International Journal of Oral Biology, Vol. 33, No. 3, September 30 2008, p. 91~95
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International Journal of Oral Biology

Osteogenic Differentiation Potential in Parthenogenetic Murine Embryonic Stem Cells

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(Received June 5, 2008 ; Revised August 13, 2008 ; Accepted August 21, 2008)

Embryonic stem cells have a pluripotency and a potential to differentiate to all type of cells. In our previous study, we have shown that embryonic stem cells (ESCs) lines can be generated from murine parthenogenetic embryos. This parthenogenetic ESCs line can be a useful stem cell source for tissue repair and regeneration. The defect in full-term development of parthenogenetic ESCs line enables researchers to avoid the ethical concerns related with ESCs research. In this study, we presented the results demonstrating that parthenogenetic ESCs can be induced into osteogenic cells by supplementing culture media with ascorbic acid and ß-glycerophosphate. These cells showed morphologies of osteogenic cells and it was proven by Von Kossa staining and Alizarin Red staining. Expression of marker genes for osteogenic cells (osteopontin, osteonectin, alkaline phosphatase, osteocalcin, bone-sialoprotein, collagen type1, and Cbfa1) also confirmed osteogenic potential of these cells. These results demonstrate that osteogenic cells can be generated from parthenogenetic ESCs in vitro.

Keywords: Embryonic stem cell, Parthenogenesis, Osteogenic cell

Introduction

In dentistry, adequate development of cranium and the quality of maxillary or mandibular alveolar bone is crucial for maintaining normal dentition. Especially with the rise of dental implant prosthesis of recent decades, the importance of dental alveolar bone has been rising. The extent and level of osseointegration of implant fixture to dental alveolar bone is decisive factor for the ‘primary fixation’, which is considered as the success criteria for implant surgery (Szmukler-Moncler *et al*, 2000). Thus, nowadays skeletal repair has been great issue in medical and dental clinic. Many of the current strategies to repair skeletal damage include the use of autogenous or allogenic bone grafts as well as various prosthetic implant devices (Damien *et al*., 1991; Crane *et al*., 1995). However, these approaches has many insufficiencies including availability of suitable bone graft material, and also issues of biocompatibility and failure of implant prostheses have created the need to develop alternative strategies (Buttery *et al*., 2001). In this regard, regenerative skeletal repair using molecular and cellular regulation has been great issue in medical and dental clinic.

An important consideration for regenerative tissue repair is the ability to generate appropriate type of cells with sufficient numbers (Langer *et al*., 1993; Minuth *et al*., 1998; Caplan *et al*., 2000). In this regard, the ESCs lie in the center of attention in clinical osteogenic-regenerative study as it has pluripotency which means that potential to differentiate into all fetal cell types. However, it is difficult to induce differentiation into intended cell type, and also application potential of ESCs to cell therapy has the limits caused by immune rejection responses (Bradley *et al*, 2002). To avoid these problems, it may be useful to use tissues having the patients' own genetic information. In this regard, adult mesenchymal stem cells (MSCs) research came to an interest. MSCs, isolated from bone marrow aspirates, have been used to facilitate bone repair in various osseous defect

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models (Jaiswal *et al*., 1997; Ohgushi *et al*., 1989; Caplan *et al*, 1997; Cummine *et al*., 1983).

As MSCs has multipotency with patients' own genetic information, these cells can deal with several problems related to tissue repair. However, MSCs has some limitations on clinical usage. These cells have relatively low frequency of occur in the marrow stroma, with perhaps as few as 1 in 100,000 cells being a true stem cell (Quarto *et al*., 1995; D'Ippolito *et al*., 1999). They also have a finite capacity for self-renewal (McCulloch *et al*., 1991), and with increasing age, both the potential to differentiate to specific cell type is diminished (Bourne *et al*., 2004).

