Kafkas Univ Vet Fak Derg
20 (5): 711-718, 2014
DOI: 10.9775/kvfd.2014.10953

Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

RESEARCH ARTICLE

The Differentiation of Neuronal Cells from Mouse Embryonic Stem Cells [1][2]

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[1] *This study was funded by the Commission for Celal Bayar University Scientific Research Projects (Project no: TIP 2008-090)*
[2] *This study presented as poster in the " Xth National Histology and Embryology Congress, 17-20 May 2010, Çeşme, İzmir-Turkey" and published in Cell Tissue Biology Research (Abstract Book), Vol: 2 No: 1(S)*

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Makale Kodu (Article Code): KVFD-2014-10953

Özet

With new technologies emerging today, the importance of stem cells in the cell therapy of nervous system diseases is supported by recent studies. Therefore, the development of neuronal cell differentiation protocols from stem cells is of great importance. In our study, the differentiation of neuronal and neuroglial cells from mouse embryonic stem (ES) cell line and their analysis with neuronal cell markers are aimed. Mouse ES cells were differentiated to neurogenic series cells by adding N2 and bFGF to the culture medium on coated Fibronectin dishes. For the identification of differentiated cells, they were evaluated by light microscopy using immunhistochemistry techniques and by electron microscopy. Indirect immunohistochemical staining method was performed with SSEA-1 (mouse embriyonic stem cells marker), Nestin (neural precursor cells marker), βIII-Tubulin (neuronal cells marker), MAP-2 (neuronal cells marker), GFAP (astrocyte marker), and O4 (oligodendrocyte marker). After 1 week of differentiation of cells, immunoreactivities of SSEA-1 and Nestin were detected to be negative and moderate, respectively. After 2 weeks culture time, the differentiation was still continuing and especially positive immunoreactivities of β-III Tubulin and MAP-2 and weak immunoreactivities of O4 and GFAP were supported neuronal differentiation. In conclusion, our results suggest that neuronal cell derived from mouse ES cells were differentiated particularly to neuron using N2+bFGF+fibronectin culture condition. Therefore, these differentiated cells may be used as a treatment method in degenerative diseases of the nervous system.

Anahtar sözcükler: *Mouse embryonic stem cell, Differentiation, Neuron and neuroglia*

Fare Embriyonik Kök Hücrelerden Nöronal Hücrelerin Farklılaşması

Summary

Günümüzde gelişen yeni teknolojiler sayesinde sinir sistemi hastalıklarının hücresel tedavisinde kök hücrelerinin önemi son yıllardaki çalışmalar ile desteklenmektedir. O nedenle kök hücrelerden nöronal hücrelerin farklılaştırılması protokollerinin oluşturulması büyük önem taşımaktadır. Çalışmamızda, fare embriyonik kök (EK) hücre hattından, nöron ve nöroglial hücrelerin farklılaştırılması ve nöronal hücre belirteçleri ile analizi amaçlanmıştır. Fare EK hücreler, fibronektin kaplı petrilerde kültür ortamına N2 ve bFGF ilavesi ile nörojenik seri hücrelerine farklılaştırıldı. Farklılaşmış hücrelerin tanımlanması için hücreler, immünohistokimya tekniği kullanılarak ışık mikroskobu ile ve elektron mikroskobu ile değerlendirildi. İndirek immünohistokimyasal boyama yöntemi SSEA-1 (fare ES hücre belirteci), Nestin (nöron öncülü hücre belirteci), βIII-Tubulin (nöron hücre belirteci), MAP-2 (nöron hücre belirteci), GFAP (astrosit belirteci) ve O4 (oligodendrosit belirteci) için uygulandı. Hücrelerin farklılaşmasının 1. haftasından sonra SSEA-1 ve Nestin immünoreaktivitesi sırasıyla negatif ve orta saptandı. Kültürün 2. haftasından sonra farklılaşmanın hala devam etmesi ve özellikle β-III Tubulin ve MAP-2 immünoreaktivitesinin güçlü pozitif ve O4 ve GFAP immünoreaktivitesinin zayıf olması nöronal farklılaşmayı desteklemiştir. Sonuç olarak, bizim sonuçlarımız göstermiştir ki fare EK hücrelerinden kaynaklanmış nöronal hücreler, N2+bFGF+fibronektin kullanılan kültür koşullarında özellikle nörona farklılaşmıştırlar. Bu nedenle, farklılaştırılmış bu hücreler dejeneratif sinir sistemi hastalıklarının hücresel tedavisinde kullanılabilir.

Keywords: *Fare embriyonik kök hücre, Farklılaşma, Nöron ve nöroglia*

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INTRODUCTION

The differentiation of neuronal cells from stem cells plays an important role in the cell therapy of nervous system diseases, including neurodegenerative disorders, multiple sclerosis, neurotrauma and neurodevelopmental disorders [1]. Recent clinical trials of regenerative therapy for neuronal disease have been performed with transplantation of stem cells [2]. Stem cells using neuronal disease models; somatic (adult derived) stem cells, fetal stem cells and ES cells, induced pluripotent stem (iPS) cells [3]. Numerous studies of transplantation of somatic stem cells using neuronal disease models have been reported and most studies have confirmed its efficiency in repairing these [2]. The transplantation effect of adipose derived mesenchymal stem cells has been reported to be greater than that of bone marrow mesenchymal stem cells [2]. iPS cells have been shown to differentiate towards a neuronal phenotype leading to functional improvement after their application in animal models of Parkinson's disease, stroke and other pathologies [3]. Fetal stem cells demonstrate higher proliferation, more specific differentiation, and better migration after transplantation. However, the use of these cells is limited [3,4]. ES cells are pluripotent stem cells [5]. Studies have shown that they can proliferate undifferentiated *in vitro* [6]. These cells have been shown to proliferate without differentiation in suitable culture medium, but changing the environment may lead these cells to differentiate [7-9]. Furthermore, ES cells have been reported to generate all three neural lineages: neurons, astrocytes and oligodendrocytes [10-12]. Several approaches have been used to achieve in vitro neural differentiation starting from ES cells, aimed at generating specified neural progenitors and/or differentiated neuronal and glial subtypes [10,13]. Differentiation of stem cells to neurons has usually been achieved by protocols that enrich ectoderm derivatives, as a first step in the production of neural progenitors [12,14]. These attempts to influence cell fate decisions in order to obtain cells of the ectodermal layer have utilized neuralizing signals derived from feeder cells, addition of growth factors or growth supplements to the medium [12,15-17].

