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Parthenogenesis-derived multipotent stem cells adapted for tissue engineering applications ☆

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ABSTRACT

Embryonic stem cells are envisioned as a viable source of pluripotent cells for use in regenerative medicine applications when donor tissue is not available. However, most current harvest techniques for embryonic stem cells require the destruction of embryos, which has led to significant political and ethical limitations on their usage. Parthenogenesis, the process by which an egg can develop into an embryo in the absence of sperm, may be a potential source of embryonic stem cells that may avoid some of the political and ethical concerns surrounding embryonic stem cells. Here we provide the technical aspects of embryonic stem cell isolation and expansion from the parthenogenetic activation of oocytes. These cells were characterized for their stem-cell properties. In addition, these cells were induced to differentiate to the myogenic, osteogenic, adipogenic, and endothelial lineages, and were able to form muscle-like and bony-like tissue *in vivo*. Furthermore, parthenogenetic stem cells were able to integrate into injured muscle tissue. Together, these results demonstrate that parthenogenetic stem cells can be successfully isolated and utilized for various tissue engineering applications.

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1. Introduction

For the replacement of damaged or diseased tissue, most current strategies for tissue engineering depend upon a sample of autologous cells from the diseased organ of the host [1–3]. However, in the setting of end-stage organ failure, a tissue biopsy may not yield enough normal cells for expansion and transplantation. In other instances, primary autologous human cells can not be adequately expanded from a particular organ for replacement purposes. In these situations, embryonic stem cells are envisioned as a viable source of pluripotent cells from which the desired tissue can be derived. Combining regenerative medicine techniques with this potentially endless source of versatile cells could lead to novel sources of replacement organs that would alleviate the chronic shortage of available donor organs.

Embryonic stem cells exhibit two remarkable properties: the ability to proliferate in a undifferentiated, but pluripotent state (self-renew), and the ability to differentiate into many specialized cell types [4]. However, the destruction of embryos required for embryonic stem cell research has led to limitations in the United States, where federally-funded embryonic stem cell research has been restricted to the use of a limited number of cell lines under current federal regulations [5,6]. Recent studies describing induced pluripotent stem (iPS) cells present the possibility of embryonic stem cell research without the need for destruction of embryos [7–9]. However, iPS cell research is still in its infancy, and it is still unclear whether these cells will be safe for use in clinical applications such as tissue engineering [10].

Parthenogenesis, the process by which an egg can develop into a blastocyst in the absence of sperm, has previously been identified as a potential source for embryonic stem cells [11]. The ability to have embryonic stem cells, without sacrificing conception human embryos, would be valuable for patients who require the replacement of damaged or diseased tissue. In addition, this alternative source of embryonic stem cells may avoid some of the political and ethical concerns that surround current human embryonic stem cell techniques. Previous studies have described the isolation of embryonic stem cells from parthenogenesis [11–18]; however, the adaptation of parthenogenetic stem cells for tissue engineering applications have not been previously described.

The aim of our study was to investigate whether multipotent stem cells could be isolated from parthenogenesis-derived blastocysts for tissue engineering applications. We have described a protocol for the isolation and expansion of embryonic stem

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cells derived from the parthenogenetic activation of rabbit oocytes, and we characterized these cells for their stem-cell properties. In addition, we induced these cells to differentiate into multiple lineages, and have demonstrated their survival in various *in vivo* environments. We anticipate that these methods will lead to eventual scale-up of these techniques to produce the large quantity of cells and tissue required for regenerative medicine applications.

2. Materials and methods

2.1. Retrieval and parthenogenetic activation of oocytes

Oocytes were collected from the oviducts of superovulated New Zealand rabbits approximately 16 h after intravenous injection of 500 U of human chorionic gonadotropin [19]. All procedures were approved by the institutional animal care committee.

Meiosis II oocytes were activated with two sets of electrical stimulation applied 30 min apart with an ECM 2001 stimulator (BTX, Holliston, MA). For each set, a total of three direct current (DC) pulses of 1.2 kV/cm for 60 μs each were applied while the oocytes were in a solution containing mannitol 0.3 M in water with 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$,10 mM HEPES, and 0.1% poly-vinyl alcohol (PVA). Electrically stimulated oocytes were then incubated for 2 h at 37 °C in potassium simply optimized medium (KSOM—Specialty Media, Phillipsburg, NJ) containing 5 μg/ml cyclohexamide (CHX) and 1 mM 6-D-methyl aminopyridine (DMAP), and then cultured in a 500 μl drop of KSOM medium under mineral oil in a 37 °C, 5% CO_2 environment. In general, CHX is used to maintain interphase in the cell cycle, and DMAP is a catalyst for acylation reactions.

2.2. Isolation and culture of parthenogenesis-derived stem cells

Once the activated oocytes reached the blastocyst stage, microsurgery was used to mechanically dissect the inner cell mass from the blastocyst. The inner cell mass was placed initially on mitotically inactive mouse feeder layers for expansion in Dulbecco's minimal essential medium (Invitrogen) and 15% fetal calf serum (Invitrogen) for passage P^0 [19]. The expanded cells were then microsurgically dissected and transferred to Petri dishes for further expansion (P^1 and beyond).

