Characterization of auricular chondrocytes and auricular/articular chondrocyte co-cultures in terms of an application in articular cartilage repair

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Received November 20, 2009; Accepted January 11, 2010

DOI: 10.3892/ijmm_00000394

Abstract. Cartilage injury remains a challenge in orthopedic surgery as articular cartilage only has a limited capacity for intrinsic healing. Autologous chondrocyte transplantation (ACT) is a suitable technique for cartilage repair, but requires articular cartilage biopsies for autologous chondrocyte expansion. The use of heterotopic chondrocytes derived from non-articular cartilage sources such as auricular chondrocytes may be a novel approach for ACT. The aim of the study is to evaluate whether co-cultured articular/auricular chondrocytes exhibit characteristics comparable to articular chondrocytes. Analysis of the proliferation rate, extracellular cartilage matrix (ECM) gene and protein expression (type II and I collagen, elastin, lubricin), ß1-integrins and the chondrogenic transcription factor sox9 in articular/auricular chondrocytes was performed using RTD-PCR, flow cytometry, immunofluorescence microscopy and Western blot analysis. Additionally, three-dimensional (3D) chondrocyte mono- and co-cultures were established. The proliferative activity and elastin gene expression were lower and that of type II collagen and lubricin was higher in articular compared with auricular chondrocytes. The species generally did not influence the chondrocyte characteristics, with the exception of type I collagen and sox9 expression, which was higher in porcine but not in human articular chondrocytes compared with both types of auricular chondrocytes. ß1-integrin gene expression did not differ significantly between the chondrocyte types. The type II collagen gene and protein expression was higher in articular chondrocyte monocultures and was slightly higher in co-cultures compared with monocultured auricular chondrocytes. Both chondrocyte types survived in co-culture. Despite their differing expression profiles, co-cultures revealed some adjustment in the ECM expression of both chondrocyte types.

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Abbreviations: ACT, autologous chondrocyte transplantation; CFDA-SE, 5-carboxyfluorescein diacetate succinimidylester; CMFDA, 5-chloromethylfluorescein-diacetate; CMTMR, (5-[6]-([(4-chloromethyl)benzoyl]amino)tetramethylrhodamine); 3D, three-dimensional; ECM, extracellular cartilage matrix; FCS, fetal calf serum; RT, room temperature; RTD-PCR, real-time detection polymerase chain reaction

Key words: chondrocyte, co-culture, alginate, type II collagen, sox9

Introduction

Cartilage injury remains a major challenge in orthopedic surgery since articular cartilage has only a very limited capacity for healing. ACT is a suitable technique to improve cartilage repair (1,2) whereby autologous articular chondrocytes were isolated from a small articular cartilage biopsy taken from a non-load-bearing area of the injured joint, expanded *in vitro* and then transplanted in the cartilage lesion for defect coverage. Harvesting and sufficient expansion of vital and differentiated autologous articular chondrocytes remains the major limiting factor for successful ACT. To achieve the required number of autologous chondrocytes for transplantation, chondrocytes have to be expanded in monolayer culture, where they start to proliferate. A crucial side effect of monolayer expansion culture is the rapid loss of the chondrocyte-specific phenotype (3). During this dedifferentiation process the chondrocyte expression profile gradually

shifts from the cartilage-specific matrix components type II collagen and aggrecan to non-specific and ubiquitous extracellular matrix proteins such as type I collagen, versican and fibronectin whereby cells exhibit a fibroblast-like morphology. Downregulation of the master chondrogenic transcription factor sox9 has also been reported as a key event during chondrocytes dedifferentiation (4,5). Sox9 plays a critical role in chondrogenesis and is intimately involved in the transcription of the cartilage-specific collagen types II, IX and aggrecan (6-8). Therefore, dedifferentiation of chondrocytes during prolonged *in vitro* expansion in monolayer culture remains a major limiting issue for tissue engineering. After prolonged monolayer culture chondrocytes are irreversibly dedifferentiated and no longer suitable for ACT since they do not produce a hyaline-like ECM (9-12).

