3D spheroid culture models for chondrocytes using polyethylene glycol-coated microfabricated chip

Wataru Ariyoshi¹, Michihiko Usui², Kotaro Sano², Aki Kawano¹, Toshinori Okinaga³, Keisuke Nakashima⁴, Kohji Nakazawa⁴, and Tatsuji Nishihara¹

¹ Division of Infections and Molecular Biology, Department of Health Promotion, Kyushu Dental University, Fukuoka 803-8580, Japan; ² Division of Periodontology, Department of Oral Function, Kyushu Dental University, Fukuoka 803-8580, Japan; ³ Department of Bacteriology, Osaka Dental University, Osaka 573-1121, Japan; and ⁴ Department of Life and Environment Engineering, The University of Kitakyushu, Fukuoka 808-0135, Japan

(Received 26 April 2020; and accepted 26 May 2020)

ABSTRACT
As chondrocytes fail to retain their chondrogenic potential in two-dimensional monolayer cultures, several three-dimensional culture systems have been employed for investigating the physiology and pathophysiology in articular cartilage tissues. In this study, we introduced a polyethylene glycol-coated microfabricated chip that enables spheroid formation from ATDC5 cell line, commonly used as a model for in vitro chondrocyte research. ATDC5 cells cultured in our devices aggregated immediately and generated a single spheroid per well within 24 h. Most cells in spheroids cultured in differentiation medium were viable and the circular shape and smooth surface of the spheroid were maintained up to 14 d in culture. We also detected potent hypoxia conditions, a key factor in chondrogenesis, in whole lesions of ATDC5 spheroids. Expression of chondrogenesis-related genes and type X collagen protein was significantly increased in ATDC5 spheroids grown in differentiation medium, compared with monolayer-cultured ATDC5 cells. We also demonstrated that the differentiation medium-induced Akt protein phosphorylation was upregulated in ATDC5 cells cultured in our spheroid device, suggesting that enhancement of chondrogenic potential in ATDC5 spheroids results from PI3/Akt signaling activation. These results indicated that our spheroid culture system could constitute a high-throughput strategy approach towards elucidating the molecular mechanisms that regulate chondrogenesis.

INTRODUCTION
Because of their sensitivity and susceptibility to changes in their extensive extracellular matrix (Bastow et al. 2008), articular chondrocytes, which reside in cartilage, are known to be phenotypically unstable in culture. In two-dimensional (2D) monolayer cultures, chondrocytes dedifferentiate into a fibroblast-like cell type (Holtzer et al. 1960; Benya et al. 1982). To overcome this loss of chondrogenic state in cultured chondrocytes, several three-dimensional (3D) culture systems have been developed (Mueller-Klieser and Sutherland 1984). Engineered 3D cell culture systems for production of multicellular spheroids, have attracted considerable attention as they appear to fill the gap between the use of whole animals and traditional 2D cell culture platforms. As 3D spheroids allow for cell-cell and cell-matrix contacts, these models can provide tissue-like architectures and replicable tissue function within an in vivo context. High-throughput generation of cell spheroids for basic and translational tissue research has been appealing, and a va-
riety of platforms have been developed to handle spheroid cultures, such as matrix coating on plastic substrates (Yoshii et al. 2011; Su et al. 2013), magnetic levitation of cells (Souza et al. 2010), and hanging drops (Tung et al. 2011). However, the development of analytical systems for spheroid cultures remains a major challenge. Especially, desirable would be the establishment of easy-handling platforms for 3D spheroid models that reduce the time required for routine operations and minimize the differences between experimental replicates.

