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Chick Retinal Pigment Epithelium Transdifferentiation Assay for Proneural Activities

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Abstract

We describe a cell culture system for assaying proneural activities of genes hypothesized to play instrumental roles in neuronal fate specification during vertebrate retinal development. The retinal pigment epithelium (RPE) is collected from embryonic day 6 (E6) chick to establish a primary RPE cell culture. The culture is then infected with a replication competent retrovirus RCAS expressing the gene of interest. The presence of retinal neurons in the otherwise nonneural, RPE cell culture is examined between 4 and 10 days after the administration of the virus. Taking advantage of the plasticity and the relative simplicity of RPE cells, this method offers an informative assay for proneural activities prior to planning for large-scale in vivo experiments.

Keywords

Proneural genes; Transcription factor; Transdifferentiation; Photoreceptor; RPE; Neuron; Retina

1. Introduction

Deciphering the role of a transcription factor in retinal cell fate specification during vertebrate retinal development constitutes an important area of study in the broad field of neural development. One of the main hurdles encountered in this area of research is a lack of an effective assay for the many factors implicated to play inductive roles during retinal neurogenesis. The neural retina, due to its natural expression of many neural genes, including proneural genes, and its composition of varied cell types, can be ill-suited for such a study, as it is often difficult to dissect the role of each individual gene, much less a combination of genes and their hierarchies. To address this issue, we have been exploring the possibility of using the retinal pigment epithelium (RPE) as an alternative medium to illustrate proneural activities of factors implicated in retinal neurogenesis.

The RPE consists of darkly pigmented cells organized as a single-layered, transporting epithelium with important roles in retinal physiology. Anatomically, the RPE lies immediately adjacent to neural retina and forms the outer blood–retinal barrier. Developmentally, the nonneural RPE and the neural retina originate from the same structure—the optic vesicle. The identities of RPE vs. neural retina is established during the transformation of the optic vesicle into the double-layered optic cup, with the outer layer forming the RPE and the inner layer developing into the neural retina.

Three well-known properties of the RPE render it a suitable medium for assaying proneural activities. First, the nonneural, single-layered epithelium lacks the expression of many proneural genes. Second, unlike retinal neurons, RPE cells can reenter the cell cycle upon stimulation. Third, progeny cells of RPE may, under appropriate conditions, differentiate into cell types other than RPE (1–7). Classic experiments have revealed tissue

transdifferentiation into a neural retina from embryonic chick RPE (8–13), embryonic rodent RPE (3, 14), and amphibian RPE (2, 15).

In this chapter, we describe an RPE transdifferentiation assay that uses dissociated chick RPE cell culture as a medium to reveal proneural activity through RCAS (16) retroviral transduction of a gene of interest. Over the years, we have observed the following regarding this assay. (a) The type of neural cells resulted from gene-directed RPE transdifferentiation appears to reflect, to some extent, the function of the gene in retinal neurogenesis (see Note 1). (b) Not all genes important for retinal development are effective in inducing RPE transdifferentiation (see Note 2). (c) RPE transdifferentiation can be induced by extrinsic factors (see Note 3). (d) Co-treatment with two or more factors can be applied to the assay (see Note 4). (e) The efficiency and the extent of neural differentiation show gene dependency (see Note 5).

It should be kept in mind that RPE cells are not retinal neuroblasts. In addition, while the simplicity of RPE makes it an attractive system for experimental manipulations, this very simplicity may become a drawback. Caution should be exercised when attempting to apply results from RPE experiments to the developing retina. To this end, RPE transdifferentiation assays should be complemented with and supported by experiments using the neural retina and retinal cells. Nonetheless, the RPE transdifferentiation assay is a useful tool in revealing the potential roles of genes and factors in retinal neurogenesis.

2. Materials

2.1. Chick Embryo Incubation

1. Chick egg incubator.
2. Pathogen free, fertilized chick eggs (see Note 6).
3. Gooseneck fiber light source for “candling” chick embryo in egg.

