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Research Article

The ability of mouse nuclear transfer embryonic stem cells to differentiate into primordial germ cells

Vahid Mansouri[1], Mohammad Salehi[2,3], Mohsen Nourozian[1], Fatemeh Fadaei[1], Reza Mastery Farahani[1], Abbas Piryaei[1] and Ali Delbari[4]

[1]*Department of Biology and Anatomical Sciences, Faculty of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran.*
[2]*Cellular and Molecular Biology Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran.*
[3]*Department of Biotechnology, Faculty of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran.*
[4]*Department of Anatomical Sciences, Faculty of Medicine, Sabzevar University of Medical Sciences, Sabzevar, Iran.*

Abstract

Nuclear transfer embryonic stem cells (ntESCs) show stem cell characteristics such as pluripotency but cause no immunological disorders. Although ntESCs are able to differentiate into somatic cells, the ability of ntESCs to differentiate into primordial germ cells (PGCs) has not been examined. In this work, we examined the capacity of mouse ntESCs to differentiate into PGCs *in vitro*. ntESCs aggregated to form embryoid bodies (EB) in EB culture medium supplemented with bone morphogenetic protein 4(BMP4) as the differentiation factor. The expression level of specific PGC genes was compared at days 4 and 8 using real time PCR. Flow cytometry and immunocytochemical staining were used to detect Mvh as a specific PGC marker. ntESCs expressed particular genes related to different stages of PGC development. Flow cytometry and immunocytochemical staining confirmed the presence of Mvh protein in a small number of cells. There were significant differences between cells that differentiated into PGCs in the group treated with Bmp4 compared to non-treated cells. These findings indicate that ntESCs can differentiate into putative PGCs. Improvement of ntESC differentiation into PGCs may be a reliable means of producing mature germ cells.

Keywords: differentiation, germ cells, nuclear transfer embryonic stem cells.

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Introduction

Pluripotent stem cells capable of differentiation into other cell types offer a potentially important therapeutic option for regenerative medicine (Wang *et al.*, 2010). Adult stem cells may be few in number and embryonic stem cells (ESCs) may be rejected by an immune response (Bongso *et al.*, 2008). Nuclear transfer from a somatic cell to an enucleated oocyte has been used to generate nuclear transfer embryonic stem cells (ntESCs) (Gurdon and Wilmut, 2011). These stem cells are genetically identical to the somatic donor cell and do not cause an immune reaction (Hochedlinger *et al.*, 2004).

Send correspondence to Mohammad Salehi and Fatemeh Fadaei. Department of Biotechnology, Department of Biology and Anatomical Sciences, Faculty of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran. E-mail: m.salehi@sbmu.ac.ir.

Somatic nuclear reprogramming leads to totipotency and ntESCs can be differentiated into somatic cells, including primordial germ cells (PGCs). PGCs undergo differentiation during the embryonic period of mammalian life (Hübner *et al.*, 2003). Defects in PGC differentiation may lead to infertility in adults (Dunlop *et al.*, 2014). ESC differentiation into PGCs has been investigated in mice and humans (Clark *et al.*, 2004; West *et al.*, 2006). *In vivo* fate of PGCs can contribute to the remodeling of cell cultures system, which may improve the differentiation of ntESCs into PGCs *in vitro* (Aflatoonian and Moore, 2005). In mice, PGCs are initially specified on the basis of allantois at embryonic day 7.5 (E7.5) (Ginsburg *et al.*, 1990). PGCs are associated with the hindgut wall at E9 and emerge from the dorsal side of the gut at E9.5 to migrate laterally and settle in the genital ridges. Once PGC colonization of the gonads is complete these cells are referred to as gonocytes (McLaren *et al.*, 2003). PGCs express specific genes during dif-

ferent periods of differentiation, including Prdm14 during specification, c-Kit during migration and Mvh during colonization (Seki *et al.*, 2007; Young *et al.*, 2010; Nakaki and Saitou, 2014).

Extrinsic factors such as bone morphogenetic protein4 (BMP4) and retinoic acid can induce the differentiation of PGCs from ESCs *in vitro* (Drummond, 2005; Kee *et al.*, 2006). ntESCs can differentiate into different cell types, including embryonic stem cells (Byrne *et al.*, 2007), cardiomyocytes (Lü *et al.*, 2008) and renal cells (Lanza *et al.*, 2002). However, the differentiation of ntESCs into PGCs has not been reported. In this study, we investigated the ability of ntESCs to differentiate into putative PGCs *in vitro* by examining the expression of specific genes and proteins.