Parthenogenetic ESCs is able to complement with these problems and be the alternative cell source for skeletal tissue engineering. Parthenogenetic ESCs has pluripotency as well as patient's own genetic information if the cells are originated from the oocytes from the same patient. Women are more susceptible to osteoporosis and other osteo-diseases, thus researches using the blastocyst and ESCs derived from patient's own oocytes are coming into a great issue. We have shown that ESCs lines can be generated from mouse parthenogenetic embryos (Choi *et al*., 2006). In the present study, we demonstrated successful in vitro differentiation of murine parthenogenetic ESCs toward osteogenic lineages. The osteogenic differentiation was verified by Von Kossa and Alizarin red staining. Evidence of the existing differentiated cell population after osteogenic induction was analyzed by RT-PCR, which confirmed the expression of osteogenic-specific genes-osteopontin, osteonectin, alkaline phosphatase, osteocalcin, bone-sialoprotein, collagen type1, and Cbfa1. Bone-sialoprotein, osteocalcin and Cbfa1 were used as intermediate/late osteogenic markers, while the others were used as early/intermediate ones (Zur Nieden *et al*., 2007).

Materials and Methods

Chemicals

All inorganic and organic compounds were obtained from Sigma-Aldrich Korea (Yong-in, Korea) unless otherwise stated. All media used were based on CZB and KSOM (Nagy *et al*., 2003).

Recovery of oocytes

Eight-week-old C57BL6 X DBA2 F1-hybrid mice were superovulated by intraperitoneal injections of 7.5 IU eCG and 7.5 IU hCG, given 48 h apart. Oocytes were recovered 16 h after hCG injection, and the oviducts were removed and transferred into a Petri dish containing 2 ml Hepes-buffered CZB medium (HCZB), supplemented with 300 IU/ml hyaluronidase. The oviduct ampullae were opened and the cumulus-enclosed oocytes were released. After 2 to 3 min exposure to the medium, the cumulus-free oocytes were washed twice in HCZB before activation.

Activation and in vitro culture

Recovered oocytes were immediately exposed to an activation medium, consisting of 10 mM $SrCl_2$ with 5 μg/ml cytochalasin B in calcium-free CZB for 5 h, and then cultured for 115 h in KSOM at 37°C in a humidified 5% CO_2 in air. After *in vitro* culture, zona pellucida of the blastocysts were removed by washing the embryos with acid-Tyrode solution.

Establishment of embryonic stem cell lines from parthenogenetic murine embryos

To generate ESC, thirty to fifty zona-free blastocysts were transferred onto STO feeder layer in gelatinized tissue culture plates (Nunc, Roskilde, Denmark) in ESC medium consisting of Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Paisley, UK) supplemented with 15% fetal bovine serum (FBS), 1% nonessential amino acid (NEAA), 0.1 mM β-mercaptoethanol and 1,000 units/ml ESGRO leukemia inhibitory factor (LIF; Chemicon, Temecula, USA). After 6 or 7 day of culture, the outgrown clumps derived from the ICM were counted under the stereo-microscope. Once the ICM outgrowth was evaluated, the cell clumps were further trypsinized with 0.05% trypsin-EDTA and seeded onto gelatinized new 4-well dish with fresh ES medium in order to generate parthenogenetic ESC. When the colonies appeared, they were considered as passage 0 and had been propagated gradually under the stringent culture conditions with careful monitoring and medium change to keep undifferentiated state and to avoid their differentiation.

Culture of Parthenogenetic ESCs

The parthenogenetic murine ESCs were cultivated on a feeder layer of mitomycin C-treated STO cells maintained in DMEM. And the medium was supplemented with 10% (v/v) FBS, 2mM L-glutamine, 0.1 mM β-mercaptoethanol, 50 U/ml penicillin, and 50 mg/ml streptomycin. Culture medium was also supplemented with leukemia inhibitory factor (LIF, 1,000 IU/ml), and maintained at 37°C, 5% CO_2, and changed once per day.

Embryoid body (EB) formation

To make EB, ESCs on the culture plates were lightly trypsinized for 2 min at 37°C. Trypsinization was terminated by addition of DMEM containing 10% fetal bovine serum (FBS). After brief centrifugation, cells were re-suspended in medium followed by medium supplementation with 10% (v/v) FBS, 2 mM L-glutamine, 0.1 mM â-mercaptoethanol, 50 U/ml penicillin, 50 mg/mL streptomycin and 10^{-7} M retinoic acid. Cells were plated onto bacteriological grade petri-dishes in the absence of fibroblast feeder layers and LIF. ESCs were maintained for 5 days in culture, with medium being replenished on day 3. After 5 days in culture, ESCs formed free-floating aggregates or EB.

