

Optimization of a Culture System for Neurospheres Derived from Embryonic Rat Brain

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Abstract

A rare population of neural cells exhibits stem cell functions including self-renewal and multilineage differentiation. These neural stem cells (NSC) proliferate in serum-free suspension cultures to form multicellular spheres called neurospheres. While mouse NSC are expanded in these conditions for many passages, rat NSC show limited expansion and typically cannot be maintained beyond passage 5. The aim of this study is to develop culture conditions supporting prolonged and reproducible expansion and differentiation of rat NSC. Cortices from embryonic day 18 rat were dissected, mechanically dissociated into single cell suspensions and seeded in suspension cultures. We modified media formulations, tested a variety of cell seeding densities and altered the timing of passages. The modified media formulation (NeuroCult™-Rat) gave an average 15-fold expansion of rat cells from passage to passage with cell viability approaching 80%. In contrast, using a commercially available media formulated for murine NSC, NeuroCult™-Mouse (StemCell Technologies Inc), the average fold expansion from passage to passage was approximately 5-fold with 70% viability and by the 9th passage few neurospheres formed and most cells stayed attached to the substrate. The lower seeding density (1.25 x 10⁵ cells/10 mL) resulted in the higher fold expansion and viability data described above. When differentiated, both early and late passage NSCs grown in NeuroCult™ and Rat NeuroCult™ retained their potential to differentiate into neurons, astrocytes and oligodendrocytes as detected by immunostaining. With Rat NeuroCult™ and the protocol described here, the culture of rat NSC achieved the same fold expansion, number of passages and reproducibility as the culture of mouse NSC. An optimized media and culture system for embryonic rat NSC may serve as a standardized tool for generating large numbers of rat NSC for transplantation studies.

Introduction

The limited degree of cell turnover in the adult mammalian CNS has led to the assumption that the mammalian CNS has an inadequate self-repair capacity. The discovery of a rare population of neural cells exhibiting stem cell features in the embryonic through to the adult mammalian CNS raises the possibility of cell replacement and challenges the classical view of the CNS as a mitotically quiescent organ.

Due to the lack of specific markers, neural stem cells (NSC), like other stem cells, are identified based on functional criteria. The key functional determinants of a stem cell are its ability to exhibit self maintenance over an extended period of time, generate a large number of progeny and produce the primary functional progeny of the tissue from which they are obtained. To this end, cells obtained from the embryonic and adult mouse and embryonic human CNS have been shown to satisfy these criteria. While growth factor responsive cells have been isolated from the embryonic rat CNS they have yet been shown to satisfy the primary features of a stem cell - namely the ability to exhibit self maintenance over an extended period of time and therefore generate a large number of progeny (Svendsen et al, 1997; Ostenfeld et al, 2002).

The aim of the present study was to develop a defined serum-free medium, culture conditions and protocols that would allow for the reproducible long-term culturing of embryonic rat NSC and satisfy the functional criteria to identify growth factor responsive embryonic rat cells as NSC.

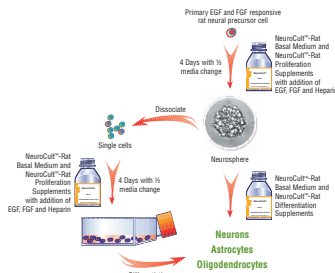
Methods

Isolation and Expansion of Cells. Dissected cortical region from E18 Sprague/Dawley rat (BrainBits Inc, Illinois, USA) was mechanically dissociated to obtain a single cell suspension. Cells were centrifuged to remove dead cells at 200g for three minutes, supernatant discarded and cells were resuspended in complete NeuroCult™-Rat media.

