Original Article

J Babol Univ Med Sci
Vol 19, Issu 10; Oct 2017. P:35-41

Genetic Changes during Differentiation of Spermatogonial Stem Cells into Oligoprogenitor Cells

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J Babol Univ Med Sci; 19(10); Oct 2017; PP: 35-41
Received: Jun 1th 2017, Revised: Sep 6th 2017, Accepted: Oct 3th 2017.

ABSTRACT

BACKGROUND AND OBJECTIVE: The culture of spermatogonial cells and the production of embryonic stem cells (ES-like cells), suggest these cells as a sufficient new source for cell therapy and for the treatment of diseases, including neurodegenerative diseases. The aim of this study is to evaluate the pattern of genetic changes during differentiation of spermatogonial cells into oligodendrocyte precursor cells.

METHODS: In this experimental study, spermatogonial cells were isolated from the testicles of 2 – 6 days old newborn mice (6 – 10 mice each time) through two stages of enzymatic digestion. The cells were divided into three groups of quasi-embryonic stem cells, neuro-progenitive and oligodendrocyte precursor. Specific markers stra8, mvh, piwil2, C-myc, Nanog, NF68, Nestin, Olig2, and NG2 were evaluated using Real Time-PCR and immunocytochemistry method at each differentiation step.

FINDINGS: Molecular evaluations showed that increase in Nestin gene expression in neuronal precursor cells was 1.3 times more than quasi-embryonic stem cells. In the molecular evaluations at the end of the second stage of differentiation, it was determined that culture at the end of the induction steps resulted in a significant increase in the expression of the genes of Olig2 and NG2 and decrease in the expression of Nestin gene ($p<0.05$). Molecular evaluations showed that this increase in oligodendrocyte-like cells was respectively 1.4 and 1.6 times more than neuronal precursor cells.

CONCLUSION: In this study, it was demonstrated that quasi-embryonic stem cells have the potential to express the NF68 and Nestin neuron markers. The quasi-embryonic stem cells of NG2 and Olig2 genes were expressed in the cells after the induction stage.

KEY WORDS: *Spermatogonia, Differentiation, Embryonic Stem Cells, Neurons, Oligodendrocytes.*

Please cite this article as follows:

Nazm Bojnordi M, Movahedin M, Tiraihi T. The Relationship between Tea and Coffee Consumption and Glioma: a systematic review. J Babol Univ Med Sci. 2017;19(10):35-41.

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Introduction

Spermatogonial stem cells are located on the membrane of the seminiferous tubes and transmit genetic information to the next generation (1). Although gametogenesis is the main role of these cells, recent findings show that other features are also relevant to these cells (2). Research results show that without the need for genetic change and after culturing spermatogonial cells under in vitro conditions, colonies of embryonic stem-like (ES-like) cells are created, which are similar to embryonic stem cells and have a self-healing ability and are differentiated into all three types of embryonic germ layer (ectoderm, mesoderm and endoderm) (3). The theory of the pluripotency of the spermatogonial stem cells was first introduced in 2004 after the culture of spermatogonial stem cell in a newborn mouse; the obtained ES-like cell colonies had common molecular properties with embryonic stem cells and were similar to these cells in terms of pluripotency (4–6).

The culture of spermatogonial cells and the production of pluripotent ES-like cells leads to increased expression of pluripotent genes such as SOX2, C-myc and Oct4 (7). Today, several cell treatment strategies are being studied using various cellular sources (9). Recent studies suggest spermatogonial stem cell as a sufficient source for cellular production for the treatment of many diseases and genetic lesions, due to abundant proliferation activity and pluripotency features (8 – 10). Spermatogonial cells are capable of producing ES-like cells that share molecular characteristics with embryonic stem cells and are similar to embryonic stem cells in terms of pluripotency, and can produce embryonic-like structures and differentiate into all types of embryonic germ layer (2, 5).

Unlike embryonic stem cells, the use of spermatogonial stem cells does not have ethical constraints (10). These spermatogonial properties allow these cells to be used for restorative therapies (3). In addition, spermatogonial stem cells can be a new and sufficient source of cell and disease therapy, including neurodegenerative diseases such as demyelination (2, 4). The production of glial cells is a key step in the treatment of neuronal demyelination. Various cellular sources have been introduced to improve myelin restoration (11, 12).

