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Embryonic cell culture in zebrafish

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Abstract

Zebrafish embryonic cell cultures have many useful properties that make them complementary to intact embryos for a wide range of studies. Embryonic cell cultures allow for maintenance of transient cell populations, control of chemical and mechanical cues received by cells, and facile chemical screening. Zebrafish cells can be cultured in either heterogeneous or homogeneous cultures from a wide range of developmental time points. Here we describe two methods with particular applicability to chemical screening: a method for the culture of blastomeres for directed differentiation toward the myogenic lineage and a method for the culture of neural crest cells in heterogeneous cultures from early somitogenesis embryos.

INTRODUCTION

While the zebrafish has proved a versatile model organism for studying *in vivo* biology, cultured zebrafish cells have also provided a platform for controlled genetic and chemical manipulation. More than 20 years ago, cells isolated from blastula stage embryos were reported to retain embryonic stem cell (ESC) characteristics in culture (Sun, Bradford, Ghosh, Collodi, & Barnes, 1995). Since then zebrafish embryonic cell culture has retained its relevance as a complementary system to *in vivo* studies, with diverse applications including chemical biology, disease modeling, and studies of cellular differentiation. While avoiding drawbacks of cell lines such as adaptation to culture, zebrafish primary cell culture allows for precise control of chemical cues received by cells, generation of homogenous populations of cells, maintenance of transient cell populations, and facile chemical screening.

Zebrafish cell cultures facilitate studies of cellular differentiation with a defined set of chemical cues (Huang, Lindgren, Wu, Liu, & Lin, 2012; Norris, Neyt, Ingham, & Currie, 2000; Xu et al., 2013). Like human ESCs, cultured zebrafish blastomeres, the cells that make up blastula stage embryos, can retain long-term pluripotency and germline competency (Fan et al., 2004; Ho et al., 2014). However, these cells can be induced to differentiate much more quickly than human ESCs and can be maintained without feeder layers (Ho et al., 2014; Huang et al., 2012; Myhre & Pilgrim, 2010; Xu et al., 2013). While

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many developmental studies can be conducted in vivo in zebrafish, blastomere cultures or cells derived from blastomere cultures provide a distinct advantage in certain situations. Studies of subcellular structure or localization, particularly in a specific tissue, can be difficult to conduct in vivo. Furthermore for studies in which a large amount of material is needed, a homogenous population of cells, for example of a specific cell type, can be generated from blastomeres. Genetic homogeneity can also be achieved by culture of blastomeres from single embryos and is essential for any study of embryonic lethal mutants in which heterozygous carriers are used (Myhre & Pilgrim, 2010).

Another advantage of zebrafish cell cultures is the potential to maintain transient cell populations. For example, neural crest progenitor cells can be isolated from early somitogenesis embryos and maintained in culture for at least 3 days (Kinikoglu, Kong, & Liao, 2013). These cells are capable of differentiation over the course of 2–3 weeks into neural crest-derived cell types including neurons, smooth muscle, pigment cells, and chondrocytes, using similar chemical cues as are used for differentiation of human ESCs or induced pluripotent stem cells (Kinikoglu et al., 2013). This culture system can be used to study the direct effects of chemicals, growth factors, or genetic mutations on neural crest development, proliferation, and migration.

Finally zebrafish cell culture allows for facile chemical screening. Unlike intact zebrafish embryos, zebrafish cells can be screened quickly in a completely automated manner with standard robotics and image analysis software. Screening can be conducted in either heterogeneous or homogeneous cell cultures. While heterogeneous cell cultures provide cell-type-specific information and do not require sorting, homogeneous cell cultures provide optimal signal strength.

Chemical screening of zebrafish blastomeres has been used to identify chemicals that promote endothelial and myogenic cell fate. Huang et al. used cells from gastrula-stage embryos to identify chemicals that promote vascular endothelial cell differentiation (Huang et al., 2012). Transgenic *flk1:GFP* embryos were plated with chemicals at 80% epiboly and cultured for 5 days before analysis of GFP fluorescence. A subset of hit chemicals from this screen were validated in promoting endothelial differentiation of murine ESCs, proliferation of human endothelial cells in culture, and angiogenesis in adult fish. The authors also demonstrated expression of transgenes marking various other cell types in cultures from late-blastula or early gastrula embryos, including *gata1* and *scf* for hematopoietic cells, *cmhc2* for cardio-myocytes, and *insulin* for pancreatic beta cells.