The use of heterotopic chondrocytes as an additional non-articular cell source might be a promising novel approach in articular cartilage repair and has currently started to attract more and more attention (13-21). Auricular cartilage contains a considerably higher number of cells and is much more easily accessible as well as being characterized by a lower donor site morbidity, compared with articular cartilage (18). During *in vitro* culture auricular chondrocytes exhibit a higher proliferative activity (18) compared with articular chondrocytes which lead in shorter time periods to high cell numbers sufficient for defect coverage. Auricular cartilage might therefore be an additional cell source to cover larger articular cartilage defects and could also possibly serve as a healthy cartilage source for cartilage repair in an inflammatory microenvironment. Overall, articular and auricular cartilage possess a similar extracellular cartilage matrix composition, however, it must be noted that elastic fibres are a typical component of auricular cartilage, but were not found in articular cartilage (22). On the contrary, lubricin is reported to be produced only by articular chondrocytes of the superficial layer. It protects joint surfaces, inhibits synovial cell over-growth and is an important lubricant for joint cartilage during motion (23,24).

However, it remains mainly unclear whether auricular chondrocytes are able to coexist with articular chondrocytes in a mixed culture system, a precondition for the use of auricular chondrocytes for articular cartilage repair. For this reason, two- and three-dimensional co-cultures were performed and analysed in the study presented.

Materials and methods

Heterotopic chondrocyte isolation. Since a previous study revealed that porcine chondrocytes are a suitable culture model and share some similarities with human chondrocytes *in vitro* (12), porcine chondrocytes were used for this study in addition to human chondrocytes; to achieve an age-matched comparison, articular (stifle joint) and auricular cartilage was harvested from the same individuals: 3-6-month-old hybrid pigs (n=7) or adult humans (n=5, aged 45-85). This study was approved by the Charité review board for experiments with human derived tissues, registration no. EA4/063/06. Articular and auricular cartilage was minced into 1-3-mm slices and enzymatically digested with 0.4% pronase (Roche Diagnostics GmbH, Mannheim, Germany, 7 U/mg) for 1 h at 37˚C and subsequently with 0.2% (w/v) collagenase (Sigma-Aldrich, Munich, Germany, 266 U/mg) for 16 h at 37˚C. Isolated chondrocytes were resuspended in growth medium [Ham's F-12/Dulbecco's modified Eagle's medium (50/50, Biochrom-Seromed, Munich, Germany) containing 10% fetal calf serum (FCS, Biochrom-Seromed), 25 μg/ml ascorbic acid (Sigma-Aldrich), 50 IU/ml streptomycin, 50 IU/ml penicillin, 2.5 μg/ml amphotericin B, essential amino acids, L-glutamine (all: Biochrom-Seromed)] and seeded at 28000 cells/cm^2 in T175 flasks.

Monolayer and alginate co-cultures. Articular chondrocytes were stained with 7.5 μM CMTMR (5-[6]-([(4-chloromethyl) benzoyl]amino)tetramethylrhodamine) and auricular chondrocytes with 7.5 μM CMFDA (5-chloromethylfluorescein-diacetate, both: MoBiTec, Göttingen, Germany - stock solutions of 10 mM in dimethyl sulfoxide were diluted in serum-free growth medium) for 45 min in serum-free growth medium in the dark at 37˚C. After rinsing in growth medium, cells were cultured in monolayers or suspended in 2.5% alginate at 2.5x10^6 cells/ml (Sigma-Aldrich) as previously described (11). Co-cultures were prepared by mixing equally 50:50 articular and auricular chondrocytes before embedding in alginate.

Chondrocyte proliferation assay. Monolayer chondrocytes (passage 1) seeded in 6-well plates at 21000 cells/cm^2 and cultivated for 24 h were rinsed in PBS before 15 min incubation in the dark with 5 μM/ml 5-carboxyfluorescein diacetate succinimidylester (CFDA-SE) solution (Molecular Probes, Invitrogen, CA, USA) at 37˚C. After removal of excessive staining by washing in PBS, the cells were further cultivated for 72 h. Cells were detached using 0.05% trypsin/0.02% EDTA solution, fixed (2% formalin in PBS) and resuspended in FACS staining buffer [1% bovine serum albumin(BSA)/PBS/0.01% NaN_3]. The CFDA-SE staining intensity was assessed by flow cytometry using a FACS-Calibur™ flow cytometer and CellQuest™ software (Becton Dickinson, Heidelberg, Germany). Further flow cytometric data analysis was performed using FlowJo™ software for PC-based computer (Tree Star Inc., Ashland, OR, USA).

