Colon Cancer Stem Cells

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ABSTRACT

This unit describes protocols for working with colon cancer stem cells. To work with these cells one must start by generating single-cell suspensions from human colon cancer tissue. These cell suspensions are sorted using flow cytometry–assisted cell sorting to fractionate the cells into tumor-initiating and nontumor-initiating subsets. Once the cells have been fractionated, they must be functionally tested to determine tumor-forming capacity, the gold standard being the in vivo xenograft assay. Methods have also been developed to grow these cells in vitro in a sphere-forming assay. This unit will describe how to isolate and functionally test colon cancer stem cells, as well as provide advice on the potential challenges of the research. Curr. Protoc. Stem Cell Biol. 7:3.1.1-3.1.12. © 2008 by John Wiley & Sons, Inc.

Keywords: human colon cancer • cancer stem cells • in vivo xenograft assay • in vitro sphere assay

INTRODUCTION

This unit describes protocols for working with colon cancer stem cells (CSC). The ability to successfully carry out this work is dependent on obtaining fresh colon cancer specimens at the time of surgical resection. Tissue fragments are processed to generate a single-cell suspension, which can then be fractionated utilizing flow cytometry to isolate subpopulations based on differential expression of cell surface markers, such as CD133 (O’Brien et al., 2007; Ricci-Vitiani et al., 2007; Todaro et al., 2007). Once these cell subsets have been fractionated, they can be tested for their tumor-forming capacity using the in vivo NOD/SCID xenograft assay. Utilizing this model it has been shown that tumor-initiating capacity exists solely within the CD133+ cell subset of colon cancer cells. The focus of this unit will be to describe the protocols for isolating, culturing (Basic Protocol 1), fractionating (Basic Protocol 2), and establishing a NOD/SCID xenograft model (Basic Protocol 3) to study colon CSC. The sphere-forming assay is also described (Basic Protocol 4).

NOTE: The following procedures are performed in a Class II biological hazard flow hood or a laminar-flow hood.

NOTE: All solutions and equipment coming into contact with live cells must be sterile, and proper aseptic technique should be used accordingly.

NOTE: All incubations are performed in a humidified 37°C, 5% CO2 incubator.

NOTE: All experiments using human tissue must be approved by the institutional committee on the ethical use of human subjects/material and tissue samples must be obtained with prior informed consent.
BASIC
PROTOCOL 1

GENERATING SINGLE-CELL SUSPENSIONS FROM HUMAN COLON CANCER TISSUE

The first step to working with colon CSC involves generating a single-cell suspension from human colon cancer tissue. The percentage of necrotic cells in human tumors varies extensively and is dependent on multiple factors including: tumor characteristics, preoperative adjuvant chemotherapy or radiation therapy, and length of operative procedure. One factor that can help in obtaining the maximum number of viable cells is to ensure that specimens are received from the operating room expeditiously after removal from the patient. It has been our experience that each sample possesses a CSC fraction; however, the percent of this fraction can vary widely between tumors and this is true whether one is using CD133 or CD44 to isolate the CSCs. It is best when isolating the CSC fraction to start with at least 1 to 2 × 10^6 colon cancer cells (use a tumor fragment ~1 × 0.5–cm in size to generate this many cells), because this will help ensure that there are enough cells in the CSC and non-CSC subsets to carry out the experiments.

**Materials**
- Colon tumor fragment
- Colon cancer stem cell medium (SCM; see recipe)
- Collagenase IV solution (200 U/ml SCM)
- Ammonium chloride: 0.8% (w/v) NH₄Cl in 0.1 mM EDTA
- Trypan blue
- 35-mm petri dishes
- Razor blade and forceps
- 5-ml disposable pipets
- 50-ml conical tube
- 45-μm cell filter
- Plunger from a 3- to 5-ml syringe
- Hemacytometer

Additional reagents and equipment for counting cells using a hemacytometer and trypan blue (UNIT 1C.3)

**Isolate colon cancer cells**

1. Place colon tumor fragment in 2 to 3 ml SCM in a 35-mm petri dish.

2. Using a razor blade and forceps, mince the tissue as much as possible.

3. Pipet tumor solution up and down 3 to 5 min with a 5-ml disposable pipet. Place the solution into a 50-ml conical tube.

   *If fragments are too large to be drawn up into a 5-ml pipet, use a pipet with an opening large enough to draw up all the tumor fragments. Note that the highest cell numbers are typically obtained when tumors are minced to yield very small pieces.*

4. Add the collagenase solution to the tumor cells. Incubate 30 to 60 min at 37°C. Pipet up and down a few times every 15 min.

   *The final concentration should be 200 U of collagenase IV per milliliter of SCM.*

5. Pass the tumor solution through a 45-μm filter. Use a plunger from a 3- to 5-ml syringe and gently mash the tumor pieces to enable more tumor cells to pass through. Wash the filter with 4 to 5 ml of SCM.
The tumors can be very different; some are soft whereas others are hard and fibrotic. The fibrotic tumors may not completely dissolve with collagenase. The fragments that remain on the filter can be resuspended in SCM with collagenase and incubated for 1 to 2 hr at 37°C. Then repeat step 5.

6. Centrifuge the tumor cell suspension 10 min at 450 \( \times \) g, 4°C.

7. Resuspend the pellet in \( \sim \) 5 ml of ammonium chloride (0.8% w/v NH\(_4\)Cl with 0.1 mM EDTA). Leave for 10 min at room temperature to lyse the red blood cells. After 10 min add an equal volume of SCM and centrifuge 10 min at 450 \( \times \) g, 4°C.

8. Resuspend the pellet in 10 ml SCM. If the solution appears clumpy then pass it through another 45-\( \mu \)m filter.

9. Count an aliquot of the cells using a hemacytometer and trypan blue (UNIT 1C.3) to determine the percentage of dead cells.

If there is a high percentage of necrotic cells, a Ficoll column can be used to remove the dead cells and debris (see Support Protocol).

**USING A FICOLL COLUMN TO REMOVE DEAD CELLS**

The high percentage of necrotic cells and debris in some samples makes it exceedingly difficult to successfully carry out techniques such as flow cytometry–assisted cell sorting and transduction. In cases where samples have \( >30\% \) dead cells this Ficoll protocol can allow for an enrichment of viable cells.

**Materials**

- Ficoll
- Colon cancer cell suspension (Basic Protocol 1)
- Colon cancer stem cell medium (SCM; see recipe)
- 15-ml conical tubes
- 5-ml pipet

1. Place 5 ml Ficoll into a 15-ml conical tube.

2. Resuspend the colon cancer cells in 5 ml of SCM. Layer this solution on top of the 5 ml of Ficoll.

divide the tumor cell suspension such that each 5 ml of medium contains no more than 4 to 5 \( \times \) \( 10^6 \) tumor cells. If the cell number is \( >5 \times 10^6 \), divide the sample into the appropriate number of Ficoll-containing tubes.

3. Centrifuge 15 min at 1000 \( \times \) g, 4°C.

4. Use a 5-ml pipet to remove 2 to 3 ml of medium off the top and then place the pipet at the interface (between the medium and Ficoll). Collect the interface, remainder of the medium, and a small amount of Ficoll. Resuspend the pellet in 5 ml SCM and save until the viable cell count is complete.

keep this solution until the cell number from the viable fraction has been counted. If the number of viable cells post-Ficoll differs significantly from the pre-Ficoll count, it is possible that some viable cells are in the pellet. In that case, repeat step 2 with the resuspended pellet.

5. Centrifuge 10 min at 450 \( \times \) g, 4°C. Resuspend the pellet in a desired volume of SCM.
FLOW CYTOMETRY–ASSISTED CELL SORTING

Flow cytometric–assisted cell sorting is an essential aspect of CSC work. It is required to fractionate the CD133\(^+\) and CD133\(^-\) cell subsets. It also allows the researcher to exclude all murine cells when sorting colon cancer xenografts, thereby avoiding any murine hematopoietic or endothelial cells contaminating the post-sort cell populations. To avoid contaminating cells in primary human colon cancer sorts one can positively select for epithelial specific antigen (ESA) expression and sort the following populations: ESA\(^+\)CD133\(^+\) and ESA\(^+\)CD133\(^-\). The initial selection on ESA\(^+\) cells allows one to exclude contaminating hematopoietic, endothelial, and stromal cells. Furthermore, flow cytometry also allows one to study other markers of interest in combination with CD133.

If flow cytometry–assisted cell sorting is not available, another option is to carry out magnetic bead cell sorting as per the Miltenyi-Biotec protocol. It has been our experience that this method can be used successfully provided the starting sample has ≤30\% dead cells. If the sample has >30\% dead cells, it can be difficult to obtain the necessary purity (≥90\% to 95\%) using the MACS bead separation. To successfully carry out MACS bead enrichment the sample should be passed through at least three Miltenyi-Biotec columns in order to best enrich the sample. One of the other main disadvantages of using the MACS beads is the inability to select on multiple cell surface markers.

Materials

Colon cancer cell suspension with <30\% dead cells
Phosphate-buffered saline, calcium- and magnesium-free (CMF-PBS) with 0.1\% bovine serum albumin (BSA; CMF-PBS/0.1\% BSA)
ESA antibody conjugated to a fluorophore
Anti–mouse antibody conjugated to a fluorophore
CD133 APC or PE (Miltenyi Biotec)
Propidium iodide (final concentration: 1 \(\mu\)g/ml of PBS with 0.1\% BSA)
5-ml polystyrene tubes

Additional reagents and equipment for flow cytometry–assisted cell sorting (Robinson et al., 2008) and counting cells using a hemacytometer (UNIT 1C.3)

1. Resuspend colon cancer cells in PBS/0.1\% BSA to a concentration of 0.5 to 1 \(\times\) 10\(^6\) cells/100 \(\mu\)l.

   During antibody staining, if you have ≤5 \(\times\) 10\(^6\) cells, you can stain all cells in the 100 \(\mu\)l per 1 \(\times\) 10\(^6\) cells. However, if you have >5 \(\times\) 10\(^6\), divide the cells into separate polystyrene tubes for staining. This is done because it is best to stain cells in a smaller volume.

