Differentiation of neuron-like cells from mouse parthenogenetic embryonic stem cells☆

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Abstract

Parthenogenetic embryonic stem cells have pluripotent differentiation potentials, akin to fertilized embryo-derived embryonic stem cells. The aim of this study was to compare the neuronal differentiation potential of parthenogenetic and fertilized embryo-derived embryonic stem cells. Before differentiation, karyotype analysis was performed, with normal karyotypes detected in both parthenogenetic and fertilized embryo-derived embryonic stem cells. Sex chromosomes were identified as XX. Immunocytochemistry and quantitative real-time PCR detected high expression of the pluripotent gene, Oct4, at both the mRNA and protein levels, indicating pluripotent differentiation potential of the two embryonic stem cell subtypes. Embryonic stem cells were induced with retinoic acid to form embryoid bodies, and then dispersed into single cells. Single cells were differentiated in N2 differentiation medium for 9 days. Immunocytochemistry showed parthenogenetic and fertilized embryo-derived embryonic stem cells both express the neuronal cell markers nestin, βIII-tubulin and myelin basic protein. Quantitative real-time PCR found expression of neurogenesis related genes (Sox-1, Nestin, GABA, Pax6, Zic5 and Pitx1) in both types of embryonic stem cells, and Oct4 expression was significantly decreased. Nestin and Pax6 expression in parthenogenetic embryonic stem cells was significantly higher than that in fertilized embryo-derived embryonic stem cells. Thus, our experimental findings indicate that parthenogenetic embryonic stem cells have stronger neuronal differentiation potential than fertilized embryo-derived embryonic stem cells.

Key Words

neural regeneration; stem cells; parthenogenesis; parthenogenetic embryonic stem cells; embryonic stem cells; neuronal cells; karyotypes; Oct4; differentiation; embryoid body; mice; grants-supported paper; photographs-containing paper; neuroregeneration

Research Highlights

(1) The differentiation potentials of parthenogenetic and fertilized embryo-derived embryonic stem cells from mice were compared. Results were determined by measuring gene and protein levels.
(2) Karyotype analysis shows parthenogenetic embryonic stem cells have a normal karyotype, carrying the female (XX) sex chromosomes.
(3) Parthenogenetic embryonic stem cells derived from autologous tissue exhibit no immunological rejection and have no ethical consequences, and therefore have promising application prospects.

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INTRODUCTION

Stem cell therapy, based on the safe and unlimited self-renewal of human pluripotent stem cells, has been investigated for its use in tissue or organ replacement after injury or disease[1]. Establishing immune-specific pluripotent cell lines may reduce or eliminate the risk of allograft rejection in cell replacement therapy. To acquire appropriate cell lines, various methods have been suggested, including establishing nuclear transfer and parthenogenetic embryonic stem cells *via* isolation of nuclear transfer and parthenogenetic blastocysts respectively; establishing adult stem cells from adult tissue and organs; and inducing pluripotent stem cells by ectopic expression of reprogramming factors[2-4]. Nuclear transfer embryonic stem cells are akin to embryonic stem cells in all characteristics and maintain self-renewal and pluripotency during long-term culture[5]. Autologous pluripotent nuclear transfer embryonic stem cells have been obtained through somatic cell nuclear transfer in many species, including mice, cows, pigs, rhesus monkeys and humans[6]. Nuclear transfer embryonic stem cells derived from immune-deficient Rag2$^{-/-}$ mice, were repaired by homologous recombination, restoring normal Rag2 gene structure[7]. The repaired nuclear transfer embryonic stem cells were differentiated into hematopoietic stem cells *in vitro*, and consequently used therapeutically in immune-deficient Rag2$^{-/-}$ mice through autologous transportation of bone marrow. Thus, nuclear transfer embryonic stem cells offer treatment potential for degenerative and genetic diseases, yet ethical issues and a shortage of human oocytes has limited nuclear transfer embryonic stem cells research in many countries[8]. More recently, Japanese scientists reported that differentiated somatic cells can be reprogrammed into pluripotent stem cells by expression of four transcription factors, producing induced pluripotent stem cells[9]. Like nuclear transfer embryonic stem cells, induced pluripotent stem cells offer therapeutic potential in immune deficient mouse models[10], showing promise for cell replacement therapy[11]. However, induced pluripotent stem cells have associated risks, namely virus protein inflammation and the insertion of mutations into the host cell's genome due to expression of exogenous genes[12]. Therefore, further research is needed before induced pluripotent stem cells are used routinely in cell replacement therapy. Adult stem cells, for example mesenchymal and epithelial, are derived from adult tissue and organs, and can potentially repair injured tissue[13-14]. However, the regenerative capacity of adult stem cells in specific body organs has been shown to gradually decline with age[15].