Gene expression analysis by RTD-PCR. To obtain semi-quantitative gene expression data on the mRNA, RTD-PCR analyses were performed. With the use of Qiagen RNeasy mini kit (Qiagen®, Hilden, Germany) chondrocyte RNA was isolated. RNA quantity and quality was evaluated with the RNA 6000 Nano assay (Agilent Technologies, Waldbronn, Germany). Equal amounts of total RNA (25 ng/μl in a volume of 20 μl) were reverse transcribed by using the Qiagen QuantiTect® reverse transcription kit (Qiagen) according to the manufacturer's instructions. Aliquots (1 μl) of the cDNA were amplified by RTD-PCR in a 20-μl reaction mixture using specific primer pairs for type II, I collagen, lubricin (all: Qiagen), ß1-integrin, elastin, sox9 (all: Applied Biosystems) and the housekeeping gene ß-actin (Qiagen). Assays were performed either using the QuantiTec gene expression assay (Qiagen) or the TaqMan gene expression assay (Applied Biosystems) in an Opticon 1 - Real-Time Cycler (Opticon™ RTD-PCR, BioRad Laboratories, Munich, Germany). The

conditions of amplification were according to the manufacturer's protocols. Relative amounts of mRNA expression for the gene of interest, and the housekeeping gene ß-actin were calculated using the ΔΔCT method (25).

Indirect immunofluorescence microscopy and confocal laser scanning microscopy. Chondrocytes (first monolayer passage) were cultured on cover slips for 48 h before being fixed in 4% paraformaldehyde for 15 min. Cells or cryosections of alginate beads were washed with Tris-buffered saline (TBS: 0.05 M Tris, 0.015 M NaCl, pH 7.6), overlaid with protease-free donkey serum (5% diluted in TBS) for 10 min at room temperature (RT), permeabilized by 0.1% Triton X-100 for 6 min, rinsed and incubated with primary antibodies [polyclonal rabbit anti-type II collagen (1:50 AcrisAntibodies, Hiddenhausen, Germany), mouse anti-aggrecan (1:20, R&D Systems, Minneapolis, MN, USA)] in a humid chamber 1 h at RT.

Controls included replacement of primary antibodies with respective isotype mouse IgG1 (Caltag, Hamburg, Germany) diluted at the same concentration as used for primary antibodies. Cells were subsequently washed with TBS before incubation with donkey anti-rabbit-Alexa-Fluor®488 or -Alexa-Fluor®648 (both: Invitrogen, Karlsruhe, Germany) coupled secondary antibodies (diluted 1:200 in TBS) respectively, for 1 h at RT. Cell nuclei were counterstained using DAPI (Roche Diagnostics GmbH). Labelled cells were rinsed several times with TBS before being mounted with fluoromount G mounting medium (Southern Biotech, Biozol Diagnostica, Eching, Germany) and examined using fluorescence microscopy (Axioskop 40, Carl Zeiss, Jena, Germany). Pictures were taken using a color view II (Olympus, Europa Holding GmbH).

Additional immunofluorescence images were scanned with a three channel confocal laser scanning microscope (Leica TCS SP-2, Leica Microsystems, Bensheim, Germany). Mean fluorescence intensity of 3 microscopic fields at x100 (type II collagen) or x400 (aggrecan) magnification was semi-quantified in three independent experiments with three different donors using the analySIS FIVE cell^{D} software [Olympus Soft Imaging Solution (SIS) GmbH].

Flow cytometrical analysis for type II collagen in monolayer and alginate co-cultures. Alginate was depolymerized after 2, 4, 7 or 14 days of alginate culture using 50 mM EDTA/10 mM HEPES (pH 7.4) solution. Chondrocytes were washed with PBS and resuspended in staining buffer (1% BSA/PBS/0.01% NaN_3) and phenotypically analysed using primary antibodies for rabbit anti-human type II collagen (1:30 in staining buffer) and their respective isotype control (mouse IgG1, 1:30 in staining buffer, Caltag, Burlingame, CA, USA) for 30 min on ice. After washing, the cells (2% fetal calf serum/PBS/100 U/ml polymyxin B sulfate) were immunolabelled with donkey anti-rabbit allophycocyanine (APC)-coupled secondary antibodies (Dianova, Hamburg, Germany) and diluted 1:200 in staining buffer for 30 min on ice. Cells were washed and fluorescence was evaluated using a FACS Calibur flow cytometer and CellQuest software (Becton Dickinson). Further flow cytometric data analysis was performed using FlowJo software version 7.1.3. (Tree Star Inc.).