Materials and Methods

Nuclear transfer embryonic stem cell (ntESC) culture

The ntESC line was obtained from the Stem Cells Technology Research Center (Farifteh *et al.*, 2014). ntESCs were maintained on mouse embryonic fibroblasts (MEF) treated with mitomycin-C (Invitrogen, USA). ntESCs were grown in ESC medium containing knockout Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% FBS (Gibco), penicillin/streptomycin (0.1 mg/mL; Sigma-Aldrich), 1% nonessential amino acids (Life Technologies), 0.1 mM 2-mercaptoethanol (Invitrogen), 1 mM sodium pyruvate (Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich) and leukemia inhibitory factor (LIF) (1000 IU/mL; Sigma-Aldrich). The culture medium was replaced every two days until ntESC colonies were obtained.

Embryoid body (EB) formation

ntESC colonies were trypsinized and the cells seeded in non-adherent six-well culture dishes containing 3 mL of ESC medium without LIF for embryoid body (EB) formation. After 24 h, EBs were observed in the center of each well.

Induction of primordial germ cell (PGC) differentiation

After the first day of EB formation, the EBs were allocated to one of two groups: a test group treated with 10 ng of BMP4/mL (recombinant mouse; R&D Systems) and a control group without BMP4. The medium was replaced every two days over an 8-day period.

RNA extraction and quantitative polymerase chain reaction (qPCR)

Total RNA was extracted from test and control group cells on days 4 and 8 for quantification of gene expression using an RNeasy kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. cDNA synthesis was done using a Prime Script First Strand cDNA synthesis kit (Qiagen) with 1 ng of total RNA. Real-time PCR reactions consisted of 40 cycles at 95 °C for 10 s and 60 °C for 35 s. Quantitative real-time PCR was done in duplicate for samples obtained from separate experiments using a SYBR Premix Ex Taq II kit (Takara, Japan). Gene expression was normalized relative to the housekeeping gene Hprt in each group and the mean normalized gene expression was compared between groups. The primer sequences used for real-time PCR are listed in Table 1.

Flow cytometry

Monoclonal antibodies were used to examine the expression of Mvh protein. The cells were dissociated using 0.25% trypsin (Gibco BRL) and then fixed with paraformaldehyde on the eighth day of culture. The cells were subsequently permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) to allow access to cytoplasmic Mvh protein and then incubated with primary antibody (mouse IgG_1 = 1:200; isotype control = 1:200; Antibodies-online GmbH, Aachen, Germany) overnight followed by a secondary anti-

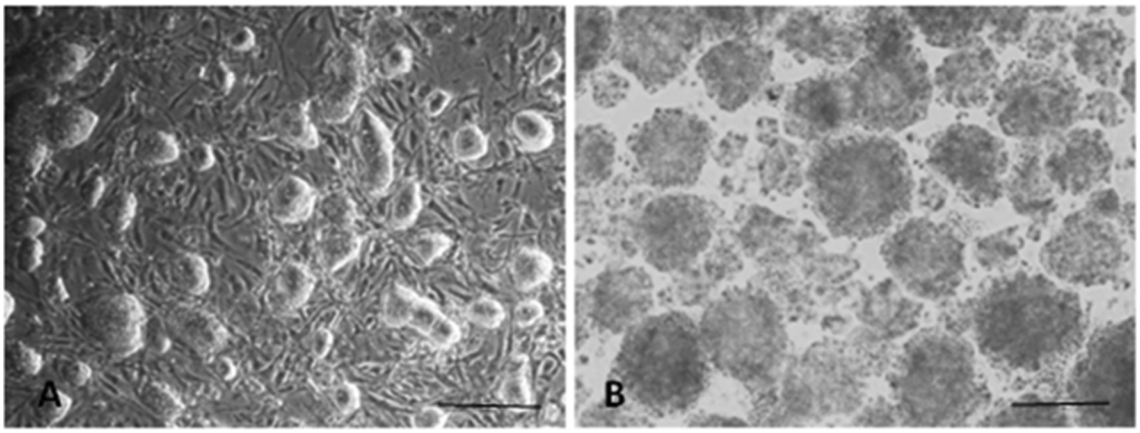

Figure 1 - Nuclear transfer embryonic stem cells and embryoid bodies (A) ntESC colonies cultured on MEF and (B) EB aggregates on day 6 of culture. Scale bars: 100 µm.