In this study, neural cell differentiation from mouse embryonic stem cells was indicated by neural and glial cells specific markers. Differentiated neuronal cells were identified with stage specific embryogenic antigen-1 (SSEA-1), nestin, βIII-Tubulin, microtubule associated protein (MAP-2), glial fibrillary acidic protein (GFAP), and oligodendrocyte 4 (O4**)** antigens. SSEA-1 is a carbohydrate antigen that can be identified in mouse and human embryonic stem cells. SSEA-1 is positive in preimplantation stage of mouse embryos while it is lost during differentiation [18]. Nestin is type VI intermediate filament protein and is synthesized in dividing cells during early development, especially in primitive neuroepithelium. Nestin is expressed by neuronal precursor cells. They are down regulated with differentiation of neuronal stem cells [19,20]. MAP-2 is responsible for microtubule formation and shaping dendritic in neurogenesis [21,22]. βIII-Tubulin is widely as an early neuronal cytoskeleton marker in neuronal development studies [23,24]. GFAP is a specific intermediate filament protein that is expressed by numerous cell types of central nervous system. It is associated with many cellular functions, such as cellular structure and movement, in neuronal cells [25-27]. O4 has been commonly used as the earliest recognized marker specific for the oligodendroglial lineage [28].

Recently, in diseases of the nervous system, many studies have been done regarding stem cell therapy. Therefore, the forming nerve cell production protocols from stem cells is essential.This study is important in constituting a study protocol for nerve cells derived from embryonic stem cell in different time periods in medium containing basic fibroblast growth factor (bFGF) and N2 on fibronectin coated plates.

MATERIAL and METHODS

The study protocols and experimental procedures were approved by the Celal Bayar University Scientific Ethics Committee (0259).

Embryonic Stem Cell Culture and Neuronal Differentiation

Mouse embryonic stem cell line (CGR8, 07032901, ECACC, Salisbury, UK) was purchased from the Celal Bayar University Medical Faculty, Histology and Embryology Department. For the feeder layer, mouse fibroblast cells (STO, 86.032.003, ECACC, Salisbury, UK) were used. When the STO cells became confluent after 1 week of culture, they were treated with 20 µg/ml of mitomycin-C (A2190, 0002, Applichem, Darmstadt, Germany) for 1.5h at 37°C with 5% CO_2 in air. Incubation of STO cells with mitomycin-C and embryonic stem cell cultures, was performed simultaneously.

Embryonic stem cells were cultured in a humidified atmosphere at 37°C in 5% CO_2 in a medium [Dulbecco's modified Eagle's medium (DMEM, F0445, Biochrom AG, Berlin, Germany) containing 4500 mg/l glucose and sodium pyruvate,15% fetal bovine serum (FBS, S0113, Biochrom AG, Berlin, Germany), 1% L-glutamine (K0283, Biochrom AG, Berlin, Germany), 1% penicillin/streptomycin (A2213, Biochrom AG, Berlin, Germany), 0.1 mM non-essential amino acid (NEAA, K0293, Biochrom AG, Berlin, Germany), 10^{-6} M β-mercaptoethanol (M7522, Sigma-Aldrich, St. Louis, MO, USA) and 1.000 IU/ml leukemia inhibitory factors (LIF, L5158 Sigma-Aldrich St. Louis, MO, USA)] on mitomycin-C treated STO cells. The medium was changed every other day. When the cells were confluent, they were routinely subcultured using trypsin-EDTA solution (L2143, Biochrom AG, Berlin, Germany). The hanging drop

method was used for forming embryoid bodies (EBs) from embryonic stem cells. On the lid of culture dishes, 20 µl drops containing 5×10^4 undifferentiated cells/ml were plated. The lid was inverted and placed over the culture dish filled with sterile phosphate buffered saline (PBS) to prevent the drops from drying [29]. The culture dishes with hanging drops were incubated in a humidified atmosphere at 37°C in 5% CO_2 for 2 days. Embryoid bodies were collected into media and then were transferred into culture dishes. Cells were cultured in a humidified atmosphere at 37°C in 5% CO_2 for 4 days in culture medium without LIF. 1% N2 solution (R&D System, SC005, USA), containing bovine insulin, human transferrin, sodium selenite, putrescine and progesterone, was added into culture media for neural stem cell expansion. When the cells were confluent, they were transferred to 1% bovine fibronectin (Neural Stem Cell Expansion Kit Monolayer Plus System, SC005, USA) coated plates. Fibronectin used to promote cell attachment and spreading. On the seventh day of the culture, some of the cells were fixed for immunohistochemical analysis, while 0.1% bFGF (Neural Stem Cell Expansion Kit Monolayer Plus System, SC005, USA) was added to the remaining cells to maintain the differentiation. These cells were passed through on the seventh, fourteenth and twenty-first days of the culture, and were fixed for immunohistochemical analysis.

Identification of Neuronal Differentiation Using Transmission Electron Microscopy

Cells were collected after 2 weeks of culture period using trypsinization technique. They were fixed with 2.5% glutaraldehyde (16210, Electron Microscopy Sciences, Hatfield, PA). After washing with buffer solution (Buffer A: 0.06 M KH_2PO_4 + Buffer B: 0.08 M Na_2HPO_4) twice, 10 min for each step, the pellets were fixed with 1% osmium tetroxide (R1015, Agar Scientific, Essex, UK) at +4°C for 1 hour. Cells were embedded in 2% agar (A 2114, AppliChem, Darmstadt, Germany) after washing with buffer solution for 10 min. They were dehydrated with propylene oxide and embedded in epon. Thin sections (120 nm) were contrasted with uranyl acetate and lead citrate. They were evaluated under Zeiss LIBRA 120 electron microscope (Oberkochen, Germany).