Western blot analysis for sox9. Western blotting was used to determine protein synthesis in 48-h old chondrocyte monolayer cultures (passage 1). Chondrocyte monolayers were washed with PBS solution (Biochrom-Seromed), whole cell proteins were extracted by incubation with lysis buffer (25 mM HEPES, pH 7.5, 0.1% Triton X-100, 5 mM $CaCl_2$, 2 mM DTT, 1 mM EGTA (Carl-Roth, Karlsruhe, Germany) and proteinase inhibitors (proteinase complete mini, Roche Diagnostics GmbH) on ice for 30 min. Cell debris was removed by centrifugation. Supernatants were stored at -70˚C until use. Total protein concentration of whole cell extracts was normalized using Bradford protein assay (Roti-Nanoquant, Carl-Roth) and BSA as standard. Samples were separated by Tris-glycine SDS-PAGE (12% acrylamide) under reducing conditions before being transferred to a nitrocellulose membrane (Carl-Roth), using a transblot apparatus (BioRad Laboratories).

Equal protein loading was controlled by the use of Ponceau S staining (Sigma-Aldrich) and ß-actin housekeeping protein expression. Membranes were blocked using blocking buffer (3% BSA/PBS/0.05% Tween-20) 1 h at RT and incubated overnight at 4˚C with the primary antibodies rabbit anti-sox9 (Chemicon International, USA) or monoclonal ß-actin (Sigma-Aldrich) diluted 1:1000 in blocking buffer. Membranes were washed with PBS/0.05% Tween-20 and incubated with horseradish peroxidase conjugated with secondary goat anti-mouse IgG antibodies (1:5000) (Dako-Cytomation, Hamburg, Germany) for 2 h at RT. Specific binding was detected by nonradioactive chemiluminescence using RotiLumin™ (Carl-Roth) and a LAS 3000 Image Reader (Fujifilm, Düsseldorf, Germany). Protein bands were semi-quantified by densitometric scanning (AlphaDigiDoc, Alpha Innotech).

Statistical analysis. All values were expressed as mean ± SEM. Data were analyzed using Student's t test (GraphPad Prism 5, GraphPad Software Inc., San Diego, CA, USA). Statistical significance was set at $p=0.05$.

Results

Morphology and proliferation capacity of articular and auricular chondrocytes. Distinct morphological differences between articular and auricular chondrocytes were evident in freshly isolated porcine and human chondrocytes. Auricular chondrocytes from both donor species had a larger cell size and exhibited more cytoplasmic processes compared with articular chondrocytes (data not shown). Using CFDA-SE proliferation assay, the proliferation capacity of porcine articular and auricular chondrocytes was compared. The proliferation assays revealed that auricular chondrocytes exhibited a significantly higher proliferation rate at 72 h compared with articular chondrocytes (data not shown).

Differences in articular and auricular chondrocyte extracellular matrix gene expression. The gene expression of the cartilage specific type II collagen, of the non-specific type I collagen and of the master chondrogenic transcription factor sox9 was considerably higher in porcine articular chondrocytes compared with auricular chondrocytes (Fig. 1A). Results for

