Published in final edited form as:

Curr Protoc Cell Biol. ; 74: 19.19.1–19.19.17. doi:10.1002/cpcb.15.

A functional microRNA screening method for organ morphogenesis

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Abstract

The increasing repertoire of microRNAs expressed during organ development and their role in regulating organ morphogenesis provide a compelling need to develop methods to assess microRNA function using various *in vitro* and *in vivo* experimental models. Methods to assess microRNA function during organ morphogenesis include transfection of microRNA inhibitors (antagomirs) and activators (mimics) into mouse embryonic explanted organs using liposomes, which can potentially result in low efficiency of transfection and off-target effects. We devised a method to assess microRNA function in explanted organs by transfecting antagomirs and mimics using peptide-based nanoparticles, increasing functional microRNA targeting efficiency, and decreasing off-target effects. Our method can be applied to a variety of embryonic organs that can be explanted, and provide an alternative to efficiently and functionally prioritize microRNAs during organ morphogenesis for further *in vivo* genetic approaches.

Keywords

microRNA; functional screening; organ morphogenesis

INTRODUCTION

The importance of microRNAs in regulating organ development (Landgraf et al., 2007; Asli et al., 2008) has created a growing demand for developing methods to assess microRNA function using *in vitro* and *in vivo* experimental models such as mouse knockouts (Park et al., 2010). Several explanted mouse embryonic organs offer, to a certain extent, reliable and reproducible alternatives to mouse genetic models, as they allow a faster functional screening of microRNAs when compared with knockout mouse models, provided that they recapitulate at least some of the important morphogenetic processes during early stages of organ morphogenesis. Among several organ models suitable for applying this protocol include submandibular salivary glands (SMGs) (Patel et al., 2006; Rebustini et al., 2009; Nedvetsky et al., 2014), lungs (Chen et al., 2015), kidneys (Costantini et al., 2011), mammary glands (Shamir et al., 2014), and tooth (Kavanagh et al., 2007) explants. In addition, organ explants allow visualizing phenotypic changes in real-time, and provide three-dimensional models for regenerative biology.

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In this unit we devise a method to functionally characterize individual microRNAs during organ morphogenesis, by employing peptide-based nanoparticle transfection to deliver microRNA inhibitors (antagomirs) and activators (mimics) inside embryonic explanted organs, and evaluate microRNA loss- or gain-of-function effects, respectively. Furthermore, we propose an optimized and direct microRNA target validation based on the use of biotinylated mimics in microRNA pulldown (miR-PD) assay, to detect and characterize target genes directly and indirectly regulated by microRNAs that yield interesting phenotypes when their function is perturbed.

This method can be adapted to a variety of ectodermal, mesodermal, and endodermal organs that develop via epithelial - mesenchymal interactions, such as the murine tooth germs and salivary glands (Kavanagh et al., 2007; Wei et al., 2007; Jumlongras et al., 2012; Jernvall et al., 2012; Tucker et al., 2004), and offers a straightforward data processing based on quantitative RT-PCR (qRT-PCR) (Schmittgen et al., 2008), uses minimal volumes of culture medium, the experimental configuration in multiwell plates allows a medium throughput analysis by assessing multiple different treatments (antagomirs and mimics) at a time, requires less cargo molecules (antagomir or mimic) during transfections, the reagents are accessible and inexpensive, the miR-PD microRNA validation method¹⁷ is relatively straightforward and requires small amounts of tissues from explanted organs, and biotinylated mimics can be used for both gain-of-function and miR-PD assays.

It is also important to carefully consider some potential limitations of this method, including limited lifespan of explanted embryonic organs and possible morphological artifacts associated with organ explants growing in culture, false positives or exclusion of authentic targets due to inaccurate predictions of microRNA target genes, and the small amounts of RNA generated in miR-PD may require scaling up the input (organ cultures) for high throughput analysis of gene expression using next generation sequencing such as RNA-Seq.

STRATEGIC PLANNING

General

The strategic planning for the successful application of this method includes carefully selecting and designing the experimental model of embryonic organ explants to perform transfections, and establishing criteria to select microRNAs of interest based on their relative expression in the organ of interest, and predicting microRNA target genes and assessing their expression in the organ of interest (further discussed on COMMENTARY).

BASIC PROTOCOL 1. TRANSFECTION OF ANTAGOMIRS AND MIMICS IN EXPLANTED ORGANS USING PEPTIDE-BASED NANOPARTICLES

The efficiency of liposome-based antagomir and mimic transfections, which we previously used to interfere with microRNA function in explanted embryonic SMGs (Rebustini et al., 2012), can be affected by the cytotoxicity of the liposomes, triggering stress-induced off-target effects, as well as by the degradation of the cargo molecules (antagomirs or mimics) via the endocytic pathway (Deshayes et al., 2010). In addition, traditional liposome-based

transfections are less efficient when targeting non-dividing, neuronal, or epithelial cells, compared to mesenchymal tissues and fibroblasts (Veldhoen et al., 2008).

This protocol describes the application of peptide-based nanoparticles to deliver antagomirs and mimics to perturb microRNA function during organ morphogenesis (Rebustini et al., 2016). To develop a robust, versatile, and reproducible protocol, and as a qualitative indication of the efficiency of peptide-based nanoparticles delivery, a preliminary step of this protocol involves optimizing transfection efficiency using a fluorescently Cy3-labeled, traceable off-target microRNA to visualize uptake in a variety of embryonic organs and tissues such as the presumptive mandible (which contains tooth germs), SMG, kidney, and lung explants (Fig. 1).

We further discuss some criteria to select microRNAs for targeting using nanoparticle-based transfections of antagomirs and mimics, and the appropriate off-target reagents (on COMMENTARY).