Parthenogensis is asexual reproduction, in which oocyte could develop a viable embryo by antomatic or artificial activation without participant of sperm. Parthenogenetic embryonic stem cells are derived from the inner cell mass of parthenogenetic pre-implantation blastocyst embryos, and are similar to embryonic stem cells[16] but with greater advantages. Parthenogenetic embryonic stem cells avoid the logical limitation of embryo research. Moreover, the major histocompatibility complex of parthenogenetic embryonic stem cells is similar to donor cells[17], and consequently they have been shown to provide a source of histocompatible tissue for transplantation[12]. Current excitement surrounds the therapeutic potential of dopamine neurons derived from parthenogenetic embryonic stem cells in the primate Parkinson's disease model[4]. Thus, parthenogenetic embryonic stem cells look promising for future clinical applications of cell therapy[17-18], although further investigation of their properties following differentiation is required.

Both fertilized embryo-derived and parthenogenetic embryonic stem cells can be differentiated into neural cells *in vitro* using neural differentiation medium[19]. Neural cells derived from embryonic stem cells contain a variety of cell types, including non-neural cells and neuronal subtypes[20]. Thus, several strategies (for example the multistep process for promotion of neuronal differentiation[19]) have been used to obtain specific neuronal cell types from embryonic stem cells. In the present study, we used retinoic acid to form embryoid bodies, subsequently disassembling the embryoid bodies into single cells to compare the neuronal differentiation ability of fertilized embryo-derived and parthenogenetic embryonic stem cells.

RESULTS

Pluripotency and karyotype analysis of fertilized embryo-derived and parthenogenetic embryonic stem cells

Karyotypes were analyzed before neural induction. Normal karyotypes were observed in parthenogenetic and fertilized embryo-derived embryonic stem cells, demonstrated by XX sex chromosomes in both types of embryonic stem cells (Figure 1).

Expression of the pluripotent gene Oct4 protein and mRNA in cells before and after neural induction

Immunocytochemistry and quantitative real-time PCR detected Oct4 protein and mRNA in fertilized embryo-

derived and parthenogenetic embryonic stem cells before neural induction (Figures 2, 3). After neural induction, Oct4 mRNA expression levels were significantly decreased ($P < 0.05$). However, comparing fertilized embryo-derived and parthenogenetic embryonic stem cells, Oct4 mRNA expression levels were not significantly different before or after induction ($P > 0.05$; Figure 3).
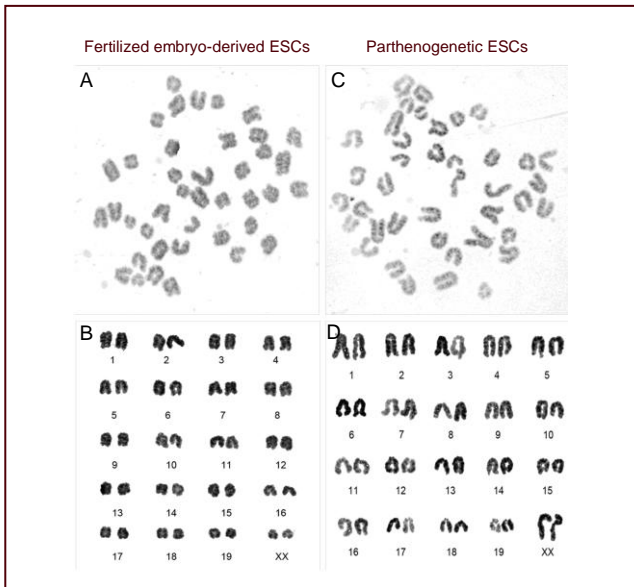

Figure 1   Karyotype analysis of fertilized embryo-derived and parthenogenetic embryonic stem cells (ESCs) from C3H mice.