The pluripotency features of spermatogonial cells and their ability to differentiate into different cells, including oligodendrocytes, suggest the use of these cells to restore and rebuild myelin in damaged nerve fibers (13). For example, Shinohara et al. (2004) differentiated spermatogonial cells into oligodendrocytes and confirmed their ability to rebuild myelin (4). In 2008, Glaser et al. showed that oligodendrocyte cells derived from spermatogonial stem cells have the ability to repair and rebuild myelin (13).

Given the increasing rate of neurodegenerative diseases, such as neuronal demyelination, there is an urgent need to identify new sources of stem cells for restoration. In this regard, cell therapy is based on the use of a variety of stem cells that can be easily obtained and can also be differentiated into the culture medium under suitable inducers in the progenitor cells of the myelin. Considering the extensive proliferation activity and pluripotency features of spermatogonial cells and the ability to differentiate them into different cells, especially the neural category, in this study, we tried to use mouse spermatogonial cells as one of the newest sources of pluripotent stem cells in the production of oligodendrocyte precursor cells.

Since this process is differentiated with a wide range of genetic changes, the aim of this study is to evaluate the genetic variation during the process of differentiation of mouse spermatogonial cells into oligodendrocyte precursor cells.

Methods

Extraction of spermatogonial cells: This study was conducted on experimental animals after being approved by the ethics committee of School of Medical Sciences, Tarbiat Modares University. In this experimental study, spermatogonial cells of 2 – 6 days old newborn mice (6 – 10 mice each time) were used. Spermatogonial cells were isolated from the testicles of the mice trough two stages of enzymatic and mechanical digestion. In this stage, after isolation of the capsule, the testicles of 2–4– day–old infant NMRI mice were mechanically sliced and incubated in a culture medium containing the enzymes necessary for digestion of the enzyme for one hour at 37 ºC. After one hour, the medium containing cells and slices of seminiferous tubes were centrifuged at 1200 RPM for 5 minutes and the deposition medium of tube was replaced with Dulbecco's modified Eagle's medium (DMEM), eliminating the intermediate tissue from the slices of testicles (10). The result of the first stage of enzyme digestion at the end of this stage was the slices of seminiferous tubes that entered the second stage of

digestion for further enzymatic digestion. In order to remove the cells from the seminal tubes, the tubes were incubated for one hour at 37°C for the first stage of digestion. At the end of the incubation period, the suspension was centrifuged at a speed of 400 RPM for five minutes for separation of undigested slices. The undigested slices of the seminiferous tubes were deposited at the bottom of the tube, and single–cells and some cellular aggregations were placed at the top of the suspension. The cellular fluid above the sediment was passed through nylon filters. The resulting suspension mainly consisted of sertoli cells, spermatogonium, and a small number of intermediate tissue cells that were separated from each other at later stages (10).

Spermatogonial cells culture: Spermatogonial cells were cultured in DMEM medium (ES medium), and after examination of the cells under a microscope, if necessary, the culture medium was changed. Spermatogonial and Sertoli cells were cultured in a medium containing 15% DMEM, FBS (Fetal Bovine Serum), LIF (Leukemia Inhibitory Factor), 1% unnecessary amino acids, 1 mM beta– mercaptoethanol and 1% penicillin/streptomycin (5). The cells were passaged twice a week and in the fifth passage, equivalent to three weeks of culture, they were used for the formation of embryoid bodies (EBs).

Formation of embryoid bodies (EBs): To produce embryoid bodies, colonies resulted from cells were isolated as separate cells and cell suspension was prepared using trypsin. After a cell count, a total of 2×10^5 cells were transferred to each well of non-sticky 6 – well plates. Cells were cultured in DMEM medium containing 15% FBS serum. LIF was removed from the culture medium and the cells were incubated for one day at 37°C. After 24 hours, embryoid bodies were formed and ready for use (5).

Differentiation of embryonic-like stem cells into oligodendrocyte-like cells: A 2-step differentiation protocol was used to differentiate embryonic-like stem cells to oligodendrocyte-like cells (5).

