Original Article

Effect of Astrocyte-Conditioned Medium, Retinoic Acid and Basic Fibroblast Growth Factor on Neural Differentiation of Mouse Embryonic Stem Cells

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Abstract

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Objective: Embryonic stem cells (ESCs) are pluripotent cells capable of extensive proliferation while maintaining their potential to differentiate into any cell type. The therapeutic potential of these cells are promising, but in many cases limited by our inability to promote their differentiation. In this study we examined the effect of inducer factors in direct differentiation of mouse ESCs into a neural fate in suspension culture systems.

Materials and Methods: Mouse ESCs (Royan B1) were cultivated in suspension to form embryoid bodies (EBs) within 2 days (2d). They were induced using astrocyte-conditioned medium (ACM), retinoic acid (RA, 1μM) and basic fibroblast growth factor (bFGF, 20 ng/ml) for 4 days (2+4d) and were cultured on poly-L-lysine coated dishes up to 5 days (2+4+5d) to differentiate. The expressions of neural specific genes were analyzed by immunocytochemistry and reverse transcription-polymerase chain reaction (RT-PCR). Differences of means for percentage significance of differentiated EBs were tested by the Mann-Whitney Test and for length and thickness of neural processes by t-test.

Results: Retinoic acid increased the percentage of EBs outgrowth with neural morphology and bFGF had synergistic effect (at least $p<0.05$), while ACM did not influence neural differentiation at all. Immunofluorescence analysis revealed the expression of β-tubulin III in neural cells and that the differentiated neural cells in RA+bFGF group were longer and thicker processes (at least $p<0.05$). The cells expressed Nestin, Pax6, NF-M, Islet-1, Lim1, and HB9.

Conclusion: The results showed that ACM had no effect on neural differentiation from ESCs, however, RA was in favor of this and that bFGF increased neural differentiation synergistically by mechanisms that remain to be defined.

Keywords: Mouse Embryonic Stem Cells, Astrocyte-Conditioned Medium, Retinoic Acid, Basic Fibroblast Growth Factor, Neural Differentiation

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Introduction

Mouse embryonic stem cells (mESCs) are pluripotent cells which are derived frequently from the inner cell mass of developing blastocysts (1, 2). The ESCs have high proliferation capacity which makes them an attractive source of different cell types for biomedical research and cell replacement therapies. Most in vitro studies on differentiation of ESCs into neural cells begin with a culture suspension of embryoid bodies (EBs), floating on serum-containing medium (3, 4). The EBs, which are formed from dissociated ESCs, resemble early post-implantation embryos in that they consist of ectodermal, mesodermal, and endodermal cells (5). The differentiation of ESCs can be stimulated by removing feeder cells and differentiation-inhibiting cytokines, or by adding specific differentiation inducing substances. In this regard, neurons can

be obtained from the ectodemal cells by retinoic acid (RA) induction (6), by growth factor-mediated lineage selection (7, 8), or by astrocyte-derived factors (9). However, these developmental processes are orchestrated by interactions among prospective precursors and morphogenes, such as basic fibroblast growth factor (bFGF), RA, and astrocyte-derived factors, secreted from surrounding tissues in unique spatial and temporal orders. On the basis of these developmental principles, the derivation of neuronal cells from ESCs *in vitro* provides an invaluable assay both for the genetic dissection of neuronal cell development and for epigenetic reprogramming. Considering the fact that the ability to induce specific and directed cell differentiation in vitro, may become a useful mechanism for deriving an unlimited source of pure population cells suitable for transplantation or for the treatment of several neuronal diseases, in the present study we attempted to induce neural differentiation of our mouse ESCs by treatment of EBs with bFGF, RA, and astrocyte-conditioned medium (ACM) and their combination and then cultivating them on an adhesive substrate.

Materials and Methods

Culture of undifferentiated ESCs

The study was carried out in the department of stem cells, Royan institute, Tehran, Iran during the year 2005. The mouse ESC (mESC) line Royan B1 (10) derived from C57BL/6 mouse strain was used throughout the present study. ESCs were kept in an undifferentiated, pluripotent state using mitomycin C (Sigma, M0503)-inactivated feeder layer of primary cultures of mouse embryonic fibroblasts. They were cultivated on gelatin (0.1%, Sigma, G2500) coated plastic flasks (Falcon) in ESC medium containing of Dulbecco's modified Eagle's medium (DMEM, Gibco, 10829-018) supplemented with 15 % fetal calf serum (Gibco, 16141-079), 0.1 mM beta-mercaptoethanol (Sigma, M7522), 2 mM glutamine (Gibco, 15039-027), 0.1 mM non-essential amino acids (Sigma, M7145) and 1000 iu/ml leukemia inhibitory factor (LIF, Chemicon, ESGRO, ESG1107).

