Chapter 10

In Vitro Explant Culture and Related Protocols for the Study of Mouse Retinal Development

Kangxin Jin and Mengqing Xiang

Abstract

The mouse retina is composed of many cell types and subtypes with distinct morphology and function; how these cells are differentiated from the multipotent progenitors is still largely unknown. Retinal in vitro explant culture has proven to be a useful tool to study the molecular and cellular mechanisms underlying retinal development. Here, we provide detailed descriptions about how to prepare retroviruses, dissect retinal cups, perform in vitro explant culture, and collect explant samples.

Key words: Retina, Explant culture, Development, Differentiation, Retrovirus, Electroporation

1. Introduction

The mouse retina is a very delicate neural tissue composed of three cellular layers and seven classes of cells. Most of the classes can be divided into subgroups based on morphological and functional differences (1, 2). For instance, the class of amacrine cells has more than 27 subgroups, each of which has distinct morphology and function. During retinogenesis, all classes of cells are derived from multipotent progenitors, and the differentiation processes are under tight control of both intrinsic and extrinsic factors, such as growth factors, membrane receptors, and most importantly transcription factors (3, 4). Loss- and gain-of-function studies have shown that a large number of factors are crucial for retinal development, but the list is far from complete (3–9).

Retinal in vitro explant culture (RIVEC) is a very important tool for both loss- and gain-of-function studies during retinal development. Homozygous mutation (or knockout) of a gene might be lethal at embryonic or early postnatal stages; yet, the differentiation of most retinal cell types has not started or been incomplete by

Shu-Zhen Wang (ed.), *Retinal Development: Methods and Protocols*, Methods in Molecular Biology, vol. 884,
DOI 10.1007/978-1-61779-848-1_10,

these early stages. Under such circumstances, RIVEC provides an important tool to investigate the differentiation defects in the mutant. As an example, *Math3*$^{-/-}$*;NeuroD*$^{-/-}$ double-mutant mice die soon after birthday; RIVEC was used to investigate the double-mutant effect on retinal development (6). The loss-of-function studies can also be achieved by RNAi technologies, with RNAi target sequence(s) cloned into plasmids or viral vectors (10). RIVEC is even more widely used in the gain-of-function studies. One or more genes, wild type, mutated, or modified, can be cloned into plasmids and driven by different promoters. The genes in plasmids can be directly delivered by the electroporation method or packaged into retroviruses or lentiviruses. In our lab, RIVEC has been successfully used to study the functions of Foxn4 (5), Brn3b (11), Nr4a2 (12), and Ebf factors (13). The RIVEC can be also applied to other studies, for instance, to test promoters/enhancers or other DNA regulatory sequences.

Any method has its own advantages and drawbacks. The major benefit of the RIVEC is that it mimics the in vivo environment and can maintain a reasonably intact tissue structure. The drawback is that retinal ganglion cells (RGCs) usually die within several days of culture due to the fact that long-term survival of RGCs requires neurotrophic factors retrogradely transported from their brain targets. Compared to other approaches such as in utero injection (14, 15), one obvious advantage of RIVEC is that different factors can be easily added to the medium or explant tissues at any time. Prior to doing any experiment, the gains and losses of each approach should be carefully weighed.

Practically speaking, there are some general principles that should be considered when adapting the RIVEC. First, the time window for generating each cell type must be considered. For example, to study whether overexpression of a gene can promote the RGC or horizontal cell (HC) fate, it would be wise to choose E13 or earlier retinas to carry out the RIVEC, since after E14, the multipotent progenitors capable of adopting an RGC or HC fate may become too few to show a detectable effect. Second, the culture period should be as short as possible, since there are more and more cells committing apoptosis as the culture progresses, especially RGCs; the retinal structure boundaries become obscure too with time. Generally, you need to design your experiment according to the developmental processes of the cell types under study.

2. Materials

Prepare all solutions with ultrapure water filtered by Millipore filters. Unless otherwise specified, all materials should be sterile or autoclaved.

