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Lymphoid lineage differentiation potential of mouse nuclear transfer embryonic stem cells

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ABSTRACT

Stem cells therapy is considered as an efficient strategy for the treatment of some diseases. Nevertheless, some obstacles such as probability of rejection by the immune system limit applications of this strategy. Therefore, several efforts have been made to overcome this among which using the induced pluripotent stem cells (iPSCs) and nuclear transfer embryonic stem cell (nt-ESCs) are the most efficient strategies. The objective of this study was to evaluate the differentiation potential of the nt-ESCs to lymphoid lineage in the presence of IL-7, IL-3, FLT3-ligand and TPO growth factors *in vitro*. To this end, the nt-ESCs cells were prepared and treated with aforementioned growth factors for 7 and 14 days. Then, the cells were examined for expression of lymphoid markers (CD3, CD25, CD127 and CD19) by quantitative PCR (q-PCR) and flow cytometry. An increased expression of CD19 and CD25 markers was observed in the treated cells compared with the negative control samples by day 7. After 14 days, the expression level of all the tested CD markers significantly increased in the treated groups in comparison with the control. The current study reveals the potential of the nt-ESCs in differentiation to lymphoid lineage in the presence of defined growth factors.

1. Introduction

Lymphocyte, as a critical cell in acquired immune system, is generated from hematopoietic stem cells (HSCs) during lymphopoiesis. The HSC differentiates to common lymphoid progenitors (CLPs) in its first phase of differentiation. The CLPs then differentiate into B cells by remaining in the bone marrow under induction with stromal cells and cytokines, or produce T cells by migrating to the thymus and induction with thymus microenvironment factors [1,2]. Nowadays, stem cells therapy has opened a new window in treatment of hematopoietic malignancy and generation of rare blood group products [3,4]. Rejection by host body immune system due to incompatibility of MHC types is considered as the most important hurdle in cell therapy [5]. Several attempts have been made to tackle such problem among which using the cells with the same MHC as reception such as nuclear transfer embryonic stem cells (nt-ESCs) and induced pluripotent stem cells (iPSCs) are the most efficient strategies [6]. Nuclear transfer process enables reprogramming of somatic cell nuclei and its transformation to embryonic cells [7]. In this way, the nucleus of the donor somatic cell is transmitted into an enucleated oocyte. After development of embryo to blastocysts, the nt-ESCs could be isolated from blastocyst inner cell mass (ICM) [8]. Following the injection into the blastocysts, the nt-ESCs settled in ICM and proliferated with a high differentiation capability and diversity, whereas these cells could

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Abbreviations

nt-ESCs	nuclear transfer embryonic stem cells
iPSCs	induced pluripotent stem cells
HSCs	hematopoietic stem cells
CLPs	common lymphoid progenitors
ESCs	embryonic stem cells
MHC	major histocompatibility complex
ICM	inner cell mass
IL	interleukin
FLT3-L	Fms-related tyrosine kinase 3 ligand
MEF	mouse embryonic fibroblast
DMEM-F12	Dulbecco's modified eagle's medium/nutrient mixture F-12
TPO	thrombopoietin
SCF	stem cell factor
M-CSF	macrophage colony-stimulating factor
BMP4	bone morphogenetic protein 4

differentiate to all three layers of mesoderm, endoderm and ectoderm [7]. Similar to other embryonic stem cells, the nt-ESCs have some unique properties such as self-renewability and pluripotency [9]. Recent studies reported some differences in characteristics of ESCs and iPSCs. On the other hand, differentiated cells derived-iPSCs show more sensitivity to apoptosis and less proliferation rate in comparison with nt-ESCs and ESCs [6,10]. Possessing the same MHC to the donor somatic cell, as a distinctive characteristic of the nt-ESCs, has made these cells a potential source for stem cell therapy and regenerative medicine [9,11]. Lymphoid cell have been produced from iPSCs and ESCs using the OP9 (mouse stromal cells) co-culture and feeder-free condition [12–14]. Several studies have reported the capability of ESCs in differentiation to both T and B lymphocytes through co-culturing with OP9 cell line [15–18]. It has been found that the ESCs differentiate to lymphocyte lineages when are cultured in feeder-free culture medium supplemented with growth factors such as interleukin-7 (IL-7) and Fms-related tyrosine kinase 3 ligand (FLT3-L), as differentiation inducers [13,14]. In the current study, we aimed to evaluate the differentiation capacity of the nt-ESCs, as a new source of stem cells therapy, in production of lymphoid linage without co-culturing with OP9 *in vitro*.

2. Materials and methods

2.1. Cell preparation and differentiation

2.1.1. nt-ESC culture

The nt-ESC line was prepared by injection of the cumulus cell nuclei into the enucleated oocytes from BDF1 mice, as described previously in a published study [19]. In brief, following injection of cumulus cells nuclei into the enucleated oocyte, blastocysts were cultured on mitotically inactivated-mouse embryonic fibroblasts (MEFs) to establish nt-ESCs lines. The expression of the ESCs markers was then determined by RT-PCR in nt-ESCs lines.

