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Spontaneous Generation of Neurospheres from Mouse Embryonic Stem Cells

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1. Introduction

Neural stem cells (NSCs) are self-renewing pluripotent cells that can produce different parts of the nervous system. NSCs were initially identified in the subventricular zone of the mouse brain (Temple, 1989) and subsequently in various regions of adult brains from human and mouse (Taupin & Gage, 2002). NSCs can be derived *in vitro* from embryonic stem (ES) cells and induced pluripotent stem (iPS) cells by employing specific culture conditions. NSCs and their progeny can be expanded for several passages as tridimensional floating aggregates named neurospheres or as monolayer cultures. This in turn allows derivation of neurons of different parts of the nervous system (Gaspard & Vanderhaeghen, 2011). NSCs derived from ES/iPS cells of various genetic backgrounds represent invaluable tools for the investigation of neurogenesis, development of neurologic diseases models, and screening of new drugs to treat neurological diseases.

Derived from ES cells, NSCs and their progeny, neural progenitors, are routinely studied *in vitro* by a method called neurosphere culture system (Reynolds & Weiss, 1992). Neurospheres can measure 100-300 mm. The zonal distribution of different cell types that compose human and murine neurospheres resembles an outside-in brain structure with nestin-positive progenitor cells in the periphery and $GFAP^+$ and β-tubulin III-positive cells in the centre (Moors et al., 2009; Campos et al., 2004).

Currently, there are two methods that allow derivation of neurospheres from mouse ES cells. Both of these methods utilize fibroblast growth factor 2 (FGF-2), epidermal growth factor (EGF), retinoic acid, and other supplements such as B27 and N2. FGF-2 is a critical component in neural differentiation protocols as it promotes proliferation of neuroprogenitor cells (Yoshimura et al., 2001). For selecting and expanding neural progenitor cells, culture media are supplemented with B-27 (containing retinoic acid) and N2 (which has a subset of component of B-27 that include insulin and transferrin).

In one method of derivation, ES cells are cultured in a specific medium supplemented with EGF, FGF-2 and N2. After 4-5 days, 50-80% of the cells undergo neural lineage specification. On day 7, these cells are dissociated and plated in suspension in uncoated plates in a differentiation medium supplemented with N2, B27, mouse EGF and human FGF-2. After a few days of culturing, neurospheres can be obtained (Conti et al., 2005).
Alternatively, in a second method, mouse ES cells are co-cultured for approximately one week with the PA6 stromal cell line to achieve efficient neuronal differentiation because of the stromal cell-derived inducing activity (SDIA) (Kawasaki et al., 2000). After 8 days of SDIA treatment, mouse ES cells are separated from PA6 stromal cells and cultured in suspension in serum-free medium supplemented with FGF-2. Under these conditions neurospheres appear within 4 days (Morizane et al., 2006).
Exemplifying stages of the neurosphere generation process are shown in figure 1.
The ability of mouse ES cells to spontaneously generate neurospheres and mature neurons in short time and in few steps, represents the strength for a powerful and reliable *in vitro* model to apply research strategies oriented to study the physiopathology of the biochemical and epigenetic mechanisms leading to the neurogenesis.
We recently observed that mouse ES cells when grown in simple culture conditions spontaneously form neurospheres. In this chapter, we describe a simple method for derivation of neurospheres from mouse ES cells without using FGF-2 and EGF and without co-culturing ES cells with stromal cells.

