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Flow Cytometry-Based Cell Type-Specific Assessment of Target Regulation by Pulmonary siRNA Delivery

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Abstract

Pulmonary siRNA delivery has attracted strong interest and has been reported to successfully mediate target gene knockdown in a number of disease models. However, the nature of the epithelial cells that eventually take up siRNA and the question if other lung cell types may also be transfected have so far been neglected. Therefore, we describe here a flow cytometry-based method using transgenic enhanced green fluorescence protein-expressing mice (EGFP mice) for the differentiation of transfected lung cell populations based on their antigen expression.

Keywords

siRNA delivery; Lung; EGFP mice; Flow cytometry; Knockdown; Epithelial cells; Type II pneumocytes; Differentiation

1. Introduction

Since its discovery and translation into functional genomics in mammalian cells (1), RNA interference (RNAi), a Nobel-Prizewinning technology, has attracted strong interest to evaluate its therapeutic potential (2). However, rapid degradation by nucleases and fast excretion following systemic injection of short interfering RNA (siRNA) (3) impede the development of RNAi-based therapies. In fact, the majority of clinically investigated siRNA-based drugs exploit local delivery routes such as intraocular, intradermal, and pulmonary administration (2). Considering the large number of reports in the literature addressing successful pulmonary siRNA delivery (4) and the variety of lung disorders ranging amongst the ten leading causes of death worldwide (5), this specific local administration route is highly promising from a translational point of view.

The advantages of local noninvasive targeting are self-evident: immediate availability, decreased dose and systemic side effects, and the fact that several obstacles such as interactions with serum proteins can be avoided by pulmonary delivery (6). Despite the favorably vast surface area and strong perfusion of the lung (7), several barriers must be overcome for efficient pulmonary siRNA delivery. These hurdles are of an anatomic nature as reflected in the pulmonary architecture, and also physiologic represented by active clearance processes and effective immune responses (8). While mucociliary clearance, cough clearance, and the clearance by macrophages constantly eliminate foreign material from the lung (9), respiratory mucus (10) and airway surface liquid (11) additionally hinder efficient siRNA delivery into lung tissue.

To achieve specific therapeutic effects, siRNA not only needs to be directed to its target region (12), but it also has to be delivered into its target cell populations (13).

Despite the availability of several animal models of pulmonary diseases, little attention has been paid to the elucidation of the nature of the epithelial cells that are transfected, and whether other lung cell types also take up siRNA after pulmonary delivery (14). Transgenic

Actin-enhanced green fluorescence protein (EGFP)-expressing mice, often used in the literature (15–18), are an animal model almost predestined for such investigations. While in the first reports EGFP knockdown after intranasal delivery of siRNA and short interfering locked nucleic acids (siLNA) was only determined by counting EGFP-positive cells in paraffin sections (17) or by means of a physical fractionator probe (16), there are currently two reports where the success of EGFP knockdown after intratracheal administration of siRNA was measured by flow cytometry in lung homogenates (13, 16) obtained as previously described (19). A recent study employed cell sorting of CD45-positive cells from lung homogenates of EGFP-expressing mice to differentiate the knockdown in leukocytes and lung macrophages (20). Accordingly, a flow cytometry-based analysis can be utilized to differentiate myeloid from endothelial cells and type II alveolar epithelial cells (AEC-II) (21) to determine which cell types in the lung take up siRNA for functional RNAi. In parallel, the knockdown efficiency can be measured by quantifying the EGFP fluorescence in the FITC-channel for each population.

2. Materials

2.1. siRNA Formulation

Due to increased stability and high activity accompanied by decreased immunogenicity, 2'-*O*-methylated 25/27mer DsiRNA targeting EGFP (22) and negative control DsiRNA from Integrated DNA Technologies (Leuven, Belgium) are recommended for in vivo use.

1. siEGFP: Sense: 5'-pACCCUGAAGUUCAUCUGCACCA-CdCdG, antisense: 3'-mAmCmUGmGGmACmUUmCmA GmUAmGAmCGUGGUGGC.
2. siNegCon: Sense: 5'-pCGUUAUUCGCGUAUAAUACGCG UdAdT, antisense: 3'-mCmAmGCmAAmUUmAGmCGmC AmUAmUUmAUGCGCAUAp.
3. A cationic lipid or a polymer for nanoformulations of siRNA, for example 25 kDa branched polyethylenimine (PEI; BASF, Ludwigshafen, Germany).