Differentiation procedure

In order to promote directed differentiation of mESC into neural and cardiac fate, the procedure was performed according to a previously published protocol, as outlined below, in 11 groups (Fig 1) (9). (i) Formation of EBs (stage 1, 2 days) by culturing 1x 10^5 cell/ml in 3 ml medium in non-adhesive bacterial dish supplemented with 15% FBS. (ii) Induction of differentiation using bFGF (20 ng/ml, Sigma, F0291) in the presence or absence of heparin to increase the bFGF effect, RA (1 µM, Sigma, R2625), and ACM (see bellow) and cultured for 4 days (stage 2, day 2+4) supplemented with 10% FBS according to figure 1. (iii) Cultivating EBs onto poly-L-lysine-coated dishes supplemented with 5% FBS for 5 days for further differentiation of precursor cells into mature neurons (stage 3, day 2+4+5). Note that in stage (ii) EBs of group 8 were first induced by RA within 2 days (2+2d) and then induced for another 2 days (2+4d) by bFGF. Conversely, in group 9 first EBs were induced by bFGF within 2days (2+2d) and then induced for another 2 days (2+4d) by RA.

Astrocyte preparation

Cell suspensions of mouse fetal hippocampus (NMRI, Razi institute, day 17-18) were prepared by mechanical dissociation in phosphate-buffered saline (PBS). Cells were plated onto flask plastic surfaces at a cell density of 2.5–3×10^4 cell/cm^2. The cultures were grown in DMEM/F12 medium supplemented with, 15% FCS, 2mM glutamine, and penicillin (100 U/ml)/ streptomycin (100 µg/ml) in humidified air atmosphere containing 5% CO2, at 37°C for two weeks. The culture medium was changed twice a week. The primary cultures were trypsinized weekly and replated onto flask, according to the experimental design. To enrich asterocytes, the differential adhesion and replating were used to isolate them from neuronal cells. Moreover, asterocytes were isolated from oligodendrocytes due to short-time trypsinization (2-3 min). After

immunocytochemical staining, positive cells were counted blindly in 10–15 random fields of view and the percentage of positive cells was calculated. All assays were performed in triplicate.

Astrocyte conditioned medium preparation

Astroglial conditioned medium (24 h) was collected from astroglial monolayers grown in 25 cm^2 flask in DMEM/F12 medium supplemented with 1% N2 and 2 mM glutamine when the cells reach about 60-70% confluency. In group 10, ACM was prepared in presence of RA (1μM) (RA.ACM). The ACM and RA.ACM (1v:1v) was supplemented with 1/2 fresh culture medium and transferred immediately onto EBs.

Immunocytochemical staining

Differentiated neural Cells at day 2+4+5 were washed twice with PBS and fixed with 4% Paraformaldehyde for 24h at 4˚C. The cells were permeabilized and blocked in PBS containing 0.2% Triton X-100 and 10% goat serum for 10 min and 30 min, respectively and thereafter were incubated in primary antibody diluted in 0.5% bovine serum albumin (BSA) at 37˚C for 1h.

