Video Article

Growth and Differentiation of Adult Hippocampal Arctic Ground Squirrel Neural Stem Cells

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Abstract

Arctic ground squirrels (*Urocitellus parryi*, AGS) are unique in their ability to hibernate with a core body temperature near or below freezing¹. These animals also resist ischemic injury to the brain *in vivo*^{2,3} and oxygen-glucose deprivation *in vitro*^{4,5}. These unique qualities provided the impetus to isolate AGS neurons to examine inherent neuronal characteristics that could account for the capacity of AGS neurons to resist injury and cell death caused by ischemia and extremely cold temperatures. Identifying proteins or gene targets that allow for the distinctive properties of these cells could aid in the discovery of effective therapies for a number of ischemic indications and for the study of cold tolerance. Adult AGS hippocampus contains neural stem cells that continue to proliferate, allowing for easy expansion of these stem cells in culture. We describe here methods by which researchers can utilize these stem cells and differentiated neurons for any number of purposes. By closely following these steps the AGS neural stem cells can be expanded through two passages or more and then differentiated to a culture high in TUJ1-positive neurons (~50%) without utilizing toxic chemicals to minimize the number of dividing cells. Ischemia induces neurogenesis⁶ and neurogenesis which proceeds via MEK/ERK and PI3K/Akt survival signaling pathways contributes to ischemia resistance *in vivo*⁷ and *in vitro*⁸ (Kelleher-Anderson, Drew *et al.*, in preparation). Further characterization of these unique neural cells can advance on many fronts, using some or all of these methods.

Video Link

The video component of this article can be found at <http://www.jove.com/video/2199/>

Protocol

1. AGS Neural Stem Cell and Media Preparation

- Adult hippocampal Arctic ground squirrel (AGS) neural stem cells isolated in a manner previously described⁹ can now be obtained commercially as cryopreserved vials. Cells are shipped overnight in insulated packages containing dry ice to ensure that the cells remain in a cryopreserved state. To maintain integrity, unpack the cells immediately upon receipt and store at lower than -150°C, or in the vapor phase of a liquid nitrogen Dewar.
- Prepare agsNSC Basal Medium + 5% FBS by adding 5 mL FBS (LS-1012) to 95 mL agsNSC Basal Medium
- Prepare agsNSC Expansion Medium by adding 500 µL B-27 Supplement (Invitrogen) to 50 mL agsNSC Basal Medium. Divide the remainder of the B27 into 1mL aliquots and store at -20°C until use.
- Warm 25 mL of agsNSC Basal Medium + 5% FBS and 20 mL of agsNSC Expansion Medium in a 37°C water bath.
- Coat flasks used for expansion with poly-L-ornithine
 - Thaw the poly-L-ornithine (10 µg/mL) working solution. Store the solution between 2-8°C for up to one month once thawed.
 - Add 8 mL Poly-L-Ornithine working solution to one vented T-75 flask and evenly distribute the solution over the entire culture surface of the flask. (Adjust amount of working solution for other culture vessels that may be used. Use 0.5 - 1.0 mL Poly-L-Ornithine working solution per 10 cm² culture surface.)
 - Incubate flasks at 37°C for a minimum of 24 hours. (Use of an incubator without added CO₂ is preferred for incubation of Poly-L-Ornithine coated culture vessels.) Coated flasks can be stored for up to two weeks in the incubator.
 - Prior to use, aspirate Poly-L-Ornithine working solution from the flask and wash twice with sterile tissue culture grade water. Aspirate liquid until the flask is nearly dry before use.
- Remove a vial from the Dewar and check that the vial cap is securely sealed. Hold the top of the vial leaving the bottom half of the vial in a 37°C water bath for approximately 60-90 seconds or until vial is almost completely thawed; a small amount of ice should still be visible. To avoid potential contamination, keep the vial cap out of the water. Do not over thaw, as doing so may damage the cells.
- Spray the exterior of the vial with 70% ethanol or isopropanol, then place the vial in a biological safety cabinet. Remove the cap carefully to avoid contamination or splatter.

8. Gently re-suspend the cells in the vial by adding 1 mL of agsNSC Basal media + 5% FBS using a sterile pipette. Transfer the re-suspended cells to 25 mL of warm agsNSC Basal Medium + 5% FBS in a 50 mL centrifuge tube.
9. Rinse the cryo-vial once with diluted cell suspension. Centrifuge the cells at 150 x g for 6 minutes. For best results, calculate the speed for each individual centrifuge type.
10. While the cells are spinning down, add 10 mL of agsNSC Expansion Medium to the dry poly-L-ornithine coated T-75 flask and add 40 μ L of rh FGF-basic (40ng/mL). Following centrifugation, aspirate the supernatant fluid taking care to not aspirate the pellet. Re-suspend the cells in 10 mL of agsNSC Expansion Medium. Transfer the re-suspended cells in agsNSC Expansion Medium into the media in the T-75 flask.
11. Gently rock the culture vessel side-to-side to evenly distribute cells within the vessel.
12. Place seeded culture vessel in a 37°C, 5% CO₂ incubator. Cells should attach to the flask and begin to form processes within two hours of seeding. If the cells remain floating and balled up within the medium, then centrifuge cells in 5% FBS and run steps 1.8-1.10 with fresh medium and flask.

