Original Article

Effects of Different Doses of Bone Morphogenetic Protein 4 on Viability and Proliferation Rates of Mouse Embryonic Stem Cells

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Abstract

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Objective: In this study, we examined the effect of different doses of bone morphogenetic protein 4 (BMP4) on CCE mouse embryonic stem cells (ESCs) viability and proliferation rates in order to improve the outcome of induction processes and make a system with highest viability and proliferation rates for further studies on BMP4 roles in multiple developmental stages.

Materials and Methods: Expression of Oct-4 was studied and confirmed in this cell line immunocytochemically. Also, in order to evaluate the proliferation and viability rates in BMP4-treated cells, ESCs were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing different doses of BMP4 (0, 0.01, 0.1, 1, 5, 25, 50 and 100ng/ml). The mean number of whole cells and living cells were considered as proliferation and survival rates respectively. Data analysis was done with ANOVA test.

Results: The results showed that there were significant differences between the mean percent of viability between 1ng/ml and 0 ng/ml (control) and 50 and 100 ng/ml BMP4 ($p \leq 0.01$), as well as between 5 ng/ml and 0, 0.01, 0.1, 25, 50 and 100 ng/ml BMP4 ($p \leq 0.02$). Also, significant differences were observed in proliferation rates between 5 ng/ml and 0, 0.01, 0.1, 1, 25 and 100 ng/ml BMP4 ($p \leq 0.01$), 25 ng/ml and 0.01, 1 and 5 ng/ml BMP4 ($p \leq 0.01$), as well as between 50 ng/ml and 0.01 and 0.1 ng/ml BMP4 ($p \leq 0.001$).

Conclusion: The results suggest that addition of 5ng/ml BMP4 had the best effects on the proliferation and viability rates of CCE mouse ESCs.

Keywords: Embryonic Stem Cells, BMP4, Proliferation, Viability

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Introduction

Bone Morphogenetic Proteins (BMPs) are signaling molecules from the transforming growth factor b (TGFb) superfamily (1) and are thought to be released from extraembryonic tissues (2).

SMAD proteins (Sma and Mad-related proteins) are downstream signaling mediators for the TGFb superfamily members (3-5). BMPs signals act through type I and II serine–threonine kinase receptors that induces phosphorylation of the BMP-specific smads (Smads1, 5, 8). Phosphorylated Smads link with Smad4 and translocate into the nucleus for transcription of BMP-target genes (6, 7). However, the signaling networks that regulate ES cell biology such as proliferation and viability are complex, and our knowledge of these regulations is rather presently primitive.

Transforming growth factor b (TGF-b) superfamily proteins are important extracellular signaling proteins participating in many developmental and physiological processes (8-10). In the mouse, the roles of BMPs in the formation of skeletal system (11-14), heart, nervous system, urogenital system, mesoderm induction (15-19), as well as formation and early proliferation of primordial germ cells (PGCs) (20-22) have confirmed with targeted or spontaneous mutations in various BMPs, their receptors and Smads.

Members of BMP family play diverse roles in the formation, development and function of various vital systems during fetal life (1). However, the outcome of mutations in many BMPs is early embryonic lethality that disturbs the analyses of BMP roles during organogenesis or tissue homeostasis in the adult mouse (23). This finding reinforced by other researcher's studies that observed the mutations in the BMP type I receptors (Bmpr1a and Acvr1) (15, 16, 24), the BMP type II receptor (Bmpr2) (18), and signaling component, Smads, (Smad1, Smad5,

and Smad4) (25-27) precludes analysis of BMP signaling during organogenesis due to the early embryonic lethality. Also, BMP4 in organ or tissue culture acts in a dose dependent manner (1, 28-37), but little is known about the effects of different studied concentrations of this inducer in cell culture systems (38-40). To bypass these uncertainties, we used cell culture systems to evaluate the effect of different doses of BMP4 in order to improve the outcome of induction processes and make a system with highest viability and proliferation rates for further studies on the BMP4 roles in multiple developmental stages.

For the investigation of BMP4 effects, a system with pluripotent potential which resembles the epiblast is needed. Pluripotent stem cells derived from embryonic sources are well-defined cell types, and as a result have a profound potential for multilineage differentiation (41). Embryonic stem cells (ES cells) are derived from the inner cell masses of preimplantation embryos (42,43) and are capable of maintaining pluripotency even after being extensively cultured in-vitro (44) through exposure to leukemia inhibitory factor (LIF) in the culture medium (42, 45). LIF suppresses the differentiation of ES cells through a cell surface complex composed of LIF receptor ß (LIFRß) and gp130. These factors can activate the transcription factor STAT3. LIF removal from culture medium leads to the formation of embryo-like aggregates known as embryoid bodie (EBs) in a similar process of activation occurring in blastocyst stage embryos (46).