Several applications of microchip techniques, including microcontact printing, photolithography, and microstencil technique have been reported for effective spheroid formation in various types of cells (Otsuka et al. 2004; Park et al. 2007; Tamura et al. 2008). Sakai et al. (2010) established an effective cell microsphere production system for rat hepatocytes, human hepatocellular carcinoma cells, mouse embryonic stem cells, and mouse neural progenitor/stem cells by using a specially prepared microchip, which possesses a polyethylene glycol (PEG)-modified non-adhesive surface. This system can produce uniformly sized spheroids which are controlled by microwell diameters of the chips. Moritani et al. (2018) also found that in-chip spheroid cultures of mesenchymal stem cells derived from human periodontal ligament exhibited enhanced stemness and osteogenesis, compared with 2D cultures. These data suggested that chip technique mentioned above is a promising cellular platform for tissue engineering, regenerative medicine, pharmacology, toxicology, and fundamental cell biology studies.

We have developed a fabricated microchip that enables spheroid size control and immobilization at a defined location, for use in molecular biological analysis to estimate the differentiation status of chondrocytes. In this study, we prepared spheroids of the ATDC5 murine cell line, commonly used as a model for in vitro chondrocyte research. Therefore, the aim of this study was to establish protocols for stable ATDC5 spheroid cultures and accentuate the differences in their features with respect to 2D cell culture methods.

MATERIALS AND METHODS

Reagents. Anti-COL10A1 polyclonal antibody was obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-phospho-Akt monoclonal and anti-Akt polyclonal antibodies were obtained from Cell Signaling Technology Inc. (Beverly, MA, USA), and anti-β-actin monoclonal antibody was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture. ATDC5 cells were maintained and cultured as previously described (Tabe et al. 2017). For chondrogenic differentiation, cells were cultured in medium supplemented with 50 ng/mL ascorbic acid (Wako Pure Chemical Industries, Osaka, Japan) and 1% insulin-transferrin-selenium (ITS; Thermo Fisher Scientific Life Sciences, Rockford, IL, USA) according to the reported protocol (Tabe et al. 2017).

Hanging drop culture. Drops of the culture medium (10 μL) containing 1 × 10^3 to 1 × 10^5 of ATDC5 cells were pipetted onto the lid of 10 cm dishes and were inverted over dishes containing 10 mL phosphate-buffered saline (PBS, pH 7.2). Hanging drop cultures were incubated for 7 d at 37°C in an atmosphere of 5% CO₂. Resultant cell aggregates were observed under a microscope (Olympus Corporation, Tokyo, Japan) at 1, 3, 5, and 7 d of culture.

Spheroid device fabrication. Figure 1 illustrates the design of a spheroid device for ATDC5 cells. Basic chip design included a multi-wells structure (4 mm in diameter) were prepared on the center of a polymethylmethacrylate (PMMA) plate (24 × 24 mm). The entire surface of the plate, including multi-wells, was coated with a thin platinum layer. The plate was immersed in 5 mM PEG-SH in ethanol solution to render its surface non-adhesive to cells. The spheroid device was prepared according to the
Spheroid for chondrocytes

Spheroid preparation. The spheroid device was placed in a 35-mm dish. ATDC5 cells were cultured in DMEM/F-12 containing 5% FBS at a concentration of $3 \times 10^6$ cells per spheroid device well. Briefly, a cell suspension was inoculated into the well of the spheroid device at a density of $3 \times 10^5$ cells/well/20 μL. After the cells had settled into the wells, the 3 mL of culture medium was added in the 35-mm dish. Spheroid formation of ATDC5 cells was performed in the presence (Induction) or absence (Control) of differentiation medium. Medium was changed every 2 d. The diameter of each ATDC5 spheroid was measured under a microscope (Keyence, Osaka, Japan) at 1, 3, 5, 7, 10 and 14 d of culture.

Cell viability assay. The viability of spheroids was evaluated on day 7 of culture by staining using a Live/Dead Viability/Cytotoxicity Kit (Thermo Fisher Scientific) according to the manufacturer’s protocol. Images of the spheroids were obtained under a BZ-9000 fluorescence microscope (Keyence). Images were captured digitally in real-time and processed using the BZ-II imaging software (Keyence). The percentage areas of live cells and dead cells present within a total spheroid area were determined.