2. Expansion of AgsNSC

1. Two days (~48 hours) after thawing and seeding the cells, a full medium replacement should be performed using 20 mL of agsNSC Expansion Medium plus a 40 μ L spike of rh FGF-basic.
2. Spike flasks with 40 μ L of rh FGF-basic on the day following inoculation. The cells will be ready to trypsinize when they have reached 75-80% confluence by the third or fourth day after inoculation. Culturing the undifferentiated stem cells for longer than 4 days after inoculation is not recommended because longer incubation will allow cells to begin to differentiate to neurons and neurons may be injured by trypsinization during step 3. The rh FGF-basic can be stored at 4°C for up to one week and still maintain activity. The rh FGF-basic can also go through one additional freeze/thaw at -20°C if more time is needed.

3. Trypsinization of Cells

1. agsNSC should be passaged 3 to 4 days after inoculation. Prepare a poly-L-ornithine coated flask between 24 hrs and one week before passaging if the agsNSC will be expanded again.
2. Aspirate the medium from the culture vessel. Add 2.5 mL of 0.05% Trypsin/0.02% EDTA (CM-0017) to the vessel for each 75 cm² of surface area. Swirl gently to ensure all cells are coated with the Trypsin/EDTA. Cells normally begin to detach from the surface within 60 seconds, depending on the confluence of the cells. Do not over trypsinize, as doing so will damage the cells. Gently tapping the culture vessel from several sides will encourage detachment.
3. Once the cells become detached, add 10 mL of agsNSC Basal Medium + 5% FBS to the flask. Gently swirl to ensure all of the trypsin solution is neutralized. Using aseptic laboratory techniques, pipette the cells into a sterile centrifuge tube containing 10 mL of agsNSC Basal medium +5% FBS. Repeat by washing the flask again with an additional 10 mL of agsNSC Basal Medium + 5% FBS and combine with the first wash.
4. Centrifuge cells at 150 x g for 6 minutes as described above. After centrifugation, the cells should form a clean loose pellet. Aspirate neutralized trypsin and medium from the centrifuge tube and re-suspend the cell pellet in 10 mL (per T-75 flask) of pre-warmed agsNSC Basal Medium + 5% FBS by gently pipetting up and down 2X with a 10 mL pipette. Perform a cell count as described below.

4. Standard Calculation for Plating of Cells

1. Using aseptic technique, swirl the cell suspension and transfer 15 μ L of the cell suspension to a microcentrifuge tube. Add 15 μ L of 0.4% Trypan Blue. Mix gently and load 10 μ L of cell suspension to each of the chambers of a clean hemacytometer such as a Bright Line Counting Chamber (catalog #1490: Hausser Scientific, <http://www.hausserscientific.com/hausserbrightlinedirect.htm>).
2. Count a minimum of 4 quadrants on the hemacytometer. The blue cells that are positive for Trypan Blue are dead and should not be counted. For accurate cell counts, the optimal number of cells per quadrant should be 25-75 cells.
3. After counting the cells, calculate the average of the 4 quadrants. Multiply the cell count average by 10,000 and by the dilution factor (2 if using the recommended volumes) to determine the number of cells per mL. Note that if total percentage viability is less than 92% there may be a number of causes. One reason for low viability may be over trypsinization, prior to trypsin neutralization with FBS. Another reason may be that the concentration of serum (FBS) to trypsin is too low and the cells continue to be trypsinized. Thirdly, the cells may have been too confluent, causing stem cells to differentiate due to cell-cell contact, leading to injury during trypsinization. If this method is followed closely a yield of 3 or more doublings may be expected, giving a population of >4,000,000 cells from an initial number of ~500,000.

