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Redifferentiation of In Vitro Expanded Adult Articular Chondrocytes by Combining the Hanging-Drop Cultivation Method With Hypoxic Environment

Inigo Martinez,* Jan Elvenes,* Randi Olsen,† Kjell Bertheussen,‡ and Oddmund Johansen*

*Department of Orthopaedic Surgery, Institute of Clinical Medicine, University of Tromsø, 9037 Tromsø, Norway
†Department of Electron Microscopy, Medicine Faculty, University of Tromsø, 9037 Tromsø, Norway
‡Department of Obstetrics and Gynaecology, University Hospital of Northern Norway, N-9038 Tromsø, Norway

The main purpose of this work has been to establish a new culturing technique to improve the chondrogenic commitment of isolated adult human chondrocytes, with the aim of being used during cell-based therapies or tissue engineering strategies. By using a rather novel technique to generate scaffold-free three-dimensional (3D) structures from in vitro expanded chondrocytes, we have explored the effects of different culture environments on cartilage formation. Three-dimensional chondrospheroids were developed by applying the hanging-drop technique. Cartilage tissue formation was attempted after combining critical factors such as serum-containing or serum-free media and atmospheric (20%) or low (2.5%) oxygen tensions. The quality of the formed microtissues was analyzed by histology, immunohistochemistry, electron microscopy, and real-time PCR, and directly compared with native adult cartilage. Our results revealed highly organized, 3D tissue-like structures developed by the hanging-drop method. All culture conditions allowed formation of 3D spheroids; however, cartilage generated under low oxygen tension had a bigger size, enhanced matrix deposition, and higher quality of cartilage formation. Real-time PCR demonstrated enhanced expression of cartilage-specific genes such us collagen type II and aggrecan in 3D cultures when compared to monolayers. Cartilage-specific matrix proteins and genes expressed in hanging-drop-developed spheroids were comparable to the expression obtained by applying the pellet culture system. In summary, our results indicate that a combination of 3D cultures of chondrocytes in hanging drops and a low oxygen environment represent an easy and convenient way to generate cartilage-like microstructures. We also show that a new specially tailored serum-free medium is suitable for in vitro cartilage tissue formation. This new methodology opens up the possibility of using autogenously produced solid 3D structures with redifferentiated chondrocytes as an attractive alternative to the currently used autologous chondrocyte transplantation for cartilage repair.

Key words: Cartilage; Chondrocytes; Hypoxia; Serum-free medium; Spheroids; Transplantation

INTRODUCTION

Cartilage degeneration is a frequent health problem that often develops in the absence of an identifiable reason (4). Clinical interventions to induce biological self-repair of damage tissue are currently in use to treat full-thickness cartilage defects. For instance, several marrow-stimulating procedures directed at the recruitment of bone marrow cells have been widely used to treat local cartilage defects. Approaches based on transplantation of autologous cartilage cells (ACI) are also extensively used in the clinic. However, the quality of the newly developed tissue in a significant number of patients does not reach the requirements of functional hyaline cartilage, and fibrocartilage formation may degenerate easier over time than native articular cartilage (23). A mix of hyaline and fibrocartilage is the most common resulting tissue after transplantation of autologous chondrocytes, and this outcome is comparable to the results obtained by microfracture (12,13). Dedifferentiation of chondrocytes during the in vitro growth phase represents a serious concern for the use of ACI treatments. It remains unresolved whether variations in the methodology used to culture chondrocytes would influence the capacity of these cells to make new tissue after transplantation.

To date, the culture of chondrocyte aggregates, or the so-called "pellet" culture system has been reported as an alternative method for overcoming biomaterial-derived

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Address correspondence to Inigo Martinez, Department of Orthopaedic Surgery, Institute of Clinical Medicine, University of Tromsø, 9037 Tromsø, Norway. Tel: +47 77 64 46 86; Fax: +47 77 62 71 64; E-mail: inigo.martinez@fagmed.uit.no

problems (1,19,24). This culture system allows cell–cell interactions similar to those that happen in cartilage formation during embryonic development. Although this system facilitates the redifferentiation of the cells and the formation of hyaline-like cartilage microstructures, some limitations are encountered for its clinical application because of the high number of cells needed to generate each pellet, and the low throughput production inherent to this technique, in which a single cell micromass is formed from each tube by centrifugal forces.

Other interesting approaches for large-scale and rapid generation of chondrocyte aggregates have been established by rotational culture systems (6). This method, however, only provides the formation of three-dimensional (3D) structures of heterogeneous sizes. Cartilage-specific genes and proteins have not been analyzed in constructs generated in this way, and the effects of important environmental factors such as oxygen, serum, or mechanical stimuli have not been evaluated in this culture system.

In the search of a biomedical solution for reparation or replacement of injured cartilage, many experts in the field consider that cartilage tissue engineered at the laboratory represents an attractive alternative (14,18). However, at the present time laboratory-engineered cartilage still does not satisfy the demands for functional cartilage restoration (22). We still do not know enough about how the cells respond when they are place in artificial biomatrices, nor the optimal combination of environmental factors that would enable good quality and durable hyaline cartilage formation.

In our study we have made an attempt to find optimal culture conditions by evaluating in vitro 3D cartilage formation after combination of different culture environments. We have used a rather new methodology to generate cartilage microtissues, based on a traditional method used for generation of 3D tissue-like structures with different cell sources, the hanging-drop method. This method does not use additional cell supports and allows the formation of many units of tissue from a single biopsy. We demonstrate that dedifferentiated articular chondrocytes are able to reestablish their chondrogenic potential when the cells are cultured by the hanging-drop methodology. Additionally, we also describe the effects on chondrocytes physiology and cartilage tissue formation by modulating culture environments, including the oxygen tension in the presence or absence of serum.

MATERIALS AND METHODS

Isolation and Propagation of Human Articular Chondrocytes

All experiments were performed with human articular chondrocytes obtained from surplus cells from patients undergoing autologous chondrocyte transplantation. Mean age for individuals who donated cells for this study was 36 years old, ranking from 26 (younger) to 45 (older). Chondrocytes from 11 different patients were used in this study, and the results presented are representative experiments performed with chondrocytes from at least three different randomly selected patients. The patients participated with informed consent, and the study was approved by the Regional Ethical Committee. Initial biopsies were mainly taken from non-weight-bearing areas where macroscopically normal cartilage could be obtained. Cartilage biopsies were kept in 0.9% NaCl for approximately 2 h, and then cut in 1–1.5 mm^3 pieces. They were kept for 18 h in 2–5 ml DMEM/Ham's F-12 (Cat. No. T 481-50, BioChrom Labs, Terre Haute, IN) containing collagenase (Cat. No. C-9407, Sigma-Aldrich, Norway AS, Oslo, Norway) at a final concentration of 0.8 mg/ml. The enzyme solution was removed after centrifugation at $200 \times g$ and by consecutive washing steps with DMEM/Ham's F-12. Thereafter, the pellet was resuspended in fresh growth medium (DMEM/Ham's F-12 supplemented with 10% human autologous serum). Cultures were further expanded by trypsinization (Cat. No.T-3924, Sigma), and after repeated washing, resuspended in DMEM/Ham's F-12 supplemented with 10% human serum.