Osteogenic induction of EBs

Osteogenic induction protocol was modified from the method of Buttery *et al* (2001). Briefly, The EBs were resuspended in alpha-modified Eagle's medium (a-MEM) containing 10% FBS, 50 U/ml penicillin, and 50 mg/ml streptomycin and allowed to adhere to six-well culture plates at a density of three EB/well. In cultures, medium was additionally supplemented with 50 mg/ml ascorbic acid and 10 mM β-glycerophosphate. The EBs were maintained in culture for 21 days and medium was replaced every 3 days.

Cell staining

For Von Kossa staining, the culture plates were rinsed off in PBS for twice and then fixed for 30 min in 70% ethanol. After being washed twice with double distilled water (DDW), 5% silver nitrate solution (60 watt lamp/UV for 20 min) was added until calcium turned black followed by three times of rinsing with DDW. Nuclear fast red were applied for 5 min to the cells.

Alizarin red staining of mineralized colonies was started with rinsing off culture plates with PBS for twice. The cells were then fixed with 0.2% glutaraldehyde for 5 min and washed off three times with DDW then 1% Alizarin Red S solution was applied for 10 min onto the cell culture plates. Excessive staining materials were washed off with DDW and the plates were then air dried (Bielby *et al*., 2004).

RT-PCR analysis

RNA extract of ostegenic cells derived from parthenogenetic ESCs was prepared. The cells were lysed with 1 ml of TRI reagent, and the lysate was homogenized by repetitive pipetting. These cell homogenate was transferred to a new tube and stored for 5-15 min at room temperature. Then 0.2 ml of chlorform was added per 1 ml of TRI agent. After vortexing for 15 s, the homogenate was stored at room temperature until aqueous layers were appeared. After centrifugation at 4°C, upper colorless aqueous phase containing RNAs and lower phenol-chloroform phase and interphase that contains DNA with protein were separated. Then aqueous phase were transferred into a new tube for isolation of RNAs and kept at −70°C. RNAs from parthenogenetic ESCs cells were used as the control group. As next, each RNA extract was carried out reverse transcription reaction. After the reverse transcription reaction, the final volume was increased to 50 ìl with ultra-purified water and then stored at −20°C until RT-PCR analysis. The analyses were performed in a final volume of 50 µl. The reactions contained 1 µl of the cDNA sample, 2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris- HCl (pH 8.3), 2.5 mM of each dNTP, 20 ìM of each primer (Table 1), and 1 ìl of rTaq polymerase (TaKaRa Korea, Seoul, Korea). After a first denaturation step of 5 min at 94°C, 35 amplification cycles were performed. Each cycle involved denaturation at 94°C for 40 s, annealing at 55°C for 40 s, and extension at 72°C for 40 sec. A final extension step of 7 min at 72°C was performed to complete the PCR. The PCR product was electrophoresed on a 1% agarose gel, stained with ethidium bromide, and visualized

Table 1. Osteogenic specific gene RT-PCR primer

	Sense	Antisense
Osteocalcin	5'-CCGGGAGCAGTGTGAGCTTA-3'	5'TAGATGCGTTTGTAGGCGGTC3'
Osteonectin	5'-ATCCAGAGCTGTGGCACACA-3'	5'-GGAAAGAAACGCCCGAAGA-3'
Bone sialoprotein	5'-CAGAGGAGGCAAGCGTCACT-3'	5'-CTGTCTGGGTGCCAACACTG-3'
Osteopontin	5'-GATGCCACAGATGAGGACCT-3'	5'-CTGGGCAACAGGGATGACAT-3'
Collagen type I	5'-GCATGGCCAAGAAGACATCC-3'	5'-CCTCGGGTTTCCACGTCTC-3'
Alkaline phosphatase	5'-GTGCCCTGACTGAGGCTGTC-3'	5'-GGATCATCGTGTCCTGCTCAC-3'
Cbfa1	5'-GTGCGGTGCAAACTTTCTCC-3'	5'-AATGACTCGGTTGGTCTCGG-3'

