The Differentiation of Radial Glial Cell Line C6 in vitro

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Abstract:

Radial glia are cells found in the ventricular zone of the brain in the embryonic and neonatal neuroepithelia. These cells then differentiate into astrocytes, ependymal cells, and B-cells (sub-ventricular zone astrocytes) in the adult brain. Radial glia are hypothesized to have the capability to differentiate into oIPCs (intermediate progenitor cells that generate oligodendrocytes) and nIPCs (intermediate progenitor cells that generate neurons).[4] This research was an independent, experimental project to test these hypotheses. Using the radial glial cell line C6, cultures were grown to differentiate into astrocytes (as a control for cell potency) and oIPCs and nIPCs. This study showed that the C6 cell line does indeed have the potential for differentiation into oligodendrocytes and neurons in vitro, therefore lending evidence to their hypothetical role in the development of the central nervous system in vivo.

Introduction

Neuronal and glial cells were long thought to differentiate from different stem cell lines. Recent research, however, shows that these two cell types are much more closely related than previously thought.[4] Some glial cells, such as the sub-ventricular zone (SVZ) astrocytes, have shown the capacity to differentiate into neurons in the adult mammalian brain.[1] Radial glial cells (RG cells) give rise to these SVZ astrocytes, and it is hypothesized that RG cells are in fact pluripotent primary progenitor cells, meaning that they are capable of giving rise to a variety of neuronal and glial cell types. RG cells are derived from the neuroepithelium during development and are therefore found throughout the central nervous system (CNS) until birth. However, RG are thought to persist in the CNS through adulthood as well.[4] This work shows that radial glial cells have the capability to differentiate into astrocytes, oligodendrocytes, and neurons, and are therefore pluripotent cells. As these cells may be found throughout the central nervous system, exploiting their pluripotency could have powerful implications for brain and nerve repair. The capability to differentiate into almost any type of CNS cell could make repair to almost any CNS tissue possible.

Methods

Cell Culture

Cells were cultured in American Type Culture Collection (ATCC) complete growth medium, Dulbecco's Modified Eagle Medium (DMEM)/F12 (1:1) (ATTC), Fetal Bovine Serum (FBS) to a final concentration of 2.5%, horse serum to a final concentration of 15%, and Penicillin-Streptomycin to a final concentration of 1%. The cells were then incubated at 37.0°C in a 5% carbon dioxide (CO_2) incubator. Next, the C6 cells (ATTC CCL-107, radial glial cells isolated from the brain of a rat glial tumor induced by N-nitrosomethylurea) were passaged every 3-4 days at a 1:2 or 1:3 ratio. This was accomplished by removing and discarding the culture medium, adding 2.0 to 3.0 ml of Trypsin-EDTA solution to the flask, and incubating at 37.0°C for 5 -15 minutes. Following this treatment, 6.0 to 8.0 ml of complete growth medium was added and the cell suspension was aspirated by gently pipetting. Then, appropriate aliquots of the cell suspension were added to new culture vessels and incubated at 37° C. Stock cultures of the C6 cells were frozen in culture medium (95%) and Dimethyl Sulfoxide (DMSO) (5%) and stored at -150°C. All cells were taken through 13 passages before the first 24-well plate treatment. When cell cultures were split, the flask with higher viability was chosen for continued passaging. Viability was determined by a Nexcelcom

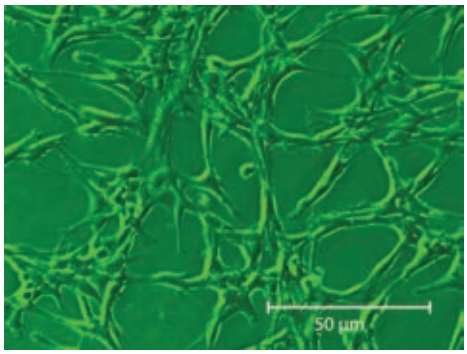

Figure 1: C6 cells cultured in a T-25 flask, observed on an inverted microscope and photographed. The elongated shape is typical of a fibroblast and the thin processes being characteristic of RG cells.

