Hypoxia Combined with Spheroid Culture Improves Cartilage Specific Function in Chondrocytes

Journal:	*Integrative Biology*
Manuscript ID:	IB-ART-11-2014-000273.R2
Article Type:	Paper
Date Submitted by the Author:	12-Jan-2015
Complete List of Authors:	Shi, Yang; Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Ma, Jingyun; Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Zhang, Xu; Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Li, Hongjing; The First Affiliated Hospital of Dalian Medical University, Department of Orthopedics Jiang, Lei; Dalian institute of chemical physics,Chinese Academy of Sciences, Qin, Jianhua; Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Department of Biotechnology

Journal Name

RSCPublishing

ARTICLE

Hypoxia Combined with Spheroid Culture Improves Cartilage Specific Function in Chondrocytes

Cite this: DOI: 10.1039/xoxxoooooox

Yang Shi[a], Jingyun Ma[a], Xu Zhang[a], Hongjing Li[b], Lei Jiang[a] and Jianhua Qin[a]*

Received ooth January 2012,
Accepted ooth January 2012

DOI: 10.1039/xoxxoooooox

www.rsc.org/

Abstract

Controlling the chondrocyte phenotype and function in physiologically relevant microenvironment remains a major challenge for cartilage repair in tissue engineering applications. This work presents a straightforward strategy to create a high throughput concave microwells array used for generating multicellular spheroids of chondrocytes and facilitating the maintenance of articular chondrocyte phenotype and function by combining 3D spheroid culture with hypoxia. The polydimethylsiloxane (PDMS) concave microwells were simply produced from a concave SU-8 template fabricated by soft-lithography approach and easily adopted for size-controlled spheroids culture. 3D spheroid culture was observed to facilitate the cartilage-specific phenotype and function maintenance as compared to 2D monolayer culture. By combining hypoxia with spheroid culture markedly increased the expressions of cartilage-specific collagen II and aggrecan at protein and mRNA levels. The hypoxia-inducible factors (HIFs) signaling pathway was found to get involved in phenotype maintenance, metabolism and differentiation of chondrocytes by regulating HIF-1α and HIF-2α, respectively. The established approach provides a useful platform for a wide range of applications in the field of cartilage biology, stem cell research and high throughput 3D drug testing in cancer.

Introduction

Articular cartilage is the specialized connective tissue of diarthrodial joints, which functions to protect the underlying bone by withstanding joint loading[1]. It mainly consists of cartilage matrix and chondrocytes sparsely distributed in the fibrous cartilage matrix without nerves or blood vessels[2] and limits in self-healing after injury. As the only cell type in this tissue, the chondrocytes are solely responsible for producing, sustaining and degrading the cartilage extracellular matrix (ECM) that gives cartilage mechanical integrity. The cartilage ECM is predominantly composed of type II collagen fibers and aggrecan with the specific function to maintain tensile strength and resistance to compressive loads[3]. It has long been recognized that the chondrocyte phenotype is unstable and very prone to loss during *in vitro* monolayer culture, in which chondrocytes tend to differentiate into fibroblasts with concomitant loss of function[4-6]. Moreover, chondrocyte phenotypic alterations are often observed in cartilage pathology, such as occurs with acute joint injury and osteoarthritis. Therefore, both autologous chondrocyte implantation and matrix assisted chondrocyte implantation require the culture of autologous chondrocytes displaying retention of phenotype with function at the time of clinical application.

Recently, culturing of chondrocytes in 3D scaffolds has been proposed to facilitate sustainable expressions of cartilage-specific genes, supporting re-differentiation of de-differentiated chondrocytes to assemble native 3D structure of cartilage. Such methods include embedding chondrocytes in either agarose[7-9], fibrin glue[10, 11], alginate beads[12-14] or pellet culture[6, 15]. However, these approaches are still limited in terms of reduced cell viability, cumbersome manipulations, and un-degradable scaffolds properties. Moreover, the critical hypoxic factor involved in physiological cartilage microenvironment is mostly overlooked.

In vivo, articular cartilage function is specifically maintained in a low oxygen environment throughout its life due to the absence of vasculature. Within this avascular tissue, oxygenation gradients have been estimated to drop from 6-10% at the surface to less than 1% at the deepest layers, indicating a physiologically hypoxic microenvironment is necessary for maintenance of articular cartilage homeostasis[16]. Although some works have made attempts to consider hypoxic condition and induce specific phenotypes in chondrocytes maintained within hanging drop culture[17] or 3D porous scaffolds[18], an extended period of time (~7 days) in culture is necessary for phenotype induction. Additionally, the mechanism underlying hypoxia-mediated chondrocyte function remains poorly understood.