Identification of Neuronal Differentiation Using Indirect Immunoperoxidase Staining

For immunohistochemical analysis, cells were stained using the indirect immunohistochemical method. The differentiated cells were fixed with 4% paraformaldehyde in PBS at 4°C for 30 min. Subsequently, they were washed with PBS. Endogenous peroxidase activity was quenched with incubation with 3% hydrogen peroxide (H_2O_2, K31355100, Merck, Darmstadt, Germany) for 10 min at room temperature. Cells were then washed with PBS, and incubated on ice for 15 min with 0.1% Triton-X (A4975,0100, Applichem, Darmstadt, Germany) for permeabilization. Afterwards, cells were incubated with blocking solution (K023, DBS, California, USA) for 1 h. Cells were then washed with PBS, and incubated with primary antibodies: anti-SSEA-1 (1:5 dilution, R&D System, MAB2155, USA), anti-Nestin (1:10 dilution, (R&D System, SC 013, USA), anti-βIII-Tubulin (1:10 dilution, R&D System, SC 013, USA), anti-GFAP (1:100 dilution, R&D System, SC 013), anti-O4 (1:10 dilution, R&D System, SC 013, USA), and anti-MAP-2 (1:100 dilution, Gene-Tex, GTX 48032, USA), all for overnight at room temperature in a humidified chamber. After removing the primary antibodies, the cells were incubated with biotinylated IgG (both anti-mouse and anti-goat supplied ready to use by Zymed, San Francisco, CA, USA) for 30 min, followed by three washes in PBS and then with streptavidin-peroxidase conjugate (supplied ready to use by Zymed) for 30 min (Histostain-Plus Bulk Kits; Zymed) and washed with PBS three times. They were incubated with a solution containing 1:9 concentration of diaminobenzidine (supplied ready to use by Zymed) with 0.3% H_2O_2 (Histostain-Plus Bulk Kits; Zymed), 50 µl for each sample, for 5 min to visualize immunolabeling. Samples were then mounted with mounting medium (AML060, Scytek, Logan, UT, USA). Subsequently, they were viewed using an IX71 inverted microscope (Olympus, Tokyo, Japan). The negative controls received the same treatment as described above, but they were incubated with rabbit IgG instead of the primary antisera [29]. Labelling in all negative control cases was negative. Immunolabelling was evaluated semi-quantitatively using an additive immuno reactive score reflecting signal intensity, as negative (–), mild (+), moderate (++) and strong (+++).

Statistical Analysis

Comparable data groups were evaluated using ANOVA. $P<0.05$ was considered significant [30]. Graph-Pad InStat statistic program (GraphPad Software, USA) was used for analyses.

RESULTS

It was observed that mouse embryonic stem cells were round-shaped with bright nuclei and they formed inexplicit-contoured colonies on the confluent mitomycin treated STO *(Fig. 1A, B)*. Both the outer and the inner layer cells were distinguishable within the embryonic bodies formed from the embryonic stem cells after the hanging drop method. It was observed that the outer layer cells were composed of cubic surface ectoderm-like single layer cells significantly *(Fig. 2A)*. The peripheral cells were seen to have proliferated forward as well *(Fig. 2B)*.

For neuronal differentiation, N2 medium was added into culture condition at fourth day of culture. Some of the cells were observed as fusiform-shaped, while the others had round bodies and axon-dendrite-like extensions just like neurons on the 7th day of differentiation *(Fig. 3A)*.

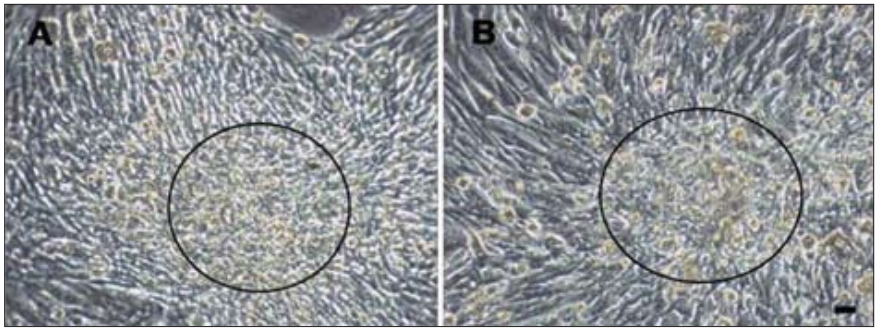
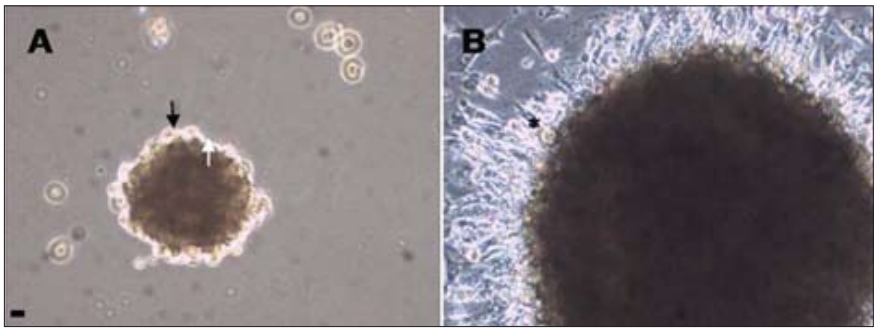


Fig 1. The embryonic stem cell colonies which were marked with the circles on confluent mouse fibroblast cells treated with mitomycin-C **(A, B)**, x200, Scale bar: 25 µm

Şekil 1. Mitomisin-C uygulanmış fare fibro-blast hücreleri üzerindeki daire ile işaretlenmiş embriyonik kök hücre kolonileri **(A, B)**, x200, Ölçek çubuğu: 25 µm

Fig 2. First **(A)** and fourth **(B)** days of culture condition of embryonic bodies. After EB formation, both the outer *(black arrow)* and the inner *(white arrow)* layer cells were distinguishable, and proliferating cells (*) were also observed on the 4th day of the culture time, x100, Scale bar: 25 µm

Şekil 2. Kültürün 1. **(A)** ve 4. **(B)** günündeki embriyonik cisimler. Embriyonik cisim oluşumun-dan sonra hem iç *(siyah ok)* hem dıştaki *(beyaz ok)* hücre tabakası ayırtedilebiliyor, bununla beraber kültürün 4. gününde farklılaşan hücreler (*) de gözleniyordu, x100, Ölçek çubuğu: 25 µm