2.2. Tissue Dissection

1. Dissecting microscope with gooseneck fiber light source.
2. Sterile (autoclaved) dissecting tools: Dumont #7 (curve), #3, and #5 tweezers (see Note 7).

¹When ectopically expressing *neuroD*, the RPE cells begin to express a photoreceptor phenotype, an observation consistent with *neuroD* expression in young photoreceptor cells and their precursors and its selective promotion of photoreceptor production in the retina (17–20). On the other hand, ectopic expression of *neurogenin2* (*ngn2*) in RPE cell culture induces de novo appearance of molecularly and morphologically different types of cells, including photoreceptor cells, retinal ganglion cells (RGCs), and in a smaller number, amacrine cells (21). This is consistent not only with *ngn2*'s expression in proliferating, multipotent progenitors but also with a fate mapping study using the Cre-ERTM—LacZ system (22). Yet, another proneural bHLH gene, *ash1*, induces RPE cells to transdifferentiate into cells resembling neither photoreceptor cells nor RGCs, but rather amacrine cells (23). This agrees with the observation that *ash1* mis- or overexpression in the developing chick retina increases amacrine cell production while causing photoreceptor deficiency (24).

²We have observed that not all bHLH genes that are expressed in the developing retina and are homologous to *Drosophila* proneural genes are capable of initiating detectable RPE transdifferentiation toward retinal neurons. These “ineffectual” bHLH genes include *NSCL1* and *NSCL2*. In addition, several homeodomain genes well known for their roles in eye/retina development, such as *Rax*, *RaxL*, and *six3*, also appear “ineffectual.” The negative outcome, however, does not undermine the importance of these genes/factors in retinal development. Rather, it shows their ineffectiveness in inducing neurogenesis in the context of RPE cells.

³Under the induction of bFGF, which is believed to potentiate RGC fate (12), RPE cells begin to transdifferentiate in the direction of becoming RGCs (25), although the extent of transdifferentiation is very limited.

⁴The extent of bFGF-induced transdifferentiation towards RGCs can be enhanced by RCAS transduction of *ath5* and *NSCL1* (26), bHLH genes expressed in developing RGCs, and to a greater extent by their cotransduction (27).

⁵Under the induction of *ngn1* or *ngn3*, over 80% of the cells present in a dish may display a noticeable neural trait (28). The neural differentiation initiated by *ngn1* can proceed to advanced stages examined at the molecular, morphological, and physiological levels (28). On the other hand, transdifferentiation initiated by *sox2* seems to stall at primitive stages (29).

⁶Pathogen-free chick embryos are important for experiments using retrovirus RCAS as a vehicle for gene transduction.

3. Sterile 60-mm dishes.
4. 15-ml Sterile, disposable centrifuge tubes.
5. Sterile, plastic transfer pipette.

2.3. Cell Culture

1. Equipment:
 - Cell culture hood (i.e. biological safety cabinet).
 - 37°C cell culture incubator with 5% CO₂.
 - Countertop centrifuge.
 - Inverted microscope.
2. Cell culture dishes (35-mm diameter) or six-well culture plates.
3. Cell culture medium (see Note 8):
 - Medium 199.
 - Medium 199 supplemented with 10% fetal calf serum (199 + 10% FCS).
 - Knock-out D-MEM supplemented with 20% serum replacement (KO/SR).
 - 0.25% Trypsin/EDTA.
 - Hank's balanced salt solution (HBSS).
 - Ca²⁺, Mg²⁺-free HBSS (CMF).
4. Concentrated RCAS (16) retrovirus expressing a gene of interest and RCAS virus expressing a control gene, such as GFP, with a titer of $1-5 \times 10^8$ pfu/ml (see Note 9).