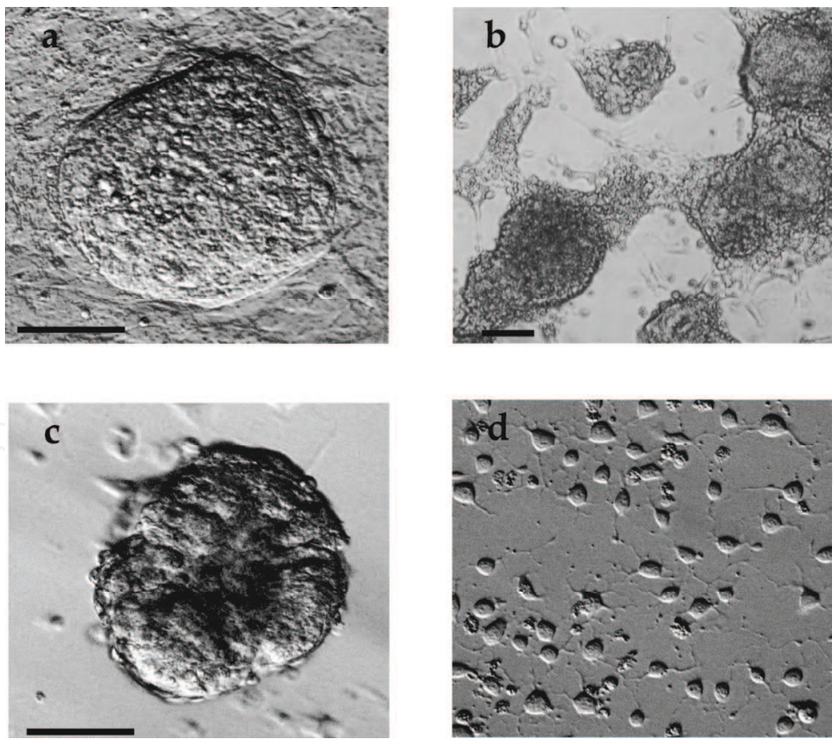

Fig. 1. Generation of neurospheres from mES cells. a) Microphotograph of a mES colony; b) typical clusters of cells generated after culturing R1 mES for three passages in a DMEM/FBS medium; c) bright field of a neurosphere; d) progenitors migrating from neurospheres. [Images taken by authors.] Scale bar 1s 100 mm.

2. Reagents and equipment

The reagents and equipment needed for the methods described in this chapter can be found listed in the tables below.

Equipment	*Company*	*Catalog #*
0.22-µm 500 ml bottle top filter	Corning	430513
0.22-µm 250 ml filter system	Corning	430767
10 cm cell culture dish	Corning	439167
24-well plates (Transwell-Clear permeable support 0.4 µm	Fischer	07-200-154
6-well ultra low attachment plate	Costar	3471
15-ml polypropylene conical tubes	BD Falcon	352096
50-ml polypropylene conical tubes	BD Falcon	352070
Transfer pipettes	Samco	262-1S
0.22-µm bottle top filters	TPP	99505
0.45-µm bottle top filters	Nalgene	165-0045
12 mm circular cover slips	Fisher	01-472A
Hemocytometer	Hycor Kova	87144
1000-ml media bottles		
Kimwipes	Kimberly-Clarks	34155
Humidified tissue culture incubator (37°C, 5% CO_2)	-	-
Centrifuge	-	-
Water bath (37°C)	-	-
Scanning confocal microscope	Olympus	FluoView 100
DM IRM inverted microscope (fitted with Leica HS N Plan BD 50X oil immersion objective)	Leica	-
75W xenon lamp-based monochromator	Ushio, Japan	-
CCD camera	Orca (Hamamatsu, Shizouka, Japan)	-
535 ± 40nm bandpass filter	Omega Optical	-
500 nm longpass dichromatic mirror	-	-
Simple PCI	Compix, Inc (Cranberry, PA)	-

Table 1. Equipment needed for the methods described in this chapter.

