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Transplantation and Homing of Mouse Embryonic Stem Cells Treated with Erythropoietin in Spleen and Liver of Irradiated Mice

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ABSTRACT

Background: The present study was designed to evaluate the homing potential of mouse embryonic stem cells (ESC) treated with erythropoietin (EPO) in hematopoietic organs such as spleen and liver after transplantation using morphological and immuno-histochemical techniques. **Methods:** Day-four embryoid body (EB)-derived cells were dissociated and re-plated in medium in the presence and absence of EPO for three days. The EPO- and untreated differentiated cells were labeled with 5-bromo-2 deoxyuridine (BrdU) before transplantation and analyzed using flow cytometry and reverse transcription-PCR methods. BrdU-labeled cells were injected via the tail vein into irradiated adult mice in both groups. The spleen colony-forming unit assay (CFU-S) was performed 12 days after transplantation. Immuno-histochemistry was also carried out to trace transplanted cells. **Results:** The percentage of CD34 positive cells was 5.51 ± 1.06% in the EPO-treated group and 1.63 ± 0.225% in untreated group. The RT-PCR analysis showed that the EPO-treated cells expressed ε globin, βH1 globin, RUNX1 and EPO receptor genes, but the beta-major globin gene was not expressed. The number of colonies formed in the spleens of treated group (17.33 ± 4.726) was significantly different from the control group (6 ± 1). The population of BrdU positive cells in spleen of EPO-treated cell-transplanted group was higher than that of the control group. Also, BrdU positive cells were observed in the central vein of the liver sections of EPO-treated and control groups but were not observed in the liver parenchyma. There were not BrdU positive cells in the spleen and liver sections of the sham group. **Conclusion:** Our results confirm that ESC have the ability to home and form colonies in spleen after transplantation and EPO-treated EB-derived cells caused an increase in the number of colonies in spleen after CFU-S. *Iran. Biomed. J. 13 (2): 87-94, 2009*

Keywords: Embryonic stem cells (ESC), Erythropoietin, Transplantation, Homing, Spleen colony assay

INTRODUCTION

Embryonic stem cells (ESC) have the potential to differentiate into several cell types, such as hematopoietic cells [1-4]. Differentiation of embryonic stem cells are a suitable source of hematopoietic progenitors for both basic research and clinical applications [1, 4, 5].

In vitro differentiation of ESC into hematopoietic stem cells (HSC) and genetically modified HSC is important for the establishment of therapeutic clones against a variety of hematological disorders [6-8]. Growth factors, cytokines and hormones are used for differentiation of ESC to hematopoietic cells, including erythropoietin (EPO) [9-11]. EPO is a primary growth factor that regulates proliferation and differentiation of erythroid progenitor cells and promotes erythropoietic development [12-14]. Mechanisms by which EPO elicits its various biologic effects on erythroid cells remain unclear, but the initial event is binding of EPO to specific receptors on the surface membrane of target cells

*Corresponding Author; Tel: (+98-21) 8801 1001 (3562); Fax: (+98-21) 8801 3030; E-mail: mogdeh@dr.com. **Abbreviations:** ESC, embryonic stem cells; BrdU, 5-bromo-2 deoxyuridine; CFU-S, colony-forming unit assay; EB, embryoid body; HSC, hematopoietic stem cells; IMDM, Iscove's modified Dulbecco's medium; LIF, leukemia inhibitory factor; DAB, diaminobenzidine; MTG, monothioglycerol; EPOR, erythropoietin receptor; EPO, erythropoietin.

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[12]. Semiquantitative RT-PCR technique has shown that the EPO receptor gene was detected by erythroid like cells derived from embryonic stem in the presence of EPO in the simple and co-culture system with bone marrow stromal cells [10]. Also, in this study, the colony assays demonstrated that almost 44-55% of erythroid colonies were formed *in vitro* [10].

Another investigation showed that when EPO was added to the cultured media of HSC, a 220-fold increase in erythropoiesis occurred between 10 and 15 days of culture [11].

There are some controversially reports about the homing and colony formation of ESC-derived hematopoietic progenitors after transplantation [15-20]. Schuringa *et al.* [16] showed that embryonic stem-derived HSC could contribute to hematopoiesis *in vivo* [16]; whereas, in another study, it was shown that hematopoietic cells derived from ESC are ineffective in reconstituting hematopoiesis in irradiated animals [20].