Hypoxia analysis. The hypoxia level of spheroids was evaluated on day 5 and 7 of culture using the hypoxia probe LOX-1 (Medical & Biological Laboratories, Nagoya, Japan) according to the manufacturer’s instructions. LOX-1 (2 μM) was added to the culture medium 24 h prior to detection. Images of hypoxic lesions in ATDC5 spheroids were obtained using the BZ-9000 fluorescence microscope. The percentage of LOX-1 positive hypoxic cell area present within a total spheroid area was determined.

Quantitative real-time reverse transcription polymerase chain reaction (real-time RT-qPCR). Total RNA from cultured ATDC5 spheroids was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) on day 7 and 14, respectively. Gene expression was analyzed by real-time RT-qPCR according to the reported protocol (Ariyoshi et al. 2017). Sequences of the primers used for real-time RT-qPCR were as followed: glyceraldehyde 3-phosphate dehydrogenase (Gapdh), forward 5’-GACGGCCGCATTCTTCT-3’ and reverse 5’-CACACCGACCTTTATGTT-3’; collagen type II (Col II), forward 5’-AAGTCAGTACACACTCAGA-3’ and reverse 5’-AAATGCGAGCAGGGTTCTTG-3’; aggrecan (Acan), forward 5’-ACGCCCAGGGGAA GGT-3’ and reverse 5’-CCGGATTCCGTAGGTTCT CA-3’; collagen type X (Col X), forward 5’-TGCA ATCATCGGACTCAGAGA-3’ and reverse 5’-CA GAGGCTAGGCGCGGTGTTGA-3’; hyaluronan synthase 2 (Has-2), forward 5’-AAAGACCTATGG TTGAGGTTGT-3’ and reverse 5’-CATCTCCAG AGGACCCTAT-3’.

Western blot analysis. Following treatments for 1, 2, 3 and 14 d, total protein was extracted from cultured ATDC5 spheroids using Cell Lysis Buffer (Cell Signaling Technology) supplemented with a protease inhibitor mixture (Thermo Fisher Scientific) and phosphatase inhibitor mixture (Nacalai Tesque, Kyoto, Japan). Western blot analysis was performed following the previously reported protocol (Ariyoshi et al. 2017) with primary antibodies against COL10A1, phospho-Akt, Akt and β-actin. Image Lab® 2.0 software (Bio-Rad, Hercules, SA) was used to measure the relative density of the comparable results. The relative band density values were normalized to changes in the β-actin or total Akt of the same sample.

Statistical analysis. All data were analyzed using JMP® software, version 10.0.2 (SAS Institute Inc., Cary, NC, USA) and expressed as mean ± SD from 3 independent experiments. The statistical analyses were performed by one-way analysis of variance (ANOVA) followed by Dunnett’s or Tukey’s test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

ATDC5 cells showed higher aggregation ability in hanging drops
To evaluate the ability of ATDC5 cells for cell-cell adhesion, we performed the commonly-used hanging drop technique. As shown in Figure 2, ATDC5 cells in a hanging drop at a concentration of $5 \times 10^4$ cells/drop, began to converge and formed a spheroid in less than 24 h. Among conditions tested, the higher aggregation ability was observed in ATDC5 cells cultured in the presence of differentiation medium (Induction). An increase in the number of ATDC5 cells seeded in the hanging drop led to formation of spheroids of increased size.
ATDC5 cells aggregated and formed a single spheroid in fabricated devices

We assessed the formation of spheroids from ATDC5 cells at a density of $3 \times 10^5$ cells per well, using our fabricated devices, which were PEG-coated to prevent cell attachment to the surface of the chip during spheroid generation. ATDC5 cells cultured in these devices spontaneously aggregated and generated a single spheroid per well within 1 d (Fig. 3A). Next, we measured the diameter of the generated ATDC5 spheroids in culture. We noted that the mean diameter of the formed ATDC5 spheroids was of approximately 700 μm (Fig. 3B). Even though the diameter of ATDC5 spheroids gradually decreased over time, the smooth surface circular shape of the spheroids was maintained up to 14 d of culture. The average diameter of spheroids in the presence of differentiation medium (Induction) was higher than that of normal medium (Control), although there was no significant statistical difference.

