



Development of CPP-Based Methods for Delivery of miRNAs into the Skin and Airways: Lessons from Cell Culture and Mouse Models

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Abstract

microRNAs (miRNAs) have capacity to modulate numerous biological processes and therefore synthetic oligonucleotides mimicking or inhibiting particular miRNA have potential in the development of novel types of therapeutics. We have elaborated several methods for safe and efficient overexpression of miRNAs using self-forming nanocomplexes of PepFect or NickFect type of cell-penetrating peptides (CPPs) and oligonucleotides mimicking well-characterized anti-inflammatory miR-146a. We focus on chronic inflammatory diseases affecting epithelium, such as atopic dermatitis and asthma harnessing respective cell cultures and mouse models. Here we provide protocols for miRNA transfection into primary human keratinocytes, primary human bronchial epithelial cells, and human monocyte-derived dendritic cells using CPPs PepFect14, NickFect70, NickFect71, and NickFect72. In addition, we provide protocols for application of CPP-miRNA nanocomplexes in *in vivo* mouse models of irritant contact dermatitis and allergic airway inflammation.

Key words microRNA, Allergy, Non-coding RNA, siRNA, Inflammation, Transfection

1 Introduction

microRNAs (miRNAs) have capacity to bind through imperfect complementarity to target mRNA and induce its degradation or inhibition of translation [1, 2]. As single miRNA can target multiple mRNAs encoding factors from the same pathway, miRNAs together regulate the majority of all biological processes in health and diseases [3]. For example, miR-146a has been shown to have anti-inflammatory properties in numerous cell types and conditions as it inhibits multiple genes from the NF- κ B pathway, including IL-1 receptor-associated kinase 1 (IRAK1) [4], caspase recruitment domain-containing protein 10 (CARD10), and chemokine CCL5 [5–8]. Considering the importance of miRNAs in various diseased

conditions, it has been proposed that synthetic oligonucleotides mimicking or inhibiting particular miRNA may be used as novel types of therapeutics [9]. However, only a few miRNA-based candidate therapeutics have reached to clinical trials, since similarly to all oligonucleotides, miRNA mimics and inhibitors are relatively unstable and difficult to deliver into the target cells and tissues due to their high negative charge [9, 10]. To enhance delivery efficiency and stability, packaging into different types of nanocomplexes and chemical modifications of oligonucleotides are used [9–11]. Accordingly, several liposome-based delivery methods as well as introduction of particular nucleoside modifications have led to revelation of miRNA effects in target cells and tissues. However, the majority of these methods often trigger side-effects, including toxicity and the activation of immune responses [9]. Such side-effects may be even beneficial in cancer treatment and during the development of vaccines, however, they should be avoided in treating immune system-related diseases and inflammation. Therefore, for the development of miRNA-based treatment modalities for inflammatory conditions, for example for allergic diseases, the delivery system and oligonucleotide itself should not aggravate the inflammation or immune responses.

One promising class of carriers, which we have shown to be tolerated well by multiple human primary cell types are cell-penetrating peptides (CPPs) [5, 12–14]. To prove the applicability of a particular set of CPPs in delivery of miRNAs in cell cultures and *in vivo*, we have used self-forming nanocomplexes of PepFect (PF) 14 or NickFect (NF) type of CPPs, NF70, NF71, NF72 and mimicking oligo for a well-characterized anti-inflammatory miR-146a [5, 13, 14]. PF14 was chosen because of its efficiency and enhanced capacity to release the cargo from the complex in delivery of siRNAs shown in previous studies [15, 16]. As compared to its predecessor CPP, TP10, PF14 contains multiple ornithines instead of lysines enabling stronger binding to nucleic acids, and N-terminal stearyl, which ensures more efficient release from the endosome [16]. NF70, NF71, and NF72 are very recently designed CPPs containing multiple histidines to optimize pH-sensitivity and N-terminal fatty acids to improve the release of cargo from the nanocomplex and endosome [17].