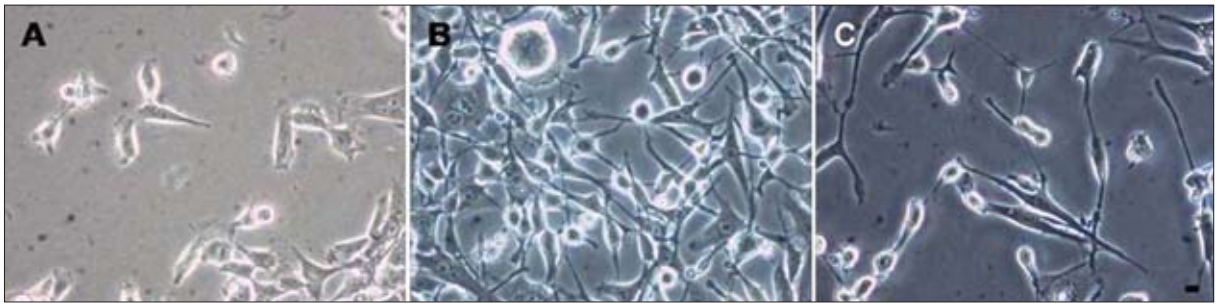


Fig 3. The cells cultured within the N2 added media on the 7th **(A)**, 14th **(B)** and 21st **(C)** days of differentiation, x100 Scale bar: 25 µm

Şekil 3. Farklılaşmanın 7. **(A),** 14. **(B)** ve 21. **(C)** günlerinde kültür ortamına N2 ilavesi ile kültüre edilmiş hücreler, x100, Ölçek çubuğu: 25 µm

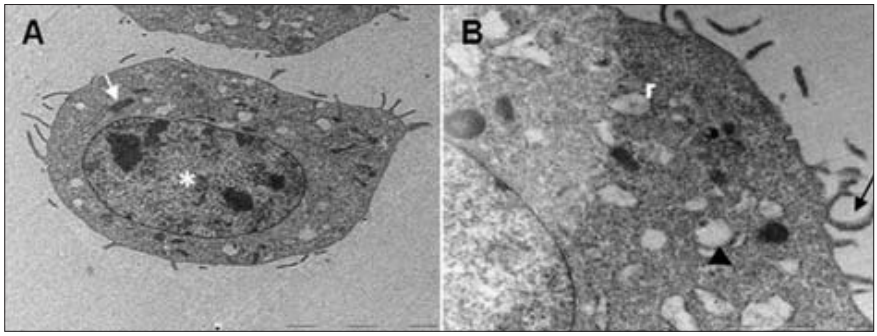


Fig 4. Electron micrograph of differentiated neuron-like cells. **A-** heterochromatin nucleus (*), mitochondria with few cristae *(white arrow)* and polyribosomes (r), Scale bar: 5.000 nm, **B-** Phagocytotic vesicles *(arrow head)* indicative of autophagocytosis, and cross sections of microvilli *(black arrow)*, Scale bar: 5.000 nm

Şekil 4. Farklılaşmış nöron benzeri hücrelerin elektron mikroskobik görüntüsü. **A-** hetero-kromatin çekir-dek (*), bir kaç kristası *(beyaz ok)* olan mitokondri ve poliribozomlar (r). Ölçek çubuğu: 5.000 nm, **B-** Oto-fagositozun göstergesi fagositik kesecikler *(ok başı)* ve mikrovilli kesitleri *(siyah ok)*, Ölçek çubuğu: 5.000 nm

Further culture of differentiated cells including bFGF on fibronectin coated plates showed that most of the cells started to become polarized apparently with extensive neurit like structures. To further promote the maturation, heterogeneous cell populations consisting cells at different stages of maturation were seen at 14th and 21st days of culture condition *(Fig. 3B,C)*. The fusiform shape of cells were accepted as undifferentiated cells, and the neuron-like cells were determined as an early, intermediate and terminal differentiated neurons.

Transmission Electron Microscopy Results: Differentiated cells were morphologically similar to the neuron-like cells, especially after 21st days of culture in N2+bFGF medium *(Fig. 4)*. After TEM analyses, the cells which were cultured in N2+bFGF medium for 9 days consisted lamellipodia, large euchromatic nuclei and sparse endoplasmic reticulum and golgi *(Fig. 4A)*. Neuron-like cells have mitochondria which have more prominent cristae and distinguishable microtubules which is an indicator of mature neurons *(Fig. 4B)*.

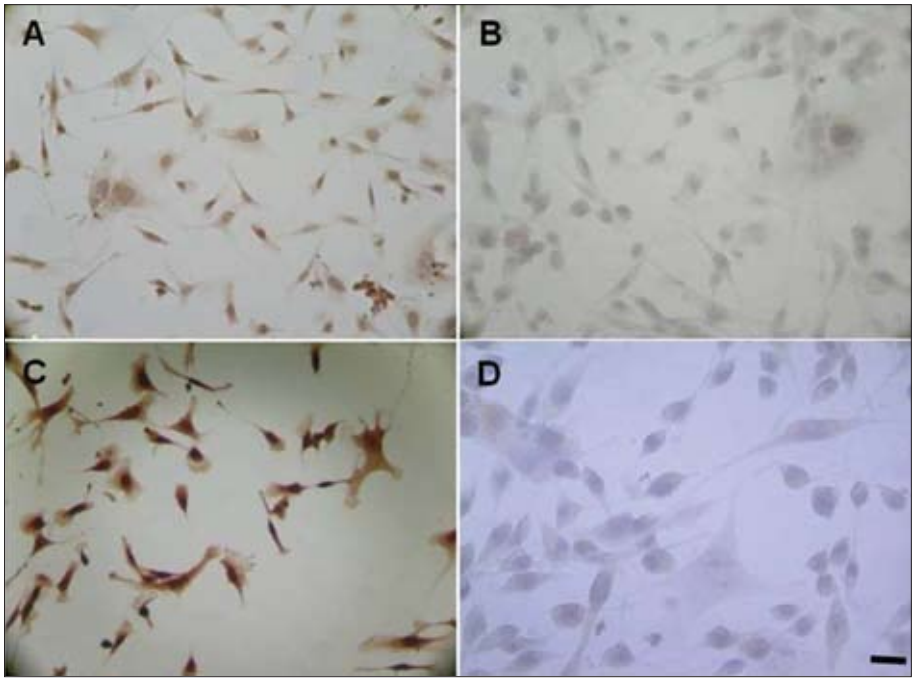


Fig 5. Distributions of SSEA-1 **(A,B)** and Nestin **(C,D)** immunoreactivities of differentiatied cells on the 7th and 14th days of differentiation, x400, Scale bar: 25 µm