2. Add fluorophore-conjugated anti-CD133 and anti-mouse or anti-ESA antibodies to the sort sample.

   When sorting human samples, an antibody against ESA should be used. When sorting xenografts, use an anti-murine IgG antibody to exclude murine cells.

   Check with the FACS facility to determine whether isotypes and individual antibody control stains are required. All antibodies should be titrated to determine the required amount.

   Ask the FACS facility whether they prefer that the sort sample be placed into a polypropylene or polystyrene tube for sorting.

3. Incubate cells with antibodies 30 to 45 min at 4°C, protected from light.

4. Add 4.5 ml CMF-PBS/0.1\% BSA to each tube and centrifuge 5 min at 450 \(\times\) g, 4°C. Discard the supernatant. Repeat three times to wash the cells.
5. Following the last wash, resuspend the cells in 1 to 2 ml CMF-PBS/0.1% BSA that contains 1 μg/ml propidium iodide.

At this point the cells are ready to be sorted.

Propidium iodide stains dead cells thereby allowing them to be excluded.

6. Sort the ESA⁺ CD133⁺ cells (Robinson et al., 2008) into 2 ml SCM.

7. Count an aliquot of the post-sort cells with a hemacytometer (UNIT 1C.3) to confirm the cell yield.

The flow cytometry facility will provide a post-sort cell count for each population; however, it is important that the researcher confirm the cell count.

### IN VIVO XENOGR AFT ASSAY

The utilization of animal models is crucial in CSC work. The ideal choice is always an orthotopic model in which cancer cells are injected into the same tissue from which they are derived. However, when carrying out CSC research the most important factor is to identify an animal model that has the greatest reliability for xenograft formation. This is essential because if the tumor take rate for the model you choose is only 50% it becomes impossible to determine whether the absence of a xenograft is due to the lack of a CSC or simply the limitation of the animal model. Published colon CSC work to date have used two models: subcutaneous (Ricci-Vitiani et al., 2007; Todaro et al., 2007) and subrenal capsule (O’Brien et al., 2007) injections. It has been our experience that only ~30% of colon cancer cell suspensions have a reliable take rate in the subcutaneous site. In our hands, injection of colon cancer cell suspensions under the renal capsule provided the most reliable results with almost all tumors tested giving rise to xenograft formation (overall take rate of ~90%).

Aside from the choice of injection site a decision must also be made about the type of immunocompromised mouse to be used. Published studies in colon CSC research have used either NOD/SCID or SCID mice (Dalerba et al., 2007; O’Brien et al., 2007; Ricci-Vitiani et al., 2007). It is difficult to make a direct comparison between the efficiency of the NOD/SCID versus SCID mice for use in CSC work because no head-to-head comparison has been carried out between the two strains using the same marker set and injection site. It is also important to acknowledge that an increasing number of immunocompromised mouse strains are becoming available and may represent new options for carrying out this work.

Irradiating the mice prior to the procedure can also improve the xenograft take rate; however, the marginal improvement in xenograft formation must be weighed against the radiation sensitivity of NOD/SCID mice. Irradiation should be carried out the day before or the day of the procedure and the dose should be 300 rad. It is best to carry out a trial of irradiation to determine both the potential benefit to xenograft take rate and the radiation sensitivity of the mice in the colony. Death related to radiation sensitivity usually occurs 6 to 7 weeks post-irradiation.

### Materials

- Matrigel
- ~10 μl sorted cell suspension in SCM (Basic Protocol 2)
- Stem cell medium (SCM; see recipe)
- NOD/SCID or SCID mice (8- to 10-week-old)
- Iodine-based solution (e.g., Betadine)
- 70% ethanol
Normal saline, sterile
Pain medication (e.g., buprenorphine)
1-ml syringe without needle
1-ml insulin syringes with a 29-G needle, 1/2-in. long
Heating pad
Clippers
Sterile gauze
Scissors
Forceps
Sutures or surgical clips (Roboz)

Additional reagents and equipment for rodent anesthesia using isoflurane (UNIT 1B.4)

1. Thaw an aliquot of Matrigel and draw it up into a 1-ml syringe without needle. Then remove the plunger from a 1-ml insulin syringe with a 29-G needle, 1/2-in. long and insert a small amount of Matrigel into the back of the syringe (Fig. 3.1.1A).

   It is difficult to be exact on the amount of Matrigel—one should aim for it to be 25 to 50 μl (closer to 25 μl is better). The best way to estimate the amount of Matrigel is to look at the markings on the side of the syringe.

   Keep undiluted Matrigel aliquoted and frozen at −20°C, ~100 to 200 μl per microcentrifuge tube.

2. Use a pipet to inject the cell suspension (aim to resuspend the cells in 10 μl of SCM) into the middle of the Matrigel (Fig. 3.1.1B). Then reinsert the plunger into the syringe and push the mixture to the top of the syringe (Fig. 3.1.1C,D).

   Once the syringes are prepared they should be injected in a timely fashion. It is best to keep the syringes on ice, because at room temperature Matrigel will set.

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Figure 3.1.1 Preparing syringes for injection. (A) Inserting the Matrigel into the back of the insulin syringe. (B) Using a pipet place the 10 μl of cell solution into the middle of the Matrigel. (C) Reinsert the insulin syringe plunger. (D) Using the plunger push the Matrigel and cell solution to the top of the syringe, it is now ready for injection.
3. Handle mice using sterile technique and anesthetize using inhalational anesthesia (UNIT 1B.4). Place the mice on a heating pad during the procedure.

4. Position the mouse left side up. Use clippers to shave the area ~0.5-cm below the costal margin on the left side. Wash the clipped area sequentially with iodine-based solution and 70% ethanol solution and then dab dry with sterile gauze.

5. Using scissors make an ~0.5-cm long incision on the flank, just below the costal margin on the left side (Fig. 3.1.2A).

   *Only inject into the left kidney. Due to the anatomy it is very difficult to inject into the right kidney.*
6. Gently pull the kidney from the abdominal cavity using two pairs of forceps. Then take the syringe containing the cells to be injected and place the needle just under the kidney capsule and push the needle until just before it comes out the opposite pole. At this point, inject the cells as you slowly pull back on the needle.

   The cell solution should be completely injected before the needle exits the kidney (Fig. 3.1.2B,C,D).

7. Deliver the kidney back into the abdomen and close the abdominal wall.

   There is the choice to close using sutures or surgical clips (Roboz) both of which provide equivalent results. The choice may depend on the requirements of the animal research facility at your particular institution.

8. Prior to awakening the mouse from anesthesia administer a 1 ml subcutaneous bolus of sterile normal saline and a dose of pain medication (e.g., 0.01 to 0.05 mg/kg buprenorphine).

   At the commencement of this work an antibiotic (Baytril) was added to the drinking water of all mice post-procedure for 2 weeks. The mice became dehydrated and as a result weak; therefore, the practice was stopped. There were no deleterious effects from discontinuing Baytril.

9. Assess the mice for tumor development every week starting 2 weeks post procedure. This can be done by holding the mouse and gently palpating in the area of the kidney. Over time, you will start to appreciate a fullness (the tumor will feel like a firm nodule) in this area.

   The rate of tumor development will differ depending on the tumor. Some xenografts will appear in 6 to 8 weeks; however, others can take up to 30 weeks to develop.

**BASIC PROTOCOL 4**

**CULTURING COLON CANCER CELLS AS SPHERES**

The ability to culture colon CSC requires the utilization of a serum-free stem cell medium. Using this protocol colon CSC will grow as spheres (Fig. 3.1.3A,B) in a non-adherent manner. The addition of serum to this medium results in the differentiation of the colon cancer cells and their growth as an adherent layer. Although much of the current work in the cancer stem cell field has been carried out using in vivo models, the ability to culture the cells as spheres can be used to complement the in vivo work. It is important to keep a record of the passage number for each tumor in vitro.

**Materials**

- Colon cancer cell suspension, sorted (Basic Protocol 2)
- Stem cell medium (SCM; see recipe)
- Trypsin/EDTA
- Ultra-low attachment surface dishes (Corning)
- 5-ml disposable pipet
- 45-μm filter
- Additional reagents and equipment for counting cells using trypan blue (UNIT 1C.3)

1. Plate sorted colon cancer cells at a density of 30,000 to 50,000 cells/ml of SCM.

   For best results the spheres should be grown in ultra-low attachment surface dishes (Corning). Any size dish can be used depending on the cell number; the most important point is to plate at a density of 30,000 to 50,000 cells/ml of SCM.

2. Passage the cells approximately every 4 days.

   There is some variability between tumors and, therefore, each tumor must be evaluated daily to follow sphere formation. Passaging requires the disruption of the spheres to generate a single-cell suspension. There are two possible approaches to sphere disruption: mechanical or enzymatic.
Mechanical sphere disruption

3a. Centrifuge the colon cancer cell solution 10 min at $450 \times g$, $4{^\circ}C$.

4a. Resuspend the pellet in 3 ml of SCM and pipet up and down for 10 min with a 5-ml disposable pipet.

   After pipetting for 10 min look at the solution. There should no longer be any visible spheres. If visible spheres remain, continue to pipet for another 5 to 10 min.

5a. Pass solution through a 45-μm filter, count an aliquot of the cells (UNIT 1C.3), and resuspend in the desired volume of SCM for replating.

   Best results are obtained when cells are plated at a density of 30,000 to 50,000 cells/ml of SCM.

Enzymatic sphere disruption

3b. Centrifuge the colon cancer cell solution 10 min at $450 \times g$, $4{^\circ}C$.

4b. Resuspend the pellet in 3 to 5 ml of 1× trypsin/EDTA and pipet up and down for 3 min with a 5-ml disposable pipet. At this point, place the tube in the incubator 10 min at $37{^\circ}C$.