In 2013, Xu et al. identified signaling pathways that promote the myogenic differentiation of pluripotent cells, which were then validated in human iPS cells. Blastomeres from *myf5:GFP;mylz2:mCherry* fish were plated with chemicals, and fluorescence was determined after only one day, identifying basic fibroblast growth factor (bFGF), inhibitors of GSK3 β , and forskolin as promoting muscle differentiation. Previous attempts to differentiate iPS cells into skeletal muscle had met with limited success, but using a combination of the factors identified in the zebrafish screen, engraftable myogenic progenitors were generated. The success of using zebrafish cell culture in these cases can be attributed to the ability to quickly screen many compounds, including combinations of

growth factors and chemicals. Using such culture systems, signals required for differentiation can be addressed in an unbiased manner.

This chapter describes methods for the culture of zebrafish blastomeres, in particular for directed differentiation to muscle, and culture of cells from early somitogenesis embryos, in particular for maintenance of neural crest progenitor cells in heterogeneous cultures. Both of these methods are ideal for application to chemical screening.

1. METHODS

1.1 BLASTOMERE CELL CULTURE

1.1.1 Overview—Blastomeres can be used for short- or long-term studies of pluripotent cells or for differentiation to a desired cell type. Here we describe a simple general method for blastomere culture in which the whole embryo is plated at the oblong stage (3.7 hpf). Addition of 1 ng/mL bFGF to the culture medium at the time of plating results in differentiation into muscle, as presented in representative results.

1.1.2 Materials and reagents

E3 embryo medium (Nüsslein-Volhard & Dahm, 2002)
0.05% bleach solution in E3
2.5 mg/mL pronase solution in E3
agarose-coated petri dishes
100 μ m cell strainer
0.1% gelatin solution, suitable for cell culture
tissue culture plates of desired size
Zebrafish embryonic stem cell (zESC) medium (Table 1)

1.1.3 Plating zebrafish blastomeres

1. Fish were allowed to mate and embryos were collected, cleaned, and incubated at 28.5°C for 3–4 h.
2. zESC medium was prepared as indicated in Table 1 by combining additives and bringing the solution to the final volume with base medium. Media was sterile filtered and used within 1 week of preparation.
3. Gelatin-coated cell culture plates were prepared. Gelatin solution was warmed to 37°C and used to cover the bottom of tissue culture plates. Plates were incubated in a cell culture incubator at 37°C for 30 min, at which point the gelatin solution was aspirated. Plates were used immediately or stored in a sterile environment.
4. Embryos were bleached for decontamination. Embryos were transferred to a clean petri dish and incubated with 0.05% bleach solution in E3 for 2 min. Embryos were then washed three times with E3.

5. Embryos were dechorionated using pronase. When embryos had reached the high stage (~3.3 hpf), unfertilized eggs were removed and the desired number of embryos were transferred to agarose-coated petri dishes to avoid adherence of embryos to the surface of the dish. Embryos were incubated in a 2.5 mg/mL pronase solution in E3 at 28.5°C for 5 min and then washed three times with E3.
6. Embryos were counted, and proper staging was verified. For myogenic cultures, embryos should be homogenized at the oblong stage and plated at a density of 1 embryo per 5.6 mm² (1 well of a standard 384 well plate). For example, for plating a full 384 well plate, 400 embryos would be homogenized in 12 mL of zESC medium, and 30 µL of the resulting cell suspension would be plated in each well.
7. Embryos were transferred to an appropriate tube, ie, a 1.5-mL microcentrifuge tube for small volumes, or 15/50 mL Falcon tube for larger volumes. E3 was removed, along with any remaining chorions. Transferring chorions should be avoided, as they will clog the filter. For large numbers of embryos, they can be homogenized in a smaller volume and brought up to the final plating volume after filtering. However, no more than 500 embryos should be homogenized in every milliliter of medium.
8. zESC medium was added to the embryos, and they were dissociated by rapidly inverting the tube approximately 5 times or until a homogenous cell suspension was observed, ie, no clumps of cells were visible. For directed differentiation toward muscle, zESC medium containing 1 ng/ml recombinant human bFGF should be used.
9. The cell suspension was passed through a 100 µm cell strainer and brought up to the desired final volume.
10. The cell suspension was pipetted into gelatin-coated tissue culture plates and incubated at 28.5°C, 5% CO₂ in a humidified chamber until analysis.