Experimental Culture Conditions

Chondrocytes were initially grown during 3–4 weeks in autologous serum to meet the requirements for ACI treatment. After surgery, surplus cells used for experimentation were maintained on DM 110 basal medium supplemented with heat-inactivated human serum (pooled Normal Human Serum, Innovative Research, Southfield, MI, USA) to avoid experimental variations due to individual serum differences. Three-dimensional growth of chondrocytes was conducted in the presence of 10% human serum (S) or in serum-free media (SF). Concomitantly, the two groups were exposed to either high-oxygen environment (HO), equivalent to 20% O_2, or in low-oxygen environment (LO) equivalent to 2.5% O_2.

Tissue SS (Synthetic Serum) for Biotechnology. The Tissue SS supplements for biotechnology (Medi-Cult A/S, Möllehaven 12, DK-4040 Jyllinge, Denmark) contain the components of SSR 2 (2) as well as a wide range of additional components for the complete replacement of serum in cell culture: a stable metal ion buffer with chelators, iron and trace elements, selenium, insulin, a synthetic transferrin replacement (aurintricarboxylic acid), a surfactant regulating surface tension, synthetic adherence promoters (for adherent cells), some well-defined lipid components of low MW including cholesteryl acetate (500 μg/L), a synthetic lipid stabilizer, a buffer of toxic waste products, dexamethasone,

and protective agents against mechanical damage. All components of Tissue SS are contained within one single solution, and the size of the components are MW 10,000 or below. There are no proteins or peptides, except for a low concentration of recombinant human insulin (500 μg/L). SS shows high degree of stability and reproducibility in cell cultures. The recommended basal medium for use with Tissue SS is DM 110. One liter of DM 110 was composed of 6.0 g DME powder (Sigma D 5648), 6.8 g MCDB 110-powder (Sigma M 6520), 2.6 g $NaHCO_3$, and antibiotics (penicillin, streptomycin, and gentamycin). The final medium was DM 110 supplemented with 5% Tissue SS, 500 nM hydrocortisone (180 ng/ml), and 50 μg/L cholic acid.

Hanging-Drop Cultivation

Freshly isolated articular chondrocytes (ACs) were expanded for 3–5 weeks prior to the 3D growth in standard growth medium (DMEM/F-12 supplemented with 10% autologous human serum). AC monolayers were dissociated by trypsination and the cell number determined on a hemacytometer. The cell suspension was used to initiate hanging-drop cultures as previously described (11). Drops of 40 μl were dispensed into each well of a 48-well lid (Nunc) and the lid was inverted. Titration experiments ranging between 500 and 100,000 cells/drop were performed to determine optimal cell density. Best results were obtained with 20,000 cells/drop, so this cell density was used for experimentation in this work. The different culture conditions were applied from the starting point of the hanging-drop culture. Viability of cells at all different time points was monitored by trypan blue exclusion assay.

Pellet Culture

Chondrocyte micromasses or pellets were created as previously described (24). Briefly, 2.0×10^5 cells from passage 1 after ACI surgery were placed into a polypropylene Eppendorf tube with 0.5 ml of the previously described serum-free medium. The cells were centrifuged at $500 \times g$ during 10 min and maintained at 37°C in 5% CO_2/95% air. After initial culture of pellets in tubes during 48 h, pellets were transferred to low binding 24-multiwell plates (Nunc). Incubation was curried out during 5 additional days, and half of the medium was changed every second day.

Histology and Immunohistochemistry

For histological and immunohistochemical staining, 3D spheroids and native cartilage were fixed in PBS containing 4% paraformaldehyde for 2 h at 20°C, included in a block of agar to facilitate the handling of the spheroids, dehydrated in alcohol, washed in acetone, and infiltrated with paraffin. Paraffin sections (3–4 μm) were dried, deparafinized using XEM-200 (Vogel, Giessen, Germany), rehydrated in alcohol, pretreated with 2 mg/ml of hyaluronidase (Merck, Darmstadt, Germany) for 15 min at 37°C, and subsequently with 1 mg/ml of pronase (Roche Diagnostics) for 30 min at 37°C. Nonspecific background was blocked using PBS containing 5% BSA for 30 min. Sections were incubated overnight at 4°C with a polyclonal rabbit anti-human type II collagen (ICN Biomedicals, Aurora, OH) in PBS containing 1% BSA. Following washing with Tris-buffered saline, reactivity was detected using biotinylated donkey anti-rabbit secondary antibody (1:200) and streptavidin-peroxidase kit (Dako, Glostrup, Denmark) for 30 min at 20°C, and hematoxylin for 2 min at 20°C. Sections were permanently mounted with Aquatex (Merck) and examined by light microscopy.

Transmission Electron Microscopy

Three-dimensional cell cultures and 1-mm^3 cartilage biopsies were immersion fixed in Karnovsky fixative overnight, washed in Karnowsky buffer, and postfixated in 1% OsO_4 for 1 h. Samples for transmission electron microscopy (TEM) were enblock stained in 2% uranylacetate before dehydration in a graded series of ethanol before embedding in glycidether/araldite (Serva) according to standard procedure. Sections were made on a Leica Ultracut E (Vienna, Austria) with a diamond knife from Diatome (Switzerland). Micrographs were taken on a JEOL 1010 electron microscope with a Morada Camera.