Şekil 5. Farklılaşmanın 7. ve 14. günlerinde farklılaşmış hücrelerin SSEA-1 **(A,B)** ve Nestin **(C,D)** immünoreaktivitelerinin dağılımları, x400, Ölçek çubuğu: 25 µm

Immunohistochemical Results: According to the immunohistochemical analyses performed in order to characterize the cells on the 7th and 14th days of differentiation, SSEA-1 immunoreactivity, which is a mouse embryonic stem cell marker, was weak and negative (0.9±0.17), (0.1±0.19) respectively *(Fig. 5A,B)*, whereas the immunoreactivity of Nestin, which is an anti- body specific to the intermediate filament proteins of neural precursor cells, was strongly and weakly positive on the 7th and 14th days of the culture within N2 medium, (2.8±0.18), (1.08±0.10) respectively *(Fig. 5C, D)*. SSEA-1 and Nestin immunoreactivites in both 7th and 14th were statistically different (P<0.001), (P<0.001) respectively. In particularly, statistical significance of nestin immuno-reactivity supported that cells were derived from embryonic cells differentiated to neuron precursor at the 7th day of culture in our differentiated condition.

Table 1. *Immunolabelling intensity of SSEA-1, Nestin antibodies at the 7th and 14th days of differentiation cultures*

Tablo 1. *Farklılaşma kültürünün 7. ve 14. günlerinde SSEA-1, Nestin antikorlarının immünişaretleme yoğunluğu*

Days	Antibody Immunolabelling Intensity[a]	
	SSEA-1	Nestin
7	+	+++
14	-	+

Table 2. *Immunolabelling intensity of MAP_2, βIII-Tubulin, GFAP and O4 antibodies at the 14th and 21st days of differentiation cultures*

Tablo 2. *Farklılaşma kültürünün 14. ve 21. günlerinde MAP_2, βIII-Tubulin, GFAP ve O4 antikorlarının immünişaretleme yoğunluğu*

Days	Antibody Immunolabelling Intensity[a]			
	MAP-2	βIII-Tubulin	GFAP	O4
14	+++	+++	+	+
21	+++	+++	+	+

[a] *Immunolabelling intensity was graded on the following scale: negative (-), mild (+), moderate (++), and strong (+++)*

On the 14th and 21st days of culture, the neuronal cell markers MAP-2 *(Fig. 6A,B)* and ßIII-Tubulin *(Fig. 6C,D)* immuno-reactivites were strongly positive, whereas the neuroglial cell markers GFAP *(Fig. 6E,F)* and O4 *(Fig. 6G,H)* immuno-reactivities were weakly positive. MAP-2 and βIII-Tubulin immunoreactivites were statistically different when compared to the GFAP and O4 immunoreactivites in 14th and 21st days (P<0.001). This supported the fact that cells differentiated into neuronal cells rather than neuroglial cells.

Intensities of SSEA-1 and Nestin immunoreactivity at the 7th and 14th days of differentiation cultures are shown in *Table 1*. Intensities of MAP-2, βIII-Tubulin, GFAP and O4 immunoreactivity at the 14th and 21st days of differentiation cultures are shown in *Table 2*.

DISCUSSION

The nervous system is controlled by a series of epi-genetic and cellular endogeneous signals during embryo-genesis and early postnatal life, and differentiates from the neural tube derived from the configuration of the neuro-ectoderm [31]. All the central nervous system elements (neurons, glial cells, ependymal cells, etc.) are inside this structure. The structural elements of the nervous tissue are completed by the formation of certain cells and structures by the crista neuralis, which is derived from the neuroectoderm. The neurons and neuroglia cells that were derived from their precursors during neurogenic