Multicellular spheroid provides an attractive option to recapitulate the 3D tissue *in vivo*, which can establish the cell-cell and cell-matrix interactions required for maintaining cellular viability, function and phenotype which are often lost in monolayer culture. In this work, we present a simple approach to produce and culture high throughput spheroids of chondrocyte with controllable sizes in micro-fabricated concave microwells. By combining 3D spheroid culture with hypoxic condition creates an *in vivo*-like cartilage microenvironment producing a matrix network resembling hyaline cartilage. We demonstrated the positive effects of combined 3D spheroid and hypoxic culture on the cartilage-specific phenotype maintenance in primary chondrocytes by increasing the expressions of collagen II and aggrecan at both the protein and mRNA levels. The time required for the phenotype induction is only 3 days, which is less than other reported method. The subunits (HIF-1α and HIF-2α) of hypoxia inducible factors (HIFs) were found to get involved in the regulation of phenotype maintenance, metabolism and the responses to hypoxia in chondrocytes. These findings support that HIFs might serve as a critical signaling pathway in regulating chondrocytes phenotype and function, representing potential new therapeutic targets for cartilage repair and autotransplantation.

Materials and Methods

Design and fabrication of the concave microwells

The Poly-dimethylsiloxane (PDMS, Sylgard 184, Dow Corning, America) concave microwells were simply fabricated by using negative photoresist SU-8(3035, MicroChem, America) as a template. The schematic diagram of the fabrication process was shown in **Figure 2A**. Briefly, a layer of SU-8 polymer was initially spin-coated on the surface of a glass substrate, which was pre-baked for 60min and then exposed to UV light for 60s. Next, the template was immersed for 5 min in ethyl lactate for an incomplete development, and heated at 85℃ for 5 min to produce the mold structure with concave configuration, and later exposed to UV again for fixing the concave structure. With the SU-8 template, the PDMS layer (PDMS-1) with convex structures could be fabricated using soft lithographic technology. The PDMS layer were treated by 2% w/v PF-127 solution for 4h to modify the surface and then heated to remove the water. After that, we poured new PDMS pre-polymers on the PDMS-1 layer surface, after 80℃ for 40min, we peeled off the top PDMS layer, and obtained the PDMS concave microwell device (PDMS-2).

Primary culture of rat chondrocytes

Animal care and treatment were conducted in accordance with institutional guidelines, national and international laws and policies. Articular cartilages were isolated from the humeral heads, femoral heads and femoral condyles of a male Sprague Dawley rattusnorregicus weighing 80-120g under sterile conditions, as previously described[19]. The chondrocytes were obtained from at least four rats at one time, Briefly, cartilage tissues were cut into small pieces and chondrocytes were isolated by digestion with 2% trypsin for 30 min at 37℃ ,followed by digestion in 0.15% type II collagenase (Worthington, America) for 16 hours in a water bath under constant vibration (THZ-82, JintanHuafeng instrument Co., Ltd., China). Isolated cells were suspended in Dulbecco's modified Eagle's medium (DMEM)/F-12 (Hyclone, America) containing 10% fetal bovine serum (Hyclone, America), 50mgml^{-1} ascorbicacid-2-phosphate (Sigma, America), 100 unitsml^{-1} penicillin and 100 unitsml^{-1} streptomycin (Hyclone, America) and counted. The culture medium was changed every 2 days. We used the third passage of cells for each experiment.

Chondrocyte culture under normoxia and hypoxia

Under normoxic condition, chondrocytes were seeded and cultivated in monolayer culture for 3 days in 25mm dishes at a seeding density of 10^6 cellsml^{-1}. Statistics of proliferation, fluorescent staining and quantitative real-time PCR of monolayer chondrocytes were performed.