5b. After 10 min remove the cells from the incubator and add an equal volume of SCM. Pass the cells through a 45-μm filter. Centrifuge the colon cancer cell solution 10 min at $450 \times g$, $4{^\circ}C$.

6b. Resuspend the cell solution and count the number of viable cells using trypan blue (UNIT 1C.3).

   The criticism associated with the use of enzymatic digestion is that it may interfere with the expression of cell surface markers. Enzymatic digestion can be used; however, it should be tested initially against mechanically digested cells from the same tumor. This testing should be carried out for each cell surface marker to determine whether enzymatic digestion has any deleterious effect on the expression of the cell surface markers being studied.
REAGENTS AND SOLUTIONS

For culture recipes and steps, use sterile tissue culture-grade water. For other purposes, use deionized, distilled water or equivalent in recipes and protocol steps. For suppliers, see SUPPLIERS APPENDIX.

Growth factor mix, 10×

For 200 ml of growth factor mix:
100 ml DMEM/F12
4 ml 30% (w/v) glucose
200 mg transferrin
50 mg insulin in 20 ml of water (add 2 ml of 0.1 N HCl to dissolve, then add 18 ml of water)
19.33 mg putrescine in 20 ml water
200 μl 0.3 mM sodium selenite
20 μl 2 mM progesterone
H₂O to 200 ml

Divide the growth factor mix into 2-ml aliquots and store indefinitely at −20°C

Stem cell medium (SCM)

500 ml of a 1:1 ratio of DMEM/F12 (Invitrogen)
1% penicillin-streptomycin (1 × from a 100 × stock purchased from Invitrogen)
2 ml 50 × B27 supplement (Invitrogen)
4 μg/ml heparin
1% (w/v) of non-essential amino acids
1% (w/v) of sodium pyruvate
1% (w/v) of L-glutamine
Store up to 1 week at 4°C
Just before use, add
5 ml growth factor mix (see recipe)
10 ng/ml fibroblast growth factor
20 ng/ml epidermal growth factor

COMMENTARY

Background Information

The existence of a CSC fraction was first studied in the context of human leukemia. Lapidot et al. demonstrated that acute myelogenous leukemias possess a CSC subset capable of recapitulating the disease in a SCID mouse model, whereas the non-CSC cells were incapable of generating the disease (Lapidot et al., 1994). More recently, it has been shown that a wide variety of solid tumors also possess a CSC subset including breast (Al-Hajj et al., 2003), brain (Singh et al., 2004), and colon cancers (O’Brien et al., 2007; Ricci-Vitiani et al., 2007).

The identification of a CSC population in human colon cancer was first published in 2007, when two groups established that fractionation of colon cancer cells based on CD133 expression identified a subset of CD133⁺ cancer cells that was capable of initiating tumor growth in murine xenograft models. In contrast, the CD133⁻ cancer cells were unable to initiate tumor growth. The limiting dilution analyses in one of the studies demonstrated that ~1 in 262 CD133⁺ colon cancer cells represented a CSC, for the ten tumors tested in the series, thereby demonstrating that CD133 expression enriches for tumor initiating capacity but does not identify a pure CSC population (O’Brien et al., 2007). More recently, another publication demonstrated that CD44 and CD166 (ALCAM) expression could also be utilized to enrich for a CSC subset in colon cancers (Dalerba et al., 2007). It is important to appreciate that the markers identified to date enrich for CSC; however, they do not identify a pure population. The field remains at a very nascent stage, and advancement will depend in large part on the identification of new CSC markers that can be used in conjunction with markers, such as CD133 and CD44, to provide further enrichment of the CSC fraction.
Limiting dilution analysis (LDA) represents an essential tool in carrying out CSC work because it provides the ability to calculate the frequency of CSC within a population of cancer cells. LDAs require the injection of a range of doses with multiple mice being injected per dose (Porter and Berry, 1964). The gold standard is to carry out an LDA of both bulk and fractionated cancer cells for each individual tumor. This allows for the calculation of a CSC frequency in both the bulk tumor cell population and the fractionated subpopulations (e.g., CD133+ versus CD133−). The limiting factor in these experiments is often the paucity of cell number. One method to circumvent issues of cell number is to initially inject unsorted cells into four to five mice to expand the cell number and then to use these xenografts to carry out the bulk and fractionated LDAs in mice. It has been our experience and the experience of others that the cell surface phenotype is maintained following passage in mice (Dalerba et al., 2007; O’Brien et al., 2007; Ricci-Vitiani et al., 2007). However, it is very important to determine the cell surface phenotype for each tumor prior to passage in mice and then after each passage. This will allow you to confirm for each tumor that the subpopulations are remaining stable following passage in vivo. It has been our experience that at high levels of in vivo passage (7 and above) we do start to see some tumors that change their cell surface phenotype; however, the changes are not predictable. It is for these reasons that it is crucial to check the tumor cell surface phenotype with each passage.

There is very limited data on the propagation of colon CSC as sphere-forming units. One recent publication identified that in a series of colon cancers only approximately half could be propagated in vitro as spheres (Todaro et al., 2007). There is also the suggestion that CSC marker expression may change with in vitro propagation (A. Kreso and C.A. O’Brien, unpub. observ.). Therefore, the sphere-forming assay represents a surrogate; however, it does not eliminate the need to carry out the gold standard, functional in vivo assays. The sphere-forming assay requires further study to clearly establish its role within CSC work and to determine how closely it recapitulates in vivo models. Furthermore, caution must be exercised when using cancer cells following serial passages in vitro because to date it has not been clearly established whether the cells maintain the same functional phenotypes. Until these questions have been answered, the best approach when using a sphere-forming assay is to functionally test the CSC and non-CSC fractions with in vivo assays at a minimum of every other in vitro passage.

**Critical Parameters and Troubleshooting**

The ability to successfully sort the cells using flow cytometry will depend in large part on the flow facility. It is important when starting this work to determine the level of expertise at your flow facility for sorting solid tumor cells. If the facility does not have expertise in this area, it is important to contact a flow facility that regularly sorts solid tumor cells to establish the instrument settings that result in the highest yield, both with respect to purity and viability of the cells post-sorting. It is also essential to count cells post-sorting to confirm the cell yield. There can be a discrepancy between the stated and actual cell yields; having an accurate cell count is essential when carrying out LDA experiments.

**Anticipated Results**

The in vivo protocol will result in the generation of xenografts, which recapitulate the phenotype of the original tumors. At the time of sacrifice a fragment of each xenograft should be saved for histological assessment. This will allow the researcher to confirm that the xenograft recapitulates the original tumor with respect to differentiation status and tumor subtype.

The in vitro protocol will result in expansion of the colon cancer cells; however, as previously stated there is a proportion of the cancers that cannot be successfully passaged in vitro. At this time we are unable to predict which tumors will grow well in vitro. As more work is carried out in the field, it will hopefully lead to a better understanding of the in vitro requirements to maintain colon cancer cells in serum-free culture conditions.

**Time Considerations**

The initial culturing of colon CSC from an unpassaged primary human specimen may take >4 to 5 days. If this is the case, one should leave the cells in the incubator for 7 to 14 days, adding EGF and FGF to the medium every 4 days. If the tumor has not formed spheres by 14 days it is unlikely to do so.

The time for xenograft formation varies between tumors, ranging from 6 to 24 weeks. To do the in vivo experiments it is important to use NOD/SCID mice between the ages of 8 and 10 weeks. Mice utilized before 8 weeks...
have an increased chance of succumbing to the stress of the procedure. Using older mice (>10 weeks) can be difficult because as NOD/SCID mice age there is a natural attrition rate; therefore in the case of tumors that take 24 weeks to appear it is possible that the mice of interest will die before the appearance of xenografts.

The primary tissue is often received from the operating room at the end of the workday and it is important to note that once the cell suspension is generated these cells can be safely left at 4°C overnight in SCM and injected or sorted the next day.

Acknowledgements
We thank Sean McDermott for his contributions to the Ficoll protocol and other members of the Dick laboratory for helpful discussions. We also acknowledge members of Peter Dirks’ laboratory, especially Ian Clarke, for technical suggestions. We thank John E. Dick for his invaluable help and guidance.

Literature Cited


In Vivo Evaluation of Leukemic Stem Cells through the Xenotransplantation Model

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ABSTRACT

The xenotransplantation model has been instrumental for the identification and characterization of human leukemic stem cells. This unit describes our current method for the engraftment of human leukemic patients’ samples in the xenotransplanted mouse model. We concentrate uniquely on the model of acute myeloid leukemia, as it was the first type of leukemia for which the xenotransplantation model was developed. Nevertheless, the Basic Protocol could be applied to other sorts of blood disorders. Curr. Protoc. Stem Cell Biol. 7:3.2.1-3.2.11. © 2008 by John Wiley & Sons, Inc.

Keywords: hematopoietic stem cell (HSC) • xenotransplantation • immunodeficient mice • leukemic stem cell (LSC)

INTRODUCTION

The adaptation of xenotransplantation assays to examine the propagation of acute myeloid leukemia (AML) in vivo has been fundamental in the identification and characterization of leukemia-initiating cells (Lapidot et al., 1994; Bonnet and Dick, 1997). Transplantation of primary AML cells into NOD/SCID mice led to the finding that only rare cells, termed AML-initiating cells (AML-IC), also known as leukemic stem cells (LSC), are capable of initiating and sustaining growth of the leukemic clone in vivo, and serial transplantation experiments showed that AML-IC possess high self-renewal capacity, and thus can be considered to be the leukemic stem cells.

The Basic Protocol below describes the most common and simple method to test for the presence of leukemia-initiating cells; this method can also be used to characterize blood disorders. Support protocols describe methods for further purification of leukemic stem cells and the intra-bone injection procedure.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must follow officially approved procedures for the care and use of laboratory animals.

NOTE: All of the following procedures using human samples should be performed in a level 2 safety tissue culture unit using sterile and proper aseptic techniques.

