**Original Article**

# Effects of Treatment with Bone Morphogenetic Protein 4 and Co-culture on Expression of Piwil2 Gene in Mouse Differentiated Embryonic Stem Cells

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Abstract

**Background:** Specific growth factors and feeder layers seem to have important roles in *in vitro* embryonic stem cells (ESCs) differentiation. In this study,the effects of bone morphogenetic protein 4 (BMP4) and mouse embryonic fibroblasts (MEFs) co-culture system on germ cell differentiation from mouse ESCs were studied.

**Materials and Methods:** Cell suspension was prepared from one-day-old embryoid body (EB) and cultured for four days in DMEM medium containing 20% fetal bovine serum (FBS) in the following groups: simple culture (SC), simple culture with BMP4 (SCB), co-culture (CO-C) and co-culture with BMP4 (CO-CB). Expression of piwi-like homolog 2 (Piwil2), the germ cell-specific gene, was evaluated in the different study groups by using quantitative real time polymerase chain reaction (RT-PCR). Testis was used as a positive control.

**Results:** The maximum and minimum Piwil2 expression was observed in SC and SCB groups, respectively. A significant difference was observed in Piwil2 expression between SCB and other study groups ($p<0.05$).

**Conclusion:** The findings of this study showed that neither the addition of BMP4 in culture medium nor the use of MEFs as a feeder layer have a positive effect on late germ cell induction from mouse ESCs.

***Keywords:*** Embryonic Stem Cell, Germ Cell, BMP4, Piwil2, Co-culture

## Introduction

Germ cells form a highly specialized cell population required for species maintenance (1). Specification of this cell type occurs through two distinct ways: inheritance of preformed germ plasm and segregation of germ cells from somatic cells. The first mode has been proven to occur in some organisms, including drosophila, caenorhabditis elegans and xenopus (2) and the second occurs in mammals (3). The initial cells identified as germ cells emerge from the epiblast immediately before gastrulation. This specification is induced by extrinsic factors such as bone morphogenetic protein 4 (BMP4) secreted by extraembryonic cells (4, 5). It is reported that BMP4 induces germ cells specification from human embryonic stem cells (ESCs) (6). In mice, targeted disruption of the BMP4 gene of 7.5-9.5 days post coitum (dpc) epiblasts (7), culture of 6-6.25 dpc epiblasts or mouse ESCs on top of the BMP4 producing cells (8-10) and addition of BMP4 to 5-6.5 and 7.5 -8.5 dpc epiblasts (11) have confirmed the role of BMP4 [members of the TGF-b family of signaling molecules (7)] in germ cell formation. Germ cell differentiation also occurs by co-culture of mouse ESCs with STO feeder layer cells (12). Furthermore, derivation of germ cells from human ESCs has been observed under co-culture with embryonic fibroblasts and CF1 embryonic fibroblast feeder layers (13, 14). In the last few years, several groups have reported that mouse and human ESCs can differentiate into germ cells (6, 10, 12, 13, 15). Germ cell differentiation from mouse and human bone marrow stromal cells (BMSCs) and teratocarcinoma cells have been reported as well (16-18). Generally, two different methods have been used for germ cell differentiation; monolayer differentiation (19) and embryoid body (EB) formation (6, 10, 12, 13, 15, 20). Germ cell development from stem cells

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is accompanied by the shift in gene expression pattern (12, 20). Molecular studies have shown some of the genes related in germ cell development. Piwi-like homolog 2 (Piwil2), known also as the Mili gene, is a known molecular marker of spermatogonia and participates in many processes such as gametogenesis and spermatogonial stem cell self-renewal (21). Hence, Piwil2 gene expression in different culture systems is required for an improvement in culture conditions a requisite step in optimal spermatogenesis (22). The key role of BMP4 in germ cell induction from mouse ESCs has not been shown *in vitro* until now. In this study, the effects of BMP4 and one type of feeder layer, mouse embryonic fibroblasts (MEFs), on Piwil2 gene-expression was studied quantitatively in order to find germ cell development from ESCs.

## Materials and Methods

### *Cell culture medium*

CCE mouse ESCs (Fig 1A), established from 129/Sv mouse strain (a kind gift from Dr. John Draper, Stem Cell Center, Sheffield University), were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing high concentrations of glucose, pyruvate and glutamine (Gibco, UK). Supplementation of cell culture medium was done using 20% fetal bovine serum (FBS; Gibco, UK), 3.7 gr/l $NaHCO_3$ (Sigma Aldrich, USA), 100 u/ml penicillin and 100 µg/ml streptomycin (Gibco, UK), 0.1 mM β-mercaptoethanol (Sigma Aldrich, USA), 1% nonessential amino acids (Sigma Aldrich, USA) and $10^3$ U/ml leukemia inhibitory factor (LIF; Sigma Aldrich, USA). The cells were cultured at 37°C, 5% $CO_2$ and 95% humidity and the medium was renewed daily.