1.1.4 Representative results—As shown in Fig. 1, blastomeres cultured without bFGF form clusters after 1 day of culture and do not express muscle-specific reporters such as *myf5:GFP*, marking myogenic progenitors and *myl2:mCherry*, marking differentiated muscle cells. Blastomeres cultured with 1 ng/ml bFGF have a more flattened morphology and express *myf5:GFP* and *myl2:mCherry*. Yolk droplets are also visible as uniform spheres in both cultures. As Fig. 2 demonstrates, myogenic cells differentiate in culture over a 2-day period. As determined by qPCR, *myf5* expression peaks during day 1 of culture, while *myl2* expression peaks during day 2.

1.2 NEURAL CREST CELL CULTURE

1.2.1 Overview—Zebrafish neural crest cells can be grown in heterogeneous cultures to facilitate studies of the effects that chemicals or growth factors have on neural crest migration, differentiation, proliferation, and survival. This technique avoids costly and time-consuming cell sorting and retains information about cell-type specificity. Furthermore relatively few embryos can be used: 250 embryos provide enough material for a full 384

well plate. Here we describe a method for the short-term culture of neural crest cells in heterogeneous cultures from early somitogenesis embryos. *Crestin:GFP* reporter fish can be used to identify neural crest cells in the culture.

1.2.2 Materials and reagents

E3 embryo medium (Nüsslein-Volhard & Dahm, 2002)

0.05% bleach solution in E3

2.5mg/mL pronase in E3

40 µm cell strainer

rotor-stator homogenizer (OMNI International TH-115) and sterilized probes

tissue culture plates of desired size

500 µg/mL rat tail collagen type I or 10 µg/mL fibronectin (optional)

sterile phosphate-buffered saline (PBS)

neural crest medium (Table 2)

1.2.3 Plating zebrafish neural crest cells

1. Fish expressing *crestin:GFP* or another transgenic marker were allowed to mate and embryos were collected, cleaned, and incubated at 28.5°C for 10–11 h. For convenience, embryos may also be incubated at room temperature for 16–18 h.
2. Neural crest medium was prepared as indicated in Table 2 by combining additives and bringing the solution to the final volume with base medium. Media was sterile filtered and used within 1 week of preparation.
3. Coated cell culture plates were prepared (optional). Neural crest cells can be cultured in heterogeneous cultures with or without collagen or fibronectin coating. Coating of plates with collagen or fibronectin results in a more adherent culture. Without coating, neural crest cells, as marked by *crestin: GFP*, are attached and highly migratory, but can be easily washed away. Collagen or fibronectin solution was warmed to 37°C, used to cover the bottom of tissue culture wells, and incubated in a cell culture incubator at 37°C for 30 min. The solution was aspirated, and wells were washed twice with sterile PBS. Plates were used immediately or stored in a sterile environment.
4. Embryos were bleached for decontamination. When embryos had reached the two somite stage, they were transferred to a clean petri dish and incubated with 0.05% bleach solution in E3 for 2 min. Embryos were then washed three times with E3.
5. For dechoriation, embryos were incubated in a 2.5 mg/mL pronase solution in E3 at 28.5°C for 5 min and then washed three times with E3.
6. Embryos were counted, and proper staging was verified. Neural crest cells can be plated from 5 to 15 somite-stage embryos at a density of 0.6–1 embryo per 5.6

mm² (1 well of a standard 384 well plate). For example, for plating a full 384 well plate, 250 embryos would be homogenized in 12.3 mL of neural crest medium, and 30 μ L of the resulting cell suspension would be plated in each well. Embryo number can also be estimated by volume. For example, 500 chorionated embryos take up approximately 0.9 mL.

7. Embryos were transferred to appropriate tube for mechanical homogenization, eg., a round-bottom FACS tube. E3 was removed along with any remaining chorions. Transferring chorions should be avoided, as they will clog the filter. For large numbers of embryos, embryos can be homogenized in a smaller volume and brought up to the final plating volume after filtering. However, no more than 500 embryos should be homogenized in every milliliter of medium.
8. Embryos were homogenized in neural crest medium using a rotor–stator homogenizer at 5000 rpm for 10–15 s. The resulting cell suspension was pipetted up and down twice to break up clumps. During this step, the cell suspension was observed to confirm that the embryos had been sufficiently homogenized. If clumps of cells were observed, the suspension was homogenized for another 10–15 s. If embryos have not been sufficiently homogenized, cells will be removed during filtering and clog the filter.
9. The cell suspension was passed through a 40- μ m cell strainer. The homogenization tube and cell strainer were then rinsed with an additional 1 mL of neural crest medium. The cell suspension can also be diluted to facilitate passage through the cell strainer.
10. The cell suspension was brought to the desired final volume, pipetted into tissue culture plates, and incubated at 28.5°C, 5% CO₂ in a humidified chamber until analysis. Neural crest cells cultured in this manner should remain viable for at least 3 days.