Viable cells persisted in ATDC5 spheroids under chondrogenic differentiation conditions

To elucidate cell viability in cultured spheroids, we performed a cell viability assay using ATDC5 spheroids cultured for 7 d. Ethidium homodimer-1 positive red fluorescence indicated dead cells, and positive green fluorescence indicated live cells. In ATDC5 spheroids cultured $3',6'-\text{Di(O-acetyl)-2',7'-bis}[\text{N,N-bis (carboxymethyl) aminomethyl}]$ fluorescein tetraacetoxyethyl ester (Calcein-AM) in the absence of differentiation medium (Control), a number of dead cells were observed ubiquitously. Under chondrogenic differentiation conditions (Induction), despite some dead cells being spotted on the surface of the spheroids, most cells in ATDC5 spheroids
were viable (Fig. 4).

Hypoxic state was markedly induced in ATDC5 spheroids
To further characterize ATDC5 spheroids formed in our device, ATDC5 spheroids cultured for 5 and 7 d were visualized using the red fluorescence emitted by the hypoxia LOX-1 probe (Fig. 5). Regions inside ATDC5 spheroids stained with red fluorescence indicated hypoxia. As shown, strong hypoxic signals were detected in whole regions of the ATDC5 spheroids. We speculated that oxygen condition was affected by several factors including spheroid size and differentiation medium. However, no significant effect was observed on the hypoxia level of ATDC5 spheroids when differentiation medium was used.
that enhanced synthesis of type X collagen protein was induced. This type X collagen protein enhancement was more prominent in ATDC5 cells cultured in the spheroid device compared with monolayer cultured cells (Fig. 6B).

**Enhancement of chondrogenic differentiation in ATDC5 spheroids**

To examine their chondrogenic potential, ATDC5 cells were cultured in monolayer or fabricated devices in the presence (Induction) or absence (Control) of differentiation medium for 7 or 14 d. mRNA expression of the chondrogenic differentiation markers, Coll II, Coll X, Acan, and Has2 was upregulated in monolayer-grown ATDC5 cells, under chondrogenic differentiation conditions. Furthermore, mRNA expression of the Coll II, Coll X and Has2 in ATDC5 spheroids was significantly increased in comparison with monolayer cultures (Fig. 6A). We also examined the protein expression levels of chondrogenic differentiation markers under specified conditions. After culturing ATDC5 cells with differentiation medium for 14 d, we observed that enhanced synthesis of type X collagen protein was induced. This type X collagen protein enhancement was more prominent in ATDC5 cells cultured in the spheroid device compared with monolayer cultured cells (Fig. 6B).

**Activation of Akt phosphorylation induced by chondrogenic medium in ATDC5 spheroids**

PI3K/Akt pathway is a principal pathway that regulates chondrogenic differentiation of ATDC5 cells induced by insulin (Hidaka et al. 2001, Starkman et al. 2005). To further determine the role of spheroid cultures in chondrogenesis, we investigated the activation of the PI3K/Akt signaling pathways in ATDC5 spheroids and monolayer cultures by western blot analysis. As shown in Figure 7, the ascorbic acid and ITS-induced phosphorylation of Akt protein was significantly enhanced in ATDC5 cells cultured in the spheroid device.
DISCUSSION