As our focus is inflammatory diseases affecting epithelium, such as atopic dermatitis and asthma, we use normal human epithelial keratinocytes (NHEKs), normal human bronchial epithelial (NHBE) cells and monocyte-derived dendritic cells (moDCs) as test cell cultures and corresponding mouse models [5, 12–14]. We specifically are focused on inflamed epithelium, as in the case of inflammation, the epithelial barrier is damaged and tight junctions between the cells are opened making the tissue more accessible to CPP-miRNA nanocomplexes [18, 19]. Accordingly, we have shown that subcutaneously injected CPP-miRNA nanocomplexes penetrate into inflamed but not into the healthy skin tissue in mice

[13]. We also have demonstrated that particular CPPs support delivery of miRNA mimics into dendritic cells without activation of immune responses [14]. More particularly, we provide protocols for transfection of miRNA mimics into NHEKs, NHBE cells, and moDCs using CPPs PepFect14, NickFect70, NickFect71, NickFect72 and miR-146a and a control mimic as examples. In addition, we provide protocols for administration of CPP-miRNA nanocomplexes using miR-146a mimics as example *in vivo* in mouse models of irritant contact dermatitis (ICD) and allergic airway inflammation (AAI). The biological validation and effect of miR-146a in these cell cultures are described previously [5, 13, 14, 20].

2 Materials

2.1 Cell-Penetrating Peptides (CPPs)

Prepare all solutions in sterile conditions (cell culture grade).

For transfection experiments in cell cultures, counting of cells is necessary. Depending on the chosen method, trypan blue and hemocytometer with light microscope or an automated cell counter, for example Countess 3 Automated Cell Counter (Thermo Fisher Scientific) with cell counting slides are needed.

1. PepFect14 (PF14) Stearyl-AGYLLGKLLLOOLAAAALLOOLL-NH₂.
2. NickFect70 (NF70) Arachidyl-HHHHHYHHGO*ILLKALKALAKAIL-NH₂.
3. NickFect71 (NF71) Stearyl-HHYHHGO* ILLKALKALAKAIL-NH₂.
4. NickFect72 (NF72) Arachidyl-HHHHHHHYHHGO*ILLKALKALAKAIL-NH₂.

CPPs were synthesized as described previously [17] or can be bought from companies specialized in CPP synthesis (*see Note 1*). *Glycine is coupled to the amino group on the side chain of ornithine.

2.2 MicroRNA Mimics

1. miRIDIAN microRNA Mimic Negative Control #1 (cont).
2. miRIDIAN microRNA hsa-miR-146a mimic (miR-146a).
3. DyLight547-labeled miRIDIAN microRNA Mimic Transfection Control #1 (Dy547-cont).

All miRNA mimics can be ordered from Thermo Fisher Scientific, USA (*see Note 2*).

2.3 Epithelial Cell Cultures

1. Pooled primary normal human epidermal keratinocytes (NHEKs) (Promocell, Germany), serum-free keratinocyte-SFM medium supplemented with 5 ng/mL human recombinant epidermal growth factor (rEGF), 50 µg/mL bovine

pituitary extract (BPE) (all from Life Technologies, CA, USA) and 5 U/mL penicillin, 5 U/mL streptomycin (both from Lonza, Switzerland).

2. Normal human bronchial epithelial (NHBE) cells (Lonza, Switzerland), Bronchial Epithelial Cell Growth Medium (BEGM™) supplemented with BPE, insulin, hydrocortisone, gentamicin-amphotericin-B mixture, retinoic acid, transferrin, triiodothyronine, epinephrine, and rEGF (Lonza, Switzerland).
3. 2× Trypsin-EDTA (0.05%) solution and Trypsin Neutralizer solution (Gibco™).
4. 1× Phosphate Buffered Saline (PBS).
5. 15 mL and 50 mL Falcon tubes.
6. Serological pipette and tips.

2.4 Isolation of CD14+ Monocytes from Peripheral Blood “Buffy Coat” Fraction and Culturing of moDCs

1. Peripheral venous anticoagulated blood sample or “buffy coat.”
2. 1× PBS.
3. PBS-EDTA buffer: Dilute 360µL of 0.5 M EDTA (pH 8.0) in 90 mL of PBS (pH 7.2). Store at 4 °C.
4. Ficoll-Paque (any type, Merck, NJ, USA).
5. Human CD14 microbeads (Miltenyi Biotec, Germany).
6. MACS separator and MS or LS columns with plunger (both from Miltenyi Biotec, Germany).
7. RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100µg/mL streptomycin, and 10% FBS (all from PAA Laboratories, Canada) in the presence of 50 ng/mL GM-CSF and 25 ng/mL IL-4 (both from PeproTech, UK).

