The Assessment of Apoptosis during Mouse Embryonic Stem Cells Differentiation towards the Primordial Germ Cells in Alginate Microspheres

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Introduction: Previous studies have shown the differentiation of Primordial germ cells (PGCs) from embryonic stem cells (ESCs). However, some PGCs die during the differentiation. Alginate biopolymers have shown to closely mimic an *in vivo* environment and they are suitable for stem cells viability. The aim of the present study was to asses ESCs apoptosis during differentiation into PGCs in alginate microspheres. **Materials and Methods:** Mouse ESCs were cultured in ESCs medium, alginate biopolymer and bone morphogenetic protein 4 (BMP4) were utilized as a differentiation factor. The expression of genes B-cell lymphoma 2 (Bcl2), BCL2 Associated X Protein (BAX), octamer-binding transcription factor 4 (Oct4), and VASA as a specific gene for PGCs, were analyzed in the time of differentiation. **Results:** Expression of apoptosis gene, BAX, in cells entrapped in alginate microspheres; in addition, they were significantly down-regulated and expression of anti-apoptosis gene, Bcl2, was not up-regulated in the entire groups. VASA gene expression was significantly up-regulated in alginate groups. The highest ratio of cells differentiated to putative PGCs belonged to the cells in alginate group without BMP4; based on the analysis of the flow cytometry. Immunocytochemistry staining as well demonstrated differentiation in these cells. **Conclusion:** It was magnificently attained that down-regulation of apoptosis gene and up-regulation of differentiation gene in alginate beads revealed the survival ability of the putative PGCs in alginate compare to ESCs medium. It was obtained that the improvement of alginate microspheres could assist ESCs differentiation towards the putative PGCs.

Key words: Embryonic stem cells; Differentiation; Primordial germ cells; Alginate

Introduction

Germ cells are the only cells able to produce gametes. In fact, the absence of these cells and their precursors lead to infertility. The essential issue mentioned in the conjunction with germ cell precursors in the embryo is limited numbers (1). During the development in utero and just after birth, some germ lines screen cell death, so that the population of these cells would reduce (2). This phenomenon has attracted many researches and it still has uncovered points (3). B-cell lymphoma 2 gene (BCL2) associated X protein (Bax) is the precursor of germ cell death program and has been shown to cause cell death (3). BCL2is one of the antagonists of Bax and activates the surviving of germ cells (4). With increased expression of Bax and no change in the expression of Bcl-2, apoptosis occurs in germ cells in mouse ovary (5). Bcl-2 expression in transgenic mice during fetal oocytes (6) or after birth (7) has been shown to cause apoptosis modulation.

Quite different laboratories have pursued the goal to differentiate stem cells into germ cells, their precursors, and primordial germ cells (PGC). As of now, by adding differentiation factors; such as, bone morphogenetic protein 4 (BMP4) to medium of stem cells, they can be stimulated to differentiate into germ cells (8). Moreover, biopolymers with the simulation of conditions inside the body have shown to be suitable for differentiation (9). The three-dimensional environment should prevent apoptosis or cell death if necessary and provide suitable conditions for proliferation and differentiation of cells.

Alginate is a biopolymer which its biocompatible properties makes it satisfactory for cell culture, also it is cost sufficient and available (9, 10). Sodium alginate salt could absorb plenty of water and make a homogenized solvent suitable for ESCs culture; even in the form of microspheres. The aim of the present investigation was to evaluate the apoptosis during differentiation of ESCs towards the PGCs in alginate microspheres and using BMP4 as a differentiation factor.

Materials and Methods

Mouse embryonic fibroblast (MEF) preparation

Maintenance of ESCs for proliferation without differentiation requires ESCs culture in mouse embryo fibroblasts (MEF). To

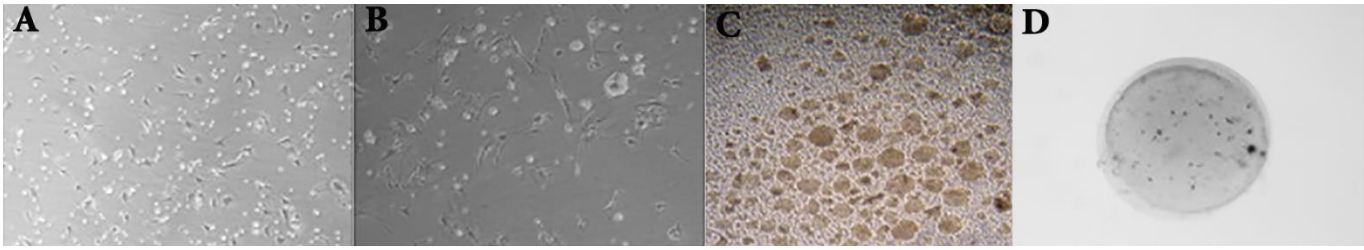

Figure 1. (A) ESCs on MEF; (B) Colonies of ESCs; (C) Embryoid Bodies; (D) Alginate Bead with EBs inside