Materials

N-TER Peptide (Nanoparticle-Forming Solution, Sigma, cat.no. N2913-1ML)
 Antagomirs and Biotinylated Mimics (QIAGEN and Dharmacon)
 Off-target miR-Cy3 microRNA (QIAGEN)
 0.7 mL sterile plastic tubes (Eppendorf or similar)
 Rainin Pipet Plus (Rainin, P1000, P200, and P10)
 Sterile Filter Pipette Tips (1000 µL, 200 µL, 10 µL)
 Fetal Bovine Serum (Stasis Stem Cells, cat.no. 100–125, or similar reagents)
 mirVana™ total RNA Isolation Kit, with Phenol (AMBION, cat.no. AM1560)
 iScript™ cDNA Synthesis Kit (BIORAD, cat.no. 720001180)
 iQ™ SYBR® Green Supermix (BIORAD, cat.no. 170-8880)
 miScript II RT Kit (QIAGEN, cat.no. 218160)
 miScript SYBR® Green PCR Kit (QIAGEN, cat.no. 218073)
 qPCR primers:
 Mature miRNA expression: miScript Primer Assays (QIAGEN)
 miRNA targets and other genes (Invitrogen primers)

Preparation of Nanoparticle Forming Solution

- 1 Use the adapted Trowell-type procedure (Munne et al., 2012) to culture mouse E13.5 mandibular explants (further instructions are provided on REAGENTS AND SOLUTIONS), or use floating-filter system to culture embryonic SMGs, lungs, and kidneys explants, prior to set up transfections (Rebustini et al., 2016).

Prepare Trowell-type and floating filter explant systems by following the SUPPORT PROTOCOL 1.

- 2 Dilute the antagomirs and mimics to 20 μM stock solutions in H_2O , make 10–50 μL aliquots in sterile 0.7 mL tubes, and store the aliquots at -20°C . The stock antagomirs and mimics solutions can be used for preparation of Nanoparticle-based Forming Solution (NFS) for transfections.
- 3 For NFS preparation, use three 1.7 mL tubes, and label them as Solution 1, Solution 2, and the corresponding final NFS tube.
- 4 Prepare 50 μL of diluted antagomirs or mimics at 5 μM from stock solutions at 20 μM . In the tube labeled as Solution 1, pipette 52 μL of diluted antagomir or mimic, 148 μL of N-TER dilution buffer (provided in the N-TER reagent kit), and vortex for few seconds. Place the tube on ice.
- 5 In the tube labeled as Solution 2, pipette 32 μL of N-TER peptide, 168 μL of water, and vortex for few seconds. Place the tube on ice.

The ratio of N-TER peptide dilution was empirically tested and optimized for organ explants enlisted in this methodology (Rebustini et al., 2016). Further optimizations testing different ratios of N-TER peptide and cargo molecules (antagomirs or mimics) may be necessary for adapting this protocol to organ explants not tested in this methodology.

- 6 Pipette the solutions 1 and 2 in the tube labeled as NFS. Carefully label NFS corresponding to off-target control and antagomirs and mimics corresponding to the microRNAs of interest. Gently mix the solution by pipetting up and down 10 times.
- 7 Incubate NFS tubes at room temperature for 20 minutes.

The NFS tubes can be frozen and stored at -20°C for as long as a month, or kept at 4°C for as long as one week.

Unlike transfections using liposomes (that require increasing liposome amounts with cargo molecule concentrations), the ratio of N-TER peptide and antagomirs or mimics remains the same all the time. Use different volumes of NFS solution at 650 nM when different concentrations of antagomirs or mimics are required. Critical point: do not replace more than 20% of the culture medium by the NFS solution (organ explants growth and the corresponding morphogenetic parameters of interest may be compromised if their culture medium is too diluted).

Transfection of nanoparticle-forming solution in organ explants

- 8 Prepare a multiwell 12 wells plate containing the metal grids or the floating filters (Fig. 1), and dissect the embryonic mandibles (as described in SUPPORT PROTOCOL 1). Add a final volume of 2.0 mL of culture medium per well.

- 9 Pipette out 510 μ L from each one of the wells previously prepared to contain the organ explants.
- 10 Pipette 310 μ L of NFS and add in each well of the multiwell 12 wells plate.

Note that the final concentration of antagomir or mimic will be 100 nM, typically used for most organ explants described in here. Concentrations of antagomirs and mimics lower than 50 nM may not work properly, and may require further organ explant volumes optimizations.

- 11 Pipette 200 μ L of Fetal Bovine Serum (FBS) into each well to a final dilution of 10% of FBS in the culture medium. Mix gently by pipetting up and down the culture medium.

Typically, SMG organ explants do not require FBS, unlikely tooth germs (or mandible explants).

- 12 Incubate the embryonic organs in the tissue incubator with 5% CO₂ for up to 96 h, and monitor the organ cultures at daily basis.

Dedicate an incubator for embryonic organ explants only, as the explants are prepared in non-sterile conditions.

- 13 Culture the embryonic mandibular explants for 24 h for RNA extraction and microRNA or gene expression analysis, and 48 h for immunofluorescence.

For RNA preparation, multiple protocols and kits are available (we recommend RNA preparation using mirVana™ total RNA Isolation Kit). For immunofluorescence, multiple protocols are available (Rebustini et al., 2009; Costantini et al., 2011; Rebustini et al., 2009-2). For RT-qPCR analysis of microRNA and target gene expression, we recommend BioRad, QIAGEN and Invitrogen reagents (primers may be custom made and requested according to the target microRNA or gene of interest).

