µg/ml.
7. Dulbecco's modified eagles' medium (DMEM) supplemented with 10% fetal calf serum (FCS). Keep at 4 °C.
8. Trypsin/EDTA.
9. 150 mM Sodium Chloride (NaCl) solution. Sterilize by filtration through a 0.22 µm filter or by autoclaving.
10. jetPEI™ (Polyplus-transfection).
11. 12-mm-diameter glass coverslips. Store in 95% ethanol and air dry before use.
12. Phosphate-buffered saline (PBS): dissolve 8 g NaCl, 0.2 g KCl, 0.61 g Na_2HPO_4 and 0.2 g KH_2HPO_4 in 800 ml distilled water. Adjust volume to 1 liter with distilled water. Sterilize by autoclaving. The pH should be about 7.4 and does not need to be adjusted.
13. Cold absolute methanol. Store at -20 °C.
14. 1% Bovine serum albumin (BSA) in PBS.
15. 9E10 mouse anti-*c*-myc monoclonal antibody (mAb) (*see* **Note 14**).
16. Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG secondary antibody (Abcam #ab6785).

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17. Mowiol coverslip mounting solution: add 6 g glycerol and 2.4 g Mowiol 4-88 to 6 ml distilled water and leave at room temperature for 2 h. Add 12 ml 0.2 M Tris (pH 8.5) and incubate at ~55 °C until Mowiol has dissolved. Clarify by centrifugation at 5000 g for 15 min. Store in 1 ml aliquots at -20 °C.
18. FACS buffer: PBS- 0.1% BSA- 0.01% sodium azide (Na_2N_3). Store at 4 °C.
19. 3.7% formaldehyde diluted in FACS buffer.
20. 0.5% saponine diluted in FACS buffer.

3. Methods

The following protocol assumes that a scFv clone of interest has been first isolated from a library constructed in a pCANTAB or pHEN derived phagemid vector ***(8)***.

3.1. Subcloning of scFv into eukaryotic expression vector pCMV/myc/cyto (*see* Note 3)

1. The scFv sequence of interest is obtained by digesting the selected pCANTAB6 plasmid by *Nco*I and *Not*I enzymes as follows (*see* **Note 4**): 1 µg DNA, 5 µl 10x NEBuffer 3, 0.5 µl BSA 100x, 1 µl *Nco*I (10 U/µl), 1 µl *Not*I (10 U/µl), and H_2O to 50 µl. Incubate for 3 h at 37 °C.
2. Digest also 1 µg of recipient plasmid pCMV/*myc*/cyto with the same enzymes (*see* **Note 5**).
3. Purify the 750 bp scFv fragment and recipient plasmid on an agarose gel using a commercial DNA purification kit.
4. Set up the ligation reaction with a slight excess of insert (1:3 vector:insert molar ratio). Mix 100 ng digested and purified recipient plasmid, 40 ng digested and purified scFv gene, 2 µl 10x ligase buffer, 0.4 µl T4 DNA ligase (5 Weiss U/µl) and H_2O to 20 µl. Incubate 1h at room temperature (*see* **Note 6**).
5. Inactivate the T4 DNA ligase by heating for 10 min at 65 °C.

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6. Transform competent *E. coli* cells with 10 µl of the ligation reaction, plate on LB with 100 µg/ml ampicillin and incubate overnight at 37 °C.
7. Test individual colonies for the presence of the insert either by plasmid DNA preparation followed by digestion with *Nco*I and *Not*I, or by PCR using as template bacterial colonies. Primers used for the amplification are:

   pCMV-Forward: CGCAAATGGGCGGTAGGCGTG

   BGH-Reverse: TAGAAGGCACAGTCGAGG
8. Perform a large-scale plasmid DNA extraction of a positive clone (*see* **Note 7**).

3.2. Cell culture and Transient Transfection of Hela cells **(*see* Note 8)**

Human cervix carcinoma Hela cells are grown in DMEM supplemented with 10% heat inactivated FCS at 37 °C in a 5% CO_2 humidified atmosphere. Cells should be seeded 24 h before transfection.

