Lymphoid Genes Expression in Mouse Embryonic Stem Cells with Differentiation on Feeder Free Condition

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Abstract

Embryonic stem cells (ESCs) as pluripotent stem cells have the potential to give rise to all three germ layer cells. Also, these cells have the self-renewing ability. ESCs could differentiate into most of cell lineages. Lymphocytes generated from hematopoietic stem cells. We designed a feeder free culture condition to generate lymphoid lineage cells from ESCs. We demonstrated that IL-7 plays critical role in differentiation of ESCs into lymphoid lineage and expression of specific cell markers such as CD3, CD19 and CD25 after 14 days of differentiation by RT-PCR.

Keywords: Genes Expression, Mouse Embryonic Stem Cells, Differentiation, Feeder Free Condition

Introduction

Mouse embryonic stem cells (mESCs), which known as pluripotent stem cells ,were first established in 1981 from inner cell mass (ICM) of day 3.5 blastocyte-stage embryos,(1, 2) and more recently from totipotent blastomeres.(3, 4) Mouse embryonic stem cells distiguised by two unique characteristics. First, the ability to give rise to all body cell types and organs. Second, the potential to undergo indefinite self-renewing and maintained the undifferentiated state for extended period with stable karyotype.(5-7) Differentiation of these cells could provide a model system to study the signaling and the development of ESCs into various cells and lineages.(8, 9) ES cells can differentiated into most of cell lineages in Vitro (10). Lymphoid lineage consist of T, B and natural killer (NK) cells. In order to maintained murine ES cells in an undifferentiated state, the cells usually co-cultured on feeder layer, typically mouse embryonic fibroblast (MEF), in the presence of leukemia inhibitory factor (LIF). In most studies in order to differentiate ES cells into leukocyte, researchers cultivated ESCs on OP9 stromal cells, which is Macrophage colony-stimulating factor (M-CSF)deficient,in addition to IL-7 and FLT-3 ligand.(8, 10-12) IL-7 is charecterized as a lymphopoietic cytokine and is critical in differentiation of HSCs into lymphoid lineage.(13) To control the differentiation of ESCs to lymphoid lineage and eliminated unnecessary factors secreted by OP9 stromal cells, we used a feeder free condition to differentiate ESCs toward Lymphoid lineages.

Methods and Materials

Feeder Layer Preparation: Primary Embryonic Fibroblasts were used to cocultivated ES Cells in undifferentiated State. MEFs were isolated from mouse embryos at day 14 of gestation.Isolated embryos washed once in PBS Then individual embryos dissected to remove the head and soft tissues. The remaining tissues were minced until they become pepetable. Following that cells were suspended in 5 ml of 0.25% Trypsin-EDTA 1X (Invitrogen,USA) and incubated at 37ºC and

humidified atmosphere containing 5% CO_2 for 30 min. Then10 ml of complete medium were added to cell suspension and centrifuged at 1700g for 5 min. Then cells were passed from 70µm cell Strainer (Beckton Dickinson, USA). Finally cells were plated by centrifuging for 5 min at 1700g. plated cells were suspended in High Glucose Dulbecco's Eagles Medium (DMEM) (Gibco, USA) supplemented with 10% Heat inactivated Fetal Bovine Serum (FBS) (Gibco, USA) and 100U/ml Streptomycin and 100µg/ml penicillin (Gibco, USA). Fibroblasts were established after three to four passages. In order to transfer the ES cells on the MEFs, Feeder Layer mitotically inactivated with mitomycin C (Sigma, USA).

Embryonic Stem cell Culture: Mouse embryonic cell line CGR8, was provided by Stem Cell Technology Research Center (Tehran, Iran). Cells were cultured on mitotically inactivated MEFs in T25 Flasks. ES cells cultured in DMEM-F12 medium containing 10% ES qualified FBS (Gibco, USA), 100U/ml streptomycin and 100µg/ml penicillin, 2mM glutamine, 5 µM 2-mercaptoethanol and 1 ng/ml LIF. Cells were keptat 37ºC and humidified atmosphere containing 5% CO_2. ESC were subcultured every 3-4 days.