Chondrocytes have a chondrogenic potential in 3D culture. To provide a 3D environment to cells, a pellet culture system supporting the differentiation of chondrocytes (Hassan et al. 2017) is commonly used. However, in this pellet culture system, production of large number of chondrocyte aggregates has been difficult and the cultivation technique has been complicated. Therefore, 3D hydrogel cultures supplemented with several extracellular matrix (ECM) components, including collagen, chondroitin sulfate, hyaluronic acid and its complex have been used to mimic the natural ECM composition of cartilage tissue. Although these ECMs provide physical, biochemical and biomechanical signals to chondrocytes via cell surface receptors (van der Kraan et al. 2002), structural stability and several factors which can alter cellular activity of artificial ECMS have been sometimes considered to be problematic. To overcome these disadvantages, we fabricated a device equipped with a PEG-coated non-adhesive surface. Use of this device allows for easy handling of the cultivation technique and enables the production of multiple, scaffold-free and homogeneous chondrocyte spheroids.

In this study, we used ATDC5 cells derived from mouse teratocarcinoma cells to elucidate the effects of 3D spheroid cultures on the induction of chondrocyte differentiation. As this cell line exhibits a
compared to other cell types including islet cells (200-500 cells/drop) and renal carcinoma cells (500 cells/drop) (Cavallari et al. 2007; Matak et al. 2017). It should be noted that cell density and cell numbers are considered the most important factors for the development of 3D spheroid cultures. Based on these results, we fabricated a multi-well device allowing for stable spheroid formation from a large number of ATDC5 cells ($3 \times 10^5$ cells/well). The number of cells required for spheroid formation was comparable to a previous report which produced 3D micromass pellet from $2.5 \times 10^5$ cells (Wu et al. 2017). Furthermore, the spheroid formation in our devices showed a high reproducibility compared to other cell types including islet cells (200-500 cells/drop) and renal carcinoma cells (500 cells/drop) (Cavallari et al. 2007; Matak et al. 2017). It should be noted that cell density and cell numbers are considered the most important factors for the development of 3D spheroid cultures. Based on these results, we fabricated a multi-well device allowing for stable spheroid formation from a large number of ATDC5 cells ($3 \times 10^5$ cells/well). The number of cells required for spheroid formation was comparable to a previous report which produced 3D micromass pellet from $2.5 \times 10^5$ cells (Wu et al. 2017). Furthermore, the spheroid formation in our devices showed a high reproducibility.
and almost all the seeded ATDC5 cells generated a single spheroid per well with smooth surface circular shape.

As oxygen supply is the important factor in maintaining cell viability, cell death induced by lack of oxygen in the core of the large spheroids (central necrosis) has been considered as a limiting factor for spheroid generation. A previous study revealed that central necrosis was induced in hepatocyte-composed spheroids with a diameter of 200 μm (Glicklis et al. 2004). We also investigated survival conditions in ATDC5 spheroids by Live/Dead Viability/Cytotoxicity Kit and revealed that most cells in day 7 spheroids cultured with differentiation medium were viable even in the core of spheroids (Fig. 4). Moreover, a large number of cells were dead in ATDC5 spheroids cultured without differentiation medium, suggesting that chondrogenic induction by differentiation medium stimulated the cell viability of ATDC5 spheroid. From these results, we speculated that cell viability in the spheroid core was dependent on cell type and differentiation status.

To confirm whether hypoxic conditions were induced in the ATDC5 spheroid core, ATDC5 spheroids cultured for 5 and 7 d were visualized using the hypoxia LOX-1 probe, a phosphorescent light-emitting iridium complex. Phosphorescence of this probe is quenched by oxygen, and it is decreased in response to the oxygen level. In this study, strong hypoxic conditions were observed in ATDC5 spheroids regardless of the ITS and ascorbic acid stimulation (Fig. 5). Due to the lack of blood vessels and the subsequently induced hypoxic microenvironment, less than 5% oxygen is present in the cartilage tissue (Pfander and Gelse 2007). Chondrocytes are well adapted for survival and are capable of maintaining their metabolism in the hypoxic microenvironment. Previous studies also suggested that hypoxia was an important factor for the growth and survival of chondrocytes (Meyer et al. 2010). Our results indicated that low oxygen tension might be responsible for the long-term survival of differentiated ATDC5 cells in 3D spheroids.