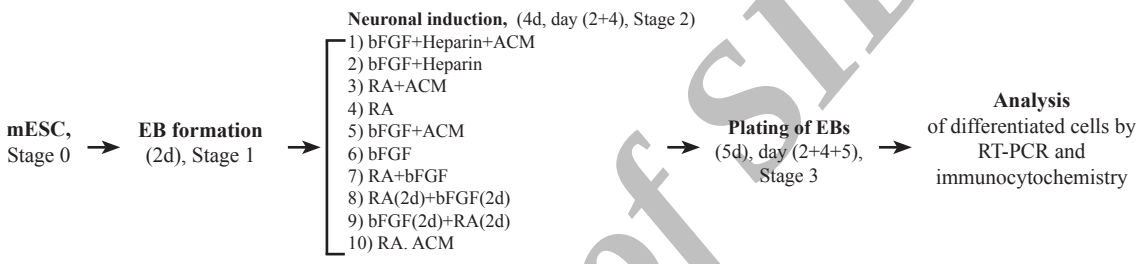

Fig 1: The protocol of mESCs neural induction with different treatments. The protocol includes: (i) formation of EBs (stage 1, 2 days) by culturing 1x 105 cell/ml in 3 ml medium in non-adhesive bacterial dish supplemented with 15% FBS ; (ii) induction of differentiation using bFGF in presence or absence of heparin to increase the bFGF effect, RA, and ACM for 4 days (stage 2, day 2+4) with 10% FBS. Note that in stage (ii) EBs of group 8 were first induced by RA within 2 days (2+2d) and then induced for another 2 days (2+4d) by bFGF. Conversely, in group 9 first EBs were induced by bFGF within 2days (2+2d) and then induced for another 2 days (2+4d) by RA. (iii) cultivating EBs onto poly-L-lysine-coated dishes with 5% FBS for 5 days for further differentiation of precursor cells into mature neurons (stage 3, day 2+4+5).

Table 1: Primers and the reaction conditions of reverse transcription-polymerase chain reaction of differentiated cells

References	Annealing Temperature	Size (bp)	Primer sequences (5'3-')	Genes
NM-016701	54	351	F:TCGAGCAGGAAGTGGTAGG R:TTGGGACCAGGGACTGTT A	Nestin
NM-013627	59	494	F:GAAATCCGAGACAGATTATTATCCGAG R:CCATTTGGCCCTTCGATTAGA	Pax6
NM-008691	62	74	F:GCACTACTTGGAACAACAGAACA R:CGTGCGAGGCCTGCTT	NF-M
NM-021459	64	259	F:TATCCAGGGGATGACAGGAAC R:GCTGTTGGGTGTATCTGGGAG	Islet1
NM-008498	65	254	F:CAAGGAGCGAAGGATGAAACAG R:CAGATGATGGCACAAAGGGTAG	Lim1
NM-019944	64	456	F:CAAGGAGCGAAGGATGAAACAG R:CAGATGATGGCACAAAGGGTAG	HB9
NM-009309	63	432	F:AGTATGAACCTCGGATTCACATCG R:GCAGATGAATTGTCCGCATAGG	Brachyury
NM-008092	60	413	F:CTCTATCACAAGATGAACGGCA R:CTGCTGTGCCCATAGTGAGA	GATA4
NM-013633	71	317	F:GGCGTTCTCTTTGGAAAGGTGTTC R:CTCGAACCACATCCTTCTCT	Oct-4
NM-011655	63	317	F:GGAACATAGCCGTAAACTGC R:TCACTGTGCCTGAACTTACC	b-tubulin

The antibodies used in this study were anti-β-tubulin III (1:250; sigma, T5293) and glial fibrillary acidic protein (GFAP) (1:50; chemicon, MAB3402). At the end of the incubation period, the cells were washed 2X with PBS+0.05% tween20 and incubated with the fluorescence isothiocyanate (FITC)-conjugated anti-mouse IgG (1:250; sigma, F9006) diluted in 0.5% BSA for 60 min at 37 ˚C. After washing twice with PBS+0.05% tween20, specimens were examined under fluorescence microscope (BX51, Olympus, Japan). The length and thickness of neural processes were measured by Olysia Bioreport software (Olympus, version: 5.1.2600.2180).

RNA extraction and RT-PCR analysis

Total RNA was extracted from the ES cells, EBs (2d), EBs (2+4d) and plated EBs (2+4+5d) using NucleoSpin® RNA II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. Then, the extracted RNA was treated by DNase I (Roche Diagnostic, Germany) to avoid cross contamination of RNA by genomic DNA and 0.5µg of RNA was reverse transcribed by random hexamer priming using Revert Aid™ First Strand cDNA Synthesis Kit (Fermentas, Germany) based on the protocol of the manufacture. The cDNA samples were subjected to PCR amplification with DNA primers specific to the mouse genes using a pair of oligomers, each from a different exon. Amplification conditions were as follows: initial denaturation at 93ºC followed by 35 cycles of denaturation at 93ºC, annealing at 54-71ºC (depending on the primers), extension at 72˚C and a final polymerization at 72ºC. PCR products were analyzed by gel electrophoresis on 1.5% agarose and stained with ethidium bromide, visualized and photographed on a UV transluminator (Uvidoc, UK). A description of primers and the sizes of final products are described in table 1.