IDENTIFICATION OF LEUKEMIA STEM CELLS THROUGH XENOTRANSPLANTATION

Xenotransplantation of human cells obtained from AML patients allows identification and characterization of leukemic stem cells.

Materials

- Immunodeficient mice: NOD/SCID, NOD/SCID-β₂ microglobulin null (β₂m⁻/⁻), or NOD/SCID IL2R gamma null (Jackson Laboratory)
- Acidified water: a solution of HCl at a final pH 2.8 to 3.2
AML sample: peripheral blood or bone marrow
Phosphate-buffered saline, calcium- and magnesium-free (CMF-PBS; see recipe)
Ammonium chloride solution (Stem Cell Technologies)
Fetal bovine serum (FBS; Stem Cell Technologies, cat. no. 06471)
Antibodies against human CD45, CD34, CD38, CD33, and CD19 (BD Biosciences Phamrmingen)
100 ng/ml 4′,6-diamidino-2-phenylindole (DAPI; UV excited, Sigma-Aldrich) or
TOPRO-3 [HeNe (633-nm) excitable, Molecular Probes]

Irradiator: Cesium source is recommended, but an X-ray system or Cobalt source can also be used
29-G, ½-in. needle and insulin syringe (Tyco Healthcare)
Dissection tools: scissors and forceps
5-ml snap-top polystyrene tubes
Benchtop centrifuge equipped with swing-out bucket rotor for 15- and 50-ml conical tubes
Hemacytometer
Fluorescent-activated cell sorter, e.g., FACSaria (BD Biosciences) and/or a Moflow (Dako) equipped with 488-nm, 633-nm, and 404-nm lasers
440/40 bandpass (bp) filter for analysis of DAPI, a 530/30 bp filter for FITC, a 575/26 bp for PE, a 695/40 bp for PerCP, and a 660/20 bp for TOPRO-3
Mouse depletion kit (e.g., StemCell Technologies, cat. no. 13066)

Additional reagents and equipment for assessing for AML engraftment (Support Protocol 3), parenteral injections (Donovan and Brown, 2006a), euthanasia of mice (Donovan and Brown, 2006b), and performing a cell count using a hemacytometer (UNIT 1C.3)

Prepare the immunodeficient mice
1. Keep the animals in a pathogen-free environment.

   All the NOD/SCID animals used have some impairment of their immune system and may succumb to infections not affecting normal mice. They thus should be kept in pathogen-free status within barrier systems to protect them from current infections.

2. Treat the mice for at least 8 days with acidified water before irradiation.

3. Set the sublethal irradiation dose (between 300 and 375 cGys) on the irradiator used.

   Success indeed depends on the dose rate/minute of irradiation. If the dose rate/minute is initially too high, try to reduce it by using appropriate shielding. This will reduce the damage to internal organs.

4. Sublethally irradiate the mice before the adoptive transfer of cells. For best results, perform the irradiation 24 hr before the adoptive transfer of cells.

   The dose of irradiation depends on the mouse strains used and also on the source and irradiator used. It usually varies from 300 to 375 cG (see notes above).

5. Maintain any mouse receiving irradiation on acidified water for 2 weeks following the irradiation dose to prevent diarrhea or weight loss possibly arising due to epithelial damage of the intestines.

   Any animal showing persistent weight loss >20% of body weight and/or other signs of illness (e.g., rough fur, loss of appetite, inability to groom, and immobility) should be sacrificed. Experience shows that with these measures, the above side effects rarely arise.

Prepare AML cells
6. Isolate mononuclear cells (for an example procedure, see Bonnet and Dick, 1997) from fresh peripheral blood or bone marrow samples.

   Frozen samples can also be used (see Support Protocol).
7. For prescreening, inject 5 to 10 × 10^6 mononuclear cells per mouse (4 to 5 mice are tested).

   Not all AML samples at diagnosis engraft. Thus, for all new samples that arrive in the laboratory, we test the capacity to engraft first by injecting 5 to 10 × 10^6 viable cells/mouse using intravenous (tail vein) injections. Injection of the cells is done using under 100 to 200 μl/mouse. Usually, cells are resuspended in PBS/2% FBS.

8. After 8 to 12 weeks, sacrifice the mice by cervical dislocation (Donovan and Brown, 2006b) or terminal anesthesia and assess for AML engraftment (see Support Protocol 3).

   If the AML sample engrafts, purification of the AML-initiating cells (AML-IC) can be performed using Lin, CD34, and CD38 expression and cell sorting (see Support Protocol 3).

Transfer the cells adoptively

9. Subject mice to sublethal irradiation prior to injection of cell preparations (unpurified or purified cell fraction, genetically modified or not).

   This may be performed on the same day as, or up to 3 days after irradiation.

10. Inject between 10^6 and 10^7 cells intravenously via the tail vein (Donovan and Brown, 2006a; maximum volume 1% body weight) using a syringe with a 29-G, 1/2-in. needle.

   In some cases, an intra-bone marrow injection (see Support Protocol) might be preferred, especially if a decrease in homing efficiency of the cell transferred is suspected.

Analyze the engraftment

11. Sacrifice mice between 4 to 14 weeks after transplantation using either cervical dislocation (Donovan and Brown, 2006b) or terminal anesthesia.

   When work under terminal anesthesia is involved, the level of anesthesia should be maintained at sufficient depth for the animal to feel no pain.

   Blood sampling is not informative as blood samples do not usually match the level of engraftment present in the bone marrow. Indeed, usually few AML cells circulate in the periphery, except in some AML samples (usually samples from patients with poor prognosis) where the AML infiltrates solid organs like spleen and liver. In these cases, the animals become sick and will need to be sacrificed potentially before 10 to 12 weeks.

12. Dissect the femurs, tibias, and iliac crests from the mice and store at room temperature in CMF-PBS before flushing. Remove all connective tissue around the bone.

Prepare bone marrow

13. Place 1 ml of room temperature CMF-PBS in a 5-ml snap-top polystyrene tube.

   This will be used to flush the bone marrow (see step 15, below).

14. Cut both ends of each bone to provide openings.

15. To flush, insert the 1-ml CMF-PBS-containing insulin syringe into one end of each bone and wash the lumen of the bone with medium pressure. Repeat twice for both ends of the bone or until the bone appears white.

Prepare the cells for FACS analysis

16. To lyse red blood cells, first cool the cell suspension 5 min on ice. Following the cooling of the suspension, add 3 ml of cold ammonium chloride solution to the 1-ml CMF-PBS/cell suspension, mix, and leave for 5 min at 4°C.

17. Add 0.5 ml FBS and centrifuge cells 5 min at 380 × g, 4°C.

18. Remove the supernatant and resuspend the cells in 1 ml of cold CMF-PBS containing 2% (v/v) FBS.
Figure 3.2.1 (legend at right)
19. Count the cells using a hemacytometer (UNIT 1C.3). Store on ice until ready for antibody labeling.

**Stain the cells**

20. Prepare a mix of human-specific FITC-conjugated anti-CD19, PE-conjugated anti-CD33, and PerCP-conjugated anti-CD45 (5 μl/sample/antibody for all stains and compensation/isotype controls). Also, prepare FITC, PE, and PerCP single-color compensation control tubes (5-ml snap-top polystyrene tubes as before) and a combined FITC/PE/PerCP matched isotype control tube.

21. Distribute 15 μl of the antibody mix into each tube of a fresh set of 5-ml tubes for antibody labeling.

22. Dispense 40 μl of each cell suspension into the appropriate antibody labeling tube and leave to label for 30 min at 4°C.

23. Wash cells in 2 ml PBS/2% FBS and resuspend in 500 μl of PBS/2% FBS supplemented with a cell impermeant DNA dye for live/dead discrimination, either 100 ng/ml 4′,6-diamidino-2-phenylindole or TOPRO-3.

**Perform FACS analysis**

24. To analyze this combination of fluorochromes set up the FACS device with a 488-nm excitation source and either a UV or HeNe (633-nm) source depending on your choice of live/dead discriminator.

25. For emission collection ensure you have a 440/40 bandpass (bp) filter for analysis of DAPI, a 530/30 bp filter for FITC, a 575/26 bp for PE, a 695/40 bp for PerCP and a 660/20 bp for TOPRO-3 in place.

26. During FACS analysis, set the photomultiplier gains so that the background signal from the combined isotype control gives 1% to 5% positive cells in each collection channel.

27. Set the compensation amount according the detected spectral overlap.

28. To analyze the engraftment, draw four dotplots as in panels A, B, C, and D in Figure 3.2.1 [440/40 nm versus side-scatter (SSC), forward scatter (FSC) versus SSC, 695/40 nm versus SSC, and 530/30 nm versus 575/26 nm].

29. First, exclude dead cells from the analysis via a region (R1) around the live, unstained cells as in Figure 3.2.1A.

30. Next, display these cells on a FSC versus SSC plot and select the lymphoid and myeloid cells for further analysis but exclude debris via a region (R2) as in Figure 3.2.1B.

31. Display cells that fall into the first two regions on a 695/40-nm versus SSC plot and draw a generous region around the CD45-PerCP positive cells as in Figure 3.2.1C (R3).

**Figure 3.2.1 (at left)** For analysis of the engraftment by FACS, first the dead cells are excluded using DAPI staining and a live cells region (R1) is drawn (A). Next, these cells are displayed on an FSC versus SSC plot and lymphoid and myeloid cells are selected for further analysis, but debris is excluded via region R2 (B). A generous region is drawn (R3) around the CD45+ cells (C). These CD45+ cells are further analyzed for the expression of CD33 and CD19 (D). The number of events that fall within these regions may be used to calculate the percentage of live, debris-free cells (R2) that are human cells. In addition, the scatter characteristics of cells may be confirmed as consistent with myeloid [high FSC and SSC; (E)] and lymphoid [low FSC and SSC; (F)]. In panel F, there are no CD19+ cells. Engraftment is classed as myeloid leukemia if a population of CD45+/CD33+ cells is present without an accompanying CD45−CD19+/CD33− cell population.
32. Display these CD45^+ cells on a CD19-FITC versus CD33-PE (530/30-nm versus 575/26-nm) dotplot and draw a quadrant to define FITC^+ /PE^- cells and FITC^- /PE^+ cell subsets as in Figure 3.2.1D.