Embryoid Bodies Formation and In vitro Differentiation: For embryoid bodies (EBs) formationES cells harvested from flasks and suspended in ES medium in the absence of LIF. EBs formed by hanging drop method. Briefly, 400-1000 cells were platted in 20-30 µl drops on the lid of petri dishes in regular arrays. Then, the lid inverted and placed over the petri dish filled with sterile Phosphate Buffer Saline (PBS) (invitrogen, USA). Petri dish was incubated at 37ºC for 48 h. Eventually drops collected and transferred to non-treated dishes and kept in incubator for 5-7 days. EBs harvested and transferred into 0.2% gelatine coated 24 wells plates. Experiment were performed in four various groups as follows:

1) ES medium containing 20 ng/ml Interleukin7 (IL-7)

2) ES medium containing 50 ng/ml FLT-3 Ligand
3) a combination of IL-7 and FLT-3 Ligand

4) A Control Group without any growth factors. EBs were cultivated in culture condition for 14 days. Medium was replaced every 48 h.

RNA extraction and RT-PCR: Total RNA were extracted with Trizol reagent (Invitrogen, USA) according to Manufacturer's instructions. cDNA were synthesized with PrimeScript 1st Strand cDNA Synthesis Kit (Takara, Japan). RT-PCR was performed with specific primers for CD3, CD19, CD25 and IL-7Rα as lymphoid markers at day 7 and 14 of differentiation. Thefollowing primers were used: CD3 Forward (Fw), 5′-ATGGCCAAGAGCTGC -3′ and CD3 Reverse (Rev), 5′-AGAATACAGGTCCCGCT-3′; CD19Fw, 5′- TTGGGGGTCTCTTCTGCTTC -3′, CD19 Rev, 5′-TCATTCGCTTCCTTTTCCTTC-3′; CD25 (IL-2R)Fw, 5′-AGCAGGATGGAGAATTACAG-3′andCD25 (IL-2R) Rev, 5′-TCAGAGCCCTTTAGTTTAC-3′; IL-7Rα Fw, 5′-AGCAACTGGACGCATGTATC-3′ and IL-7RαRev, 5′-TCACCATCTCTGTAGTAGG-3′; and GAPDH Fw, 5′- GACAAGCTTCCCGTTCTCAG-3′ and GAPDH Rev, 5′-GGCATGGACTGTGGTCATGAG-3′.

Results

EB formation and in vitro differentiation: Formed ES clones on Feeder cellsand embryoid bodies in non-treated dish are shown in figure- 1.