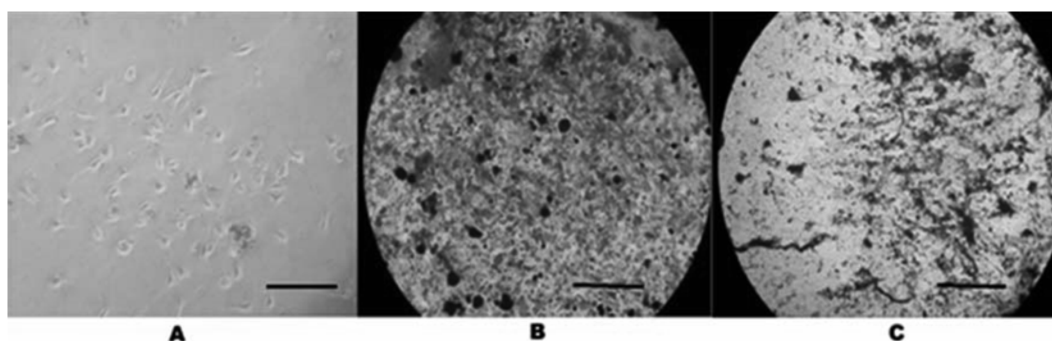

Fig. 1. The morphology of colonies formed after osteogenic induction of parthenogenetic embryonic stem cells was monitored and photographed. (A) The cells displays distinctive morphology of osteogenic cells. (B) Alizarin Red staining: Calcium turns out to be orange-red. The end product of Alizarin red staining is an Alizarin Red-calcium complex. It demonstrated the existence of calcium content in tissue sections which is the characteristic of the calcified osteoblasts. (C) Von Kossa staining: Nuclei appear to be red, cytoplasm as pink, and calcium deposition turns out to be black. Scale bar: A- 1 mm, B and C- 2.5 mm.

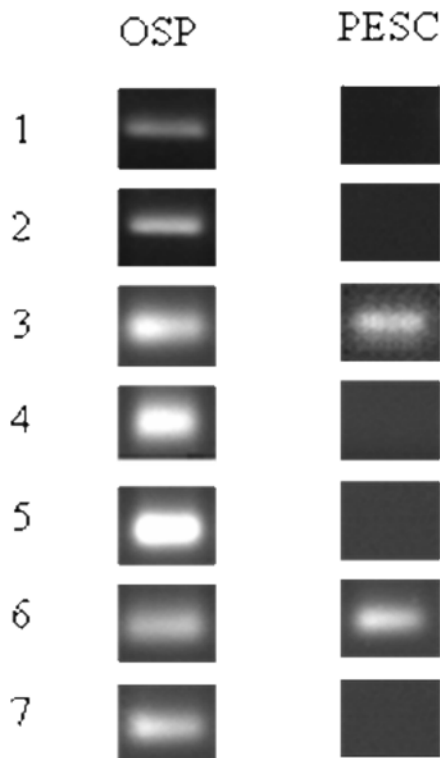

Fig. 2. Comparison of gene expression between the osteogenic cells derived from parthenogenetic embryonic stem cells (OSP, Left), and undifferentiated parthenogenetic embryonic stem cells (PESC, Right) was performed by RT-PCR. 1. osteopontin, 2. osteonectin, 3. alkaline phosphatase, 4. osteocalcin, 5. bone-sialoprotein, 6. collagen type1, 7. Cbfa1. All osteogenic marker genes were expressed in OSP whereas only alkaline phosphatase and collagen type1 genes were expressed in PESC.

Animal ethics

All animal experiments were approved and performed under the guidelines of the Institutional Animal Care and Use Committee of Seoul National University.