SUPPORT PROTOCOL 1 (optional). EMBRYONIC ORGANS EXPLANTED IN TROWELL-TYPE OR FLOATING FILTERS

In a further refinement of the Trowell-type organ culture (Jernvall et al., 2012; Munne et al., 2012), multiple explanted embryonic mandibles containing both molar and incisor tooth germs can be placed directly on top of a metal grid without the Nuclepore filters, with no apparent adverse effects (Fig. 1). This final streamlined procedure, which is applicable to explants significantly larger than the grid mesh size, obviates both the need to dissect the tooth germs from the mandibles and the need to place them on the top of floating filters. It also preserves the anatomical orientation of the embryonic mandibles and of the molar and incisor tooth germs, and facilitates the preparation of frozen and paraffin embedded sections and the analysis of morphogenetic changes via histology and immunofluorescence

microscopy. For branching organs such as salivary glands, kidneys, and lungs, floating-filter systems are the ideal choice (Rebustini et al., 2009-2).

Materials

DMEM-F12 (GIBCO, cat.no. 11330-032)

Penicillin and streptomycin (PS) antibiotics (GIBCO, cat.no. 15140-122)

Bovine Serum Albumin (BSA, Fisher, cat.no. BP 1600-100)

Fetal Bovine Serum (Stasis Stem Cells, cat.no. 100-125)

Ethanol 70% (spray bottle, 70% of ethanol and 30% of distilled water)

Ethanol, molecular biology grade (many sources)

Phosphate Saline Buffer (PBS, Thermo Scientific, cat.no. SH30378.02)

Dispase Neutral Proteases (Invitrogen, cat.no. 17105-0412q)

Rainin Pipet Plus (Rainin, P1000, P200, and P10)

Sterile Filter Pipette Tips (1000 μ L, 200 μ L, 10 μ L)

Sterile 1.7mL Tubes (Eppendorf and other sources)

Parafilm (Sigma-Aldrich, cat.no. P7793)

Bench Top Centrifuge Eppendorf 5148 (Eppendorf)

Laboratory Rocker (many sources)

Table Vortex Mixer (many sources)

Plastic Metric Pipettes (5.0, 10.0, and 25.0 mL, many sources)

CO₂ incubator Thermo Scientific NAPCO Series 8000 WJ

12 Wells Multiwell Tissue Culture Plates (Falcon, cat.no. 353043)

WhatmanTM NucleporeTM Track-Etched Polycarbonate Membrane Filter, diameter: 1.3cm, pore size: 0.1 μ m (Capitol Scientific, cat.no. 110405)

Petri dishes (100 mm and 150 mm) (VWR, cat. no. 25384-342 and 25384-139)

Metal mesh (corrosion-resistant stainless steel, 0.7 mm mesh size, many sources)

Sterilized 60 mm Corning Glass Dish (many sources)

Surgical Instruments:

- Forceps, Fine Dressing 12.5 cm (Dumont, cat.no. 503283)
- Forceps no. 5 CO (Dumont, cat.no. 11295)
- Tweezers Dumont no. 7, 12 cm, Inox (Dumont, cat.no. 14097)
- Forceps, Iris 10 cm, CVD Serrated (Dumont, cat.no. 15915)
- Scalpel Handle no. 4, 14 cm (Dumont, cat.no. 500237)

Scalpel Blade no. 11 (Sterling, cat.no. 500240)

Scissors for Tenotomy, 10 cm (Dumont, cat.no. 14396)

Perforated Round Spoon, 15 mm (Dumont, cat.no. 10370)

Stereomicroscope Leica M80 and camera Leica IC80 HD

Stereomicroscope Zeiss Discovery.V8

Embryonic organ dissections

1. The embryonic stages for organ harvesting for this comparative analysis are typically E11.5 for lungs and kidneys, and E13.5 for salivary submandibular glands (SMGs) and for molar tooth germs, which represent early stages of morphogenesis for these organs (Fig. 1)
2. Working on a bench space sterilized with Ethanol 70%, place the corresponding pregnant and euthanized mice on their back in a 150 mm Petri dish.
3. Spray liberally the mouse fur with 70% ethanol.
4. Surgically open, using 70% ethanol-sterilized scissors and a serrated forceps, the abdomen of the mouse to expose the uterus.
5. Remove the uterus and place it in a 150 mm Petri dish containing 15–20 mL of DMEM-F12/PS. Using scissors, cut the embryos from the uterus, separating each individual embryo.

At E11.5 the embryos are very small, and the uterus tends to contract and swell when removed from the pregnant mouse, making it difficult to separate individual embryos without damaging them. Work fast to remove the embryos after harvesting the uterus from the pregnant mice.

6. Wash the embryos, typically 12–16 embryos per litter, with 15–20 mL of DMEM-F12/PS, placing the dish in the rocker for 5 minutes. Repeat this washing step.
7. Under a dissection stereomicroscope, place the embryos in a 100 mm Petri dish containing 10–15 mL of DMEM-F12/PS. Start releasing the embryos from the uterus using scissors and collecting the embryo with a perforated spoon.

Use one 150 mm Petri dish per embryos litter, and keep the litters in separated Petri dishes to avoid mixing organs harvested at different embryonic stages, and sort out the outlier embryos (too small or too big).

8. Transfer the embryos using a perforated sterile spoon to a new 100 mm Petri dish containing 10–15 mL of DMEM-F12/PS. Under the stereomicroscope, dissect each organ by following specific methods of harvesting molar tooth germs (Munne et al., 2012), SMGs (Rebustini et al., 2009-2), lungs (del Moral et al., 2010) and kidneys (Costantini et al., 2011; Barak et al., 2011) as previously described.