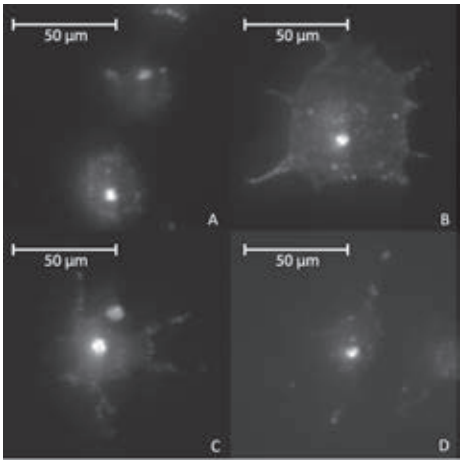

Figure 2: Calcein AM stain after 4 days of treatment. A) Cells cultured in DMEM/F12 (1:1) with 20ng/mL FGF-2 and 1% N2 for 2 days, then for an additional 2 days after withdrawal of FGF-2 for Oligodendrocyte differentiation. B) Cells cultured in DMEM:F12 (1:1) with 20ng/mL FGF-2, 50 ng/mL BMP-2, 50 ng/mL LIF, and 1%FCS for 4 days for Astrocyte differentiation. C) Cells cultured in DMEM/F12 (1:1) with 20ng/mL FGF-2, 1% N2 supplement, 1uM RA (300.4 ng/mL) and 5uM forskolin for Neuronal differentiation. D) Control cells cultured in normal culture media. Note the distinct morphological differences of A, B, and C when compared to D, the control. Each image is to scale respectively.

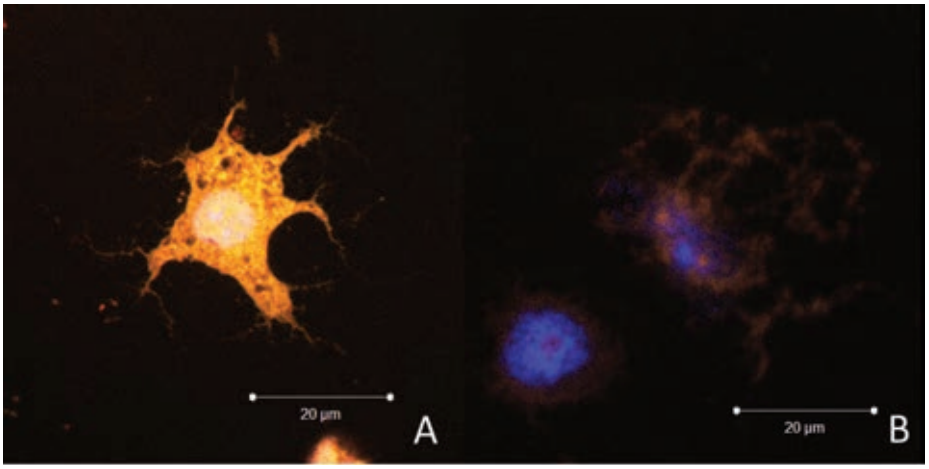

Figure 3: Immunofluorescent stain using phalloidin (red), anti-MAP2 (yellow), and DAPI (blue) visualized under a fluorescent confocal microscope. Cells cultured in DMEM/F12 (1:1) with 20ng/mL FGF-2 and 1% N2 for 2 days, then for an additional 2 days after withdrawal of FGF-2 for Oligodendrocyte differentiation. A) Characteristic morphology of an immature Oligodendrocyte. The cell appears yellow-orange due to high levels of MAP2. B) Characteristic morphology of a mature Oligodendrocyte. The cell body is reduced and is surrounded by many fibrous dendrites. The cell appears more reddish as MAP2 is no longer highly expressed after formation of the dendrites.