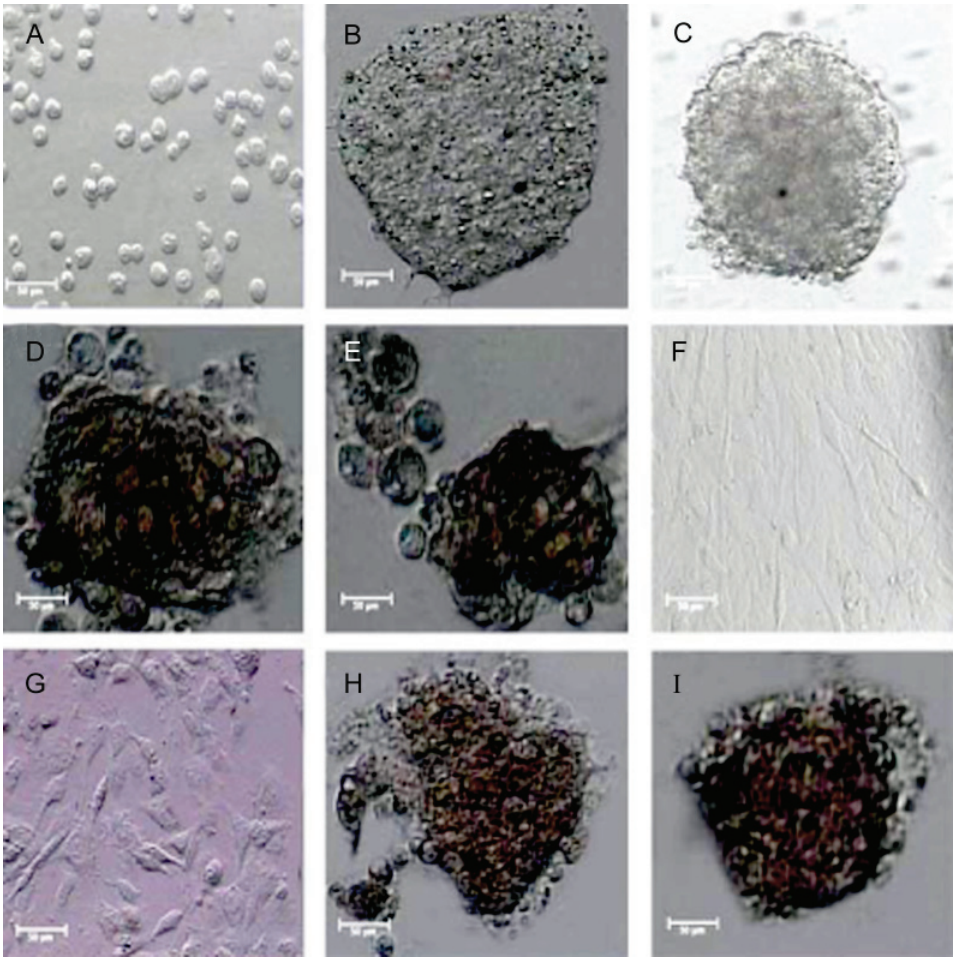


***Fig 1: Light microscope photograph of A: CCE mouse embryonic stem cells (ESCs), B: Undifferentiated colony of CCE mouse ESCs, C: One-day-old embryoid body (EB), D: Mouse embryonic fibroblasts (MEFs), E: Mitotically inactivated MEFs after treatment with mitomycin C. Cluster of CCE cells formed on F: Simple culture (SC) group, G: Simple culture with BMP4 (SCB) group, H: Indirect MEFs co-culture (CO-C) group and I: Indirect MEFs co-culture with BMP4 (CO-CB) group. Pictures were taken with the indicated resolutions.***

Undifferentiated ESCs (Fig 1B) were passaged every two days by trypsin (0.25%; Merck, Germany)/ EDTA (1 mM; Sigma Aldrich, USA) dissociation.

***Embryoid body formation***

Adherent mouse ESCs were tripsinized in order to form a single cell suspension. Cells were seeded ($2\times10^5$ cells) onto low-attachment 6-well culture dishes containing 3 ml of ES medium without LIF in order to EB formation (Fig 1C). After 24 hours, EB cells were tripsinized and replated ($8\times10^4$ cells) onto a 96 well microplate for continuation of the induction protocol.

***BMP4 treatment for germ cell induction***

The cells cultured in DMEM medium containing BMP4 (Sigma Aldrich) were considered as the SCB group (simple culture with BMP4; Fig 1D) and cells cultured in the absence of BMP4 were labeled as the SC group (simple culture; Fig 1E). BMP4 was added at a concentration of 5ng/ml (23). Culture duration was for four days and the medium was changed daily.

