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Protocols for Investigating microRNA Functions in Human Neural Progenitor Cells

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Abstract

Human embryonic stem cells and induced pluripotent stem cells offer great hope for studies of pathogenic mechanisms of disease and cell-based therapies. One powerful approach to manipulate the behaviors of human stem cells and their progeny is through microRNAs (miRNAs), a class of small noncoding RNAs that regulate gene expression at the posttranscriptional level. Each miRNA may target up to hundreds of mRNAs; some are specifically expressed in progenitor cells and affect multiple cellular processes. Here we present experimental protocols for investigating the endogenous functions of specific miRNAs in the proliferation, survival, and migration of human neural progenitor cells derived from embryonic stem cells. These methods may be applicable to protein factors and neural progenitor cells derived from patient-specific induced pluripotent stem cells.

Keywords

microRNA; Human stem cells; Neural progenitor cells; Proliferation; Migration; Apoptosis; Transplantation

1. Introduction

Human embryonic stem cells (hESCs) can develop along specific lineages into progenitor cells that in turn differentiate into diverse cell types, such as neurons, skin cells, and muscle cells (1, 2). Thus, hESCs and their progeny hold great promise for understanding human development and for regenerative medicine. This prospect is greatly enhanced by a technological breakthrough—the ability to use only a few transcription factors to directly reprogram human cells into induced pluripotent stem cells (iPSCs) (3). Now, patient-specific iPSCs with global gene expression profiles similar to those of hESCs can be generated with relative ease (4, 5).

Both hESCs and hiPSCs can be differentiated into human neural progenitor cells (hNPCs) and subsequently into various types of post-mitotic neurons using different protocols (6–8). Many attempts have been made to transplant hNPCs into animal models of human diseases, such as stroke (8), retinal degeneration (9), and Parkinson's disease (10). This therapeutic approach remains highly challenging, in part because we do not fully understand the characteristics and cellular behaviors of hNPCs. Much remains to be learned about the molecular mechanisms that control the proliferation, survival, migration, and differentiation of hNPCs *in vitro* and *in vivo*.

Among the important regulators of hNPCs are microRNAs (miRNAs), a class of noncoding RNAs of ~21–23 nucleotides that regulate mRNA translation and stability through base-

pairing, mostly with sequences in the 3' untranslated region (11, 12). miRNAs are excellent targets for molecular manipulations in hNPCs because of their small size and unique modes of action. For instance, although each miRNA is predicted to target hundreds of mRNAs (13), the effects of many miRNAs are mediated by one or a few key targets in a specific developmental process (14). Here, we describe in detail experimental approaches to knock down the expression of endogenous miRNAs in hNPCs and examine their physiological functions in the proliferation, survival, migration, and differentiation of hNPCs in vitro and ex vivo.

2. Materials

2.1. hNPC Culture and miR-9 Knockdown

1. Human ESCs (H9 line, WiCell Research Institute).
2. Mouse embryonic fibroblasts (MEFs) (PMEF-CFL), previously inactivated with mitomycin C (Roche Applied Science).
3. Human ESC medium: Dulbecco's modified Eagles's medium (DMEM)/F12 (Gibco) supplemented with 20% KnockOut Serum Replacement (Gibco), 1× nonessential amino acids (Gibco), 4 ng/mL human basic fibroblast growth factor 2 (FGF2; Gibco), and 0.1 mM 2-mercaptoethanol.
4. Neural induction medium (NIM) consisting of 33% F12 nutrient mixture (Gibco), 66% DMEM (Gibco), 1× N2 supplement (Gibco), 1× nonessential amino acids solution, 10 ng/mL FGF2, and 2 µg/mL heparin (Sigma).
5. Dispase (Gibco) is prepared at 1 mg/mL in DMEM/F12 and incubated at 37°C for 15 min to dissolve completely. It should be filter-sterilized and used fresh.
6. Poly-L-ornithine solution (Sigma).
7. Laminin (Sigma) is dissolved at 1 mg/mL in distilled water.
8. 24-well plates coated with Ultra-Low Attachment Surface (Corning).
9. miRCURY LNA microRNA inhibitor hsa-anti-miR-9, 5 nmol (Exiqon) and miRCURY LNA microRNA inhibitor negative control A, 5 nmol (Exiqon).
10. Lipofectamine 2000 (Invitrogen).
11. Opti-MEM I reduced-serum medium (Invitrogen).
12. miRNeasy kit (Qiagen).
13. Turbo DNA-free kit (Ambion).
14. TaqMan microRNA reverse transcription kit (Applied Biosystems).
15. Reagents for TaqMan microRNA assays: hsa-miR-9 and RPL21/small nucleolar RNA (snoRNA) (Applied Biosystems). Each TaqMan assay kit provides primers for RT (5×) and qPCR (20×).
16. TaqMan Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems).