Materials and Methods

Cell line

CCE is a mouse embryonic stem (ES) cell line derived from the 129/Sv mouse strain and has been provided for research use only. This cell line has been adapted to grow on gelatin-coated culture plates and with the appropriate medium does not require a primary embryonic fibroblast (PEF) feeder layer. This cell line gift from Dr. John Draper, Stem Cell Center, Sheffield University and This work approved by ethical committee of Tarbiat modares University

Cell Culture

Mouse ES cells were cultured in gelatinized tissue culture plates. They were cultured in Dulbecco´s modified Eagle's medium (DMEM) with high levels of glucose, pyruvate and L-Glutamin (GIBCO), and were supplemented with 15% heat-inactivated FBS (GIBCO), 3.7 g/L NaHCO3 (Sigma Aldrich), 0.1 mM ß-mercaptoethanol (Sigma Aldrich), 1% nonessential amino acids (Sigma Aldrich), 100 µg/ml penicillin and 100 µg/ml streptomycin (GIBCO). 10^3 U/ml LIF (Sigma Aldrich) was added to this medium to maintain the undifferentiated state of the ES cells. The cells were incubated in 5% CO_2 and 95% atmospheric air at 37º C, and the medium was renewed every day.

Passage of mouse embryonic stem cells

For passaging, the medium was aspirated, and the dishes were rinsed once with PBS. Trypsin-EDTA (0.25%) was added enough to cover the surface of the tissues in the culture dish which was incubated at room temperature (20ºC) until the cells lifted off the plate and were pippeted. To inhibit Trypsin-EDTA, 15% FBS was added to the DMEM. After pipeting, the cell suspension was centrifuged at 250 gravity for 5 minutes at room temperature, and the cells were split into new tissue culture dishes for subsequent experiments. For immunocytochemistry, the cells were cultured on gelatinized coverslip dishes for one and two days and LIF was removed to induce embryonic body (EB) formation.

Immunocytochemistry

After EB formation, the cover slips were transferred onto slides. Adherent EBs were fixed with freshly prepared 4% paraformaldehyde (Sigma–Aldrich) for 20 minutes at room temperature, washed with PBS, incubated with HCl (2N) for 30 minutes at room temperature for antigen retrival, rinsed with burate buffer twice, permeabilized with 3% Triton X-100 in PBS, and nonspecific reactions were blocked with 10% normal goat serum. EBs were incubated for 1 hour with Oct 4 antibody (Chemicon) diluted 1:100 in phosphate buffered saline. After 3 PBS washes, the preparations were incubated for another 30 minutes with a 1:50 dilution of secondary FITC anti-rabbit IgG antibody (Chemicon) and finally were dried and mounted. The intensity of the reaction (intensity of positive staining) was determined based on the arbitrary scale from Gong et al. from 0 to 4 (No Reaction Too Strong) (47).

BMP4 treatment

CCE ES cells from passage 2 were tripsinized and a cell suspension was prepared. The cells were counted and cultured in a 96-well microplate. Each well of this plate contained $3X10^4$ cells in 20% FBS in DMEM media. The cells were incubated for one day, washed with PBS, and then cultured for one day in DMEM containing different doses of BMP4 (0.01, 0.1, 1, 5, 25, 50 and 100 ng/ml) as experimental groups. The control group was cultured in a BMP4-free medium. ES cells were incubated at 37°C overnight, washed with PBS, tripsinized and a cell suspension was prepared separately from each well. In order to investigate the viability and proliferation rates of CCE ES cells, staining with Trypan blue and counting were done. The mean number of whole cells and living cells were considered as proliferation and survival rates respectively.

Data analysis

For evaluation of viability and proliferation rates of BMP4-treated cells in different concentrations, the data is presented as mean±standard deviation. Each data point represents the average of three separate experiments with five repeats in each experiment. The one way ANOVA and Tukey post-tests were used to determine the statistical significance of observed differences

in the mean values derived from the SPSS statistical software (SPSS 15.0 Production Mode Facility). P value <0.05 indicated statistical significance.