33. Use the number of events that fall within these regions to calculate the percentage of live, debris-free cells (R2) that are human cells. In addition, evaluate the scatter characteristics of cells to confirm them as consistent with myeloid (high FSC and SSC, example in Fig. 3.2.1E) and lymphoid (low FSC and SSC, example in Fig. 3.2.1F).

   In this example, there are no CD19^+ cells. Engraftment is classed as myeloid leukemia if a population of CD45^+ /CD33^- cells is present without an accompanying CD45^+ /CD19^- /CD33^- cell population.

   To confirm the leukemic origin of the myeloid cells present in the bone marrow of engrafted mice and if the original AML sample has a known translocation, it is possible to sort human CD45^+ cells and perform fluorescent in situ hybridization analysis of the cells or any PCR analysis in search of a fusion product or a mutated gene (NPM, Flt3L, c-kit, WT1, CEBPa). The description of the procedures for FISH or PCR analysis is beyond the scope of this unit.

**Perform serial transplantation**

34. To test for self-renewal capacity, perform secondary transplantation. Sort human CD45^+ cells from either the first primary recipients or enrich them using a mouse depletion kit following the manufacturer’s instructions.

35. Inject the recovered cells into a second recipient using the same protocol as described before (see steps 9 and 10).

36. After 6 to 12 weeks sacrifice the mice and analyze for human cells engraftment the same way as for primary transplantation (see steps 12 to 33).

**INTRA-BONE MARROW INJECTION**

To exclude stem cell homing interference and focus on the intrinsic capacity of a cell to self-renew, a few groups recently developed a highly sensitive strategy based on direct intra-bone marrow (IBM) injection of the candidate human stem cell (Mazurier et al., 2003; Wang et al., 2003; Yahata et al., 2003). IBM injection was found to be a more sensitive and adequate means to measure human HSC capacity. The intra-bone injection technique is performed under a short general anesthesia following a method originally described by Verlinden et al. (1998).

**Additional Materials (see Basic Protocol)**

- Anesthetic solution (see recipe)
- Post-operative analgesic (Vetergesic; Alstoe Animal health), diluted 1/10 in PBS and injected at 100 μl subcutaneously per mouse
- 29-G, ½-in. needle (or 25-G needle) and insulin syringe (Tyco Healthcare)

**Anesthetize the mice**

1. Inject the mice intraperitoneally with a dose of 0.2 to 0.25 ml of anesthetic solution.

   General anesthesia suppresses the heat-regulating mechanisms of the body. This is overcome by intra- and post-operative maintenance of body temperature in appropriate thermostatically controlled incubators or by other heat sources.

2. Insert a syringe with a 25-G needle (maximum) into the joint surface of the right or left tibia/femur, and inject up to 40 μl cell suspension into the bone marrow cavity of the tibia or femur.
3. During recovery, keep the animals under regular observation until full mobility is regained.

4. At this stage, provide at least one dose (100 μl) of post-operative analgesic (Vetergesic diluted 1/10) subcutaneously following bone marrow injection.

THAWING AML CELLS

While freshly obtained AML cells are desirable for xenotransplantation, thawed frozen cells are also suitable, and it may be more convenient to collect and freeze the cells until one is ready for the transplantation experiment.

Materials

- AML cells, frozen in 1.8- to 2-ml cryovials
- DNase (Sigma, cat. no. D4513), thawed
- Fetal bovine serum (FBS; Stem Cell Technologies, cat. no. 06471) or any other suppliers
- Phosphate-buffered saline, calcium- and magnesium-free (CMF-PBS; see recipe)
- 37°C water bath
- 50-ml centrifuge tubes, sterile
- Table-top centrifuge equipped with swing-out bucket rotor for 15- and 50-ml conical tubes
- Cell strainer

Additional reagents and equipment for counting cells (UNIT 1C.3)

NOTE: Before thawing the samples, ensure the water bath is at 37°C.

NOTE: Before starting, ensure that the DNase is completely thawed.

1. Rapidly thaw the AML cells (cryovial of 1.8- to 2-ml) in the 37°C water bath. 

   There are no commercial suppliers of frozen AML. These samples can be obtained from clinics after informed consent from the patients has been obtained.

2. Add 100 ml DNase (1 mg/ml) dropwise into the cryovial.

3. Mix gently, wait 1 min, and transfer cells into a sterile 50-ml centrifuge tube.

4. Gently add 1 ml heat-inactivated, pure FBS dropwise, mix gently, and wait 1 min.

5. Slowly add 10 ml CMF-PBS/2% FBS and wait 1 min.

6. Slowly add up to 30 ml CMF-PBS/2% FBS to fill the tube.

7. Centrifuge 5 min at 200 × g, 4°C.

8. Resuspend in 1 ml CMF-PBS/2% FBS.

9. Filter using a cell strainer if needed (if cells are clumping).

10. Count viable cells (UNIT 1C.3) and use for purification or adoptive transfer protocols.

PURIFICATION STRATEGY

Mononuclear cells from AML samples can be stained using a combination of antibodies. The most commonly used are the lineage cocktail antibodies (BD, cat. no. 340546), anti-CD34 and anti-CD38. Stain the cells 25 to 30 min at 4°C in the presence of 5 μl/million of each of these antibodies. After the incubation period, centrifuge the cells 5 min at 380 × g, 4°C. Discard the supernatant, resuspend the cells in 1 ml CMF-PBS/2% FBS. Repeat this wash procedure one more time. After washing, the cells are ready to be sorted through a cell sorter (FACS Aria, BD, or equivalent).
REAGENTS AND SOLUTIONS

*For culture recipes and steps, use sterile tissue culture-grade water. For other purposes, use deionized, distilled water or equivalent in recipes and protocol steps. For suppliers, see SUPPLIERS APPENDIX.*

**Anesthetic solution**

Mix 1 ml of Ketase solution (Fort Dodge Animal Health) with 0.5 ml of 2% Rompun solution (Bayer plc) and dilute with 8.5 ml of CMF-PBS. Store up to 1 month at 4°C.

**Phosphate-buffered saline, calcium- and magnesium-free (CMF-PBS)**

Prepare 10× stock with 1.37 M NaCl, 27 mM KCl, 100 mM Na2HPO2, and 18 mM KH2PO4 (adjust to pH 7.4 using HCl, if necessary) and autoclave. Store up to 2 months at room temperature. Prepare working solution by dilution of one part with nine parts water.

COMMENTARY

**Background Information**

AML-IC can be prospectively identified and purified as CD34+/CD38− cells in AML patient samples, regardless of the phenotype of the bulk blast population, and these cells represent the only AML cells capable of self-renewal (Bonnet and Dick, 1997). The phenotype of AML-IC has been extended to include the expression of CD123 but the absence of CD71, HLA-DR, and CD117 (Blair et al., 1997, 1998; Blair and Sutherland, 2000; Jordan et al., 2000). In a recent study, this phenotype was further extended to include expression of CD33 and CD13 on AML-IC for the vast majority of patients (Taussig et al., 2005). Hence, the immunophenotype of the leukemic stem cell as defined by in vivo propagation is CD34+/CD38−/CD71−/HLA-DR−/CD117−/CD33+/CD13+/CD123+. However, the exclusivity of some of these markers is debatable. For instance, CD123 is indeed expressed on AML-IC, but it is also expressed on the vast majority of AML blasts (D. Bonnet, unpub. observ.) from most patients, and hence could be excluded from the above phenotype of AML-IC.

Considerable heterogeneity within the AML-IC compartment exists. Lentiviral gene marking to track the behavior of individual LSCs, following serial transplantation, has revealed heterogeneity in their ability to self-renew, similar to what is seen in the normal HSC compartment (Hope et al., 2004). Furthermore, using the NOD/SCID IL2Rγnull mice (see Anticipated Results) pretreated with anti-CD122, we show that in some patients the LSC activity can be detected in a CD34+/CD38− population (Taussig et al., 2008). Thus, there is not a universal protocol to purify for LSC in AML. Each AML patient sample should be tested first for its ability to engraft (prescreening) and secondly for identifying the nature of the cells responsible for repopulating ability. Secondary transplantation should also be used to test for the self-renewal ability of the LSC.

**Critical Parameters**

By LSC, we refer to a cell that has self-renewal and differentiation potential and is able to reinitiate the leukemia when transplanted into NOD/SCID mice. This definition does not preclude the nature of the cells being transformed.

The confusion regarding the origin of the AML-IC may be due to the extreme heterogeneity of AML. Given the various possible routes to AML from a normal hematopoietic cell, it is not surprising that there is great heterogeneity in AML. Indeed, AML may be thought of as a large collection of different diseases that merely share similar characteristics. Indeed, the most effective risk stratification approach so far has been to examine the genetic abnormalities associated with a particular case of AML and compare it to previous experience with AML cases with the same abnormality (Grimwade et al., 1998, 2001). Although cytogenetic analysis allows the definition of the hierarchical groups with favorable, intermediate, and poor prognosis, the intermediate risk group contains patients with variable outcomes. Assessing the prognosis of this large group of patients is currently difficult.

**Troubleshooting**

It is usually straightforward to detect AML engraftment. The human cells present express
human CD45+ and the pan-myeloid marker CD33 without detection of lymphoid markers (CD19). Nevertheless, it happens that in some cases a chimeric engraftment can be detected, indicating that both human normal and leukemic cells are present in the engrafted mouse. In this case, it is usually important to confirm the leukemic origin of the CD33 subfraction by performing either FISH analysis or PCR for the mutations present in the original patient samples. Human CD45+ can be sorted based on CD19 and CD33, and both fractions should be tested for the presence of leukemic cells. Thus, it is not sufficient when screening for human AML engraftment to only test for the presence of human CD45+ cells, as in some cases these human cells could be exclusively normal cells.