2.2. Cell Proliferation and Viability Assays

1. In Situ Cell Death Detection Kit, TMR Red (Roche Applied Science).
2. Matrigel (BD Bioscience).
3. Fixation solution: 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4, freshly prepared.

4. Permeabilization solution: 0.1% Triton X100 in 0.1% sodium citrate, freshly prepared.
5. DNase I recombinant (Roche Applied Science) solution: 300 U/mL in 50 mM Tris-HCl, pH 7.5, 1 mg/mL bovine serum albumin.
6. Glass coverslip.
7. Vectashield mounting medium (Vector Laboratories).
8. CytoTox 96 nonradioactive cytotoxicity assay (Promega).
9. 5-Bromo-2'-deoxyuridine (BrdU) is dissolved at 1 mg/mL in water. Combine 10 mg of BrdU with 10 mL of water and filter sterilize with a 0.22- μ m filter. Dispense in 1-mL aliquots and freeze at -20°C.
10. PBS/1% Triton X-100.
11. 1 M and 2 M HCl.
12. 0.1 M borate buffer: Prepare 200 mL of 0.2 M sodium borate and 200 mL of 0.2 M boric acid. Add boric acid to sodium borate solution until pH 7.4 is reached.
13. Blocking solution: Combine 0.1 g bovine serum albumin (BSA), 0.5 mL donkey serum (Sigma), and PBS/Triton X-100 to make 10 mL final volume.
14. BrdU antibody (Abcam).
15. Alexa Fluor 594 donkey anti-mouse (Molecular Probes).
16. Hoechst 33258 (Invitrogen) is dissolved at 1 mM in distilled water.
17. Cell proliferation reagent WST1 (Roche Applied Science).

2.3. Migration Assays

1. GFP-FUGW plasmid (Addgene).
2. PEG-it Virus precipitation solution (SBI System Biosciences).
3. E14.5 C57BL/6 pregnant mice (Charles River Laboratories).
4. Autoclaved dissecting tools.
5. Penicillin-streptomycin solution.
6. Rinse solution: Hanks' balanced salt solution (HBSS) (Gibco) containing 100 U/mL penicillin/streptomycin.
7. Embedding medium: 2.5% low-melt agarose (Sigma) in slicing medium.
8. Slicing medium: DMEM/F12 (Gibco) and glucose (final concentration, 6.5 mg/mL).
9. Vibratome for brain slices. Razor blade cleaned with ethanol before use.
10. Millicell cell culture insert (Millipore).
11. Nutrition medium: DMEM/F12, 10% Characterized Fetal Bovine Serum (HyClone) and 100 U/mL penicillin/streptomycin.
12. Standard Wall Borosilicate Tubing (Sutter Instrument).