Results

Expression of Oct-4

As ES cells are considered to model epiblast cells, it was necessary to prove that this cell line has an undifferentiated state. Thus, as our first step, the marker of pluripotency, Oct-4, was studied and the pluripotency of CCE mES cells was confirmed with a positive Oct-4 immunocytochemistry reaction. This reaction diminished in 2 day-old EBs relative to 1day-old EBs (Fig 1).

Evaluation of Viability percent

The mean percent of living cells showed that BMP4 in 5 (69.86 ± 11.87) and 100 (35.34 ± 10.03) ng/ml concentrations had the best and the worst effects on the viability percent of ES cells respectively. The results showed that there were significant differences among the mean percent of viability in 1ng/ml and control (0 ng/ml) medium concentrations compared to that of 50 and 100 ng/ml BMP4 concentrations ($p \leq 0.01$). Also, significant differences were observed between 5 ng/ml in comparison with 0, 0.01, 0.1, 25, 50 and 100 ng/ml BMP4 concentrations ($p \leq 0.02$) (Table 1 and Fig 2).

Table 1. The mean viability percent and proliferation rate of CCE mouse embryonic stem cells after exposure to different doses of BMP4.

BMP4 dose (ng/ml)	Viability percent (Mean±SD)	Proliferation rate (Mean±SD)
0	40.36 ± 9.87 (a, b)	0.19 ± 0.34 (c)
0.01	43.56 ± 9.43 (b)	-0.55 ± 0.08 (c, d, e)
0.1	39.8 ± 0.95 (b)	-0.57 ± 0.11 (c, d, e)
1	59.42 ± 12.53	0.36 ± 0.24 (c)
5	69.86 ± 11.87	1.38 ± 0.89 (d)
25	45.54 ± 10.6 (b)	0.59 ± 0.54 (c)
50	40.28 ± 14.17 (a, b)	0.82 ± 0.42
100	35.34 ± 10.03 (a, b)	0.15 ± 0.26 (c)

a: Significant differences in viability percent with 1 ng/ml BMP4 ($p \leq 0.01$), b: Significant differences in viability percent with 5 ng/ml BMP4 ($p \leq 0.02$), c: Significant differences in proliferation rate with 5 ng/ml BMP4 ($p \leq 0.01$), d: Significant differences in proliferation rate with 25 ng/ml BMP4 ($p \leq 0.01$), e: Significant differences in proliferation rate with 50 ng/ml BMP4 ($p \leq 0.001$)