Quantitative Gene Expression Analysis by Real-Time PCR

RNA Preparation and First-Strand cDNA Synthesis. Cartilage was homogenized in liquid nitrogen before RNA was extracted using Trizol Reagent (Invitrogen, Norway), according to the manufacturer's protocol. The extracted RNA was then purified by the Quiagen RNeasy Mini kit according to the manufacturer's protocol and nucleic acid concentrations were measured with a spectrophotometer (NanoDrop ND-1000, Saveen & Werner AS, Norway). Total RNA from spheroids and cell layers was extracted directly by the Quiagen RNeasy Mini kit and then quantified. First-strand cDNA was generated by incubating a mixture of 250 ng of RNA, 1 μl of 0.5 μg/μl oligo$(dT)_{12\text{-}18}$ primers, and 1 μl of 10 mM dNTP mix at 65°C for 5 min. The mixture was cooled on ice and 4 μl of 5 × First Strand Buffer, 1 μl of 0.1 M DTT, 1 μl of 40 U/μl RNaseOUT™, and 1 μl of 200 U/μl Superscript III™ reverse transcriptase (Invitrogen) were added. The reaction was then carried out at 50°C for 40 min and 70°C for 15 min on a MJ

Research PTC-200 DNA Engine thermal cycler (MJ Research, Waltham, MA).

Quantitative RT-PCR (qRT-PCR). Quantification was performed by using the ABI Prism 7900 Fast Sequence Detection System (Applied Biosystems) according to the comparative C_T method. The real-time PCR reactions were performed by using 2 µl of each reverse transcribed sample. Cycle temperatures and times as well as primers and probes used for the reference gene (GAPDH) and the genes of interest (collagen type I, collagen type II, aggrecan, and versican) were as previously described (5). Because all probes are designed over an exon–exon junction, genomic DNA can be excluded as a template in the real-time PCR reaction.

Data Analysis. The amounts of targets were analyzed by using the comparative C_T method, where the threshold cycle (Ct) values of each target sequence are given by the $2^{-\Delta\Delta CT}$ formula (16). We present the data as log fold change in gene expression normalized to the endogenous reference gene (GAPDH) and relative to the expression in the monolayer cultured cells.

RESULTS

3D Cartilage Tissue Formation by Hanging Drops

The hanging-drop method has been commonly used for cultivating embryoid bodies (9), and more recently adapted to grow multicellular tumor spheroids with a wide variety of cell lines (11). In this work we have attempted to generate cartilage microtissues by the use of the hanging-drop method with isolated adult human chondrocytes. To aid handling and to enhance throughput, 40 µl hanging-drop cultures were performed in inverted 48-well lids. Attempts of hanging drops with higher number of cells hampered the spontaneous cell assembling and the resulting structures were less solid and little consistent. As shown in Figure 1, after 24 h the cells already accumulated at the bottom of the drop in all culture conditions. However, the chondrocytes cultured in TSS-supplemented medium (HOSF, LOSF) presented a tighter and more homogeneous distribution than the serum-cultivated cells. By day 3 the chondrocytes were more tightly packed, but a more defined and solid structure could be observed in serum-free cultured cells. At day 7 all the culture conditions allowed the formation of homogenous, well-rounded, and solid cell aggregates. Of note, a larger diameter of spheroids was readily observed in the low-oxygen culture conditions (LOS and LOSF). Thereafter, nonsignificant changes were observed in the shape and size of the formed structures, indicating a plateau in their growth during these time intervals. Viability of cells remained high (>92%) up to day 21 of culture, measured by the trypan blue exclusion assay (data not shown).

Morphological Examination and Analysis of Extracellular Matrix Proteins Expression by Histology and Immunohistochemistry

Parafin-embedded chondrospheroids and pellets were analyzed by histological and immunohistochemical methods. Histological examination of the specimens revealed homogeneous and well-rounded structures developed in all culture conditions, with a diameter ranging from 200 to 600 µm. However, cell density was considerably higher in high-oxygen-treated cultures. Conversely, more intercellular space was developed in low-oxygen tension, resulting in structures of larger diameter. Expression of sulphated proteoglycans was monitored by staining of spheroids with alcian blue and safranin-O (Fig. 2, upper panels). The results revealed significantly more glycosaminoglycans expression in spheroids formed at low oxygen than at high oxygen, and similar amounts between low-oxygen-formed spheroids and cell pellets. Specific polyclonal antibodies against collagen type II showed a strong immunoreaction in high- and low-oxygen-treated spheroids as well as in ACs pellets (Fig. 2, botton panels), and the average intensity was equivalent to that observed also in adult cartilage. Cell proliferation was also evaluated by expression of the nuclear marker Ki-67 (data not shown). Cell division was nearly undetectable in any of the conditions, including serum-treated samples, as well as in native cartilage, suggesting that the chondrocyte aggregation developed by this culture system induces cell growth arrest without hampering cell viability.

Ultrastructural Examination of Chondrospheroids by TEM

Ultrastructural analysis of intermediate cell layers of chondrospheroids also revealed the differences in cell density encountered between normal and low-oxygen-treated microtissues examined by light microscopy. Intercellular space and matrix deposition were more evident in spheroids developed under low-oxygen tension than in atmospheric oxygen (Fig. 3a–d). Chondrocytes in all culture conditions were characterized by oval shape or variably elongated cells, frequently containing a plurilobated nucleus, and heavily loaded with lipid granules. Chondrocytes in the 3D structures showed no signs of cell deterioration or apoptosis at any depth of the spheroids. The cells displayed intact cell membranes, no chromatin condensation, and well-developed organelles. Interestingly, the amount of mitochondria per cell unit was apparently more elevated in high-oxygen-treated spheroids than in low-oxygen-treated ones. In low-oxygen-maintained spheroids, chondrocytes were surrounded by a complex matrix network, where abundant proteoglycans, collagen fibrils, and matrix vesicles could be identified in the cell vicinity (Fig. 3c, d). The fibrils and other matrix molecules were sparse and ran-