3. Methods

3.1. In Vitro Differentiation of Neural Precursors from hESCs

1. Human ESCs are cultured on an MEF feeder layer (Fig. 1); the medium should be refreshed daily. To obtain efficient and reproducible differentiation of hESCs and to prevent premature differentiation or cell death, colonies must be split before they become too large or start to fuse with each other.
2. When it is time to split the colonies, remove the medium from three wells of a 6-well plate containing hESC colonies (see Note 1).
3. Add 0.5 mL of hESC medium and 0.5 mL of dispase (final concentration, 0.5 mg/mL) to each well.
4. Incubate in a CO₂ incubator for 5–10 min until the colonies start to curve up.
5. Remove the dispase and add 1 mL of hESC medium to each well. Gently scrape cells with a cell lifter and pool the suspension into a 15-mL conical tube. Be careful to not break up the colonies.
6. Centrifuge at $200 \times g$ for 2 min at the room temperature.
7. Aspirate supernatant from hESC pellet. Resuspend pellet in hESC medium without FGF2 and centrifuge again.
8. Remove medium and resuspend cell pellet in 8 mL of hESC medium without FGF2.
9. Culture cells for 4 days in a T25 flask; change half of the medium daily (see Note 2). Human ESC cell aggregates will form embryoid bodies (EBs) in 24 h.
10. On day 5, collect the EBs into a 15-mL tube and centrifuge at $50 \times g$ for 1 min at room temperature.
11. Wash EBs with 5 mL of NIM and centrifuge again.
12. Resuspend EBs in 24 mL of NIM and dispense 8 mL of the suspension per T25 flask, each freshly pre-coated with poly-L-ornithine/laminin (see Note 3).
13. EBs attach to the pre-coated plastic substrates in 2 days. After that, replace 5 mL of culture medium with fresh NIM every other day. Cells will begin to migrate out from the EBs, and in a few days elongated cells will appear and form rosettes.
14. On day 16–18 of differentiation culture (11–13 days after plating the EBs), isolate neuroepithelial cells in the rosettes from the surrounding cells. Wash the rosette culture once with PBS and add 4 mL of NIM and 1 mL of dispase (final concentration, 0.2 mg/mL).
15. Incubate in a CO₂ incubator for 15 min. Retracted rosette clumps will begin to come off the plate; if necessary, tap sides of the flask and incubate for additional 5–10 min.
16. Collect rosette clumps from the three T25 flasks into a 15-mL tube and triturate them with a 5 mL pipette but without breaking up the clumps. Centrifuge at $50 \times g$

¹For simplicity, all the volumes in this section are given for three wells of a 6-well plate. We recommend using at least a full 6-well plate. The procedure for isolating hNPCs was adapted from ref. (15).

²Floating hESC aggregates are transferred to new flasks in the first 2 days to eliminate contaminating MEFs that attach to the flask.

³T25 flasks are first coated with 2.5 mL of poly-L-ornithine at 37°C, overnight, washed three times with 5 mL of PBS, and coated with 20 µg/mL laminin (1 mL) for 4 h at 37°C. Flasks are washed three times with PBS just before use.

for 1 min at room temperature and aspirate medium. Add 10 mL of NIM and centrifuge again to wash away the dispase and single cells.

17. Resuspend the cells in 6 mL of NIM, transfer to a T25 flask, and incubate in a CO₂ incubator for 2–3 h to allow nonneural cells to attach to the flask.
18. Collect the floating cells into a 15-mL tube and centrifuge at $50 \times g$ for 1 min. Resuspend the pellet in 3 mL of NIM.
19. Mechanically dissociate them into smaller clumps (around ten cells) with a 5 mL pipette (see Note 4). Divide cells in suspension into six wells of a 24-well plate coated with Ultra-Low Attachment Surface.

3.2. Transfection with Anti-miR-9 LNA Probe (See Note 5)

1. Prepare LNA/Lipofectamine complexes. For one well of a 24-well plate: mixture A, combine 2 μ L of hsa-anti-miR-9 or scrambled control (see Note 6) (final concentration, 100 nM) and 50 μ L of Opti-MEM I. For mixture B, in a separate tube, combine 2 μ L Lipofectamine with 50 μ L of Opti-MEM I. Mix well and incubate 5 min at room temperature.
2. Add mixture A to mixture B, mix well, and incubate for 20 min at room temperature.
3. Apply the complexes to the cells. Incubate at 37°C in a CO₂ incubator overnight.
4. The next day, add 0.5 mL of NIM. Move the plate back to the CO₂ incubator. Assay for miR-9 knockdown 24 h later and at later stages (see Note 7).