2.2. In Vivo Transfection

1. C57BL/6-Tg(CAG-EGFP)10sb/J Actin-EGFP-expressing mice, 6 weeks old, both genders.
2. EGFP-negative mice (e.g., C57BL/6) as control.
3. One tubus for each formulation made of the flexible part of a BD Insite 24-gauge catheter (BD Biosciences, Heidelberg, Germany) attached to a 24-gauge needle (BD Biosciences, Heidelberg, Germany). Alternatively, the Mouse Intubation Pack (Hallowell Engineering & Manufacturing Corporation, Pitts field, MA, USA) can be used.
4. Tweezers wrapped with Para film™.
5. Intubation stand, e.g., Rodent Tilting Work Stand (Hallowell Engineering & Manufacturing Corporation, Pitts field, MA, USA).
6. Syringes, 1 ml (BD Biosciences, Heidelberg, Germany).
7. Microliter pipet adjustable or fixed at 50 µl, corresponding pipet tips.
8. Cold Light Illumination Source (e.g., Leica L2).

2.3. Bronchoalveolar Lavage

1. Surgery table and surgical instruments.

2. Venflon catheters, 18-gauge (BD Biosciences, Heidelberg, Germany).
3. Centrifuge cups, 1.5 ml, and tubes, 15 ml.
4. Syringes, 1 and 20 ml.
5. PBS buffer containing 2 mM EDTA.
6. Complete Protease Inhibitor Cocktail (Roche Diagnostics, Mannheim, Germany): Dissolve one tablet in 50 ml PBS buffer.

2.4. Lung Homogenates

1. Low melting point agarose (Promega, Mannheim, Germany): 1% in PBS, boiled in a microwave oven.
2. Dispase (Roche, Mannheim, Germany), working concentration: 2 U/ml in HEPES-buffered saline (50 mM HEPES/KOH, pH 7.4, 150 mM NaCl).
3. DNase (Roche, Mannheim, Germany), working concentration: 100 U/ml in RPMI (PAA, Cölbe, Germany) containing 25 mM HEPES.
4. Wash buffer: PBS supplemented with 2 mM EDTA and 0.5% v/v fetal calf serum.
5. Cell culture plates, 6-well.
6. Petri dishes, 6 cm.
7. Nylon Cell Strainers, 100 and 40 μ m (BD Falcon, Heidelberg, Germany).
8. Tubes, 50 ml.
9. Venofix™ 0.8 $\times$ 20 mm (B. Braun, Melsungen, Germany) butterfly tube attached to a 20 ml syringe.
10. PBS buffer.
11. CasyTon™ isotonic salt solution (Schaerfe Systems, Reutlingen, Germany).
12. Casy TT™ cell counter (Schaerfe Systems, Reutlingen, Germany) (see Note 1).

2.5. Cell Fixation and Permeabilization

1. Paraformaldehyde: 1% v/v in PBS buffer, freshly prepared.
2. Wash buffer: PBS supplemented with 2 mM EDTA and 0.5% v/v fetal calf serum.
3. Saponin buffer: 0.3% w/v saponin (Carl Roth, Karlsruhe, Germany) in PBS buffer.
4. FACS tubes.

2.6. Antibodies

1. Rat anti-mouse CD16/CD32 (Fc-Block; BD Biosciences, Heidelberg, Germany), 1:100 dilution in 0.3% saponin buffer.
2. Rabbit polyclonal anti-mouse pro-surfactant protein-C (proSP-C) antiserum, reactive with human and mouse proSP-C (Abcam, Cambridge, UK), 1:500 dilution in 0.3% saponin buffer.
3. Rat anti-mouse CD45-PerCP/Cy5.5, clone 30-F11 (BioLegend, Uithoorn, The Netherlands), 1:50 dilution in 0.3% saponin buffer.
4. Rat anti-mouse CD31-PE, clone MEC13.3 (BioLegend, Uithoorn, The Netherlands), 1:100 dilution in 0.3% saponin buffer.

5. Secondary antibody: Alexa Fluor® 647 F(ab')₂ fragment of goat anti-rabbit IgG (H + L) × 2 (Invitrogen, Karlsruhe, Germany) 1:500 dilution in 0.3% saponin buffer.
6. Isotype controls: Rabbit immunoglobulin, rat IgG2a -PE (Biolegend), rat IgG2b PerCP/Cy5.5 (Biolegend).
7. FACS Canto II (BD Biosciences, Heidelberg, Germany) or a similar flow cytometer equipped with the following lasers and filter settings: GFP: excitation: 488 nm, emission filter: 530 nm/30; PerCP/Cy5.5: excitation: 488 and 635 nm, emission filter: 670 nm/LP; PE: excitation: 488 nm, emission filter: 585 nm/42; Alexa Fluor®: excitation: 635 nm, emission filter: 670 nm/20.