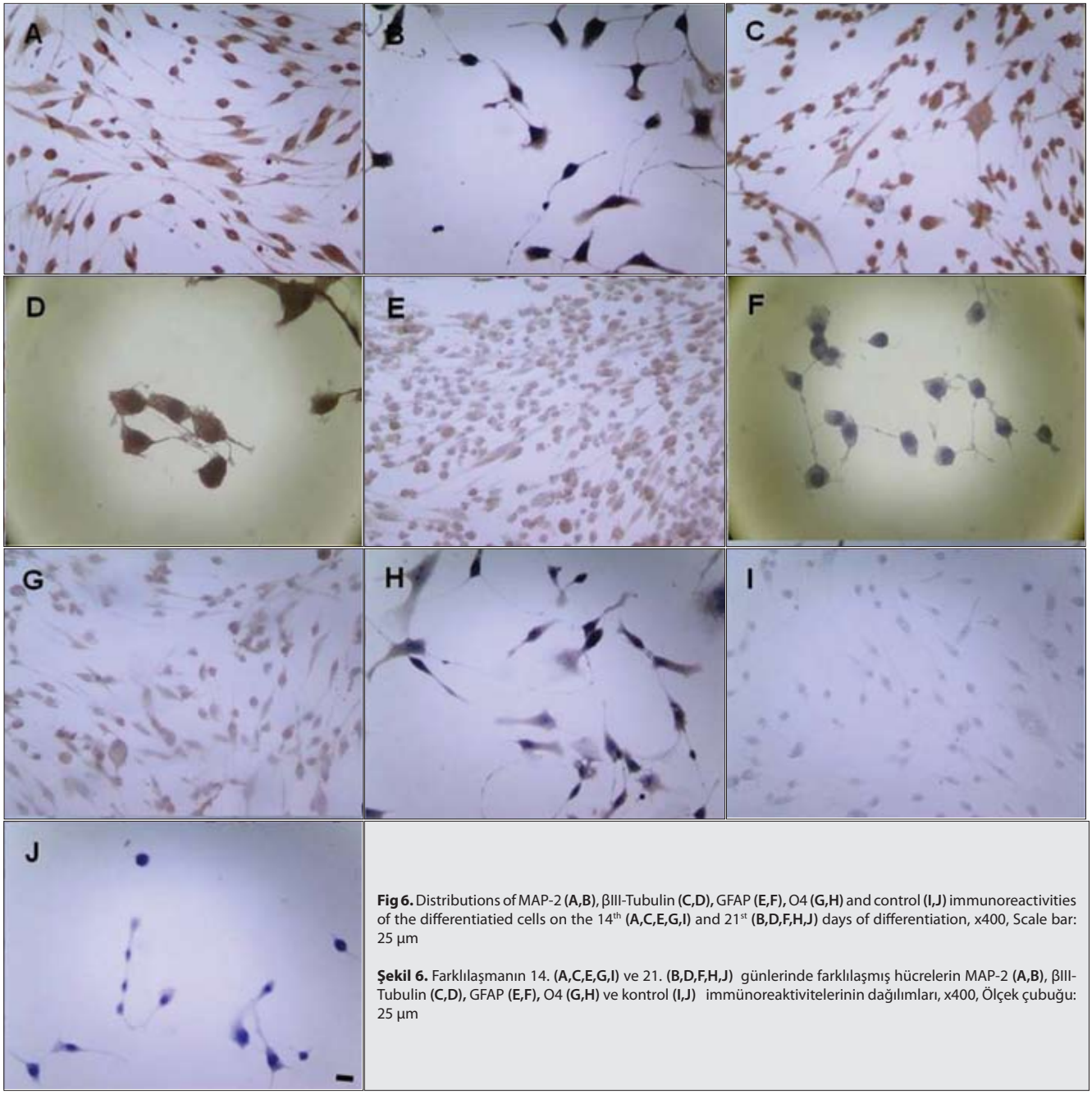


Fig 6. Distributions of MAP-2 **(A,B)**, βIII-Tubulin **(C,D),** GFAP **(E,F),** O4 **(G,H)** and control **(I,J)** immunoreactivities of the differentiatied cells on the 14th **(A,C,E,G,I)** and 21st **(B,D,F,H,J)** days of differentiation, x400, Scale bar: 25 µm

Şekil 6. Farklılaşmanın 14. **(A,C,E,G,I)** ve 21. **(B,D,F,H,J)** günlerinde farklılaşmış hücrelerin MAP-2 **(A,B)**, βIII-Tubulin **(C,D),** GFAP **(E,F),** O4 **(G,H)** ve kontrol **(I,J)** immünoreaktivitelerinin dağılımları, x400, Ölçek çubuğu: 25 µm

development complete their differentiation in the embryonic period. The extreme differentiation of the nerve cells makes them the ultimate controller of all the other cells in the organism. The nerve cells are ultimately developed during the embryogenic development. This situation eliminates their regeneration and proliferation ability in adult nerve cells. This is why the degenerative disorders of the nervous system cannot be amended [32].

Recently, many studies have aimed to clarify the mechanisms that will enable the replacement of the damaged tissues and perform nervous transmission. Thus, nervous cells that may be differentiated both from embryonic or extraembryonic derived stem cells are of great importance.

One of the most important factors determining the development and differentiation of stem cells during the embryonic development is the environment. The cell is differentiated depending on its environment and the signal molecules it is exposed to. The signal molecules build up the micro-environment of the cell [10]. Depending on the settlement within the micro-environment of the cell and certain intrinsic factors, stem cells may enlarge the cellular pool with divisions, turn into different cell lines and progress into programmed cell death [33]. Multiple different protocols exist for achieving neural induction and differentiation of ES cells. For example, the pluripotent cells can differ depending on the altering cell culture condition such as culturing on matrigel, mouse embryonic

fibroblast, and fibronectin coated plates. In addition, neural induction methods from embryonic stem cells also vary which can include embryoid bodies, adherent monolayers, and rosette formation of ES cells. In our condition, we used fibronectin as an extracellular matrix component, bFGF as an inductive factor and N2 as a supportive medium to neuronal lineage differentiation.

Neural differentiation was first experienced in embryonal carsinoma cells. Following the retinoic acid in vitro exposure of P19 (EC cell line) and embryo derived pluripotential stem cell line, they differentiated into neural cells [10,11,34,35]. Different studies have utilized other signal molecules such as βNGF, FGF, Wnt, TGF-β and N2 with different protocols in order to promote neural differentiation [14,36,37].

In our study, a micro-environment was created by mouse fibroblast cells as a feeder layer for mouse embryonic stem cells. The physical conditions of the cells were changed using N2 and bFGF, and their differentiation into neuronal cells and proliferation were promoted. The N2 medium provides the growth of neural stem cells in vitro, and contains insulin, transferrin, sodium selenite, putrescine, and progesterone. bFGF is a mitogen agent that is used in order to promote the proliferation of the neural precursor cells [38-43]. The media supplemented with N2 and bFGF induced embriyonic stem cells to differentiate into more complex neuron-like cells.

At the beginning of the study, the maintenance of the pluripotent properties of the ES cells was provided using nutritive cell layers (STO fibroblast cells) and LIF. After culturing EB cells derived from ES cells in the absence of LIF in culture dishes until the fourth day of the experiment, N2 was added into the ESC differentiation culture medium. The positivity of SSEA-1 and nestin immunoreactivity in the culture at the end of the seventh day showed that these were embryonic stem cells and precursor neuro-genic cells. The lower immunoreactivity of SSEA-1 on the 7th day showed that the embryonic stem cells had begun to differentiate. The positive immunoreactivity of nestin on the7th day indicated that the cells had begun to differentiate into neuron precursor cells in accordance with the decrease in the SSEA-1 immunoreactivity. This result suggested that this culture condition lead to the differentiation of mouse embriyonic stem cells into neuro-genic progenitor cells.

The results of immunohistochemical analyses after 14 days of culture time, the strong positivity of βIII-Tubulin and MAP-2 immunoreactivities, which were neuronal cell indicators, and the weak positivity of O4 and GFAP immuno-reactivities, which were oligodentrocytes and astrocytes markers, were observed respectively. The data suggested that the cells had begun to differentiate into neurogenic (neurons, oligodendrocytes, astrocytes etc.) cells from the precursor cells. The increased immunoreactivities of βIII-Tubulin and MAP-2 compared to those of O4 and GFAP on the 21st day suggested that the differentiation protocols provided a particular differentiation into neuronal cells.

The protocol used in our study induced the differentiation of a higher number of embryonic stem cells into neurons, while providing a lower potential of differentiation into neuroglial cells. Therefore, it may be concluded that the protocol used is appropriate for neuronal differentiation, and that a different mediator should be added into the culture medium in order to promote the activation of astrocyte and/or oligodendrocyte differentiation. Barres et al.[44] have demonstrated that retrieval of FGF from the medium after the 7th day of culture and continuation of the culture with T3 addition resulted in a higher oligo-dendrocyte differentiation from embryonic stem cells.

