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Scaffold-free three-dimensional culture systems for mass production of periosteum-derived progenitor cells

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Mesenchymal stem cells (MSCs) are capable of self-renewal and can differentiate into various types of cells for therapeutic purposes. MSCs are frequently cultured in a two-dimensional (2D) system. However, MSCs can lose their differentiation capacity over time in this culture system. In addition, the available surface area for the propagation of cells is limited. Therefore, various three-dimensional (3D) culture systems have been developed. In this study, we developed the scaffold-free 3D culture systems for the expansion of periosteum-derived progenitor cells (PDPCs) as spheres. The spheroid formation of PDPCs was induced using a rotation platform. The spheres maintained their viability and proliferation ability. Moreover, expression levels of the stemness marker genes and proteins were higher in cells grown on 3D culture system than in 2D culture system. In conclusion, a simple and economical 3D culture system has been developed that can increase the potential of PDPCs for clinical use.

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[**Key words:** Mesenchymal stem cells; Periosteum-derived progenitor cells; Three-dimensional culture system; Scaffold-free culture; Spheroid formation]

Stem cells are undifferentiated cells that can regenerate themselves and produce specialized cell types. They have emerged as an attractive source of cells for clinical treatment, as stem cell-based products for transplantation and regenerative medicine have been increased (1). In the early 1900's, it was found that some stem cells could generate blood cells. In 1968, the first bone marrow transplant was performed using stem cells (2). Proving their popular use as therapeutics, a recent search in the ClinicalTrials.gov database indicates many clinical trials of stem cells under current investigation.

Mesenchymal stem cells (MSCs) exist in adult tissues and are important tools for cell therapy because of their immunosuppressive properties and homing effects. The International Society for Cellular Therapy (ISCT) defines MSCs according to three properties: (i) an adherence to plastic; (ii) the positive expression of specific surface markers (CD73, CD90, and CD105) with the negative expression of hematopoietic markers (CD14, CD34, and CD45); and (iii) multipotent differentiation (3). Periosteum-derived progenitor cells (PDPCs) are one of the diverse MSCs. They have a high proliferation capacity and are easily isolated from tissues. The PDPCs have a capacity for mesodermal differentiation (4). In addition, they can differentiate into insulin-producing cells (IPCs) for the treatment of type 1 diabetes (5).

MSCs are typically cultured on the flat surface of a conventional two-dimensional (2D) culture. During 2D culture, they lose the primitive properties of stem cells and require the large surface for cell expansion. For these reasons, various methods have been developed for the three-dimensional (3D) culture of MSCs (6). MSCs should be obtained in sufficient quantities (10^8–10^9 cells) for transplantation. The propagation of MSCs through 2D culture is unsuitable due to limitations in surface area. Furthermore, cell proliferation and differentiation capacity are limited during 2D culture expansion (7). Cell aging, associated with actin accumulation and reduced adhesion molecules, is affected as passage number increases in 2D culture. On the other hand, 3D cultures can easily obtain sufficient numbers of cells for clinical use.

The aggregation of stem cells in 3D culture also has advantages in terms of biological functions that closely represent conditions *in vivo*. The spheres, formed by aggregation, can enhance cell–cell interactions and promote their therapeutic potential by stimulating stemness marker genes (8). Numerous 3D cultures have been studied using spheres (9). In particular, embryonic stem cells (ESCs) have been grown as spheres called embryoid bodies (EBs). There are a variety of techniques for inducing EBs and these techniques can be applied to MSCs (10).

The 3D culture using biocompatible a scaffold such as collagen and some polyesters has additional processes and manufacturing costs of scaffolds. Therefore, spheroid culture system that is induced by aggregation of stem cells has been developed in scaffold-free condition. Scaffold-free 3D culture is simple methods to form a spontaneous spheroid formation. These methods for aggregation of stem cells, for example hanging drop culture, suspension culture in stirred reactor system, cultivation on low-adherence plate, overcome the attachment problem (6).

In this study, we developed the scaffold-free 3D culture system for the expansion of PDPCs as spheres. The spheroid formation of

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PDPCs was easily induced by a rotating platform. The rotation system provides a sufficient nutrient supply for the growth of the spheres. As a result, the 3D culture system has potential for the economical production of stem cells and can increase the efficiency of differentiation.