3. Methods

3.1. In Vivo Transfection

1. Prepare siRNA containing nanoparticles or complexes according to your standard protocol. For example, mix 50 µg siRNA with 40 µg PEI for a charge ratio of 6 PEI amines per phosphate. Pipet vigorously and then incubate for 20 min at room temperature. The maximum volume that can be instilled per mouse is 50 µl. The nanoparticle formulation should therefore contain the desired dose of siRNA in a total volume of 50 µl (see Note 2).
2. Deeply anesthetize EGFP-mice intraperitoneally with Xylazine/Ketamine and fix one mouse after another in a supine position. Place the gooseneck light on the lower part of the thorax, gently pull the tongue out with Para film™-wrapped tweezers, and clear the throat so that the trachea can be recognized as a light spot. Use magnification if necessary and move the light source until the trachea clearly appears as light spot. Gently intubate the mouse (see Note 3).
3. Pipet up to 50 µl into the tubus, then attach a syringe filled with 100 µl air, and push the air into the tubus, thereby instilling the material into the mouse lung.
4. Check for vital signs, return the mouse into the cage, and instill the next mouse as described.
5. The treatment can be performed repeatedly, e.g., on day 1 and day 3.
6. Treat at least 5–8 animals per group (siEGFP, scrambled control sequence, vehicle only, and EGFP-negative mice).

3.2. Bronchoalveolar Lavage

1. Sacrifice animals 48 h after the last treatment by cervical dislocation and exsanguination. Fix the mouse in a supine position and cut open the thorax without affecting the heart or the lung. Remove the thymus and all tissue surrounding the trachea. Gently cut a 2 mm hole in the trachea (see Note 4) and cannulate the trachea using an 18-gauge Venflon catheter.
2. Rinse the lung with 1 ml fresh PBS/cOmplete via the tracheal catheter and store the bronchoalveolar lavage (BAL) fluid (BALF) of each animal on ice. Spin the BALF down for 5 min at 350 g (see Note 5).
3. To perfuse the lung, first cut the left atrium, then insert the butterfly into the right ventricle (only insert ~3 mm), and gently inject 20 ml PBS until lung appears white.

3.3. Flow Cytometric Quantification of EGFP Knockdown

1. To obtain lung homogenates, inflate the lungs with 1 ml dispase (2 U/ml) followed by 0.5 ml 1% low-melting-point agarose (alternatively lungs may be prepared for histological analysis, see Note 6).
2. Secure the trachea and excise the lungs and incubate them in an additional 3 ml dispase per lung for 30 min at 37°C in a 6-well plate.
3. Place the lungs into a 6 cm petri dish filled with 6 ml RPMI containing 100 U/ml DNase and 25 mM HEPES. Remove the lung tissue from the trachea by gentle tapping with the back of a pair of tweezers (see Note 7).
4. Incubate for 10 min at room temperature with slight agitation.
5. Add 4 ml medium with a 10 ml serological pipet.
6. To further disarticulate the tissue, homogenize by pipetting up and down with a 1 ml pipet (see Note 8).
7. Pipet the lung homogenate up and down again with a 10 ml serological pipet and transfer it to a 100 µm nylon cell strainer placed on a 50 ml tube. Add 5 ml medium without DNase and process the tissue through the cell strainer.
8. Pass the lung homogenate through the 40 µm cell strainer into a fresh 50 ml tube.
9. Centrifuge the single cell suspension for 10 min at 350 g.
10. Discard the supernatant and resuspend the pellet in 5 ml fresh medium.
11. To determine the amount of cells, dilute the suspension 1:2,000 in 10 ml CasyTon™ and count the cells using a Casy TT cell counter (see Note 1).
12. Centrifuge the single cell suspension for 10 min at 350 g.
13. Discard the supernatant and resuspend the pellet in 4 ml fresh 1% paraformaldehyde solution and incubate for 15 min on ice.
14. Centrifuge the fixed cells for 10 min at 350 g.
15. Discard the supernatant and resuspend in 5 ml wash buffer.
16. Centrifuge cells for 10 min at 350 g.
17. Discard the supernatant and resuspend the pellet in 4 ml wash buffer.
18. Transfer about 200,000 cells per lung sample into FACS tubes.
19. Prepare additional samples as blank and isotype controls.
20. Centrifuge the aliquots of 200,000 cells per FACS tube for 10 min at 350 g.
21. Discard the supernatant, add 100 µl 0.3% saponin buffer per sample for permeabilization, mix, and incubate for 15 min at 4°C.
22. Centrifuge FACS tubes for 5 min at 350 g.
23. Discard the supernatant, add 10 µl diluted Fc-block, add 20 µl of the appropriate dilutions of the antibodies against CD45, CD31, and pro-SPC (or isotype controls), vortex, and incubate for 25 min at 4°C in the dark.
24. Centrifuge the stained cells for 5 min at 350 g.
25. Discard the supernatant, wash with 100 µl 0.3% saponin buffer twice, and centrifuge the cells a third time for 5 min at 350 g.