2.1. Instruments (Keep Clean, Unnecessary to Be Sterile)

1. Zeiss Stemi SV6 dissecting microscope (for tissue dissection).
2. Schott ACE I light source (for tissue dissection).
3. BTX Electro Square Porator ECM 830 (for electroporation).
4. Beckman L8-70M ultracentrifuge and SW-28 rotor (for retrovirus preparation).

2.2. Dissecting Tools

These tools can be acquired from Fine Science Tools Inc. or other similar sources.

1. 10-cm Straight forceps.
2. 10.5-cm Fine iris scissors.
3. Dumont #7 forceps with large radius curved shanks.
4. Dumont #5 or 55 forceps (for fine dissection).
5. Extra delicate mini-Vannas style iris spring scissors.
6. Micro-curette, diameter 1 mm (for electroporation).

2.3. Tissue Dissection

1. 70% Ethyl alcohol.
2. DMEM medium (Invitrogen).

2.4. Electroporation

1. Nepagene Micro Electroporation Chamber Model CUY532.
2. Electroporation solution containing 1× PBS (pH 7.4) or Hanks buffer (see Note 1), 0.5–3 μg/μl of your plasmid (see Note 2).

2.5. Retrovirus Preparation and Transfection

1. 100 mm × 20 mm and 150 mm × 25 mm Cell Culture Dish (Corning).
2. 0.25% Trypsin–EDTA (Invitrogen).
3. pBMN-GFP vector and/or derivatives with your gene insertions (Gentaur Molecular Products) (see Note 3).
4. Phoenix Eco packaging cell line (Gentaur Molecular Products) (see Note 4).
5. OPTI-MEM I medium (Invitrogen) (see Note 5).
6. Lipofectamine (Invitrogen).
7. 1,000× Puromycin (5–10 mg/ml in H_2O) (see Note 6).
8. Culture medium: DMEM medium,10% FBS, 1× penicillin/streptomycin/glutamine.
9. Screening medium: Culture medium with 1× puromycin (see Note 6).
10. 10× Polybrene (50 μg/ml) (see Note 7).
11. 250-ml Filter System (0.45 μm, from Corning) (see Note 8).
12. Beckman Ultra Clear Centrifuge Tubes (25 mm × 89 mm) (see Note 9).

2.6. Explant Culture

1. Falcon 6-well cell culture plate.
2. Millipore Millicell-CM Low Height Culture Plate Inserts (0.4-μm pore size) (see Note 10).
3. Explant culture medium (see Note 11): 42.5% DMEM, 42.5% F-12 Nutrient Mixture, 15% Fetal Bovine Serum, 1× penicillin/streptomycin/L-glutamine (Invitrogen), 5 mM Forskolin (optional), and 1× serotonin/transferrin/insulin (optional, Invitrogen).

2.7. Tissue Collection and Treatment (Unnecessary to Be Sterile)

1× PBS, pH 7.4 (sterile).
30% sucrose in 1× PBS (sterile).
Tissue-Tek O.C.T. Compound.
Tissue-Tek Cryomold Standard (25 mm × 20 mm × 5 mm).
Fresh 4% paraformaldehyde (PFA) (see Note 12), prepared as following:

(a) Heat 90 ml ddH_2O to 50–70°C.
(b) Add 30 μl 5 N NaOH.
(c) Add 4 g PFA and shake by hand until dissolved (see Note 13).
(d) Cool down immediately on ice (see Note 14).
(e) Add 30 μl 5 N HCl.
(f) Add 10 ml 10× PBS (pH 7.4).
(g) Use filter paper to remove the undissolved particles (optional).
(h) Store at 4°C and use it on the same day (see Note 15).