The process of chondrocyte spheroids formation was as follows: before cell seeding in the PDMS concave microwell, the surface of the device was modified with 1% PluronicF-127 (Sigma-Aldrich, America) solution for 4 hours, and then washed by sterile water and PBS twice, respectively. The polymer adsorbed to the surface of the PDMS device, thus preventing cell attachment. 500μl chondrocyte suspension of the third passage was directly seeded on the top of the whole PDMS concave microwells slice with cell density at 6×10^6 cellsml^{-1}. After gently shaking and standing for 30 minutes, the cells were allowed to be trapped within concave microwells. A flow of culture medium was gently added to remove cells which did not sink in the microwells. The chondrocyte spheroids were formed less than 12h and cultured 3 days in the concave microwell device in humidified air with 5% CO_2(normoxia) at 37℃. Fluorescent staining and quantitative real-time PCR of chondrocyte spheroids were carried out. Under hypoxic assay, the cells were seeded on concave microwells and then put into hypoxic chamber under the oxygen concentration of 5% condition. The set up of cultured device under hypoxic condition was shown in **Figure 2B.** The device cultured with cells was placed in a multi-gas incubator with air condition of 5% O_2 and 5% CO_2 at 37℃. Low oxygen tension was maintained through the controlled supply of N_2 gas to the incubator. The culture medium was changed daily.

HIFs inhibitor assay in chondrocyte spheroids under hypoxia

HIF-1α inhibitor (Methyl 3-[[2-[4-(2-adamantyl)phenoxy]acetyl]amino]-4-hydroxy-benzoate, Santa Cruz Biotechnology, Inc., America) or HIF-2α inhibitor (Methyl-3-(2-(cyano(methylsulfonyl)methylene)hydrazino)thiophene-2-carboxylate(Calbiochem, Germany) was added to the culture medium at the time spheroid culture was initiated, using a working concentration of 30μM. The concentration of selected inhibitors was referred to the operation manual. Effects of HIFs inhibitors on the gene expressions of HIF-1α and HIF-2α were shown in **supplemental figure 4.** Chondrocyte spheroids cultured in the presence of HIF-1α or HIF-2α inhibitor were maintained for 3 days under hypoxia (5% O_2) without change of medium.

Fluorescence staining and imaging

Chondrocyte spheroids harvested from the microwell device were transferred to a culture dishpre-coated with type I collagen(Rat tail collagen, BD Biosciences, America) for 2h to facilitate cell attachment before staining. Monolayer chondrocytes were stained directly as described. Briefly, monolayer cells and spheroids were both treated with 4%

paraformaldehyde(Sigma, America), 0.1% Triton-X100 (Sigma, America) and blocked with normal goat serum for 20 min, and then incubated with primary antibody collagen II (bs-10589R, Bioss, China) and aggrecan(bs-1223R, Bioss, China)(1:100) at 4℃ overnight. Negative controls were set using PBS instead of primary antibody. After rinsing with PBS, cells were incubated with FITC-labeled anti-rabbit IgG (ZSGB, China) and TRITC-labeled anti-rabbit IgG (ZSGB, China) for 45 minutes at room temperature. DAPI staining solution (Sigma, America) was used to stain nuclei for indication of cell positions. Fluorescent photographs were taken by fluorescence microscope (Olympus IX-71, Japan) and confocal laser scanning biological microscope (Olympus FV1000, Japan).

Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was used to analyze the expressions of collagen II, aggrecan, collagen I, Glut-1 and Sox-9. Briefly, total RNAs were extracted from chondrocytes in monolayer culture or spheroids using RNAiso Plus (Takara, China). Total RNAs were reversely transcribed into cDNA using PrimeScript® RT reagent Kit (Perfect Real Time, Takara, China). Real-time PCR was performed with Mx3000P QPCR System (Agilent Technologies, America) using SYBR®Premix Ex Taq™ II (Perfect Real Time, Takara, China). β-actin, a common housekeeping gene in cells, was used as the internal control gene to normalize the quantities of target gene expressions. Thermocycling conditions were as follows: 95℃ for 30 seconds 40 cycles of denaturation (95℃, 5s), annealing (60℃, 30s) and extension (72℃, 30s). The primer sequences used for qRT-PCR were listed in **Table.1**.

Table.1
Primer sequences for qRT-PCR.

Target gene	Forward primer sequence	Reverse primer sequence
Col1a2	5'-TCCAGGGCTCCAACGAGA-3'	5'-CTGTAGGTGAATCCACTGTTGC-3'
Col2a1	5'-CCCCTGCAGTACATGCGG-3'	5'-CTCGACGTCATGCTGTCTCAGG-3'
Aggrecan	5'-GGCCTTCCCTCTGGATTTAG-3'	5'-CCGCACTACTGTCCAAC-3'
β-actin	5'-GATCATTGCTCCTCCTGAGCG-3'	5'-TGCTGATCCACATCTGCTGGA-3'
HIF-1α	5'-CCAGATTCAAGATCAGCCAGCA-3'	5'-GCTGTCCACATCAAAGCAGTACTCA-3'
HIF-2α	5'-GCTGTCCACATCAAAGCAGTACTCA-3'	5'-ATCACCGTCTTGGGTCACCAC-3'
Glut-1	5'-GACCCTGCATCTCATTGGTCTG-3'	5'-CCACAATGAACCATGGAATAGGA-3'
Sox-9	5'-GAAGACCACCCCGATTACAAG-3'	5'-AAGATGGCGTTAGGAGAGATGTG-3'