First stage of differentiation: the induction of neural progenitor cells

The second stage of differentiation: the induction of oligodendrocyte-like cells

In the first step, retinoic acid was used to induce neural precursor cells. The cells were cultured in a DMEM medium containing one µM retinoic acid for four days and then cultured again in a retinoic acid-free DMEM medium for another four days. For induction of precursor oligodendrocyte, the neuronal precursor cells were cultured for 8–12 days in a Neurobasal medium containing 10 ng/ml EGF (Epidermal Growth Factor), and 20 ng/ml bFGF (basic Fibroblast Growth Factor).

Molecular analysis of differentiation to oligodendrocyte -like cells: At the end of the first stage of differentiation, the Nestin gene was examined for the identification of neural precursor cells. At the same time, changes in the expression of specific spermatogonial genes (stra8, mvh, and piwil2) and Nanog and C-My pluripotent markers were also studied. At the end of the second stage of differentiation, specific oligodendrocyte genes, including NG2 and Olig2 were studied along with specific spermatogonial genes (stra8, mvh, and piwil2), Nanog and C-Myc genes and Nestin gene.

Immunocytochemistry study of differentiation to oligodendrocyte-like cells: At the end of the first stage of differentiation, the ability to express the neuronal progenitor markers, NF68 and Nestin, and at the end of the second stage of differentiation, specific markers of oligodendrocytes including NG2 and Olig2 were examined by immunocytochemistry. In the immunocytochemistry study, the stages of the research were similar.

Statistical analysis: All values were provided in Mean±SEM. Data were analyzed by Student T-test, one-way ANOVA and TUKEY tests. The significance level was considered as $p < 0.05$.

Results

Evaluation of the embryonic stem-like cells differentiation into the phenotype of retinoic acid-induced neural precursor cells: The results of this study showed that spermatogonial cells were able to produce embryoid bodies in non-sticky plates in the absence of an embryonic germ layer (Fig 1a). At the end of the induction with retinoic acid, the embryonic stem-like cells take a neuron-like structure, so that their cellular bodies are elevated and have dendrites and axons (Fig1b). However, without retinoic acid, a small percentage of cells exhibited this cell phenotype as a result of self-differentiation; but the number of these cells increased significantly as a consequence of the inductive effect of retinoic acid.

Evaluation of the differentiation under the inductive effect of retinoic acid by immunocytochemistry: To evaluate the degree of differentiation after treatment with retinoic acid, an immunocytochemistry study was

performed for Nestin, and NF68 antibodies. The results of the study indicated the expression of the above markers in differentiated neuronal cells (Fig 2).

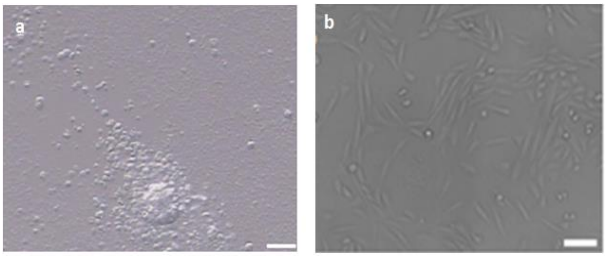

Figure 1. a: Reverse microscope image of embryoid bodies produced from culturing spermatogonial cell under in vitro conditions. b: Scale bar=50 µm. Phenotype of neural precursor cells at the end of retinoic acid induction stage. Scale bar = µm 100.

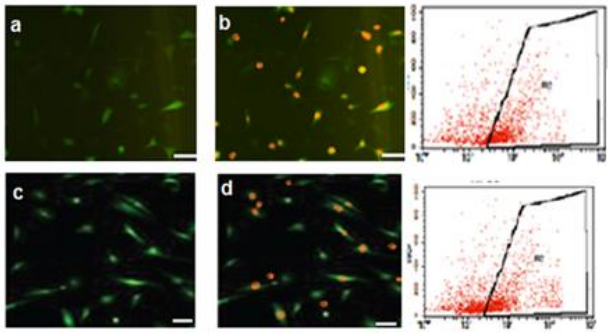

Figure 2. Immunocytochemistry staining of Nestin and NF68 in differentiated nerve cells after treatment with retinoic acid. Immunocytochemistry findings: The expression of Nestin (a) and NF68 (c) proteins in differentiated cells. Differentiated staining of the cells of the nucleus with ethidium bromide (b,d). Scale bar=100µm.