3. Methods

3.1. Retrovirus Preparation (Optional, Only if Retroviruses Are Needed)

1. Culture the Phoenix Eco cells in a 100-mm dish or split them into multiple dishes if you have multiple plasmids, and wait until the cells are 40–60% confluent (see Note 16).
2. Prepare the following solutions (see Note 17):
   (a) A: Dilute 10 μg of pBMN-GFP (or derivatives) into 1 ml of OPTI-MEM I, and mix well.
   (b) B: Dilute 50 μl of lipofectamine into 1 ml of OPTI-MEM I, and mix well.
   (c) Combine A and B, add 1 ml of OPTI-MEM I, mix gently, and incubate for 30 min to allow DNA–lipofectamine complexes to form at room temperature.
3. Discard the culture medium and wash cells with OPTI-MEM I medium.
4. Overlay the DNA–lipofectamine complexes onto the washed cells, and put the cells back into a cell culture incubator at 37°C for 5 h.

5. Remove the DNA–lipofectamine complexes, add 10 ml of Culture Medium, and incubate the cells for 1 day.
6. Remove the Culture Medium, add 10 ml of Screening Medium, and continue culturing until cells grow to confluence. Change Screening Medium every 2 days.
7. Transfer the cells into a 150-mm culture dish and add 20–25 ml of Screening Medium. Refresh Screening Medium every 2 days until cells grow to confluence (see Note 18).
8. Split the cells into three 150-mm culture dishes and continue culturing with the Screening Medium until cells grow to confluence.
9. Remove the Screening Medium and wash once with 1× PBS to get rid of the residual puromycin; add 13 ml of Culture Medium and continue culturing.
10. After culturing for 24 h, collect the medium (containing the viruses) into a 50-ml tube and store at 4°C; add 13 ml of Culture Medium, and collect again after 24 h (see Note 19).
11. Combine the collected medium, remove the cell debris through a 0.45-μm filter, transfer the medium into ultracentrifuge tubes, place the tubes into a SW-28 rotor, and centrifuge at 21,000 rpm (~79,000×*g* force) for 3 h at 4°C using the Beckman L8-70M Ultracentrifuge (see Note 20).
12. Pour off the supernatant and aspirate the last drop from the lip of the tube. Seal the tube with parafilm and shake on ice for 1–2 h (see Note 21).
13. Aliquot 10–20 μl to each Eppendorf tube and store at −80°C (see Note 22).

3.2. Retinal Cup Preparation

1. Embryonic stage:
   (a) Expose the mother to carbon dioxide inhalation in a sealed chamber to euthanize the mouse. It usually takes 1–3 min (see Note 23).
   (b) Clean its abdominal area with 70% ethanol.
   (c) Cut the abdomen open with scissors. Now you should be able to see the embryos. Take the embryos out of wombs with the Dumont #5 (or #55) forceps very carefully, and put them into cold DMEM medium in the Petri dish (see Note 24).
   (d) Carefully isolate the eyeball with the Dumont #5 (or #55) forceps, and transfer it to a new Petri dish with fresh cold DMEM.
   (e) Under a Zeiss dissecting microscope, carefully remove the sclera, choroid, and other structures wrapping the retina, and then remove the lens using the Dumont #5 (or #55) forceps. Now you have a retinal cup.

2. Neonatal stage:
   (a) Euthanize the pup by decapitation (see Note 23).
   (b) Clean the area around the eyes with 70% ethanol.
   (c) Take out the eyeball with the Dumont #7 forceps and place it into cold DMEM medium (see Note 25).
   (d) Dissect under a Zeiss dissecting microscope. First, punch a hole on the iris–retina midline with forceps, rip a small fissure along the line with two pairs of forceps, and use scissors to cut open the sclera along the iris–retina midline (see Note 26 and Fig. 1a); and then remove the remaining sclera and choroid using the Dumont #5 (or #55) forceps; remove the lens last.

3.3. Electroporation (Optional Step, Only for Plasmid Transfection)

1. Add about 30 μl of electroporation solution (containing the plasmids) to Nepagene Micro Electroporation Chamber (see Note 27); transfer the retina into the chamber using forceps, and adjust the orientation of the retina so that the RGC layer faces the positive electrode (see Note 28, Fig. 1c).
2. Electroporation conditions: 10–12 V, 50 ms duration, 950 ms interval, five pulses (see Note 29). Push the start button to electroporate (see Note 30). Then, transfer the retina back into cold DMEM medium using the micro-curette (see Note 31).