1. Aspirate and discard culture medium and rinse the cells with Trypsin/EDTA.
2. Add enough trypsin/EDTA to cover the cell monolayer and incubate at 37 °C for about 4 minutes until cells become round and start to float.
3. Neutralize the trypsin with a two-fold excess of supplemented culture medium.
4. Centrifuge the cells at 1000 rpm and resuspend the pellet in fresh medium.
5. Count the cells and plate 4 x 10^5 cells/well in 6-well plates containing 12-mm-diameter glass coverslips (*see* **Note 9 & 10**).
6. The day of transfection, make sure that cells reached 50-60% confluency. Replace the culture medium with 2 ml fresh DMEM 10% FCS.
7. For each well, dilute in one tube, 3 µg of DNA into 50 µl of 150 mM NaCl, and in another tube, 6 µl jetPEI™ solution into 50 µl of 150 mM NaCl. Vortex the tubes. Add the 50 µl jetPEI™ solution to the 50 µl DNA solution, vortex, and incubate for 30 min at room temperature.

8. Add the 100 µl mixture dropwise in a well of the 6-well culture plate and homogenize by gently swirling the plate.
9. Incubate cells for 5 h at 37 °C, then replace the medium with 3 ml fresh culture medium.

3.3. Immunofluorescence Microscopy

1. Twenty-four to 48 hours post-transfection, recover the coverslips with a forceps and place each one, cells side up, in a well of a 12-well plate.
2. Wash the cells once with PBS.
3. Immerse coverslips in cold absolute methanol (about 1 ml), then incubate for 10 minutes at -20 °C (*see* **Note 11 & 12**).
4. Gradually rehydrate the cells by adding PBS in aliquot of about 500 µl (*see* **Note 13**). Finally wash cells once in PBS.
5. Cover the cells with 100 µl of PBS-1% BSA to minimize non-specific adsorption of the antibodies on the coverslips. Incubate 30 min at room temperature.
6. Remove the blocking solution by aspiration and incubate the cells with 100 µl of the 9E10 anti-*c*-myc primary antibody solution (1:10 dilution in PBS-1% BSA) for 1 h at room temperature (*see* **Note 14**).
7. Wash the cells 3 times with PBS during 5 min.
8. Incubate the cells with 100 µl of a FITC-labeled anti-mouse antibody solution (1:2500 in PBS-1% BSA) for 30 min at room temperature in the dark.
9. To visualize DNA, add cell-permeant Hoechst dye to the secondary antibody solution to a final concentration of 5 µg/ml. Incubate 10 minutes at room temperature in the dark.
10. Wash the cells 3 times with PBS during 5 min.

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11. Place 5 µl of Mowiol mounting medium on the surface of a clean glass microscope slide. With a forceps, invert the coverslip and place it cell-side-down onto the Mowiol medium. Leave the slide 1 h at room temperature before examining the cells under a fluorescent microscope.

3.4. FACS analysis of intrabody expression level

1. Harvest the transfected cells as described above. Count and transfer 5 x 10^5 cells per sample in a FACS tube. Pellet the cells by centrifugation, 5 min at 1000 rpm.
2. Discard supernatant and resuspend the pellet with 1 ml of PBS-0.1% BSA. Centrifuge the cells as before.
3. Resuspend gently the pellet in 200 µl PBS-0.1% BSA containing 3.7% formaldehyde. Incubate for 10 min at room temperature.
4. Wash cells with 1 ml PBS-0.1% BSA.
5. Resuspend the cells in 200 µl PBS-0.1% BSA containing 0.5% saponine. Incubate for 10 min at room temperature.
6. Wash the cells once and add 200 µl of the 9E10 antibody solution (1:10 diluted in PBS-0.1% BSA-0.5% saponine). Incubate for 30 min at 4 °C (*see* **Note 14**).
7. Wash cells twice with 1 ml PBS-0.1% BSA.
8. Add 200µl of the FITC-labeled anti-mouse antibody solution (1:2500 diluted in PBS-0.1% BSA-0.5% saponine). Incubate for 30 min at 4 °C in the dark.
9. Add 1 ml of PBS-0.1% BSA, centrifuge, resuspend cells in 0.5 ml FACS buffer, and analyze immediately or store at 4 °C in the dark.