3.3. Analysis of miR-9 Expression

1. Extract total RNA with the miRNeasy kit according to the manufacturer's instruction.
2. Purify RNA with the Turbo DNA-free kit following manufacturer's instruction.
3. Determine RNA concentration by measuring absorbance at 260 nm in a spectrophotometer (see Note 8).
4. Reverse transcribe 50 ng of total RNA with the TaqMan microRNA reverse transcription kit and miR-9-and RPL21/snoRNA-specific RT primers (TaqMan microRNA assays) in the same RT reaction (see Note 9).

⁴Breaking up the clumps is necessary for better transfection efficiency; however, disaggregated individual cells will die. Use a pipette to gently break up the clumps and watch the suspension closely to obtain best transfection condition.

⁵It is important to transfect the multipotent hNPCs immediately after isolating the rosettes and generating the small-clump suspension, before the clumps form neurospheres, which are more difficult to transfect.

⁶Proper controls are important for any knockdown experiment. It is always necessary to verify that the observed phenotype is not reproduced when using a negative control (scrambled) LNA knockdown oligonucleotide. A custom-designed mismatch control LNA knockdown oligonucleotide can be used to assess the specificity of the miRNA knockdown.

⁷In the early stages of planning miRNA knockdown experiments, it is important to profile its expression and ensure that the miRNA is actually expressed in the cells (See ref. [8] for details on miR-9 expression in hNPCs). At 16 days of hESC differentiation, hNPCs are more homogenous since they were derived shortly after dissociation from the rosette structures. The ability to study miR-9 function in early-stage hNPCs is an advantage of using hESC-derived hNPCs in culture. First, one can determine the specific roles of miR-9 at particular developmental stages. Second, the lower expression level of miR-9 than at later stages does not diminish its functional significance at this particular developmental stage. The same miRNA may have different functions at different developmental stages of various NPCs. Ultimately it is important to determine the efficiency and the time point of the transient knockdown. Knockdown studies, including miRNA-target interactions and rescue experiments, are more suitable to reveal miRNA endogenous and physiologically relevant functions. Overexpression of miRNAs in certain cellular contexts can have dramatic nonphysiological effects.

⁸Besides absorbance at 260 nm (A₂₆₀), the A₂₆₀/A₂₈₀ ratio should be calculated to verify the purity of the sample. This value should be in the range 1.8–2.0. A value <1.8 indicates contamination that could interfere with the qPCR.

5. Incubate the reaction tube at 16°C for 30 min, 42°C for 30 min, and 85°C for 5 min in the thermal cycler. Store reaction tubes at –20°C if not proceeding to the next step.
6. For qPCR amplification, prepare a qPCR master mix for each primer. For each 20 µL reaction, add 1 µL of TaqMan MicroRNA Assay (20×; forward primer, reverse primer, and probe), 10 µL of TaqMan Universal PCR Master Mix (2×) and 4 µL of nuclease-free water.
7. Dilute the RT reaction 1:12 (see Note 10).
8. Transfer 5 µL of diluted RT reaction into each well of a 96-well qPCR plate (each sample should be run in triplicate). A no-template control reaction should also be prepared to evaluate background signal.
9. Add 15 µL of the master mix (miR-9 or RPL21) to each well.
10. Seal the plate with the optical adhesive cover and centrifuge the plate for 2 min.
11. Load the plate into the instrument and run the following thermal cycling conditions: 95°C for 10 min followed by [95°C for 15 s and 60°C for 60 s] for a total of 40 cycles. Quantify at the threshold detection line (Ct value) and normalize the Ct of miR-9 to that of human snoRNA located within an RPL21 gene intron.