Anticipated Results

From AML patients’ samples at diagnosis, the capacity to engraft in the xenotransplantation model is usually ∼65% to 70%. Thus, there are still 0.25% to 30% of patients for which no engraftment could be detected after 10 to 12 weeks. The ability of a particular AML to engraft in the xenotransplantation model is related to the prognosis of individual AML cases (Pearce et al., 2006). Specifically, examination of the follow-up results from younger patients with intermediate-risk AML revealed a significant difference in overall survival between NOD/SCID-engrafting and non-engrafting cases. No differences have been detected between engrafting and non-engrafting cases in various engraftment variables including: homing ability, AML-IC frequency, and immune rejection by the host or alternative tissue sources. Hence, the ability to engraft NOD/SCID recipients seems to be an inherent property of the cells that is directly related to prognosis. Other mouse models have been developed to support the growth of human hematopoietic cells but less is known about the ability of these new models to sustain AML engraftment. The NOD/SCID-β2 microglobulin null (β2m−/−) mouse has an additional defect in NK cell activity and is more tolerant of human grafts than the NOD/SCID model (Christianson et al., 1997; Kollet et al., 2000; Glimm et al., 2001). However, the percentage of AML samples that engraft in β2m− is similar to the level achieved using the NOD/SCID mice. Thus, it does not appear that the β2m− is superior for the engraftment of AML samples (Pearce et al., 2006). Furthermore, both NOD/SCID and β2m− are susceptible to developing lymphomas over time, limiting their lifespan and preventing long-term reconstitution assessment. These hurdles have recently been overcome in three new strains: NOD/Shi-Scid IL2Rgnull (Yahata et al., 2002; Hiramatsu et al., 2003), NOD/SCID IL2Rgnull (Ishikawa et al., 2005; Shultz et al., 2005), and BALB/c-Rag2null IL2Rgnull (Traggiai et al., 2004), which all lack the IL-2 family common cytokine receptor gamma chain gene. The absence of functional receptors for IL-2, IL-7, and other cytokines may prevent the expansion of NK cells and early lymphoma cells in NOD/SCID IL2Rgnull mice, resulting in better engraftment of transplanted human cells and longer lifespan of the mice. It was reported recently that human HSCs and progenitor cells engraft successfully in these mice and produce all human myeloid and lymphoid lineages. T and B cells migrate into lymphoid organs and mount HLA-dependent allogeneic responses, and generate antibodies against T cell–dependent antigens such as ovalbumin and tetanus toxin (Traggiai et al., 2004; Ishikawa et al., 2005). However, preliminary testing in our group seems to indicate that the NOD/SCID IL2Rgnull mice are not superior for leukemic engraftment to NOD/SCID or the NOD/SCID-β2m− mice. Thus, intrinsic properties of AML cells will dictate whether or not the cells will engraft.

Time Considerations

It usually takes 4 to 6 weeks to detect engraftment. Nevertheless, we have seen in the past that the kinetics of AML engraftment, in contrast to normal stem cells, is usually slower, and thus it is better to wait 10 to 12 weeks for estimating the engraftment of AML samples. It sometimes happens, especially with poor cytogenetic samples that the mice get sick after only 3 to 4 weeks due to an infiltration of AML cells into solid organs (like spleen, liver, kidney). In this case, the mice have to be sacrificed earlier.

Acknowledgments

The author would like to thank Dr. Daniel Pearce for his assistance in the preparation of this manuscript.

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Culture and Isolation of Brain Tumor Initiating Cells

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ABSTRACT

This unit describes protocols for the culture and isolation of brain tumor initiating cells (BTIC). The cancer stem cell (CSC) hypothesis suggests that tumors are maintained exclusively by a rare fraction of cells that have stem cell properties. We applied culture conditions and assays originally used for normal neural stem cells (NSCs) in vitro to a variety of brain tumors. The BTIC were isolated by fluorescence activated cell sorting for the neural precursor cell surface marker CD133. Only the CD133+ brain tumor fraction contains cells capable of sphere formation and sustained self-renewal in vitro, and tumor initiation in NOD-SCID mouse brains. Therefore, CD133+ BTICs satisfy the definition of cancer stem cells in that they are able to generate a replica of the patient’s tumor and they exhibit self-renewal ability through serial retransplantation. This established that only a rare subset of brain tumor cells with stem cell properties are tumor-initiating, and, in this unit, we describe their culture and isolation. Curr. Protoc. Stem Cell Biol. 11:3.3.1-3.3.10. © 2009 by John Wiley & Sons, Inc.

Keywords: brain tumor initiating cells (BTICs) • tumor sphere culture • CD133 • cell sorting • cancer stem cell (CSC)

INTRODUCTION

In this unit, protocols for the culture and isolation of brain tumor initiating cells (BTICs) are described. The cancer stem cell (CSC) hypothesis suggests that tumors are maintained exclusively by a rare fraction of cells that have stem cell properties. Here, we discuss the methods that we first used to prospectively identify and enrich for a subpopulation of human BTICs that exhibit the stem cell properties of proliferation, self-renewal, and differentiation in vitro (Singh et al., 2003) and in vivo (Singh et al., 2004). We applied culture conditions and assays originally used to characterize normal neural stem cells (NSCs) in vitro (Reynolds and Weiss, 1992; Tropepe et al., 1999) to a variety of pediatric and adult brain tumors. The BTIC were exclusively isolated by fluorescence activated cell sorting for the neural precursor cell surface marker CD133 (Yin et al., 1997; Yu et al., 2002). Only the CD133+ brain tumor fraction contains cells that are capable of sphere formation and sustained self-renewal in vitro, as well as tumor initiation in NOD-SCID mouse brains. Therefore, CD133+ BTICs satisfy the definition of cancer stem cells in that they are able to generate a replica of the patient’s tumor and they exhibit self-renewal ability both in vitro and in vivo through serial retransplantation (Bonnet and Dick, 1997; Reya et al., 2001). This formally established that only a rare subset of brain tumor cells with stem cell properties are tumor-initiating.

This unit begins with a method for the culture of tumor spheres from human brain tumors (Basic Protocol 1) and follows with a protocol for the prospective isolation of BTICs from these cultures by magnetic bead sorting (Basic Protocol 2) or fluorescence activated sorting (Alternate Protocol) for CD133.

NOTE: The following procedures are performed in a Class II biological hazard flow hood or a laminar-flow hood.
NOTE: All solutions and equipment coming into contact with live cells must be sterile, and proper aseptic technique should be used accordingly.

NOTE: All incubations are performed in a humidified 37°C, 5% CO₂ incubator unless otherwise specified.

CULTURE OF TUMOR SPHERES FROM HUMAN BRAIN TUMORS
This protocol is adapted from those previously established for isolation of neural stem cells as neurospheres (Reynolds and Weiss, 1992, 1996; Reynolds et al., 1992), and has been applied to the culture of human brain tumors. We use this culture method specifically to select for cell populations within human brain tumors that possess stem cell properties. Serum-free medium (SFM) allows for the maintenance of an undifferentiated stem cell state, and the addition of bFGF and EGF allows for the proliferation of multipotent, self-renewing, and expandable tumor spheres. The medium on these tumor sphere cultures should be changed every other day, and when primary tumor spheres reach a critical size of >100 μm, they may be passaged to secondary spheres based on the principles of the neurosphere assay. The frequency of the stem cell population within the brain tumor can be determined by primary sphere formation assay, and the minimal frequency of repopulating tumor sphere cells within the culture can be estimated by serial sphere formation through limiting dilution analysis (Tropepe et al., 1999).

Materials
Hi/low aCSF (see recipe)
95% O₂/5% CO₂
Enzyme digestive mix for tumors (see recipe) or high-performance liquid chromatography–purified collagenase/dispose cocktail (e.g., Liberase Blendzyme 3 from Roche)
Human brain tumor specimen
Tumor sphere medium (see recipe)
Soybean trypsin inhibitor (from Glycine max; Sigma, cat. no. T9003)
10 ng/μl leukemia inhibitor factor (LIF) stock solution (see recipe)
10 ng/μl recombinant human basic fibroblast (bFGF) stock solution (see recipe)
10 ng/μl recombinant human epidermal growth factor (EGF) stock solution (see recipe)
0.22-μm pore size, 150-ml filter system (Corning)
Oxygen tank setup in laminar flow hood to allow for sterile oxygenation of solutions
15- and 50-ml conical centrifuge tubes
0.22-μm syringe filter units (Millipore)
10-cm² tissue culture–grade plates (Falcon) or 100-mm Ultra Low Attachment Culture dishes (Corning)
Fine sterile scissors and forceps
Incubator-shaker (VWR Scientific)
Tabletop centrifuge
70-μm cell strainer (BD Falcon)

Dissociate human brain tumor tissue
1. Prepare 125 ml of hi/low aCSF as described in Reagents and Solutions, and filter sterilize using a 0.2-μm filter and 150-ml filter system.

2. Bubble aCSF with sterile 95% O₂/5% CO₂ for 15 min and place in 37°C water bath.
3. Freshly prepare the enzyme digestive mix for tumors (see Reagents and Solutions) by weighing out the enzymes (and kynurenic acid) into separate 15-ml centrifuge tubes. Resuspend each enzyme/reagent in 10-ml of hi/low aCSF, vortex thoroughly, and filter all of the components through a 0.22-μm syringe filter into one sterile 50-ml centrifuge tube. Alternatively, measure high performance liquid chromatography–purified collagenase/dispase cocktail (Liberase Blendzyme 3) according to the manufacturer’s instructions, and resuspend in aCSF.

If using enzyme digestive mix for tumors, final working concentrations are: trypsin 1.33 mg/ml, hyaluronidase 0.67 mg/ml, and kynurenic acid 0.1 to 0.17 mg/ml. If using the alternate method with Liberase Blendzyme 3, the final working concentration is 0.2 Wünsch units/ml in a total of 15 ml aCSF.

