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Isolation and Propagation of Glioma Stem Cells from Acutely Resected Tumors

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Abstract

Gliomas are characterized by striking intratumoral cellular heterogeneity. A consistent method to isolate undifferentiated GSC fraction from clinical specimens provides an opportunity to establish in vitro models that more closely mirror the in vivo state compared to traditional serum containing culture methods. Here we describe techniques involved with isolation, identification, and expansion of human malignant glioma-derived stem cells using a serum-free growth condition.

Keywords

Glioma stem cell; Glioblastoma multiforme; GBM; Neural stem cell; N2 medium; Serum-free cell culture

1 Introduction

The suggestion that cancers have properties of stemness (undifferentiated, high capacity for self-renewal) dates back many years [1]. This hypothesis driven by histopathologic similarities between undifferentiated developing tissue and poorly differentiated cancers was extended by subsequent investigators who proposed that cancer represents a disrupted developmental program in which tissues fail to appropriately differentiate to instructive specification [2, 3]. Benefiting from improved understanding of development and technical improvements, leukemic stem cells were identified [4]. Subsequently, the presence of the so-called “cancer stem cell” or “cancer initiating cell” has been identified in a variety of solid tumors [5–11]. Although some investigators take issue with the term “cancer stem cell,” and suggest alternative names such as cancer recapitulating cell, cancer initiating cell, or stem-like cell, the argument is mostly semantic since the cancer stem cell hypothesis does not necessarily imply that all cancers originate from stem cells, nor does it assert that even some cancers are derived from the direct malignant transformation of stem cells. The conceptual importance may be that certain cancers are maintained by a population of malignant cells that exhibit stem-like properties of self-renewal and capacity to differentiate into specialized lineage(s), irrespective of the cell of origin which may be a progenitor cell or a differentiated somatic cell that has reacquired the stem-like properties.

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Ideal culture system should be able to preserve the functional capacities and self-renewal activity of the parental tumor cells. Standard serum-dependent cell culture techniques alter the cellular phenotype by inducing artificial genetic and epigenetic changes. The N2 medium based serum-free method is largely adopted from techniques used for the study of in vitro rodent neural stem cells [12, 13]. Also, the strictly defined condition of the medium excludes all uncharacterized additives. We discuss methods to establish initial primary culture from tissue, composition of the N2 medium, protocols to identify GSC, techniques to passage and freeze cells for storage, and preparation for in vivo implantation into immunodeficient hosts.

2 Materials

2.1 Plate Preparation for Propagation as Monolayer

- 100 mm tissue culture dishes.
- Poly-L-ornithine (Sigma-Aldrich, cat. no. P-3655).
    - **a.** Prepare 5 mg/mL stock solution in sterile water and store at −20 °C for up to 6 months.
    - **b.** For working solution, dilute stock solution to 0.5 mg/mL with water and filter sterilize. Prepare 6 mL solution per 100 mm tissue culture dish.
    - **c.** This diluted solution can be stored at 4 °C for up to 4 weeks.
- Fibronectin (R&D Systems, cat. no. 1030-FN).

2.2 Dissection of Glioma Patient Tissue

Dissection-related general instruments such as blade, forceps, clean tissue culture hood, and occasionally dissection microscopy.

- N2 medium: Combine N2 supplements, which are 50 mg apo-transferrin (Sigma-Aldrich, cat. no. T-2036), 12.5 mg insulin (Sigma-Aldrich, cat. no. I-0516), 50 μL putrescine (Sigma-Aldrich, cat. no. P-5780), 30 μg sodium selenite (Sigma-Aldrich, cat.no. S-5261), 100μL progesterone(Sigma-Aldrich, cat. no. P-8783), and penicillin/streptomycin/Fungizone antibiotic/antimycotic in 500 mL of DMEM/F12 (Thermo Fisher Scientific, cat. no. 11320–033; including high glucose, L-glutamine, and phenol red). Adjust pH to 7.2. Filter the medium through a 0.2 μm filter. The medium can be stored up to 4 weeks at 4 °C.
- Sterile EBSS (Earle's Balanced Salt Solution; Thermo Fisher Scientific, cat. no. 14155063) with bicarbonate and phenol red. Refrigerate between each use (*see* Note 1).
- Papain solution containing L-cysteine and EDTA (Worthington Biochemical Cooperation, papain dissociation system, cat. no. LK003150). 5 mL EBSS solution should contain 20 units of papain per mL, 1 mM L-cysteine, and 0.5 mM EDTA. Short incubation is necessary for full solubility and activity.