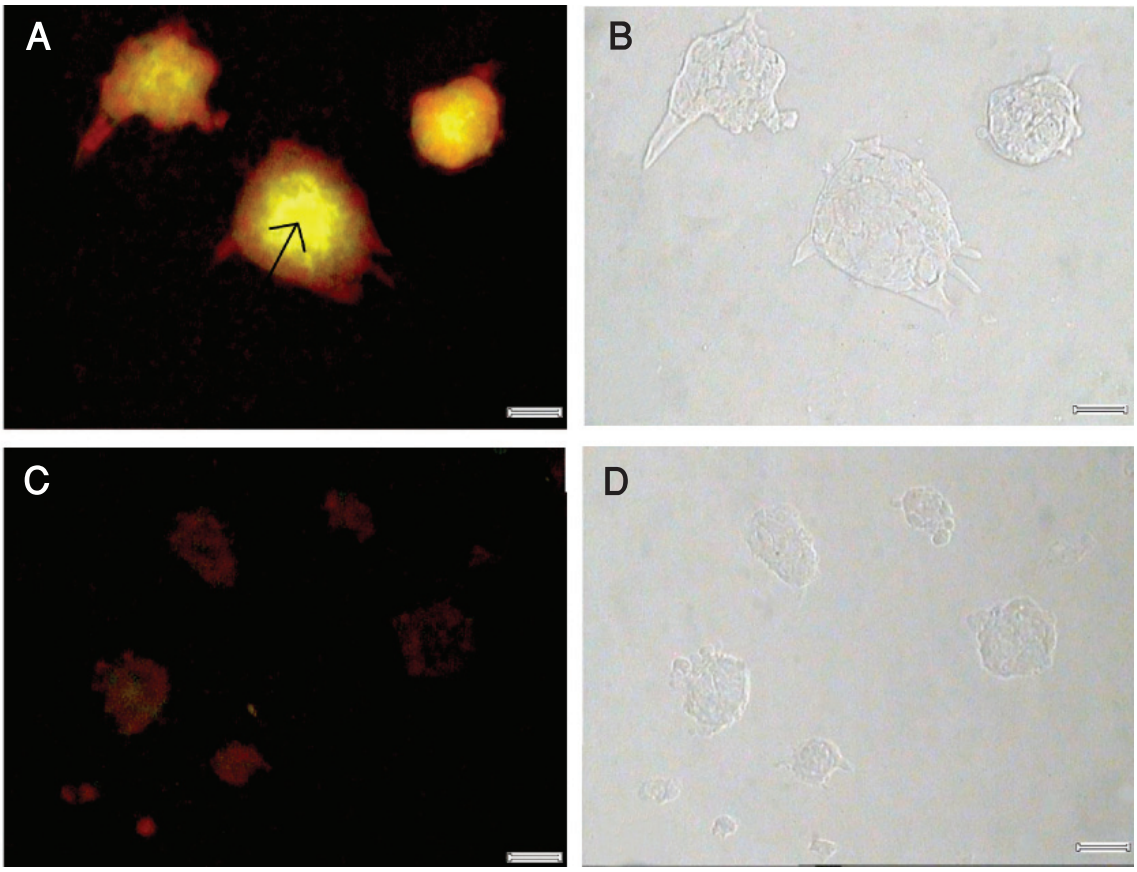

Fig 1: Oct-4 immunocytochemistry reaction (arrow): A. 1-day old EBs, B. phase contrast of (A), C. 2-days old EBs, D. phase contrast of (C), Scale bar=30 µm.

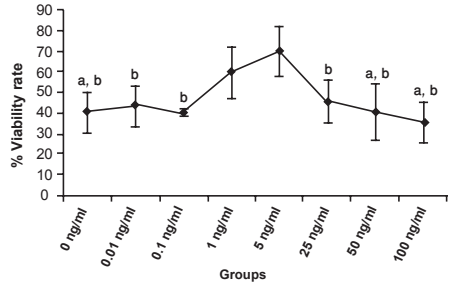

Fig 2. The comparison between the mean percent of viability rates in experimental (0.01, 0.1, 1, 5, 25, 50 and 100 ng/ml BMP4) and control (0 ng/ml BMP4) groups.
a: Significant differences with 1 ng/ml BMP4 (p≤0.01)
b: Significant differences with 5 ng/ml BMP4 (p≤0.02)

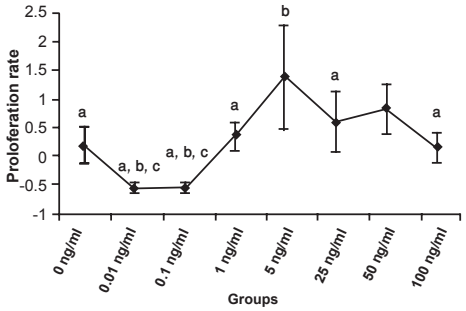

Fig 3: Comparison between the mean proliferation rates in experimental (0.01, 0.1, 1, 5, 25, 50 and 100 ng/ml BMP4) and control (0 ng/ml BMP4) groups.
a: Significant differences with 5 ng/ml BMP4 (p≤0.01)
b: Significant differences with 25 ng/ml BMP4 (p≤0.01)
c: Significant differences with 50 ng/ml BMP4 (p≤0.001)

Evaluation of Proliferation Rate

Statistically analyzed data showed that the best and the worst effects on the proliferation rate were seen in 5 (1.38 ± 0.89) and 0.1 (-0.5 ± 0.11) ng/ml BmP4 respectively. Also, when the mean proliferation rates in different BMP4 concentrations were compared with each other, the following results were found: significant differences were observed when 5 ng/ml BMP4 was compared to 0, 0.01, 0.1, 1, 25 and 100 ng/ml BMP4 concentrations (p≤0.01). Significant differences were also found when 25 ng/ml BMP4 was compared to 0.01, 1 and 5 ng/ml BMP4 concentrations (p≤0.01). Also, significant differences were seen between 50 ng/ml and 0.01 and 0.1 ng/ml BMP4 concentrations (p≤0.001) (Table 1 and Fig 3).

Discussion

Initially in this study, Oct-4 immunocytochemistry was done for CCE mouse ES cells. Mouse embryonic stem (ES) cell self-renewal depends upon extrinsic signals from leukemia inhibitory factor (LIF) that activate the nuclear localization of the latent transcription factor STAT3 and, the Oct4, a member of the POU family of homeodomain proteins, as an intrinsic factor (48, 49). Pluripotent cells differentiate to somatic lineages at the blastocyst stage and the expression of Oct-4 downregulate simultaneously. Embryonic stem cell lines express Oct-4 only if they remain undifferentiated and when are triggered to differentiate, Oct-4 is downregulated thus providing a model in the developing embryo for the early events linked to somatic differentiation (50, 51). Experiments indicate that an Oct-4 expression in necessary for self-renewal and maintenance of ESC pluripotency (52).