To our knowledge, there is no report regarding the capacity of EPO-treated ESC to home to hematopoietic organs after transplantation in the irradiated animal model. The present study was designed for the first time to evaluate the homing potential of ESC treated with EPO in hematopoietic organs such as spleen and liver after transplantation using morphological and immuno-histochemical techniques. The results of the present study could direct us to a new approach in cell therapy.

MATERIALS AND METHODS

Cell culture media. CCE mouse ESC (a kind gift from Dr. John Draper, Stem Cell Center, Sheffield University, Sheffield, UK) were cultured in DMEM (Invitrogen, Paisley, UK) with 20% fetal bovine serum (FBS, Invitrogen), 0.1 mM non-essential amino acids (Invitrogen), 0.1 mM β-mercaptoethanol (Sigma, St. Louis, MO, USA) and 1000 U leukemia inhibitory factor (LIF, Sigma)/mL. Undifferentiated ESC were passaged every two days by trypsin (0.25%, Merck, Frankfurt, Germany)/EDTA (1 mM, Sigma) dissociation and cultured at 37 °C, 5% CO_2, and 95% humidity [10].

Immuno-cytochemistry for Oct-4. The undifferentiated state of ESC was confirmed immuno-cytochemically. The cells were fixed in 4% paraformaldehyde in PBS and blocked with 10% normal goat serum (Sigma, Germany) in PBS. After permeabilizing with 0.3% Triton X-100 (Sigma), the cells were incubated with primary antibody against Oct-4 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and then with secondary antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology). Finally, the samples were developed with diaminobenzidine (DAB, Sigma), and mounted onto slides with gelatin.

Embryoid body (EB) formation. Two days prior to their differentiation, ESC were cultured in Iscove's modified Dulbecco's medium (IMDM, Sigma, Germany) with FBS (15%) and monothioglycerol (MTG) (1.5×10^{-4} mol/L, Sigma), ascorbic acid (50 ng/mL, Sigma), and L-glutamine (2 mmol/L, Gibco, UK). After reaching confluence, the cells were dissociated with trypsin/EDTA and plated on plastic culture dishes for 4 days in the absence of LIF to promote EB formation (5×10^3 cells/mL).

Cell Preparation for transplantation. The four-day-old EB cells were dissociated and re-plated in IMDM medium. One milliliter of culture media containing FBS (15%), MTG (4.5×10^{-4} mol/L, Sigma, Germany), ascorbic acid (12.5 ng/mL, Sigma), L-glutamine (2 mmol/L, Gibco, UK), and 20 ng/mL of EPO (R and D system, Minneapolis, MN, Germany) was added to cells for three days [10]; the media added to the cells without EPO were considered the control. Cells were labeled with 10 µg/mL 5-bromo-2 deoxyuridine (BrdU, Sigma) before transplantation and incorporation of BrdU into cells are tested using immuno-cytochemistry.

Flow cytometry analysis. To detect CD34 positive cells, flow cytometry was performed. The single cells were harvested from EPO-treated and non-treated groups (these experiments were performed three times). They were washed with PBS and filtered with 70 µm mesh. Cells were then incubated with an antibody directed against CD34-FITC (Miltenyi Biotech, Auburn, CA, USA) and its corresponding IgG isotype for one hour. Next, a conjugated single cell suspension with CD34-FITC was prepared and retained on ice until analysis by flow cytometry. All fluorescence-activated cell sorter analyses were performed on a PAS III flow cytometer (Partec, Germany), and data was analyzed using Partec Flomax.

Gene expression analysis by RT-PCR. The EB-derived ESC were collected after 3 days of culturing in IMDM medium with or without EPO treatment as

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Table 1. Sequence of the primer.

Gene	Sense	Antisense	Size (bp)
β2M	5'TGACCGGCTTGTATGCTATC-3'	5'CACATGTCTCGATCCCAGTAG-3'	316
εglobin	5'GGAGAGTCCATTAAGAACCTAGACAA-3'	5'CTGTGAATTCATTGCCGAAGTGAC-3'	122
βH1	5'AGTCCCCATGGAGTCAAAGA-3'	5'CTCAAGGAGACCTTTGCTCA-3'	265
RUNX1	5'AAACAAAACTGACCCGCAAG-3'	5'CAAAGTCAAATGCCCAACAG-3'	217
βMajor	5'CTGACAGATGCTCTCTTGGG-3'	5'CACAACCCCAGAAACAGACA-3'	578
EPOR	5'GGACACCTACTTGGTATTGG-3'	5'GACGTTGTAGGCTGGAGTCC-3'	452

described above. Their total RNA were extracted (Qiagen, Valencia, CA, USA) and treated with DNase. RT-PCR was carried out with the following conditions: 95°C for 5 min, followed by amplification at 95°C for 30 s, 58°C for 30 s (annealing), and 72°C for 50 s (extension) for 35 cycles [21]. The specific primers used for RT-PCR are listed in Table 1 [22-24]. The PCR products were analyzed on a 1.5% agarose gel (Isolab, Wertheim, Germany) and visualized by ethidium bromide staining.