Evaluation of the degree of retinoic acid – induced differentiation using Real time-PCR: The quantitative analysis of gene expression by Real time PCR showed that treatment of cells with retinoic acid resulted in increased expression of Nestin gene and decreased expression of Nanog and C-Myc pluripotent genes in differentiated cells (a neural precursor). Molecular studies showed that the increase in Nestin gene expression in neural precursor cells was 1.3 times as much as embryo stem-like cells (Fig 3).

Immunocytochemistry study at the end of the second stage of differentiation: At the end of the second phase of the differentiation protocol, immunocytochemistry tests were performed to express the Olig2 and NG2 antibodies. The results indicated that the differentiated cells were immunopositive at the end of the induction stage compared to the olig2 and NG2 glial markers (Fig 4).

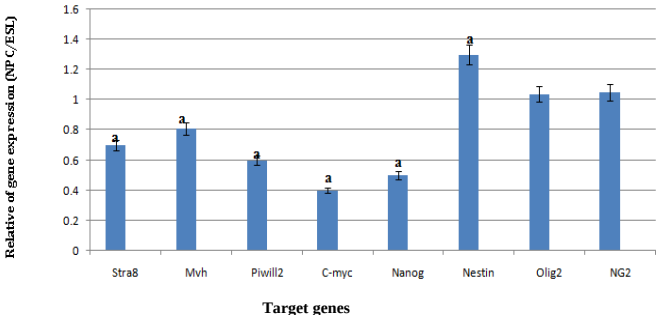

Figure 3. Changes in the expression of Nestin gene, Nanog and C-Myc pluripotent genes, and specific spermatogonial genes of piwil2, stra8, mvh in neural precursor cells compared to embryonic stem-like cells. Each column shows an average of 3 repetitions and $p \leq 0.05$ is considered as the significant difference level. The degree of expression of the above genes in embryonic stem-like cells is considered to be 1. a: Significant increase or decrease in neural precursor cells compared to embryonic stem cells

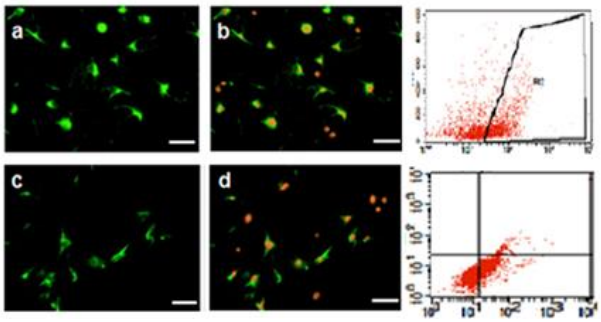

Figure 4. Immunocytochemistry staining of Olig2 and NG2 in differentiated glial cells at the second stage of differentiation. Immunocytochemistry findings: The expression of Olig2 (a) and NG2 (c) proteins in differentiated cells. Differential staining of cells with ethidium bromide. (b,d) Scale bar=100 µm

Quantitative analysis of genes at the end of the second stage of differentiation: The results of the polymerase chain reaction indicated the expression of Olig2 and NG2 genes in oligodendrocyte-like cells. In the molecular study at the end of the second stage of differentiation, it was determined that culture resulted in a significant increase in the expression of Olig2 and NG2 genes and decreased expression of the Nestin gene at the end of the induction stages ($p<0.05$). Molecular studies showed that the increase in oligodendrocyte-like cells was 1.4% and 1.6%, respectively, compared to neural precursor cells (Fig5).