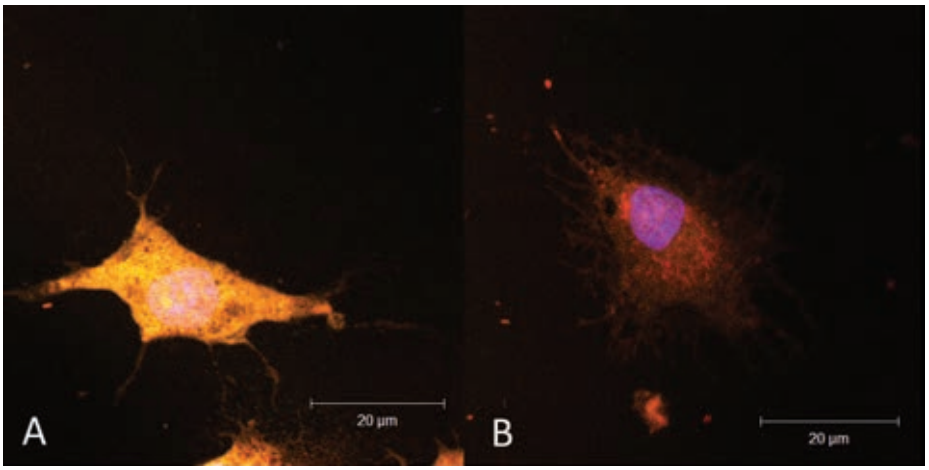

Figure 4: Immunofluorescent stain using phalloidin (red), anti-MAP2 (yellow), and DAPI (blue) visualized under a fluorescent confocal microscope. Cells cultured in DMEM:F12 (1:1) with 20ng/mL FGF-2, 50 ng/mL BMP-2, 50 ng/mL LIF, and 1%FCS for 4 days. A) Immature astrocytes expressing high levels of MAP2. B) Mature astrocytes with the characteristic bushy appearance.

Auto T4 cell counter. Once treatment began, the cells were observed each day using a stereomicroscope. Cells thawed from the tenth passage were used for the second 24-well plate treatments. These cells were treated in two 24-well plates. For astrocyte differentiation, cells were cultured in DMEM:F12 (1:1) with 20ng/mL Fibroblast Growth Factor 2 (FGF-2) (Sigma), 50 ng/mL Bone Morphogenic Protein 2 (BMP-2) (Sigma), 50 ng/mL Leukemia Inhibitory Factor (LIF) (Sigma), and 1% Fetal Calf Serum (FCS) for 4 days. For neuronal, differentiation cells were cultured in DMEM/F12 (1:1) with 20ng/mL FGF-2, 1% N2 supplement, 1µM Retinoic Acid (RA) (300.4 ng/mL) (Sigma), and 5µM forskolin (Sigma). For oligodendrocyte differentiation, cells were cultured in DMEM/F12 (1:1) with 20ng/mL FGF-2 and 1% N2 (R+D Systems) for 2 days, then for an additional 2 days after withdrawal of FGF-2.[5] The media was changed at the second day for all treatments.

Calcien AM staining

The media was removed with a micropipette and then the cells were washed with 150µL of Phosphate Buffered saline (PBS). Next, 200µL of 2µM Calcein AM solution was added to the cells, followed by incubation for 30 minutes at 37°C and 5% CO_2. The cells were rinsed three times with PBS, and then observed with a fluorescent microscope.

Immunofluorescence

Cells were cultured on poly-L-lysine coated coverslips in a 24-well plate, at approximately $1.74x10^4$ cells per well. After 4 days of treatment (described above), the cells were fixed with 4% paraformaldehyde. The cells were then permeabilized with 0.5% Triton X-100 in PBS for exactly 2 minutes. The primary antibody was rabbit α-MAP2 (1:500) (Sigma) in 1x block solution (20% goat serum, 2% BSA in PBS). The secondary antibody was Alexa Fluor 647 (Far Red) goat α-rabbit (1:1000) (Sigma) in 0.5x block solution. Phalloidin (1:1000) (Sigma) in 0.5x block solution was used to stain the actin cytoskeleton. Coverslips were mounted onto slides with Prolong Gold with DAPI. The cells were then observed with a Zeiss LSM 510 laser scanning confocal a microscope at 630x magnification. The slides were stored at 4°C.