2.4. Neural Detection

1. Fixation solution: ice-cold, 4% paraformaldehyde in phosphate buffer saline (PBS), pH 7.4.
2. Antibodies or antisense RNA probes specific for retinal neurons, and buffers and reagents for routine immunochemistry or in situ hybridization.

3. Methods (See Note 10)

1. In a cell culture hood (biological safety cabinet), add 1 ml of KO/SR to each 35-mm culture dish (see Note 11). Place the dishes in a 37°C cell incubator (for seeding cells later of the day).
2. Warm the following solutions in a 37°C water bath for 10–20 min:
 - Medium 199 (see Note 12).

⁷Tweezers with fine tips are vital for isolating the single-layered RPE.

⁸These solutions must be free of viral contamination.

⁹In our hand, a viral stock with a high titer, $1-5 \times 10^8$ pfu/ml, plays an important role in the success of the transdifferentiation assay. For method on producing high titer RCAS virus, see the chapter by Yan and Wang in this volume.

¹⁰Because the delicacy of the tissue being handled, investigators may refrain from caffeine intake before and during dissection.

¹¹The investigator will decide on number of dishes needed for one particular experiment. Depending on the specifics of each experiment, 6-well plates may work better than individual 35-mm dishes. Cell culture vessels with smaller culture area may be used, but they often produce larger variations in cell densities. It is important to minimize variations in cell densities among the dishes in a given experiment to avoid undue complication in data interpretation.

Medium 199 + 10% FCS.

CMF HBSS.

0.25% Trypsin/EDTA.

3. Wipe the outside of the bottles with 70% ethanol and Kimwipes, flame the mouth of the bottles, and place them in a cell culture hood.
4. Add 10 ml of Medium 199 to each of three 60-mm dishes (for dissection), and add 3 ml of Medium 199 + 10% FCS to a 35-mm dish (for collecting isolated RPE tissue).
5. Transfer the dishes to a dissecting bench surface-cleaned with 70% ethanol.
6. Candle chick eggs at E6 (see Note 13) to select for those with viable embryos and mark the position of the embryo with a permanent marker. Lay the chosen ones horizontally on an egg carton.
7. Clean egg top with 70% ethanol. Gently knock at the center-top of shell with a pair of tweezers to produce cracks in shell, and remove shell pieces with a pair of sterilized Dumont #7 tweezers. With another pair, break the vitelline membrane and scoop out the embryo. Place the embryo in a 60-mm dish.
8. Decapitate the embryo, enucleate the eyes (see Note 14), and place the eyes in the first of the three 60-mm dish with 10 ml of Medium 199.
9. Under a dissecting microscope, remove the sclera using Dumont #3 tweezers. Transfer the RPE-retina-vitreous-lens into the second 60-mm dish with 10 ml of Medium 199 (see Note 14).
10. Using Dumont #5 tweezers, make an incision in the RPE + retina along the ora serrata to rid of the ciliary epithelium, the associated periphery retina, and the lens. Place the RPE-retina-vitreous into the third 60-mm dish with 10 ml of Medium 199.
11. Separate RPE from the retina and vitreous. Make sure that no retinal tissue is attached to the RPE, particularly at the periphery region. Place the isolated RPE in the 35-mm dish with 199 + 10% FCS (see Note 15).
12. After enough RPE tissues have been collected (see Note 16), take the 35-mm dish with RPE to the cell culture hood. Transfer the RPE into a sterile 15-ml tube using a sterile, plastic transfer pipette.
13. Let the tissue sink down, and take out the residual solution. Rinse the RPE twice with 5–10 ml of CMF.
14. Add trypsin/EDTA, triturate the tissue about 20 times with a plastic transfer pipette, and watch carefully not to over-trypsinize the tissue (see Note 17).

¹²Although we routinely use Medium 199 for chick cells, we have found that other cell culture medium, such as D-MEM:F12 or HBSS, can also be used.

