Iranian Red Crescent Medical Journal

ORIGINAL ARTICLE

Assessment of Piwil2 Gene Expression Pattern upon Germ Cell Development from Mouse Embryonic Stem Cell

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Abstract

Background: In order to improve culture conditions for optimal spermatogenesis, quantitative assessment of the male germ cell gene expression profile upon spontaneous ES cell differentiation is necessary. In this study, the quantitative expression profile of Piwil2, germ-line specific marker, during the early stage of embryoid body (EB) formation and differentiation (0-3-day-old EB) was studied.

Methods: CCE mouse embryonic stem cells (ESCs) were cultured in DMED containing 20% fetal bovine serum (FBS) for 1, 2 and 3 days. The total RNA was isolated from ESCs, 1-3-day-old EBs, and adult testis as positive control. cDNA was prepared and quantitative real-time PCR was done for Oct-4 to study the pluripotency of this cell line. Also, the molecular pattern of Piwil2 expression in developing EB was investigated.

Results: Our quantitative results confirmed the pluripotency of CCE mouse ESC line and showed that Piwil2 was expressed in undifferentiated CCE mouse ESC line. Our results also showed that expression of Piwil2 increased significantly during the process of EB formation and differentiation up to 2-day-old EB and decreased non- significantly in 3-day-old EB.

Conclusion: Our results suggest that EB provide a cellular environment similar to the early embryonic microenvironment and cause the efficient and progressive germ cell lineage differentiation in this system.

Keywords: Embryonic stem cell; Germ cell; Embryoid body; Piwil2

Introduction

Embryonic stem cells (ESCs) are pluripotent cells isolated from the inner cell mass of blastocyst-stage embryos. The capacity of ESCs in differentiation into diverse cell lineages such as germ cells provided a new way in the study of the mechanisms of in- vitro germ cell development.[1-3] Embryonic stem cells can be differentiated into germ cell lineage in suitable culture conditions.[1-10] Kee *et al.* showed that addition of bone morphogenetic protein 4 (BMP4) into the medium of human embryonic stem cells facilitates the germ cell differentiation.[6] The differentiation of germ cells was also observed when mouse ESCs were cultured on the top of the different cell types, e.g. STO cell,[3] BMP4 producing cell,[1] and CF1 mouse embryonic fibroblast feeder layer.[7] Additionally, germ cell differentiation was reported from other sources of pluripotent cells such as teratocarcinoma cells,[8] human, and mouse bone marrow stromal cells (BMSCs).[9,11] Above all, there are two different methods reported for germ cell differentiation, namely monolayer differentiation[12] and embryoid body (EB) formation.[1-3,6,7,10] Differentiation of germ cell in 3-9-day-old-EB was reported by Geijsen *et al.*[3] and West *et al.*,[2] using non-quantitative gene expression analysis.

Germ cell differentiation from the stem cell is accompanied by the alteration in the gene expression pattern.[2,3] Genetic studies have identified several

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Received: February 4, 2009 Accepted: May 17, 2009

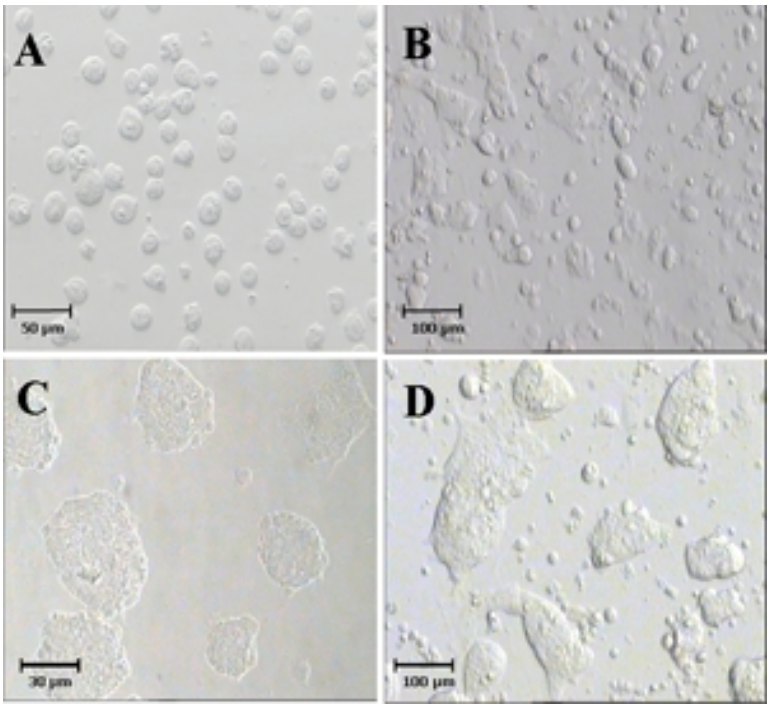
genes involved in germ cell development. One of the most important germ cell specific genes is piwi-like homolog 2 (Piwil2), also known as Mili gene. Piwil2 is one of the three mouse homologues of Piwi. The Piwi family genes are highly preserved during development and participate in many processes like gametogenesis, stem cell self-renewal, and RNA interference in diverse organisms. Piwil2 is expressed in the germ cells of adult testis and plays an essential role in spermatogonial stem cell self-renewal.[13] Thus, the study of Piwil2 gene expression may show the pattern of germ cell production. But the ratio of Piwil2 expression at the early stage of EB formation and differentiation has not been studied quantitatively up to now. In this study, we examined the Piwil2 gene-expression profile quantitatively in order to evaluate the efficiency of EB system without any induction in germ cell development at days 0-3 of EB formation and differentiation.

