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Effects of Exogenous Insulin-like Growth Factor 2 on Neural Differentiation of Parthenogenetic Murine Embryonic Stem Cells

Young-Ju Choi, Sangkyu Park, Hoin Kang and Sangho Roh[†]

Cellular Reprogramming and Embryo Biotechnology Lab, Dental Research Institute and CLS21, Seoul National University School of Dentistry, Seoul 110-749, Korea

ABSTRACT

Differential capacity of the parthenogenetic embryonic stem cells (PESCs) is still under controversy and the mechanisms of its neural induction are yet poorly understood. Here we demonstrated neural lineage induction of PESCs by addition of insulin-like growth factor-2 (Igf2), which is an important factor for embryo organ development and a paternally expressed imprinting gene. Murine PESCs were aggregated to embryoid bodies (EBs) by suspension culture under the leukemia inhibitory factor-free condition for 4 days. To test the effect of exogenous Igf2, 30 ng/ml of Igf2 was supplemented to EBs induction medium. Then neural induction was carried out with serum-free medium containing insulin, transferrin, selenium, and fibronectin complex (ITSFn) for 12 days. Normal murine embryonic stem cells derived from fertilized embryos (ESCs) were used as the control group. Neural potential of differentiated PESCs and ESCs were analyzed by immunofluorescent labeling and real-time PCR assay (Nestin, neural progenitor marker; Tuj1, neuronal cell marker; GFAP, glial cell marker). The differentiated cells from both ESC and PESC showed heterogeneous population of Nestin, Tuj1, and GFAP positive cells. In terms of the level of gene expression, PESC showed 4 times higher level of *GFAP* expression than ESCs. After exposure to Igf2, the expression level of *GFAP* decreased both in derivatives of PESCs and ESCs. Interestingly, the expression level of *Tuj1* increased only in ESCs, not in PESCs. The results show that IGF2 is a positive effector for suppressing over-expressed glial differentiation during neural induction of PESCs and for promoting neuronal differentiation of ESCs, while exogenous Igf2 could not accelerate the neuronal differentiation of PESCs. Although exogenous Igf2 promotes neuronal differentiation of normal ESCs, expression of endogenous *Igf2* may be critical for initiating neuronal differentiation of pluripotent stem cells. The findings may contribute to understanding of the relationship between imprinting mechanism and neural differentiation and its application to neural tissue repair in the future.

(Key words : Igf2, Parthenogenesis, Embryonic stem cells, Neural differentiation, Mouse)

INTRODUCTION

Embryonic stem cells (ESCs) are pluripotent and can differentiate into all somatic cell types. ESCs are permanent cells, which can be isolated from the inner cell mass of blastocysts in mice. These cells are pluripotent and are capable of self-renewal in specific culture environments. Upon withdrawal of leukemia inhibitory factor (LIF) or feeder cell supports, ESCs will differentiate into variable cell mass complexes called embryoid bodies (EBs). Differentiation within these cell aggregates occurs in a defined temporal manner with the initial formation of all three germ layers, followed by further differentiation to terminally differentiated cell lineages (Ling and Neben, 1997; Desbaillets *et al*., 2000). A variety of differentiated cell types have been generated from ESCs *in vitro* including neural cells, hematopoietic cells, cardiomyocytes, and osteoblasts (Yamashita *et al*., 2000; Buttery *et al*., 2001; Boheler *et al*., 2002). However, it is not easy to control ESC differentiation into specific cell types, and the use of ESCs is limited by the immune rejection response as well as the ethical debate surrounding embryo destruction (Bradley *et al*., 2002). To avoid these problems, the use of adult stem cells (ASCs), which have the same genetic information of the patient they were derived from, has been suggested. ASCs have been shown to facilitate bone repair in various osseous defect models (Ohgushi *et al*., 1989; Caplan and Weintraub, 1997; Jaiswal *et al*., 1997).

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[†] Corresponding author : Phone: +82-2-740-8681, E-mail: sangho@snu.ac.kr

These cells have multipotent differentiation capacity and can address several problems related to tissue repairing. However, very few ASCs are present in the body and it is very difficult to isolate these cells. As result, it is very difficult to obtain sufficient quantities of these cells for therapeutic purposes (Quarto *et al*., 1995). In addition, the self-renewal capacity and the potential of specific cell type differentiation of ASCs decreases with the age increasing (McCulloch *et al*., 1991; Quarto *et al*., 1995; Bourne *et al*., 2004).