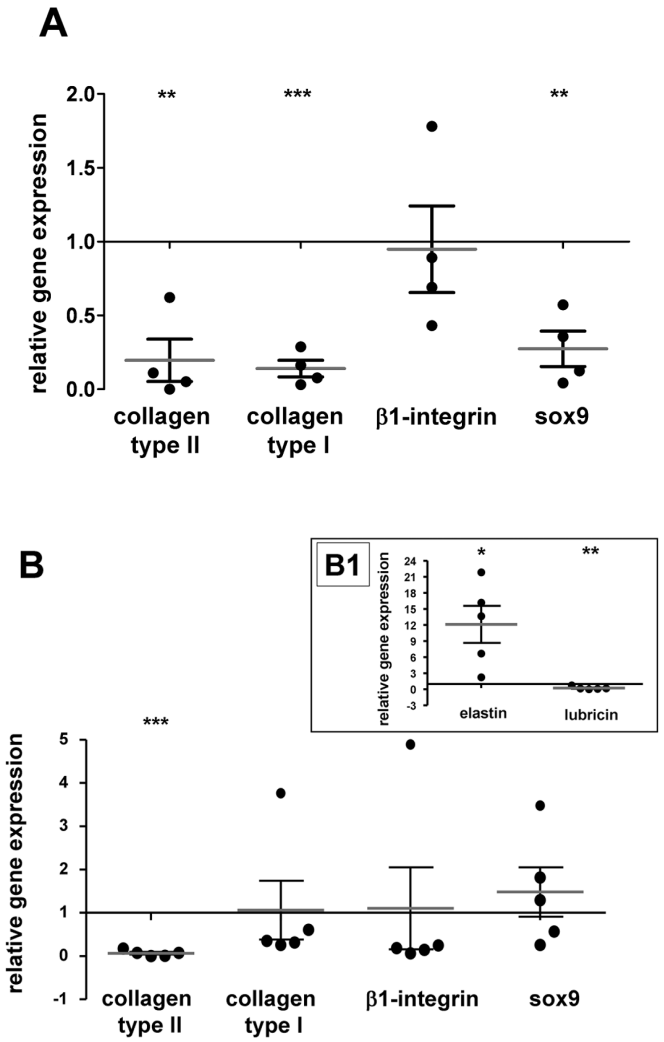

Figure 1. Extracellular matrix gene expression in human and porcine heterotopic chondrocytes. Type II and type I collagen, ß1-integrin and sox9 gene expression was determined in porcine (A) and human (B) auricular and articular chondrocytes (passage 1, 48 h) by RTD-PCR. The gene expression of the articular chondrocytes was normalized (A, B and B1). In human chondrocytes additionally elastin and lubricin gene expression was evaluated (B1). Porcine articular and auricular chondrocytes derived from the same donor whereas human heterotopic chondrocytes derived from different donors. Porcine chondrocytes: n=4, human chondrocytes: n=5.

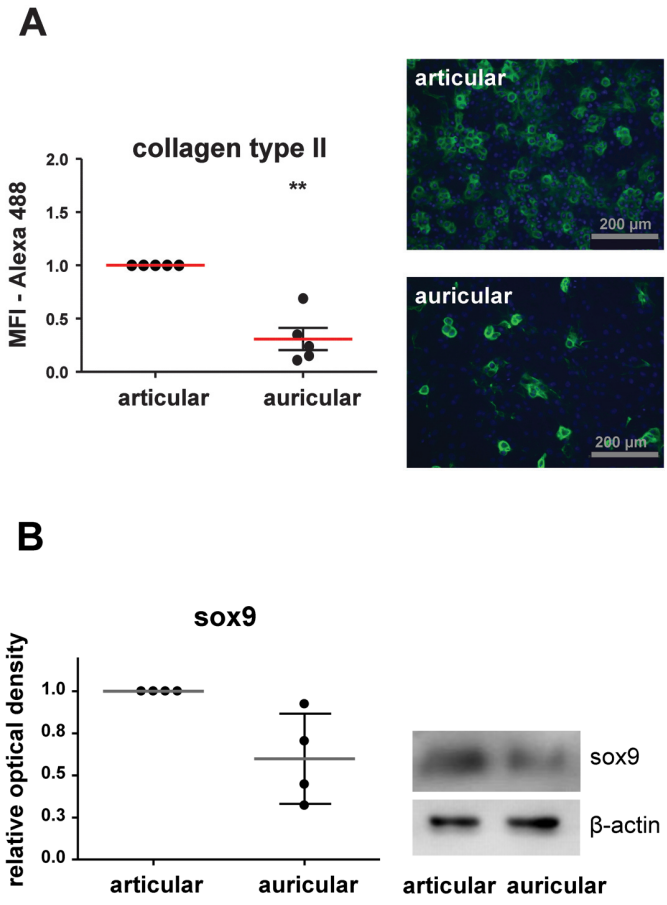

Figure 2. Extracellular deposition of type II collagen and expression of the transcription factor sox9 in porcine heterotopic chondrocytes. (A) Porcine chondrocytes of the first passage were double-immunolabelled extracellularly with specific type II collagen antibodies and Alexa®488 coupled secondary antibodies (green). Densitometric evaluation of mean fluorescence intensity (MFI) deriving from 5 different donors is shown. Chondrocyte cell nuclei were counterstained using DAPI (blue). Scale bars: 200 μm. (B) Whole cell proteins of porcine heterotopic chondrocytes (passage 1) were extracted at day 2 of culture, separated by SDS-PAGE and immunoblotted using specific antibodies directed against sox9. Blots were reprobed with an antibody directed against the housekeeping protein ß-actin as an internal reference. The results of densitometric evaluations of 4 independent experiments using chondrocytes from 4 different donors are shown.

the type II collagen gene expression in porcine chondrocytes was confirmed using human chondrocytes deriving from 5 different donors (Fig. 1B). In regard to the type I collagen and sox9 gene expression, no significant differences were found in the investigated chondrocyte species (articular/auricular) of the studied human donors. In human chondrocytes the gene expression of the glycoprotein lubricin, which is responsible for lubrication of joint cartilage and elastin, which is an important component of elastic fibres, was also evaluated. Lubricin was expressed in significantly higher amounts by articular chondrocytes compared with auricular chondrocytes, whereby the elastin gene expression was superior in auricular chondrocytes (Fig. 1B1). Gene expression levels of the cell matrix receptor ß1-integrin, which is the most common integrin subunit in cartilage, did not show major differences between articular and auricular chondrocytes of human and porcine specimens (Fig. 1A and B).