The culture medium for growing rat neurospheres is a modified media formulation of the NeuroCult™ NSC Basal Medium - Mouse (StemCell Technologies Inc, Catalog #05700). NeuroCult™-Rat (StemCell Technologies Inc, Catalog #05770) was supplemented with NeuroCult™ NSC Proliferation Supplement (StemCell Technologies Inc, Catalog #05701), consisting of a defined hormone and salt supplement, human Epidermal Growth Factor - EGF (20 ng/mL), basic Fibroblast Growth Factor (FGF-b) (10 ng/mL) and Heparin (20 ng/mL) to produce the final complete NeuroCult™-Rat Proliferation medium (Figure 1).

Primary cells were plated at a density of 6 x 10⁴ cells/mL and 1.2 x 10⁵ cells/mL in T-25 cm² tissue culture flasks (Corning). Passaged cells were plated at 1.25 x 10⁴ cells/mL and 2.5 x 10⁴ cells/mL in T-25 cm² flasks. Neurospheres were collected on the 4th day after plating, mechanically dissociated into single cell suspension by trituration (15-20 times) with a P200 Gilson pipette tip and re-plated at 1.25 x 10⁴ cells/mL and 2.5 x 10⁴ cells/mL. Fold expansion was calculated as total number of viable cells (Trypan Blue exclusion assay) in each passage divided by total viable cells in the previous passage. The sub-culturing protocol of rat cells consisted of passaging every four days with a half media change every second day (Figure 1).

Figure 1. Expansion and differentiation of rat neural precursor cells in NeuroCult™-Rat Media and Supplements



Differentiation. Neurospheres were dissociated into a single cell suspension and plated onto Poly-D-Lysine-coated 8-well chamber slides (BioCoat BD #354632) at a density of 1 x 10⁴ cells/well in NeuroCult™ NSC Basal Medium - Rat supplemented with serum containing NeuroCult™ NSC Differentiation Supplements. Cells were cultured for eight days and then processed for indirect double-label immunocytochemistry with antibodies directed against neurons (β -tubulin and MAP-2), astrocytes (GFAP) and oligodendrocytes (O4 and MBP). Antibodies were labeled with species and fragment specific secondary antibodies (Jackson Immunochemicals) and counterstained with DAPI. The frequency of neurons, astrocytes and oligodendrocyte was calculated based on counts of positive stained cells divided by total number of cells. For each experiment eight fields were counted per three separate wells.

Results

A. Proliferation and Expansion of Embryonic Rat NSC

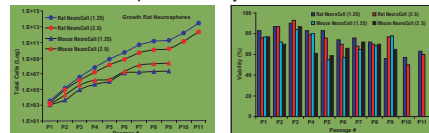


Figure 2A. Expansion of rat neural precursor cells in NeuroCult™-Rat and NeuroCult™-Mouse media in the presence EGF, FGF and Heparin

Figure 2B. Cell viability of rat neural precursor cells in NeuroCult™-Rat and Mouse media in the presence of EGF, FGF and Heparin

Table 1. Fold expansion and viability of rat neural precursor cells (1.25x10⁴ cells/mL) in NeuroCult™-Rat media (n=3)

Passage #	Fold Expansion	Viability (%)
P0 → P1	4	81
P1 → P2	56	87
P2 → P3	34	88
P3 → P4	13	80
P4 → P5	6	77
P5 → P6	5	62
P6 → P7	5	73
P7 → P8	4	81
P8 → P9	8	77
P9 → P10	9	76
P10 → P11	16	79
Average ± SE	15 ± 5	78 ± 2

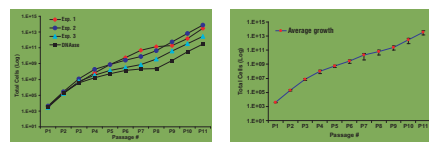
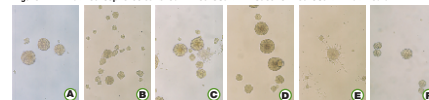


Figure 3A. Expansion of rat neural precursor cells in NeuroCult™-Rat media

Figure 3B. Average expansion of rat neural precursor cells in NeuroCult™-Rat media (n=3)