The cells were cultured on mitotically inactivated MEFs, as a feeder layer, in Dulbecco's Modified Eagle's medium/Nutrient Mixture F-12 (DMEM- F12) (Gibco, USA) supplemented with fetal bovine serum (FBS) (15%) (Gibco, USA), Penicillin (100 IU/mL), Streptomycin (100 μg/mL) (Sigma Aldrich, USA), nonessential amino acids (0.1 mM) (Gibco, USA), 2-Mercaptoethanol (0.1 mM) (Sigma–Aldrich; Munich, Germany), L-Glutamine (2 mM) (Sigma–Aldrich; Munich, Germany), ESGRO Leukemia Inhibitory Factor (LIF) (1000 IU/mL) (Millipore, USA). The cells were incubated at 37 °C with 95% humidity and 5% CO_2 saturation and the medium was changed every day. The cells were passaged after reaching to ~70% confluence by adding 0.5 mL of 0.25% trypsin/0.2% EDTA in PBS, and after neutralize the trypsin with nt-ESCs medium, the cells were added to gelatinized coated dish to remove the feeder cells. The suspended cells were then collected and transferred to new mitotically inactivated MEFs.

2.1.2. Embryonic body (EB) formation

To prepare the EBs, the nt-ESC clones were harvested and then transferred to a non-adherent six well plates. The EBs culture medium was the same as that of the nt-ESC medium, except for the addition of LIF. The EBs were formed 2 to 3 days after culturing.

2.1.3. Lymphocyte differentiation

After 2 to 3 days, EBs were transferred to 6 well cultures plates and cultured in the ESC medium supplemented with 20 ng/mL IL-7, 5 ng/mL thrombopoietin (TPO) (both purchased from ProSpec-Tany TechnoGene, Rehovot, Israel), 15 ng/mL interluekin-3 (IL-3) and 20 ng/mL FLT3-ligand (both purchased from PeproTech, Rocky Hill, NJ). The cells cultured in medium lacking aforementioned growth factors served as control. The culture medium was changed every two days up to 14 days. At day7 and 14, the differentiated cells were trypsinized and used for the following characterization assays by flow cytometry and quantitative Real-Time PCR.

2.2. Characterizations

2.2.1. Real-Time PCR

The total RNA of the differentiated cells were extracted based on Qiagen Rneasy kit (Qiagen, Hilden, Germany) and followed by synthesis of cDNA using Fermentas cDNA synthesis kit (Fermentas, Opelstrasse, Germany). The gene expression levels were measured by quantitative Real Time-PCR. The PCR mix contained 1 μL of cDNA, 6.5 μL of SYBR green ER master mix (Takara, Japan), 4.5 μL of nuclease-free water, and 0.5 μL each of forward and reverse primer pairs for each gene (final volume of 13 μL). The program used for this stage was as follows: denaturing step of 3 min at 95 °C followed by 45 cycles of PCR (95 °C for 15 s, annealing at 60 °C for 20 s, and extension at 72 °C for 20 s) and the final step is melting curve. At the end of PCR reactions, melt curve analysis was performed for all the genes. All reactions were carried out using the Rotor Gene 6000 (Corbett Life Science, Sydney, Australia).The samples were analyzed in duplicate and the average value of the duplicate was used for quantification. Data were analyzed using the Delta CT method. The geometric mean of the internal reference gene (Hprt1) was used to correct the raw values for the genes of interest. The mouse thymus was used as a positive control. Total RNA from thymus was extracted by Qiagen Rneasy kit (Qiagen, Hilden, Germany) and followed by synthesis of cDNA using Fermentas cDNA synthesis kit (Fermentas, Opelstrasse, Germany). The endogenous control (Hprt1) and the targeted genes were amplified with the following PCR cycle program: 94 °C for 1 min (denaturation), 98 °C for 5 s (denaturation), 55 °C for 5 s (annealing), and 72 °C for 40 s (extension) followed by 35 cycles and final extension 72 °C for 1 min. The PCR products were run in a 2% agarose gel electrophoresis. The products were stained with SYBR safe (Qiagen, Hilden, Germany) and then visualized under a short wavelength UV. The following primers have been used for development and differentiation analysis: 5′-TTGGGGGTCTCTTCTGCTTC-3′ and - 5′-TCATTCGCTTCCTTTTCCTTC-3′ for CD19, 5′-ATGGCCAAGAGCTGC-3′and 5′-AGAATACAGGTCCCGCT-3′ for CD3, 5′-AGCAGGATGGA-GAATTACAG-3′ and 5′-TCAGAGCCCTTTAGTTTTAC-3′ for CD25, 5′-

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