Karyotypes of fertilized embryo-derived (A, B) and parthenogenetic (C, D) ESCs are normal. The sex chromosomes are identified as XX, consistent with the derivation of both types of ESCs from female mice.
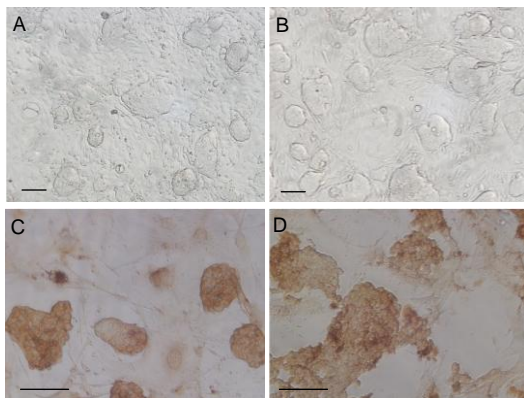

Figure 2   Expression of Oct4 protein in fertilized embryo-derived and parthenogenetic embryonic stem cells from C3H mice (immunocytochemistry; scale bars: 100 μm).

Fertilized embryo-derived and parthenogenetic embryonic stem cells maintain colony-like growth (A, B), and express Oct4 protein (C, D). Images were obtained with an inverted microscope.

Expression of neuronal proteins in fertilized embryo-derived and parthenogenetic embryonic stem cells after neuronal differentiation

Fertilized embryo-derived and parthenogenetic embryonic stem cells were differentiated for 9 days in neural differentiation medium and immunocytochemistry performed to determine expression of neuronal marker proteins. Nestin, βIII-tubulin and myelin basic protein were detected in differentiated neuronal cells from fertilized embryo-derived and parthenogenetic embryonic stem cells. Myelin basic protein shows stronger expression in parthenogenetic embryonic stem cells than in fertilized embryo-derived embryonic stem cells, where it was only weakly detected (Figure 4).
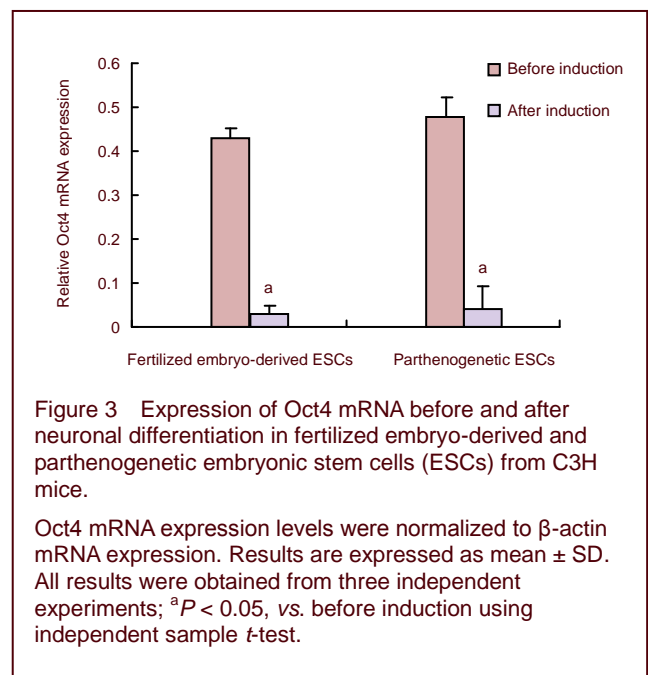

Figure 3   Expression of Oct4 mRNA before and after neuronal differentiation in fertilized embryo-derived and parthenogenetic embryonic stem cells (ESCs) from C3H mice.

Oct4 mRNA expression levels were normalized to β-actin mRNA expression. Results are expressed as mean ± SD. All results were obtained from three independent experiments; [a]$P < 0.05$, *vs.* before induction using independent sample *t*-test.

Expression of mRNAs involved in neurogenesis in fertilized embryo-derived and parthenogenetic embryonic stem cells after neuronal differentiation

Quantitative real-time PCR was performed to determine expression of genes involved in neuronal development. Our results show that Sox-1, nestin, GABA, Pax6, Zic5 and Pitx1 mRNA were expressed in neuronal cells derived from both fertilized embryo-derived and parthenogenetic embryonic stem cells. Nestin and Pax6 expression was significantly higher in parthenogenetic embryonic stem cells than in fertilized embryo-derived embryonic stem cells ($P < 0.05$; Figure 5).