3.4. Explant Culture

1. Now you have retinal cups from either Subheading 3.2 or 3.3 in cold DMEM. Make four incisions from the margin of the eye cup half way through toward the bottom at 0, 3, 6, and 9 o'clock positions. The retina looks like a four-petal flower after the incisions (see Fig. 1d).
2. Use a pipette to transfer up to three to four retinas onto a Millipore Millicell Cell Culture Insert; use forceps to adjust retinal orientation and position so that the RGC layer faces up and all four petals spread outwards. Carefully remove the medium along the boundary with a 200-μl pipette (see Note 32, Fig. 1e).
3. Place the insert into a 6-well plate with 1 ml of culture medium in each well (see Note 33). And transfer the plate into a cell culture incubator (37°C, 5% CO_2).
4. Virus infection (optional step, for retrovirus only):
   (a) After 5 h of culturing, infect retinas with virus in the following steps.
   (b) Add 1 μl of 10× polybrene (see Note 7) to every 10 μl of concentrated virus and mix well.
   (c) Add 2–3 μl of the mixture to each retina. Repeat once after 10 min.
   (d) Continue culturing in the incubator.
5. Change the culture medium every 1–2 days until collection of the explant tissue.

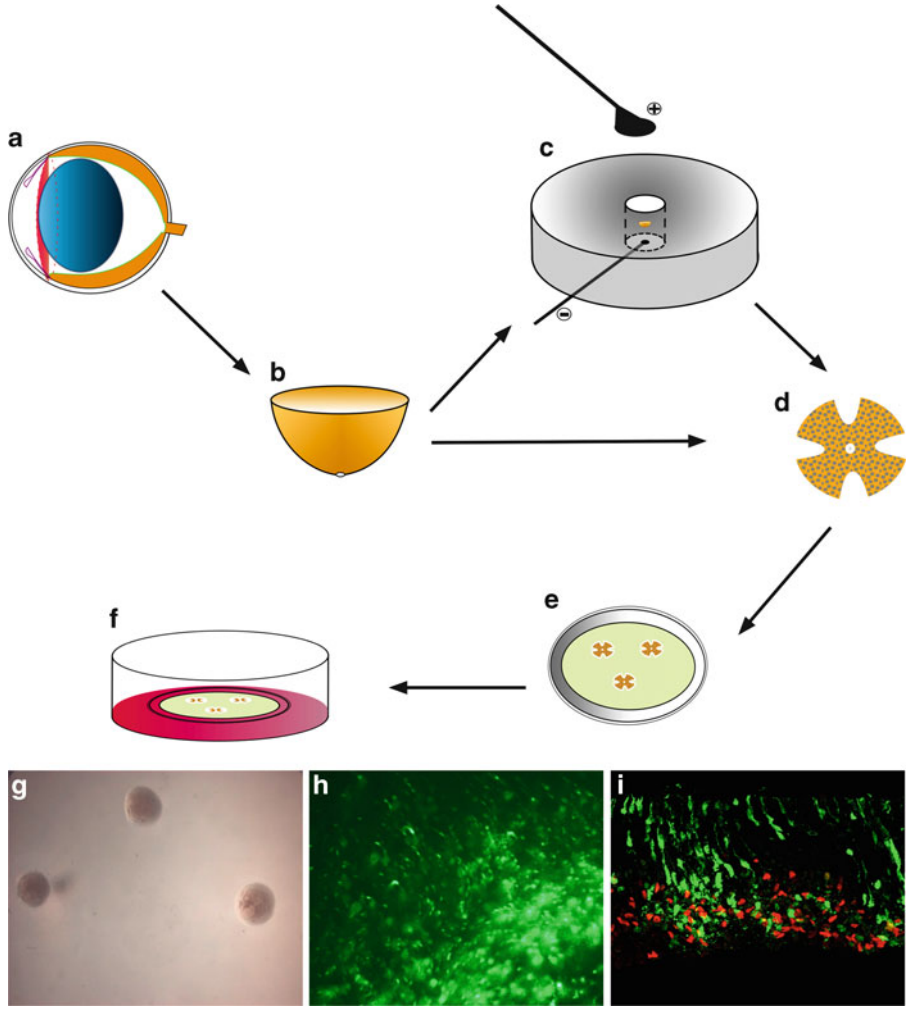

Fig. 1. Illustration of the retinal in vitro explant culture (RIVEC) protocol. (**a**) Structure of the mouse eye. The *orange color* represents the retina. When trying to separate postnatal stage retinas, use scissors to cut along the *dashed red line* to get rid of cornea, sclera, and other tissues. (**b**) A complete retinal cup after removing other tissues. (**c**) Electroporation in the Nepagene Micro Electroporation Chamber. (**d**) The retina looks like a four-petal flower after incisions. (**e**) Retinal explants were transferred onto a culture plate insert. (**f**) The insert was placed into one well of a 6-well plate with culture medium. (**g**) Retinal explants growing on the insert filter. (**h**) A microscopic image showing numerous GFP-positive cells in an electroporated explant. (**i**) A confocal microscopic image of an explant section showing cells immunoreactive for GFP (*green*) and Brn3a (*red*).

3.5. Sample Collection and Treatment for Immunohistochemistry

1. Depending on your experimental design, collect retinal explant samples following 2–14 days of culture.
2. Prepare fresh 4% PFA (see Note 12).
3. Collecting samples:
   (a) Remove the culture medium.