Parthenogenetic ESCs (PESCs) are one of the useful cell sources in that they have only maternal genetic materials which enable to avoid ethical concern, and their high chances in major-histo-compatibility matching when they were transplanted, whilst retaining the capacity for self-renewal in culture and the ability to differentiating into various cell types in sufficient numbers. Moreover, in female patients, if the PESCs are derived from oocytes, then they will have that patient's genetic information. In a previous study, we demonstrated that murine PESCs can be differentiated into osteogenic cells *in vitro* (Kang *et al*., 2008). However, differential capacity of the PESCs is still under controversy and the mechanisms of its neural induction are yet poorly understood. The expression of imprinting genes related to growth and organ formation such as *Igf2* is abnormal in parthenogenetic embryos, as these genes are only expressed by the paternal genome, which is absent in parthenotes. As an imprinting gene that is only expressed in paternal-oriented genomes, *Igf2* have been shown to play an important role in regulating placental development and fetal growth (Baker *et al*., 1993; Eggenschwiler *et al*., 1997; Burns *et al*., 2001; Constancia *et al*., 2002; Carter *et al*., 2006). In the recent publication, our research group reported that supplementation of the culture media of PESCs with exogenous Igf2 induces these cells to differentiate into an osteogenic lineage although, for autogenous graft, this system may be limited to the patients who can provide her own oocytes (Kang *et al*., 2012). By applying same strategy, here we present data demonstrating that neural differentiation of PESCs by addition of exogenous Igf2, an important factor for embryo organ development but not expressed correctly in PESCs.

MATERIALS AND METHODS

Chemicals

All inorganic and organic compounds were obtained from Sigma-Aldrich Korea (Yong-in, Korea) unless otherwise stated.

Formation of EBs

To induce EBs formation, ESCs or PESCs on the culture plates were trypsinized for 2 min at 37℃. Trypsinization was terminated by addition of DMEM (Invitrogen, USA) containing 10% FBS (Invitrogen). After a brief centrifugation, cells were re-suspended in medium followed by medium supplementation with 10% FBS, 2 mM L-glutamine, 0.1 mM β-mercaptoethanol, 50 U/ml penicillin, 50 mg/ml streptomycin, 10^{-7} M retinoic acid. Cells were plated onto bacteriological-grade petri-dishes in the absence of STO feeder layers and LIF. ESCs and PESCs were maintained in culture for 7 days and the medium was replaced every 2 days. After 7 days of culture, ESCs or PESCs formed free-floating aggregates or EBs. To test the effect of exogenous Igf2, 30 ng/ml of Igf2 was supplemented to EBs induction medium and thus experimental groups were divided by four (Igf2-treated ESCs, Igf2-naive ESCs, Igf2-treated PESCs and Igf2-naive PESCs; Fig. 1).

In Vitro Neural Induction of EBs

Then neural induction was carried out with serum-free medium containing insulin, transferrin, selenium, and fibronectin complex (ITSFn) for 12 days on a plate coated with poly-L-ornithine and fibronectin (Fig. 1). Normal murine ESCs derived from *in vivo* derived blastocysts were used as the control group. Neural potential of differentiated PESCs and ESCs were analyzed by immunofluorescence labeling and real-time PCR assay (Nestin, neural progenitor marker; Tuj1, neuronal cell marker; GFAP, glial cell marker).

Immunofluorescence Staining of Differentiated EBs

Differentiated derivatives of EBs that were grown on the cover slip coated with 0.1% gelatin were fixed in 4% paraformaldehyde in PBS for 20 min. Fixed cells were then permeabilized with 0.2% Triton X-100 in PBS for 10 min and washed three times. After blocking with 1% bovine serum albumin in PBS for 30 min, cells were incubated with the first antibody. Antibodies used in our experiments were: mouse monoclonal Nestin (Santa Cruz Biotechnology, USA; 1:100), Tuj1 (1:100) and GFAP (1:100). After washes with PBS, primary an-

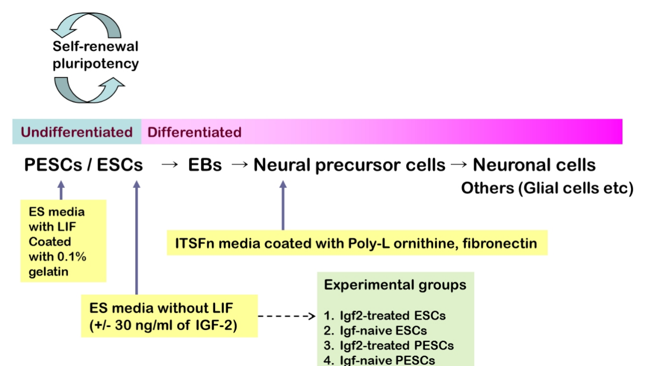

Fig. 1. Experimental scheme: Neural induction of parthenogenetic embryonic stem cells in mice.

tibodies were applied using FITC-conjugated goat anti-mouse (Jackson Immunoresearch, USA; 1:200).