Preparation of irradiated mice and transplantation. Ten 6-8-week-old male NMRI mice were cared for and used according to the university Guide for the Care and Use of Laboratory Animals. The experimental procedure was approved by the Committee for Animal Research of University. Nine of mice were given single dose of 7.5 Gy gamma-irradiation (whole body) for 6 min (Theratron 780C, Canada) [25] and then they were divided into experimental (N = 3), control (N = 3) and sham groups (N = 3) randomly. One hour after irradiation, cell transplantation was performed as follows [25].

In the experimental group, 2×10^6 EPO-treated and BrdU marked cells in 300 µL of PBS were injected via tail vein [25]. The same concentration of untreated ESC was injected into the control group and 300 µL PBS was injected into the sham group for host-derived spleen colonies. Transplanted mice were maintained under sterile conditions for 12 days after transplantation [25]. Experiments were carried out under Institutional Animal Care and Use Committee approval.

Spleen colony assay. The animals were sacrificed by cervical dislocation and their spleens and livers were removed. The spleens were scored for the colony-forming units of spleen (CFU-S) under stereomicroscope [25]. Then, the tissues were fixed in 4% formaldehyde, processed and embedded in paraffin wax for light microscopic study. The paraffin serial sections were prepared and morphological staining using hematoxylin and eosin was performed. Also, some of the sections (n = 10 in each groups of study) were randomly analyzed by immuno-histochemistry. Moreover, the spleen and liver tissues of un-transplanted mice were used as controls for the morphological study. For each group, 3 mice were analyzed.

Immuno-histochemistry for BrdU. Immuno-cytochemistry for BrdU was done before and after cell transplantation. BrdU-labeled cells were prepared before transplantation. They were fixed with 4% paraformaldehyde in PBS and blocked with 10% normal goat serum (Invitrogen) in PBS. Then they were permeabilized with 0.3% Triton X-100 (ICN). Following incubation with primary antibody against BrdU (1/300; Sigma, Germany), sections were incubated with secondary antibody conjugated with DAB (Sigma).

After transplantation, the paraffin sections of spleen and liver were placed in a 60°C oven for 2 hours, and then the sections were incubated with formamide (1/4 in 2XSSC, Sigma) at 60°C for 2 hours. Sections were then permeabilized and blocked with 0.3% Triton X-100 (ICN) and 10% normal goat serum (Invitrogen) in PBS for 30 min. They were incubated with primary antibody against BrdU (1/300, Sigma, Germany) at room temperature for 1 hour and then were incubated with secondary antibody conjugated with FITC (1/150, Sigma) at 4°C for 24 hours. Finally, the sections were observed under fluorescence microscope.

Statistical analysis. Statistical analysis was done using the SPSS 13.0 software. Data are shown as mean ± SD or as indicated. Spleen colony assays were repeated three times and the results were compared by a non-parametric (Mann-Whitney) test ($P \leq 0.05$).

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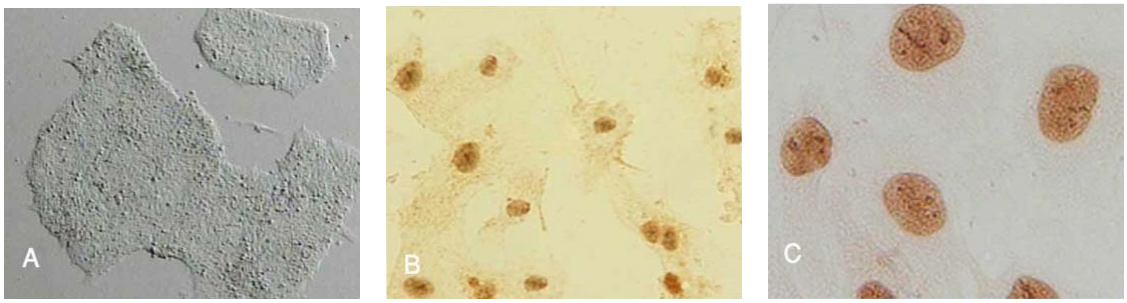


Fig. 1. (A) Light microscope photograph of undifferentiated colony of CCE ESC; **(B)** Oct4-positive ESC cells after immunocytochemistry (×100 magnification); **(C)** The BrdU-labeled cells were stained with DAB before transplantation (×400 magnification).