2.5 Transfection Experiments in Cell Culture

1. 12-well plates suitable for cell culture.
2. Sterile 15 mL or 50 mL Falcon tubes.
3. Sterile 1.5 mL tubes.
4. Sterile, filtered MilliQ grade water (MQ).
5. Serological pipette and according 10 mL tips.
6. Vortex mixer.

2.6 Mouse Model of ICD

1. 8–10-week-old C57BL/6 wild-type mice.
2. Phorbol 12-myristate 13-acetate (PMA) solution. Prepare 5 mg/mL PMA stock in acetone, then dilute to 2.5 mg/mL PMA concentration with sterile 1× PBS.
3. Acetone (ACS reagent, ≥99.5%, Merck, NJ, USA).

4. Sterile 1 × PBS.
5. 10% glucose solution in MQ.
6. 29G insulin syringe.
7. 100μL pipette and according tips.
8. Digital Vernier caliper (Mitutoyo, Japan).
9. Isoflurane suitable for veterinary use.
10. Tabletop isoflurane anesthesia system with small induction box.

2.7 Mouse Model of AAI

1. 8–10-week-old C57BL/6 wild-type mice (at least 5 mice per each study group, when experiment is repeated at least two times).
2. Sterile 1 × PBS.
3. House dust mite (*Dermatophagoides pteronyssinus*) extract (HDM) (Stallergenes Greer, USA). Use sterile 1 × PBS to prepare HDM sensitization solution (25μg/mL) or HDM challenge solution (250μg/mL).
4. Isoflurane suitable for veterinary use.
5. Tabletop isoflurane anesthesia system with small induction box.
6. 1 × PBS.
7. 100μL automatic pipette and according tips.

3 Methods

3.1 Peptide Synthesis and Preparation of Stock Solutions

PF14 and NF70, NF71 and NF72 peptides can be synthesized in-house or ordered from specialized companies. In current study, automated peptide synthesizer (Biotage Initiator+ Alstra, Sweden) and fluorenylmethyloxycarbonyl (Fmoc) solid-phase peptide synthesis strategy was used as previously described [17]. Synthesized peptides were purified by reverse-phase liquid chromatography on C4 column (Phenomenex Jupiter C4, 5μm, 300 Å, 250 × 10 mm) using a gradient of acetonitrile/water containing 0.1% trifluoroacetic acid (TFA). The molecular weight of the peptides was determined by MALDI-TOF mass spectrometry (Bruker Microflex LT/SH, USA).

3.2 Primary Human Epithelial Cell Cultures

Prior to thawing and culturing epithelial cells, prepare suitable cell culture media (*see Note 3*) and pre-warm 7 mL of appropriate media in 15 mL falcon tube to 37 °C. For NHEK, supplemented keratinocyte-SFM media should be used. To culture NHBE cells, use supplemented BEGM™ media.

1. Thaw frozen epithelial cells in the hand and by pipetting pre-warmed cell culture media to the cells. Carry cells over to 15 mL Falcon tube containing 7 mL of the pre-warmed media.

2. Suspend cells briefly by pipetting and centrifuge at $250 \times g$ for 5 min at 20 °C.
3. Aspirate the supernatant, re-suspend the cell pellet in 10 mL of warm cell culture media, and transfer cell suspension to a 100 mm cell culture dish.
4. Incubate cells at 37 °C in humidified atmosphere with 5% CO₂. Exchange cell culture media every other day until 70–80% of confluency is achieved, then split the cells (*see Note 4*).
5. To split cells, aspirate culture medium, add 2 mL of pre-warmed 2× trypsin, and incubate cells at 37 °C for 5 min.
6. Add 4 mL of trypsin neutralizer solution to the cells and collect the cell suspension to a new 15 mL falcon tube.
7. Wash the plate with 5 mL of 1× PBS and transfer all obtained liquid to Falcon tube that contains cell suspension and centrifuge at $250 \times g$ for 5 min at 20 °C.
8. Take 3 new 100 mm cell culture dishes and add 7 mL of pre-warmed cell culture media to each dish.
9. Following centrifugation, aspirate the supernatant and re-suspend the cell pellet in 9 mL of warm cell culture media.
10. Transfer 3 mL of obtained cell suspension to each 100 mm cell culture dish with media. Mix and grow cells at 37 °C, with 5% CO₂.