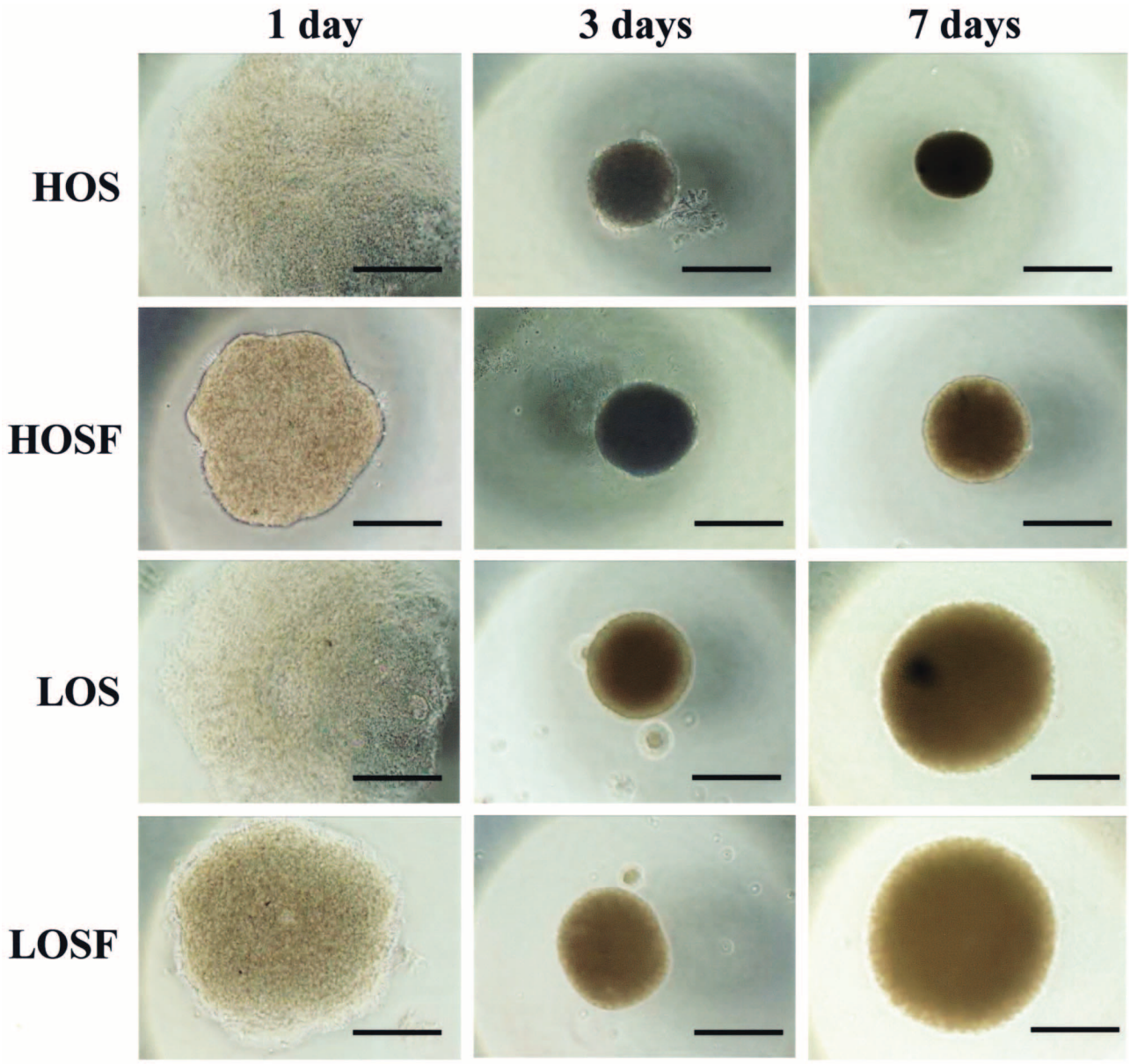

Figure 1. Time course formation of chondrocyte spheroids by the hanging-drop method. Human articular chondrocytes expanded during 3 weeks in monolayers were trypsinized and then dispensed into 40-µl drops at a concentration of 20,000 cell/drop. Spheroid formation over time was monitored during different culture conditions, including high oxygen (HO) or low oxygen (LO), along with human serum-supplemented medium (S) or serum-free medium (SF). Scale bars: 250 µm.

domly oriented in between the cells. Occasionally, focal organization of matrix into fibril meshwork could be observed (Fig. 3e). However, the fibril network in spheroids was still rather loose and more disorganized when compared with that in native tissue (Fig. 3e, f).

Quantitative Gene Expression of Extracellular Matrix Molecules

The expression of cartilage signature genes was used to monitor the degree of chondrocyte differentiation and the development of a hyaline cartilage phenotype. All the experiments were initiated with dedifferentiated articular chondrocytes previously expanded in monolayer cultures during 3–5 weeks. Thus, results of the different extracellular matrix components obtained by real-time quantitative PCR were expressed as ratios to values of gene expression obtained in monolayers. The results revealed that chondrocytes in hanging drops recovered a more differentiated phenotype as revealed by upregulation of characteristic hyaline cartilage genes such as col-