RESULTS

Morphological and immuno-histochemical studies of ESC before transplantation. Cultured mouse embryonic stem cells (CCE) formed colonies with irregular borders of different shape and sizes (Fig. 1A). The cells in these colonies were Oct-4 positive, indicating that the cells were undifferentiated (Fig. 1B). Day four EB were spherical with irregular borders. The cells were analyzed before transplantation for BrdU labeling. The BrdU-labeled cells were stained with DAB and their nuclei were stained brown (Fig. 1C).

Analysis of differentiated cells before transplantation. To determine the homogeneity and differentiation of the ESC derived population, the expression of the CD34 marker was analyzed using flow cytometry after 3 days. The small number of cells in EPO-treated group was expressed the CD34 marker (in three replicate). The percentage of CD34 positive cells in the untreated sample was 1.63 ± 0.22% (Fig. 2A) and this percentage in EPO-treated group was 5.51 ± 1.06% (Fig. 2B). This difference

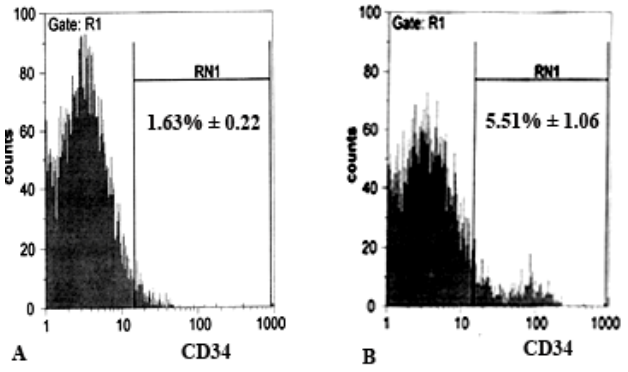


Fig. 2. Flow cytometry analysis of non-treated **(A)** and EPO-treated ES cells **(B)** for CD34 marker. The percentage of CD34 positive cells was higher in EPO-treated group than in the untreated sample and this difference was statistically significant (P<0.05).

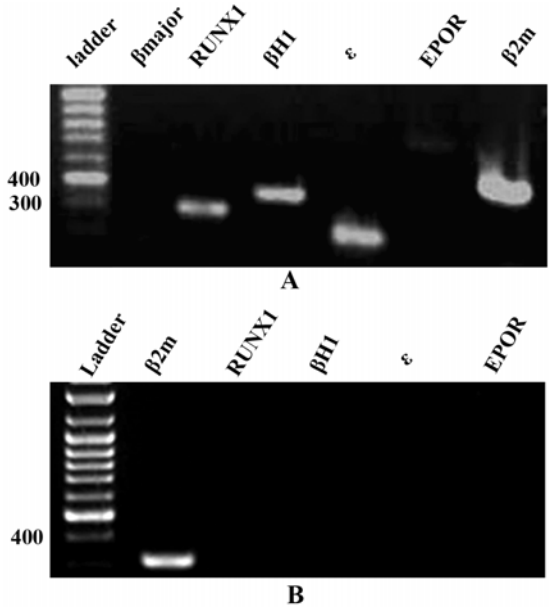


Fig. 3. RT-PCR analysis of hematopoietic-specific genes expression in ES cells treated with EPO **(A)** and in undifferentiated CCE cells as untreated control **(B)**. β2m gene (316 bp), ε globin gene (122 bp), βH1 gene (265 bp), RUNX1 gene (217 bp), beta-major globin (578 bp) and EPOR gene (452 bp).

was significant between these groups (P<0.05).The RT-PCR analysis demonstrated that in EPO-treated cells specific erythropoietic related genes such as ε globin, βH1 globin, RUNX1 and erythropoietin receptor (EPOR) were expressed, but the beta-major globin gene was not expressed (Fig. 3A). There were not shown any erythropoietic related gene expression in undifferentiated CCE cells as control (Fig. 3B).