mRNA Extraction and cDNA Synthesis

RNA was extracted from osteogenic induced cell of each group for 20 days using Trizol reagent (Invitrogen). The induced cells of each group (6.0×10^5 cells/ml) were harvested by centrifugation, resuspended in 1 ml of Trizol reagent by vortexing and then incubated for 15 min in ice for the cells were lysed. Then 0.2 ml of chloroform was added, mixed by smooth shaking and incubated for 15 min. After centrifugation at 12,000 rpm for 15 min at 4℃, the colorless upper aqueous phase was transferred to new tubes containing 0.5 ml of isopropanol and by smooth shaking and incubated for 15 min. Total RNA pellet was obtained by centrifugation at 12,000 rpm for 10 min at 4℃, air-dried, and re-suspended in diethyl pyrocarbonate-treated water. The isolated RNA samples were used for real-time PCR analysis. For the synthesis of cDNAs, reverse transcription was performed for 1 hr at 42℃ in a final reaction volume of 25 µl containing the total RNA, 5 µl of 5 × reaction buffer (Promega, USA), 5 µl of dNTP (each 2.5 mM), 2.5 µl of 10 µM synthesis primer, 0.5 µl of RNasin Plus RNase Inhibitor (40 U/ml; Promega), and 1 µl of M-MuLV reverse transcriptase (20 U/µl; Roche, Germany). cDNAs were diluted by the addition of 50 µl of RNase-free ultra-purified water.

Real-Time PCR

Expression levels of *Nestin*, *Tuj1* and *GFAP* were measured by real-time PCR in four experimental groups (Igf2-treated ESCs, Igf2-naive ESCs, Igf2-treated PESCs and Igf2-naive PESCs groups). Primers for real-time PCR were designed using Primer Express software (Applied Biosystems, USA). Real-time PCR was performed using the ABI PRISM 7500 system and SYBR Green PCR Master Mix (Applied Biosystems). The primers list of target genes was showed in Table 1. The extraction of mRNAs and the synthesis of cDNAs were performed twice, and all samples were run in triplicate to obtain technical replicates. In each run, 1 µl cDNA was used as a template added to 5 µl double-distilled water, 2 µl forward and reverse primers (20 pmol/ml), and 10 µl SYBR Green PCR Master Mix. The following amplification procedure was employed: denaturation stage (95℃ for 10 min), amplification and quantification stage repeated 40 times (94℃ for 15 sec, 60℃ for 1 min with single fluorescence measurement) and dissociation curve stage (temperature increments of 0.1 ℃ per 30 sec from 60 to 95℃ with fluorescence measurement). Gene expression was always related to expression of murine *Gapdh* as housekeeping gene, which is known to be a good reference gene for normalization of target genes expression levels. Quantification was performed using the $^{\Delta\Delta}$CT method. Non-template control was used as the negative control. The paired samples *t*-test was performed to compare the differences among the experimental groups.

RESULTS

The differentiated cells from PESCs showed heterogeneous population of Nestin, Tuj1, and GFAP positive cells (Fig. 2). In terms of the level of gene expression, PESCs showed 4 times higher level of *GFAP* expression than ESCs, however, after exposure to exogenous Igf2, the level of *GFAP* expression decreased both in derivatives of PESCs and ESCs. In particular, the level of *GFAP* in PESCs derivative was decreased to the level in ESCs. Interestingly, after exogenous Igf2 supplementation during the formation of EBs, the expression level of *Tuj1* was significantly increased only in ESCs, not in

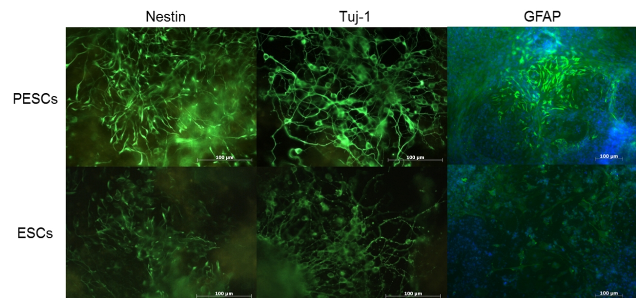

Fig. 2. Immunofluorescence staining of cells cultured for 12 days in the medium containing insulin, transferrin, selenium and fibronectin complex. Immunofluorescence images of differentiated parthenogenetic embryonic stem cells (PESCs) and normal embryonic stem cells (ESCs).