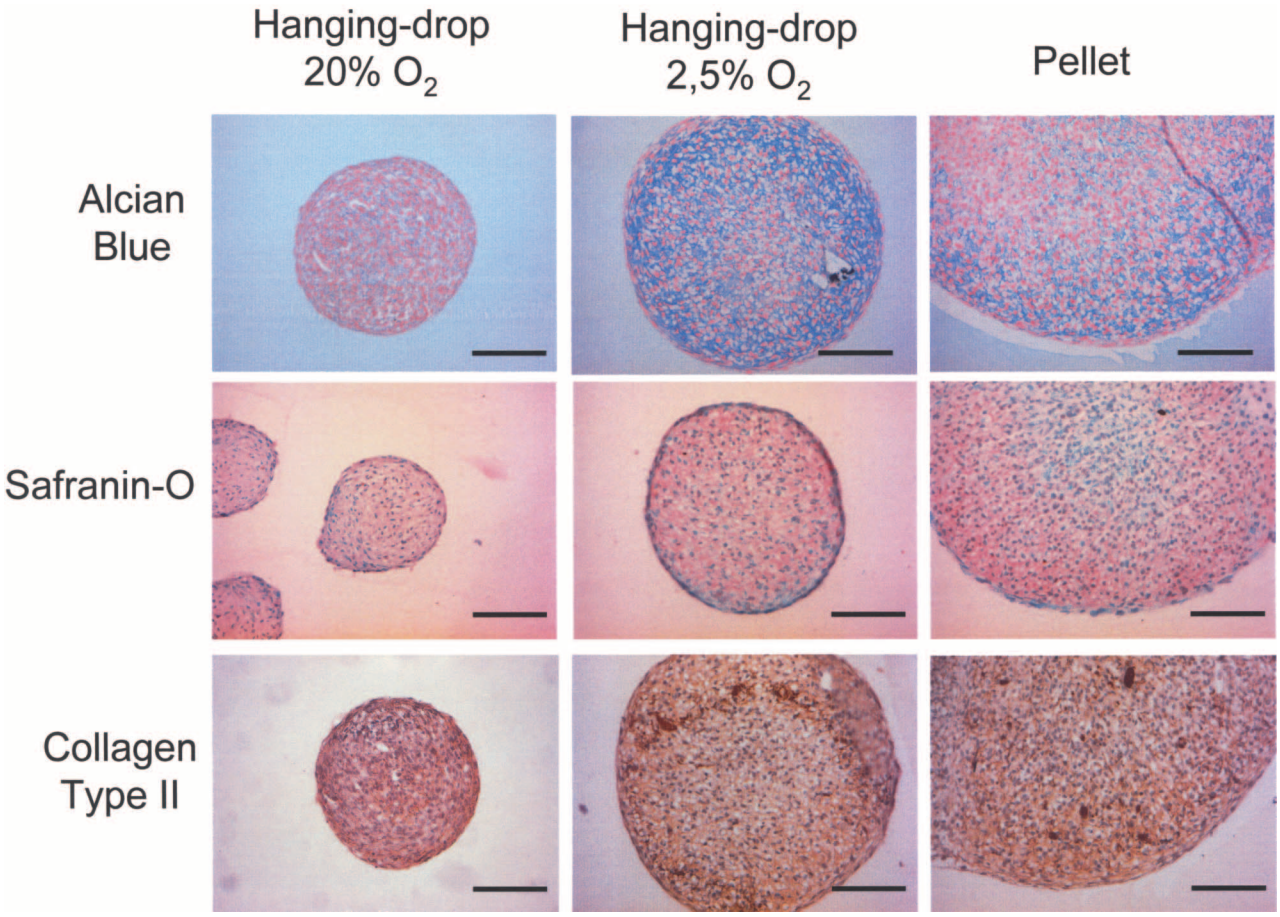

Figure 2. Histologycal and immunohistochemical analysis of glycosaminoglycan production and type II collagen expression in chodrocyte spheroids. Seven-day-old ACs spheroids developed in hanging drops and ACs pellets were fixed and prepared for histological examination. Alcian blue and safranin-O were used for specific staining of sulphated glycosaminoglycans (GAGs). The expression of collagen type II was examined by immunostaining with specific polyclonal anti-collagen type II antibodies. Scale bars: 100 μm.

lagen type II and aggrecan, and downregulation of fibrocartilage-related genes such as collagen type I and versican (Fig. 4). Except for the case of HOSF-cultured spheroids, the expression pattern of the four different messengers studied revealed no significant differences between spheroids formed under high- or low-oxygen tension. However, collagen type II and aggrecan mRNA expression were considerably higher in serum-containing medium. To ascertain if the newly developed system to create cartilage microtissue is comparable to previously described methods to induce redifferentiation of chondrocytes, expression of cartilage-associated gene was compared between hanging-drop-generated spheroids and the pellet culture system. Results revealed a very similar pattern of gene induction and repression between hanging-drop-developed spheroids or ACs pellets (Fig. 5). Despite the significant induction of gene expression by both methods towards a cartilage phenotype, native cartilage still show a higher expression profile of both collagen type II and aggrecan.

DISCUSSION

In the present study we show the establishment of well-formed and solid microtissues ranging from 200 to 600 μm, derived from chondrocyte suspensions, which shares morphological and phenotypic similarities with native hyaline cartilage. This has been achieved without using biopolymers or biomatrices as cell carriers for tissue development.

The precise effects induced on chondrocytes by artificial environments like industrially produced biomatrices are unknown, and so are the consequences that these effects could have on the quality of the resulting tissue (17). In our laboratory we favor the idea of avoiding

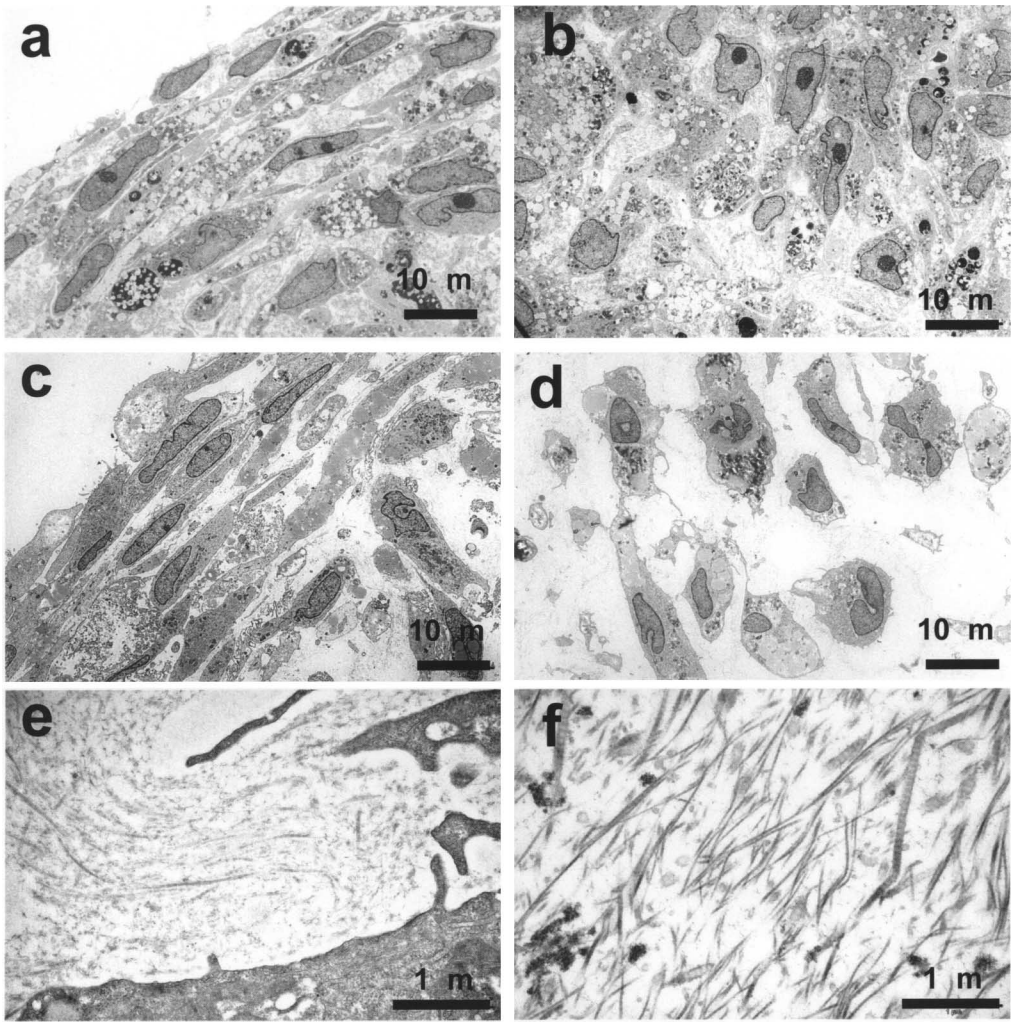

Figure 3. Ultrastructural examination of chondrocyte spheroids by transmission electron microscopy. Seven-day-old spheroids cultured under different oxygen tensions were fixed and prepare for electron microscopy visualization. ACs located at peripheral (a, c) and internal (b, d) layers of spheroids developed by hanging drops show a variable shape, mostly elongated, with plurilobulated nucleus. Matrix deposition is more evident in low-oxygen-treated spheroids (c, d) than in high-oxygen-treated specimens (a, b). Scale bars: 10 μm. Both proteoglycan masses and collagen fibrils can be observed at the cell vicinity in low-oxygen-cultured chondrocyte spheroids (e). Collagen fibrils in spheroids are organized in reticular networks in the cell proximity or in close relationship with other ECM components (e). Substantially more abundant and more organized matrix is observed, though, in normal adult cartilage (f). Scale bars: 1 μm.