3.3 Preparation of CPP-miRNA Nanocomplexes and Transfection into Cultured Epithelial Cell

We have developed the following protocol previously to describe the functions of miR-146a in NHEKs and NHBE cells and miR-10a in NHEKs using PF14 [5, 13, 14, 21, 22] or PF14, NF70, NF71, and NF72 [14]. Main steps in the procedure are visualized in Fig. 1a. Please *see also Note 5*.

24 h prior to transfection of CPP-miRNA nanocomplexes, cultured epithelial cells should be counted and seeded to 12-well plates in 1 mL of cell culture media. For transfection experiments with NHEKs, seed 3×10^4 and for NHBE cells 4×10^4 cells per well.

CPP-miRNA complexes should be prepared in 1/10th of final volume of media at charge ratio 1.5–3:1 and at 30 nM final concentration of miRNA mimics. miRNA amount and charge ratio optimum may vary depending on the peptide, miRNA mimic, and type of cells. The following example is for charge ratio 2:1 (molar ratio 17:1 in case of PF14) and for 1 well of 12-well plate with final volume of 1 mL. For that, 100 µL of solution of CPP-miRNA complexes should be prepared as follows:

1. In a 1.5 mL tube, mix 1.5 µL of 20 µM miRNA mimics (for example miR-146a, any other specific miRNA or negative control) with 93.38 µL of sterile MQ.

