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# Characterization and evaluation of the differentiation ability of human adipose-derived stem cells growing in scaffold-free suspension culture

YAN-HSIUNG WANG[1,2,*], JYUN-YI WU[3,*], PEI-JUNG CHOU[3], CHUNG-HWAN CHEN[2,4,5], CHAU-ZEN WANG[2,6,7], MEI-LING HO[2,6,7], JE-KEN CHANG[2,4,8], MING-LONG YEH[3] & CHIA-HSIN CHEN[9,10,11]

[1]*School of Dentistry, College of Dental Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan, Republic of China,* [2]*Orthopaedic Research Center, College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan, Republic of China,* [3]*Department of Biomedical Engineering, National Cheng Kung University, Tainan, Taiwan, Republic of China,* [4]*Department of Orthopedics, College of Medicine, Kaohsiung Medical University Hospital, Kaohsiung, Taiwan, Republic of China,* [5]*Departments of Orthopedics, College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan, Republic of China,* [6]*Department of Physiology, College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan, Republic of China,* [7]*Graduate Institute of Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan, Republic of China,* [8]*Department of Orthopedics, Kaohsiung Municipal Ta-Tung Hospital, Kaohsiung, Taiwan, Republic of China,* [9]*Department of Physical Medicine and Rehabilitation, Kaohsiung Medical University Hospital, Kaohsiung, Taiwan, Republic of China,* [10]*Department of Physical Medicine and Rehabilitation, Faculty of Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan, Republic of China, and* [11]*Department of Physical Medicine and Rehabilitation, Kaohsiung Municipal Ta-Tung Hospital, Kaohsiung, Taiwan, Republic of China*

**Abstract**

*Background aims.* Human adipose-derived stem cells (hADSCs) have become a popular stem cell source because of their abundant supplies, high differentiation ability and the fact that they present few ethical concerns. Suspension culture, a type of three-dimensional culture, is a more suitable model for mimicking cell-cell and cell–extracellular matrix interactions than is two-dimensional monolayer culture. The aim of this study was to determine the effects of suspension culture on the viability and differentiation potential of hADSCs. *Methods.* Different densities of hADSCs were cultured in ultra-low–attachment surface plates. The morphology and mean diameter of the resultant aggregates were determined by means of microscopy. The viability of the aggregates was evaluated with the use of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt, lactate dehydrogenase and live/dead assays. To detect osteogenesis, chondrogenesis and adipogenesis in hADSCs in suspension culture, cell aggregates were stained to determine cell function, and the expression of specific markers was evaluated through the use of real-time reverse transcriptase–polymerase chain reaction. *Results.* The hADSCs remained viable in suspension culture and formed cell aggregates. The diameter of the majority of the aggregates was in the range of 50–200 μm, regardless of cell density. The aggregation of the hADSCs served to maintain cell survival. In addition, the results of the histomorphometric and gene expression analyses showed that the hADSCs were more efficiently induced to differentiate into osteoblasts, chondrocytes and adipocytes in suspension culture than in two-dimensional monolayer culture. *Conclusions.* Suspension culture can be used to maintain cell viability and contributes to the effective differentiation of hADSCs, providing an alternative cell growth strategy for application to stem cell–based regenerative medicine.

**Key Words:** *adipose-derived stem cells, cell viability, differentiation, suspension culture, tissue engineering*

## Introduction

The aim of tissue engineering and regenerative medicine is restoration of the function of damaged tissue, which requires synergy between stem cells, scaffolds, and cytokine growth factors. The availability of a dependable source of stem cells is important for the regenerative process, and these cells have been harvested from various tissues throughout the body, including the skin, muscle, bone marrow, brain, umbilical cord, adipose tissue

*These authors contributed equally to this work.
Correspondence: **Chia-Hsin Chen**, MD, PhD, Department of Physical Medicine and Rehabilitation, Kaohsiung Medical University Hospital, 100 Tzyou 1st Road, Kaohsiung 807, Taiwan, ROC. E-mail: chchen@kmu.edu.tw