Reagent	*Company*	*Catalog #*
R1 ES cells	Mutant Mouse Regional Resource Centers	011979-MU
Non-essential amino acid solution (100X)	Millipore	TMS-001
Nucleosides (100X)	Millipore	ES-008-E
L-glutamine (100X)	Millipore	TMS-002-C
Penicillin/Streptomycin (100X)	Millipore	TMS-001
2-Mercaptoethanol (100X)	Millipore	ES-007-E
Sodium pyruvate (100mM)	Cellgro	25-00-CI
Trypsin-EDTA (100X)	Gibco	15400
Dulbecco's phosphate buffered saline (10X) (PBS)	Sigma	P-3813
Gelatin from porcine skin (Type A)	Sigma	G1890
Dulbecco's modified Eagle's medium (DMEM)	Millipore	SLM-220-B
Fetal bovine serum (ES cell grade) (FBS)	Gibco	16141-079
Knockout Serum Replacement (KSR)	Invitrogen	10828028
Neurobasal medium	Invitrogen	21103-049
Phosphate buffered saline (PBS)	Gibco	14040141
Poly-D-lysine (PDL) hydrobromide	Sigma	P6407
Leukemia inhibitory factor (LIF) (10^7)	Millipore	ESG1107
B27	Invitrogen	17504-044
N2 supplement	Invitrogen	17502-048
FGF-basic Recombinant Mouse	Invitrogen	PMG0035
Laminin	Sigma	L2020
BisBenzimide H 33342 trihydrochloride	Sigma	B-2261
Mounting solution	Gelvatol	-
Paraformaldehyde (16% in premixed PBS buffer at pH7 [below])	Electron Microscopy Sciences	15710-6
PBS buffer at pH7 (1X)	Roche	11666789001
10% Normal Donkey Serum (NDS)	Jackson ImmunoResearch	711-165-152
0.01% TritonX-100	Fluka	BP151-100
Tubulin III	R&D Systems	MAB1195
Rabbit Anti-NMDAR1	Abcam	Ab68144
Bovine albumin	Sigma	A-6003
Fura-2, AM cell-permeant	Invitrogen	F-1221
Cy3 AffiniPure Donkey Anti-Rabbit	Jackson ImmunoResearch	711-165-152
AffiniPure Donkey Anti-Mouse	Jackson ImmunoResearch	715-175-151

Table 2. Reagents needed for the methods described in this chapter.

3. Reagent and equipment setup

The setup for the reagents and equipment listed is as follows.

Reagent	*Setup*
ES medium	For 500 ml of ES medium, add the following components: 400 ml DMEM, 75 ml FBS, 5 ml Pen/Strep, 5 ml 2ME (100 mM), 5 ml L-glutamine, 5 ml NEAA, 5 ml nucleosides, 5 ml 2ME, and 50 µl LIF.
EB medium	For 500 ml of EB medium, add the following components: 450 ml DMEM, 25 ml KSR, 5 ml sodium pyruvate, 5 ml Pen/Strep, 5 ml 2ME (100 mM), 5 ml L-glutamine, and 5 ml NEAA.
DMEM/FBS medium	For 500 ml of feeder cells medium, add the following components: 440 ml DMEM, 50 ml FBS, 5 ml NEAA, and 5 ml Pen/Strep.
N2 medium	For 100 ml of N2 medium, add 96 ml DMEM/F12, 1 ml N-2, 1 ml Pen/Strep, 1 ml L-glutamine, 1 ml NEAA, 4 µl FGF-2, and 100 µl laminin.
B27/Neurobasal medium	For 500 ml of B27/Neurobasal medium, add the following components: 480 ml Neurobasal medium, 10 ml B27, 5 ml L-glutamine 100 X, and 5 ml Pen/Strep.
Gelatin 0.1%	Dissolve 100 mg of gelatin in 90 ml warm (~ 60 ^{o}C) distilled water. Sterilize by autoclaving and store at room temperature.
25x FGF stock solution (100 µg ml^{-1})	Dissolve 25 mg recombinant mouse FGF in 250 ml of sterile PBS. Store at ~20 °C.
5x Poly-D-Lysine	Dissolve 50 mg poly-D-lysine in 333.3 ml distilled water and rinse the package with the water. Filter the solution with a 0.22-µm bottle top filter. Aliquot 5 ml into 15-ml conical tubes and store at -20 °C.
Hepes-buffered salt solution (HBSS)	Prepare stocks of the composition described in table 4 below. To obtain the HBSS basic, combine the solutions in the following order: 10.0 ml Solution #1, 1.0 ml Solution #2, 1.0 ml Solution #3, 1.0 ml Solution #5, 86.0 ml distilled H_2O, 1.0 ml Solution #4. Prior to use, prepare the following solution: 9.8 ml HBSS basic, 0.1 ml Stock #5, 0.1 ml Stock #6. The final HBSS composition is 137 mM NaCl, 5.4 mM KCl, 0.6 mM Na_2HPO_4, 0.6 mM KH_2PO_4, 20 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$, 10 mM $NaHCO_3$, 5.5 mM glucose.

