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A comprehensive analysis of human dental pulp cell spheroids in a three-dimensional pellet culture system

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ABSTRACT

Objective: Three-dimensional (3D) cell culture methods are of high importance to studies of biological processes. This is particularly the case with spheroid cultures, which create 3D cell aggregates without the use of exogenous materials. Compared to conventional monolayer cultures, cellular spheroid cultures have been demonstrated to improve multilineage potential and extracellular matrix production. To address this issue in depth, we present a more comprehensive analysis of 3D human dental pulp cell (hDPC) spheroids.

Design: hDPC spheroids were fabricated by the pellet culture method and were cultured without adding any reagent to induce differentiation. The gene-expression profiles of the 3D and two-dimensional (2D) cultured hDPCs were compared by complementary DNA microarray analysis. Odontoblastic and osteoblastic differentiation marker gene expression was evaluated by quantitative real-time PCR (RT-qPCR). Hematoxylin-eosin (HE) staining and transmission electron microscopy (TEM) were applied to examine the morphology of hDPC spheroids and extracellular matrix components.

Results: Compared with 2D monolayer culture, microarray analysis identified 405 genes and 279 genes with twofold or greater differential expression after 3 days and 28 days of 3D culture, respectively. In 3D hDPC spheroids, gene ontology analysis revealed upregulation of extracellular matrix-related genes and downregulation of cell growth-related genes. RT-qPCR analysis showed higher expression levels of osteocalcin, dentin sialophosphoprotein, and alkaline phosphatase. TEM revealed the morphological characteristics of the fibrillar collagen-rich matrix and cell-cell interactions.

Conclusions: The present findings provide clues to understanding the mechanisms of pellet-cultured hDPCs and contribute to future research in the comparative studies of different 3D culture methods.

1. Introduction

Research in cell biology often requires establishing in vitro cell culture models to investigate biological processes or cellular mechanisms under specific experimental conditions. Two-dimensional (2D) monolayer cell culture methods have been widely used and have undergone intensive development since they create a well-controlled cell environment and sustain various types of cell proliferation. However, cells grown on 2D substrates show a simplified morphology and property changes. Such limitations result in conditions that greatly differ from those of the natural microenvironment (Fennema, Rivron, Rouwkema, van Blitterswijk, & de Boer, 2013; Knight & Przyborski, 2015). To mimic in vivo conditions more accurately, three-dimensional (3D) culture techniques were applied to basic cell studies. The complex communication network of cell-cell and cell-extracellular matrix is the highlight characteristic of 3D culture. Scaffold-based culture and scaffold-free culture are two general ways to fabricate 3D microtissue. The former is the most commonly used approach in 3D models. Each type of scaffold has its specific mechanical and biological properties and facilitates seeded cells to migrate, proliferate, differentiate, and come in contact with each other (Zhang, Zhuang, Gu, Zhou, & Fan, 2016). Nevertheless, scaffolds also have negative effects on cell behaviors, such

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as stability during the culture period and the break down rate during new tissue formation. Alternatively, if cells grow and create 3D microtissue without exogenous materials, the cells would assemble endogenous extracellular matrix and generate their favored microenvironment. Therefore, the scaffold-free culture method, also known as spheroid culture, better reflects physiological conditions in self-contained 3D microtissue (Fennema et al., 2013).

Regenerative medicine is generally performed by using mesenchymal stem cells (MSCs) because of their self-renewal and multilineage differentiation potential. In humans, MSCs can be harvested from bone marrow, adipose tissue, umbilical cord blood and other tissue resources (Gronthos et al., 2003; Jin et al., 2013). Among these MSCs, dental pulp cells (DPCs) are relatively easily obtained and have similar but not identical morphological and multilineage potential (Huang, Gronthos, & Shi, 2009). It has been demonstrated that DPCs have an even greater proliferation rate, increased mineralization capacity, and higher cell number than bone marrow stem cells (Alge et al., 2010). Thus, DPCs could be a suitable cell candidate for studying 3D cell culture techniques. Some previous studies have revealed significant differences between 2D and 3D MSC culture models. The latter generally exhibits enhanced osteoblastic differentiation (Hildebrandt, Büth, & Thielecke, 2011; Yamaguchi, Ohno, Sato, Kido, & Fukushima, 2014), adipogenic differentiation (Frith, Thomson, & Genever, 2010), and neuronal differentiation (Anghileri et al., 2008). DPC spheroids have also been demonstrated to have similar multilineage differentiation capacities (Iohara et al., 2004; Lee et al., 2017; Syed-Picard, Ray, Kumta, & Sfeir, 2014; Yamamoto et al., 2014). However, the inner mechanisms of cell spheroids have not been studied in detail. To address this issue in depth, we present a genome-wide gene expression analysis of 3D culture accompanied by gene level comparison, histological analysis, and morphological evaluation.