3.4. Evaluation of Cell Viability with the TUNEL Assay

1. The day after transfection (or up to 6 days after; see Subheading 3.2), transfer hNPCs (whole or dissociated neurospheres) to Matrigel (1:100)-coated wells (24-well plate) and allow cells to attach for 3–4 h (see Note 11).
2. Remove medium from cells and rinse with PBS.
3. Fix cells in 0.3 mL of 4% paraformaldehyde for 1 h at room temperature (see Note 12).
4. Rinse cells with PBS and incubate with 0.3 mL of permeabilization solution for 2 min on ice.
5. Rinse cells twice with PBS.
6. Prepare positive control: incubate two wells with DNase I recombinant for 10 min at room temperature to induce DNA strand breaks. Rinse with PBS.
7. Prepare TUNEL reaction mixture (500 µL) by adding total volume of enzyme solution (vial 1, In Situ Cell Death Detector Kit) to 450 µL of Label Solution (vial 2, In Situ Cell Death Detector Kit). Mix well with a pipette.
8. Add 50 µL of TUNEL reaction mixture to each well (including positive control). For the negative control, add 50 µL of label solution (vial 2) to each well (prepare two wells).

⁹The endogenous control for real-time quantification of miRNA with TaqMan microRNA assays we chose shares similarities with the miRNA, such as RNA stability and size. This assay uses human snoRNA located within an intron of the RPL21 gene. The suitability of this snoRNA as endogenous control was validated across a wide variety of tissues and cell lines (Applied Biosystems Application Note).

¹⁰The dilution factor must be adapted depending on the level of expression of the miRNA studied.

¹¹As an alternative to Matrigel-coated plates, poly-D-lysine and laminin-coated coverslips (BD Bioscience) can be used. This assay is easier, and better pictures can be obtained, if cells are attached to the plate/coverslip. To facilitate the counting labeled cells, neurospheres can be mechanically dissociated with dispase before the assay.

¹²Paraformaldehyde is toxic and carcinogenic and should be disposed properly.

9. Cover plate with parafilm and incubate in a humidified atmosphere for 1 h at 37°C in the dark.
10. Rinse three times with PBS.
11. Add a drop of mounting medium and place a glass coverslip on top. Samples are ready to be analyzed under a fluorescence microscope (see Note 13 and ¹⁴) See example in Fig. 2a.

3.5. Evaluation of Cell Viability with the LDH Assay (See Note 15)

1. The day after transfection (or up to 6 days; see Subheading 3.2), remove medium and add 0.5 mL of fresh NIM to each well. Add medium to an empty well as a background control.
2. Twenty-four hours later, move the contents of each well (medium and neurospheres) to a 1.5-mL centrifuge tube and centrifuge at $5,000 \times g$ (Microcentrifuge) for 2 min.
3. Collect each supernatant in a new tube (released LDH) and add 0.5 mL $1\times$ lysis buffer (CytoTox 96 Cytotoxicity Assay kit) to each cell pellet (intracellular LDH). Mix well.
4. Incubate the tubes containing the cell pellet at -70°C for 20 min. Thaw at 37°C for 2–3 min and centrifuge the tubes at $10,000 \times g$ (Microcentrifuge) for 4 min.
5. Transfer 50 μL of supernatant/cell lysate of each sample to a 96-well flat-bottom plate. For the background controls, transfer 50 μL of the medium/ $1\times$ lysis buffer. All samples should be done in duplicate.
6. Add 12 mL of room temperature Assay Buffer (CytoTox 96 Cytotoxicity Assay kit) to a bottle of Substrate Mix (CytoTox 96 Cytotoxicity Assay kit). Invert and shake gently to dissolve the substrate. Protect the substrate solution from light and use immediately.
7. Add 50 μL of the substrate solution to each well of the plate. Protect plate from light and incubate at room temperature for 30 min.
8. Add 50 μL of Stop Solution (CytoTox 96 Cytotoxicity Assay kit) to each well. Read absorbance at 490 nm.
9. Subtract background values from the sample readings and determine cytotoxicity by calculating the percent of LDH released into the medium out of the total LDH activity (the sum of intracellular and released LDH activity) (Fig. 2b).

3.6. BrdU Staining

1. The day after transfection (or up to 6 days after; see Subheading 3.2), transfer hNPCs to Matrigel-coated wells (24-well plate) and allow cells to attach for 3–4 h (see Note 11).
2. Dilute BrdU stock solution in NIM to obtain a 1 mM BrdU solution.