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- DNase (Deoxyribonuclease I; Worthington Biochemical Cooperation, papain dissociation system, cat. no. LK003150). Reconstitute with 0.5 mL of EBSS at 2000 units of DNase per mL. Avoid vigorous vortexing.
- Ovomucoid protease inhibitor with bovine serum albumin (Worthington Biochemical Cooperation, papain dissociation system, cat. no. LK003150). Prepare to 10 mg/mL with EBSS. [1] Cell strainer (70 μm filter; Falcon, cat. no. 352350).
- PBS (phosphate buffered saline; HyClone, cat. no. SH3002802) without calcium and magnesium.
- Incubator preset for 95 % O_2: 5 % CO_2 equilibration for EBSS solution.

2.3 Identification of GSC

- CD133 MicroBeads kit (Miltenyi Biotec, cat. no. 130–050-801).
- Magnetic-activated cell sorting (MACS) separator and column (*refer* **Miltenyi Biotec homepage**; http://www.miltenyibiotec.com/).
- Basic F12/DMEM (Thermo Fisher Scientific, cat. no. 11320–033).

2.4 Passaging

- HBSS (Hank's balanced salt solution; HEPES buffer, Media-Tech, cat. no. 20–021-CV). Mix 700 mL water with 100 mL of 10 Ca^{2+} and Mg^{2+}-free (CMF) HBSS. Weigh 3.7 g $NaHCO_3$ and 3.9 g HEPES and transfer to mixture. Adjust pH to 7.1–7.2 with 1 N HCl. Make up to 1 L with water. Sterilize with filter.
- Stock solution for EGF (20 μg/mL, epidermal growth factor; R&D Systems, cat. no. 236-EG) and (20 μg/mL, bFGF, basic fibroblast growth factor; R&D Systems, cat. no. 233-FB). Final working concentration should be 20 ng/mL.
- N2 medium (*see* Sect. 2.2).

2.5 Freezing Cell Stocks in 10 % DMSO Plus N2 Medium Without Serum

- DMSO (Dimethyl sulfoxide; Sigma-Aldrich, cat. no. D-2650).
- Cryo-freezing vials (Corning Life Sciences, cat. no. 430487).
- Propanol.
- Liquid nitrogen tank for cell storage.

2.6 Induction of Differentiation Using Serum or CNTF

- CNTF (Ciliary neurotrophic factor; R&D Systems, cat. no. 557-NT/CF).
- FBS (Fetal bovine serum; Gibco, cat. no. 10437–028).

2.7 Neurosphere Culture and Daily Maintenance with EGF and bFGF

- Non-treated tissue culture dishes (100 mm dish).

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- Accutase (Sigma-Aldrich, cat. no. A6964).
- N2 medium, EGF, bFGF, and HBSS (*please see* Sects. 2.2 and 2.4).
- Cell counter.

2.8 Preparation of Cells for Establishing Animal Models

- Cell counter.
- PBS (*please see* Sect. 2.2) and ice.

3 Methods

3.1 Plate Preparation for Propagation as Monolayer

1. Coat 100 mm dish with 6 mL of poly-L-ornithine overnight in cell culture incubator. This should be done at least 2 days before day of tissue processing.
2. On the following day, aspirate poly-L-ornithine and wash twice for 5 min with 10 mL PBS.
3. Coat the surface of the dish with fibronectin (1:250, in PBS) and incubate overnight (or at least 2 h if urgent).
4. Aspirate fibronectin next day (day of tissue processing), and wash with PBS twice. Incubate the fibronectin-coated dish coated with PBS used in washing step in the cell culture incubator until dissociated cells from the tumor tissue is ready for plating.
5. Dish coated with poly-L-ornithine can be stored in the incubator for up to 2 weeks. The dish double-coated with poly-L-ornithine and fibronectin can be stored for up to 1 week (*see* Note 2).