9. Transfer freshly dissected molar germs, SMGs, lungs, and kidneys, using a P1000 Pipette, from the 100 mm Petri dish to a sterilized 60 mm Corning glass dish containing 1 mL of sterile PBS solution. Collect dissected organs using a P1000 Pipette and transfer to a sterile 1.7 mL tube, placed on ice.

Cut off approximately 2 mm of the end of the 1000 tip in order to easily aspirate the embryonic organs in a minimum DMEM-F12/PS volume (> 100 μ L).

10. Reserve the separated embryonic organs for RNA extraction or for use in explanted organ cultures.

For RNA extraction, immediately freeze the intact organs in dry ice, with or without RNA Lysis buffer (typically 100–200 μ L), and store the tubes containing the frozen organs at -80°C up to 12 months. Proceed with miRvana RNA extraction kit protocol, or any RNA methodology of choice, for further microRNA or gene expression analysis.

11. Using fine forceps, transfer the embryonic organs to floating filters placed in the 12 wells multiwell plate, or transfer the embryonic mandibles (containing molar and incisor germs) on the top of the metal meshes placed in the multiwell plate.

When using embryonic mandibular explants, they should not float on the top of the metal grid, and the height of the metal grid may vary slightly from well to well. Pipette out extra culture medium and correct the final volume to allow the embryonic explants to rest on the top of the metal grid, and at the same time being completely covered with enough culture medium to avoid exposing the surface of the oral epithelium directly to the air. By setting up your organ cultures this way, you ensure proper capillarity exchange of nutrients from culture medium, and CO_2 /oxygen exchange between culture medium and air interface (remember: there is no nutrients circulation system in this organ explant system).

BASIC PROTOCOL 2. MICRORNA PULL DOWN (miR-PD)

An additional feature of this protocol is the validation of predicted microRNA target genes using an adapted pulldown assay (miR-PD) (Lal et al., 2011). We recommend this assay in select cases where the morphogenetic loss-of-function and gain-of-function assays reveal compelling and consistent phenotypes. Moreover, in the ideal case, the mimic selected for miR-PD will have already been transfected in its unmodified form, analyzed, and shown to confer an expected or interesting morphological phenotype. In fact, biotinylated and non-biotinylated mimics exhibit almost indistinguishable effects²¹, which allows using biotinylated mimic for both microRNA gain-of-function (similarly to over-expressing the corresponding mature microRNA) and miR-PD.

A workflow for the miR-PD protocol for organ explants (Fig. 1) and a corresponding RT-qPCR calculation flowchart are provided. The goal is to determine whether the predicted

microRNA target genes are enriched in the pulldown fraction when using biotinylated mimic after capturing it using streptavidin beads.

Materials

Dynabeads® MyOne™ Streptavidin T1 (Invitrogen, cat.no. 2021-09)

miR-PD Lysis Buffer

Blocking Buffer

Nuclease-free water (many sources)

Biotinylated microRNA mimics (Dharmacon)

Nanodrop 2000 Spectrophotometer (Thermo Scientific)

Bench Centrifuge Eppendorf (for PCR strip tubes)

Thermal Cycler BIORAD CFX96 C100

MagnaRack for Microcentrifuge Tubes (Invitrogen, cat.no. CS15000)

Homogenizer (many sources)

96-well PCR Plate, Non-Skirted, 25 plates, Low Profile (cat.no.)

Combitips Advanced, 0.5 mL, Standard

Microseal Sealing Tape for 96 wells PCR plate (BIORAD, cat.no. MSB1001)

Parafilm (Sigma-Aldrich, cat.no. P7793)

microRNA pulldown (miR-PD)

1. Dissect the E13.5 mandibles or SMGs, lungs and kidneys from embryos, and prepare organ explants (SUPPORT PROTOCOL 1) and transfections of biotinylated mimics as described above (BASIC PROTOCOL 1).
2. On the day of the miR-PD assay, collect the explanted organs previously transfected with biotinylated mimics in a new 12 wells multiwell culture plate containing 200 µL of PBS.

Note that you can place up to 10–12 embryonic SMGs or 5–6 mandible explants per well. The purpose of this step is washing the culture medium from the organ explants prior to dissociation with lysis buffer. Count how many wells or different treatments you have in your experimental set up. You will prepare the same number of tubes containing Dynabeads for each condition of your experiment.

3. Meanwhile, prime the Dynabeads using Blocking Solution previously prepared (see REAGENTS AND SOLUTIONS), as follows:
4. Pipette 100 µL of magnetic streptavidin beads into a sterile 1.7 mL tube, and place the tube on ice.

Cut approximately 2 mm off the end of a P1000 tip prior to pipetting the Dynabeads, and make sure to mix the beads very well by pipetting up and down the stock solution or vigorously using a vortex before using.

5. Remove the tubes from ice, and place them in a MagnaRack. Incubate the tubes for 2 minutes at room temperature, and pipette the supernatant out using a P200 pipette.

The purpose of this step is to wash the stock buffer and recover Dynabeads. Remove the supernatant slowly and avoid touching the Dynabeads.

6. Remove the tubes from the MagnaRack, and add 100 μ L of miR-PD Lysis Buffer (see recipe on REAGENTS AND SOLUTIONS). Mix gently by pipetting up and down Dynabeads 10 times. Add the tubes back in the MagnaRack. Incubate tubes on MagnaRack for 2 minutes, at room temperature. Remove supernatant from tubes.

7. Repeat the washing process described on 5 and 6. Place tubes containing Dynabeads on ice.

Make sure to remove as much supernatant as possible without disturbing the Dynabeads while tubes are in the MagnaRack. Try to avoid dilution of further Blocking Solution due to remaining washing solution left in the Dynabeads.