MATERIALS AND METHODS

Isolation and 2D culture of PDPCs Periosteum was obtained from the Good Shepherd Hospital in Seoul, Korea. The isolation of PDPCs has been described in a previous report (11). Briefly, the cambium side of minced periosteum explants was plated onto a 100-mm culture dish (SPL Lifescience, Pocheon, Korea) in Dulbecco's Modified Eagle's Medium-low glucose (DMEM; Gibco, NY, USA) containing 10% fetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin (P/S; Gibco). The detached cells were then washed and incubated for 20 min at 4°C with conjugated monoclonal antibodies against CD9, CD45, CD90, CD105 and CD166. Triple positive cells (CD9, CD90 and CD166), called PDPCs were sorted using FACSVantage flow cytometer (Becton Dickinson, NJ, USA). The isolated PDPCs were seeded in T-flasks (SPL Lifescience). Cultures were maintained at 37°C in a humidified atmosphere with 5% CO_2. The culture medium was changed every 3 days.

Scaffold-free spheroid culture of PDPCs In the scaffold-free spheroid culture, spheres were formed by aggregation. The PDPCs were seeded at a density of 2×10^5 cells/ml in 35-mm non-adhesive culture dishes (SPL Lifescience). The total volume was 2 ml and the cells were cultivated at a rotation rate of 60 rpm using an orbital shaker (Vision Scientific Co., Daejeon, Korea). The 3D cultures were maintained at 37°C in a humidified atmosphere with 5% CO_2 in DMEM containing 10% FBS and 1% P/S.

Cell proliferation The proliferation assay is based on the cleavage of tetrazolium salt to water-soluble formazan by succinate-tetrazolium reductase. The spheres were harvested and plated on a 96-well plate (Nunc, Roskilde, Denmark) with 100 μl of fresh culture medium and 10 μl EZ-Cytox (iTSBIO, Seoul, Korea) was added to each well. The spheres were Incubated for 1 to 2 h in dark conditions. The absorbance was measured on a BiotrackII plate reader (Amersham, Buckinghamshire, UK) at 450 nm.

Live/Dead assay The spheres consisting of PDPCs were washed five times with phosphate buffered saline (PBS; Gibco). After washing, they were co-incubated with 10 μl of 1 mg/ml Calcein-AM (Sigma, MO, USA) for live cell staining and 10 μl of 1 mg/ml Propidium iodide (PI; Sigma) as dead cell fluorescent marker for 10 min. Next, the spheres were washed again in PBS five times. The treated spheres were observed with the BX51 fluorescence microscope (Olympus, Tokyo, Japan).

Flow cytometry analysis The spheres were dissociated with 0.05% trypsin-EDTA (T/E; Gibco) for 20 ~ 30 min. Dissociated spheres were washed with PBS and then incubated with anti-human antibodies for 30 min at 4°C in dark conditions. The anti-human antibodies used were CD9-FITC, CD90-APC, CD166-PE, CD45-PerCP, and PI (all from Becton Dickinson). The PDPCs were analyzed on a fluorescence-activated cell sorter (FACS Calibur; Becton Dickinson). Data analysis was conducted using CellQuest Pro and WinMDI 2.8 software.

RNA isolation and reverse transcription-polymerase chain reaction Total RNA from the spheres was isolated using the RNeasy plus mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. 1 μg of RNA was reverse-transcribed using the Maxime RT PreMix Kit (iNtRON, Seongnam, Korea). The cDNA was then synthesized using the 2720 Thermal Cycler (Applied Biosystem, CA, USA). The reverse transcription-polymerase chain reaction (RT-PCR) reaction mixture consisted of cDNA, DEPC water, AccuPower PCR Premix (Bioneer, Daejeon, Korea), and primer pairs. Each primer used is shown in Table 1. The mixture was amplified using the 2720 Thermal Cycler for 20 cycles as follows: denaturation at 95°C for 20 s; annealing at 60°C for 20 s; and extension at 72°C for 30 s. The PCR products were electrophoresed on a 2% agarose gel.

TABLE 1. List of primers for PCR.

mRNA	Sense/Antisense sequences	Size (bp)
GAPDH	5′-GCTCTCCAGAACATCATCCCTGCC-3′	346
	5′-CGTTGTCATACCAGGAAATGAGCTT-3′	
Nanog	5′-CAGAAAAACAACTGGCCGAA-3′	253
	5′-GGCCTGATTGTTCCAGGATT-3′	
Sox2	5′-AATGCCTTCATGGTGTGGTC-3′	357
	5′-GTTCATGTGCGCGTAACTGT-3	
KLF4	5′-CCCAATTACCCATCCTCCCT-3′	309
	5′-ACGGTACTGCCTGGTCAGTT-3′	
Oct4	5′-AAGCGATCAAGCAGCGACTA-3′	237
	5′-CCTCAGTTTGAATGCATGGG-3′	
Sox9	5′-CAGAAAAACAACTGGCCGAA-3′	213
	5′-GGCCTGATTGTTCCAGGATT-3′	
Osteopontin	5′-ACTCGAACGACTCTGATGATGT-3′	224
	5′-GTCAGGTCTGCGAAACTTCTTA-3′	
Leptin	5′-AGATCCTCACCAGTATGCCTT-3′	179
	5′-CTCTGTGGAGTAGCCTGAAGC-3′	

Real time-quantitative PCR The real time-quantitative PCR (RT-qPCR) reaction mixture consisted of cDNA, DEPC water, iQTM SYBR Green Supermix (Bio-Rad, CA, USA), and primer pairs (sequences listed in Table 1). The RT-qPCR was performed with a Chromo4 System (Bio-Rad) for 45 cycles. The cycles for PCR were as follows: denaturation at 95°C for 20 s; annealing at 54°C for 20 s; and extension at 72°C for 30 s.