¹³If a coated glass coverslip was used in Subheading 3.4, step 1, add a drop of mounting medium to a glass slide and place the coated glass coverslip with cells facing down on top of the slide.

¹⁴Use an excitation wavelength of 520–560 nm (maximum 540 nm; green) and detection at 570–620 nm (maximum 580 nm, red).

¹⁵The amount of LDH released into the medium is proportional to the number of dead cells.

3. Add 10 μL of the 1 mM BrdU solution per mL of culture medium to each well (final concentration, 10 μM) (e.g., if the well volume is 0.5 mL, add 5 μL to each well). Incubate for 4 h.
4. Remove medium from cells and rinse once with PBS.
5. Fix cells with 4% paraformaldehyde for 30 min at 4°C.
6. Wash cells three times for 5 min each with PBS/1% Triton X100.
7. Incubate cells in 1 M HCl for 10 min on ice. This step breaks open the DNA structure of the labeled cells.
8. Incubate in 2 M HCl for 10 min at room temperature followed by 20 min at 37°C.
9. Remove HCl and add 0.1 M borate buffer for 12 min at room temperature.
10. Wash cells three times for 5 min each with PBS/1% Triton X-100.
11. Incubate with blocking solution for 30 min at room temperature.
12. Dilute anti-BrdU antibody 1:400 in blocking solution and incubate at 4°C overnight.
13. Wash cells three times for 5 min each with PBS/1% Triton X-100.
14. Dilute Alexa Fluor 594 anti-mouse antibody 1:500 in blocking solution and incubate at room temperature for 2 h. Protect plate from light.
15. Wash cells for 5 min with PBS/1% Triton X-100 and twice for 5 min each with PBS.
16. Incubate cells with Hoechst (final concentration, 1 μM in PBS) at room temperature for 5–10 min.
17. Rinse cells with PBS.
18. Add a drop of mounting medium and place a glass coverslip on top. Samples are ready to be analyzed under a fluorescence microscope (Fig. 2c).

3.7. WST1 Proliferation Assay

1. Twenty-four hours after transfection (see Subheading 3.2), collect hNPCs and seed them in a 96-well plate (8–16 wells per condition, see Note 16) in a final volume of 100 μL /well. Add medium to an empty well to be used as a background control. Prepare a new plate for each time point (see Note 17).
2. Add Cell Proliferation Reagent WST1 (10 μL /well) and incubate the cells for 1 h in the CO₂ incubator. During the incubation, viable cells convert WST1 to a water-soluble formazan dye.
3. Shake the plate for 1 min on a shaker.
4. Read absorbance at 450 nm.
5. Subtract background value (medium without cell) from the sample readings. Normalize the average absorbance for each time point to the value on day 1 after transfection. The absorbance is directly correlated with the cell number (Fig. 2d).

¹⁶Several assays must be performed for each condition, as hNPCs cannot be dissociated into single cells for this proliferation assay. Variability is observed due to distribution of hNPC aggregates in suspension.

¹⁷As a good start, perform the WST1 proliferation assay every 24 h for 4–5 days after transfection.

3.8. In Vitro: Three-Dimensional Cell Migration Assay (See Note 18)

1. Dilute Matrigel (see Subheading 2.2, Item 2) 1:1 in NIM (see Note 19).
2. Put 70 μ L of Matrigel mixture in each well of a 96-well plate. Incubate at 37°C overnight to polymerize.
3. Three days after transfection (see Subheading 3.2), collect hNPCs forming neurospheres from each condition into 15-mL tubes and centrifuge at $50 \times g$ for 1 min at room temperature.
4. Resuspend neurospheres in a small volume of NIM, just enough to keep them in suspension and place a small volume (not more than 5 μ L) on top of the polymerized Matrigel. Rock the plate back and forth. Check plate under the microscope to make sure the neurospheres are evenly distributed, with no more than three per well.
5. Apply 70 μ L of Matrigel mix (see Note 19) on top of the hNPC layer. Incubate at 37°C.
6. Take pictures of the hNPCs at desired time points (e.g., 2.5 h, 24 h, 48 h, 96 h) (see Note 20). The migration phenotype can be quantified by measuring the distance from the hNPC to the nucleus of the most distant cell at various times after transfection or/and by counting cells that migrated out of the hNPCs, see example in Fig. 3.