8. Pipette 200 μ L of miR-PD Blocking Solution, previously prepared (REAGENTS AND SOLUTIONS), into each tube containing beads. Seal the tubes with Parafilm.

9. Place tubes in a rocker and incubate for 2 h at 4 °C. While the beads are being blocked, proceed as follows:

10. Carefully transfer the organ explants from the multiwell plate to 1.7 mL sterile tubes, using fine forceps.

11. Pipette 200 μ L of miR-PD Lysis Buffer into the tubes containing organ explants.

12. Homogenize organ explants on ice using an automatic portable homogenizer for 10 seconds, gently moving the tip of the homogenizer up and down, for 10–12 times.

Place the tubes on ice during the homogenization process to avoid over heating.

13. Seal tubes with Parafilm and incubate them on ice (5 minutes).

14. Centrifuge tubes (8,500Xg, 10 minutes, 4°C), and carefully collect the supernatant (containing cytoplasmic lysate). This is the Input Fraction. Pipette the supernatant to a sterile 1.7 mL tube, and keep the tubes on ice.

Tubes containing the pellets (nuclei fraction and cellular debris of explanted organ previously homogenized) can be discarded, or frozen and store at -80°C for further additional analysis.

15. Remove tubes containing Dynabeads from the rocker at 4°C (step 9) and place them in a MagnaRack. Pipette out Blocking Solution, remove the tubes from MagnaRack and place them on ice.
16. Transfer the Input Fraction (step 14) from the tubes sitting on ice directly into tubes containing blocked Dynabeads. Seal the tubes containing Dynabeads with Parafilm.
17. Place tubes in a rocker at 4°C and incubate for 4 h.

Note that 4 h incubation was optimized specifically for the organ explants described in this protocol, in order to recover molecules that bind to the corresponding biotinylated mimic. Longer or short periods of incubation time may have to be optimized according to tissue or organ input amount. Also, longer incubation times may generate false positives in your experiment (further discussed in COMMENTARY).

18. Remove tubes from the rocker at 4°C , and place tubes in a MagnaRack. Pipette the supernatant into a new sterile 1.7 mL tube for immediate use, or freeze the supernatant. This corresponds to Input – PD Fraction, and contains total RNA and cytoplasmic contents that do not interact with the biotinylated mimic.
19. Pipette 200 μL of ice-cold miR-PD Lysis Buffer into the tubes containing the beads. The Dynabeads contain the PD (pulldown) Fraction. Remove tubes from MagnaRack, mix Dynabeads gently by pipetting the beads up and down 10 times. Place the tubes in the MagnaRack, incubate for 2 minutes at room temperature, and discard supernatant.
20. Repeat washing process (step 19) for 5 times, and collect the cleaned Dynabeads using MagnaRack. Dynabeads or your PD Fraction can be used immediately for final analysis or frozen in dry ice.

The Input-PD fraction, and the PD fraction (Dynabeads) can be stored at -20°C for 6 months prior to final analysis. Washing step must be performed 5 times to ensure that the beads containing the PD Fraction are clean from the Input-PD Fraction components. Less washing steps may potentially generate false positives due to contamination of mRNAs that do not bind to mimics captured in the Dynabeads.

21. Proceed to RNA extraction using an aliquot of 50 μL of Input-PD Fraction (collected in step 18) and Dynabeads or PD Fraction (collected in step 20).
22. Pipette 100 μL of RNA Lysis Buffer from the mirVanaTM total RNA Isolation Kit, to each Input-PD and PD Fraction, and perform RNA extractions following kit specifications.

23. Analyze the expression of the microRNA target genes using RT-qPCR using method of choice.
24. Use the flow chart (Fig. 1) and any method of RT-qPCR analysis, to calculate the percentage of enrichment of mRNAs that bind to the microRNA of interest (mimic).

REAGENTS AND SOLUTIONS

Culture medium

5 mL of Penicillin and Streptomycin (PS)

500 mL of DMEM-F12

FBS (10% for mandible explants, lungs and kidneys. Not necessary for SMGs)

Dispase Solution

5 mg/mL of Dispase in PBS.

10% Bovine Serum Albumin

10% (m/v) Bovine Serum Albumin diluted in PBS

miR-PD Lysis Buffer

Tris pH 7.5 20 mM

KCl 100 mM

MgCl₂ 5 mM

NP-40 0.3%

Prior immediate use, add Rnase-OUT (stock solution at 40U/μL) to have a final concentration of 70 u/mL, and add 1 tablet of Complete Mini Protease Inhibitor Cocktail per 10 mL. Place the complete miR-PD Lysis Buffer on ice. Use the complete miR-PD Lysis Buffer immediately, and store the miR-PD Lysis Buffer (without Rnase-OUT and Protease Inhibitors) for as long as 6 months at 4 °C. For typical experiments using 6–12 different experimental conditions (antagomirs and mimics treatments), prepare 10.0 mL of miR-PD Lysis Buffer, and keep on ice for as long as 6 hours. Prepare fresh miR-PD Lysis Buffer (do not store at 4°C or freeze it).

miR-PD Blocking Solution

BSA 10 mg/mL

Yeast t-RNA (10 mg/mL)

Prepare these dilutions in miR-PD Lysis Buffer

Metal grids for Trowell-type organ cultures

Instructions on how to prepare the metal grids are described (Mune et al., 2012). Commercially available organ culture dishes (Falcon, Becton Dickinson) with metal grids can also be used. Briefly, for the purpose of this method, when using a 12 wells tissue culture dish, prepare an octagonal-shaped mesh by bending the edges of a 17 mm by 17 mm square-shaped piece of metal mesh (Fig. 1). The height of the grid should be 3 or 4 mm (when using 2.0 mL of final culture medium volume). Place the mandibles with the oral epithelium facing up, on top of the metal mesh, and cover with culture medium (DMEM-F12/PS, with 10% FBS), or simply place floating-filters (shining side up) on top of the culture medium (DMEM-F12/PS, with 10% FBS, for lungs and kidneys, or simply DMEM-F12/PS, for SMGs).