DISCUSSION

We have previously shown that parthenogenetic embryonic stem cells have pluripotent differentiation
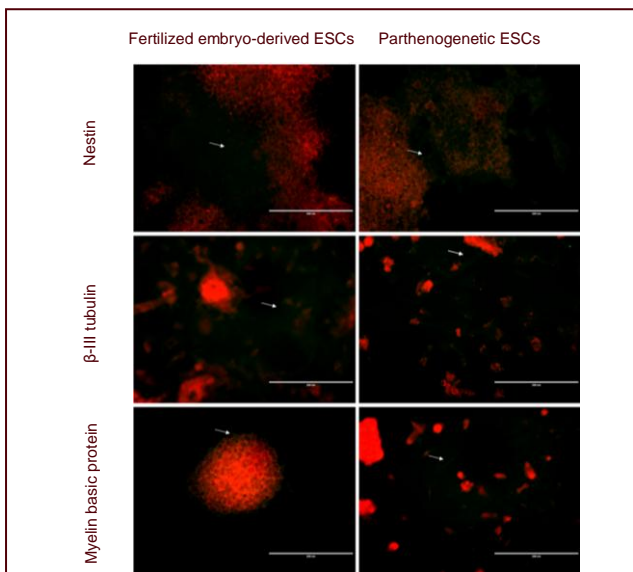
capacities in mouse models[21].

Figure 4 Expression of neuronal specific proteins in fertilized embryo-derived and parthenogenetic embryonic stem cells (ESCs) from C3H mice after induction in neuronal differentiation medium (immunocytochemistry; scale bars: 100 μm).

Differentiating neuron-like cells derived from fertilized embryo-derived and parthenogenetic ESCs express nestin, βIII-tubulin and myelin basic protein. Marker proteins (green) and the nucleus (red) are shown. Images were obtained by imaging fluorescence through an inverted microscope.
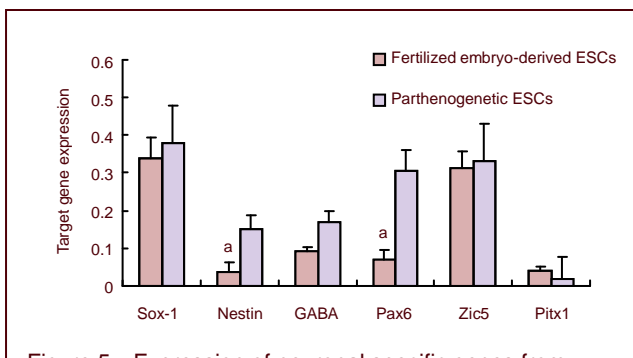

Figure 5 Expression of neuronal specific genes from fertilized embryo-derived and parthenogenetic embryonic stem cells (ESCs) from C3H mice after induction.

Target gene expression levels were normalized to β-actin mRNA expression. Results are expressed as mean ± SD, and all results were obtained from three independent experiments. $^a P < 0.05$, *vs.* before induction using independent sample *t*-test.

Moreover, parthenogenetic embryonic stem cells have specific advantages and can be used as seed cells for cell replacement therapy. For example, mitochondria are transferred through the female line, and as parthenogenetic embryonic stem cells are derived from the donor female, the surface antigens of parthenogenetic embryonic stem cells are reduced, compared with embryonic stem cells, without

participation of the paternal genetic background[17]. Thus, for cell transplantation, the immunogenicity of parthenogenetic embryonic stem cells is weaker than that of embryonic stem cells, potentially improving transplantation safety. The developmental competence of parthenogenetic embryos is similar to that of normal embryos, and the isolation of parthenogenetic embryonic stem cells needs less oocytes than nuclear transfer embryonic stem cells[22]. Growing evidence suggests that parthenogenetic embryonic stem cells are promising seed cells for cell therapy. However, the expression profile of certain genes, for example imprinting genes, differs between parthenogenetic and fertilized embryo-derived embryonic stem cells[23], and gene expression differences may influence the differentiation efficiency of parthenogenetic embryonic stem cells. In the present study, we detected nestin, βIII-tubulin and myelin basic protein in fertilized embryo-derived and parthenogenetic embryonic stem cells following neuronal differentiation. βIII-tubulin is an early neuronal marker and is also expressed in neural stem cells[24], while myelin basic protein is expressed in differentiated neuronal cells[25]. Therefore, our results show that parthenogenetic embryonic stem cells have the potential to differentiate into neuronal cells.