artificial cell supports and developing a system where the cells are programmed to create cartilage matrix by themselves. With this goal in mind we have made use of the hanging-drop culture technique to generate small 3D tissue structures. This culturing technique has been previously applied for generation of 3D structures with a variety of cell types including mammary gland epithelial cells, cardiac epithelial cells, embryoid bodies, and a wide range of tumor cells (10,25–27).

Chondrocyte aggregation established as micromass cultures has already been used by some laboratories to generate hyaline-like cartilage (1,19,24), thus avoiding the use of scaffolds. This methodology has proven to be satisfactory to generate small pieces of good quality cartilage. The hanging-drop method shares the principle of chondrocyte condensation, but allows the formation of good quality microtissues with a considerably lesser amount of cells. In this study we have done a side-to-side comparison of the two systems, concluding that the characteristics of the final constructs and the expression of cartilage-specific genes and proteins are very similar. In our study we show that by this method the chondrocyte phenotype is stabilized, the growth rate is slowed down, and cartilage-specific genes and proteins are rees-

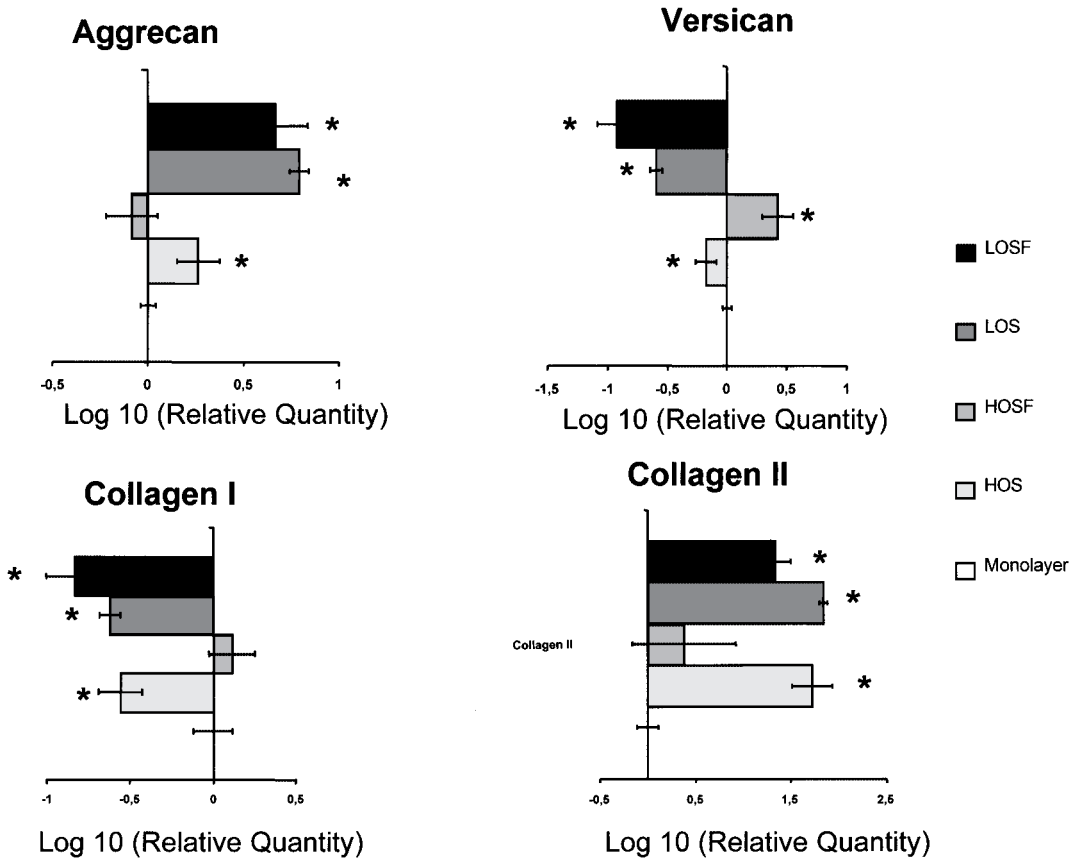

Figure 4. Determination of cartilage signature gene expression by real-time PCR in hanging-drop-developed spheroids relative to the expression in monolayer culture. ACs spheroids were developed in hanging drops during 7 days under different culture conditions: 1) 10% human serum and high-oxygen level (HOS), 2) serum free and high-oxygen level (HOSF), 3) 10% human serum and low-oxygen level (LOS), or 4) serum free and low-oxygen level (LOSF). The spheroid culturing system (except HOSF condition) of chondrocytes increased gene expression of collagen type 2 and downregulated collagen type 1 when compared to the mRNA levels measured in monolayers. Aggrecan was also upregulated in all spheroid conditions (but not the HOSF condition). GAPDH was used as endogenous reference gene, which the values were normalized against. Values are shown as log *n*-fold differences compared to monolayer culture levels, and are means from three different patients (each analysis performed in duplicate). Error bars represent SDs.

tablished. However, because the size of the structures generated by hanging drops are in a scale that probably does not allow the self-formation of oxygen gradients, to observe good intercellular matrix formation the chondrocyte fusion reaction had to take place in a low-oxygen environment. Recent reports have demonstrated that hypoxia is a crucial factor in cartilage differentiation and endochondral bone development (8,20). Cartilage is indeed an avascular tissue, and the chondrocytes have thus adapted their metabolism to low-oxygen tension (21). In vitro experiments performed in chondrocyte monolayers have demonstrated also that chondrocyte-specific genes encoding structural and regulatory proteins are enhanced during low-oxygen exposure of cells (7). The oxygen requirements for optimal in vitro propagation and cultivation of cartilage cells should in theory get close to the hypoxic conditions encountered in native tissue. We demonstrate here that matrix deposition is increased when the chondrospheres are incubated in low oxygen. Nevertheless, our results suggest that chondrocyte aggregation is by itself enough to induced cartilage-specific genes. Gene expression of matrix molecules seems to be equal under high- and low-oxygen tensions; however, we observe more matrix deposition in low-oxygen-treated spheroids. Matrix formation is the result of a complex multistep process including posttranslational modification of extracellular matrix precursors and the correct balance between matrix-forming and matrix-degrading factors. Thus, although we see similar expression of extracellular matrix precursors at the mRNA level, unmeasured factors may still play important roles in the final outcome, and may explain the logic behind

the clear variations observed by morphology between high- and low-oxygen environments.