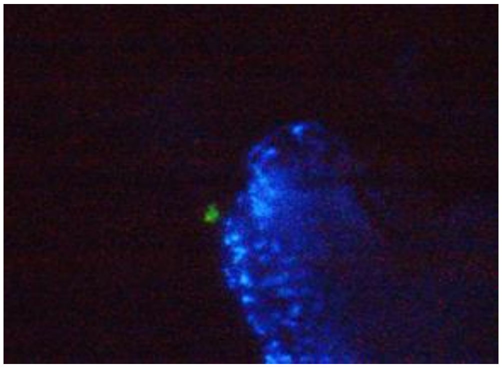

Figure 2. Immunocytochemistry staining of differentiated PGCs in yellow green (HOCHST) and ESCs in blue (DAPI)

obtain MEF, male & female mice mated to observe vaginal plaque in female in the first day of gestation. Female mouse were sacrificed on day 12 to isolate mouse embryos. The embryos were dissociated and then trypsinized to produce single-cell suspensions. MEF were proliferated and activated with mitomycin- C (Invitrogen, USA).

ESCs culture and embryoid body (EB) formation

ESCs were obtained from the Stem Cells Technology Research Center Bonyakhteh in Tehran, Iran. ESCs were maintained as inactivated MEF. ESCs, then were proliferated on MEF supplemented with ESCs culture medium containing DMEM supplemented with 10% FBS (Gibco), 1% nonessential amino acids (Life Technologies), 0.1 mM 2-mercaptoethanol (Invitrogen), 1 mM sodium pyruvate (Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich), penicillin/streptomycin (0.1 mg/mL; Sigma-Aldrich), and LIF (1000 IU/mL; Sigma-Aldrich) for undifferentiated state of ESCs (Figure 1A). Proliferation of ESCs led to formation of ESCs colonies (Figure 1B)**.** Indeed, for the formation of embryoid bodies (EBs), colonies of ESCs were trypsinized and cultured in ECSs medium without LIF. EBs were formed after 24 hours in 4 well non adherent dishes, as ECSs groups, with and without BMP4 (Figure 1C).

Alginate microsphere formation

Na-Alginate 1.5% and Cacl2 100mM solutions (Sigma Aldrich) were prepared. Na-Alginate solution was filtered through a 0.2 mμ syringe filter (Sartorius, Germany) and warmed up to 25 ˚C. ESCs (10^6 cells) then, they were cultured in ESCs medium without LIF and centrifuged in 1200 rpm for 5 minutes. ESCs were plated on the tip of the centrifuged tube and mixed gently with Na-Alginate solution to obtain Na-alginate - ESCs uniform mixture (Al-ESC). The Al-ESC was transferred into an insulin syringe with gauge 31(BD Ltd USA) in 25 ˚C. As the syringe placed over a petri dish containing Cacl2, Al-ESC droplets started to drop in Cacl2 solution for formation of AL-ESC beads. The beads were hardened sufficiently when remained in Cacl2 solution for roughly 10 minutes. The beads with ESCs inside were considered as test groups and remained in 4 well dishes.

Differentiation of ESCs

BMP4 (R&D Systems, USA) as a differentiation factor was added to ESCs and Al-ESC beads medium after 24 hours (10 ng/ml). In fact, the medium was changed every 3 days.

Cell groups

There were 4 groups of ESCs for differentiation into putative PGCs. (1) ESCs culture in ESCs medium, without BMP4 (Group C-); (2) ESCs culture in ESCs medium, with BMP4 (Group C+); (3) ESCs cultured in alginate beads, without BMP4(Group A-); and ESCs cultured in alginate beads, with BMP4 (Group A+).

RNA extraction and q-PCR

The primer sequences used for q-PCR are listed in Table1. RNeasy kit (Qiagen, Hilden, Germany) was used to extract total RNA from ESCs of different groups. Prime Script First Strand cDNA synthesis kit (Qiagen, Hilden, Germany); in addition, with 1ng of total RNA were used to synthesis cDNA. PCR reactions were consisted of 40 cycles at 95 ˚C for 10 s and 60 °C for 35 s. SYBR Premix Ex Taq II kit (Takara, Japan) were utilized for q-PCR analysis with two reactions in cells which were obtained from separate groups. Hypoxanthine-guanine phosphoribosyl transferase (Hprt) gene was considered as a housekeeping gene and expression of genes were normalized in each group according to Hprt.