As stem cells differentiate into specific cell lineages *in vitro*, the cell morphology alters and many related genes are expressed at the mRNA level[26]. Expression of specific mRNAs is used as a marker of cellular differentiation. In the present study, Sox-1, nestin, GABA, Pax6, Zic5 and Pitx1 mRNAs were expressed in neuronal cells derived from fertilized embryo-derived and parthenogenetic embryonic stem cells after 9 days of differentiation. Sox1, nestin and Pax6 are involved in early differentiation of neuronal cells[27-29]. Embryonic stem cells are differentiated into neuronal cells *in vitro*, and undergo the intermediate stage of neural progenitor/stem cells[30]. These neural progenitor/stem cells maintain their own characteristics through continuous self-renewal and suppression of differentiation, in which Sox1 plays an important role. Sox1 carries out its function *via* the prospero-related homeobox 1-mediated pathway[31]. Nestin is another important gene for neuronal cell development. Nestin, a type VI intermediate filament protein, is abundantly expressed in neural stem cells and has been used as a marker for neural progenitor cells[32-33]. Regulation of nestin expression is complicated, and involves many transcription factors, including Sox2, Brn1 and Brn2. Pax6 is also involved in embryonic stem cell differentiation into neural stem cells. In humans, Pax6 is

specifically expressed in the fetal neural plate and plays an important role in the neuroectoderm, repressing expression of pluripotent genes and activating neural development genes[34]. In mouse, Pax6 is involved in embryonic stem cell differentiation. High Pax6 levels promote differentiation of embryonic stem cells into neural stem cells, and Pax6 expression then sharply declines with differentiation of neural stem cells into differentiated cell types[35]. Therefore, high Pax6 levels maintain the quantitation of neural stem cells and low Pax6 levels promote the differentiation of neural stem cells[36]. Of these three early neuronal marker genes, Sox1 expression was not significantly different in differentiated neuronal cells derived from fertilized embryo-derived and parthenogenetic embryonic stem cells, but nestin and Pax6 expression were relatively low in fertilized embryo-derived embryonic stem cells, suggesting parthenogenetic embryonic stem cells have greater potential to differentiate into neural stem cells than fertilized embryo-derived embryonic stem cells.

Neural stem cell differentiation is a complex process, and involves expression of a series of genes, including GABA and Zic5. GABA is a neurotransmitter secreted by neurons[37] known as GABAergic neurons. GABA is a marker of mature neurons. GABA expression levels show no significant difference between differentiating neuronal cells from fertilized embryo-derived and parthenogenetic embryonic stem cells, demonstrating that both embryonic stem cells, have the same potential to differentiate into GABAergic neurons. Zic5 is a member of the zinc finger proteins, responsible for the human brain malformation syndrome and involved in generation of mouse neural crest tissue[38], thereby suggesting Zic5 regulates differentiation of neural stem cells. In the present study, GABA and Zic5 expression were not altered, suggesting there is no significant difference between fertilized embryo-derived and parthenogenetic embryonic stem cells in differentiation of neural stem cells into differentiated neuronal cells.

Parthenogenetic embryonic stem cells offer an alternative source of histocompatible cells with therapeutic potential for replacement of cells, tissues and organs[17]. Differentiation of parthenogenetic embryonic stem cells is complicated, relying on a series of gene expression. Expression of early neuronal marker genes, nestin and Pax6, is high in neuronal cells derived from parthenogenetic embryonic stem cells. However, expression of GABA and Zic5 genes indicative of a mature neuronal phenotype is similar in both fertilized embryo-derived and parthenogenetic embryonic stem

cells. Overall, our results show that with regard to gene expression, parthenogenetic embryonic stem cells have greater potential to differentiate into neural stem cells, and can contribute to the neuronal lineage in differentiation medium *in vitro*. Thus, parthenogenetic embryonic stem cells offer greater therapeutic potential for nervous system diseases.

MATERIALS AND METHODS

Design
A controlled observational study examining cell cytology.