In the study of Okabe et al.[15], ES cells were increased in number with the addition of N2 and FGF factors into the medium and they differentiated into neuronal precursor cells, which were shown by the detection of nestin, MAP-2, GFAP and O4 positive cells. These results were similar to the results in our study. However, following the retrieval of FGF from the medium after differentiation, the number of nestin-positive cells in the culture decreased, MAP-2 positive cells increased and GFAP and O4-positive cells were visualized.

In the study of Li et al.[45], retinoic acid was used for the neural line specification of the embryonic stem cells. Neural progenitor cells were formed on the 7th day of the culture, neuronal cells were formed on the 10th day and neuronal line specification and functional neuron formation were completed by the 17th day, similar to our study. Sox1, Sox 2 and ß Galactosidase indicators were used as neuroepithelial markers in this study in addition to nestin, Pax3, 6 and Islet expressions which were examined as neural markers. Although there are many studies on the use of embryonic stem cells for the differentiation of neuronal cells, it has been demonstrated that mesenchymal stem cells and multipotent adult progenitor cells are also shown to form neurons, oligodendrocytes and astrocytes in humans, mice and rats *in vitro* [8,46-51].

In conclusion, using the protocols that use the differentiation potentials of embryonic stem cells and considering the cell differentiation criteria of ectodermal serial cells, the cells were demonstrated to start differentiating on the 7th day and this continued until the 21st day. It was also observed that, the neuron-like cell differentiation was to a higher extent in particular. It is necessary to analyze the *in vivo* usability and functionality of the cells that are derived in vitro. This study is important in constituting a study protocol for the analysis of the usability of cells derived from embryonic stem cell differentiation treating nerve cell injuries and degenerative disorders of the nervous system.

REFERENCES

1. Compagnucci C, Nizzardo M, Corti S, Zanni G, Bertini E: *In vitro* neurogenesis: Development and functional implications of iPSC technology. *Cell Mol Life Sc,* 20, 1623-1639, 2013.

2. Kanno H: Regenerative therapy for neuronal diseases with transplantation of somatic stem cells. *World J Stem Cells*, 5 (4): 163-171, 2013.

3. Sykova E, Forostyak S: Stem cells in regenerative medicine. *Laser Ther,* 22 (2): 87-92, 2013.

4. MJ Evans, MH Kaufman: Establishment in culture of pluripotential cells from mouse embryos. *Nature,* 292, 154-156, 1981.

5. HJ Rippon AE: Bishop embryonic stem cells. *Cell Prolif*, 37, 23-34, 2004.

6. KS O'Shea: Self-renewal vs. differentiation of mouse embryonic stem cells. *Biol Reprod*, 71, 1755-1765, 2004.

7. Wobus AM, Boheler KR: Embryonic stem cells: Prospects for developmental biology and cell therapy. *Physiol Rev,* 85, 635-678, 2005.

8. Sato M, Nakano T: Embryonic stem cell. *Intern Med,* 40 (3): 195-200, 2001.

9. Doss MX, Koehler CI, Gissel C, Hescheler J, Sachinidis A: Embryonic stem cells: A promising tool for cell replacement therapy. *J Cell Mol Med*, 8, 465-473, 2004.

10. Bain G, Kitchens D, Yao M, James E. Huettner JE, Gottlieb DI: Embryonic stem cells express neuronal properties *in vitro. Dev Biol,* 168 (2): 342-357, 1995.

11. Fraichard O, Chassande G, Bilbaut C, Dehay P, Savatier P, Samarut J: *In vitro* differentiation of embryonic stem cells into glial cells and functional neurons. *J Cell Sci,* 108 (3): 181-188, 1995.

12. Abranches E, Bekman E, Henrique D: Expansion and neural differentiation of embryonic stem cells in adherent and suspension cultures. *Biotechnol Lett,* 25 (9): 725-730, 2003.

13. Abranches E, Silva M, Pradier L, Schulz H, Hummel O, Henrique D, Bekman E: Neural differentiation of embryonic stem cells *in vitro*: A road map to neurogenesis in the embryo. *PLoS One,* 4 (7): e6286, 2009.

14. Schuldiner M, Eiges R, Eden A, Yanuka O, Itskovitz-Eldor J, Goldstein RS, Benvenisty N: Induced neuronal differentiation of human embryonic stem cells. *Brain Res,* 913 (2): 201-205, 2001.

15. Okabe S, Forsberg-Nilsson K, Spiro AC, Segal M, McKay RD: Development of neuronal precursor cells and functional postmitotic neurons from embryonic stem cells *in vitro*. *Mech Dev,* 59 (1): 89-102, 1996.

16. Liour SS, Kraemer SA, Dinkins MB, Su CY, Yangisawa M, Yu RK: Further characterization of embryonic stem cell-derived radial glial cells. *Glia*, 53 (1): 43-56, 2006.

17. Kang SM, Cho MS, Seo H, Yoon CJ, Oh SK, Choi YM, Kim DW: Efficient induction of oligodendrocytes from human embryonic stem cells. *Stem Cells*, 25 (2): 419-424, 2007.

18. Henderson JK, Draper JS, Baillie HS, Fishel S, Thomson JA, Moore H, Andrews PW: Preimplantation human embryos and embryonic stem cells show comparable expression of stage-specific embryonic antigens. *Stem Cells*, 20 (4): 329-337, 2002.

19. Guérette D, Khan PA, Savard PE, Vincent M: Molecular evolution of type VI intermediate filament proteins. *BMC Evol Biol*, 13 (7): 164-174, 2007.

20. Michalczyk K, Ziman M: Nestin structure and predicted function in cellular cytoskeletal organisation. *Histol Histopathol,* 20 (2): 665-671, 2005.

21. Neve RL, Harris P, Kosik KS, Kurnit DM, Donlon TA: Identification of cDNA clones for the human microtubule-associated protein tau and chromosomal localization of the genes for tau and microtubule-associated protein 2. *Brain Res,* 387 (3): 271-280, 1987.

22. Kalcheva N, Albala J, O'Guin K, Rubino H, Garner C, Shafit Zagardo B: Genomic structure of human microtubule-associated protein 2 (MAP-2) and characterization of additional MAP-2 isoforms. *Proc Natl Acad Sci U S A*, 92 (24): 10894-10898, 1995.

23. Katsetos CD, Herman MM, Mörk SJ: Class III beta-tubulin in human development and cancer. *Cell Motil Cytoskeleton,* 55(2):77-96, 2003.