Results

Morphological and histochemical characteristics

The morphology of colonies formed in the cells after osteogenic induction was checked and photographed. The differentiated parthenogenetic ESCs displayed distinctive morphology of osteogenic cells (Fig. 1A), and histochemical characteristics were verified by Alizarin Red and Von Kossa staining (Fig. 1B and C, respectively). In Alizarin Red staining, calcium turns out to be orange-red. In Von Kossa staining, nuclei appear to be red, cytoplasm as pink, and calcium deposition turns out to be black.

RT-PCR analysis

The genes of osteopontin, osteocalcin, osteonectin, bone-sialoprotein, collagen type1, Cbfa1 and alkaline phosphatase were strongly expressed in the osteogenic cells differentiated from parthenogenetic ESCs while only alkaline phosphatase and collagen type1 genes were expressed in the undifferentiated parthenogenetic ESCs. The expression of intermediate/late osteogenesis markers of Cbfa1, bone-sialoprotein and osteocalcin means that fully differentiated osteogenic cell population, not progenitors, existed in the experimental group.

Discussion

In this study, we presented in vitro data demonstrating that mouse parthenogenetic ESCs can differentiate into osteogenic cells lineage. Von Kossa staining and Alizarin red staining have confirmed calcium deposition in the differentiated cells. Basically, bone is a mineralized connective tissue consisting of mainly type I collagen and other non-collageneous matrix proteins, such as bone sialoprotein, osteocalcin, osteonectin, osteopontin, and proteoglycans that determine characteristics of osteoblasts (Nanci, 2003). Osteopontin, a non-collagenous bone matrix molecule, is associated with osteoblastic cell adhesion and abundantly expressed during the early stages of osteoblast differentiation (Bourne *et al*., 2004), was expressed in the parthenogenetic ESCs in our study. The expression of intermediate/late osteogenesis markers such as Cbfa1, bone-sialoprotein and osteocalcin also showed existence of fully differentiated osteogenic cell population as well as osteogenic progenitors. These results strongly suggest that osteogenic induction of parthenogenetic ESCs was driven into the osteogenic lineage, which might be an alternative embryonic stem cell source for the study of skeletal repair and regeneration. Especially the defects in full-term development of this cell lines have ability to enableresearchers to avoid the ethical concerns related embryonic stem cell research. Although there are many remarkable researches on tissue engineering and regenerative medicine using cellular supports (Daar *et al*., 2007; Freymana *et al*., 2001), few of those are related on hard tissue regeneration using parthenogenetic ESCs and their derivatives. Although osteogenic differentiation of normal murine ESC was once reported (Buttery *et al*., 2001), studies on parthenogenetic ESCs focused on the histocompatibility of ESCs and their potential of non-specific three-germ layer differentiation after EB induction (Kim *et al*., 2006; Lengerke *et al*., 2007).

Integrity of alveolar bone affects significantly on successful osseointegration and primary fixation of dental implant fixture, thus it plays crucial part on success of dental implant prosthesis (Hujaa *et al*., 2004; Smith *et al*., 1989). If the osteogenic cells generated from parthenogenetic ESCs could enhance bone regeneration, it will help alveolar socket healing of female patients with regenerative problems, and can help edentulous people to recover anatomic integrity of alveolar ridge that would enable them to get the retentions for full arch

denture prosthetics, and enhance primary osseointegration of implant fixture to dental alveolar bone as well as less concern about histocompatibility. This kind of cell therapy can also help female patients of innate malformations and defects, and patients under oromaxillo-facial cancer therapy. Further in vivo studies are needed to confirm the hard tissue regeneration potential of parthnogenetic ESC derivatives.

In summary, we demonstrated that parthenogenetic ESCs can be generated into osteogeneic lineage by supplementing defined inducing factors to culture medium. It may enable researchers to avoid the ethical concerns related with ESCs researches. As demands on regenerative medicine and dentistry related to bone disease and defects are increasing, approaches using parthenogenetic ESCs could be one of the alternative solutions for the hard tissue regeneration and skeletal tissue repair.

Acknowledgement

This study was supported by the Korea Science and Engineering Foundation (KOSEF) grant funded by the Korea government (MOST; M10641000001-06N4100-00110 and R01-2007-000-10316-0).

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