COMMENTARY

Background Information

Methods for functional microRNA perturbation—Methods for functional microRNA perturbation *in vivo* such as the injection of chemically modified microRNA inhibitors (Krützfeldt et al., 2005), and viral infections using microRNA constructs (Xie et al., 2012), are mostly limited to postnatal development and can generate systemic off-target effects. Genetically targeting microRNAs using knockout mouse models, although allowing direct assessment of *in vivo* microRNA function (Park et al., 2010), are costly and time-consuming, and potential microRNA functional redundancy and unpredictable compensatory mechanisms can make the *in vivo* genetic screening daunting. The use of peptide-based nanoparticles transfection was originally employed in siRNA gene targeting (Deshayes et al., 2010; Morris et al., 1999) and to deliver DNA oligonucleotides in antisense gene therapy (Falzarano et al., 2014), to overcome the problems of liposome cytotoxicity and endocytic degradation. Cell-penetrating amphiphilic peptides possess a self-assembling property when in presence of nucleic acids, and have relatively higher affinity for single and double strand nucleic acids compared to liposomes. The mechanism of delivery cargo molecules using peptide-based nanoparticles still remains fairly elusive; however, the delivery occurs independently of endocytosis and probably involving pinocytosis. To date, the commercially available N-TER peptide (Sigma) is the most commonly used peptide for nucleic acid delivery in a variety of applications.

Antagomirs and mimics: formal definitions—The antagomir used in this methodology incorporates a chemical modification termed Locked Nucleic Acid (LNA) (Kauppinen et al., 2005) into an oligonucleotide that is designed to interfere with microRNA function. The LNA features a 2', 4' methylene-bridge in the ribose that forms a bicyclic nucleotide with higher affinity binding to its complementary microRNA. This allows the use of short LNA-modified oligonucleotides in multiple applications including microRNA *in situ* hybridization (Silahtaroglu et al., 2007; Obernosterer et al., 2007) and knockdown studies (Galardi et al., 2007). Conversely, a microRNA mimic corresponds to an oligonucleotide that shares exactly the same sequence corresponding to an 18–21 nucleotides mature microRNA of interest.

Choosing the experimental model of embryonic organ explant to perform

transfections—A number of embryonic organ explant methods are compatible with this protocol, among them: 1. Explanting individual embryonic organs that are cultured directly on Nuclepore floating filters, using methods similar to those established for SMG (Rebustini et al., 2009-2), lung (Carraro et al., 2010) and kidney (Barak et al., 2011) explants; and 2. Use of the classic Trowell-type system that typically involves explanting embryonic organs on Nuclepore filters suspended on a wire mesh with bathing of the explant by capillarity (Munne et al., 2012). In our experience, epithelial branching organs are best studied using Nuclepore floating filters where whole mount techniques can be used to visualize morphogenesis, while the early morphogenetic events of odontogenesis, for example, when detailed histology is required, are best assessed using the Trowell-type organ culture. The Trowell-type organ culture system is also useful in cases where it is desirable to explant larger tissue blocks such as intact E11.5-13.5 mouse embryonic mandibles that contain smaller and more difficult to handle early stage tooth germs. In this case, molar and incisor tooth germ development may be analyzed *in situ*, by tissue sectioning (paraffin-embedding or frozen sections) and immunohistochemistry, for example.

Establishing criteria to select microRNAs of interest—High and low throughput expression profiling have generated microRNA expression databases for different developing vertebrate organs (Landgraf et al., 2007; Rebustini et al., 2012; Rebustini et al., 2016; Michon et al., 2010; Oommen et al., 2012). Hence, an initial step that provides the input for this protocol consists of selecting the microRNAs for functional studies. This can be accomplished by establishing sets of differentially expressed microRNAs in representative ectodermal (*e.g.*, tooth germs and SMGs), endodermal (*e.g.*, lungs (Cushing et al., 2011)), and mesodermal (*e.g.*, kidneys (Potter et al., 2014)) organs during early organogenesis, either based on relative abundance within an individual organ (generating microRNA expression profiles), or after comparison of microRNAs among different organs to identify a set of microRNAs that are enriched in a particular organ (microRNA expression signatures) (Rebustini et al., 2016). Either RNA-Seq (Pritchard et al., 2012) or qPCR-based TaqMan Low Density Arrays (TLDA) (Wang et al., 2011; Hennessy et al., 2011) can enable fast and robust microRNA expression screening. Good protocols currently exist for RNA-Seq which is now commonly used, while TLDA, although limited to previously annotated mature microRNAs, has also been used to generate microRNA expression profiles for SMG morphogenesis (Rebustini et al., 2012). Starting from any of these microRNA expression datasets, we employ Bioinformatics and annotation-based criteria to select which microRNAs to functionally target in our experimental model.