Statistical and morphological analysis

For statistical analysis, nearly 100 EBs were observed in each experiment and three or more replicates were performed. Neural cells with more than 5% of the outgrowths were investigated. All values are expressed as mean ± SD. At day 2+4+5, differences of means for the percentage significance of the differentiated EBs into beating cardiomyocytes and neural cells were tested by the Mann-Whitney test, while student *t*-test (SPSS software) was used for measuring the length and thickness of neural processes. The significant differences between the treatments (p value) were defined as $p<0.05$.

Results

More than 95% of the isolated astrocytes were GFAP positive (Fig 2).

The influence of different supplements (bFGF, RA, and ACM) and their combinations on the percentage of EB outgrowths with neural morphology was evaluated (Fig 3).

ACM and bFGF, either individually or both, did not effect neural differentiation (groups 1, 2, 5, and 6). In contrast, RA favoured neuronal differentiation (at least $p<0.05$), whereas the addition of bFGF and ACM in the presence of RA did not enhance neuronal differentiation significantly (groups 3, 4, 7, 8, and 9). However, they influence neural differentiation in the control group (without treatment). Therefore, we compared the length and thickness of the processes of ESC-derived neurons in group 4 (only RA) and group 7 (RA+bFGF), the groups with high neural differentiation potential on day 4 (stage 2). ESC-derived neurons in group 7 have longer and thicker processes in comparison with group 4. Moreover, the RT-PCR analysis of differentiated cells showed the expression of nestin, Pax6, and motoneuron-specific elements (ISL1, Lim1 and HB9) (Fig 4). The NF-M was expressed at stage 3, when RA was added into the medium. Differentiated neurons expressed neuron-specific tubulin III, a marker of postmitotic neuron as shown by immunocytochemistry (Fig 2). Immunocytochemistry with anti-β-tubulin III showed that the processes in group 7 were longer (>2 folds) and thicker (>2 folds) than group 4 (Fig 3 C, D).

In the absence of RA treatment, the majority of EBs flattened shortly after plating and

developed rhythmically pulsating cardiac muscle cells (Fig 3). The differentiation of ESCs to cardiomyocytes was independent of the neuronal differentiation elicited by FCS. Addition of bFGF increased the percentage of EBs with beating cardiomyocytes and heparin has a synergistic effect as well. In contrast, ACM treatment reduced the cardiomyocytes (group 2>6>11>5 and 1).

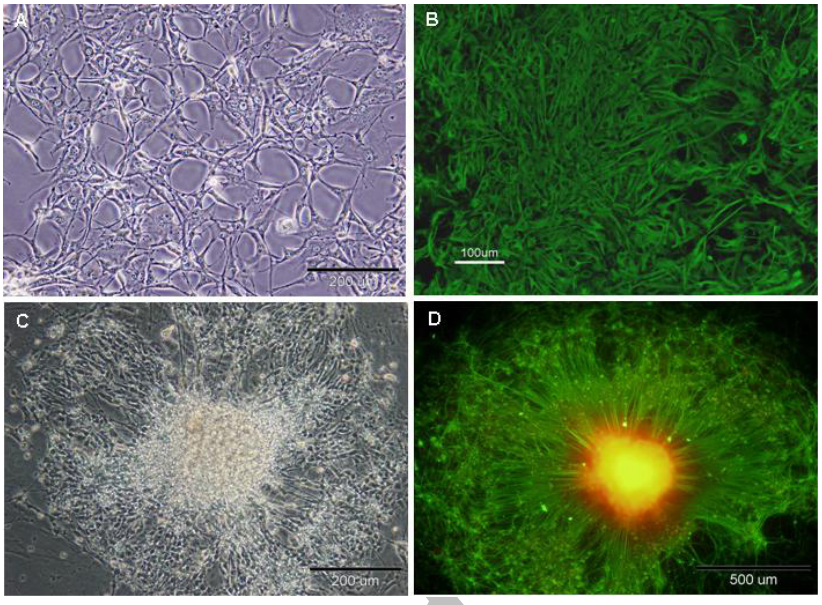

Fig 2: Isolated astrocytes and ESC-derived neual cells. Isolated astrocytes with phase contrast (A) and immunocytochemistry of isolated astrocytes with anti GFAP (B)..ESC-derived neural cells with phase microscopy (C) and immunocytochemistry with anti β-tubulinIII (D) after treatment with RA (group 4).