For immunoisolation, selection for c-kit^{pos} cells was performed with magnetic cell sorting (Mini-MACS-Miltenyi Biotec Inc., Auburn, CA) as previously described [20]. Magnetic sorting with the Mini-MACS system was utilized to minimize the contamination risk to the cells.

2.3. Flow cytometry

Cells were trypsinized, washed with PBS, then incubated with antibodies against oct-4, bmp-4, cmet, ckit, tra-1-60, and tra-1-80 (Santa Cruz Biotechnology, Santa Cruz, California), as well as ssea-3 and ssea-4 (Developmental Studies Hybridoma Bank).

For cell cycle analysis, cells were fixed in 90% ethanol, then incubated in a 50 μg/ml propidium iodide solution to stain the DNA.

All analyses were performed with a FACScalibur flow cytometer (Becton–Dickinson).

2.4. Karyotype

Cells were cultured in T-25 culture flasks (flask method) and/or on glass coverslips (*in* situ method) for 3–5 days. Standard cytogenetic harvesting, fixation, and GTG banding techniques were used.

2.5. Induction toward multiple cell lineages

The cells were induced to osteogenic [21], myogenic [22,23], and adipogenic [24] lineages according to previously described protocols. For endothelial differentiation, the cells were maintained in EGM-2 endothelial cell medium (Cambrex Corporation, East Rutherford, NJ) supplemented with 10% fetal bovine serum (FBS), and basic fibroblast growth factor (bFGF).

2.6. Immunocytochemistry and histochemistry

Single and double immunolabelling was performed using indirect immunofluorescence. Cell cultures were fixed in methanol for 10 min, incubated in PBS with 10% of appropriate serum for 45 min to block non-specific antibody binding, incubated overnight at 4 °C with primary antibodies, washed three times with PBS, and incubated for 30 min at room temperature with secondary antibodies. Primary antibodies used were desmin, myo D, actinin, sarcomeric tropomyosin, osteocalcin, and osteopontin (Santa Cruz Biotechnology, Santa Cruz, CA), KDR (Imclone Systems, New York, NY), P1H12 (Chemicon, Temecula, CA), and Von Willebrand Factor (VWF) (Dako, Denmark). Secondary antibodies were biotinylated anti-mouse and biotinylated anti-goat immunoglobulins (Vector Laboratories, Burlingame, CA). The secondary biotinylated antibodies were incubated with Fluorescein–Avidin D (Vector).

Von Kossa staining was used to detect the presence of mineralization in cell culture. The cell culture plates were fixed with 10% formaldehyde for 1 h, incubated with 2% silver nitrate solution for 10 min in the dark, washed thoroughly with deionized water, and then exposed to ultraviolet (UV) light for 15 min.

For Oil Red O staining, cells were fixed with 2% paraformaldehyde for 10 min at room temperature and rinsed with PBS. Samples were incubated in Oil Red O solution (Sigma) for 15 min and rinsed with distilled water. Hematoxylin was used for counterstaining.

2.7. Reverse-transcriptase polymerase chain reaction (RT-PCR)

RNA was isolated from cultured cells and cell pellets with RNA-Bee RNA Isolation Reagent (Tel-Test Inc., Friendswood, TX) according to the manufacturer's protocol. RNA (2 μg) was processed for c-DNA synthesis with Superscript II reverse transcriptase with random hexamers (Invitrogen). c-DNA was used for each PCR reaction, in a final volume of 50 μl with 10 mM dNTP, 15 pM of each primer, 0.4 U *Taq* DNA-polymerase, reaction buffer, and MgCl2 (Invitrogen), in a Mastercycler (Eppendorf). The cycling conditions consisted of annealing at various temperatures for 30 s, elongation at 72 °C for 1 min, and denaturation at 92 °C for 45 s. Cycle numbers varied between 28 and 35 cycles and were chosen in the exponential phase of the RT-PCR. Primer sequences and fragment sizes are listed in Table 1. All primers were obtained from Invitrogen. PCR products were resolved on a 2% agarose gel and stained with ethidium bromide. The amplified DNA was visualized under UV light.

2.8. In vivo placement of labeled-cell-seeded scaffolds

The outer membrane of undifferentiated cells were labeled using PKH26 Red (Sigma) according to the manufacturer's protocol with the exception of using 40 μl dye with each ml of diluent. For the myogenic cells, polyglycolic acid (PGA) scaffolds measuring $0.5 \times 0.5 \times 0.5$ cm were seeded with undifferentiated cells, and for the osteogenic cells, hydroxyapatite–collagen scaffolds measuring $0.5 \times 0.5 \times 0.5$ cm were seeded with undifferentiated cells, both at a density of 60 million cells per cm^3. The cell-seeded scaffolds were incubated in their respective differentiation media for 14 days, then implanted into the dorsal subcutaneous area of athymic mice. After in vivo formation, tissue was surgically retrieved

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