Materials and Methods

CCE mouse ESCs (a kind gift from Dr. John Draper, Stem Cell Center, Sheffield University) were derived from 129/Sv mouse strain and adapted to grow on gelatin-coated culture plates without requiring a primary embryonic fibroblast (PEF) feeder layer[14] (Figure 1). This work was confirmed by Ethics Committee of Tarbiat Modares University.

CCE mouse ESCs were cultured in Dulbecco's modified Eagle's medium (DMEM) with high glucose, pyruvate and L- Glutamin (Gibco, UK) supplemented with 20% fetal bovine serum (FBS; Gibco, UK), 0.1 mM b-mercaptoethanol (Sigma, USA), 0.1 mM non-essential amino acids (Sigma, USA), 100 u/ml penicillin, 100 µg/ml streptomycin (Gibco, UK), 3.7 gr/l NaHCO3 (Sigma, USA), and 1000 U/ml leukemia inhibitory factor (LIF; Sigma, USA). Undifferentiated ESCs were cultured at 37ºC with 5% CO2 and 95% humidity. The medium was changed every day.

Undifferentiated mouse ESCs were passaged every 2 days. After reaching confluence, the medium was aspirated and the dishes were rinsed with PBS. Trypsin (0.25%; Merck, Germany)/EDTA (1 mM; Sigma, USA) was added sufficiently to cover the surface of the tissue culture dish. Incubation was performed at room temperature (20ºC) until the cells lifted off from the plate and pippeting was done for cell suspension preparation. In order to inhibit Trypsin-EDTA,



Figs.1: Morphology of the A: CCE mouse embryonic stem cell (ESC), B: 1-day-old EB, C: 2-day old EB and D: 3-day old EB. Pictures were taken with the indicated magnifications.

DMEM supplemented with 15% FBS was added and cell suspension was centrifuged at 1200 rpm for 8 min at room temperature. The supernatant was removed, the pellet was dissolved in DMEM supplemented with 15% FBS, and cell suspension was divided into new tissue culture dishes.

The mouse ESCs were tripsinized and 2×10^5 cells were cultured in 6-well culture plates. For EB formation, ESCs was incubated for 1, 2 and 3 days in the presence of 20% FBS in DMEM and the medium was changed daily (Figure1).

The total RNA was isolated from the testis (positive control), ESCs and 1-3-day-old EBs in three separate experiments, using an RNX-Plus™ (Cinnagen). Genomic DNA contamination was removed from extracted RNA, using DNase I (Fermentase) and RNA concentrations were determined using UV spectrophotometer (DPI-1, Kiagen). Treated RNA was reverse transcribed, using RevertAidTM first strand cDNA synthesis kit (Fermentase) with oligo dT primer according to the recommended protocols. PCR master mix (Cinnagen) and SYBR Green were used for PCR reactions in a Rotor-Gene3000 thermocycler. Oligonucleotide PCR primers specific for Piwil2, Oct-4 and β2m (internal control) genes were adapted from others[15-17] and synthesized by Cinnagen Company. Cycling conditions were as follows: 94°C for 20s, 58-60°C for 30s and 72 °C for 30s. Melt-curve analysis was used to confirm the accuracy of the predicted fragments. The standard curve was prepared, using serial dilution of testis cDNA to determine the efficiency. The ratio of gene expression was determined, using comparative CT (cycle threshold) method.[18] The sequences of the specific primers used for RT-PCR are listed in Table 1.