Most newly developed tissue engineering protocols make use of media that contain animal- or human-derived serum (15). Autologous human serum is also used for chondrocyte propagation during cell-based treatments (3). However, the use of serum in culture media holds some concerns to be considered. Animal and human serum harbors proteins and factors in variable and unknown concentrations that may interact with exogenously added growth factors in an unpredictable way. From the clinical applicability point of view, the use of xenogenous serum in culture media introduces a substantial risk for immunologic rejection and transmission of animal-borne pathogens. On the other hand, serum is a nonphysiological medium for chondrocytes. Ideally, a culture medium deprived of animal or human blood derivatives should be use for cartilage regeneration in vitro. With all these considerations in mind we have tested the production of cartilage tissue in a quite newly developed serum-free culture medium (2). This medium contains no proteins and has a similar formulation to other serum-free media used for chondrocyte culture such as ITS, Nutridoma®, or others (see Materials and Methods). Our results show good quality tissue formation in the serum-free and low-oxygen conditions with no significant histomorphological differences when compared with the serum-containing groups. However, cartilage-specific genes seem to be more elevated in the presence of serum; thus, complete avoidance of serum could be still a matter of debate when it comes to strategies for tissue engineering or culturing chondrocytes for transplantation.

In conclusion the hanging-drop method used with articular chondrocytes allows the formation of cartilage-like microstructures, and induces redifferentiation of chondrocytes previously expanded in monolayers. Some environmental factors like low-oxygen tension contribute positively to extracellular matrix deposition. Nevertheless, native cartilage tissue still shows a more developed and more organized matrix than any in vitro-developed scaffold-free 3D cartilage constructs. Better quality car-

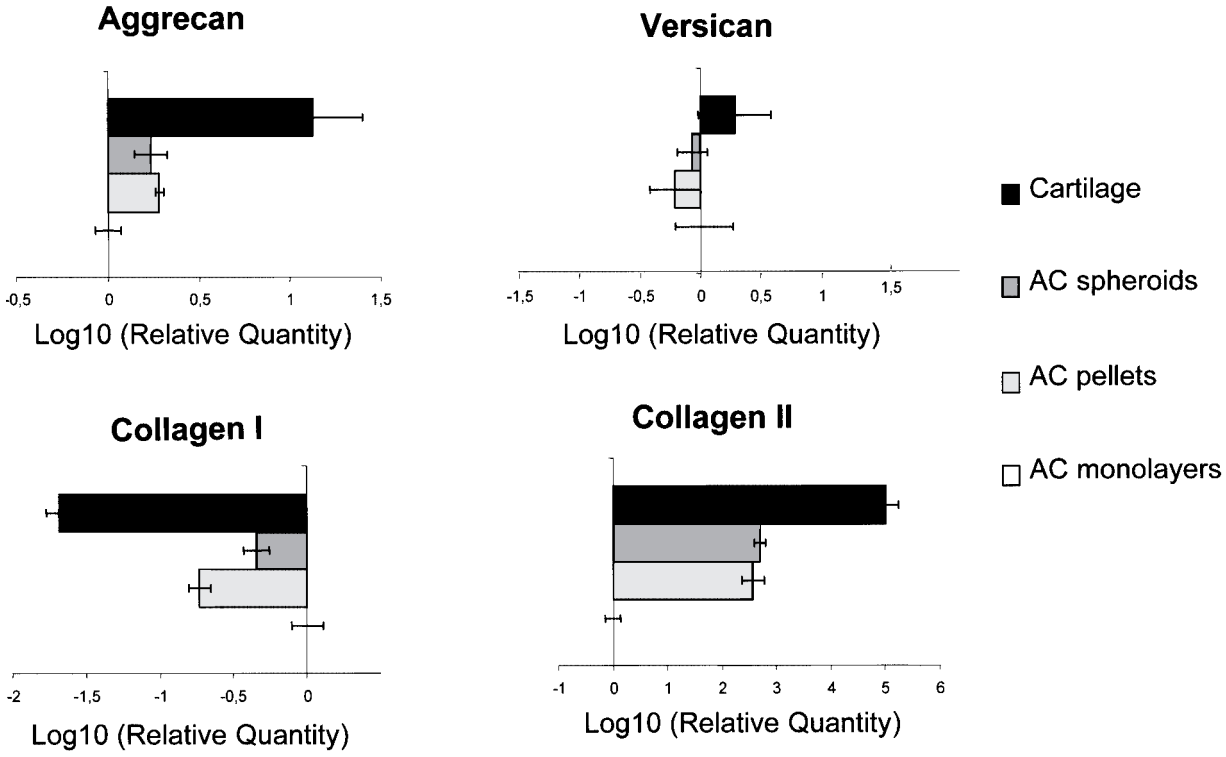

Figure 5. Determination of cartilage signature genes expression by real-time PCR in native cartilage, 3D spheroids, and pellet cultures, relative to the gene expression level in monolayer cultures. Hanging-drop-derived spheroids and pellets were developed during 7 days in serum-free medium at low- and high-oxygen tension, respectively The hyaline cartilage-associated genes aggrecan and collagen II were significantly ($p < 0.05$) increased in both spheroids and pellet cultures, whereas versican was unchanged. Collagen I was significantly ($p < 0.05$) decreased in hanging-drop-developed spheroids and pellet cultures. All gene expression data were normalized against GAPDH. Values are shown as log n-fold differences compared to monolayer culture levels, and are means from two different patients (each analysis performed in triplicate). Error bars represent SDs.

tilage-like tissue could have been developed if other environmental factors such as mechanical stress had been added to the experimental settings. Generation of larger pieces of cartilage using adult articular chondrocytes in a scaffold-independent manner still represents a challenge for the scientific community. In summary, chondrosopheroids generated by hanging drops in combination with low oxygen represents a convenient means of studying chondrocyte biology in a 3D context, and brings up the possibility of using well-formed solid microcartilage with differentiated chondrocytes as an alternative for the standard chondrocyte transplantation technique, or for developing engineered cartilage at the laboratory.