In the present study, the pluripotency of CCE mouse ES cells was confirmed with a positive Oct-4 immunocytochemistry reaction. Also, the intensity of the reaction decreased in 2 day-old EBs in comparison to 1 day-old EBs. These results indicate that the pluripotency of ES cells decreased along the time of LIF removal, and that the contributions of both LIF and Oct-4 pathways provide maximal self-renewal efficiency. They also indicate that 1 day-old EBs are more suitable for the induction process.

Our results also showed that the viability percent and proliferation rate of CCE mouse embryonic stem cells change in a dose dependent manner upon the addition of BMP4 in the culture. In line with our results, several other investigators also reported that BMP4 in organ or tissue culture acts in a dose dependent manner (1, 28-37). In an experiment, Dudley observed that BMP signaling affected both PGC numbers and motility in an organ culture assay in a dose dependent manner (1). In addition, Hayashi et al. demonstrated that the average number of PGCs from epiblasts cultured with extraembryonic ectoderm was comparable to that of the epiblasts cultured with recombinant human BMP4 at 100ng/ml and that the treatment of epiblasts with 500ng/ml recombinant human BMP4 gave rise to more PGCs (29). In other investigations, dose dependent effects of BMP4 also were shown in somatic tissue cultures such as in skeletal system formation (34, 35), neurogenesis (39), hair cell development in otocytes (36) and in hematopoietic progenitor cell formation (37).

Different levels of BMP signaling may have different effects on cell behavior (1). It has been shown that low doses of BMP4 can induce differentiation of hematopoietic stem cell precursors, whereas high doses promote self renewal (53). Our results demonstrated that the medium BMP4 dose (5 ng/ml) promoted while low and high doses inhibited the proliferation and viability rates of mouse embryonic stem cells. These findings are in agreement with the result of other studies on optimum doses of BMP4 in tissue cultures. For example, Kiyono and Buckley showed that a concentration of 50 ng/ml or higher of BMP4 was required to induce the apoptosis of A549 lung adenocarcinoma and HUVEC capillary endothelial cells (31, 32). Dudley observed that low-dose (5 ng/ml) of BMP4 increased PGC numbers, whereas higher doses (50 and 500 ng/ml) had no effect or actually reduced PGC numbers. (1). The results of Liu demonstrated that low BMP4 doses (5 ng/mL) promoted while high dose (100 ng/mL) inhibited the proliferation of SVZa neural stem cells (39). Li also reported the 5ng/ml BMP4 as the optimum dose which increases the number of hair cells in otocytes (36). Thus, it seems that different doses of BMP4 signaling may also have different effects on ES-cell behavior. In other words,

the proliferation and viability rates are associated with addition of BMP4 in the culture in a dose dependent manner. Hayashi found that BMP4-induced differentiation of in vitro epiblasts was fully dependent on the existence of phosphorylated SMAD1 (29). Ying showed that in presence of BMP4, phosphorylation of SMAD1 increases in embryonic stem cells (54). BMP4 proteins may phosphorylate SMAD proteins in ES cells and consequently ES cells themselves, acquiring SMAD (1, 5, 8) activation above a certain threshold, and through this concentration induce the expression of a particular set of genes involved in proliferation and viability. As Wilson and Dosch reported, it is likely that different sets of genes may be induced at different concentrations of BMPs (29, 55, 56).

Also, BMP4 stimulation may increase the expression of certain genes, such as p38, which are involved in the self-renewal process in a dose dependent manner. The expression of these genea depends on the activation of kinasis proteins in the extra cellular signal regulated kinase (ERK) pathway which is involved in mitosis (53). In summery, the signaling networks regulating ES cell biology are complex, and our knowledge of these regulations is presently rather primitive. Hence, effects of BMP4 on embryonic stem cell biology need to be further investigated.

Conclusion

In conclusion, our results confirmed the CCE mouse ES cell-line pluripotentability, and showed that 5ng/ml BMP4 produced the best effects on its proliferation and viability rates. Therefore, 5ng/ml BMP4 is proposed to be the optimal dose among the different doses studied in this research in order to increase the success rate of BMP4 induction in specific differentiation patterns.

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