Table 3. Setup of reagents needed for the methods described.

Stock	*Preparation*
1	In 90ml of distilled H_2O, dissolve 8.0 g NaCl. 0.4 g KCl, and fill to 100 ml with distilled H_2O.
2	In 90ml of distilled H_2O, dissolve 0.41 g Na_2HPO_4 (anhydrous), 0.80 g KH_2PO_4, and fill to 100 ml with distilled H_2O
3	Add 0.72 g $CaCl_2$ to 50ml of distilled H_2O
4	Add 1.23 g $MgSO_4x7H_2O$ to 50ml of distilled H_2O
5	Add 4.76 g Hepes to 10ml of distilled H_2O
6	Add 0.9 g Glucose to 10ml of distilled H_2O
7	Add 0.84 g $NaHCO_3$ to 10ml of distilled H_2O

Table 4. Preparation of the stock solutions needed to prepare HBSS. The combination of these stock solutions in described in table 3.

Equipment	*Setup*
Gelatin-coated plate	Add 5 ml of 0.1% (wt/vol) gelatin solution into a plate so that it covers the entire bottom of the plate. Incubate the plate for 20 min at room temperature.
Poly-D-lysine (PDL) cover slips	Autoclave cover slips. Place sterilized cover slips into the wells of a 24-well plate. Dilute the 5 x poly-D-lysine to 1 x with sterile distilled water plate except for the outermost wells. Add 0.5–1 ml of the 1 x poly-D-lysine solution to cover the surface of the cover slips. After an overnight incubation, remove the PDL and wash the plates with sterile deionized water and dry in a cell culture hood.

Table 5. Setup for the equipment needed for the methods described.

4. Methods

The generation of neurospheres and subsequent neuronal differentiation from ES cells is achieved through the stages described in the protocols below.

4.1 Maintenance of ES cells

Place R1 ES cells (Nagy et al., 1993) on 10 cm gelatin-coated dishes with 12 ml of ES medium in a humidified chamber in a 5% CO_2/air mixture at 37°C. Change the medium every day. When the cells reach 80-90% confluence split them to two dishes as follows: wash the cells with 1x PBS twice, then add 2 ml of 1x trypsin-EDTA. Rock the dish to ensure that the solution covers all the cells. Incubate the dish for 5 min at 37°C. Pipette up and down to break up clumps and obtain a good dissociation of cells. Pipette 8 ml ES medium to inhibit further tryptic activity and transfer to 15-ml conical tubes. Spin at 1000 rpm for 3 min. Aspirate the medium from cells and resuspend the pellet in 6 ml of fresh ES medium.
Transfer 3 ml of cell suspension into a new 10 cm gelatin-coated dish containing 9 ml of fresh ES medium.

4.2 Generation of embryoid bodies

Aspirate the medium, add 2 ml of 1x trypsin-EDTA and incubate for 5 min at 37 °C. Dissociate the cells by thoroughly pipetting several times with a P1000 Pipetman. Monitor

the trypsinization under an inverted microscope. If the separation of the cells is not complete, continue the treatment with trypsin for additional 2-3 min.
Transfer the cell suspension into a 15-ml sterile conical tube. Pellet the cells centrifugation for 5 min at 1200 rpm; then resuspend the cell pellet in 10 ml EB medium.
Count the cells with a cell counter and plate $5x10^5$ ES cells per well in 6-well ultra low attachment plates in EB medium. Embryoid bodies should appear by overnight incubation.
Culture plates for 7 days. Change the medium every other day as follows: Collect the EBs in suspension from the plate and transfer into a 15 ml conical tube. Leave the conical tube for 15 minutes to allow the EBs to settle to the bottom of the tube. Aspirate the medium and add 12 ml of fresh EB medium. Transfer the EBs suspension into a new 6-well ultra low attachment plates.