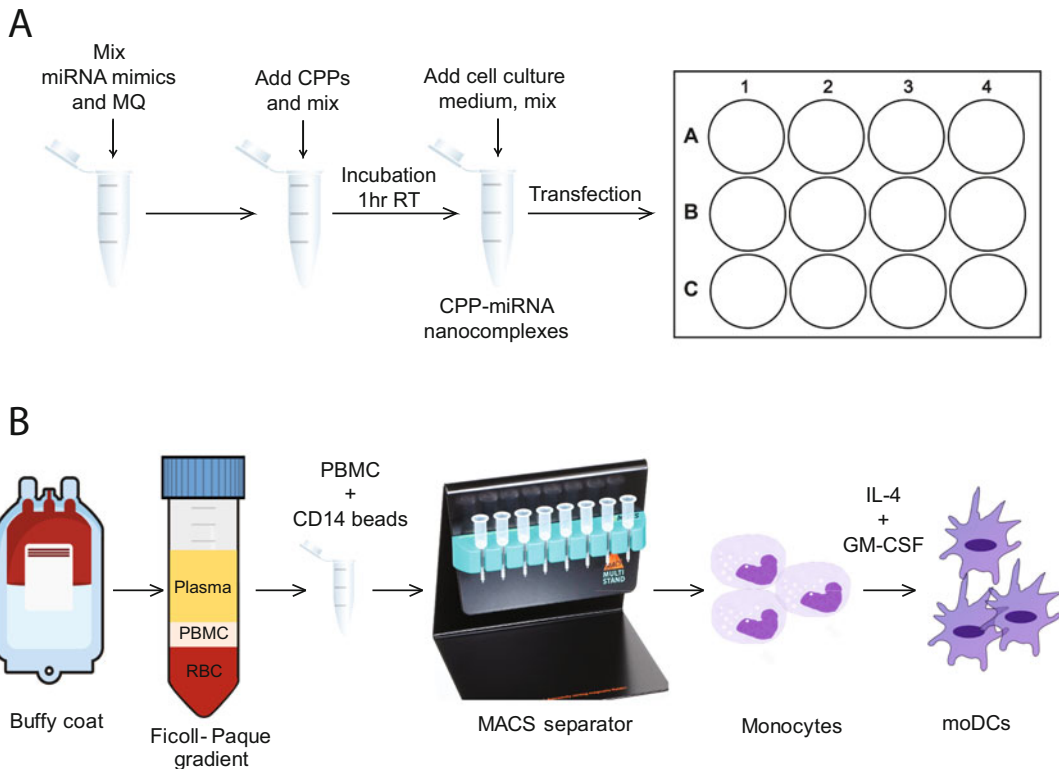


Fig. 1 Procedures associated with the transfection of CPP-miRNA nanocomplexes in cell cultures. **(a)** Preparation of CPP-miRNA nanocomplexes and transfection of epithelial cell culture. Appropriate amount of miRNA mimics are diluted in MilliQ water (MQ) in a sterile 1.5 mL tube, then CPPs are added, mixed and incubated for 1 h at room temperature. Fresh cell culture medium is added to self-formed CPP-miRNA nanocomplexes and resulting solution is transferred to plated epithelial cells for transfection. **(b)** Generation of monocyte-derived dendritic cells (moDCs). First, peripheral blood mononuclear cells (PBMCs) are isolated from the buffy coat by Ficoll-Paque gradient method, which enables to separate PBMCs from red blood cells (RBC) and plasma. Second, PBMC fraction is incubated with CD14 microbeads followed by capture of CD14-positive cells with MACS separator. Purified CD14 positive monocytes are cultured in the presence of interleukin 4 (IL-4) and granulocyte-macrophage colony-stimulating factor (GM-CSF) to derive moDCs

2. In a separate 1.5 mL tube, dilute 1 mM CPP stock to a 100 μ M CPP working solution in MQ.
3. Add 5.12 μ L of 100 μ M CPP to a miRNA mimic's solution in MQ and mix well.
4. Incubate the CPP-miRNA complexes at room temperature for 1 h.
5. Transfer 900 μ L of appropriate supplemented epithelial cell culture media to 100 μ L of CPP-miRNA complexes and mix well.
6. Transfect the epithelial cells by replacing old media on plated epithelial cells with 1 mL of freshly prepared cell culture media with CPP-miRNA nanocomplexes.

7. Incubate the cells at 37 °C in cell culture incubator for 24 h.
8. When needed, the transfected epithelial cells can also be challenged with stimulant of choice for up to 48 h without replacing the media.
9. Harvest the cells and measure the effect of miR-146a (or any other specific miRNA) on target genes at mRNA level after purification of total RNA. Alternatively, the effect on protein levels can be measured using western blot, flow cytometry, or immunofluorescence.

3.4 Isolation of CD14+ Monocytes and Culture of moDCs

The protocol is developed based on [23] and main procedures are schematized in Fig. 1B. All the following steps have to be performed in sterile conditions.

First, peripheral blood mononuclear cells (PBMCs) should be isolated from the peripheral blood or “Buffy coat” (points 1–9), second CD14+ cells are purified (points 10–13) and third, CD14+ monocytes are plated and differentiated into moDCs. The following example is for “Buffy coat.”

1. Dilute “Buffy coat” with ice-cold PBS-EDTA buffer using 1:1 ratio and mixing gently by pipetting up and down.
2. Per one 50 mL Falcon tube, carefully layer about 35 mL of diluted blood on the top of the 10 mL bed of Ficoll-Paque reagent (*see Note 6*).
3. Centrifuge at $400 \times g$ for 35 min at 20 °C without the brake (*see Note 7*).
4. Aspirate upper layer (plasma and platelets) without disturbing the mononuclear cell layer which contains lymphocytes, monocytes, and thrombocytes.
5. Carefully transfer the mononuclear cell layer into a new 50 mL falcon tube and mix with 30 mL of ice-cold PBS-EDTA buffer.
6. Centrifuge at $200 \times g$ at 20 °C for 15 min and discard the supernatant. Suspend the pellet with 20 mL of PBS-EDTA buffer and count the cells.
7. Transfer cell suspension into new Falcon tube and centrifuge at $300 \times g$ for 10 min at 20 °C.
8. Discard the supernatant and re-suspend the cell pellet with 80 μ L of PBS-EDTA buffer per 10^7 cells. For example, add 1600 μ L of buffer to 20×10^7 cells.
9. Add 20 μ L of CD14 microbeads suspension per 10^7 cells in 80 μ L of PBS-EDTA buffer. For example, to 20×10^7 cells, add 400 μ L of microbeads, mix and incubate the suspension at 4 °C for 15 min. Mix the suspension in every 5 min gently.
10. Place a suitable MS or LS column in the magnetic separator (*see Note 8*). Equilibrate the column with PBS-EDTA buffer and

pass the cell suspension through the column. Add new buffer only after the column reservoir is empty.