3.9. Ex Vivo: Migration of Transplanted hNPCs in Embryonic Brain Slices (See Note 21)

1. Prepare GFP-FUGW lentivirus (see Note 22). Cotransfect the vector encoding GFP, the HIV-1 packing vector Δ 8-9, and the VSVG envelope glycoprotein into 293TN producer cells. Concentrate the pseudoviral particles using PEG-it virus precipitation solution following manufacturer instructions to obtain ultrahigh titer preps without toxic reactions.
2. Transfect hNPCs as indicated in Subheading 3.2, Items 1–3. At the same time, transduce the cells with high titer virus to obtain at least 30% infectivity (see Note 23).
3. Incubate for 24 h and replace the medium with fresh NIM.
4. Two days after transfection, anesthetize a pregnant female and sacrifice by cervical dislocation. Remove the embryos and place them in ice-cold PBS.
5. Carefully remove the brains from the embryos and transfer them to a Petri dish containing ice-cold rinse solution and rinse brains once.

¹⁸Cellular behavior in the three-dimensional (3D) migration assay more accurately reflects the in vivo situation.

¹⁹Allow the Matrigel aliquot to thaw overnight at 4°C. Tips for pipetting Matrigel and the medium used for dilution must be at 4°C to avoid polymerization of the Matrigel.

²⁰Filamentous processes will start to come off of the hNPCs within the first 2.5 h when miR-9 activity is inhibited. Under control conditions, that will happen within the first 24 h. After 24 h, cells will migrate from the neurospheres into the matrix, in chains, or as individual cells.

²¹Organotypic culture is more relevant, both anatomically and physiologically, than cell culture in dish to study neuronal migration. The maintenance of cell–cell interactions in brain slices allows analysis of cell migration under conditions that resemble those in vivo.

²²Transducing hNPCs with the gene encoding green fluorescent protein (GFP) makes it possible to analyze details of differentiation, morphology, and direction control mechanisms of cells migrating in embryonic brain slices.

²³Infectivity is determined after 72 h by fluorescence microscopy for GFP expression. Optimization must be performed depending on the number of hNPCs and the virus preparation. It is important to check the transduction efficiency by plating a portion of neurospheres from each condition in a Matrigel-coated Petri dish. Determine the percentage of GFP-positive hNPCs at the time you look for their migration in the forebrain slices.

6. Embed brains in embedded medium in a Petri dish and immediately put them on ice.
7. Cut out a block containing a single embedded brain. Orient the embedded brain and mount it on the Vibratome stage with cyanoacrylate (e.g., SuperGlue) so that coronal forebrain sections will be made.
8. Cut coronal forebrain slices 250 μm thick with the Vibrotome (at a high frequency and low speed) and keep them in ice-cold slicing medium.
9. Transfer the slices to culture dishes with ice-cold slicing medium. Select the three best forebrain slices from each brain under the microscope (use ~5 embryos per condition). Wash them twice with sterile slicing medium and once with the nutrition medium. With a cut 1 mL disposable transfer pipette, individually place slices onto the membrane of a tissue culture insert in a 6-well plate containing 1.2 mL of nutrition medium per well (see Note 24). Up to six slices may be placed on a single membrane.
10. Place plates in the CO₂ incubator for ~1 h. Meanwhile, prepare the neurospheres for transplantation by washing them twice in PBS and once in nutrition medium.
11. Prepare transfer pipettes by pulling glass pipettes (see Note 25). Cut pipettes by hand with a sharp scalpel blade under a dissecting microscope where the diameter is ~150 μm . A clean neat break is essential.
12. Under dissection microscope, carefully select neurospheres on the basis of size and load one at a time into a pipette. Make sure that the tip of the pipette does not touch anything to avoid contamination.
13. Transfer a membrane insert containing the forebrain sections to a 10-cm Petri dish without culture medium and place on the dissection microscope stage. Blow gently into the mouth capillary until a single neurosphere has been expelled into the medial ganglionic eminence.
14. Replace the insert into the 6-well plate and incubate in CO₂ incubator. Refresh the medium every other day for 4 days (see Note 26).
15. Visualize under the fluorescence microscope at desired time points. The migration of transplanted hNPCs in mouse brain slices can be quantified as the maximum distance covered. For each transplanted neurosphere, select the five cells that migrated farthest from the neurosphere and measure the distance to the edge of the neurosphere. Calculate the average.