Table 1 - Primer sequences used in quantitative real-time PCR.

Gene	Primer sequences
Oct4	F: 5'CACCATCTGTCGCTTCGAGG3' R: 3'AGGGTCTCCGATTTGCATATCT5'
Prdm14	F: 5'CTCTTGATGCTTTTCGGATGACT3' R: 3'GTGACAATTTGTACCAGGGCA5'
C-Kit	F: 5''TGTCTCTCCAGTTTCCCTGC3' R: 3'TTCAGGGACTCATGGGCTCA5'
Mvh	F: 5'ATGCACCACCGGCAATTTTG3' R: 3'TCCTGCTAATACAATGGGAAT5'
Hprt	F: 5'TCAGTCAACGGGGGACATAAA3' R: 3'GGGGCTGTACTGCTTAACCAG5'

body (rabbit polyclonal IgG = 1:50; Antibodies-online GmbH) in the dark for 30 min. The cells were then stored in 10% paraformaldehyde at 4 °C and analyzed with an Attune flow cytometer (Applied Biosystems, USA) using FlowJo 7.6 software.

Immunocytochemistry

EBs were fixed in 4% paraformaldehyde at 4 °C for 20 min on day 8 and then washed twice with phosphate-buffered saline (PBS) containing 0.05%Tween 20 (Life Technologies) for 5 min. The cells were subsequently placed in 0.4% Triton X-100 (Life Technologies) for 30 min, after which they were washed with PBS and non-specific binding sites were blocked with 10% goat serum + 1 mg of BSA/mL (Gibco) at 25 °C for 45 min. Subsequently, the cells were incubated overnight in 1% BSA in PBS containing primary antibody (rabbit anti Mvh; Antibodies-online GmbH) followed by secondary antibody (rat anti-rabbit IgG = 1:100; Antibodies-online GmbH) for 2 h and then washed with PBS to remove excessive antibodies and allow the visualization of endogenous Mvh protein. Cell nuclei were stained for 30 s with DAPI solution (Sigma-Aldrich) and then visualized and photographed using an immunofluorescent microscope (Nikon Eclipse 2000).

Statistical analysis

Real-time PCR data were analyzed with REST 2009 software (Qiagen). Numerical data were reported as the mean ± SD. Student's *t*-test was used to compare the flow cytometry results. All data analyses were done with SPSS 17.0 software (SPSS, Chicago, IL, USA), with $p < 0.05$ indicating significance.

Results

Embryoid body (EBs) formation

ntESC colonies formed on the feeder layer (Figure 1A) and the detachment of ntESCs from the MEF layer followed by replacement of the cell culture medium with EB culture medium resulted in EB formation (Figure 1B). Bmp4 was added to EB medium on the second day after EB formation in the test group but was omitted from the control group.

Quantitative real-time PCR

Expression of the Oct4, Prdm14, C-kit and Mvh genes in the test group was compared to the control group on days 4 and 8 (Figure 2). Oct4 expression was down-regulated in the test group compared to the control group on day 4, whereas there was no significant difference in the gene expression of Prdm14, C-kit and Mvh at this interval. On day 8, the gene expression of Prdm14 was significantly upregulated in the test group compared to the control group whereas the Mvh gene was significantly down-regulated in the test group. The gene expression of Oct4 and C-kit was also down-regulated in the test group on day 8, but the decrease was not significantly different from that in the control group.

The gene expression of Oct4, Prdm14, Mvh and C-kit in the test and control groups on days 4 and 8 was also compared. Oct4 expression was significantly up regulated on day 8 in both groups (Figure 3). As shown in Figure 3A, Prdm14 expression was down-regulated in the control