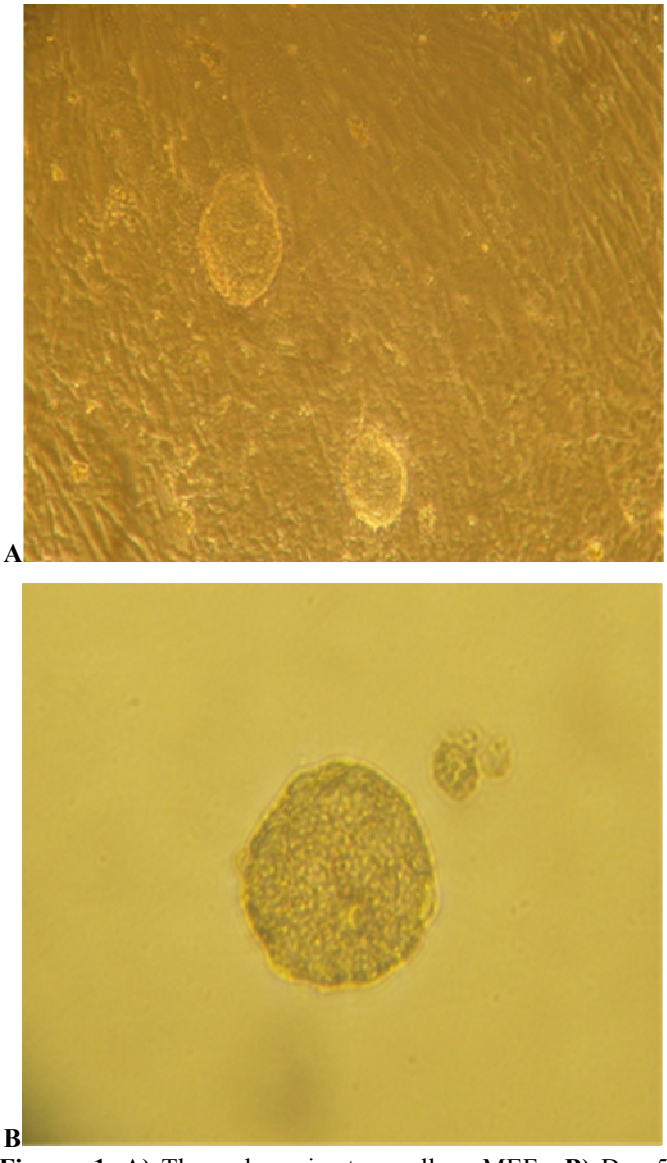


Figure- 1. A) The embryonic stem cell on MEFs. **B)** Day 5 EBs.

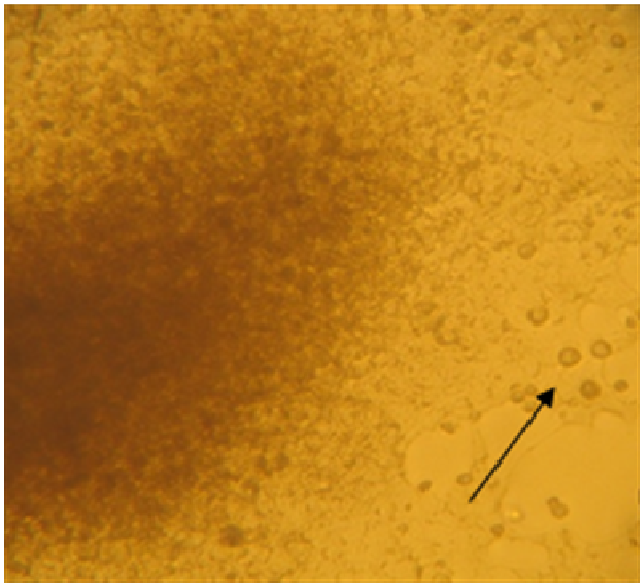


Figure- 2. Rounded cells showed with arrow are hematopoietic cells. These round cells growing from a EB which is attached to the plate.

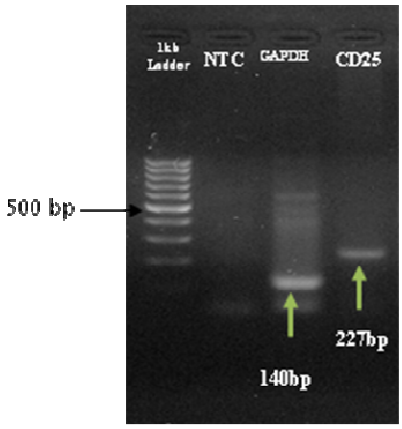
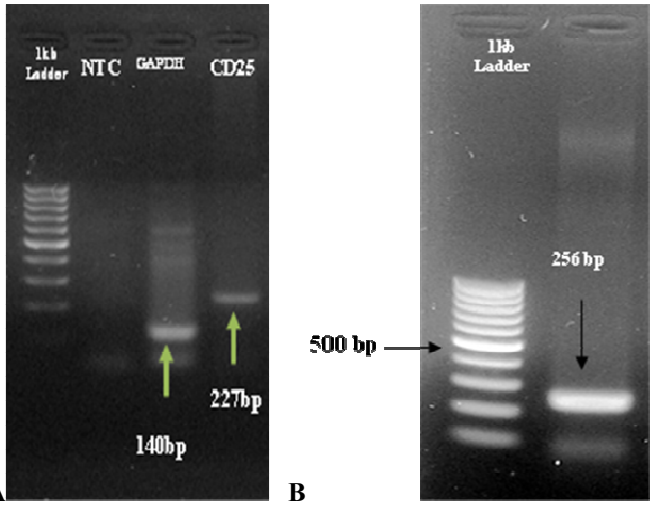


Figure- 3. Positive Expression of CD25 . GAPDH is used as Internal Control .