4. Notes

1. The plasmid used here for scFv expression in eukaryotic cells is the 5.9 kb pCMV/*myc*/cyto vector from Invitrogen (#V82020), designed for cytoplasmic

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expression. It includes the CMV promoter for constitutive expression of the protein. The expressed scFv will be tagged at its C-terminus with the *c*-myc epitope recognized by mAb 9E10 (*see* **Note 14**). Alternative vector with the stronger EF-1α promoter is also available from Invitrogen (#V89020).

2. To target your recombinant scFv to a specific intracellular location in mammalian cells (nucleus, mitochondria, endoplasmic reticulum), vectors containing a targeting signal are also available from Invitrogen (for example pCMV/*myc*/nuc, pCMV/*myc*/mit, pCMV/*myc*/ER vectors).
3. The *Nco*I site contains the ATG initiation codon. If you wish to also include the tag, make sure to clone the *c*-myc epitope in frame at the *Not*I site. This is the case if your scFv is cloned from pCANTAB or pHEN vectors.
4. The scFv sequence can also be obtained by PCR using appropriate oligonucleotides. Design primers to introduce a *Nco*I site at the 5' end and a *Not*I site at the 3' end of the gene in the correct frame (*see* **Note 3**). Digest PCR product, and purify on silica-based columns.
5. Recircularization of partially digested plasmid can be avoided by dephosphorylation of the 5' ends with calf intestinal alkaline phosphatase (CIP). Add 0.5 U of CIP per µg of plasmid directly to the digestion reaction and incubate 1 h at 37 °C.
6. You can also incubate overnight at 16-20 °C.
7. It is necessary to streak at least once on a LB-Amp plate the positive clone before making the large plasmid preparation to minimize the risk of having two different plasmids. Then choose one colony for the large-scale DNA preparation. It is of good practice to sequence the insert to make sure that no mutation has appeared during the cloning procedure.
8. The protocol uses jetPEI™ as transfection reagent, but any transfection method can be used (electroporation, lipofectamine …).

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9. Cells can also be grown and transfected on glass chamber slides. Adapt the transfection conditions according to the manufacturer's instructions.
10. Hela cells are adherent. If you wish to perform immunofluorescence staining on non-adherent cells, coat coverslips with poly-L-lysine (0.01% solution) for 10 min at room temperature. Remove the poly-L-lysine solution and let coverslips air dry.
11. Do not use a pipette to distribute methanol to keep temperature as low as possible. Pour directly cold methanol from the bottle onto the cells and incubate immediately at -20 °C.
12. Methanol fixes the cells by dehydration. It precipitates proteins and permeabilizes the cells at the same time, so that soluble proteins from the cytoplasm may be extracted. Use therefore methanol fixation for nuclear and cytoskeletal protein detection. Another method is to fix the cells in formaldehyde which cross-links proteins and permits detection of soluble proteins. Incubate the cells for 20 min at room temperature in 3.7% formaldehyde in PBS, then permeabilize for 5 min at room temperature with 0.2% Triton X-100 in PBS.
13. Add 500 µl of PBS, mix, then remove 500 µl before adding a new 500 µl aliquot of PBS. Repeat 3 times (final Methanol will be about 30%). Alternatively, aspirate methanol and let coverslips air dry. You can store coverslips at -20 °C for months or immediately rehydrate cells by incubating in PBS for 5 min.
14. 9E10 hybridoma is available from ATCC (#CRL-1729) and ECACC (#85102202). If using culture supernatant from hybridoma cell line 9E10, the suitable dilution must be first determined by testing 1:10, 1:20 and 1:50 dilutions. Purified 9E10, conjugated or not, can also be purchased from several suppliers (Santa-Cruz, Abcam, Sigma) and is generally used at a 1 µg/ml concentration for immunofluorescence experiments and by adding 1 µg per 10^6 cells for FACS analysis.