Table 1. Primers sequences used for real-time PCR analysis

Gene	Sense	Antisense
Nestin	5′-GGAGAAGCAGGGTCTACAG-3′	5′-AGCCACTTCCAGACTAAGG-3′
Tuj1	5′-TCCGCCTGCCTTTTCGTCTCTA-3′	5′-AGTTGCCGCTGGGGTCTATGC-3′
GFAP	5′-CATGCCACGCTTCTCCTTGTCTC-3′	5′-GCTCGCTCGCCCGTGTCT-3′
Gapdh	5′-GCATGGCCTTCCGTGTTCCTA-3′	5′-CTTCAGTGGGCCCTCAGATGC-3′

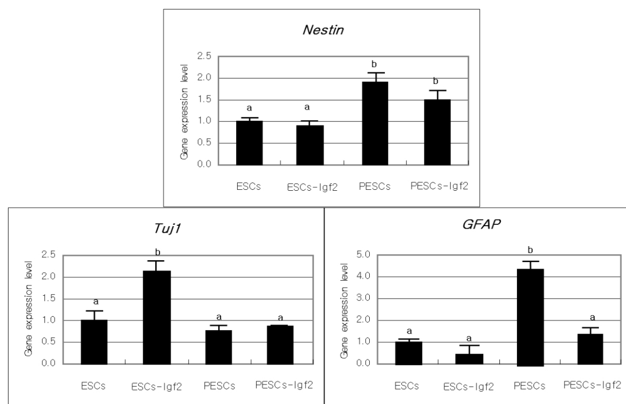

Fig. 3. Neural-specific gene expression in Igf2-naive ESCs (ESCs), Igf2-naive PESCs (PESCs), Igf2-treated ESCs (ESCs-Igf2) and Igf2-treated PESCs (PESCs-Igf2) after neural differentiation induction. Igf2-treated PESCs were treated with exogenous Igf2 for 7 days during the period of embryoid body formation before neural induction. The extraction of mRNAs and the synthesis of cDNAs were performed twice, and all samples were run in triplicate to obtain technical replicates. Gene expression was related to expression of Murine *Gapdh* for normalization of target genes and the value in ESCs group was used as the control (value = 1.0). Values with different superscripts are significantly different (a, b; $p<0.05$, paired samples *t*-test).

PESCs. There was no difference in the expression level of *Nestin* between Igf2-treated and Igf2-naive groups regardless of the origin of cells (Fig. 3).

DISCUSSION

ESCs have been induced to differentiate the wide variety of cell types by supplementing the culture medium with specific factors. Cultures of ESCs in defined media containing various proteins and small molecules have been used as a strategy to investigate ESC differentiation *in vitro* (Rathjen and Rathjen, 2001; Hwang *et al*., 2008a; Hwang *et al*., 2008b). In particular culturing cells under defined conditions in medium containing several factors is a good strategy for discovering factors that are critical for inducing or improving specific cell lineages. We have shown previously that PESCs, which are derived from parthenogenetic blastocysts, can be induced to differentiate into an osteogenic lineage by supplementation of the culture medium with defined induction factors (Kang *et al*., 2008). However, the differentiation potential of PESCs is limited compared to that of ESCs (Allen *et al*., 1994; Morali *et al*., 2000). The limited differentiation potential of PESCs relative to ESCs may be related to the abnormal expression of imprinting genes in PESCs. In the next series of the experiments, we hypothesized that the addition of exogenous Igf2 could potentially enhance the osteogenic cell differentiation of PESCs. As described above, Igf2 is a paternally expressed imprinting gene that is therefore not expressed in PESCs (Gong *et al*., 2009). In this series of experiment, we evidenced that exogenous Igf2 promoted osteogenic lineage differentiation of PESCs and concluded that the lack of endogenous *Igf2* expression can be compensated for by exogenous supplementation with this factor, resulting in osteogenic cell differentiation (Kang *et al*., 2012). Thus, here we applied same strategy for neural differentiation of PESCs focusing on neuronal lineage induction.

Interestingly, the result above shows that Igf2 supplementation could not induce neuronal differentiation of PESCs although the same factor stimulated neuronal differentiation of ESCs in which endogenous *Igf2* is also expressed. This result is far from our previous report demonstrating promoted osteogenic lineage differentiation of PESCs by Igf2 supplementation (Kang *et al*., 2012). In the present study, derivatives of PESCs after neural differentiation induction showed heterogeneous population of Nestin, Tuj1, and GFAP positive cells like the cells from normal ESCs. However, PESCs tended to be differentiated into glial cells, not neuronal cells in the aspect of gene expression. On the other hand, Igf2 showed a positive effect on suppression of over-expressed glial differentiation during neural differentiation induction of PESCs, although exogenous Igf2 could not activate neuronal differentiation of PESCs. Endogenous *Igf2* expression may be an essential factor for neuronal differentiation of embryo-derived pluripotent stem cells. In addition, imprinting genes other than *Igf2* may have critical roles for neurogenesis in mammalian development. The finding may contribute to understanding of the relationship between imprinting mechanism and neural differentiation and its application to neural tissue repair in the future.

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