Results

While in culture in the T-25 flasks, the cells remained highly confluent with typical fibroblast morphology (Fig. 1). Viability of the cells remained high throughout passaging, often well above 90% viability. When observed, the treated cells appeared to have lower confluency and viability than when cultured in the normal culture media. On the fourth day of treatment, the cells were stained with Calcein AM and observed. The living cells absorbed the Calcein AM stain and the morphology of the cells was easily observed (Fig 2). The cells treated to differentiate into oligodendrocytes displayed an oligodendrocyte like morphology from the original fibroblast. The oligodendrocyte like cells were rounded and considerably smaller, with many small processes along the periphery of the cell. The astrocytes displayed an enlarged cell body with several processes along the periphery of the cell. The neurons did not show a distinct change in the size of the cell body, but did however, possess morphologically distinct processes. The control cells retained the fibroblast morphology suggesting no differentiation took place. The Calcein stain confirmed the earlier observations of apparently lower viability, as many of the cells did not absorb the dye. DAPI was used to ensure that the Calcein was not auto fluorescing. The images in Figure 2 are not representative images and do not encompass all of the morphologies observed. In order to better observe the differences in morphology a Zeiss LSM 510

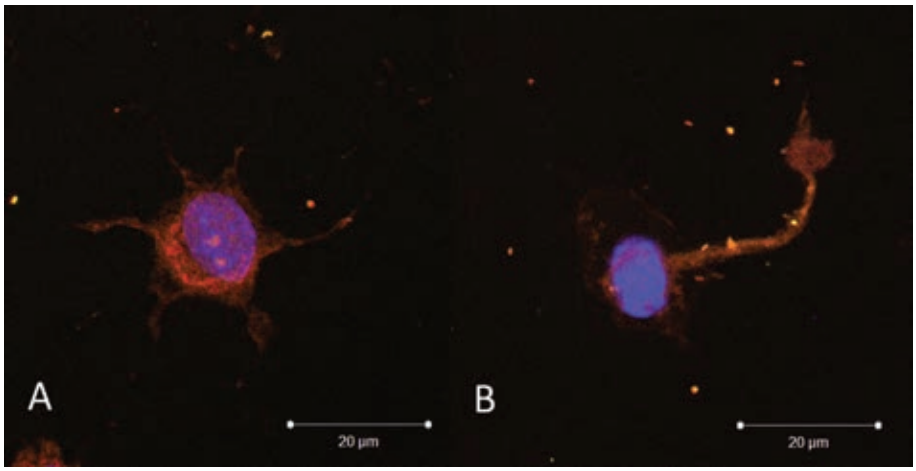

Figure 5: Immunofluorescent stain using phalloidin (red), anti-MAP2 (yellow), and DAPI (blue) visualized under a fluorescent confocal microscope. Cells cultured in DMEM/F12 (1:1) with 20ng/mL FGF-2, 1% N2 supplement, 1uM RA (300.4 ng/mL) and 5uM forskolin. A) Typical multipolar neuron morphology with dendrites extending radially from the cell. B) Bipolar neuron morphology; dendrites extending from the cell body near the nucleus and a long axon at one end.