   (b) Wash with 1× PBS once.
   (c) Carefully wash off the explant (see Fig. 1g) with 1× PBS from the insert along the boundary.
   (d) Fix the explant in fresh 4% PFA for 10–15 min on ice.

(e) Wash the explant in 1× PBS twice.

(f) Soak the tissue in 30% sucrose (see Note 34) and shake gently at 4°C overnight or until the explant sinks to the bottom.

(g) Remove the sucrose solution, add O.C.T. (see Note 35), and shake gently at 4°C for 1–2 h; transfer the explant and O.C.T. into Tissue-Tek Cryomold and adjust the position of the explant (see Note 36); place the bottom half of the mold in dry ice–alcohol bath until O.C.T. is frozen completely (see Note 37).

(h) Store the embedded samples in −80°C freezers.

4. Notes

1. Dilute your DNA using 10× PBS or Hank's buffer as the buffer is necessary to provide ions required for electric conduction.
2. Depending on your experimental design, for example how many cells you want to transfect, dilute the plasmid to desired concentration, and test if it is optimized. Different plasmids can be combined together before electroporation.
3. pBMN-GFP is an MMLV-based retroviral expression vector commercially available. Depending on your purpose, you may use pBMN-Z or other MMLV-based vectors and adapt your protocol accordingly.
4. Phoenix cell lines, Phoenix-Eco and Phoenix-Ampho, are retrovirus producer lines based on the 293 T-cell line that are capable of producing gag-pol and envelope proteins for ecotropic and amphotropic viruses.
5. Opti-MEM® I Reduced Serum Medium is modified from Eagle's Minimum Essential Medium, and optimized to improve the transfection efficiency.
6. pBMN-GFP vector contains the puromycin-resistant gene that can be used to screen transfected cells. Phoenix cells without the resistant gene will be killed and float in the culture medium.
7. Polybrene is a small, positively charged molecule that binds to cell surfaces and neutralizes surface charge. It increases retrovirus gene transfer efficiency by enhancing receptor-independent virus adsorption on target cell membranes (16). Attention: Target cells could be killed by high concentration of polybrene. This is especially a known problem with some B- and T-cell lines. Lower the concentration of polybrene if necessary.
8. Filters with 0.45-μm pore size are recommended since smaller pores increase the chance of damaging the retrovirus surface proteins when they pass through the pores.