Statistical analysis was performed, using SPSS software (version 13.0, Chicago, IL, USA). The results were compared through one way ANOVA and LSD post-test and a significance level of $P\leq0.05$ was considered. Also, we used Partial Eta Squared (η_p^2) as the effect size as follows: The values under 0.2, 0.2-0.5, 0.5-0.8 and higher than 0.8 were regarded as weak, moderate, large and very large effect size, respectively.[19]

Results

The undifferentiated state of CCE mouse ES cell line was proved with Oct-4 mRNA expression, using RT-qPCR. This result confirmed the pluripotency of this cell line as a model for germ cell development during EB formation and differentiation.

Expression of Piwil2 in CCE mouse ES cell line and 1-3-day-old EBs stages was evaluated, using *RT-qPCR*. In ESCs, Piwil2 expression was normalized in comparison to house keeping gene. But in each stage of 1-3-day-old EBs, the target gene/house keeping gene ratio of Piwil2 expression was calculated and calibrated to the previous stage. So, the mean calibrated expression of Piwil2 gene was compared in the different stages. The value of η_p^2 for Piwil2 was large (about 0.69).

In CCE mouse ESC, expression of Piwil2 was observed. The mean normalized expression of Piwil2 was 2×10^{-2} (Figure 2). One-day-old EB gene expression analysis showed 1.82 folds increase of Piwil2 in comparison to ES cells (Figure 2). This upregulation was statistically significant ($p\leq0.05$). In the two-day-old EB, quantitative PCR showed 14.31 folds increase in Piwil2 mRNA expression (Figure 2) in comparison to 1-day-old EBs. The increase in the ratio of Piwil2 gene expression was statistically significant compared to 1-day-old EBs and ESC ($p\leq0.05$). RT-qPCR analysis in the three-day-old EB displayed no significant decrease in Piwil2 expression in comparison to the 2-day-old EBs (0.06 fold) (Figure 2).

Discussion

In this study, pluripotency of CCE mouse ESC line was confirmed and spontaneous differentiation of the male germ cell during the process of EB formation

Table 1: Quantitative RT-PCR Primer Sequences

Gene	Primer (forward/reverse)	Significance
Oct-4	5′-CTTCTCGCCCCCTCCAGGT-3′ 5′-AAATAGAACCCCCAGGGTGAGC-3′	Pluripotency marker
Piwil2	5′-ATGCTTCCATCAGGTAGAGG-3′ 5′- GATCCATCAAACGCAGTGAC-3′	Germ cell marker
β_2m	5′-TGACCGGCTTGTATGCTATC-3′ 5′-CACATGTCTCGATCCCAGTAC-3′	Internal control

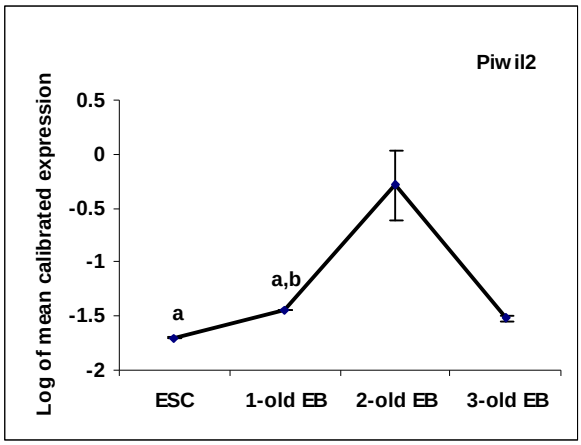


Fig. 2: The profile of mean calibrated Piwil2 expression (y-axis) was shown in embryonic stem cell (ESC) and during days of embryoid body (EB) formation and differentiation (x-axis). B2m was used as normalizer and Bars indicate standard deviations. a shows significant difference with ESC and b indicates significant difference with 2-day-old-EB.