Time and setting
Experiments were performed from March 2011 to June 2012 in the Shaanxi Provincial Key Laboratory of Biotechnology, Key Laboratory of Resource Biology and Biotechnology in Western China, Ministry of Education, College of Life Sciences, Northwest University, China.

Materials
Female (4–6 weeks old) and male (6–8 weeks old) Kunming mice were purchased from the Experimental Animal Center at the Fourth Military Medical University of Chinese PLA, China (license No. SCXK (Military) 2002-005). C3H female mice (4 weeks old) were purchased from Vital River Laboratory (license No. SCXK (Beijing) 2007-0001) in China. Animal treatments were performed in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*[39].

Methods
Isolation of parthenogenetic and fertilized embryo-derived embryonic stem cells
Female Kunming mice were intraperitoneally injected with 10 IU of pregnant mare serum gonadotropin (Sigma, St. Louis, MO, USA), and at 48-hour intervals intraperitoneally injected with 10 IU of human chorionic gonadotropin (Sigma). Immediately following human chorionic gonadotropin injection, female mice were mated (1:1) to male mice of proven fertility and the same strain[40]. Metaphase II oocytes were collected from oviducts of C3H female mice that were superovulated and artificially activated by chemical methods[22]. Blastocysts were assembled by culturing parthenogenetic embryos *in vitro*. Fertilized blastocysts were derived from natural matings of C3H female mice at 3.5 days post-coitum. The zona pellucida of parthenogenetic and fertilized blastocysts were removed using acid Tyrode's solution (Sigma), and the zona-free blastocyst was cultured on a feeder layer of mouse

embryo fibroblasts treated with mitomycin C (Sigma) in gelatin-coated 4-well multi-dishes. Cells were cultured in knock-out Dulbecco's minimal essential medium (Gibco, Carlsbad, CA, USA), supplemented with 0.1 mM β-mercaptoethanol, 1% (v/v) non-essential amino acids, 2 mM L-glutamine, 1% (v/v) penicillin and streptomycin, and 2 000 U/mL mouse leukemia inhibitory factor (Chemicon, Temecula, CA, USA). After 3 days culture, colonies derived from the inner cell mass were dissociated with accutase (Gibco) and subcultured on gelatin-coated 4-well multi-dishes in the presence of mouse embryo fibroblast feeder cells. When cultured embryonic stem cells had obvious colonies, subcultures were conducted at 3–4 day intervals with the same protocols, as previously reported[41]. Both embryonic stem cell types were demonstrated to be pluripotent by embryoid body formation, differentiation of three germ layers and expression of pluripotent genes (data not shown).

Karyotype analysis of parthenogenetic and fertilized embryo-derived embryonic stem cells

Karyotype analysis of parthenogenetic and fertilized embryo-derived embryonic stem cells was performed as described previously[42]. Briefly, there were at least three passages in feeder-free conditions, aimed to remove cells derived from mouse embryo fibroblasts. Parthenogenetic and fertilized embryo-derived embryonic stem cells were incubated in colchicine (10 μg/mL; Sigma) for 2 hours. Subsequently, cells were dissembled into single-cell suspensions, followed by incubation for 15 minutes in 0.075 mM KCl. Cells were fixed in a methanol/acetic acid mixture at room temperature, at least 3 times (the last time, temperature of fix solution is at –20°C), then spread over slides and stained with Giemsa solution (Sigma). Karyotypes were observed and imaged using a Digital Microscope (Leica, Heidelberg, Germany).

Embryoid body formation and differentiation into neuronal lineages

For embryoid body formation, 3×10^6 parthenogenetic and fertilized embryo-derived embryonic stem cells were plated on non-adherent bacterial dishes in embryoid body medium, without leukemia inhibitory factor. The medium was changed every 2 days, and 5 μM retinoic acid (Sigma) was added after 4 days, with continued culture for 8 days. Embryoid bodies were dissociated using accutase and cells (1×10^4/mL) plated on polyornithine-coated plates in N2 medium (Invitrogen, Carlsbad, CA, USA). The medium was changed every 2 days, as in previous reports[20].