Results

Fabrication of Concave Microwells for Chondrocyte Spheroid Generation and Culture

In order to culture chondrocytes in spheroids format, the PDMS microwells array with concave configuration was simply replicated from a concave SU-8 template fabricated by soft-lithograpic approach. As shown in **Figure 2A**, the concave configuration was produced from the SU-8 layer via continuous steps including elective exposure, incomplete development and hot melting. After twice replications based on SU-8 template, a PDMS device with microwell concaves was formed. It is noted that, the depth and curvature of the microwells can be adjusted controllably simply by changing the development and hot melting time during SU-8 molding. In order to investigate the effect of microwell size on the cellular spheroid formation, the microwell arrays (20 × 20) with diameters of 400μm and 600μm (the depth of microwell was 200μm) were tested respectively. No significant difference in colony size was observed in both of the sizes (as shown in **supplemental figure 3**). As the microwells with diameter of 600μm facilitated the rapid formation of cellular spheroids, it was selected for the following experiments. The established fabrication approach is quite simple and easy to realize the massive production in an high throughput manner. As shown in **Figure 2A** (middle picture), the concave array containing 3600 wells in a total area of 25 cm^2 could be easily produced with a controllable dimension. Prior to spheroid formation, the concave PDMS surface was initially modified with 0.2% w/v PF-127 to inhibit the adhesion of cultured chondrocytes. After the mono-dispersed cell suspension of chondrocytes was seeded into the concave microwells array, the cells could physically aggregate and assemble into 3D multicellular spheroids with uniform diameter (~180μm) after 3 days culture. The cells spheroids exhibited good viability with proliferation ability in the concave microwells after one week(as shown as **supplemental figure 1**), verifying the feasibility of this concave microdevice for rapid formation of cell spheroids of chondrocytes.

Cartilage Specific Phenotype and Gene Expression in 3D Spheroid Format

In cartilage tissue, chondrocytes are distributed in fibrous cartilage matrix in the form of 3D construction as shown in **Figure 1**. Forming multicellular spheroids *in vitro* can establish cell-cell contact resembling the physiologically relevant extracellular matrix, which is supposed to preserve cellular viability and function. To investigate the effect of 3D spheroid structure on cartilage phenotype maintenance, we exploited the expressions of cartilage phenotype associated markers type II collagen and aggrecan. The distinct expressions of these matrix synthesis proteins were evaluated by immune-fluorescent staining and qRT-PCR analysis, respectively. As shown in **Figure 3A**, the cells exhibited enhanced expressions of type II collagen (green) and aggrecan (red) in 3D spheroid format after only 3 days, which is much stronger than that of monolayer culture. Furthermore, qRT-PCR appeared to show that the expressions of cartilaginous marker genes for Col2a1 and

aggrecan were significantly up-regulated in spheroids culture, which were 6-folds to 7-folds higher than that in 2D monolayer culture **(Figure 3B)**, indicating the enhanced synthesis of extracellular matrix in chondrocytes spheroids. Importantly, the chondrocytes maintained in spheroid culture re-expressed the cartilage specific phenotype after only 3 days, which greatly facilitated the formation of immature cartilage tissue *in vitro*. Notably, there was no significant difference in the expression of Col1a2 between spheroid and monolayer culture, further supporting the role of 3D spheroids format in favor of cartilage-specific phenotype maintenance.