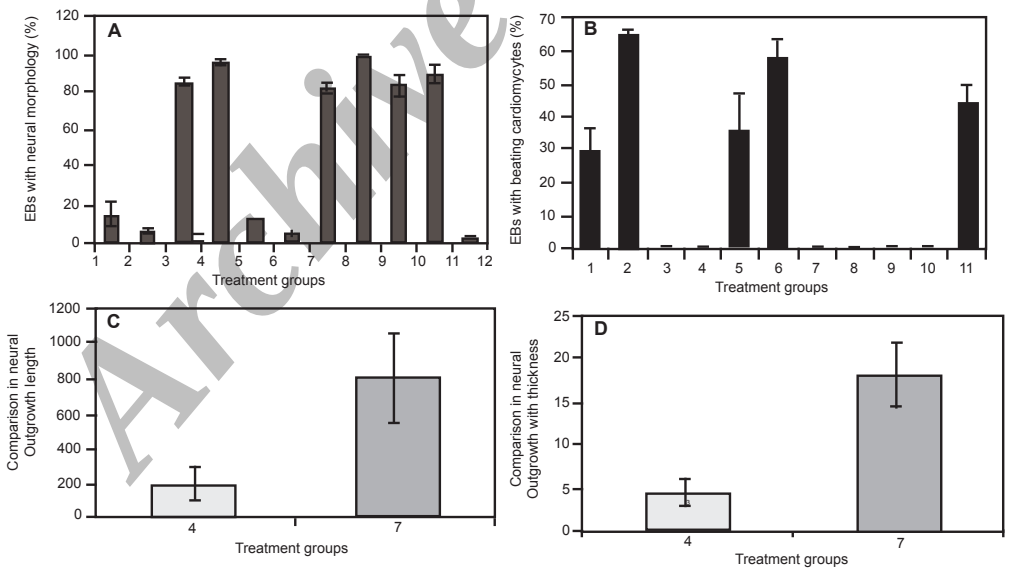

Fig 3: The percent of EB outgrowth with (A) neural and (B) beating cardiomyocyte morphology using different treatments. Change of neural length (C), and thickness (D) by signaling molecules RA alone (group 4) and RA+ bFGF (group 7) (p<0.05). (A) In the absence of RA treatment, the majority of EBs flattened shortly after plating and developed rhythmically pulsating cardiac muscle cells. Addition of bFGF increased the percent of EBs with beating cardiomyocytes and heparin has a synergistic effect as well. In contrast, ACM treatment reduced the cardiomyocytes (group 2>6>11>5 and 1). ACM and bFGF, either individually or both, did not effect neural differentiation (groups 1, 2, 5, and 6). In contrast, RA favored neuronal development in general (at least P<0.05), whereas the addition of bFGF and ACM in presence of RA did not enhance neuronal differentiation (groups 3, 4, 7, 8, and 9). Therefore, we compared the length and thickness of processes of ESC-derived neurons in group 4 (only RA) and group 7 (RA+bFGF), the groups with high neural differentiation potential on day 4 (stage 2). ESC-derived neurons in group 7 have longer and thicker processes in comparison group 4.