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REFERENCES

1. Battistelli, M.; Borzi, R. M.; Olivotto, E.; Vitellozzi, R.; Burattini, S.; Facchini, A.; Falcieri, E. Cell and matrix morpho-functional analysis in chondrocyte micromasses. Microsc. Res. Tech. 67:286–295; 2005.
2. Bertheussen, K. Growth of cells in a new defined protein-free medium. Cytotechnology 11:219–231; 1993.
3. Brittberg, M.; Lindahl, A.; Nilsson, A.; Ohlsson, C.; Isaksson, O.; Peterson, L. Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. N. Engl. J. Med. 331:889–895; 1994.
4. Buckwalter, J. A.; Mankin, H. J.; Grodzinsky, A. J. Articular cartilage and osteoarthritis. Instr. Course Lect. 54: 465–480; 2005.
5. Demarteau, O.; Wendt, D.; Braccini, A.; Jakob, M.; Schafer, D.; Heberer, M.; Martin, I. Dynamic compression of cartilage constructs engineered from expanded human articular chondrocytes. Biochem. Biophys. Res. Commun. 310:580–588; 2003.
6. Furukawa, K. S.; Suenaga, H.; Toita, K.; Numata, A.; Tanaka, J.; Ushida, T.; Sakai, Y.; Tateishi, T. Rapid and large-scale formation of chondrocyte aggregates by rotational culture. Cell Transplant. 12:475–479; 2003.
7. Grimshaw, M. J.; Mason, R. M. Modulation of bovine articular chondrocyte gene expression in vitro by oxygen tension. Osteoarthritis Cartilage 9:357–364; 2001.
8. Hirao, M.; Tamai, N.; Tsumaki, N.; Yoshikawa, H.; Myoui, A. Oxygen tension regulates chondrocyte differentiation and function during endochondral ossification. J. Biol. Chem. 281:31079–31092; 2006.
9. Keller, G. M. In vitro differentiation of embryonic stem cells. Curr. Opin. Cell Biol. 7:862–869; 1995.
10. Kelm, J. M.; Ehler, E.; Nielsen, L. K.; Schlatter, S.; Perriard, J. C.; Fussenegger, M. Design of artificial myocardial microtissues. Tissue Eng. 10:201–214; 2004.
11. Kelm, J. M.; Timmins, N. E.; Brown, C. J.; Fussenegger, M.; Nielsen, L. K. Method for generation of homogeneous multicellular tumor spheroids applicable to a wide variety of cell types. Biotechnol. Bioeng. 83:173–180; 2003.
12. Knutsen, G.; Drogset, J. O.; Engebretsen, L.; Grøntvedt, T.; Isaksen, V.; Ludvigsen, T. C.; Roberts, S.; Solheim, E.; Strand, T.; Johansen, O. A randomized trial comparing autologous chondrocyte implantation with microfracture. Findings at five years. J. Bone Joint Surg. Am. 289:2105–2112; 2007.
13. Knutsen, G.; Engebretsen, L.; Ludvigsen, T. C.; Drogset, J. O.; Grontvedt, T.; Solheim, E.; Strand, T.; Roberts, S.; Isaksen, V.; Johansen, O. Autologous chondrocyte implantation compared with microfracture in the knee. A randomized trial. J. Bone Joint Surg. Am. 86-A:455–464; 2004.
14. Kuo, C. K.; Li, W. J.; Mauck, R. L.; Tuan, R. S. Cartilage tissue engineering: Its potential and uses. Curr. Opin. Rheumatol. 18:64–73; 2006.
15. LeBaron, R. G.; Athanasiou, K. A. Ex vivo synthesis of articular cartilage. Biomaterials 21:2575–2587; 2000.
16. Livak, K. J.; Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25:402–408; 2001.
17. Lu, L.; Zhu, X.; Valenzuela, R. G.; Currier, B. L.; Yaszemski, M. J. Biodegradable polymer scaffolds for cartilage tissue engineering. Clin. Orthop. Relat. Res. S251–S270; 2001.
18. Nesic, D.; Whiteside, R.; Brittberg, M.; Wendt, D.; Martin, I.; Mainil-Varlet, P. Cartilage tissue engineering for degenerative joint disease. Adv. Drug Deliv. Rev. 58: 300–322; 2006.
19. Park, K.; Huang, J.; Azar, F.; Jin, R. L.; Min, B. H.; Han, D. K.; Hasty, K. Scaffold-free, engineered porcine cartilage construct for cartilage defect repair—in vitro and in vivo study. Artif. Organs 30:586–596; 2006.
20. Pfander, D.; Cramer, T.; Schipani, E.; Johnson, R. S. HIF-1alpha controls extracellular matrix synthesis by epiphyseal chondrocytes. J. Cell Sci. 116:1819–1826; 2003.
21. Rajpurohit, R.; Koch, C. J.; Tao, Z.; Teixeira, C. M.; Shapiro, I. M. Adaptation of chondrocytes to low oxygen tension: relationship between hypoxia and cellular metabolism. J. Cell Physiol. 168:424–432; 1996.
22. Sittinger, M.; Perka, C.; Schultz, O.; Haupl, T.; Burmester, G. R. Joint cartilage regeneration by tissue engineering. Z. Rheumatol. 58:130–135; 1999.
23. Smith, G. D.; Knutsen, G.; Richardson, J. B. A clinical review of cartilage repair techniques. J. Bone Joint Surg. Br. 87:445–449; 2005.
24. Tare, R. S.; Howard, D.; Pound, J. C.; Roach, H. I.; Oreffo, R. O. Tissue engineering strategies for cartilage generation—micromass and three dimensional cultures using human chondrocytes and a continuous cell line. Biochem. Biophys. Res. Commun. 333:609–621; 2005.
25. Timmins, N. E.; Dietmair, S.; Nielsen, L. K. Hanging-drop multicellular spheroids as a model of tumour angiogenesis. Angiogenesis 7:97–103; 2004.
26. Timmins, N. E.; Harding, F. J.; Smart, C.; Brown, M. A.; Nielsen, L. K. Method for the generation and cultivation of functional three-dimensional mammary constructs without exogenous extracellular matrix. Cell Tissue Res. 320:207–210; 2005.
27. Yamada, T.; Yoshikawa, M.; Kanda, S.; Kato, Y.; Nakajima, Y.; Ishizaka, S.; Tsunoda, Y. In vitro differentiation of embryonic stem cells into hepatocyte-like cells identified by cellular uptake of indocyanine green. Stem Cells 20:146–154; 2002.