1.2.4 Representative results—As shown in Fig. 3A, the percentage of *crestin:GFP*+ cells increases over a period of 1 day in the culture conditions described. While at the time of plating, GFP + cells represent 3% of the total, after 1 day of culture, GFP + cells represent 18% of the total. *Crestin:GFP*+ cells are adherent, migratory, and extend projections in heterogeneous whole embryo cultures (Fig. 3B).

CONCLUSION

Zebrafish embryonic cell culture has many uses, including generating hypotheses for in vivo validation, performing mechanistic experiments in a controlled environment, and facilitating chemical screening. Zebrafish embryonic cells can be cultured from blastula-, gastrula-, or somitogenesis-stage embryos in either a homogeneous or heterogeneous context. As described in this chapter, zebrafish blastomeres can be cultured for differentiation into myogenic progenitors, and zebrafish neural crest cells can be maintained in a heterogeneous context without the need for sorting. These and similar cell culture techniques promise to inform stem and progenitor cell biology in the future.

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FIGURE 1.

Addition of basic fibroblast growth factor (bFGF) to zebrafish embryonic stem cell medium alters the morphology and reporter gene expression of cultured blastomeres. Cells were plated using the described method and cultured for 26 h. While bFGF-treated cultures express both *myf5:GFP* and *myl2:mCherry* and appear flattened and elongated, cultures without bFGF do not express these transgenes and form clusters of rounded cells. Yolk droplets are visible as uniform spheres in the cultures. Scale bar represents 100 μ m.

FIGURE 2.

Muscle gene expression changes over the course of a 2-day culture. Blastomeres were plated as described with or without basic fibroblast growth factor for the indicated period of time. Expression of *myf5* or *myl2* was determined in whole cultures by qPCR and normalized to β -actin. Data points represent the average and standard error of the mean of three technical replicates.

FIGURE 3.

(A) FACS analysis of *crestin:GFP* embryos before and after 1 day of culture demonstrate promotion of neural crest cell fate in neural crest medium. Cells were plated as described at the 10 somite stage at a density of 0.6 embryos per 5.6 mm². FACS plots show GFP (x-axis) versus PE (y-axis) to control for autofluorescence. (B) *Crestin:GFP* + cells are adherent and extend projections in heterogeneous cultures. Left panel: GFP and bright field overlay, 24 h culture. Right panel: magnification of GFP only, 13 h culture. Scale bar represents 100 μm. (See color plate)

Table 1**Zebrafish Embryonic Stem Cell Medium**

Base Medium	Stock Concentration	Volume (Per 100 mL)	Final Concentration
Leibowitz's L-15 medium, with sodium pyruvate, no phenol red	1x	35 mL	35%
DMEM high glucose, HEPES, no phenol red	1x	21 mL	21%
DMEM/F12 1:1 high glucose, HEPES, no phenol red	1x	14 mL	14%
RTS34st conditioned medium ^a	1x	30 mL	30%
Additives			
Sodium bicarbonate	75 g/L	24 µL	0.18 g/L
HEPES	1 M	500 µL	5 mM
L-glutamine	200 mM	1 mL	2 mM
Sodium selenite	1 µM	1 µL	10 nM
Primocin (InVivoGen)	100%	200 µL	0.2%
N2 supplement	100%	1 mL	1%
B27 supplement	100%	2 mL	2%

Unless otherwise indicated, media components were obtained from Invitrogen.

^aPrepare by incubating Leibowitz's L-15 plus 15% FBS on a confluent culture of RTS34st cells for 3 days.

Table 2

Neural Crest Medium

Base Medium	Stock Concentration	Volume (per 100 mL)	Final Concentration
DMEM/F12 1:1 with L-glutamine and 2.438 g/L sodium bicarbonate	1x	100 mL	100%
Additives			
FBS	100%	12 mL	12%
N2 supplement	100%	1 mL	1%
Recombinant human insulin (Gemini Bioproducts)	2 mg/mL	1 mL	20 µg/mL
Recombinant human EGF (R&D systems)	500 µg/mL	4 µL	20 ng/mL
Recombinant human fibroblast growth factor basic (R&D systems)	100 µg/mL	20 µL	20 ng/mL
Primocin (InVivoGen)	100%	200 µL	0.2%

Unless otherwise indicated, media components were obtained from Invitrogen.