Figure 4A-F. Rat neurospheres cultured in NeuroCult™-Mouse or NeuroCult™-Rat media



A. Rat neurosphere at P2 cultured in NeuroCult™-Rat; **B.** Rat neurospheres at P2 cultured in NeuroCult™-Mouse; **C.** Rat neurosphere at P8 cultured in NeuroCult™-Mouse; **D.** Rat neurospheres at P8 cultured in NeuroCult™-Rat; **E.** Neurospheres at P9 cultured in NeuroCult™-Mouse; **F.** Neurospheres at P10 cultured in NeuroCult™-Rat

Summary. Neurospheres derived from rat cortical region can be passaged in the NeuroCult™-Rat media beyond passage 10 (over 40 days) with an average 15-fold expansion and 78% cell viability in NeuroCult™-Mouse, rat neurospheres could only be passaged up to P9 (36 days) with a significant decrease in fold expansion after P5. Rat cells started to attach to the flask and differentiated in NeuroCult™-Mouse media after P5. The average fold expansion of rat neurospheres in NeuroCult™-Mouse media from passage to passage was 5-fold with an average viability of 70%. Rat neurospheres can be maintained beyond passage 10 in the NeuroCult™-Rat media. A critical phase in the growth curve occurs between P6-P8 (24-32 days) in culture, when a significant drop was observed in terms of fold expansion.

B. Differentiation of Embryonic Rat NSC

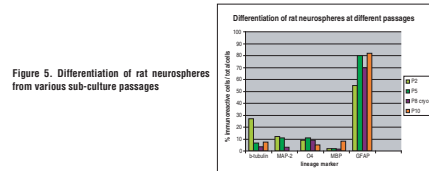
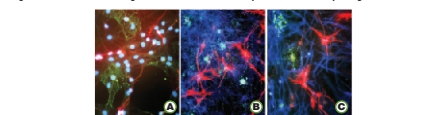


Figure 5. Differentiation of rat neurospheres from various sub-culture passages

Figure 6A-C. Immunostaining of differentiated rat neurospheres at different passages



A. Double staining with DAPI counterstain (blue), anti- β Tubulin (red) and anti-MBP (green) for rat neural precursor cells at P2 in NeuroCult™-Rat differentiation media; **B.** Triple staining (O4 - green, GFAP - blue, β -tubulin - red) of differentiated rat neural precursor cells at P2 in NeuroCult™-Rat differentiation media; **C.** Triple staining (O4 - green, GFAP - blue, β -tubulin - red) of differentiated rat neural precursor cells at P10 in NeuroCult™-Rat differentiation media. Magnification A-C: 400X.

Summary. Early (P2, P5) and late (P8, P10) passaged neurospheres grown in complete NeuroCult™-Rat are able to produce neurons, astrocytes and oligodendrocytes when allowed to differentiate in NeuroCult™-Rat Differentiation Supplements. This indicates that NeuroCult™-Rat media is a suitable media for culturing rat NSC and for allowing the maintenance of stem cell characteristics of these cells for over 40 days. Complete NeuroCult™-Rat is suitable for cryopreserving rat neurospheres as stem cell properties are maintained upon thawing and sub-culturing in this medium.

Conclusion

- NeuroCult™-Rat media allows efficient expansion of rat neurospheres (average of 15-fold between each passage) and high viability (78 %) over 40 days in culture.
- The differentiation assay demonstrated that rat neurospheres cultured in NeuroCult™-Rat media are able to produce neurons, astrocytes and oligodendrocytes.
- Cultured rat neurospheres offer a good source of NSC for transplantation studies in animal models and for *in vitro* studies screening cytokines, inhibitors and other exogenous compounds for effects on stem cell function.

Future Studies

- Proliferation and differentiation studies in NeuroCult™-Rat media of adult rat CNS and different regions of embryonic CNS.
- Clonal analysis to demonstrate multipotentiality of individual cells at >10 passages.
- BrdU analysis for labeling of proliferating cells.

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