9. Those tubes are able to hold around 30 ml of liquid.
10. For E12 or earlier stages, the retina can be suspended in the culture medium for a few days. The major drawback is tissue hypoxia and cell death. The problem can be partially solved by using culture inserts that allow for studying three-dimensional explant structures.
11. Forskolin is commonly used to raise the levels of cyclic AMP (cAMP) and thus promotes cell survival. Serotonin/transferrin/insulin are also used to promote cell survival. All these factors are optional.
12. PFA is a suspected carcinogen. All operations should be carried out under the ventilation hood.
13. There are always some unsolvable white small particles, which usually do not affect your experiment. Those particles can be filtered out in the following steps.
14. PFA will be decomposed to formaldehyde at high temperature.
15. Some labs tend to store concentrated PFA (8–20%) at –20°C, and dilute it when needed. We prefer 4% PFA freshly prepared to guarantee consistent results, especially for antibodies that are highly sensitive to fixation conditions.
16. Pass the Phoenix Eco cells at a split ratio of 1:4–5 when they reach 70–80% confluence. They should reach 40–60% confluence before transfection; however, some people suggest that the cells should reach 70–80% confluence to obtain higher transfection efficiency.
17. Diluting plasmid and lipofectamine separately helps to mix and distribute DNA–lipofectamine complexes uniformly in the transfection mixture.
18. You should be able to see the dead cells floating in the medium. Under an inverted fluorescence microscope, you should clearly see the GFP-positive green cells adhering to the surface of the plate.
19. Due to the exhaustion of nutrition and pH value change, the medium should become yellowish instead of pink.
20. The speed of SW-28 at 21,000 rpm is equivalent to around 79,000 $\times g$ force, in case you use other ultracentrifuge equipment.
21. After the last drop aspirated from the lip of the tube, there is still some residual supernatant left, usually in the range of 100–150 μl. The virus concentration will be diluted if there is more supernatant left.
22. Aliquot according to the volume you need later. Avoid repeated freeze–thaw cycles which decrease the titer of the virus dramatically.
23. Depending on the protocol approved by your institute, the mice can be euthanized by other methods.

24. Generally, a C57BL6/J mother will have 5–9 embryos and a CD1 mother will have 10–17. To avoid tissue degradation caused by the lack of oxygen and other factors, dissect out retinas as quickly as you can.
25. Tip: Rip and push down the eyelids with two fingers, and the eyeball will protrude out; use forceps to clamp the bottom of the eyeball and pull it out.
26. The sclera becomes more and more tenacious after P0. Scissor cutting helps to keep better integrity of the retina.
27. The small chamber can hold about 30 μl liquid; however, the solution needs to be replaced after several cycles of electroporation, since the fluid becomes very viscous with broken tissues. Prepare more solution in advance.
28. The negative electrode is at the bottom of the chamber, so the plasmid migrates from the bottom to the top. Try not to bring extra fluid into the chamber when transferring the retinas. The retina looks like "sticking" to the tip of the forceps while being transferred.
29. Early-stage retina is very fragile and higher voltage tends to damage the retina.
30. You should hear one "beep" sound for each pulse of electroporation; there are also numerous bubbles forming in the buffer. After five pulses, you need to push the "start" button again to reset to the original state.
31. If the retina sticks to the Micro-curette, use a pipette to wash it down carefully.
32. The trick is that when you use the pipette to "suck" the fluid, as a result, the "petals" spread naturally.
33. Do not use more than 1 ml of medium; otherwise, the medium would seep through the filter and "flood" the explant, leading to hypoxia.
34. Prepare the sucrose solution in 1× PBS. Sucrose helps to keep better tissue morphology in later steps.
35. Some labs prefer to freeze the tissues in a mixture of O.C.T. and 30% sucrose at a 50:50 ratio. Remember to label the blocks properly using a permanent marker pen.
36. Tissues should be oriented in the block appropriately for sectioning (cross sections, longitudinal sections, etc.); label the orientation as well if necessary, for example, to check the cone cells in the future.
37. Add 90% or higher concentration of ethyl alcohol to the dry ice, which makes the freezing process much faster. The alcohol can be reused repeatedly for this purpose. Alternatively, if dry

ice is not available, freeze the mold in liquid nitrogen. Place the bottom half of the mold into liquid nitrogen until O.C.T. is completely frozen.