24. Katsetos CD, Legido A, Perentes E, Mörk SJ: Class III beta-tubulin isotype: A key cytoskeletal protein at the crossroads of developmental neurobiology and tumor neuropathology. *J Child Neurol*, 18 (12): 851-866, 2003.

25. Fuchs E, Weber K: Intermediate filaments: structure, dynamics, function, and disease. *Annu Rev Biochem*, 63, 345-382, 1994.

26. Bongcam-Rudloff E, Nister M, Betsholtz C, Wang JL, Stenman G, Huebner K, Croce CM, Westermark B: Human glial fibrillary acidic protein: Complementary DNA cloning, chromosome localization, and messenger RNA expression in human glioma cell lines of various pheno-types. *Cancer Res*, 51 (5): 1553-1560, 1991.

27. Reeves SA, Helman LJ, Allison A, Israel MA: Molecular cloning and primary structure of human glial fibrillary acidic protein. *Proc Natl Acad Sci USA*, 86 (13): 5178-5182, 1989.

28. Baumann N, Pham-Dinh D: Biology of oligodendrocyte and myelin in the mammalian central nervous system. *Physiol Rev*, 18 (2): 871-927, 2001.

29. Vatansever HS, Turkoz Uluer E, Aydede H, Ozbilgin MK: Analysis of transferred keratinocyte-like cells derived from mouse embryonic stem cells on experimental surgical skin wounds of mouse. *Acta Histochem,* 115, 32-41, 2013.

30. Ergun G, Aktas S: Comparisons of sum of squares methods in ANOVA models. *Kafkas Univ Vet Fak Derg,* 15 (3): 481-484, 2009.

31. MB Luskin: Neuronal cell lineage in the vertebrate central nervous system. *The FASEB J,* 8, 722-730, 1994.

32. McKay RDG: The origins of cellular diversity in the mammalian central nervous system *Cell,* 58, 815-821, 1989.

33. Reeves SA, Helman LJ, Allison A, Israel MA: Molecular cloning and primary structure of human glial fibrillary acidic protein. *Proc Natl Acad Sci USA*, 86 (13): 5178-5182, 1989.

34. Guan K, Chang H, Rolletschek A, Wobus AM: Embryonic stem cell derived neurogenesis, retinoic acid induction and lineage selection of neuronal cells. *Cell Tissues Res,* 305, 171-176, 2001.

35. Strübing C, Ahnert-Hilger G, Shan J, Wiedenmann B, Hescheler J, Wobus AM: Differentiation of pluripotent embryonic stem cells into the neuronal lineage *in vitro* gives rise to mature inhibitory and excitatory neurons. *Mech Dev*, 53 (2): 275-287, 1995.

36. Calhoun JD, Lambert NA, Mitalipova MM, Noggle SA, Lyons I, Condie BG, Stice SL: Differentiation of rhesus embryonic stem cells to neural progenitors and neurons. *Biochem Biophys Res Com*, 306, 191-197, 2003.

37. Schulz TC, Palmarini GM, Noggle SA, Weiler DA, Mitolipova M, Condie BG: Directed neuronal differentiation of human embryonic stem cells. *BMC Neurosci*, 4, 27-40, 2003.

38. Sommera L, Raob M: Neural stem cells and regulation of cell number. *Neurobiol,* 66, 1-18, 2002.

39. Johe KK, Hazel TG, Müller T: Single factors direct the differentiation of stem cells from the fetal and adult central nervous system. *Genes Dev*, 10, 3129-3140, 1996.

40. Chen CW, Liu CS, Chiu IM, Shen SC, Pan HC, Lee KH, Lin SZ, Su HL: The signals of FGFs on the neurogenesis of embryonic stem cells. *J Biomed Sci,* 17 (1): 33-44, 2010.

41. John D, Calhoun JD, Lambert NA, Mitalipova MM, Noggle SA, Lyons I, Condie BG, Stice SL: Differentiation of rhesus embryonic stem cells to neural progenitors and neurons. *Biochem Biophys Res Com,* 306 (1): 191-197, 2003.

42. Schuldiner M, Yanuka O, Itskovitz-Eldor J, Melton DA, Benvenisty N: Effects of eight growth factors on the differentiation of cells derived from human embryonic stem cells. *Proc Natl Acad Sci U SA,* 97 (21): 11307-11312, 2000

43. Rolletschek A, Chang H, Guan K, Czyz J, Meyer M, Wobus AM: Differentiation of embryonic stem cell-derived dopaminergic neurons is enhanced by survival-promoting factors. *Mech Dev,* 105 (1-2): 93-104, 2001.

44. Barres BA, Lazar MA, Raff MC: A novel role for thyroid hormone, glucocorticoids and retinoic acid in timing oligodendrocyte development. *Development*, 120 (5): 1097-1098, 1994.

45. Li M, Pevny L, Lovell-Badge R, Smith A: Generation of purified neural precursors from embryonic stem cells by lineage selection. *Curr Biol,* 8 (17): 971-974, 1998.

46. Li H, Liu H, Corrales CE, Risner JR, Forrester J, Holt JR, Heller S, Edge AS: Differentiation of neurons from neural precursors generated in floating spheres from embryonic stem cells. *BMC Neurosci,* 24 (10): 122-134, 2009.

47. Cai C, Grabel L: Directing the differentiation of embryonic stem cells to neural stem cells. *Dev Dyn*, 236 (12): 3255-3266, 2007.

48. Abranches E, Silva M, Pradier L, Schulz H, Hummel O, Henrique D, Bekman E: Neural differentiation of embryonic stem cells *in vitro:* A road map to neurogenesis in the embryo. *PLoS One,* 4 (7): e6286, 2009.

49. O'Shea KS: Neuronal differentiation of mouse embryonic stem cells: Lineage selection and forced differentiation paradigms. *Blood Cells Mol Dis*, 2 (3): 705-712, 2001.

50. Strübing C, Ahnert-Hilger G, Shan J, Wiedenmann B, Hescheler J, Wobus AM: Differentiation of pluripotent embryonic stem cells into the neuronal lineage *in vitro* gives rise to mature inhibitory and excitatory neurons. *Mech Dev,* 53 (2): 275-287, 1995.

51. Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Kene CD, Ortiz-Gonzalez XR, Reyes M, Lenvik T, Lund T: Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature*, 418, 41-49, 2008.