4.3 Neurosphere generation and neuronal differentiation

Collect EBs and remove the medium as described in the previous step.
Count the number of EBs and prepare an EBs suspension in Feeder medium at a density of 40-50 EBs/ml.
Transfer 40-50 intact EBs to non-coated 10 cm cell culture dish containing 12 ml DMEM/FBS medium.
Culture until cells reach confluence, changing medium every 2-3 days.
Aspirate the medium, add 2 ml of trypsin-EDTA solution and incubate for 5 min at 37°C. Dissociate cells thoroughly by pipetting with a P1000 Pipetman. Pipette 8 ml Feeder medium and transfer the cell suspension to a 15-ml conical tube.
Centrifuge for 5 min at 1200 rpm. Aspirate the medium and resuspend the cells in 12 ml DMEM/FBS. Culture the cells until confluence.
Trypsinize as described in section 4.2 and culture cells for other two passages.
Transfer the neurospheres floating in the culture medium into 50-ml conical tubes and allow them to settle for 10 minutes. Collect the supernatant containing neurospheres. Trypsinize cells attached to the bottom of the plate as described in section 6.2 and culture to generate new neurospheres.

4.4 Neuronal differentiation

Resuspend the neurospheres in the appropriate volume of N2 medium and culture for differentiation in a 24-well plate (15-20 neurospheres/well) containing poly-D-lysine-coated cover slips. Replace a half volume medium every other day.
At day 7 exchange N2 medium to B27/Neurobasal medium and let the cells differentiate for 10 more days with a medium change every other day.

5. Characterization and functional analysis of neurons

Selective immunocytochemistry can be performed according to standard procedures as described in the following sections.

5.1 Cell cultures

Plate the neurospheres on PDL-treated cover slips, and culture according to experimental protocols with the same conditions described above.

5.2 Immunocytochemistry procedure

Fix mouse neurospheres-derived neurons for 20 minutes at room temperature in a 4% paraformaldehyde solution (prepared from stock 16% Electron Microscopy Sciences, 15710-6 in 1X Premixed PBS Buffer at pH 7: Roche, 11666789001)

After three 10-minutes washes in PBS at room temperature, perform a blocking step for 1 hour in 10% of Normal Donkey Serum (NDS, Jackson ImmunoResearch, 017-000-121) supplemented with 0.01% TritonX-100 (Fluka, BP151-100).

Incubate the samples overnight with primary antibodies diluted in 10% of NDS in order to detect the expression of Tubulin III (R&D Systems, MAB1195) and of the ubiquitous NMDA receptor subunit NR1. (Rabbit Anti-NMDAR1, Abcam ab68144; 1/250).

After three 10-minutes washes in PBS at room temperature, use fluorescently labeled secondary antibodies for detection (Cy3 AffiniPure Donkey Anti-Rabbit [Jackson ImmunoResearch, 711-165-152] or Cy5 AffiniPure Donkey Anti- Mouse [Jackson ImmunoResearch, 715-175-151]).

After three 10-minutes washes in PBS at room temperature, treat the cells with bisBenzimide H 33342 trihydrochloride (Sigma B-2261) for 2 minutes, to stain nuclei with 405nm fluorescence.

After two 10-minutes washes, in PBS remove the cover slip from the plates and mount on glass slide with mounting solution (Gelvatol).

Images can be acquired using a laser scanning confocal microscope (FluoView 1000, Olympus). The microscope is equipped with spectral detector technology that provides precise wavelength separation of the emitted light.

5.3 Intracellular Ca^{2+} measurement

For intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) measurements, incubate cells for 45 min at 37°C with 5 μM fura-2-AM (cell permeant; Invitrogen, F-1221) and 5 μg/ml bovine serum albumin (Sigma A-6003) in Hepes-buffered salt solution (standard HBSS).