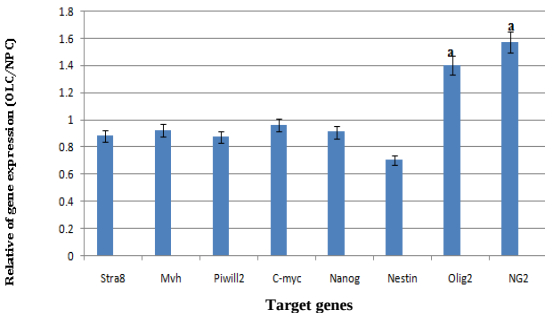

Figure 5. The rate of changes in the expression of Olig2, NG2, Nestin genes, Nanog and C-Myc pluripotent genes, and specific spermatogonial genes of piwil2, stra8, mvh in oligodendrocyte-like cells compared to neural precursor cells. Each column has an average of 3 repetitions and $p \leq 0.05$ is considered as the significant difference level. The degree of expression of the above genes in neural precursor cells is considered to be 1. a: Significant increase in neural precursor cells.

Discussion

The results of this study showed that spermatogonial cells are capable of producing embryoid bodies under in vitro conditions. The above – mentioned pluripotent cells exhibited nerve-like and glial phenotypes at the end of the induction stages, indicating their neuroglial differentiation potential. The recent studies suggest that embryonic stem-like cell colonies are created after culturing spermatogonial cells in culture media, which have the ability to self-repair and produce all three types of germ layers (4, 6). Guan reported reproduction and differentiation of cardiac cells derived from spermatogonial stem cells after transplantation in animal models (14). Subsequent developments were such that groups such as Kossak and Mizrak confirmed the emergence of embryonic stem-like cells after culturing human spermatogonial cells and differentiating into the three types of embryonic germ layers (6, 15). Increased expression of genes such as Nanog and C-Myc and decreased expression of specific spermatogonial genes such as piwil2, stra8, mvh in embryonic stem cells compared with spermatogonial cells in the present study are similar to the reports by different groups, including Guan (2006) and Kossac (2009).

They examined and reported changes in a wide range of genes during the conversion of spermatogonial cells to embryonic stem-like cells (5, 15). In this study, retinoic acid was used as an inducer to differentiate embryonic stem-like cells into oligodendrocyte-like cells. The use of retinoic acid to differentiate embryonic stem-like cells to neural precursor cells is a common technique that is commonly used (16–18). Glaser reported similar results in the differentiation of nerve phenotypes using a retinoic acid inducer for differentiation of embryonic stem cells (13). In fact, retinoic acid belongs to the vitamin A family and plays an essential role in the development and differentiation of the nervous system (19, 20). Overall, the positive inductive effect of retinoic acid on the differentiation of embryonic stem-like cells in the present study confirms the similarity of the nature of these cells with embryonic stem cells and the similarity of their protocols. The results of genetic studies at the end of the second stage of differentiation show the expression of Olig2 and NG2 genes in oligodendrocyte-like cells and the reduction of the expression of the Nestin gene. In fact, Olig2 and NG2 markers are prominent markers of oligodendrocyte precursor cells, which can confirm the nature of differentiated glial cells in the present research. This study was similar to the Glaser's report regarding the induction of embryonic stem-like cells in the presence of growth factors in nerve-like and glial cells (13). In the present study, there are similarities between these two types of cells (embryonic stem-like cells and the embryonic stem cells) by different factors, such as growth factors of cytokines and retinoic acid (21 – 24). In this regard, previous studies have shown that stem cells that are induced by growth factors are differentiated into nerve-like and glial cells (25, 26). In other studies, the stem cells were differentiated into oligonucleotide-like cells in a two-step differentiation protocol (27, 28). The results of the present study showed that the process of differentiation of spermatogonial cells into oligodendrocytes precursor cells under in vitro conditions was similar to the differentiation of embryonic stem cells and is associated with a wide range of genetic changes. The potential of using these new cellular sources to treat neurodegenerative diseases, such as nerve demyelination, can open up new perspectives on restorative therapies, although further research is required (22). Spermatogonal stem cell culture leads to the formation of pluripotent embryonic stem-like cells. The spermatogonal stem cells of the mouse are differentiated into neural precursor cells by retinoic acid in the culture medium. Neural precursor cells are differentiated into oligodendrocyte-like cells by appropriate inducers.

Acknowledgments

Hereby, we express our deepest sense of gratitude and indebtedness to Stem Cell Headquarters, Vice president of Science and Technology for their financial support, Experts of the Anatomical Laboratories, Mr. Biranvand and Ms. Ebrahimi.

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