11. Discard the flow through solution that contains unlabelled cell fraction.
12. Wash the column three times with PBS-EDTA buffer (500 μ L to MS column or 3 mL to LS column).
13. Transfer column from the separator stand to a 50 mL falcon tube, add PBS-EDTA buffer (1 mL to MS column or 5 mL to LS column), and immediately flush out the cells by pushing the plunger into the column.
14. Collect the labeled cells by centrifuging at $300 \times g$ for 10 min at 20 °C. Re-suspend the monocytes using RPMI 1640 media supplemented with 25 ng/mL of IL-4 and 50 ng/mL of GM-CSF.
15. Seed 2.5×10^5 monocytes per well of a 24-well plate in 500 μ L of cell culture medium.
16. Incubate the cells at 37 °C in 5% CO₂ containing atmosphere for 6 days. Replace half of the media (250 μ L/well) with new conditioned media on every other day. During 1 week of culturing, monocytes will differentiate into moDCs.

3.5 Preparation of CPP-miRNA Nanocomplexes and Transfection of moDCs

The following protocol is developed using miR-146a and control mimics and CPPs NF70, NF71, and NF72 [14].

CPP-miRNA complexes at 2:1 charge ratio (17:1 molar ratio) are prepared in 1/10th of the final volume of media. 2.5×10^5 moDCs in the culture volume of 500 μ L in 24-well plate are transfected at final 100 nM concentration of miR-146a or control miRNA (*see Note 9*).

1. Prepare 50 μ L of CPP-miRNA complexes, by adding 2.5 μ L of 20 μ M miRNA mimics to 39.0 μ L of sterile MQ and mix. After that add 8.5 μ L of 100 μ M CPP, mix the resulting solution thoroughly and incubate at room temperature for 1 h.
2. Mix 50 μ L of CPP-miRNA solution with 250 μ L of appropriate medium (*see Note 10*).
3. Remove 250 μ L of media from moDCs. Add the prepared CPP-miRNA nanocomplexes (with miR-146a or control miRNA) into suspension of moDCs.
4. Incubate the cells with added CPP-miRNA nanocomplexes for up to 48 h.
5. Harvest the cells and measure the effect of miR-146a (or any other specific miRNA) on target genes at mRNA level after purification of total RNA. Alternatively, the effect on protein levels can be measured using western blot, flow cytometry, or immunofluorescence.