Differences in articular and auricular chondrocytes extracellular matrix protein expression. On the protein level, type II collagen (Fig. 2A) and the chondrogenic transcription factor sox9 (Fig. 2B) were expressed at a considerably higher level in articular compared with auricular chondrocytes of the same porcine donors as assessed by immunofluorescence labelling (type II collagen) or Western blot analysis (sox9) and densitometric evaluation. The aggrecan deposition (data not shown) did not show major differences between the chondrocyte species (articular/auricular) as revealed by immunofluorescence microscopy. The protein expression of the signal transduction receptor ß1-integrin and the cytoskeletal protein vinculin was more pronounced in articular compared with auricular chondrocytes as shown by Western blot analysis (data not shown).

2D and 3D co-cultures of articular and auricular porcine chondrocytes. To reveal the distribution and the survival of

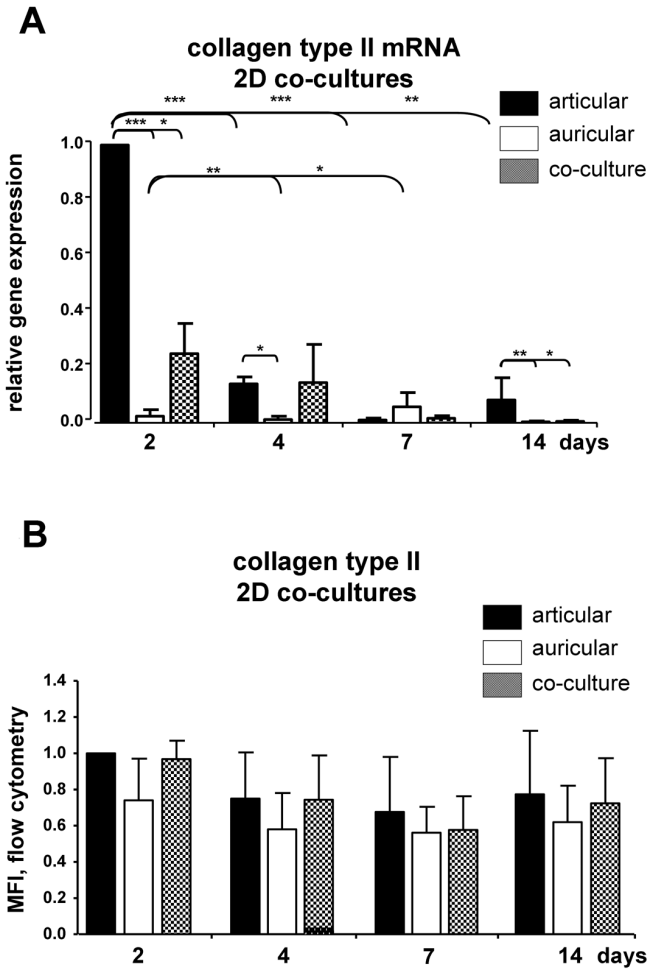

Figure 3. Type II collagen mRNA and protein expression in articular/auricular monolayer co-cultures. Porcine articular and auricular chondrocytes (passage 2) were cultured for 48 h. Gene expression was determined using RTD-PCR and protein expression was evaluated using flow cytometry. For flow cytometric analysis cells were fixed with 2% formalin before immunolabelled for type II collagen. Three independent experiments using chondrocytes from 3 different donors are included.

both chondrocyte species in the co-culture system, chondrocytes were stained with two different cell trackers (articular chondrocytes were stained in red using CMTMR and auricular chondrocytes in green using CMFDA) and analysed after 2, 4, 7 and 14 days in monolayer (2D) (Fig. 3A and B) and alginate (3D) co-cultures using flow cytometry (Fig. 4A) and at day 7 using confocal laser scanning microscopy (Fig. 4B1-3). Type II collagen expression was evaluated in co-cultured in comparison to mono-cultured articular and auricular chondrocytes under the same conditions using RTD-PCR.