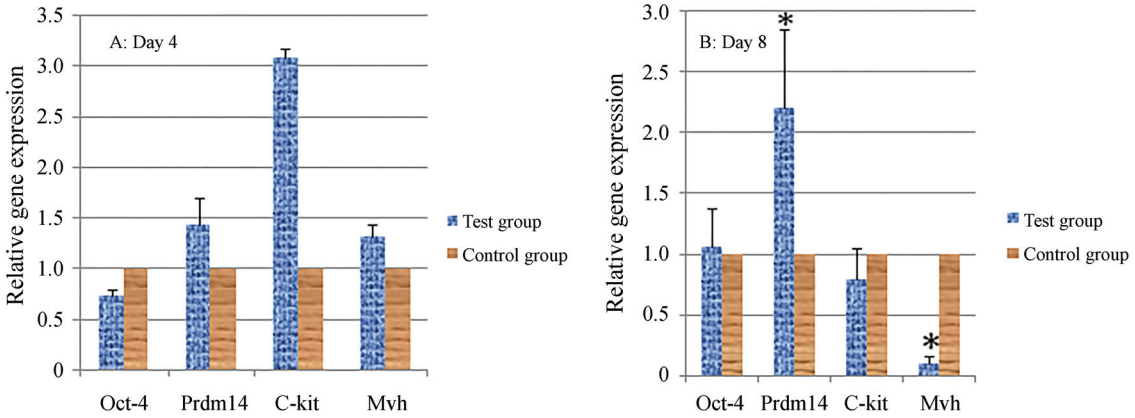

Figure 2 - Relative mRNA expression of Oct-4, Prdm14, C-kit and Mvh in test group compared to the control group on days 4 (A) and 8 (B). The columns are the mean ± SD (n = 2). *$p < 0.05$ compared to control group.

group on day 8 compared to day 4, whereas Prdm14 expression was significantly up-regulated in the test group (Figure 3B). Mvh gene expression was low in the control group and was significantly down-regulated in the test group (Figure 3B). C-Kit expression was higher in the control group compared to the test group, and was significantly down-regulated in the test group on day 8 compared to day 4.

Flow cytometry and immunocytochemistry

Flow cytometry on day 8 of EB formation revealed the presence of Mvh protein in EBs as a specific marker of PGCs. The percentage of Mvh positive cells in the total cell population was 1.69 ± 0.23% and 2.93 ± 0.04% in the control and test groups, respectively. EBs in the test group elicited a response that was significantly stronger than that in the control group (Figure 4). Immunocytochemical analysis on day 8 confirmed the presence of Mvh positive cells in EBs (Figure 5).

Discussion

Previous studies have described the ability of ESCs to differentiate into PGCs (Hamidabadi *et al.*, 2011). Our results, based on the analysis of Oct4, Prdm14, C-Kit and Mvh gene expression, also showed that ntESCs were capable of differentiating into putative PGCs.

Oct4 is expressed by ESCs as a pluripotency gene (Chew *et al.*, 2005), although Fuhrmann *et al.* (2001) reported that Oct4 is a PGC-surviving gene when differentiated from ESCs. The significant up-regulation of Oct4 expression on day 8 explained the effect of Bmp4 on ntESC differentiation into putative PGCs in the test group. Significant up-regulation of Prdm14 on day 8 in the test group revealed that ntESCs could initiate the period of specification leading to differentiation under the effect of Bmp4. Prdm14 maintains the expression of Oct4 in PGCs (Magnúsdóttir and Surani, 2014). ntESCs expressed both Prdm14 and Oct4 on days 4 and 8 in the test group treated with Bmp4 compared with significant down-regulation of Prdm14 in the control group. Prdm14 regulates a variety of genes involved in cell-cell adhesion and migration (Magnúsdóttir *et al.*, 2013).

C-kit is expressed during the migration of PGCs to the genital ridge (Seki *et al.*, 2007) and contributes to the adhesion of PGCs to somatic cells in culture (Pesce *et al.*, 1997). C-kit can prevent PGC apoptosis and stimulate their proliferation *in vitro* (De Felici *et al.*, 2005). In female mice, C-kit is abundant in proliferating germ cells, is absent in germ cells once they undergo the transition to early meiosis, and is detectable again once the oocytes enter the diplotene stage of meiosis after birth (Motro and Bernstein, 1993; Pesce *et al.*, 1993; Tanikawa *et al.*, 1998). Down-regulation of C-kit in the test group on day 8 indicated that putative PGCs may loosen cell adhesions to pass from the migration phase to early colonization phase (Gu *et al.*, 2009). Bmp4 appeared to affect the down-regulation of C-kit expression in the test group, thus confirming the presence of ntESCs in the migratory phase.

Mvh is a gene specifically associated with the period of colonization in PGCs (Toyooka *et al.*, 2000). A study by Tooyoka *et al.* (2003) demonstrated that only PGCs that differentiated from ESCs could express both Mvh and Oct4 during the colonization period. Overall changes in germ cell maturation with increasing gestation in human involve a switch from Oct4 expression in primitive germ cells (gonocytes) to Vasa (Mvh in mice) expression in more mature germ cells (oogonia) (Anderson *et al.*, 2007). Mvh gene expression is up-regulated in ESCs that differentiate to PGCs during the initiation of meiosis (Noce *et al.*, 2001). The significant down-regulation of Mvh and up-regulation of Oct4 in the test group on day 8 was particularly marked. ntESCs could enter the early colonization period of differentiation under the effect of Bmp4 and in the absence of meiosis.