Immunocytochemistry

For immunofluorescence, staining was carried out as reported previously[43]. Briefly, single cells were plated on coverslips and neuronal differentiation was continued for 9 days. Cells were fixed in 4% paraformaldehyde for 3 hours, rinsed with PBS at least three times, blocked in 10% goat serum with 0.2% Triton X-100 for 1 hour, and then washed, at least three times, with PBS and Triton X-100 (0.2%). Cells were incubated at 4°C overnight with primary monoclonal rabbit anti-mouse antibodies (all 1:100; Chemicon, Santa Cruz, CA, USA): βIII-tubulin, nestin and myelin basic protein. After at least five PBS washes, FITC-conjugated secondary antibody (goat anti-rabbit IgG; 1:150; Chemicon) was incubated at 37°C for 1 hour. 4',6-diamidino-2-phenylindole (1 μg/mL; Chemicon) was used to stain the cell nucleus.

For immunocytochemistry, fertilized embryo-derived and parthenogenetic embryonic stem cells were cultured on coverslips with a feeder cell layer. Immunocytochemistry was performed according to the manufacturer's instructions, SP-9000 General Immunocytochemical Kit (Chemicon). Briefly, cells were blocked with 10% goat serum with 0.2% Triton X-100, then incubated at 4°C overnight with primary rabbit anti-mouse Oct4 monoclonal antibody (1:100; Chemicon). Finally, cells were exposed to horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit IgG) with positive 3,3-diaminobenzidine expression detected as red- or yellow-brown[21].

Quantitative real-time PCR detection

Total RNA was extracted from fertilized embryo-derived and parthenogenetic embryonic stem cells, and differentiating neuronal cells as described previously[22]. Reverse transcription was performed using a reverse transcription kit (Takara, Dalian, China) according to the manufacturer's instructions. For mRNA expression, PCR amplification of genes was performed using EXTaq polymerase (Takara), with a program of 94°C for 5 minutes, 35 cycles of 94°C for 30 seconds, 52–59°C for 30 seconds, 72°C for 30 seconds, and final extension at 72°C for 10 minutes. Real-time reverse-transcription PCR was performed on a Bio-Rad CFX96 Sequence Detection System (Bio-Rad, Hercules, CA, USA) using the SYBR Green PCR Mix (Takara). PCR reactions contained 10 μL SYBR Green PCR Master Mix, 1 μL forward and reverse primers (10 mM), 8.5 μL water and 0.5 μL cDNA template, in a total 20 μL volume. Sox-1, nestin, GABA, Pax6, Zic5, Pitx1 and Oct4 were detected by quantitative real-time PCR. Primer sequences are shown in Table 1. Results were

normalized to β-actin levels. Experiments were repeated three times.

Table 1   Oligonucleotide primers used for quantitative real-time PCR

Gene	Primer sequence	Product size (bp)
Oct4	U: 5'-TAT GCA AAT CGG AGA CCC TG-3' D: 5'-AAG CTG ATT GGC GAT GTG AG-3'	143
Zic5	U: 5'-CGA GAA CCT CAA GAT CCA-3' D: 5'-TGT ACG ATT TAT CAC AGC CT-3'	173
Nestin	U: 5'-GCT GGA GGC TGA GAA CTC-3' D: 5'-GAA AGG CTG TCA CAG GAG-3'	200
GABA	U: 5'-ATC ATT GGC TTA GGC AAC-3' D: 5'-ATG GGA CAG ATT CTC CTC-3'	185
Pax6	U: 5'-ACA ACG ACG AAA GAG AGG-3' D: 5'-ACA AAG ACA CCA CCA AGC-3'	209
Sox1	U: 5'-ATC ACC TTC CCC AGG ACT-3' D: 5'-CAT ACA AAA GTT GGC ATC G-3'	178
Pitx1	U: 5'-ACT CTC TTG TCC CCA ACA GGT-3' D: 5'-ATG TCG TGC GGT GGT GGT-3'	167
β-actin	U: 5'-GCG GCA TCC ACG AAA CTA C-3' D: 5'-TGA TCT CCT TCT GCA TCC TGT C-3'	120

U: Upstream; D: downstream.

Statistical analysis

Statistical analyses were performed using SPSS 11.5 software (SPSS, Chicago, IL, USA). Gene expression levels were expressed as mean ± SD and analyzed using independent sample t-tests. For all tests, statistical significance was $P < 0.05$.

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