In monolayer (2D) monocultures of articular and auricular chondrocytes, the mRNA-expression for type II collagen decreased significantly from day 2 to 7 and was barely detectable at day 14 (Fig. 3A). The mRNA expression of type II collagen was higher in articular chondrocytes compared with co-cultured and monocultured auricular chondrocytes, whereas the co-cultured chondrocytes had a slightly increased type II collagen expression in comparison to the mono-cultured auricular chondrocytes at days 2 and 4 (not statistically significant).

At the protein level a similar trend was evident; the articular chondrocytes in the 2D co-cultures, which could be distinguished from the auricular cells by cell-tracking, revealed a higher (not significant) expression of cell surface-attached type II collagen compared to the monocultured auricular chondrocytes as shown by flow cytometry (Fig. 3B).

Moreover, the staining with the cell trackers indicated that both chondrocyte populations survived in 2D and 3D co-cultures during the observation time of 14 days. Analogous with the results of the 2D cultures; cell-surface attached type II collagen was most pronounced in 3D cultures of monocultured articular chondrocytes. The co-cultured chondrocytes revealed a slightly increased type II collagen expression compared to the auricular chondrocyte monocultures on day 2-7 (not significant, Fig. 4A).

Confocal laser scanning microscopy revealed a pronounced deposition of type II collagen around all of the chondrocyte populations in 3D alginate culture and the presence of both chondrocyte types in co-culture. The type II collagen deposition in alginate was on the same level in the 3D co-cultures and the 3D monocultures (Fig. 4B1-3).

Discussion

The use of heterotopic chondrocytes as an additional cell source for ACT to improve articular cartilage repair is a promising novel approach (15-20). To assess possible limitations of this strategy, a detailed characterization of heterotopic chondrocyte biology is necessary. Several studies exist comparing the *in vitro* behaviour of heterotopic chondrocytes such as auricular with articular chondrocytes derived from different species which lead to divergent results. Henderson *et al* (26) found, after investigating heterotopic rabbit chondrocytes, that auricular cartilage samples showed more consistent staining for proteoglycans and type II collagen and had significantly higher glycosaminoglycan and collagen contents than articular chondrocyte samples. Therefore, they suggested to prefer auricular chondrocytes for generation of tissue-engineered cartilage suitable for *in vivo* testing in laryngotracheal reconstruction. In another study by Tay *et al* (27) human auricular, nasoseptal, and rib chondrocytes were compared with respect to their suitability to generate autologous cartilage grafts for non-articular reconstructive surgery.

In conclusion, post-expansion cell yields suggested that all three sources investigated could be used to generate autologous grafts of a clinically relevant size. However, auricular and nasoseptal chondrocytes displayed a superior post-expansion chondrogenic potential and were supposed as a preferred cell source for cartilage tissue engineering compared to rib-derived chondrocytes (27). Bovine chondrocytes from heterotopic origins (articular and auricular) were cultured and examined to determine relative cell proliferation rates, type II collagen and aggrecan gene expression, as well as extracellular matrix production (28). Type II collagen and aggrecan gene expression was higher in articular compared with auricular chondrocytes (28) which corresponds with our results with porcine chondrocytes. In another study, the biomechanical properties of the engineered cartilage made with porcine auricular or costal chondrocytes were found to be superior to those of cartilage