Then, wash cells 3 times for 5 min each with standard HBSS and place the cover slips onto a perfusion chamber on a DM IRM inverted microscope (Leica) fitted with a Leica HC N PLAN BD 50X oil immersion objective. $[Ca2^{+}]_i$ can be monitored in single cells using excitation light provided by a 75W xenon lamp-based monochromator (Ushio, Japan). Emitted light can be detected using a CCD camera (Orca; Hamamatsu, Shizouka, Japan). Alternatively, illuminate cells with 340 and 380 nm light for fura-2. Emitted fluorescence can be passed through a 500 nm longpass dichromatic mirror and a 535 ± 40 nm bandpass filter (Omega Optical).

Analyze acquired data using software such as Simple PCI (Compix, Inc., Cranberry, PA) as the 340/380 ratio. Measure fluorescence in 15–25 individual neurons for each cover slip. Subtract background fluorescence, determined from three or four cell-free regions of the cover slips, from all signals prior to calculating the ratios. Choose the excitation light exposure time and a neutral density filter to avoid saturation of the fluorescence signal.

6. Conclusion

Neural induction from pluripotent stem cells often yield heterogeneous cell populations that can hamper quantitative and comparative analyses. There is a need for improved differentiation and enrichment procedures that generate highly pure populations of neural stem cells (NSC), glia and neurons. The method described here allows an efficient generation of neurospheres from mES cells. On day-in vitro 16 after neural induction about 50% of the cells derived from neurospheres show a neural morphology, a significant

expression of neuronal markers such as Tuj1 and the ubiquitous NMDA receptor subunit NR1 (figure 2), and a functional NMDAR-dependent calcium influx mediated by exogenous glutamate administration (figure 3).

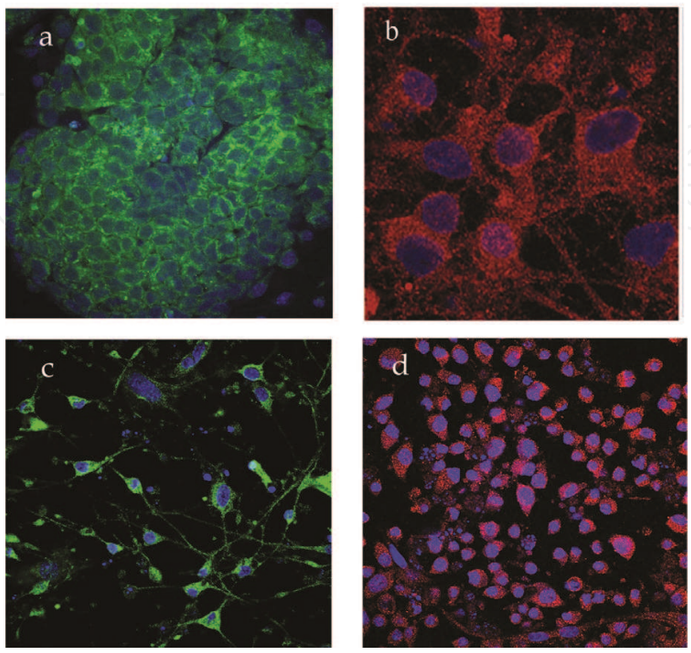

Fig. 2. Neurosphere differentiation. a) Immunostaining of a neurospheres with antibody specific to NMDA receptor (NR1 sub-unit, green). b-c) Staining of neurosphere-derived neurons with (b) NMDA receptor (NR1 sub-unit, red) and (c) β-tubulin III (green). d) staining of astrocytes differentiated from neurospheres with GFAP (red). Nuclei were counterstained with DAPI (blue). [Images taken by authors.]