3.2 Disassociation of Human Glioma Tissue

1. Place acutely resected tumor tissue in 100 mm dish with ~5 mL of N2 medium containing EGF and bFGF (20 ng/mL) in tissue culture hood (*see* Note 3).
2. Aspirate medium and then wash several times with N2 medium.
3. Chop and mince tissue using forceps and scalpel, removing membranes, blood vessels, and necrotic portions of the tumor.
4. Prepare a premixture of DNase (350 μL) and papain solution containing L-cysteine and EDTA. Add this solution and gently disassociate with 10 mL pipette until a homogenous suspension is formed. Keep remaining solution of DNase on ice for later use.
5. Incubate the dish at 37° C. Approximately every 10 min, blend the suspension with 5 mL pipette, and then inspect with inverted microscope for presence of single cells. Repeat this step until single cells are visualized. This step should not exceed 60 min. Total time is dependent on density and tumor size. (*see* Note 4).

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6. Prepare two 50 mL conical tubes with 70 μm cell strainer while tumor cells are in incubator.
7. Transfer the slurry onto the filter in the conical tube. Rinse plate with N2 medium and continue to filter.
8. Repeatedly triturate the slurry mixture on filter and continue to wash with N2 medium.
9. Centrifuge at 300 relative centrifugal force (RCF) for 5 min.
10. During centrifugation, prepare a mixture containing 2.7 mL EBSS with ~300 μL ovomucoid protease inhibitor with albumin, and 150 μL DNase.
11. Gently aspirate the supernatant and resuspend the cell pellet in above solution (**step 10**).
12. Layer the cell suspension in 5 mL of the remaining ovomucoid protease inhibitor plus albumin, and then centrifuge at 200 RCF for 5 min. The interface between the two gradient layers should be clearly visible. Dissociated cells remain at the bottom of the tube and membrane fragments at the interface.
13. Resuspend the cells with 10 mL of N2 medium, and incubate overnight at 37 °C.
14. Next day, centrifuge the cells at 300 RCF for 3 min.
15. Wash the cells with 10 mL PBS and centrifuge at 300 RCF for 3 min.
16. Resuspend the collected cells in N2 medium containing EGF and bFGF, and incubate at 37 C (*see* Note 5).

3.3 Selection of CD133 Expressing GSC

1. This section concerns isolation of GSC positive for expression of CD133 surface marker.
2. Centrifuge collected cells from above, at 300 RCF for 5 min.
3. Remove supernatant and wash with basic F12/DMEM medium.
4. Count number of cells (*see* Note 6).
5. Centrifuge at 300 RCF for 5 min.
6. Remove supernatant and resuspend with N2 medium (up to3×10^7 cells per 300 μL).
7. Turn off light source in cell culture hood and add 100 μL of blocking reagent per 3×10^7 cells.
8. Label the cells with 100 μL of the CD133 microbeads per 3×10^7 cells (Total volume is 500 μL per 3×10^7 cells).
9. Suspend the mixture and incubate for 1 h in dark at room temperature.
10. Invert mixture every 10 min.

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11. Wash with medium.
12. Centrifuge at 200 RCF for 5 min.
13. Aspirate supernatant and resuspend cell pellet in 500 μL of medium per 1×10^7 cells.
14. Place column on magnet with a 50 mL conical tube under the column to collect CD133-negative cells.
15. Equilibrate the column by rinsing with 3 mL DMEM/F12 (*see* Note 7).
16. Wash the column with 1 mL DMEM/F12 and collect in the same tube as prior step. Label these as first pass CD133-negative cells.
17. Place a fresh 50 mL conical tube under the magnet and wash the column with 3 mL DMEM/F12 three times (*see* Note 8).
18. Firmly flush out the CD133-positive fraction containing the magnetically labeled cells using the supplied plunger into CD133-positive conical tube.
19. Collect CD133-positive cell in N2 medium.
20. Send the first pass CD133-negative cells through a new column and keep only the cells that pass through the column initially. Keep these cells as CD133-negative fraction (*see* Note 9).

In our hands, we are unable to maintain and propagate pure population of CD133-positive cells. A fraction of this pool appears to revert to CD133-negative state. Nevertheless, this method may be useful for short-term experiments that call for CD133-positive cells.