There are now some different spheroid-forming techniques, such as nonadherent surface, hanging drop, pellet culture, micromolding, and rotary systems (Lin, Lin, & Chang, 2008). The nonadherent surface technique, as the name suggests, is set up on low-attachment substrates, allowing cells to grow and aggregate into spheroids. However, the size and shape of spheroids can be varied. The hanging drop technique is a gravity-enforced self-assembly method with precise spheroid size control but difficult mass production. Advanced bioreactors have been applied to generate 3D spheroids and have promoted production effectiveness and better cell culture conditions. However, specialized equipment is required, limiting the application of this method (Fennema et al., 2013; Knight & Przyborski, 2015; Lin et al., 2008; Zhang et al., 2016). Here, we applied the pellet culture method, which fabricates cell spheroids by one-step centrifugation. Its prominent advantages are its cost-effectiveness, simplicity of performance and rapid fabrication with only a small of amount cells (Handschel et al., 2007; Johnstone, Hering, Caplan, Goldberg, & Yoo, 1998). This method requires only sterile polypropylene conical tubes and a benchtop centrifuge, available in most biological laboratories. The pellets obtained by one-step centrifugation were also characterized by long-term culture and differentiation abilities (Bellotti, Duchi, Bevilacqua, Lucarelli, & Piccinini, 2016; Muraglia et al., 2003).

In the present study, complementary DNA microarray was used to compare the gene-expression profiles of 2D and 3D cultured cells to explain their inner mechanisms, such as cell proliferation, odontoblastic and osteoblastic differentiation, and extracellular matrix production. The results were subsequently evaluated by reverse transcription quantitative real-time PCR, histological analysis and transmission electron microscopy. These findings may provide a more detailed and comprehensive understanding of 3D hDPC spheroids.

2. Materials and methods

2.1. Dental pulp cell isolation

Healthy human impacted third molars were obtained from RWTH Aachen University, School of Dentistry and had been extracted because of orthodontic and prophylactic reasons. The teeth were collected from patients aged between 16 and 30 years. To avoid contamination with possible existing stem cells from the dental apical papilla (SCAP), only teeth with certainly completed root development were used. Tooth surfaces were cleaned, and the attached gingival and periodontal tissues were cut off. The teeth were split by a hammer, and the pulp tissue was separated from the crown and roots. The pulp tissue was minced into pieces and digested in a solution of 3 mg/ml collagenase types I and 4 mg/ml dispase II (both from Sigma, Steinheim, Germany) for 1 h at 37 °C. Single-cell suspensions were obtained and filtered through a 70-µm strainer (BD, Heidelberg, Germany) to remove large tissue fragments. Next, DPCs were seeded in T25 culture flasks containing Dulbecco's modified Eagle's medium (DMEM; Gibco, Karlsruhe, Germany) supplemented with 20% fetal bovine serum (FBS; PAA, Cölbe, Germany) and 50 mg/ml gentamycin (Gibco, Karlsruhe, Germany). Cells between the second and fifth passages were used for experiments.

2.2. Fabrication of 3D pellet culture

3D pellet culture of the hDPCs was performed following a published protocol (Johnstone et al., 1998). Briefly, adherent hDPCs were trypsinized, washed in medium, and then resuspended in standard medium. Next, 1-ml aliquots of 2.5×10^5 cells were centrifuged at 500*g* for 5 min in 15-ml polypropylene conical tubes. Condensed cells were maintained at 37 °C and 5% CO_2 with loosened caps to permit gas exchange. After 24-h incubation, spherical aggregates were observed at the bottom of each tube. The medium was replaced every 3 days, and aggregates were harvested at days 3, 10, 21 and 28.

2.3. Complementary DNA microarray analysis

Complementary DNA (cDNA) microarray analysis was used to identify a set of genes that were differentially expressed on hDPC pellets compared with cells in monolayer culture (control). The samples were prepared according to the manufacturer's instructions. Total RNA was isolated from 3D and 2D models using an RNeasy mini Kit (Qiagen, Hilden, Germany). RNA quality was assessed with an RNA 6000 Nano Assay (Agilent Bioanalyzer), and RNA quantity was assessed with a NanoDrop 1000.

As recommended by the manufacturer's protocol, for each sample, 500 ng of total RNA was converted to double-stranded cDNA. A random hexamer oligonucleotide tagged with a T7 promoter sequence (5′-GAATTGTAATACGACTCACTATAGGGNNNNNN-3′) was used in reverse transcription. Antisense cRNA was generated from the double-stranded cDNA. Thereafter, random hexamers and dNTPs containing dUTP were used to reverse transcribe the cRNA into single-stranded sense strand cDNA. Next, uracil DNA glycosylase and apurinic/apyrimidinic endonuclease 1 fragmented the cDNA. The fragment size was optimized in the range of 50–200 bp with a Bioanalyzer (Agilent). Fragmented biotin-labeled cDNA was hybridized to an Affymetrix GeneChip Human Exon 1.0 ST Array (1.4 million probe sets covering > 1 million exon clusters). After hybridization, the chips were stained and washed in a GeneChip Fluidics Station 450 using the FS450-0001 program (Affymetrix) and were scanned using a GeneChip Array scanner 3000 G7 (Affymetrix). Gene expression data were generated using Affymetrix Command Console software version 1.1 (Affymetrix).