Acknowledgment

This protocol is adapted from a number of procedures of different labs, and we would like to specifically thank Dr. Constance Cepko's lab at the Harvard Medical School for their tremendous original work. We thank Kamana Misra for critical reading of the manuscript. This work was supported by the National Institutes of Health grants EY012020 and EY020849 (to M.X.).

References

1. Masland RH (2001) The fundamental plan of the retina. Nat Neurosci 4:877–886
2. Masland RH (2001) Neuronal diversity in the retina. Curr Opin Neurobiol 11:431–436
3. Cepko CL (1999) The roles of intrinsic and extrinsic cues and bHLH genes in the determination of retinal cell fates. Curr Opin Neurobiol 9:37–46
4. Livesey FJ, Cepko CL (2001) Vertebrate neural cell-fate determination: lessons from the retina. Nat Rev Neurosci 2:109–118
5. Li S, Mo Z, Yang X, Price SM, Shen MM, Xiang M (2004) Foxn4 controls the genesis of amacrine and horizontal cells by retinal progenitors. Neuron 43:795–807
6. Inoue T, Hojo M, Bessho Y, Tano Y, Lee JE, Kageyama R (2002) Math3 and NeuroD regulate amacrine cell fate specification in the retina. Development 129:831–842
7. Furukawa T, Morrow EM, Li T, Davis FC, Cepko CL (1999) Retinopathy and attenuated circadian entrainment in *Crx*-deficient mice. Nat Genet 23:466–470
8. Fujitani Y, Fujitani S, Luo H, Qiu F, Burlison J, Long Q, Kawaguchi Y, Edlund H, Macdonald RJ, Furukawa T, Fujikado T, Magnuson MA, Xiang M, Wright CV (2006) Ptf1a determines horizontal and amacrine cell fates during mouse retinal development. Development 133:4439–4450
9. Wang SW, Kim BS, Ding K, Wang H, Sun D, Johnson RL, Klein WH, Gan L (2001) Requirement for *math5* in the development of retinal ganglion cells. Genes Dev 15:24–29
10. Matsuda T, Cepko CL (2004) Electroporation and RNA interference in the rodent retina in vivo and in vitro. Proc Natl Acad Sci USA 101:16–22
11. Qiu F, Jiang H, Xiang M (2008) A comprehensive negative regulatory program controlled by Brn3b to ensure ganglion cell specification from multipotential retinal precursors. J Neurosci 28:3392–3403
12. Jiang H, Xiang M (2009) Subtype specification of GABAergic amacrine cells by the orphan nuclear receptor Nr4a2/Nurr1. J Neurosci 29:10449–10459
13. Jin K, Jiang H, Mo Z, Xiang M (2010) Early B-cell factors are required for specifying multiple retinal cell types and subtypes from postmitotic precursors. J Neurosci 30:11902–11916
14. Petros TJ, Rebsam A, Mason CA (2009) In utero and ex vivo electroporation for gene expression in mouse retinal ganglion cells. J Vis Exp. (31):e1333, doi:10.3791/1333
15. Garcia-Frigola C, Carreres MI, Vegar C, Herrera E (2007) Gene delivery into mouse retinal ganglion cells by in utero electroporation. BMC Dev Biol 7:103
16. Davis HE, Morgan JR, Yarmush ML (2002) Polybrene increases retrovirus gene transfer efficiency by enhancing receptor-independent virus adsorption on target cell membranes. Biophys Chem 97:159–172