3D culture environments are essential for metabolism, proliferation, and differentiation in several types of cells. Chondrocytes cultured in a 2D microenvironment were reported to have altered morphology and activity (Rottmar et al. 2014), and an in vitro 3D microenvironment was required for hypertrophic chondrocytes formation via the chondrogenic differentiation process induced by the cell-matrix interactions (Foster et al. 2015). As we expected, a comparison of the outcomes of the spheroid cultures with those of the monolayer cultures revealed that...
3D cultures grown in our devices provided higher sensitivity to chondrogenic responses of ATDC5 cells (Fig. 6). The expression of cartilage specific extracellular matrix, type II collagen which provides structural rigidity was stimulated during the process of chondrogenic differentiation. In the ATDC5 spheroids, induction of type II collagen synthesis was observed after 14 d cultivation in chondrogenic differentiation medium, while ATDC5 cells in monolayer cultures showed reduced ability to induce type II collagen expression during the same time period. These data suggested that our spheroid culture system positively regulates chondrogenesis and has a potential to retain the chondrocyte phenotype compared to the monolayer cultures.

Previous studies also demonstrated that hypoxia induced chondrocyte differentiation in 3D culture models. The hypoxia-inducible factor-1 (HIF-1) is regulated by the intracellular oxygen concentration. Under hypoxic conditions, HIF-1 induced chondrogenesis through activation of transcription factors such as activating protein 2 epsilon (Atsumi et al. 2015). However, HIF-1α expression in ATDC5 spheroids was comparable to that observed in monolayer cultures (data not shown). Öztürk et al. (2017) reported that hypoxic conditions generated in a 3D culture using alginate hydrogels suppressed Rho and canonical Wnt signaling, followed by upregulation of type II collagen. Further studies are needed to elucidate the signaling mechanisms involved in the induction of the chondrogenic differentiation phenotype under hypoxic conditions in ATDC5 spheroids.

A previous study using a gene-trap approach revealed that PI3K/Akt signaling was involved in the early stages of chondrogenesis (Ihara-Watanabe et al. 2004). We found that the Akt protein phosphorylation during chondrogenesis was increased in 3D spheroids (Fig. 7). These results indicated that the 3D microenvironment provided by our spheroid device stimulated ITS/ascorbic acid-induced chondrogenesis via activation of PI3/Akt signaling. In rat mesenchymal stem cells, hypoxia rapidly induced Akt phosphorylation followed by subsequent stabilization of HIF-1α and enhanced cartilage formation (Kanichai et al. 2008). To fully elucidate the molecular mechanism governing chondrogenesis in ATDC5 spheroids under hypoxic conditions, the activation and interaction of intracellular signaling induced in 3D spheroid cultures are currently under investigation in our laboratory.

To develop treatment strategies for cartilage regeneration, understanding the mechanisms underlying chondrogenesis is required. Although traditional 3D micromass pellet cultures could maintain a chondrocyte phenotype, chondrogenic screening analysis performed in such culture systems revealed them to be relatively low-throughput. Our novel 3D spheroid model could potentially provide a high-throughput approach to help elucidate the molecular mechanisms, and to assist in the discovery and evaluation of chondrogenic treatments, and in the development of regenerative medicine approaches for cartilage repair. We have to assess the long-term stability of ATDC5 spheroid cultured in our devices. Further histological and biochemical analyses should be performed to illustrate the comparison of chondrogenic capacity (e.g. lacunae formation and ECM synthesis) between our spheroid culture system and other 3D cultures.

Acknowledgements

We would like to thank Editage (www.editage.com) for English language editing. This work was partially supported by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (No. 18K09797 and 16K11838).

CONFLICT INTEREST

The authors declare that they have no conflict of interest.