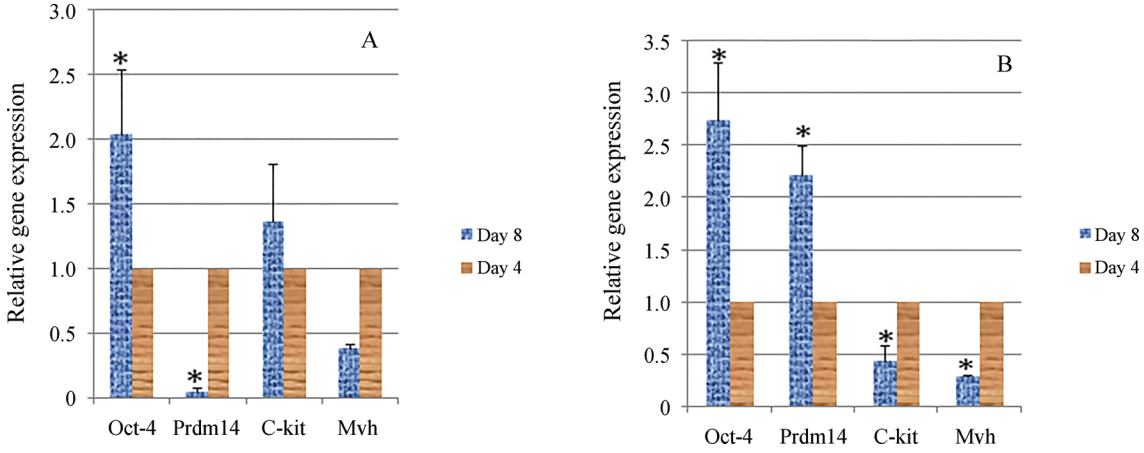

Figure 3 - Relative mRNA expression of Oct-4, Prdm14, C-kit and Mvh in control (A) and test (B) groups on day 8 compared to day 4 as analyzed by real-time PCR. The columns are the mean ± SD (n = 2). *p < 0.05.

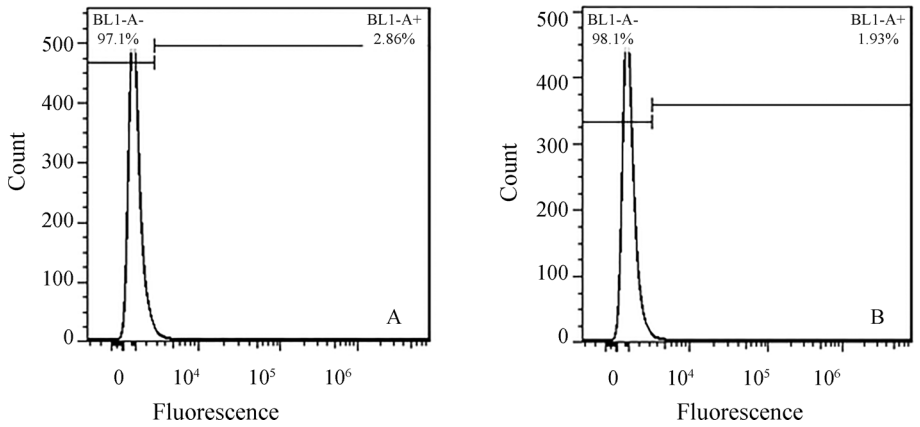

Figure 4 - Flow cytometry showing the ratio of Mvh-positive cells in the test (A) and control (B) groups.