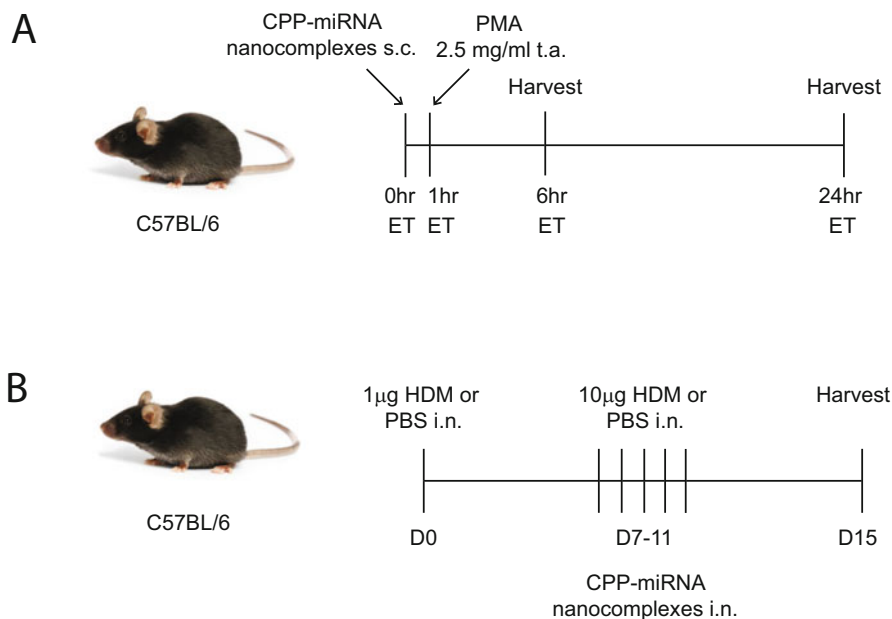


Fig. 2 Schematic of application of CPP-miRNA nanocomplexes in mouse models of irritant contact dermatitis (ICD) and house dust mite (HDM)-induced allergic airway inflammation (AAI). **(a)** Procedures in mouse model of ICD. 60 pmoles of miR-146a or control miRNA in complex with CPP (molar ratio 1:28) should be injected subcutaneously (s.c.) into mouse ears. After 1 h, Phorbol 12-myristate 13-acetate (PMA) should be topically applied (t.a.) on the ears to induce ICD. Ear thickness (ET) should be measured at 0 h, 6 h, and 24 h time points. At 6 h and 24 h time points, ear tissue should be harvested to measure the effect of particular miRNA in mRNA and protein level. **(b)** Procedures in mouse model of AAI. Mice should be lightly anesthetized and 1µg HDM extract in 40µL PBS should be administered intranasally (i.n.) to the mice on day 0 (D0). On days 7–11, mice should be daily challenged with 10µg of HDM extract in 40µL PBS and 2 h later 60 pmol of miR-146a or control miRNA mimic nanocomplexes with CPP (molar ratio 1:28) should be administered i.n. At day 15, lung tissue and bronchoalveolar lavage can be harvested to measure the effect of CPP-miRNA nanocomplexes

3.6 Preparation of CPP-miRNA Nanocomplexes and Application in a Mouse Model ICD

The protocol of a mouse model of ICD and biological effect of CPP-miRNA complexes using NF71 and miR-146a mimics is published previously [14]. Per each injection, 60 pmol of miRNA at CPP-miRNA molar ratio 28:1 in 20µL is used. Schematic representation of the procedures is shown in Fig. 2a.

1. Prepare a fresh working stock of PMA at 2.5 mg/mL (*see Note 11*).
2. Prepare CPP-miRNA nanocomplexes in 1/2 of final volume. For 5 mice, 100µL of injection solution is required. First, prepare 50µL of CPP-miRNA complexes in MQ. For that, mix 15µL of 20µM miR-146a and corresponding control with 26.6µL of sterile MQ.
3. Add 8.4µL of 1 mM CPP to miRNA mimics' MQ solution and mix well.
4. Incubate mixture at room temperature for 1 h to form the CPP-miRNA nanocomplexes.

5. Add 50 μ L of 50% glucose solution in water to CPP-miRNA nanocomplexes and mix the solution well.
6. Place one mouse at a time into the induction box of the tabletop anesthesia system and lightly anesthetize the mouse with 3% isoflurane (*see Note 12*).
7. Measure the thickness of mouse ear by using digital caliper (*see Note 13*).
8. Inject 20 μ L of CPP-miRNA nanocomplexes subcutaneously into ear of mice. One ear receives 60 pmol of miRNA mimic.
9. One hour after the injection, lightly anesthetize the mouse with isoflurane and topically apply 20 μ L of 2.5 mg/mL PMA solution on mouse ears using 100 μ L pipette tip.
10. Measure mouse ear swelling response in different time points by using digital Vernier caliper (*see Note 13*).
11. At 6 h or 24 h time points, ear tissue is harvested to measure the effect of miR-146a (or any other miRNA) on target genes at mRNA and/or protein level. In addition, histological analysis can be prepared.

3.7 Preparation of CPP-miRNA Nanocomplexes and Application in a Mouse Model of AAI

A mouse model of AAI is developed based on [24]. Biological effect of PF14-miR-146a complexes in this model is described in another study [20]. Per each administration, 60 pmol of miRNA at CPP-miRNA molar ratio 28:1 in 40 μ L is used. Schematic representation of the procedures is shown in Fig. 2b.

On day 0, prepare solution of HDM extract for sensitization of mice. For example, for 5 mice prepare solution containing 5 μ g of HDM extract in 200 μ L of PBS.