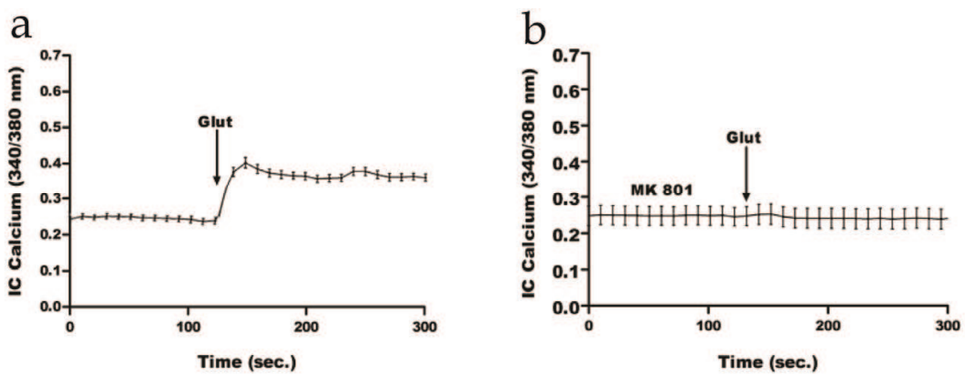

Fig. 3. Analysis of calcium influx in neurons differentiated from neurospheres indicated by glutamate. a) Administration of 10μM glutamate (glu) in neurons causes an increase in $[Ca^{2+}]_i$ (340/380 = 0.32 ± 0.05a). b) Glu-induced increase in $[Ca^{2+}]_i$ in R1 cells is blocked by treatment with MK801, an NMDAR-specific antagonist, suggesting that NMDARs are the predominant glutamate-sensitive receptors in neurospheres-derived neurons. [Data generated by authors.]

Furthermore, the rest of the cellular population show typical astroglial morphology. This chapter illustrates the utility mouse ES cells as a simple system able to develop populations of viable NSC and neurons. The method described here will enable downstream studies that require consistent and defined cell populations to study and characterize the physiopathology of the neuronal differentiation.

This procedure allows generation of a cell line derived from mouse ES cells which continually produces neuropheres in the absence of FGF-2 and EGF and coculturing. By generating neurospheres via this method, one can use them for a variety of purposes for further experiments with neurons.

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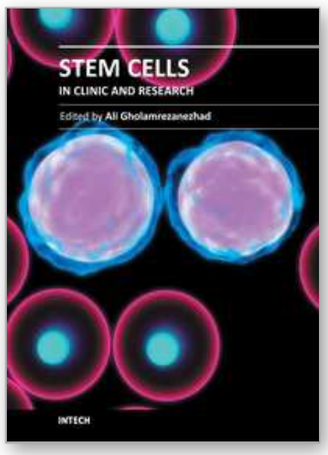

Stem Cells in Clinic and Research

Edited by Dr. Ali Gholamrezanezhad

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Based on our current understanding of cell biology and strong supporting evidence from previous experiences, different types of human stem cell populations are capable of undergoing differentiation or trans-differentiation into functionally and biologically active cells for use in therapeutic purposes. So far, progress regarding the use of both in vitro and in vivo regenerative medicine models already offers hope for the application of different types of stem cells as a powerful new therapeutic option to treat different diseases that were previously considered to be untreatable. Remarkable achievements in cell biology resulting in the isolation and characterization of various stem cells and progenitor cells has increased the expectation for the development of a new approach to the treatment of genetic and developmental human diseases. Due to the fact that currently stem cells and umbilical cord banks are so strictly defined and available, it seems that this mission is investigationally more practical than in the past. On the other hand, studies performed on stem cells, targeting their conversion into functionally mature tissue, are not necessarily seeking to result in the clinical application of the differentiated cells; In fact, still one of the important goals of these studies is to get acquainted with the natural process of development of mature cells from their immature progenitors during the embryonic period onwards, which can produce valuable results as knowledge of the developmental processes during embryogenesis. For example, the cellular and molecular mechanisms leading to mature and adult cells developmental abnormalities are relatively unknown. This lack of understanding stems from the lack of a good model system to study cell development and differentiation. Hence, the knowledge reached through these studies can prove to be a breakthrough in preventing developmental disorders. Meanwhile, many researchers conduct these studies to understand the molecular and cellular basis of cancer development. The fact that cancer is one of the leading causes of death throughout the world, highlights the importance of these researches in the fields of biology and medicine.

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