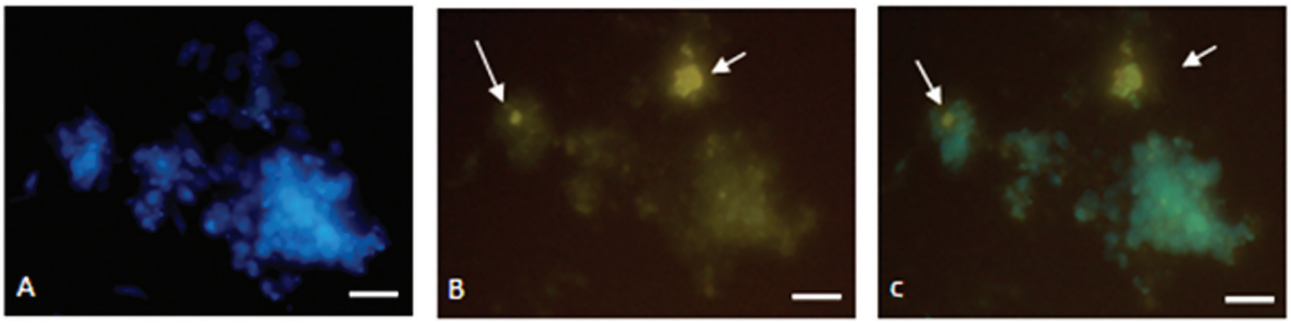

Figure 5 - Immunoflourescence images of embryoid bodies (EBs) at day 8. (A) Nuclear transfer embryonic stem cell (ntESC) nuclei stained with DAPI are shown in blue. (B) Anti-Mvh staining was detected with primary and secondary polyclonal IgG antibodies. Cell aggregates with green cytoplasm (arrows) indicate Mvh-positive cells in the EB. (C) Overlaying the images of EBs shown in panels (A) and (B) cytoplasm in green and nuclei in blue. Arrow indicates Mvh-positive cells with green cytoplasm. Scale bars: 50 μm.

In ntESCs, the transcription of germ cell-specific genes is linked to translation via the expression of Mvh protein. Flow cytometry revealed the expression of Mvh protein in ntESCs in both the test and control groups on day 8. Mvh is a specific marker of PGCs (Toyooka *et al.*, 2003). Other investigations have studied SSEA1 as a membranous marker of PGCs. The ratio of SSEA1-positive cells in previous reports was higher than for Mvh because SSEA1 is also a pluripotency marker of ESCs (Toyooka *et al.*, 2003; Saiti and Lacham-Kaplan, 2008; Geijsen *et al.*, 2004). For this reason, in this study, Mvh protein expression was chosen as a special PGC marker rather than SSEA1. The significantly higher ratio of ntESCs expressing Mvh protein in the test group compared to the control group revealed that Bmp4 could affect the ratio of Mvh positive cells in the former group.

Studies *in vitro* have shown that female germ cells differentiate from ESCs when left in contact with surrounding cells in EBs (Lacham-Kaplan *et al.*, 2006; Novak *et al.*, 2006), whereas male germ cells are derived when isolated from their surrounding cells (Toyooka *et al.*, 2003; Geijsen *et al.*, 2004). The advantage of PGC isolation is that it will enrich the limited number of PGCs, as large quantities are required for culture and transplantation. Flow cytometry could not separate PGCs from other cells in EBs. Many reports have used immunochemistry-based sorting as the common means of isolating PGCs for Mvh positive cells in EBs, as was the case in this study (Geijsen *et al.*, 2004; Nayernia *et al.*, 2006; Anderson *et al.*, 2007).

Some studies have shown that the addition of BMP4 to the medium, or simply co-culturing cells with BMP4-producing cells as feeders, does not necessarily stimulate PGC production from ESCs. Some researchers have suggested that ESCs can spontaneously differentiate to PGCs and other cells (Eguizabal *et al.*, 2009). However, other studies have emphasized the effect of different concentrations (20, 50 and 100 ng/mL) of BMP4 on PGC differentiation (Wei *et al.*, 2008), and a concentration of 10 ng/mL was used in the present work. One possible reason for the divergent results of various investigations is that the serum used in culture media includes factors that exert functions similar to BMP4 or interfere with BMP4 (Wei *et al.*, 2008). Efficient reprogramming of ntESCs may cause proper differentiation of PGCs from these cells (Yang *et al.*, 2007).

Although we have demonstrated the ability of ntESCs to differentiate to putative PGCs based on gene expression

and Mvh presentation under the effect of Bmp4, it is clear that significant improvements are required in the differentiation process whereby ntESCs remodel another cell's nucleus to restore totipotency and allow differentiation to diploid cells such as PGCs. Given the ability of *in vitro* culture systems to reproduce essential aspects of normal development, the differentiation of putative PGCs from ntESCs may provide clues to vital aspects of ntESC development into gametes. Further research should allow us to improve the differentiation of ntESCs to germ cells in the future.

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