5. Inoculation of Another Expansion Flask

1. To determine the total number of cells required to inoculate one vessel, multiply the desired seeding density (6,600 cells per cm² or 500,000 cells per T-75 flask) by the surface area (in cm²) of the vessel to be inoculated. If inoculating more than one vessel multiply the number of cells per vessel by the total number of vessels. To determine the volume (mL) of cell suspension needed to inoculate each vessel, divide the number of cells required to inoculate one vessel by the number of viable cells/mL in the cell suspension. If inoculating more than one vessel calculate the total volume of cell suspension needed by multiplying the volume needed for one vessel by the total number of vessels to be inoculated.
2. Centrifuge the desired volume of cells in agsNSC Basal Medium + 5% FBS, leaving a loose pellet. Normally 500,000 cells are brought to a total volume of 25 mL in agsNSC Basal Medium +5%FBS as described previously. Aspirate liquid and re-suspend the loose pellet in 10 mL of agsNSC Expansion Medium. This extra centrifugation step is required to remove FBS before inoculation. Add 10 mL of pre-warmed agsNSC Expansion Medium to the T-75 flask. Add the 10 mL of resuspended cell suspension to the T-75 flask then add 40 μ L of rh FGF-basic to the flask. Consider that the trypsinization and inoculation should take no longer than 1.5 hrs when deciding the maximum number of flasks to work with at any one time. One should also have enough media for complete media changes if using numerous flasks.
3. Gently rock the flask from side to side to evenly distribute the cells and place the culture vessels into a 37°C, 5% CO₂ incubator, as described previously.

6. Dilution of Cells for Inoculation of the Well Plates for Differentiation

1. Any multi-well format might be used, but inoculation into BD Biocoat Poly-L-lysine coated 96 well plates is described here. Determine the volume of cell suspension needed for your experiment by multiplying the number of wells to be inoculated by 0.2 mL per well for a 96

- multi-well plate and add approximately 10% to cover any loss in volume during the inoculation. Calculate the dilution to a cell density of 75,000 cells/mL. Determine the amount of FBS to be added to the Differentiation Medium to achieve a final concentration of 2% FBS (Table 1). Mix the FBS and Differentiation Medium. Carefully mix the cells with the calculated volume of Differentiation Medium with FBS. (note: During centrifugation the cells are in agsNSC Basal Medium + 5% FBS. While FBS protects cells during centrifugation and facilitates cell adhesion FBS inhibits differentiation. Diluting FBS to 2% will allow the adhesion of the cells to the multi-well surface. The 2% FBS is then diluted further to promote differentiation. Insulin-Transferrin-Selenium(ITS-e) is also included in the Differentiation Medium to promote differentiation.
2. In a biosafety cabinet, dispense 200 μ L of diluted cell suspension from a sterile combitip reservoir to each well of a Poly-L-lysine coated 96-well plate using a multi-channel pipette and large orifice tips. Incubate the plates for 1.5-2 hours at 37°C, 5% CO₂. After the cells have attached, after about 90 to 120 minutes, carefully remove 50% (100 μ L for 96-well plates) of the medium from each well using large orifice pipette tips. Carefully replace the volume removed with warm agsNSC Differentiation Medium, again using large orifice pipette tips. (Use of standard orifice tips may injure the cells).
 3. Two days after inoculation (~ 48 hours), perform a 50% medium change using agsNSC Differentiation Medium and large orifice pipette tips.

7. Maintenance of Neurons

1. Prepare Neuronal Maintenance Medium using NeuraLife, glutamine and B27 per the instructions. Neuronal Maintenance Medium should be prepared weekly.
2. Two days after the last agsNSC Differentiation Medium change, normally Day 4 post inoculation, perform a 50% medium replacement using Neuronal Maintenance Medium.
3. Continue to perform 50% medium replacements using Neuronal Maintenance Medium every 2 to 3 days. Neuronal processes should appear within a few days of changing to Neuronal Maintenance Medium. The cells should be used within 3 weeks of being transferred to Neuronal Maintenance Medium. Neurons can be identified using a number of commercially available neuronal markers. Astrocytes will also always be observed.
4. Culture populations typically express a greater than 40% ratio of neurons to total cell number. The use of neuron maintenance media other than NeuraLife may provide inferior cells that do not look morphologically mature and may not survive as long in culture.

8. Representative Results:

The Arctic ground squirrel adult neural stem cells will continue to double every 24 hrs for 3 to 5 days for at least two passages when seeded at 500,000-600,000 cells/75mm flask. These cells will easily differentiate with the removal of basicFGF and B27 and the presence of 1-2% FBS and 1% Insulin/Transferrin/Selenium, and will become TUJ1 (Covance) positive neurons within 14-21 days (see Figure 2). The ratio of neurons to total cells will range between 40-60%, dependent on the age of the culture (Figure 3). Neurons may be utilized for experimentation from 12 days post-seeding through at least 21 days post-seeding.

Figure 1. Photomicrograph of AGS adult hippocampal neural stem cells.

Phase photomicrograph of AGS adult hippocampal neural stem cells following 3 days of growth in agsNSC basal media in Poly-L-Ornithine coated flasks. Doubling occurs approximately every 24hrs. Photo at 100X magnification.