Western blot To prepare whole-cell extracts, cells were washed three times in cold PBS and suspended in RIPA buffer containing 50 mM Tris−HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS and supplemented with protease inhibitors such as supplemented with 30 μg/ml aprotinin, 10 μg/ml phenyl methylsulphonyl fluoride (PMSF) and 10 μg/ml sodium orthovanadate. After incubation on ice for 30 min, lysates were centrifuged and protein in the supernatants was quantified using the Bradford Protein Assay Reagent (Bio-Rad). An equal amount of protein from each sample was then separated by electrophoresis on a 12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE), followed by electrophoretic transfer to nitrocellulose membranes. Membranes were blocked with 5% Skim milk (Bio-Rad) and then incubated with the indicated antibodies at 4°C overnight. The primary anti-human Oct4 (Cell Signaling Technology, MA, USA), Sox2 (Abcam, MA, USA), KLF4 (Millipore, MA, USA), Nanog (Cell signaling Technology) and β-actin (Santa Cruz Biotechnology) antibodies were diluted to 1:1000. The secondary antibodies, anti-mouse IgG-HRP (Santa Cruz Biotechnology, CA, USA) and anti-rabbit IgG-HRP (Santa Cruz Biotechnology), were diluted in the 5% Skim milk at a dilution of 1:5000. Blots were processed with ECL Plus Western Blotting detection kit (Bio-Rad) and the signal detected using an LAS-3000 image analyzer (Fuji Photo Film Co., Tokyo, Japan).

Mesodermal differentiation For Mesodermal differentiation, the culture medium was replaced with reagents from the STEMPRO Differentiation Kit (Gibco). Adipogenic differentiation was detected by Oil Red O staining, which stains lipid-containing vacuoles. The differentiated spheres were fixed in 4% paraformaldehyde for 30 min, and then stained with 0.3% Oil Red O (Sigma) for 30 min. The stained spheres were washed with PBS. Osteogenic differentiation was evaluated by von Kossa staining, which determined mineral deposition. After osteogenic differentiation, the spheres were fixed in 4% paraformaldehyde for 30 min, cultivated in 5% silver nitrate (Sigma) and incubated in dark conditions for 30 min. The spheres were then exposed to ultraviolet light for 30 min. Next, the spheres were incubated in 1% sodium thiosulfate (Sigma). Chondrogenic differentiation was determined by Alcian blue staining which stains glycosaminoglycans in cartilage. The spheres containing PDPCs were fixed in 4% paraformaldehyde, stained in Alcian blue 8GX (Sigma) for 30 min. The stained spheres were then washed with 0.1 N HCl (Sigma).

Statistical analysis Experiments were performed in duplicates. Results were expressed as mean ± standard deviation from duplicate samples.

RESULTS

3D culture system of PDPCs The stem cells have to be obtained in large numbers for clinical applications. 2D cultures are not commercially suitable for large-scale cultivation, due to limitations in incubation area. However, the 3D culture could rapidly and easily obtain cells compared with 2D cultures (Fig. 1). Therefore, we developed the 3D culture systems for the expansion of PDPCs which are a kind of MSCs. The PDPCs were seeded in 35-mm culture dishes on a rotation platform using an orbital shaker. The spheres of PDPCs formed through spontaneous aggregation. As a result, the scaffold-free spheroid culture system could induce compact spheres (Fig. 2). The culture medium was changed every 3 days.

Spheres retain their viability and proliferation ability The PI staining was used to determine cell death through fluorescent detection. Calcein-AM permeated into the viable cells of spheres, which were confirmed through green staining. Also, cell death of the spheres was increased after day 6 (Fig. 3A). The proliferation ability in the 3D culture system increased up to 4 days. This result was similar to 2D cultures (Fig. 3B). To confirm the possibility of scale-up, spheres were transferred to 125-ml Erlenmeyer flasks. The cells steadily maintained their proliferation without the dissociation of spheres. After 9 days, we could get up to 10^7 cells (Fig. 4). In scaffold-free condition, developed 3D culture system efficiently achieve sufficient number of stem cells for transplantation.

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