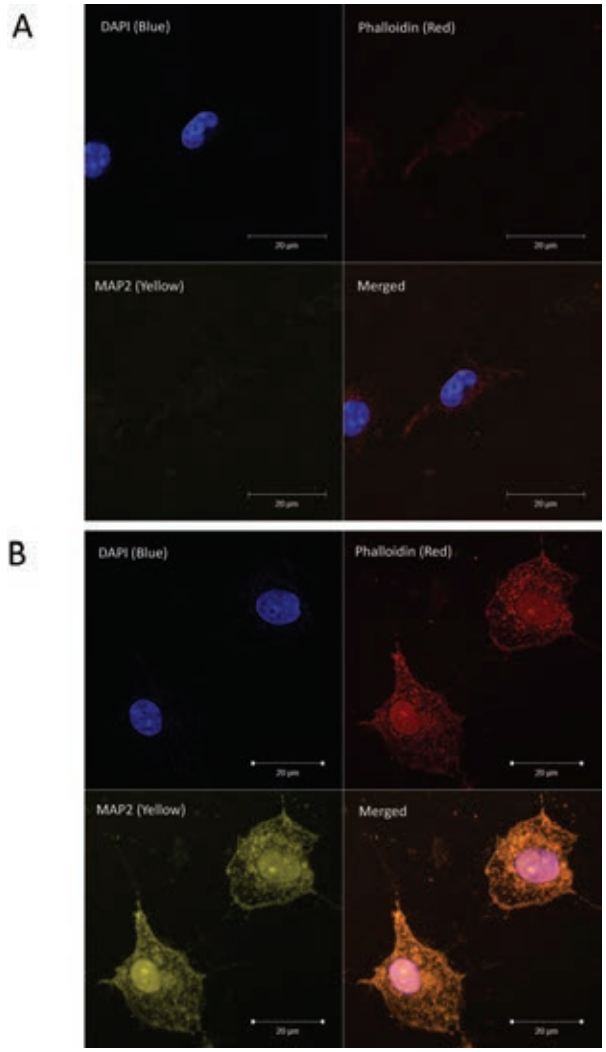

Figure 6: Immunofluorescent stain using phalloidin (red), anti-MAP2 (yellow), and DAPI (blue) visualized under a fluorescent confocal microscope.

A) Control cells cultured in DMEM/F12 (1:1) high glucose medium containing 2.5mM L-glutamine, 10% FBS, and 20ng/mL of FGF-2. Note the low levels of MAP2 (yellow) expressed and the retention of the fibroblast morphology.

B) Cells cultured in DMEM:F12 (1:1) with 20ng/mL FGF-2, 50 ng/mL BMP-2, 50 ng/mL LIF, and 1%FCS for 4 days. These cells are beginning to differentiate into astrocytes. Note the high levels of MAP2 (yellow) expressed and the distinct changes in morphology.

laser scanning confocal microscope at 630x magnification was used.

Under the confocal microscope, it was observed that the cells were in various stages of differentiation. Those in the midst of differentiation expressed high levels of Microtubule Associated Protein 2 (MAP2), as it is necessary in the building of microtubules, which are in turn necessary for the building of new dendrites. These cells appeared yellow-orange in color due to the superposition of the anti-MAP2 antibody (yellow) and the phalloidin (red) antibody (Fig 3 A). Those cells that displayed a mature morphology express lower levels of MAP2, thus appear redder in color (Fig 3 B). Like the oligodendrocytes in Figure 3, the astrocytes were observed in different phases of differentiation (Fig 4). The immature astrocytes also appeared yellow-orange in color due to high levels of MAP2 expression, with decreased expression in the mature cells.[7]

Unlike the oligodendrocytes and astrocytes, those cells treated for differentiation into neurons did not appear to be in different stages of differentiation. The neuronal cells were observed to have differing morphologies. Some neuron like cells displayed the morphology of a multipolar neuron, which is characterized by the axon and dendrites extending radially from the cell body (Fig 5A), whereas others displayed a more bipolar nerve morphology, characterized by the dendrites extending from the cell body near the nucleus with a long axon at the opposite end of the cell (Fig 5B).

While those cells that were treated for differentiation displayed distinctly different morphologies than the original fibroblast, and expressed high levels of MAP2, the control cells did not. Those cells that were grown on the poly-L-lysine coated coverslip in the normal culture media retained the fibroblast morphology. In addition, the levels of MAP2 were extremely low, especially when compared to an actively differentiating cell (Fig 6).

Discussion

The use of anti-MAP2 to detect the levels of MAP2 within the cells proved to be an effective way of testing for differentiation, and was actually more sensitive than expected. Those cells in the midst of differentiation expressed the highest levels of MAP2, as this is indicative of a cell actively building new dendrites.[8] Following differentiation the levels of MAP2 decreased, as no new dendrites were being made. This provided a method to estimate how mature

the cell type was based on morphology and MAP2 levels. The presence of immature oligodendrocytes and astrocytes suggests that longer treatments would be needed for complete maturation. The neurons however, appear to mature more rapidly as many of the cells had already matured by the end of treatment.

The lower viability observed during the Calcein staining would seem to suggest that the treatments may have been too harsh. However, the decreased viability was seen in all of the treated wells, including the control. Therefore, the decreased viability may have been due to another factor, perhaps leaving the cells exposed too long while changing media.

Despite a decrease in viability and many cells not achieving a mature morphology after treatment, this experiment suggests that radial glial cells are pluripotent cells capable of differentiating into not only astrocytes, but oligodendrocytes and neurons as well. Further experimentation will be needed in order to determine the appropriate lengths for treatments to ensure that the cells reach maturity. In addition to this, further studies with radial glial cells *in situ* and *in vivo* will be needed to determine the possibility, practicality, and applicability of the pluripotent nature of the radial glial cell in brain repair.

References

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