A B

Figure- 4. A) Expression of CD3 after 14 days of differentiation into lymphoid lineage. **B)** Expression of CD19 GAPDH used as an internal control.

After 7 days of differentiation, signs of differentiation into hematopoietic cells probably were seen according to their morphology in the cultivated cells (Figure- 2).

Differentiation into lymphoid Lineage: Collected results designated that IL-7 plays critical role in differentiation of ESCs into lymphoid lineage.After 14 days of differentiation the expression of CD25 were positive in group which is treated with IL-7 (Figure- 3). Also Expression of CD3 and CD19 were shown in Figure 4 A and B, respectively. IL-7Rα expression was negative (Data not shown).

Disscusion

In current study we focused on differentiation of mESC into lymphoid lineage in a feeder free condition with growth factors. Most of the researcher used of stromal feeder layer such as AP6, MS-5 and OP9, to induced the differentiation into hematopoietic stem cells.(14) In all experiments, in order to differentiate mESC into lymphoid lineage, researchers applied OP9 stromal cell line.(11, 15, 16) OP9 as a M-CSF deficient stromal cell line in comparison to normal stromal cell lines had more potential to induced the differentiation into hematopoietic lineages. Induced in vitro differentiation by feeder cells didn't affected by cell-cell interactions. These cells induced differentiation by secretion of unidentified factors. In order to control unwanted factors and signals we used a feeder free condition for differentiation of embryonic stem cells by using specific growth factors. The advantage of this condition is controlling the mechanism of cell signaling compared to the condition that mESCs were cocultivated on stromal feeder cells. The other benefit of using feeder free condition is to minimize the rate of infection for cell therapy and transplantation.

In 1992 Gutiereez-Ramosand co-workers, were used a system in which mESCscocultured with the stromal cell RP010 and a mixture of exogenous interleukins 3, 6, and 7, to differentiate into T and B lymphocyte progenitors and other hematopoietic precursors.(17) The differentiated cells generated from embryonic stem cells had the pro-B and pro-T source,which could reconstitute the lymphoid compartments of SCID mice.In other study which performed by de pooter and colleagues in 2003 embryonic stem cells cocultured with OP9 stromal cells. They showed that OP9 stromal cells could significantly support lymphopoiesis. These cells investigated from the expression of FLK-1 (VEGFR-2) cell surface markers.(8) In current study we used a feeder free culture condition to produce lymphoid lineages cells which were expressed CD3, CD19 and CD25 as lymphoid lineage cell surface markers.

Junas Carlos –Pflücker and co-workers reported that co-culture of ESC on OP9 stromal cell line which expressed notch receptors, Delta-1 ligand (DL-1), led to differentiation into lymphoid lineage. They demonstrated that generation of T-cell was depended on expression of DL-1.(10) In 2006 Olsen and colleagues reviewed that the only way in generatition of lymphocyte from ESCs were the cocultivation with stormal cells systems.(18)

In 2009 embryonic like cells, induced pluripotent (iPS) cells, used to generated T cells. In this experiment iPScocultivated with OP9 stromal cells to induced production of T cells.(19) IL-7 Plays critical role in genration of T-cells from embryonic stem cells in OP9-DL1 culture differentiation system.(20, 21) In other reports researches used OP9 stromal cells to generate lymphoid lineage cells.(22, 23) We designated that cytokines depedently led to generation of lymphoid lineage cells from ESCs in a feeder free condition. RT-PCR results of CD25, CD3 and CD19 confirmed the differentiation of ESCs into lymphoid lineage.

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