Flow cytometry

ESCs trypsinized (Gibco) on the eight day of culture in different groups. Then, ESCs were fixed with paraformaldehyde. ESCs were perforated with triton 1% 100X (Sigma) to achieve VASA

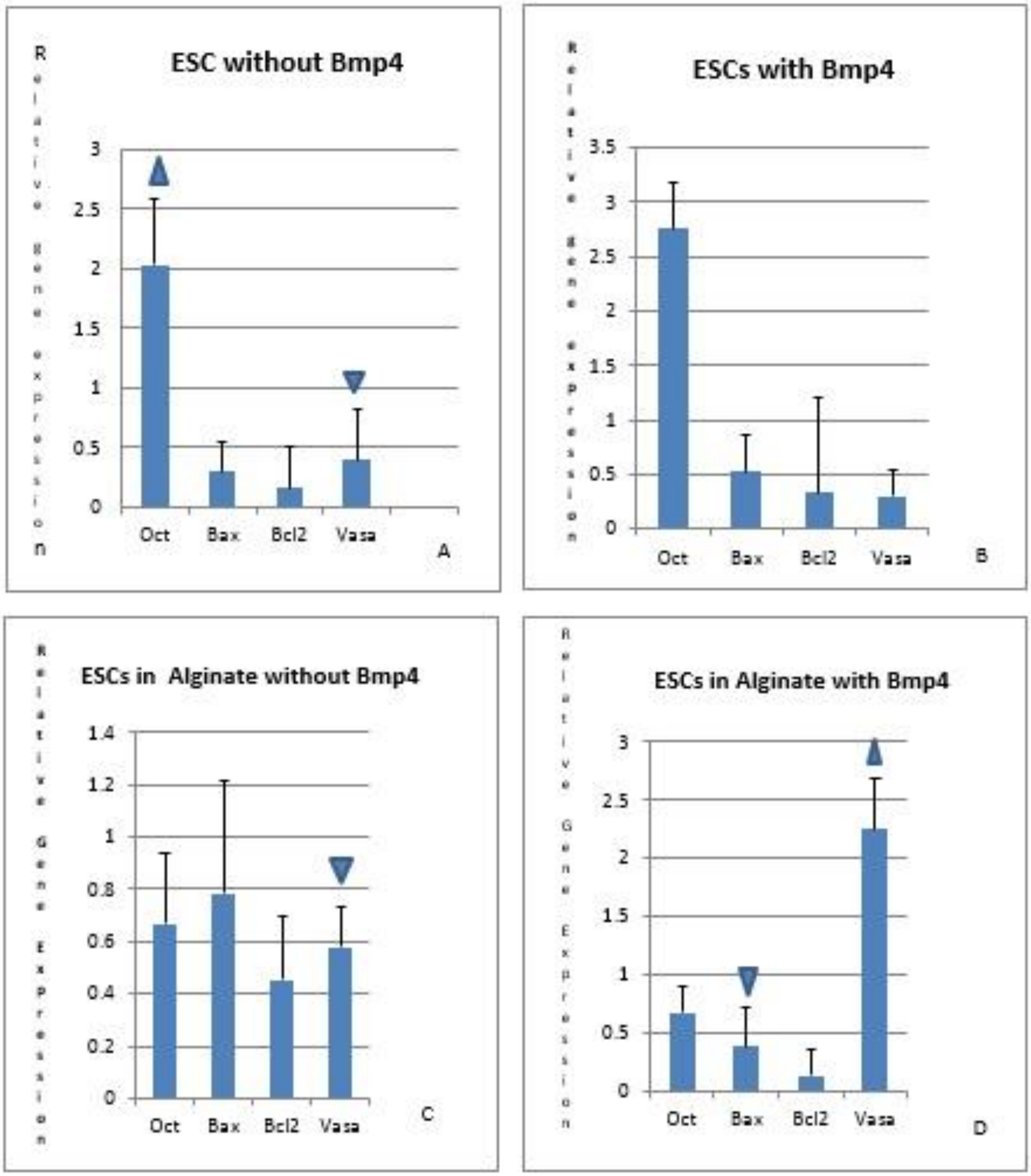

Figure 3. Relative mRNA expression of Oct-4, VASA, Bax and Bcl2 in groups (A) Control without BMP4; (B) Control with BMP4; (C) Alginate without BMP4; (D) Alginate with BMP4

protein in cytoplasm. VASA protein incubated with primary antibody (mouse IgG1=1:200; isotype control=1:200; Antibodies-online GmbH, Aachen, Germany) overnight, followed by a secondary antibody (rabbit polyclonal IgG=1:50; Antibodies-online GmbH, Aachen, Germany) for 30 min. Cells remained in paraformaldehyde 10% at 4˚C. Analysis was done by using an Attune flow cytometer (Applied Biosystems, USA) FlowJo 7.6 software.