Combined 3D Spheroids with Hypoxic Condition Enhanced Chondrocyte Phenotype and Functions

In vivo, articular cartilage is maintained in a hypoxic homoeostasis microenvironment, which is an essential factor in regulating various cell behaviors and functions. To explore the effect of oxygen tension on the maintenance of chondrocyte phenotype, primary chondrocytes were cultured as monolayers under normoxic (21%O_2) and hypoxic (5% O_2) conditions. The proliferation of chondrocytes under these conditions was investigated. It was found that monolayer cells cultured at low oxygen tension demonstrated good viability with increased proliferation as compared to that cultured under normoxic condition. Specifically, the growth rate of chondrocytes was about 2-folds greater than that of normoxic assessed 3 days after plating as shown in **Figure 4A-B**. However, no significant difference was observed in mRNA expressions for collagen II and aggrecan between hypoxic and normoxic conditions in monolayer culture as demonstrated by immune-fluorescent staining **(Figure 4C).**

As above, hypoxic condition was required for chondrocyte survival, but it was still insufficient to sustain the chondrocyte-specific phenotype in monolayer culture alone. We further probed the combined effects of hypoxia with 3D spheroid culture on sustaining chondrocyte phenotype *in vitro*. Next, primary chondrocytes were cultured in 3D spheroid format under normoxia and hypoxia for up to 3 days. The immunostaining and qRT-PCR analysis were used to measure expressions of collagen II and aggrecan at protein and gene levels. According to the immunostaining assay, the expressions of collagen II and aggrecan were increased in 5% O_2 culture condition over the 3-day period as compared with normoxia as shown in **Figure 5A**. Moreover, the hypoxic induction of matrix synthesis was also investigated at mRNA level, as assessed by qRT-PCR analysis. The real-time PCR analysis also exhibited an enhanced gene expressions of Col2a1 and aggrecan as indicated in **Figure 5B,** indicating the up-regulated expressions of Col2a1 and aggrecan in 3D spheroid culture under hypoxia. Notably, the expression of Col1a2 in spheroid format was significantly down-regulated, suggesting that combined spheroid culture under hypoxia reduced the fibroblast-like phenotype of expanded chondrocytes.

HIF-1α and HIF-2α Involved in Chondrocyte Phenotype and Functions under Hypoxia

From the previous results, the combined hypoxia with 3D spheroid culture conditions had markedly promoted the phenotype and function maintenance of chondrocytes in a combinational 3D manner. Hypoxia inducible factors have been regarded as the transcription factors, which are mainly responsible for the regulation of various cell behaviors in response to hypoxia, in which HIF-1α and HIF-2α are two subunits of the HIF-1, and differ in their biological functions. To determine whether up-regulation of chondrocyte markers were mediated by HIFs signaling pathway in this combinational manner, we further performed the phenotype analysis of multicellular spheroid by using HIF-1α and HIF-2α inhibitors under hypoxic conditions. The cells were cultured in 5% O_2 hypoxia for 3 days and treated with HIF-1α and HIF-2α inhibitors at 30μM concentration for 72h. As shown in **Figure 6**, the HIF-1α inhibitor slightly influenced the expression of Col2a1, but significantly inhibited the expression of aggrecan, while HIF-2α inhibitor markedly down-regulated Col2a1 and aggrecan expressions in chondrocytes, indicating the involvement of HIFs signaling pathway and different roles of HIF-1α and HIF-2α in regulating the chondrocyte phenotypes.

Both Glut-1 and Sox-9 are HIFs target genes involved in HIFs signal pathway for regulating the different cell behaviors and functions. Glut-1 is generally considered a housekeeping glucose transporter, exerting the functions to regulate cell metabolism and other behaviors in response to hypoxia. While Sox-9 is expressed in all chondroprogenitor cells and chondrocytes which can correlates with expressions of collagen II and aggrecan during cartilage development. Here, we further assessed the effects of combined spheroid culture with hypoxia on the expressions of HIFs target genes, Glut-1 and SOX-9 at the molecular level. The results in **Figure 7** revealed a significant up-regulated expressions of Glut-1 and SOX-9 in chondrocyte spheroids grown under hypoxia. Hypoxic conditions markedly promoted the expressions of Glut-1 and SOX-9 genes compared with that in normoxia as demonstrated by qRT-PCR analysis. Moreover, both HIF-1α and HIF-2α inhibitors were effective in down-regulating hypoxic inductions of Glut-1 and SOX-9. Notably, HIF-2α was demonstrated as a major player in inhibiting of SOX-9 gene expression compared to HIF-1α.