1. For sensitization with HDM, place one mouse into tabletop isoflurane anesthesia system's induction box and lightly anesthetize the mouse with isoflurane. Immediately administer intranasally (i.n.) 40 μ L of HDM extract solution (*see Note 14*). Repeat anesthetization and HDM administration step with each mouse.
2. On days 7–11, prepare solution of HDM extract for challenge. For example, for 5 mice prepare solution containing 50 μ g of HDM extract in 200 μ L of PBS.
3. Challenge with HDM on days 7–11: Place one mouse into tabletop isoflurane anesthesia systems induction box and lightly anesthetize the mouse with isoflurane. Instantly, administer 40 μ L of HDM extract solution i.n. (*see Note 14*).
4. Prepare CPP-miRNA nanocomplexes in 1/2 of final volume. For 5 mice 200 μ L of solution is required. First prepare 100 μ L of CPP-miRNA complexes' solution by mixing 15 μ L of 20 μ M miRNA mimics (miR146a and corresponding control) with 33.8 μ L of sterile MQ.

5. In a separate 1.5 mL tube, dilute 1 mM CPP stock to 100 μ M CPP working solution with MQ.
6. Add 51.2 μ L of 100 μ M CPP to miRNA mimics' MQ solution and mix well.
7. Incubate the mixture at room temperature for 1 h to form CPP-miRNA complexes.
8. Add 100 μ L of PBS to 100 μ L CPP-miRNA complexes solution and mix well.
9. 2 h after each HDM challenge, lightly anesthetize the mouse with isoflurane and immediately administer 40 μ L of CPP-miR-146a or CPP-control mimic nanocomplexes i.n. Sacrifice mice on day 15 and measure the effect of administered miRNA. For example, effect on cell numbers in bronchoalveolar lavage using flow cytometry and lung mRNA levels using RT-qPCR can be measured.

4 Notes

1. We recommend to prepare 1 mM stock solution of cell-penetrating peptide in RNase-free sterile MQ water and to store small aliquots at -20°C in order to avoid freezing and melting cycles. Alternatively, stock solutions should be prepared and stored according to manufacturer's guidelines.
2. To prepare 100 μ M stock solution of microRNA mimic, briefly centrifuge the tube containing miRNA mimic pellet and add RNase-free sterile MQ water. From freshly made 100 μ M stock solution, prepare suitable 20 μ M aliquots and store these at -20°C to ensure stability for at least 12 months.
3. We suggest to prepare 50 mL aliquots of supplemented BEGMTM media and to store the remaining media supplements at -20°C to ensure the stability of the components. The supplemented 50 mL BEGMTM media aliquots are stable at 4°C for up to 3–4 weeks. Thereafter we recommend to prepare fresh supplemented BEGMTM media for culturing NHBE cells.
4. The cultures of primary epithelial cells should be split at least once after thawing before proceeding with transfection experiments.
5. miRNA DyLight547-labeled miRIDIAN microRNA Mimic Transfection Control #1 (Dy547-cont) should be used for the estimation of transfection efficiency by microscopy. All experiments for assessing biological effect of particular miRNA should be performed using specific miRNA mimic and control mimic.
6. We recommend to tilt the Ficoll-Paque reagent containing tube to one side and then to slowly pipet blood on top of it.

7. It is essential to perform the centrifugation step without using break during the Ficoll-Paque gradient procedure. This avoids abrupt deceleration, which would interfere with the separation of layers in the gradient.
8. MS column is suitable for capturing up to 200 million cells, to LS column more than 200 million cells can be loaded.
9. We recommend to use 100 nM concentration of miR-146a or control miRNA for transfection of moDCs while 30 nM concentration is sufficient for transfection of keratinocytes. This difference may be due to the higher number of moDCs in the same medium volume. Do not use less cells as moDCs should be cultured at density of at least 5×10^5 per mL.
10. For the transfection of moDCs, RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% FBS (without IL-4 and GM-CSF) should be used.
11. We recommend to store the stock of 5 mg/mL PMA at -20°C and to prepare the 2.5 mg/mL PMA working solution just before the experiment.
12. Closely monitor the behavior of mouse. When the respiration rate is reduced and once they lack postural reflex, shift the mouse from induction box to nose cone.
13. To reliably estimate the thickness of the mouse ear, measurements should be performed in three different locations for one ear.
14. For intranasal administration of HDM solution or CPP-miRNA nanocomplexes, pipet the solution as a small droplet to mouse nostrils and during inhalation phase, mouse will breathe in the solution.

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