REFERENCES

Ariyoshi W, Okinaga T, Chawewannakorn W, Akifusa S and Nishihara T (2017) Mechanisms involved in enhancement of matrix metalloproteinase-9 expression in macrophages by interleukin-33. J Cell Physiol 232, 3481–3495.

Atsumi T, Miwa Y, Kimata K and Ikawa Y (1990) A chondrogenic cell line derived from a differentiating culture of AT805 teratocarcinoma cells. Cell Differ Dev 30, 109–116.

Bastow ER, Byers S, Golub SB, Clarkin CE, Pitsillides AA, et al. (2008) Hyaluronan synthesis and degradation in cartilage and bone. Cell Mol Life Sci 65, 395–413.

Benya PD, and Shaffer JD (1982) Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. Cell 30, 215–224.

Cavallari G, Zuellig RA, Lehmann R, Weber M and Moritz W (2007) Rat pancreatic islet size standardization by the “hanging drop” technique. Transplant Proc 39, 2018–2020.

Foster NC, Henstock JR, Reinwald Y and El Haj AJ (2015) Dynamic 3D culture: models of chondrogenesis and endochondral ossification. Birth Defects Res C Embryo Today 105, 19–33.

Glicklis R, Merchuk JC and Cohen S (2004) Modeling mass transfer in hepatocyte spheroids via cell viability, spheroid size, and hepatocellular functions. Biotechnol Bioeng 86, 672–680.

Hassan FM, Montazer SS and Montaseri A (2017) Conditioned medium of Wharton’s jelly derived stem cells can enhance
the cartilage specific genes expression by chondrocytes in monolayer and Mmass culture systems. Adv Pharm Bull 7, 123–130.

Hidaka K, Kanematsu T, Takeuchi H, Nakata M, Kikkawa U, et al. (2001) Involvement of the phosphoinositide 3-kinase/protein kinase B signaling pathway in insulin/IGF-I-induced chondrogenesis of the mouse embryonal carcinoma-derived cell line ATDC5. Int J Biochem Cell Biol 33, 1094–1103.

Holtzer H, Abbott J, Lash J and Holtzer S (1960) The loss of phenotypic traits by differentiated cells in vitro. I. Dedifferentiation of cartilage cells. Proc Natl Acad Sci USA 46, 1533–1542.

Ihara-Watanabe M, Uchihashi T, Miyauchi Y, Sakai N, Yamagata M, et al. (2004) Involvement of phosphoinositide 3-kinase signaling pathway in chondrocytic differentiation of ATDC5 cells: application of a gene-trap mutagenesis. J Cell Biochem 93, 418–426.

Kanichai M, Ferguson D, Prendergast PJ and Campbell VA (2008) Hypoxia promotes chondrogenesis in rat mesenchymal stem cells: a role for AKT and hypoxia-inducible factor (HIF)-1alpha. J Cell Physiol 216, 708–715.

Matak D, Brodzczewska KK, Lipiec M, Szymanski L, Szczylık C, et al. (2017) Colony, hanging drop, and methylcellulose three dimensional hypoxic growth optimization of renal cell carcinoma cell lines. Cytotechnology 69, 565–578.

Meyer EG, Buckley CT, Thorpe SD and Kelly DJ (2010) Low oxygen tension is a more potent promoter of chondrogenic differentiation than dynamic compression. J Biomech 43, 2516–2523.

Moritani Y, Usui M, Sano K, Nakazawa K, Hanatani T, et al. (2018) Spheroid culture enhances osteogenic potential of periodontal ligament mesenchymal stem cells. J Periodontal Res 53, 870–882.

Mueller-Klieser WF and Sutherland RM (1984) Oxygen consumption and oxygen diffusion properties of multicellular spheroids from two different cell lines. Adv Exp Med Biol 180, 311–321.