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²⁴Prepare the 6-well plate with membrane inserts before starting to remove the embryos. Add 1.2 mL of medium to each well and place it in the CO₂ incubator.

²⁵Hold each end of a glass capillary between your thumb and forefinger. Position the center of the capillary horizontally over the top of a Bunsen flame. Roll the capillary back and forth between your thumb and forefinger over the flame. When the glass gets red in the center, remove the glass from the flame and immediately pull your arms apart so that the micropipette lengthens.

²⁶Only a thin layer of medium is needed on the surface of the slice to keep it humid. Tissues must be well exposed to the air (CO₂ enriched) to be healthy.

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Fig. 1.

Neural differentiation of hESCs. H9 hESCs were maintained on MEF feeder cells in the presence of FGF2. Day in vitro (DIV) 0 refers to the day hESCs were first dissociated from MEFs and cultured in suspension. Neural differentiation of hESCs started with the formation of EBs, followed by rosette formation at 13–16 DIV in the presence of FGF2 and N2 supplements. Rosette structures resemble neuroectodermal cells. Neural progenitors in rosettes were positive for SOX2 and PAX6. For neurosphere formation, rosette-forming cells were dissociated and expanded in suspension cultures, and the resulting neurospheres were cultured at 16–46 DIV. For terminal neuronal differentiation, neural progenitors were dissociated from neurospheres and exposed to BDNF and GDNF at 43–60 DIV. Scale bar, 100 μm. (Reproduced from ref. (8) with permission from Elsevier Science.)

Fig. 2.

Assays for studying the survival and proliferation of hESC-derived hNPCs. **(a)** Loss of miR-9 does not affect the survival of human neural progenitors as shown by TUNEL staining. Three representative images of TUNEL staining are presented on the right. **(b)** LDH assay to measure the cytotoxic effect of miR-9 knockdown on hNPCs. Cells were transfected with scrambled LNA probe or with anti-miR-9 LNA probe at 16 DIV, and cytotoxicity was measured 48 h and 96 h after transfection. *n.s.* not significant. Values are mean $\pm$ SEM. **(c)** BrdU incorporation was measured to indicate the rate of proliferation of hNPCs with or without miR-9 knockdown. The percentage of BrdU-positive cells at 3 days after transfection is shown. Values are mean $\pm$ SEM. $**P < 0.01$ ($n = 3$). **(d)** WST-1 assay to measure the proliferation of hNPCs after transfection of the LNA-anti-miR-9 or scrambled probe at 16 DIV of neuronal differentiation. Values are mean $\pm$ SEM of 8–16 wells per time point and per conditions. $*P < 0.05$. (Reproduced from ref. (8) with permission from Elsevier Science.).

Fig. 3.

3D Matrigel migration assay to examine the migratory behaviors of hNPCs. (a–c) hNPCs transfected with scrambled LNA probe and cultured in a 3D matrix. (d–f) hNPCs transfected with anti-miR-9 LNA probe and cultured in a 3D matrix. hNPCs were dissociated from rosettes at 16 DIV, transfected, cultured for 3 days in suspension, and embedded in Matrigel. (d) Quantification of migratory behaviors of hNPCs in Matrigel. The distance from the edge of the neurosphere to the most distant nucleus of the outmigrating cells was quantified. Values are mean $\pm$ SEM. (Reproduced from ref. (8) with permission from Elsevier Science.).