Spleen colony assay. Twelve days after cell transplantation, the CFU-S was carried out in irradiated mice as follows: in EPO-treated transplanted cells (experimental group), non-treated transplanted cells (control group) and sham groups of study. The spleens of irradiated mice were smaller

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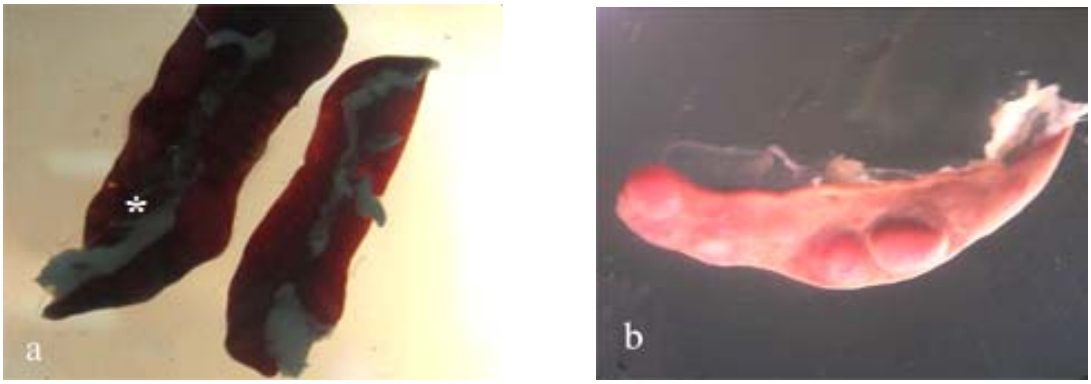


Fig. 4. Macroscopic view of the spleen in normal control and gamma irradiated transplanted-untreated cell groups. **(a)** The colonies of different sizes were seen in transplanted EPO-treated cells 12 days after transplantation **(b)**. Star shows control spleen.

than those of the non-irradiated control (Fig. 4a). Colonies of different sizes were seen in the spleens of cell-transplanted groups (Fig. 4b). The colony number in the spleens of the experimental group (17.33 ± 4.726) was significantly higher ($P \leq 0.05$) than the control group (6 ± 1), and there were no colonies in the spleens of the sham group.

Immuno-histochemistry. BrdU positive cells were observed in paraffin sections of spleen in both cell-transplanted and groups (Fig. 5). The population of BrdU positive cells in the EPO-treated cell-transplanted group (Fig. 5A) was higher than that of the untreated control group (Fig. 5B). However, there were no BrdU positive cells in the spleen sections of the sham group (Fig. 5C). The morphology and immuno-histochemical observation of liver in both cell-transplanted groups were shown in Figure 5D and 5E. BrdU positive cells were seen in the central vein of the liver sections of either EPO-treated cell- transplanted (Fig. 5D) or untreated control groups (Fig. 5E) but were not observed in the liver parenchyma. Additionally, no BrdU positive cells were observed in liver sections of the sham group (Fig. 5F).

DISCUSSION

The differentiation potential of ESC to become hematopoietic precursors *in vitro* has been shown in several investigations [2-4, 9, 26]. However, the ability of the cells to repopulate in irradiated animals models has received poor attention and remains to be demonstrated. Also, there are some technical limitations to obtain a pure population of hematopoietic differentiated cells for transplantation [27].