4. In sterile hood, wash tumor specimen in a 10-cm² plate filled with hi/low aCSF, transferring sequentially to new 10-cm² plates filled with aCSF until excess blood is thoroughly washed out.

The authors have tested 12 brain tumor subtypes (Singh et al., 2003, 2004) and they all had some proportion of BTICs; sample size can be very small and BTICs are still isolated, so there is no minimum sample size. BTIC yield directly correlates with grade of tumor, i.e., there is lower BTIC yield with benign low-grade tumors and higher yield with biologically aggressive malignant tumors (Singh et al., 2003, 2004).

5. Using sterile fine scissors and forceps, cut tumor into 1-mm³ pieces in a 10-cm² tissue culture plate with 2 to 3 ml of enzymatic digestion mixture (either the enzyme/kynurenic acid mix used in step 3, or Liberase Blendzyme 3).

6. Collect tumor pieces with 10-ml pipet, pipetting up and down and dispensing the tumor fragments into 30 ml of the enzymatic digestion mixture used in step 3 or 15 ml Liberase Blendzyme 3.

7. Digest at 37°C for 30 to 90 min (depending on tumor size) with gentle mixing in an incubator-shaker.

The incubator-shaker provides superior digestion and subsequent yield of cells compared to a rocker.

If using Liberase Blendzyme 3, the digestion period can be reduced to 15 to 30 min.

Stop enzymatic reaction
Steps 8 to 10 are skipped if using Liberase Blendzyme 3. In that case, simply filter the cell digest from step 7 through a 70-μm cell strainer and proceed to step 11.

8. Freshly prepare the trypsin inhibitor solution by resuspending 35 mg of trypsin inhibitor in 5 ml tumor sphere medium (without LIF, bFGF, or EGF) and filtering through a 0.2-μm filter.

9. Centrifuge cells 3 min at ~450 × g, room temperature, and take off as much supernatant as possible without dislodging the tumor tissue at the bottom of the tube.

10. Add the 5 ml of trypsin inhibitor solution from step 8, mix well, and filter through a 70-μm cell strainer.

Plate cells in tumor sphere medium with growth factors

11. Centrifuge cells 3 min at ~450 × g, room temperature. Aspirate all of the supernatant and resuspend in 10 to 12 ml tumor sphere medium supplemented with 10 ng/ml LIF (add from 10 ng/μl stock), 20 ng/ml bFGF (add from 10 ng/μl stock), and 20 ng/ml EGF (add from 10 ng/μl stock).

12. Plate cells in 10-cm² dishes at 2 × 10^5 cells per cm², in 10 to 12 ml of tumor sphere medium with LIF, bFGF, and EGF. Incubate in a 37°C 5% CO₂ incubator.
Figure 3.3.1  Brain tumor initiating cells forming sphere-like structures in vitro. (A) Tumor spheres of anaplastic medulloblastoma. (B) Tumor spheres of metastatic melanoma.

13. Feed cells every other day by centrifuging 3 min at 450 × g, room temperature, aspirating the medium, and replacing it with fresh tumor sphere medium supplemented with fresh LIF, bFGF, and EGF, as described in step 11.

14. When number of spheres per plate has doubled or spheres are consistently >120 μm in size, split cultures by repeating steps 11 to 12.

Figure 3.3.1 shows brain tumor initiating cells forming sphere-like structures in vitro.

**ENRICHMENT OF BTICs BY MAGNETIC BEAD SORTING FOR CD133**

This protocol is used for the prospective isolation or enrichment of BTICs from tumor sphere cultures, which constitute primary human brain tumor cells, cultured as per Basic Protocol 1. Cell sorting is performed as soon as tumor spheres begin to form in culture, and is optimally performed within 1 to 24 hr after initial cell culture. Cells must be in single-cell suspension for optimal sorting, and spheres are gently tritured or chemically dissociated prior to cell sorting, by methods detailed below. Cell sorting for CD133 can be performed either by magnetic bead cell sorting (MBCS; this protocol) or by fluorescence activated cell sorting (FACS; Alternate Protocol), and we provide methods for each of these options. Considerations of experimental timing, cell sorter availability at short notice or at night, cell viability after processing through FACS, individual tumor characteristics, overall cell number, and inter-user reliability can help to discern which method of cell sorting should be used for each tumor sphere sample.
Materials

Primary human brain tumor cells growing in culture (Basic Protocol 1)
Incubation buffer: phosphate buffered saline (PBS; Invitrogen, cat. no. 14190-144)/0.5% (w/v) BSA with (or without; see below) 2 mM disodium EDTA, pH 7.2
FeR blocking reagent (Miltenyi Biotec)
Phosphate-buffered saline (Invitrogen, cat. no. 14190-144)
MACS CD133 Cell Isolation Kit (Miltenyi Biotec) consisting of:
beads conjugated to monoclonal mouse anti–human CD133 Microbeads, Isotype IgG1 magnetic cell separator (MiniMACS column magnet)
MS separation columns
CD133-2-PE antibody
mouse IgG2b-PE isotype control antibody
Tumor sphere medium (see recipe)
4% (w/v) paraformaldehyde (optional)
15-ml conical centrifuge tubes (Falcon or equivalent)
Flame-narrowed pipets
70-μm cell strainer
6-well Ultra Low Cluster plates (Corning)
Flow cytometry tubes (BD Falcon 352058)

Additional reagents and equipment for counting viable cells by trypan blue exclusion (UNIT 1C.3) and flow cytometry (Robinson et al., 2009)

NOTE: Work quickly and keep cells/buffer (not culture media) cold (4° to 6°C).

Prepare cell suspensions

1. Place 2 × 10^5 cultured tumor spheres in a 15-ml centrifuge tube. Centrifuge cells 3 min at ~450 × g, room temperature, and remove medium. Resuspend cell pellet in 1 ml of incubation buffer.

   Do not use EDTA if working with Notch pathway molecules, due to potential interactions.

   RBC contamination will decrease purity and cause tumor cell death upon RBC lysis; to remove RBCs if specimen is vascular, treat the cells with an RBC lysis buffer (StemCell Technologies, cat. no. 67850).

2. Triturate gently with flame-narrowed pipet or micropipet tip (if not at single-cell suspension already).

3. Filter through 70-μm cell strainer, and count viable cells using a hemacytometer and trypan blue (UNIT 1C.3).

   Gently triturate and strain cells just before flow to avoid clumping. You may use a flame-narrowed or regular pipet or micropipet; we avoid excessive trituration to minimize trauma to the cells.

4. Aliquot equal amounts of cell suspension into four microcentrifuge tubes as follows:

   negative control (unstained) (A1)
   isotype control (A2)
   pre-sort CD133-1 staining of specimen (A3)
   bulk of the cells in the last aliquot, for bead sorting (A4).

Perform magnetic separation

5. Resuspend A1 and A4 in 300 μl incubation buffer and add 100 μl FeR blocking reagent to A4. If cell number in A4 is >5 × 10^6, divide into two tubes and treat both the same way. Resuspend A2 and A3 in 500 μl PBS. Keep aliquots A1, A2, and A3 on ice.
6. Add 1 μl beads per 10^6 cells to tube A4. Mix beads well in A4 and leave at 4°C (in the refrigerator in dark) for 30 min.

7. After 30 min, take the A4 aliquot and place the microcentrifuge tube into a MACS separator magnet. Use LS columns or MS columns provided with the kit, based on cell number.

8. Rinse column with 3 ml incubation buffer for LS column. Add 3 ml cell suspension onto the column, and allow negative cells to pass through. Collect negative cells in a 15-ml centrifuge tube.

9. Wash four times, each time with 3 ml incubation buffer. Pool with the negative cells in the 15-ml centrifuge tube from step 8.

10. Remove column from separator and place on a clean 15-ml centrifuge tube. Flush out positive fraction with 3 ml incubation buffer by firm application of hand to column.

11. Repeat, and apply column one more time to resuspended CD133^+ cells, to purify this population further if cell numbers permit.

   *Reapplying the cells to the column increases the purity of the CD133^+ cells to ~95%.*

12. Centrifuge the sorted A4 aliquots, CD133^- from steps 8 and 9 and CD133^+ from step 11, 3 min at 450 × g, room temperature, and remove the supernatant. Wash by adding 2 ml incubation buffer, centrifuging again as before, and removing the supernatant. Resuspend each pellet in 3 ml tumor sphere medium.

13. Plate 2–5 × 10^4 cells per well in 3 ml tumor sphere medium per well of a 6-well plate. Re-equilibrate immediately to 37°C.

**Perform a purity check by immunostaining and flow cytometric analysis**

14. Take a 10-μl aliquot from the last CD133^+ and CD133^- aliquots (step 12) and resuspend each in 500 μl PBS.

15. Add 5 μl CD133-2-PE antibody to each sample, and incubate at 4°C for 30 min.

   *These specimens will be taken to the flow cytometry lab for a purity check of the CD133^+ and CD133^- sorted populations.*

16. Add 5 to 10 μl of CD133-2-PE antibody to tube A2 and 5 to 10 μl isotype IgG2b-PE control antibody to tube A3 (see step 4). Incubate at room temperature for 15 to 30 min. Also incubate the unstained A1 control and carry it through the remaining steps.

17. Centrifuge aliquots A2 and A3 3 min at ~450 × g, room temperature, and remove the supernatants. Wash by adding 10 to 20 pellet volumes of PBS, centrifuging again as before, and removing the supernatant. Finally, resuspend in 250 μl PBS.

   *The cells should be washed as thoroughly as possible after staining; however, this must be balanced with the potential for cell loss.*

18. **Optional:** Post-fix A2, A3, and A1 aliquots and the two purity check aliquots from A4 in 2% (w/v) paraformaldehyde (i.e., add 250 μl 4% paraformaldehyde to each aliquot).

19. Triturate each sample gently and transfer to flow cytometry tubes, preferably through strainer caps to remove clumps.