¹³We prefer to use E6 RPE for easy handling of the embryos and for effective transdifferentiation. While RPE from younger embryo may be more receptive to transdifferentiation, it, nevertheless, contains fewer cells and is technically more difficult to handle during the dissection process.

¹⁴It is important to keep the eye ball intact during this step. Any puncture will result in collapsing of the eyecup during the following steps, and that will make it very difficult to isolate the PRE.

¹⁵We found that the presence of 10% FCS improves the viability of RPE cells.

¹⁶It is very important to have high quality RPE cell culture for the RPE transdifferentiation assay to be successful. To have high-quality RPE cell culture, the first step is to isolate sufficient amount of RPE tissues free from retinal contamination in a relatively short period of time. We recommend practicing RPE isolation a few times before carrying out assay experiment.

¹⁷Over-trypsinization of the tissue is evident when the tissue/cell suspension becomes slimy after a gentle swirling.

15. As soon as all big pieces of RPE tissue become small and barely visible (about 5 min in the hood), add 10 ml of 199 + 10% FCS to stop the trypsin digestion. Mix by pipetting a couple times.
16. Centrifuge at $650 \times g$ for 5 min at room temperature in a countertop centrifuge.
17. In the culture hood, flame around the cap area of the tube, and gently reverse the tube to discard the supernatant. Use the transfer pipette to take away any extra solution at the mouth of the tube.
18. Resuspend the cell pellet with 199 + 10% FCS (1.5 ml for each E6 RPE) by pipetting with a transfer pipette.
19. Seed 0.5 ml cells into each of the 35-mm dishes placed in the 37°C cell incubator and containing 1 ml of KO/SR.
20. Culture the cells and change medium every other day with 1.5 ml KO/SR (see Note 18).
21. Examine the culture with an inverted microscope. At ~50% confluency (see Note 19), add 10–20 μ l RCAS virus. Swirl the dish gently 50 revolutions. Repeat the swirling four times during the day (see Note 20).
22. On the following day, repeat the swirling (four times during the day, each with 50 revolutions).
23. Change medium every other day with 1.5 ml of KO/SR.
24. Between 8 and 10 days after the administration of the virus (see Note 21), fix the cells with ice-cold 4% paraformaldehyde in PBS for 30 min, and proceed with immunocytochemistry, in situ hybridization, or physiological (e.g. Ca^{2+} imaging; see Note 22) analysis for the presence of retinal neurons in the culture (see Note 23).

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¹⁸Maintaining healthy cells in the culture improves the experimental outcome. We recommend two simple measures: (a) keep medium as fresh as possible (take out and warm up only the amount you need each time, and never use medium older than a month) and (b) minimize the time of cells being outside the incubator.

¹⁹It usually takes 3 days for the culture to reach ~50% confluency. A culture that reaches ~50% confluency too early (e.g. in 2 days) or too late (e.g. in more than 4 days) tends not to work well.

²⁰This is to increase the chance of RCAS to attach to cells for subsequent infection.

²¹As short as 4 days after the administration of the RCAS viruses expressing a proneural gene, the culture may display visible signs of neural transdifferentiation. One such sign is lower cell density in the transdifferentiating dish than the control dish infected with RCAS-GFP. Another sign is, under an inverted microscope, transdifferentiating dish contains many clusters of cells with compact cell body and displaying long processes, reminiscent of neuronal clusters. The presence of these two foresees a highly effective neural transdifferentiation. However, the absence of these two does not necessarily indicate a lack of neural transdifferentiation. Instead, it may be a sign of relatively low efficiency due to the gene or low viral transduction.

²²Details on Ca^{2+} imaging analysis of the transdifferentiated culture can be found in refs. 28, 30.

²³This chapter focuses on steps of establishing and transducing RPE cell culture for assaying proneural activities, with a premise that neural detection is a routine procedure in a laboratory interested in the RPE transdifferentiation assay. Therefore, details in neural detection are not provided.

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