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and liver. Two major characteristics have been defined as criteria for the identification of stem cells: a self-renewal ability and the potential for differentiation (1). In recent years, the use of adult stem cells has been less controversial than the use of embryonic stem cells in various applications because they may be of autologous origin and are mostly free of ethical concerns (2). In addition to bone marrow stem cells (BMSCs), adipose-derived stem cells (ADSCs) have attracted increasing attention because they are less invasive to obtain, can be safely transplanted into an autologous or allogeneic host and exhibit higher cell activity (3–6). Moreover, ADSCs show a remarkable potential for differentiation into bone, cartilage, fat and muscle *in vitro* (7–11). Approximately $10^7$ to $10^8$ ADSCs can be isolated from 240 grams of adipose tissue (12), which is a much greater number of cells than can be obtained from isolated BMSCs. ADSCs express common mesenchymal stromal cell surface markers, such as CD9, CD10, CD13, CD29, CD34, CD44, CD49d, CD49e, CD54, CD55, CD105, CD106 and CD146 (13).

Stem cell–based tissue engineering has been confronted with several challenges in clinical applications, including large-scale expansion, the regulation of cell differentiation and adaption to the micro-environment of the recipient (14,15). It is known that the scaffold plays a crucial role in providing a substitute for the extracellular matrix (ECM) in supporting seeded stem cells (16). The disassociation of transplanted stem cells from ECM components may trigger apoptosis, leading to very low survival rates (~5%) and low differentiation efficiency in damaged tissues. A possible explanation for these issues may be that stem cells are altered by the traditionally used two-dimensional (2D) monolayer culture and differentiation methods and separated from their neighbors into single cells before transplantation into the recipient (17). This lack of interaction between the cells and environmental conditions restricts the implementation of cell therapies in clinical applications (18).

To solve the potential problems associated with traditional monolayer culture, three-dimensional (3D) culture has been considered as an alternative strategy for application to stem cell–based regenerative medicine. The advantage of 3D culture is the ease with which complicated structures are formed to communicate signals under these conditions, leading to the development of a complete tissue. Bioreactors are a general type of methodology applied in 3D culture, including perfusion stir tank bioreactors (19) and rotary microgravity bioreactors (20–24), with or without the addition of nanoparticle carriers (25–27), though most of these 3D culture methods are expensive and time-consuming. Special devices or a combination of materials and scaffolds are required for stem cell growth. Interestingly, it has been reported that the use of a low-attachment culture plate generates a suspension culture environment that allows cells to aggregate into 3D blocks (28,29). However, little is known about the growth properties and differentiation ability of stem cells growing in suspension culture. In this study, human ADSCs (hADSCs) were cultured in traditional 2D culture plates or ultra-low–attachment surface plates (ULASPs, 3D culture), and the resultant cell viability and differentiation potential were intensively investigated.

## Methods

### *Cell culture*

Human ADSCs purchased from Cellular Engineering Technologies (CET, Coralville, IA, USA) were used in this study. The hADSCs were extracted and separated from the fat tissue of a normal donor and grown in a mixture of keratinocyte serum-free medium (Gibco, Rockville, MD, USA) and Dulbecco's modified Eagle's medium-low glucose (DMEM; Gibco), as described in a previous report by our group (30). Briefly, the keratinocyte serum-free medium was supplemented with bovine pituitary extract (25 mg), human recombinant endothelial growth factor protein (2.5 μg), 0.2 mmol/L *N*-acetyl-cysteine (Sigma, St Louis, MO, USA), 0.2 mmol/L L-ascorbic acid 2-phosphate magnesium (Sigma), 0.06 μg/mL insulin (Sigma), 5% (vol/vol) fetal bovine serum (FBS; SAFC Biosciences, St Louis, MO, USA), and 1% (vol/vol) penicillin/streptomycin (P/S; Gibco). The cells were cultured in a 37°C incubator in a humidified 5% $CO_2$ atmosphere, and the medium was changed every other day.

### *Suspension culture*

ULASPs (No. 3471; Corning, Corning, NY, USA) were used as a suspension culture system in our study. These plates are designed to prevent cells from adhering to the bottom of the culture plates. Different densities of hADSCs (low [L]: $1 \times 10^5$ cells/mL; medium [M]: $5 \times 10^5$ cells/mL; and high [H]: $1 \times 10^6$ cells/mL) were cultured in the system to mimic 3D suspension culture conditions.

### *Quantitative analyses of cell aggregates*

The size distribution of cell aggregates was determined by capturing cell images by means of microscopy (Nikon/50i, DS-L1) and measuring the diameter of each cell aggregate with the use of