20. Analyze the five samples by flow cytometry (Robinson et al., 2009).

   *See Figure 3.3.2 for sample results.*
ENRICHMENT OF BTICs BY FLUORESCENCE ACTIVATED CELL SORTING

Cell preparation and staining are performed as per the magnetic separation protocol (Basic Protocol 2), with the exception that the A4 aliquot, containing the bulk of the cells, is used for fluorescence activated cell sorting (FACS; Robinson et al., 2009). This aliquot should be resuspended in 300 μl PBS with 100 μl FcR blocking reagent. If the cell number in A4 is >5 × 10^6, divide the suspension into two tubes and treat both the same way. Incubate A4 with a 1:100 dilution of CD133-2-PE antibody and leave at 4°C (in refrigerator in dark) for 30 min. Wash and centrifuge in 10 to 20 volumes of PBS as described in Basic Protocol 2, and resuspend A4 cells in 250 μl PBS. Take A4 aliquot to your FACS operator, with aliquots A1 through A3 prepared as in Basic Protocol 2 for controls.

REAGENTS AND SOLUTIONS

For culture recipes and steps, use sterile tissue culture–grade water. For other purposes, use deionized, distilled water or equivalent in recipes and protocol steps. For suppliers, see SUPPLIERS APPENDIX.

Enzyme digestive mix for tumors

40 mg bovine pancreas trypsin (Sigma, cat no. T9201)
20 mg bovine testis hyaluronidase (Sigma, cat no. H3884)
3 to 5 mg kynurenic acid (Sigma, cat no. K3375)
Resuspend these three components in 30 ml of warm (37°C) hi/low aCSF and filter through a 0.2-μm filter. This enzyme digestive mix must be made up fresh prior to use.

**Hi/low artificial cerebral spinal fluid (aCSF)**

For a total of 125 ml aCSF, combine the following:

- 7.75 ml 2 M NaCl
- 0.625 ml 1 M KCl
- 0.4 ml 1 M MgCl₂
- 21.125 ml 155 mM NaHCO₃
- 1.25 ml 1 M glucose
- 0.1157 ml 108 mM CaCl₂
- 93.73 ml H₂O

Hi/low aCSF can be prepared and stored at 4°C for up to 6 months (Dr. L. Doering, pers. comm.).

**Recombinant human basic fibroblast growth factor (bFGF) stock solution, 10 ng/μl**

Resuspend lyophilized bFGF (Invitrogen) to a final concentration of 10 ng/μl in PBS (Invitrogen, cat. no. 14190-144) containing 0.1% (w/v) BSA. Store at −30°C.

**Recombinant human epidermal growth factor (EGF) stock solution, 10 ng/μl**

Resuspend lyophilized EGF (Sigma) to a final concentration of 10 ng/μl in PBS (Invitrogen, cat. no. 14190-144) containing 0.1% (w/v) BSA. Store at −30°C.

**Tumor sphere medium**

DMEM/F12 (Invitrogen) containing:

- 1× antibiotic-antimycotic (Wisent, cat. no. 450-115-EL; http://www.wisent.ca/)
- 1× hormone mix (N2 Supplement; Invitrogen, cat. no. 17502-048)
- 10 mM HEPES (Wisent, cat no. 330-050; http://www.wisent.ca/)
- 0.6% (w/v) glucose
- 60 μg/ml N-acetylcysteine (Sigma, cat no. A9165)
- 2% (w/v) NSF-1 (neural cell survival factor; Lonza, cat. no. CC-4323)

Store for up to several weeks at 4°C

Supplement the medium just before use with:

- 10 ng/ml LIF
- 20 ng/ml bFGF (see recipe for stock solution)
- 20 ng/ml EGF (see recipe for stock solution)

**COMMENTARY**

**Background Information**

When multipotent NSCs were isolated from the mammalian neuraxis more than a decade ago, culture conditions were developed that allowed embryonic EGF-responsive cells to proliferate as floating spheres (neurospheres), which could be easily manipulated for subsequent passage and differentiation (Reynolds and Weiss, 1992; Reynolds et al., 1992). Serum-free medium (SFM) allowed for the maintenance of an undifferentiated state, and the addition of saturating concentrations of bFGF and EGF (20 ng/ml) induced the proliferation of multipotent, self-renewing and expandable neural stem cells (Reynolds et al., 1992; Reynolds and Weiss, 1996). This neurosphere culture system and analysis procedure to identify NSCs has permitted in vitro characterization of these cells, but in a retrospective fashion, as the multipotential floating clusters of cells are inferred to have been derived from clonal expansion of a single NSC (Tropepe et al., 1999). Prospective study of this cell has been previously limited by lack of cell surface markers necessary for its isolation; recent reports show NSC enrichment using antibodies against the cell surface protein CD133 (Yin et al., 1997; Yu et al., 2002). Uchida and
colleagues determined that this 120-kDa five-transmembrane cell surface receptor of unknown function could effectively sort sphere-forming cells from their non-sphere-forming counterparts in isolates of fetal human brain. Normal CD133+ human fetal brain cells not only efficiently form neurospheres in vitro, but also demonstrate the key stem cell properties of self-renewal and multilineage differentiation, and are capable of seamless lifelong engraftment and multilineage contribution to the mouse brain (Uchida et al., 2000). These findings represented the first evidence that the in vitro neurosphere-forming cell, when prospectively isolated, bore key stem cell properties both in vitro and in vivo.

We applied the neurosphere culture assay to human brain tumors of different phenotypes, in order to select for stem cell growth and functionally characterize human brain tumor cell populations. Regardless of pathological subtype, within 24 to 48 hr of primary culture most brain tumors yielded a minority fraction of cells that demonstrated growth into clonally derived neurosphere-like clusters, termed tumor spheres. Tumor spheres are defined as clonally derived nonadherent colonies of cells derived from a single tumor stem cell. The remaining majority of tumor cells exhibited adherence, loss of proliferation, and subsequent differentiation, whereas tumor spheres remained nonadherent, continuing exclusively to self-renew and expand the tumor cell culture. From these cultures, the BTIC can then be exclusively isolated by fluorescence activated cell sorting for the neural precursor cell surface marker CD133 (Yin et al., 1997; Yu et al., 2002). Only the CD133+ brain tumor fraction contains cells that are capable of sphere formation and sustained self-renewal in vitro, and tumor initiation in NOD-SCID mouse brains (Singh et al., 2003, 2004). Therefore, our characterization of CD133+ BTICs lends support for the application of the cancer stem cell hypothesis to solid tumors, and our in vitro and in vivo BTIC models will provide the foundation for a brain cell hierarchy that may begin to organize the heterogeneity of brain tumors.

**Critical Parameters and Troubleshooting**

There are many different methods of culturing brain tumor cells, and many variable applications of the neurosphere assay to human brain tumors. In culturing cells with stem cell properties in both normal and cancer tissues, there is no standardized protocol with respect to growth factors, hormones, and their concentrations (Chaichana et al., 2006). In establishing human tumor sphere cultures, we have found that our tumor sphere medium and its components have been optimized for growth of healthy spheres with good cell viability and reliable stem cell frequency across different tumor subtypes. We recommend plating the cells at a high density (e.g., $2 \times 10^5$ cells per cm$^2$), and we anticipate a large amount of cell death in the first few days of culture. This cell death can be attributed to both the elevated apoptotic activity of cancer cells and to the fact that the bulk tumor population will not survive in serum-free conditions, which does select for stem and progenitor cell growth.

Another parameter within the tumor culture protocol that requires much testing and optimization is the enzyme digestion. Both the length of time for tumor digestion and the choice of enzyme/protease mix will influence the yield of tumor cells and their viability. Length of time for tumor specimen digestion must be judged based on each individual tumor, with larger and firmer specimens requiring longer digestion. Tumor pathological subtype may also influence this decision, as some benign brain tumors have a more extensive collagen- or fibrin-based framework, and some malignant tumors may harbor a greater degree of pre-existing tissue necrosis. Under-digestion will not yield the largest possible cell number, whereas overdigestion results in DNA lysis and increased amounts of stringy white fibers and cellular debris in the culture. In terms of choice of enzyme/protease mix for digestion, we have begun to favor the use of collagenase-based cocktails, due to the fact that CD133 is trypsin-sensitive (Fukuchi et al., 2004; Schwab et al., 2008), and this receptor may be cleaved during the digestion process. If the cells are not stringently treated with antitrypsin or are not allowed to re-equilibrate for a long enough time in tumor sphere medium post-digestion, the CD133 expression level on cell sorting may be underestimated.

For sorting either with FACS or MBCS, it is often obtained to maintain a single-cell suspension, and neither normal neurospheres nor adherent tumor spheres are easily amenable to dissociation. Thus, we use a combination of very gentle mechanical trituration just prior to sorting, with filtering through cell-strainer caps into flow cytometry tubes, to prevent clumping during the sort. We also recommend treating the cells with chemical cell dissociation buffers, which may provide a more gentle alternative to mechanical dissociation.
Anticipated Results
Depending on the size of the original tumor specimen, our tissue culture protocol should yield 1–100 × 10^6 viable tumor sphere cells from brain tumors of different subtypes, which then can be subjected to a panel of in vitro stem cell assays, including primary sphere formation assays, proliferation assays, limiting-dilution assays, and differentiation assays. Our BTIC enrichment protocol should provide millions of CD133− brain tumor non-stem cells, and thousands to millions of CD133+ BTICs, depending on the clonogenic frequency and CD133 index of each tumor subtype. In general, the increased self-renewal capacity of the BTIC and the correlated CD133 index are highest from the most aggressive clinical samples of medulloblastoma and glioblastoma compared with low-grade gliomas. Both bead and FACS separation routinely yield average purities of 80% to 90% for the CD133+ cells and >99.5% purity for CD133− cells; thus, these methods should be considered as good enrichment methods, not methods for isolation to purity of BTICs.

Time Considerations
Tumor dissociation and plating of cells into tumor sphere cultures typically takes 2 to 4 hr, and cell sorting can be completed within 4 hr.

Literature Cited