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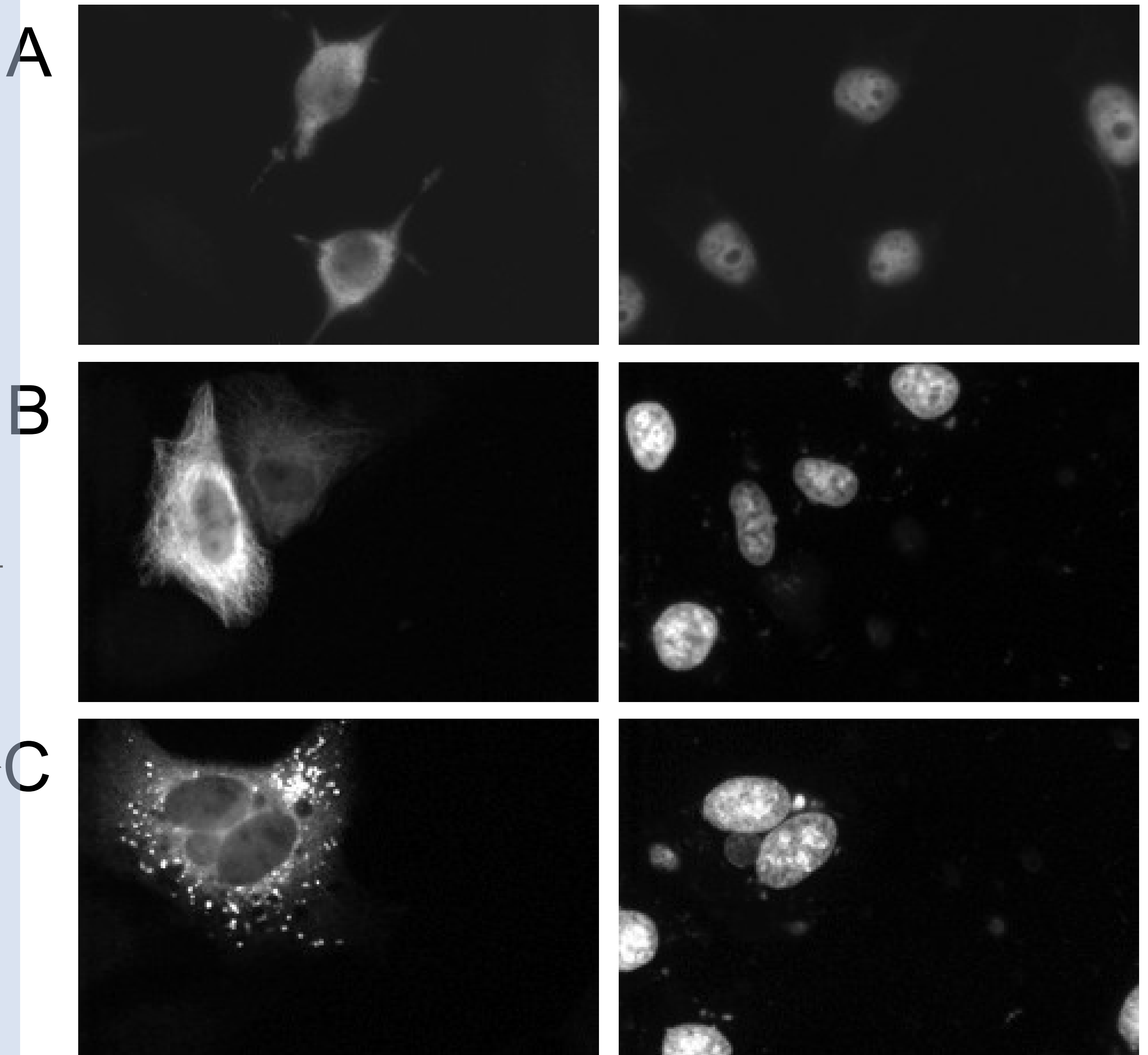

Fig. 1. Expression of scFvs in transfected Hela cells.

48 h after transfection with scFv cloned in pCMV/myc/cyto, Hela cells were fixed with methanol and stained with anti-myc antibody. The figure shows expression pattern of a soluble intrabody that does not recognize any expressed protein (*panel A*), a soluble intrabody directed against tubulin (*panel B*) and an aggregating intrabody (*panel C*). Right panels: Hoechst staining of nuclear DNA.