Discussions

In vitro expansion of autologous chondrocytes with superior phenotype and functions is essential for many clinically used cartilage repair treatments. In this work, we developed a straightforward strategy for rapid production of size controlled cell spheroids in concave microwells array and resembled the *in vivo*-like physiological environment of cartilage by culturing chondrocytes in combined 3D spheroid and hypoxic conditions. Different from the existing methods for fabricating concave microwells structure, such as ice-lithographic fabrication[20],polymer micro-sphere[21] and elastic PDMS membranedefor-mation[22], the approach we proposed is simply based on SU-8 mold, which is quite simple, low cost and easy to operate, without the requirement of complicated instrumentation and procedures. The dimensions of the concave microwells can be adjusted in a controllable way to meet the specific need, such as *in vitro* anti-cancer 3D drug testing, the size of cell spheroids is closely associated with the resistance response of the cells to drugs. This device may present a simple way to produce ultra-high throughput of cell spheroids with controlled size and less sample requirement, thus facilitating the 3D drug screening under multiple conditions simultaneously.

As a type of mesenchymal original cell, chondrocytes tend to differentiate into fibroblast-like phenotype and lose their cartilage phenotype *in vitro* monolayer. But the cells

aggregated into spheroids can resemble the native 3D extracellular matrix network supporting the cartilage specific phenotypes and functions driven by the interactions between cell-cell and cell-matrix. Specially, the chondrocytes in spheroid format induce the appearance of tissue-specific phenotype within a short time (~3 days), which is less than that cultured by other methods (~1 week or more)[6, 7, 10, 12]. In addition, articular cartilage *in vivo* is an avascular and, thus, per se hypoxic tissue and has to be adapted to low oxygen tension environment[16]. 5% oxygen is considered as the most appropriate oxygen concentration for chondrocytes[9]. Importantly, the combined spheroid culture and simulative hypoxic conditions exhibit positive effects on the sustaining of chondrocytes phenotype and expressions of collagen II and aggrecan at protein and gene levels, indicating the essential roles of biomimetic microenvironment *in vitro* in maintaining the tissue-specific functions in cartilage. Both hypoxic condition and 3D construction were prerequisite for *in vitro* cartilage engineering and tissue repair. It is also clinically necessary to select the superior chondrocytes by providing the physiologically relevant microenvironment before chondrocyte implantations. The parameters of cell spheroids were measured as shown in **supplemental figure 2**. It was observed that, the spheroids could be formed within 24h at a diameter of 200μm, and gradually shrunk in later 2 days due to cell-cell interactions at certain cell density. The trend was consistent with other reported work[23, 24].

The transcription factors-HIFs have been regarded as the chief mediators of hypoxic response in mammalian tissue by activating their downstream genes[25].Although several works reported the possible mechanism of HIFs in regulating the different chondrocyte behaviors, there are still contradictions. We demonstrated that both HIF-1α and HIF-2α were involved in the regulation of cell behaviors such as proliferation, metabolism and phenotype maintenance, but they differed in different biological functions. From real-time PCR analysis, the hypoxic condition markedly promoted the gene expressions of Glut-1 and SOX-9 in chondrocytes spheroids, which could be down regulated by using HIF-1α and HIF-2α inhibitors, indicating the roles of HIFs involved in regulating these target genes. E. Lafont et al previously reported that HIF-2α, but not HIF-1α, could mediate SOX-9 induction in chondrocytes under hypoxia[26]. Interestingly, in this work, we found that both HIF-2α and HIF-1α could take part in the regulation of SOX-9 gene expression associated with matrix synthesis, in which HIF-2α might play a major role as compared to HIF-1α. It is obvious that HIF-2α can regulate cartilage matrix genes Col2a1 and aggrecan significantly, which might be mediated by Sox-9 gene. We reveal that HIF-2α was regulated by hypoxia very similar to HIF-1α, but they might exert different biological functions in some sense.

Conclusions

In summary, we presented a scalable bio-fabrication approach for rapid production of high throughput spheroids for resembling the *in vivo*-like cartilage hypoxic microenvironment that facilitated the cartilage-specific phenotype and functions. We revealed that combined 3D spheroid culture with hypoxia conditions dramatically increased the phenotype expressions of collagen II and aggrecan at protein and mRNA levels in a combinational manner. We demonstrated that HIFs signaling pathway was involved in mediating phenotype maintenance, metabolism and differentiation behaviors of chondrocytes, but HIF-1α and HIF-2α differed in their biological functions. Both HIF-1α and HIF-2α could regulate the expression of Glut-1 target gene. HIF-1α could directly regulate the expression of aggrecan, while HIF-2α mainly regulated the expressions of Col2a1 and aggrecan by mediating Sox-9 gene. These findings represent new potential therapeutic targets for maintenance of tissue integrity and cartilage repair, which might contribute to auto-transplantation in clinical research. The presented device is simple and easy to operate, which may further be connected with microfluidic channel or external elements to realize controllable spheroid culture with enhanced functionality, thus, finding more interesting applications in cartilage biology, 3D drug testing and stem cell research.