***Preparation of mouse embryonic fibroblasts (MEFs) feeder layer***

Thirteen to fourteen day-old pregnant NMRI mice were sacrificed by cervical dislocation. MEF cells (Fig 1F) were derived from 13-14 day-old embryos according to the protocol described by Abbondonzo et al. (24). A confluent layer of MEFs (~90%) was treated with 10 µg/ml mitomycin C (Sigma Aldrich, USA) for three hours and washed with PBS 3-4 times (Fig 1G).

***Co-culture with a feeder layer***

About $3\times10^5$ cells from dissociated EBs were replated into millicell 24-well cell culture insert plates. The inserts were placed in a 24 well plate containing mitomycin-treated MEF cells and cultured for four days in the presence of 5 ng/ml BMP4 as the CO-CB group (co-culture with BMP4; Fig 1H). Co-culture system without BMP4 (CO-C groups; Fig 1I) was considered as the control. The medium of co-culture groups consisted of DMDM with 20% FBS and was renewed daily.

***Quantitative real-time polymerase chain reaction (RT-qPCR)***

Total RNA was extracted from the testis, SC, SCB, CO-C and CO-CB groups using RNX-PlusTM (Cinnagen, Iran) according to the manufacturer's recommendations. In order to remove genomic contamination, RNA was treated with DNase I using a fermentase kit (Lithuania) based on the protocol described by the manufacturers. Concentrations of RNA were determined using UV spectrophotometer (DPI-1, Kiagen).

The cDNAs were synthesized from 500 ng DNase-treated RNA samples with a RevertAidTM first strand cDNA synthesis kit (Fermentase, Lithuania) using oligo(dT) primers. For PCR reactions, primers were adapted from others (21, 25) and synthesized by Cinnagen Company (Iran, Table 1).

PCRs were done using Master Mix (Cinnagen, Iran) and SYBR Green I (Fluka, Switzerland) in a Rotor-Gene3000 thermocycler (Corbett, Australia). The PCR program started with an initial melting cycle, 4 minutes at 94ºC, to activate the polymerase and followed by 40 cycles as follows: a melting step (20 seconds at 94ºC), an annealing step (30 seconds at 57ºC) and an extension step (30 seconds at 72ºC). After completing the PCR run, the quality of the reactions was confirmed by melting curve analyses. Efficiency was determined for each gene using a standard curve (the logarithmic dilution series of testis cDNA). For each sample, the reference gene (b2M) and the target gene were amplified in the same run. Ratio of gene expression was determined using the comparative CT (cycle threshold) method (26). Testis was used as the positive control. This work was approved by the Ethical Committee of Tarbiat Modares University.

***Statistical analyses***

The statistical analysis was done with SPSS 13.0 software using one way analysis of variance (ANOVA) and Tukey's post hoc test. The Mean normalized Piwil2 expression are shown as Mean ± SE. Each point represents the average of three separate experiments.

***Table 1: Quantitative RT-PCR primer sequences***

| Gene | Primer (forward/reverse) | Significance |
|---|---|---|
| **Piwil2** | 5′-CTTCTCGCCCCCTCCAGGT-3′ | Germ cell marker |
| | 5′-AAATAGAACCCCCAGGGTGAGC-3′ | |
| **$\beta_2$m** | 5′-TGACCGGCTTGTATGCTATC-3′ | Internal control |
| | 5′-CACATGTCTCGATCCCAGTAC-3′ | |

$p<0.05$ indicated the statistical significance of observed differences. Also, partial eta squared ($\eta_p^2$) was used 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 considered as weak, moderate, large and very large effect sizes, respectively (27).

## Results

### *Piwil2 expression analysis*

Piwil2 expression in the SC, SCB, CO-C and CO-CB groups was evaluated using RT-qPCR. Expression of Piwil2 was normalized relative to the housekeeping gene in each group and the mean normalized Piwil2 expression was compared between the different groups (Fig 2). The value of $\eta_p^2$ for Piwil2 was elevated, at about 0.74. This value confirmed the significant differences in data analysis between the different groups.