Predicting microRNA target genes—The mechanism of action of a microRNA is based on the specificity of its 5'-UTR seeding region, a 6–8 nucleotide sequence that binds the complementary 3'-UTR sequence present in the corresponding target mRNA, which triggers mRNA degradation and translational downregulation (Guo et al., 2010; Zamore et al., 2005). It is possible to employ Bioinformatics to predict based on sequence homology the corresponding mRNAs (referred here as target gene) regulated by a particular microRNA. Among the available microRNAs target prediction programs (Sethupathy et al., 2006), we recommend Gene Ontology-based methods (Ritchie et al., 2009) that associate

microRNA targets with biological processes in the context of interest. To assist in this aspect, we recommend a target prediction pipeline that utilizes TargetScan (Rebustini et al., 2016; Grimson et al., 2007), a searchable homology-based prediction database that associates microRNA seeding regions with 3'-UTR sequences of potential microRNA target mRNAs. This target prediction protocol employs two selection filters: (1) expression of the potential microRNA target gene in the organ of interest using an appropriate gene expression database; and (2) target gene interaction analysis, using the Gene Ontology-based CytoScape-GeneMania plugin (Warde-Farley et al., 2010; Montojo et al., 2010). This analysis helps identify potential target mRNAs that are associated with organ-specific signaling pathways and morphogenetic processes.

Methods to validate microRNA targets—Among a number of methods to investigate microRNA:mRNA interactions (Rebustini et al., 2016), the pulldown protocol using biotinylated microRNA mimics (Lal et al., 2010) (miR-PD) provides the most straightforward means to validate microRNA-targeted mRNAs in their biological contexts. Luciferase Reporter Assays (Nicolas et al., 2011) are biochemically accurate but are limited to short sequences of mRNA 3'-UTR, and are typically performed in artificial contexts. Ribosome Profiling (Guo et al., 2010) captures the translational status of the corresponding mRNAs targeted by a microRNA, but both the experimental setup and data analysis are technically demanding and time-consuming. In contrast, miR-PD offers the advantage of avoiding the crosslinking step required in CLIP (CrossLinking ImmunoPrecipitation methodology), and of using antibodies that bind to RNAi machinery components such as Dicer and Ago2 (Moore et al., 2014). These steps often decrease the recovery of intact and non-degraded RNA.

Critical Parameters

To perform morphogenetic analysis of antagomir treated organ explants, priority should be given to morphometric parameters and/or to indices of epithelial and mesenchymal development that are quantifiable. For example, frozen sections of molars or mandibular explants are suitable for early morphometric analysis of molar development starting from the single bud stage at E13.5, continuing through the cap stage at E14.5, and concluding at the bell stage at E15.5 and E16.5⁴⁸. EdU incorporation can be assayed on frozen sections, following either pulse injection of the pregnant female, or labeling of the explant as in SMGs, and used to determine a proliferation index for the tissue of interest. Whole mount staining is another useful assay, albeit qualitative, for assessing gene expression in branching organs.

The lifespan of embryonic organ explanted in cultures must be considered. Desirable morphogenetic effects typical of early stages of organ development such as tissue proliferation (often localized in specific regions of a tissue in an given organ) usually do not recapitulate normal organ development at later times of explanted organs. The time spent between embryonic dissections and organ explants set up shows to be determinant of the quality of the morphogenetic process to be studied (plan carefully and resource literature information about best methods to handle the organ explant model of your interest).

Other critical parameters to perform the nanoparticle-based transfection of antagomirs and mimics in organ explants include the use of carefully tested and certified negative controls (off-target microRNAs), positive controls (usually, microRNAs known to play a role in the corresponding organ system), and carefully testing dose-responses for transfections starting with concentrations of antagomirs or mimics as low as 50 nM.

Troubleshooting

Several points for protocol troubleshooting should be considered, including:

1. Ratio of nanoparticles/cargo molecules (antagomir or mimics) used for transfections (step 5, BASIC PROTOCOL 1), if microRNA functional perturbation is not detected (low efficiency of microRNA expression knockdown when using antagomirs at different concentrations, for example).
2. Change transfection medium every two or three days of organ explant cultures, and attempt to changes in pH indicator (the medium becomes yellow as pH decreases), for potential sources of variation and artifacts in the experiments. Sources of variation include contamination of organ explants, too many organ explants per condition, and leaving the culture medium too long without replacing it (note that it is not recommended changing the medium every day, as the organ explants condition their culture medium)
3. For high throughput methodologies such as RNA-Seq that require more than 1 µg of total RNA, increase the number of technical replicates for a given transfection condition, in order to increase the input of starting material from miR-PD. Considering that less than 4% of total RNA corresponds to mRNA population in a cell, and within this population, only a fraction of it contains seeding regions that bind to a microRNA of interest, you should expect a very low amount of recovered RNA in the PD Fraction (step 19, on BASIC PROTOCOL 2). For the purpose of screen for predicted target genes of a microRNA using low throughput RT-qPCR platforms, the amount of RNA recovered in miR-PD (usually 20–30 ng) should be enough for a preliminary analysis
4. The onset of morphogenetic changes in organ explants occurs in a delayed time frame when compared to their corresponding normal developmental times *in vivo*. For discrete morphological changes such as during early tooth germ morphogenesis (cap stage, bell stage, etc), consider that these changes will take longer when in *ex vivo* contexts. Also consider that embryonic mandible explants are more robust and last longer culture systems (up to a week of explanting conditions), when compared with SMGs, for example.

Anticipated Results

Transfections of antagomirs and mimics in organ explants should ideally result in opposing phenotypes, but cautious interpretation must be used when analyzing the results, as there is no tissue targeting control for the transfections of cargo molecules (antagomirs and mimics). Due to tissue-specificity of some microRNAs (for example, miR-200c is expressed in

epithelial tissue of salivary glands and molar tooth germs), transfection of mimics in both epithelial and mesenchyme tissue compartments could generate undesirable artifacts, such as over expression of the target microRNA, originally abundant in the epithelium, also in the mesenchyme tissue.