26. Discard the supernatant, add 20 μ l of the appropriate dilution of the secondary antibody (including the isotype control tube), mix, and incubate for 25 min at 4°C in the dark.
27. Centrifuge the stained cells for 5 min at 350 g.
28. Discard the supernatant, wash with 100 μ l 0.3% saponin buffer twice, and centrifuge the cells a third time for 5 min at 350 g.
29. Discard the supernatant, wash with 100 μ l once with wash buffer, centrifuge the cells again for 5 min at 350 g, and resuspend them in 200 μ l wash buffer.
30. Adjust the laser power at the flow cytometer using the isotype controls and EGFP-negative samples.
31. Gate the cells to exclude debris and cell clumps. Count at least 30,000 events.
32. Compensate the fluorophores to remove the spectral overlap so that three different populations of myeloid cells (CD45+), endothelial cells (CD31+), and type II pneumocytes (pro-SPC+) can be distinguished (see Note 9).
33. Gate single populations as shown in Fig. 1. Use density plots for better differentiation, if necessary.
34. Determine EGFP expression in every single population in the FITC channel (see Note 10) and record mean fluorescence for each population as a major readout parameter as shown in Figs. 2 and 3.

4. Notes

1. Alternatively use a hemocytometer counting chamber.
2. Instillation of volumes greater than 50 μ l can drown the mice whereas it has to be noted that a certain volume is always lost in the tubus. Therefore volumes smaller than 30 μ l are not recommended either.
3. Deep intubation leads to treatment of the left lung lobes only. Therefore the tubus should only be introduced a few millimeters deep.
4. If the trachea is cut through, it easily disappears and can hardly be cannulated. Therefore, only cut a tiny hole of 2 mm in the anterior part of the trachea.
5. To determine the total number of leukocytes captured by lavage, resuspend the pellet in PBS containing 1% BSA, dilute the suspension 1:2,000 in 10 ml CasyTon™, and count the cells using a Casy TT™ cell counter. Cytospins can be prepared with aliquots of 100,000 cells spun on glass slides (100 g, 5 min) and stained for differentiation with Diff-Quick (DADE Diagnostics, Unterschleissheim, Germany). For evaluation, count 100 cells and classify concerning morphology and staining. Cytokine and chemokine secretion can be measured in the supernatant by ELISA or multiplex ELISA (e.g., Luminex System, Bio-Rad Laboratories, Germany) as previously described (15).
6. For microscopic assessment of histology and EGFP knockdown, some lungs (e.g., 2 out of 8 per group) can be prepared for histological analysis, leaving another six for flow cytometric assessment of the knockdown. For microscopy, inflate the lungs with 6% paraformaldehyde and store them in 6% paraformaldehyde in the dark for 24 h, before embedding in paraffin. Deparaffinize and slice sections of 3 μ m. Counterstain the tissue sections with 1 μ g/ml 4',6-diamidino-2-phenylindole (DAPI) (Molecular Probes, Invitrogen, Karlsruhe, Germany) or stain with

hematoxylin and eosin (H&E) for confocal laser scanning or light microscopy, respectively, as described before (13).

7. The lung tissue should not be cut or the very sensitive type II pneumocytes will be lost.
8. Slightly cutting off the tip of 1 ml pipet tip helps the homogenization step.
9. Older cytometers require compensation to be performed prior to data acquisition; in this case spectral overlap can be determined using samples stained with a single antibody.
10. As can be seen in Figs. 1 and 2, it is possible that the EGFP expression within one population varies strongly. In this case, subpopulations need to be gated to account for these differences in EGFP expression and to quantify the fluorescence in every single population correctly.

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Fig. 1.

Dot plots obtained after fluorescence compensation. Myeloid CD45-positive cells are gated in the *middle panel*, shown in *purple*. Endothelial, CD31-positive cells are gated in the *middle panel*, shown in *blue*. Type II alveolar epithelial cells (AEC-II) which are positive for pro-surfactant protein-C (pro-SPC) are gated in the *right panel*, shown in *green*.

Fig. 2. The treatment with nanoparticles containing the scrambled negative control sequence (**a** : siNegCon) or the specific siRNA sequence (**b** : siEGFP) affects the EGFP fluorescence which can be measured for every population as shown in the statistic tables below the dot plots. Amongst AEC-II, highly EGFP-expressing cells (High GFP: shown in the *right part* of the dot plot) and only slightly highly EGFP-expressing cells (Low GFP: shown in the *left part* of the dot plot) can be found and have to be evaluated separately.

Fig. 3. Mean fluorescence values of EGFP expression in every single cell population can be summarized in bar graphs. The knockdown efficiency in every single population can then be easily recognized.