Acknowledgements

This research was supported by Key Laboratory of Separation Science for Analytical Chemistry (Dalian Institute of Chemical Physics, Chinese Academy of Sciences), the Joint Research Fund of NSFC-RGC (11161160552), National Nature Science Foundation of China (No.91227123, 81201689, 81101125), Key Projects in the National Science & Technology Pillar Program in the Twelfth Five-year Plan Period of China (No.2012BAK02B00, 2012BAK02B03).

Notes and references

[a] Department of Biotechnology, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian, 116023, China
jhqin@dicp.ac.cn
[b]Department of Orthopedics, the First Affiliated Hospital of Dalian Medical University, Dalian, 116023, China

† Footnotes should appear here. These might include comments relevant to but not central to the matter under discussion, limited experimental and spectral data, and crystallographic data.

Electronic Supplementary Information (ESI) available:

Supplemental figure 1. The viability testing of chondrocytes cultured for up to 7 days using dead/live staining. Green color represented for live cells stained by Calcein AM and Red for dead cells stained by Eth-D.

Supplemental figure 2. The proliferation test of chondrocytes cultured in concave microwells for 3 days. Error bars represent the standard deviations (mean ± SD) obtained from 6 independent experiments.

Supplemental figure 3. The proliferation test of chondrocytes cultured in concave microwells with different sizes per well for 3 days. A. Two types of microwells with diameter at 400μm, and 600μm, respectively. B. The averaged diameter of chondrocytes spheroids in both sizes of microwells (mean ± SD, n=10).

Supplemental figure 4. Effects of HIFs inhibitors on the mRNA gene expressions of HIF-1α and HIF-2α under different conditions by quantitative RT-PCR. Chondrocyte spheroids were cultured for 3 days after treated with HIF-1α and HIF-2α inhibitors at the concentration of 30μM, respectively. Error bars were standard deviations, the data was shown as mean ± SD (n=3), Statistical significance was calculated by Student's unpaired t-test; **, $p<0.01$ vs normoxic group *, $p<0.05$ vs normoxic group.

1 Hasler EM, Wu JZ, Muller M, Wyss U. *Critical reviews in biomedical engineering*, 1999, **27**: 415.
2 Buckwalter JA, Mankin HJ. *Instructional Course Lecture*, 1998, **47**: 477.

3 Lin Z, Xu JK, Zheng M. *Tissue engineering*, 2006, **12**: 1971.
4 Benya PD, Padilla SR, Nimni ME. *Cell*, 1978, **15**: 313.
5 Kang SW, Kim BS. *Bio-Medical Materials and Engineering*, 2007, **17**: 269.
6 Schulze-Tanzil G, De Souza P, Villegas Castrejon H, John T, Merker HJ, et al. *Cell and tissue research,* 2002, **308**: 371.
7 Benya PD, Shaffer JD. *Cell*, 1982, **30**: 215.
8 Buschmann MD, Grodzinsky AJ, Kimura JH, Hunziker EB. *Journal of orthopaedic research*, 1992, **10**: 745.
9 Buckley CT, Vinardell T, Kelly DJ. *Osteoarthritis Research Society*, 2010, **18**: 1345.
10 Perka C, Schultz O, Lindenhayn K, Spitzer RS, Muschik M, et al. *Clinical and Experimental Rheumatology*, 2000, **18**: 13.
11 Perka C, Lindenhayn K, Sittinger M, Schultz O. *Journal of Biomedical Materials Research*, 2000, **49**: 305.
12 Hauselmann HJ, Fernandes RJ, Mok SS, Schmid TM, Block JA, et al. *Journal of Cell Science*, 1994, **109**: 17.
13 Hauselmann HJ, Masuda K, Hunziker EB, Neidhart M, Mok SS, et al. *Am J Physiol*, 1996, **271**: 742.
14 Homicz MR, Chia SH, Schumacher BL, Masuda K, Thonar EJ, et al. *Laryngoscope*, 2003, **113**: 25.
15 Stewart MC, Saunders KM, Burton-Wurster N, Macleod JN. *J Bone Miner Res*, 2000, **15**: 166.
16 Silver IA, Maroudas A. *Phil. Trans. R. Soc. Lond. B*, 1975, 2**71**: 261.
17 Martinez I, Olsen R, Bertheussen K, Johansen O. *Cell Transplantation*, 2008, **17**: 987.
18 Foldager CB, Nielsen AB, Munir S, Ulrich-Vinther M, Soballe K, et al. *Acta orthopaedic*, 2011,**82**: 234.
19 Gosset M, Thirion S, Jacques C. *Nat Protoc*, 2008, **3**: 1253.
20 Park JY, Hwang CM, Lee SH. *Biomedical microdevices*, 2009, **11**: 129.
21 Liu C, Gao D, Ding M, Lin JM. *Anal. Chem.*, 2010, **82**: 9418.
22 Choi YY, Chung BG, Lee DH, Khademhosseini A, Kim JH, et al. *Biomaterials*, 2010, **31**: 4296.
23 Jun Y, Kang AR, Lee JS, Jeong GS, Ju J, Lee DY, et al. *Biomaterials*, 2013, **34**:3784.
24 Wong SF, Noda Y, Choi YY, Kim DS, Chung BG, Lee SH. *Biomaterials*, 2011,**32**:8087.
25 Araldi E, Schipani E. *Bone*, 2010, **47:** 190.
26 Lafont JE, Talma S, Murphy CL. *Arthritis and rheumatism*, 2007, **56**:3297.