Although one can demonstrate a dose-response relationship for transfected antagomirs and mimics, one cannot accurately quantify or distinguish between the specific amounts of mimic and endogenous microRNA. Also, concentrations of mimics lower than 50 nM do not significantly alter phenotypically tissues and organs when using nanoparticles. A dose-response preliminary study using different concentrations of antagomirs and mimics higher than 50 nM is still recommended as microRNAs relative abundance may require different doses of inhibitors and activators.

Lastly, the developmental defects after functional microRNA targeting should be corroborated molecularly. Following antagomir and mimic treatment, the corresponding set of microRNA target genes are expected to be upregulated and downregulated, respectively. Relative abundance and the half-life of individual mRNAs targeted by a given microRNA, and molecular turnover of mRNAs should be taken in consideration, in case discrepancies between expected and final results occur.

Furthermore, as expected, when miR-PD is performed, predicted microRNA targets should be enriched in the pulldown fraction, and the mRNAs that do not contain sequences complementary to the microRNA seeding region, should not be enriched in the pulldown fraction. Unexpectedly, however, mRNA containing predicted binding sites for the microRNA of interest might not be enriched in the pulldown assay. A possible explanation is that the region of this mRNA that is predicted to bind the corresponding microRNA seeding region is not available due to secondary structure of the mRNA.

Time Considerations

For the execution of this protocol, consider the time to set up the embryonic organ explants prior transfections. The SUPPORT PROTOCOL 1 usually takes 6–8 hours to accomplish; the BASIC PROTOCOL 1 is faster, and usually takes 2–3 hours to accomplish. The effects of transfection of antagomirs and mimics become significantly measurable at least after 24 h of transfections. For the BASIC PROTOCOL 2, the first step of blocking the Dynabeads takes approximately 3 hours, and the incubation of Dynabeads with organ lysates 4–6 hours, and the final step of the protocol (recovery of PD Fraction and RNA preparations) takes 2 hours. Final analysis time varies depending on methodologies employed (2–4 hours for gene expression analysis using RT-qPCR, and typically 2–3 days for immunohistochemistry).

Acknowledgments

The authors received funding for this research from NIDCR 5R01DE011697-20 and 1U01DE02443-2. This protocol has been developed in the Department of Medicine, Division of Genetics, at Brigham and Women's Hospital Harvard Medical School (BWH-Harvard), under supervision of Dr. Richard L. Maas, and in collaboration with Dr. Maria Kukuruzinska at Boston University Medical Center. We would like to thank Dr. Matthew Phillip Hoffman from the Laboratory of Cell and Developmental Biology, National Institutes of Dental and Craniofacial Research (LCDB-NIDCR-NIH), for providing the TaqMan Low Density Arrays (TLDA) microfluidic qPCR plates and data.

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Significance Statement

Functional microRNA studies during organ morphogenesis provide invaluable insights into how organs develop and regenerate. In this unit, we describe a method of interfering with microRNA function, by transfecting microRNA inhibitors (antagomirs) and activators (mimics) into mouse embryonic explanted organs using peptide-based nanoparticles. This method offers an update on traditional microRNA functional targeting using liposome-based transfections, by increasing efficiency and decreasing off-target effects during microRNA functional targeting, and can be applied for a variety of organs that can be explanted.

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Figure 1.

Overview of the methodology for transfection of antagomirs and mimics in embryonic explanted organs using nanoparticles. **(A)** The method consists of Nanoparticle Preparation, Organ Explants Preparation, and microRNA Target Validation (miR-PD). Nanoparticle Preparation involves loading an amphiphilic peptide with microRNA antagomir (inhibitor) or mimic (activator) to functionally target a microRNA of interest. Organ Explants Preparation involves micro-dissection of embryonic organs of interest and explanting these organs into multiwell culture plates, either directly onto metal mesh in a modified Trowel-type system (e.g., embryonic intact mandibles containing more tissues such as tongue and salivary glands, or isolated mandibles from the tongues attached to salivary glands) or directly onto floating Nuclepore filters (e.g. salivary glands, kidneys, lungs). MicroRNA Target Validation (miR-PD) involves preparations of organ lysates and incubation of the cytoplasmic fraction with biotinylated-mimic microRNA, followed by a streptavidin bead purification step to recover the biotinylated-miRNA mimic and its target mRNA transcripts. RISC: RNA Interference Silencing Complex (necessary for miRNA:mRNA interactions). **(B)** Representative epifluorescence images of *ex vivo* organ explants transfected for 48 h using 100 nM of Antagomir-Cy3 (red). Intact E13.5 mandible explants (upper panel, left), and after dissecting the epithelium and mesenchyme tissues with Dispase incubation (upper

panel, right), shows uptake of Cy3-labeled antagomir throughout epithelium (OE: oral epithelium; DE: dental epithelium) and in the mesenchyme (M). Lower panel shows immunofluorescence using E-Cadherin antibody detection (green) and Cy3-labeled microRNA uptake in embryonic branching organs. Scale bars: 100 μ . **(C)** Preparation of Trowell-type organ cultures and metal meshes. Metal mesh grids are prepared to fit into wells of a multiwell plate (12 wells), each designed to use 2.0 mL of culture medium. **(D)** Flowchart of calculation steps using Threshold Cycle numbers from qRT-PCR analysis, after RNA extraction from fractions obtained in miR-PD assay (see BASIC PROTOCOL 2) in order to calculate enrichment of mRNA after pulldown assay (miR-PD). All images are adapted from Rebustini et al., 2016, Scientific Reports.