and differentiation was established. Self-renewal of ESC depends on extrinsic and intrinsic signals. LIF acts as an extrinsic factor and maintains the pluripotency of ESC through activation of nuclear transcription factor STAT3 and the Oct-4; a member of the POU family of homeodomain proteins acts as an intrinsic factor.[20] Expression of Oct4 in the embryonic stem cell lines is necessary for self-renewal and maintenance of ESC pluripotency. Expression of Oct-4 is down-regulated when they are triggered to differentiate; thus, a model in the EB system is made, helping to study the early events linked to different cell type differentiation.[20]

EB is composed of ESC aggregates and can make different cell types of all three germ layers. Several groups showed differentiation of EBs into primordial and more mature germ cells.[1-3] Follow of germ cell transdiferentiation in EB system needs specific male germ cell marker and quantitative assessment method to distinguish the pattern of germ cell differentiation. Finding this pattern is necessary for the improvement of culture conditions, a requisite step in optimal spermatogenesis.[21]

Recently, several groups using non-quantitatively PCR have reported the expression profiles of many genes that are involved in male germ cell proliferation and/or differentiation.[22-30] Also, some researchers showed that EBs derived from human and mouse ESCs express germ cell specific markers.[1,3,7,12] Geijsen *et al.* added retinoic acid to EBs and isolated primordial germ cell-like cells on days 3-9 EBs based on RT-PCR for germ cell-specific genes Tdrd1, Tex14, Rnf17, Rnh2 and Piwil2.[3] Toyooka *et al.* used male knock-in ESCs in which GFP or LacZ was located near the germ cell specific gene, Mvh, and isolated primordial germ cell several times (5-7-day-old EBs) based on Mvh expression. The graft of these cells into the testis resulted in fully differentiated sperm production.[1] Our quantitative results showed that Piwil2, the germ line-specific marker,[8] was expressed in undifferentiated CCE mouse ES cell line. This result was in agreement with those of Geijsen *et al.* who showed that Piwil2 was expressed in mouse ESC derived from an F_1 cross between 129SvEv and C57BL/6-TGN (ACTbEGFP) 1Osb.[3] Also, a similar result was obtained by Silva *et al.* in a study showing that Piwil2 gene was expressed in undifferentiated TL-1 Sv129 mouse XY ES cells. He claimed that there was a group of founder cells existing within undifferentiated ES cell populations subject to differentiation into germ cell lineage.[31] Furthermore, Lee *et al.* showed that Piwil2 was expressed in a wide variety of tumors and acted as an oncogene. This function happens through inhibition of apoptosis and support of proliferation via Stat3/Bcl-X_L signaling pathway.[16] Transcriptional factor Stat3 plays an essential role in stem cells proliferation.[20] Thus, expression of Piwil2 in ESCs may be related to the role of this gene in the stem cell self-renewal.

Our results also showed that expression of Piwil2 increased during the process of EB formation and differentiation up to 2-day-old EB and decreased in

3-day-old EB. It was shown that Piwil2 modulates expression of spermatogonial cell surface markers integrin alpha 6 (Itga6), CD9, Thy-1 (CD90) and spermatogonial specific markers stimulated by retinoic acid gene 8 (Stra8) and heat shock protein 90 alpha (Hsp90a). These molecules play an essential role in germ cell differentiation.[13] Cell-cycle status and differentiation of germ cells in vivo was influenced by the close contact between a variety of cells.[32] Eddy *et al.* reported that cell-cell contact within the testis controlled sequential regulation of genes and caused in vivo germ cell differentiation.[33] Similarly, Shamblott *et al*. reported that EB composed of randomly dispersed precursor cells provide an environment in which a wide variety of lineages can emerge.[21] Thus, the increased expression of Piwil2 in EB system suggests that a cellular environment similar to the early embryo be provided in EB that causes efficient and progressive germ cell lineage differentiation in this system. In general, it seems that the same events of in vivo may occur in developing EB and some differences in the expression of genes may simply reflect differences between the in vitro and in vivo microenvironments. In summary, our results suggest that efficient differentiation of germ cell can happen during EB formation and differentiation. By developing efficient in vitro methods for germ cell differentiation, genetic and epigenetic manipulation studies which were previously impractical would be possible. Also, proper sources for future transplantation therapies will be provided.

Acknowledgment

We sincerely thank the Tarbiat Modares University's Medical Research Director for providing a grant for this work. There is no conflict of interest in this study.

Conflict of interest: None declared.

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