Figure 1. The schematic representation of *in vivo* articular cartilage microenvironment containing hypoxia and 3D extracellular matrix, which might be regulated by hypoxia inducible factors (HIFs) signal pathway.

Figure 2. The procedures of fabricating concave microwells adopted for spheroid formation of chondrocytes and experimental setup for chondrocyte culture. A. Schematic diagram of fabricating concave microdevice for chondrocyte spheroid formation by using SU-8 template. Middle picture: real device fabricated with 3600 concave microwells. B. Culture of spheroid chondrocytes in hypoxic chamber. The chamber was placed in a hypoxic condition under 5% O_2.

Figure 3. Comparison of cartilage phenotype maintenance of chondrocytes in monolayer dish and 3D spheroid culture under normoxic condition. The cells were cultured in different conditions for 3 days. A. Immunostaining of collagen II and aggrecan in different culture conditions. Green represents for collagen II, red for aggrecan and blue for DAPI. B. Quantitative gene expressions of collagen II, collagen I and aggrecan by using real-time PCR analysis.The data was shown as mean ± SD(n=3).

Figure 4. The proliferation assay of chondrocytes and phenotype specific expression of chondrocytes at protein level in monolayer culture under normoxic (21% O_2) and hypoxic (5% O_2) conditions. A. Bright images of chondrocytes proliferated under different conditions. B. Quantification of chondrocyte proliferation ability under different conditions over 3days. The proliferation test of chondrocytes was performed by counting the cell numbers, and the data was shown as mean ± SD (n=3). C. The expressions of collagen II and aggrecan in monolayer chondrocytes under different conditions by immunofluorescence staining.

Figure 5. The comparison of phenotype specific proteins and gene expressions in chondrocyte spheroids under normoxic and hypoxic conditions. A. Immunostaining of collagen II and aggrecan in chondrocyte spheroids under different conditons. The chondrocyte spheroids were cultured for 3 days. Greenrepresents for collagen II, red for aggrecan and blue for DAPI. B. Quantitative real time-PCR results of collagen II, aggrecanand collagen I in chondrocyte spheroids under different conditions.

Figure 6. Effects of HIFs on the mRNA gene expressions of collagen II, and aggrecan by quantitative RT-PCR . Chondrocyte spheroids were cultured for 3 days after treated with HIF-1α inhibitor (HIF-1i) and HIF-2α inhibitor (HIF-2i) at a concentration of 30μM, respectively. Error bars were standard deviations, the data were shown as mean ± SD (n=3). Statistical significance was calculated by Student's unpaired t-test; **, $p<0.01$ vs hypoxic group *, $p<0.05$ vs hypoxic group.

Figure 7. Effects of HIFs on the mRNA gene expressions of Glut-1 (A) and Sox-9 (B) under different conditions by quantitative RT-PCR.Chondrocyte spheroids were cultured for 3 days after treated with HIF-1α inhibitor and HIF-2α inhibitor at a concentration of 30μM, respectively. Error bars were standard deviations, the data was shown as mean ± SD (n=3). Statistical significance was calculated by Student's unpaired t-test, **, $p<0.01$ vs hypoxic group *, $p<0.05$ vs hypoxic group.