3.4 Passaging

1. After several days in culture, cells should begin to form distinct colonies with cell-free space between spheres. At 80 % confluency, cells should be passaged.
2. Place the culture dish under cell culture hood and wash twice with 10 mL of prewarmed HBSS (*see* Note 10). Incubate for 5 min at 37 °C with 5 mL HBSS containing EGF (20 ng/mL) and bFGF (20 ng/mL).
3. Gently pipet HBSS over the surface of the culture dish to lift the colonies off.
4. Collect the HBSS solution including the cells in a 15 mL conical tube, and centrifuge at 300 RCF for 5 min. Remove the supernatant.
5. Resuspend the cells in 1 mL N2 medium and count the viable cells. Inoculate 1.5 million cells per 100 mm precoated dish in 10 mL N2 medium containing recombinant growth factors.

3.5 Freezing Cell Stocks in 10 % DMSO Plus N2 Medium Without Serum

1. Cells can be frozen in liquid nitrogen for long-term storage. Prepare cell freezing container with 250 mL propanol.
2. Centrifuge the cells and remove the supernatant. Harvest and count cells.

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3. Redilute to final concentration of greater than 3×10^6 cells per mL.
4. Add 100 μL of DMSO in cryo freezing vial and add 900 μL of cells in the vial. Invert twice and store in freezing container overnight at −80 °C (at least 4 h). Transfer to liquid nitrogen tank for long term storage.

3.6 Induction of Differentiation

Differentiation of GSC can be induced by addition of serum, cytokine such as CNTF, or growth factor withdrawal.

1. Add 10 % FBS or 20 ng/mL CNTF.
2. Replace with medium containing FBS or CNTF every day for 3–5 days.
3. Confirm extent of differentiation by immunocytochemistry for expression of stem and lineage markers.

3.7 Neurosphere Culture and Daily Maintenance with EGF and bFGF

GSC can be maintained in sphere form without use of precoated culture dishes. EGF signaling promotes sphere formation capacity [13]. In our experience, daily addition of growth factors appears critical in maintaining the stem state.

1. For sphere propagation, use uncoated dishes.
2. Count cell number and plate 1×10^6 cells (100 mm dish) with N2 medium containing growth factors.
3. Add 20 ng/mL EGF and bFGF every day.
4. When ready to passage or store, collect the culture medium including tumor spheres. Rinse with 5 mL of warm HBSS.
5. Centrifuge the cells for 5 min at 200 RCF and aspirate supernatant.
6. Resuspend cells with 1 mL of Accutase at 37 °C during 2 min to dissociate the cells. Mechanical trituration can be used in lieu of Accutase.
7. Centrifuge the cells for 5 min at 200 RCF and collect the cell pellet.
8. Inoculate 1×10^6 cells (100 mm dish) in N2 medium or freeze for storage.

3.8 Preparation of Cells for Establishing Animal Models

Acutely dissociated tumor cells and primary culture cells can also be used to establish orthotopic xenograft models in immunodeficient hosts.

1. We found most GSC lines to be capable of forming infiltrative tumors with 1×10^3 to 1×10^4 cells per mice.
2. Centrifuge and wash with PBS. Dissociate (Accutase or trituration) and collect cells by centrifugation.
3. Count cells and resuspend in 8 μL of PBS per each mice. Keep on ice until transplantation.

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4 Notes

1. EBSS includes a pH-sensitive dye. Color purple indicates alkaline state. Incubation medium should be well buffered at physiological pH.
2. Fibronectin is susceptible to denaturation. Care should be taken to prevent drying of tissue culture dishes.
3. Only one tumor sample should be processed at a time to avoid cross-contamination.
4. Trituration is a critical process. Too vigorous manipulation will result in damaged cells; too weak and tissue fragments will be left intact. Gentle trituration is needed using 5 mL pipette.
5. Cells should be maintained with daily addition of growth factors.
6. Dead cell should be removed to improve efficiency of the magnetic beads.
7. Do not allow column to dry out.
8. Do not use culture these cells for the following step.
9. Do not collect any other cells.
10. Do this step one dish at a time to avoid cell loss.

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