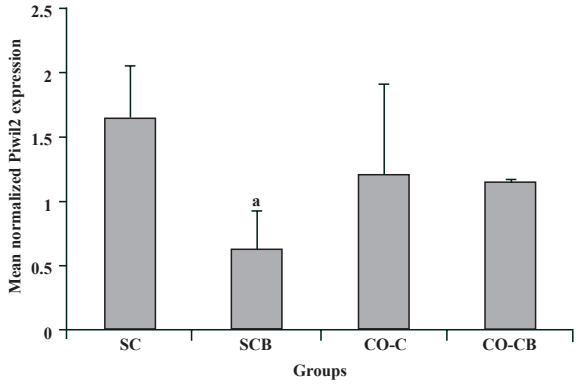


***Fig 2: The mean normalized expression of Piwil2 (y-axis) was shown in different groups of the study (x-axis) as follows: Simple culture (SC), Simple culture with BMP4 (SCB), Co-culture (CO-C) and Co-culture with BMP4 (CO-CB) groups. B2m was used as a normalizer. Bars represent standard errors. a shows significant difference with other groups ($p< 0.05$).***

The results obtained from this study showed that Piwil2 was expressed in testis. The maximum expression of Piwil2 was observed in the SC group (Fig 2). There were significant differences between Piwil2 expressions in the SC group when compared with the SCB group ($p<0.05$). The minimum level of Piwil2 expression was obtained in the SCB group (Fig 2) and showed significant differences with all other groups ($p<0.05$). The mean normalized expression of Piwil2 in the CO-C group was higher than the SCB and CO-CB groups (Fig 2). Expression of Piwil2 mRNA in the SC group was significantly different as compared to the SCB group ($p<0.05$). The Piwil2 expression in cells of the CO-CB group (Fig 2) was significantly higher relative to the SCB group ($p<0.05$).

## Discussion

Our results suggest that neither the presence of BMP4 in culture medium nor the employment of mouse embryonic fibroblasts as feeders have a positive effect on late germ cell derivation from mouse ESCs. *In vitro* germ cell development from ESCs has been demonstrated by several groups (6, 10, 12, 13, 15, 19). There are two different methods that have been reported for germ cell differentiation, monolayer differentiation (19) and EB formation (6, 10, 12, 13, 15, 20). Previous reports show that expression of Piwil2 increased in one day-old EBs relative to ESCs, suggesting that efficient differentiation of germ cells can happen during EB formation (28). Accordingly, EB was chosen as the starting point of germ cell induction in this study.

Our quantitative PCR data showed that the ratio of the Piwil2 gene concerned in germ cell development was significantly lower in BMP4-treated cells in the simple co-culture system compared to the other groups ($p<0.05$). Several investigators showed that BMP4 was specifically required for germ cell differentiation in mice (6-9, 11, 29). Lawson et al. reported that mutation in the BMP4 gene in the epiblast of a 7.5-9.5 day-old embryo resulted in no primordial germ cell (PGC) formation (7). Also, it was shown that 72 hour co-culture of a 6-6.25 day-old epiblast with BMP4 producing cells caused an increase in PGC number (8, 9). Furthermore, the appearance of PGCs in culture was reported by the addition of BMP4 to epiblast tissue (11, 29). Toyooka et al. also reported that one day co-culture of mouse ESCs that have Mvh-reporter with BMP4 producing cells resulted in Mvh positive cells (10). Kee et al. also demonstrated that addition of BMP4 to humam ESCs increased the Mvh marker (6). Mvh (VASA) is a known molecular marker of primordial germ cells, while Piwil2 is expressed in spermatogonia (16). Thus, based on our results and the above mentioned investigations, it seems that BMP4 is an efficient and potent inducer in primordial germ cell specification and not in late germ cell induction.

The molecular results of quantitative PCR also showed no significant differences in the expression of Piwil2 between co-culture systems and simple culture medium ($p>0.05$). These findings indicated that the use of MEF as a feeder has no apparent effect on late germ cell specification. *In vivo* germ cell differentiation affected by physical contact with a variety of cell types, influencing their cell-cycle status and differentiation (30). In this line, Eddy et al. showed that cell-cell contact within the testis controlled sequential regulation of genes and

caused *in vivo* germ cell differentiation (31). Hayashi et al. reported that a four day culture of 7.5-8.5 dpc epiblasts on top of the STO feeder layer in the presence of 100 ng/ml BMP4 has a positive effect on germ cell specification (29). In another investigation, West et al. assessed the effect of MEF feeders on germ cell specification from human ESCs and claimed that differentiation and enrichment of human germ-like cells was mediated by feeder cells (14). Differentiation of germ cells was also reported by co-culture of mouse ESCs with STO cells (12), or human ESCs with CF1 MEFs feeder layers (13). The important implication that arises from these results is the influence of physical contact on germ cell development. Physical contact is a requisite step in cell-cell recognition that is the basis for a functional relationship between two cell types (32). Thus, in this research the lack of interaction between two cell types due to indirect co-culture systems may be the cause of a decrease in germ cell derivation from mouse ESCs.

## Conclusion

Quantization of Piwil2, a known molecular marker for spermatogonia, in simple and MEFs co-culture systems both in the presence or absence of BMP4 showed that neither the use of mouse embryonic fibroblast as a feeder nor the addition of BMP4 in culture have positive effects on late germ cell specification from mouse ESCs.

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