Immunocytochemistry

EBs were formed on the 8th day in Alginate groups; in fact, they were fixed in 4% cold paraformaldehyde (4ºC) for 20 minutes. EBs were washed twice with PBS and Tween 20 (0.05% solution) (Life Technologies). Cells were penetrated with 0.4% Triton X-100 (Life Technologies). Non-special proteins in the cells were blocked with Goat Serum 10%+BSA (Gibco) in 25 ˚C. Cells were incubated in BSA and in PBS containing primary antibody (Rabbit MVH) (antibodies-online Gmbh, Aachen, Germany). Furthermore, cells were incubated with secondary antibody (Rat anti Rabbit IgG=1:100) (antibodies-online Gmbh, Aachen, Germany). Excessive antibodies were removed to observe endogenous MVH protein. DAPI solution (Sigma Aldrich) was used to stain cells nuclei. Observation of the cells and photography was performed with immunofluorescent microscope (Nikon Eclipse 2000).

Statistical Analysis

PCR data were analyzed by rest 2000 software (Qia gene). Numerical data reported as mean±SD. Flow cytometry results were compared with students T-test and the entire data were analyzed by ANOVA Test with $P \leq 0.05$ significant.

Results

Colonies of ESCs were formed on MEF (Figure 1B). Detachment of the colonies followed by replacement of EBs medium led to EBs formation (Figure 1C) in groups C- & C+. Differentiation factor (BMP4) was added to Groups C+ and A+ on the 2nd\day of culture. Group C- and A- lacked BMP4. ESCs were transferred into alginate beads in groups A- and A+ (Figure 1D) to form EBs (Figure 1D).

q-PCR Analysis

Expression of apoptosis related genes, Bax and Bcl2, along with Oct4 as pluripotency gene, specific PGC gene, and Vasa were compared on day 8 to day 4 in all groups. Oct 4 expression was down-regulated in groups A- and A+ on the 8th day comparing to the 4th day; and significantly up- regulated in C- group. Expression of Bax down-regulated significantly in group A+ while its expression was little in other groups without any significant differences. In contrast, Bcl2 expression was up-regulated in groups C+ and A+ on the 8th day comparing to the 4th day. VASA expression was significantly up-regulated in A+ group in the 8th day comparing to the 4th day. Nonetheless, Vasa expression was significantly down-regulated in groups C- and A- groups in the 8th day comparing to the 4th day (Figure 1C).

Flow cytometry Analysis

The expression of VASA protein in the 8th day of EBs formation analyzed by flow cytometry; as a matter of fact, it was revealed that the ratio of this specific protein in PGCs was significantly up- regulated in group C+ (2.92%) more than other groups (C- 1.58%, T- 2.71%, T+ 2.85%).

Immunocytochemistry

Immunocytochemistry staining is demonstrated in Figure 2. The VASA positive cells PGCs in EBs are shown in yellow green for confirming putative PGCs differentiation in EBs.

Discussion

The ability of ESCs differentiation into PGCs were reported in routine cell cultures (11, 12). Embryonic stem cells require specific signals to acquire eventual lineage or specific fate. External cues specifically can be the major factor which cause the ESCs differentiation towards the desired cell type. Physical cues and soluble chemical signaling could be placed in these categories. Expression of VASA gene in putative PGCs entrapped into alginate microspheres on day 8th reveals the competence of alginate biopolymer, as an appropriate medium for PGCs differentiation. Up-regulation of VASA may be due to the impact of BMP4 as differentiation factor in alginate microsphere comparing to the other groups without BMP4. It is quite essential to mention that, Oct4 as a pluripotency gene decreases the expression during PGCs differentiation (13); for instance, ESCs in alginate beads with BMP4. Furthermore, ESCs could enter into differentiation phase in alginate microspheres with the assist of BMP4 as differentiation factor comparing to the alginate group without BMP4. Up- regulation of Oct4 gene in the ESCs culture mediums with and without BMP4 indicates that ESCs could not start differentiation process and remain in pluripotency phase in those groups. Bax gene expression was not up-regulated in all groups which designates (3) ESCs could survive and do not enter into apoptosis phase; nevertheless, Bcl2 as a regulator of Bax was not up-regulated in all groups. The flow cytometry outcomes confirmed ESCs ability to differentiate into PGCs in the entire groups; such as, alginate groups and the gene expression for VASA coincided with protein expression. It appears that 1.5% Na-alginate biopolymer with special mechanical stiffness and cross-linking cation (14) could assist differentiation process of ESCs to putative PGCs; indeed, in the future studies, it could act in the improvement of quality of biopolymers and help to achieve gametes from ESCs *in vitro*.

Conclusion

In despite, down-regulation of apoptosis gene and up-regulation of differentiation gene in alginate beads revealed the survival ability of the putative PGCs in alginate comparing to the ESCs medium. It is concluded that improvement of alginate microspheres could assist ESCs differentiation towards the putative PGCs.

Acknowledgment

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Conflict of Interest: 'None declared'.

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