Niebler S, Angele P, Kujat R and Bosserhoff AK (2015) Hypoxia-inducible factor 1 is an inducer of transcription factor activating protein 2 epsilon expression during chondrogenic differentiation. Biomed Res Int 2015, 380590.

Otsuka H, Hirano A, Nagasaki Y, Okano T, Horiike Y, et al. (2004) Two-dimensional multiarray formation of hepatocyte spheroids on a microfabricated PEG-brush surface. Chembiochem 5, 850–855.

Öztürk E, Hobiger S, Despot-Slade E, Pichler M and Zenobi-Wong M (2017) Hypoxia regulates RhoA and Wnt/beta-catenin signaling in a context-dependent way to control re-differentiation of chondrocytes. Sci Rep 7, 9032.

Park J, Cho CH, Parashurama N, Li Y, Berthiaume F, et al. (2007) Microfabrication-based modulation of embryonic stem cell differentiation. Lab Chip 7, 1018–1028.

Pländer D and Gelse K (2007) Hypoxia and osteoarthritis: how chondrocytes survive hypoxic environments. Curr Opin Rheumatol 19, 457–462.

Rottmar M, Mhanna R, Guimond-Lischer S, Vogel V, Zenobi-Wong M, et al. (2014) Interference with the contractile machinery of the fibroblastic chondrocyte cytoskeleton induces re-expression of the cartilage phenotype through involvement of PI3K, PKC and MAPKs. Exp Cell Res 320,175–187.

Sakai Y and Nakazawa K (2007) Technique for the control of spheroid diameter using microfabricated chips. Acta Biomater 3, 1033–1040.

Sakai Y, Yoshiha S, Yoshiura Y, Morii R, Tamura T, et al. (2010) Effect of microwell chip structure on cell microsphere production of various animal cells. J Biosci Bioeng 110, 223–229.

Souza GR, Molina JR, Raphael RM, Ozawa MG, Stark DJ, et al. (2010) Three-dimensional tissue culture based on magnetic cell levitation. Nat Nanotechnol 5, 291–296.

Starkman BG, Cravero JD, Delcarlo M, and Loeser RF (2005) IGF-1 stimulation of proteoglycan synthesis by chondrocytes requires activation of the PI 3-kinase pathway but not ERK MAPK. Biochem J 389, 723–729.

Su G, Zhao Y, Wei J, Han J, Chen L, et al. (2013) The effect of forced growth of cells into 3D spheres using low attachment surfaces on the acquisition of stemness properties. Biomaterials 34, 3215–3222.

Tabe S, Kijiki H, Ariyoshi W, Hashidate-Yoshida T, Shindou H, et al. (2017) Lysophosphatidylcholine acyltransferase 4 is involved in chondrogenic differentiation of ATDC5 cells. Sci Rep 7, 16701.

Tamura T, Sakai Y and Nakazawa K (2008) Two-dimensional microarray of HepG2 spheroids using collagen/polyethylene glycol micropatterned chip. J Mater Sci Mater Med 2008 19, 2071–2077.

Tung YC, Hsiao AY, Allen SG, Torisawa YS, Ho M, et al. (2011) High-throughput 3D spheroid culture and drug testing using a 384 hanging drop array. Analyst 136, 473–478.

van der Kraan PM, Buma P, van Kuppevelt T and van den Berg WB (2002) Interaction of chondrocytes, extracellular matrix and growth factors: relevance for articular cartilage tissue engineering. Osteoarthritis Cartilage 10, 631–637.

Wu B, Durisin EK, Decker JT, Ural EE, Shea LD, et al. (2017) Phosphatase regulates chondrogenesis in a biphasic and maturation-dependent manner. Differentiation 95, 54–62.

Yoshii Y, Waki A, Yoshida K, Kakezuka A, Kobayashi M, et al. (2011) The use of nanoimprinted scaffolds as 3D culture models to facilitate spontaneous tumor cell migration and well-regulated spheroid formation. Biomaterials 32, 6052–6058.