Figure 2. Fluorescent micrograph of differentiated AGS adult hippocampal neural stem cells

AGS neural stem cells were subjected to differentiation media for 4 days, then maintained in Neuralife for 17 more days prior to fixing. Cells were fixed with 4% paraformaldehyde and neurons identified using TUJ1 primary antibody (Covance) and Alexa Fluor 568 secondary antibody (green) from Invitrogen. Hoechst dye 33343 was added to stain all nuclei (blue). Cell number, neuron number and neuron ratio were determined using neurite outgrowth software and the Arrayscan instrument from Cellomics. This photo represents approximately 57% neurons. 10X magnification

Figure 3. Neuron numbers and Neuron Ratio of Differentiated AGS neural stem cells at three culture ages

AGS neural stem cells were subjected to differentiation media for 4 days, then maintained in Neuralife for 12, 15 and 17 days prior to fixing. Cells were fixed and neurons identified with TUJ1 primary antibody (Covance). Hoechst dye was added to stain all nuclei for total cell identification. Cell number, neuron number and percent neuron ratio were determined using neurite outgrowth software and the Arrayscan instrument from Cellomics. Values represent mean of 15 wells per condition + SD.

Table 1. Sample calculations used in passaging and seeding

- Using 700,000 cells/mL cell suspension
- Using a cell density of 75,000 cells/mL or 15,000 cells per well of 0.2 mL volume in each well.
- Inoculating 96 wells at 0.2 mL per well = 19.2 mL or desired final volume 21 mL.
- $(\text{Final Concentration} \times \text{Final Volume}) \div \text{Initial Concentration} = \text{Initial Volume}$
 $(21 \text{ mL} \times 75,000 \text{ cells/mL}) \div 700,000 = 2.25 \text{ mL}$
- Volume of FBS in cell suspension. $2.25 \text{ mL} \times 0.05 = 0.1125 \text{ mL}$
- Volume of FBS needed in 21 mL = $21 \text{ mL} \times 0.02 = 0.42 \text{ mL}$
- Volume of FBS to add to Differentiation Medium = $0.42 \text{ mL} - 0.1125 \text{ mL} = 0.3075 \text{ mL}$
- Volume of Differentiation Medium = $21 \text{ mL} - 2.25 \text{ mL cell suspension} - 0.3075 \text{ mL 5\%FBS} = 18.44 \text{ mL}$.
- Combine 18.44 mL Differentiation Medium with 0.308 mL FBS and add 2.25 mL of cell suspension.

Discussion

AGS adult neural stem cells are robust and can be expanded for numerous passages making them an excellent source of neural stem cells for study of basic neural stem cell properties. The expansion rate is approximately one doubling every twenty-four hours, but these cells must be passaged prior to reaching confluence because contact between cells will favor differentiation to astrocytes. Contact-mediated differentiation will therefore cause the ratio of neurons to total cell number to drop dramatically, if differentiation begins prior to trypsinization and passage. Figure 1 illustrates an AGS neural stem cell culture that should be passaged within 24 hrs.

AGS neural stem cells adhere strongly to poly-L-lysine coated Biocoat plates and FBS can be reduced to 1% within 90 minutes without the cells lifting. As FBS and rhFGFbasic are removed, the cells will differentiate to about half neurons and half astrocytes. The neurons will be TUJ1 positive within two weeks (Figure 2), and MAP2 positive within three weeks post-induction (if NeuraLife Medium is used). A more complete characterization of receptor expression and function of mature neurons is pending. TUJ1 and MAP2 expression is characteristic of neuronal progenitors that are committed to a neuronal fate but retain mitotic activity¹⁰.

Response to hypoxia or oxygen glucose deprivation (OGD) changes between 2 and 3 weeks in culture. While, the number of neurons may decrease following 16 days of culturing (4 days of differentiation and 12 days of maintenance) the percentage of neurons to total cells remains near 50% (Figure 3). Nonetheless, hypoxia or OGD applied at 12 to 19 days post seeding can induce neurogenesis in these cultures of TUJ1 expressing neurons. Induced neurogenesis appears to be one mechanism by which these cells resist injury observed in differentiated human neural stem cells exposed to equivalent insults (in preparation). Better understanding of mechanisms of hypoxia and OGD-induced neurogenesis in AGS neuronal cultures may lead to development of novel therapies for treating stroke, cardiac arrest and dementia associated with limited blood supply.

Disclosures

J.A. Kelleher-Andersson is the founder of Neuronascent and is the inventor on the Arctic ground squirrel patent applications. Neuronascent receives royalties on the sale of AGS media and stem cells. R.C. McGee is employed by Lifeline, Inc. which distributes the media and AGS neural stem cells.

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