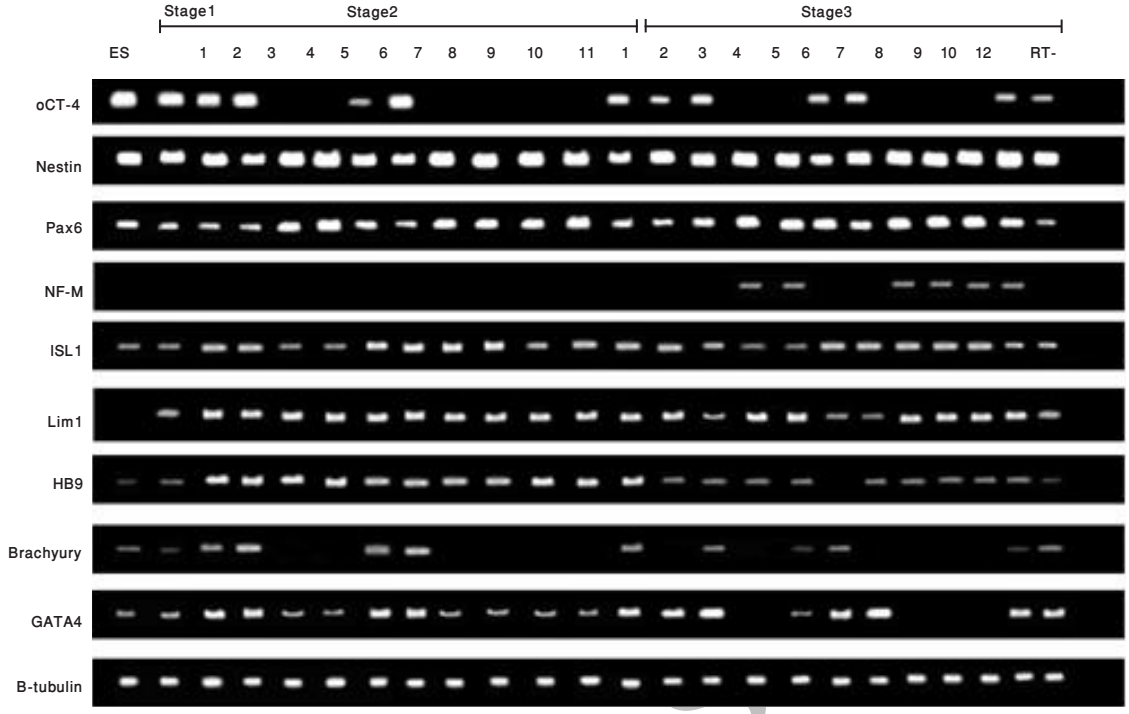

Fig 4: RT-PCR analysis of the ESC-derived cells in different stages of differentiation induction. RT- indicates no cDNA

Moreover, the mesodermal marker brachyury (11), and the early endodermal marker GATA4 (12) were detected in groups without RA treatment and even in presence of ACM at stage 3. Expression of Oct-4, a pluripotent cell marker, was detected in undifferentiated mESC but not in differentiated mESC at the final stage of differentiation (Fig 4).

Discussion

The data presented in this study provided evidence that ESCs were efficiently induced by 1 μM RA to generate neural cells and bFGF has increased this effect. On the other hand, the humoral soluble factors from astrocytes had been reported to have no influence on the differentiation of ESCs into neurons. (Kornyel et al, 2005). Moreover, the addition of RA during the induction phase prevents the differentiation to beating cardiac muscle cells. This result was surprising, since not only was RA implicated in various steps of cardiomyocyte development in the early embryo, but it had also been shown that RA treatment during ESC differentiation increased the number of cardiomyocytes in a time- and RA concentration-dependent manner (13-15). The formation of the neuronal cells was not induced with bFGF, a growth factor that is known to be effective for induction propagation of neural stem cells (16). However, bFGF with RA, but not with ACM, increased the percentage of EBs with neural morphology. Moreover, combination of bFGF and RA increased longer and thickener neural processes in comparison with RA alone.

Retinoic acid (vitamin A) is a morphogen responsible for development of the vertebrate anterior-posterior axis, patterning the central nervous system (CNS) and promoting neuronal differentiation (17). Also, it had been previously shown that bFGF could posteriorize anterior neuroectoderm, in vitro and many inferred an endogenous role in neural induction and patterning (18). It could be argued that FGF and RA act synergistically to promote posteriorization (19).

Our data demonstrate that differentiated neurons exhibited features characteristic of early postmitotic CNS neurons (NF-M and tubulin-III). Furthermore, we examined expression of the motoneuron genes to investigate the effect of RA and bFGF on motoneuron differentiation. Motoneurons generated in this study express Islet1, Lim1 and HB9 genes which are expressed

by cells in posterioventral regions of spinal cord.

Findings of this study revealed that ACM probably has not played an important role in direct differentiation of ESCs into neural cells and has only decreased their cardiac differentiation. However, astrocytes, which are cells of ectodermal origin, produce a variety of soluble and membrane-associated factors that influence many kinds of phenomena in the development and function of CNS (20-23). Astrocytes were shown to promote the survival and maturation of young neurons and neuronal precursors by many investigators (24-26). More recent reports have demonstrated that astrocytes induce neurogenesis both by adult neural (20, 27) and by ESCs in vitro (9). On the other hand, there are reports showing that astrocytes failed to instruct neuron formation by ESCs (28). Although these observations show that astroglial cells have the potential to instruct non committed stem cells to adopt a neuronal fate, little is known about the factors responsible for the instructive effect and its effect is controversial.

Conclusion

Detailed examination of neurons derived from ESCs will facilitate elucidation of molecular mechanisms that regulate survival and differentiation of neurons. These cells may provide a model system for the screening of factors involved in neuron dysregulation and pathology as well as providing the foundation for regeneration of diseased or traumatized tissue in patients.

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