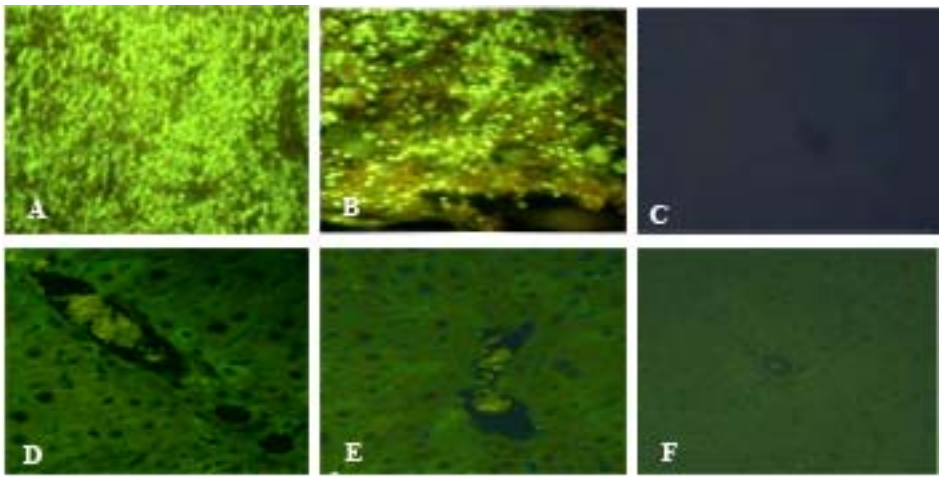


Fig. 5. Immuno-fluorescence and morphological staining of spleen sections in mice transplanted with EPO-treated cells **(A)**, in mice transplanted with untreated cells **(B)** and sham group **(C)**. The morphological staining of liver sections in mice transplanted with EPO-treated cells **(D)**, in mice transplanted with untreated cells **(E)** and sham group **(F)**. The BrdU positive cells are displayed as green (×200 magnification).

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One approach is to culture hematopoietic cells from ESC and evaluate the expression of genes involved in hematopoiesis to enhance HSC function.

Our results showed that supplementation of ESC culture media with EPO for three days caused cells to express a specific marker of hematopoietic cells, and specific erythropoietic genes such as ε globin, βH1, RUNX1 and EPOR. These results reconfirmed previous studies in other culture conditions [3, 28, 29]. Yuen *et al.* [22] demonstrated that EPO is required for both EB-primitive erythrocyte proliferation and differentiation of ESC into erythroid cells. Koury and Bondurant [30] showed that EPO is a stimulator of proliferation and differentiation of a definitive erythroid progenitor.

The result of this study also showed that, as analyzed by flow cytometry, 5.51 ± 1.06% of the population of treated ESC were CD34 positive. A similar effect was observed by Srivastava *et al.* [31] when they showed thrombopoietin can enhance generation of CD34+ cells from human ESC. By using co-culture of human ESC with OP9 bone marrow stromal cells, Trivedi and Hematti [32] were able to obtain up to 20% of $CD34^+$ cells and isolate up to 10^7 $CD34^+$ cells with more than 95% purity from a similar number of initially plated human ESC after 8 to 9 days of culture [32].

Hematopoietic progenitor and stem cells develop optimally *in vivo* through cellular interactions and paracrine effects provided by stromal cells. Furthermore, in our study, the homing of EPO-treated and untreated cells were compared in the spleen and liver of irradiated mice. Our findings demonstrated that ESC from two of the groups were able to form colonies in spleen after transplantation than liver. In the EPO-treated group, CFU-S increased 3 folds compared to the other groups. We suggest that the difference between the numbers of spleen colonies in the two groups may be due to the ability of EPO to induce erythropoiesis via the EPOR, which promotes amplification of hematopoietic genes in culture media before transplantation.

However, in the untreated EPO group, the transplanted cells had limited capacity to home in spleen and form colonies in irradiated mice. This may be due to the abundance of abnormally expressed differentiated genes. A similar result was shown by Liu *et al.* who demonstrated that a typical spleen colony was not observed after transplantation of day 12 EB cells or high proliferation potential colony forming cells (derived from ESC) into lethally irradiated adult mice [7].

In this regard, Lensch and Daley [5] showed that hematopoietic tissue is limited in its capacity to engraft due to abnormal expression of Hox A- and B-cluster genes. However, the limited colony formation of the untreated group may be due to the secretion of some factors after transplantation, or it may be due to de novo differentiation [33, 34]. The hematopoietic cells *in vivo* are in contact with a variety of molecules, including components of the extra cellular matrix, soluble and bound growth factors and adhesion molecules that influence their cell cycle status, viability, mobility and differentiation.

In the earlier reports, the percentage of dead cells in the absence of EPO was about twice as high as in the presence of EPO. This indicates that EPO is a proliferative and survival factor in culture media [35]. Another study showed that using EPO in the culture media of differentiating mouse, EB cells decreased the incidence of apoptosis [36].

Also, our results showed that BrdU positive cells were not observed in the liver parenchyma in both transplanted groups but some transplanted cells were seen in the central vein of liver. The BrdU positive cells in the vessels of liver showed the circulatory ESC. One explanation to this observation is that the liver has less potential to provide suitable microenvironment for homing the embryonic stem cells. The other is that the colonization of embryonic stem cells for hematopoiesis in liver needs more time than spleen. In conclusion, our results showed that EPO-treated EB-derived cells caused an increase in the number of colonies in spleen after CFU-S.

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