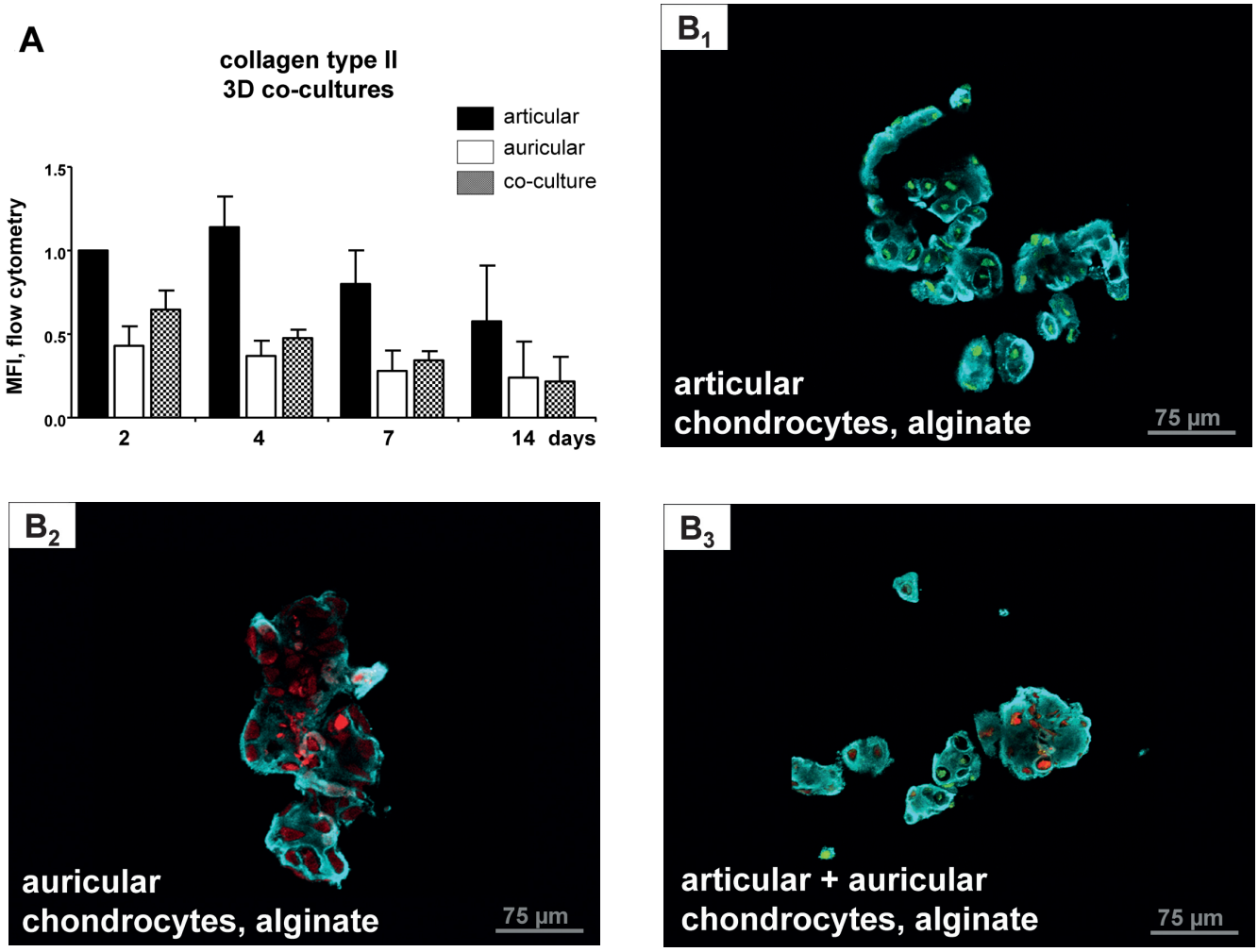

Figure 4. Type II collagen protein expression in articular/auricular three-dimensionl alginate-co-cultures. Porcine articular and auricular chondrocytes (passage 2) were stained with cell trackers and cultured for 7 days in alginate. Alginate was resolved and fixed cells (A) or cryosections of the alginate beads (B) were immunolabelled for type II collagen for flow cytometry (A) or confocal laser scanning microscopy (B1-3, turquoise). Articular chondrocytes: green, auricular chondrocytes: red. Scale bars: 75 μm. Three independent experiments using chondrocytes from 3 different donors are included.

made with articular chondrocytes as reported by Xu *et al* (29).

Since these studies which compare the behaviour of heterotopic chondrocytes *in vitro*, mostly display diverse and partly contradictory results, detailed consideration of auricular-derived and articular-derived chondrocyte synthesis patterns of extracellular cartilage matrix proteins was necessary. The results of the present study indicated a higher expression profile of cartilage specific matrix components such as type II collagen in 2D and 3D articular chondrocyte cultures harvested from two species: human and swine and were therefore in accordance with data reported by Isogai *et al* (28), and Malicev *et al* (30), but did mostly not agree with the results of Chung *et al* (31).

The reason for these inconsistencies between most studies remains unclear, however, the results seem to be strongly influenced by the differing culture conditions, supplements, effects of various biomaterials used for 3D culturing, inter-species differences (human versus rabbit, porcine, bovine, caprine) or age-/disease-related effects of cartilage donors. In some cases, different individuals were used for the isolation of heterotopic chondrocytes and therefore inter-donor variance could not be excluded. Most importantly, chondrocytes of different monolayer passages were investigated, so that a divergent velocity of dedifferentiation which depends on the cartilage source (articular and auricular) could cause the differing results [dedifferentiation is enhanced in the more rapidly proliferating auricular chondrocytes compared with slowly proliferating articular chondrocytes (30)]. Therefore, we decided to study chondrocytes harvested from swine and to compare some of these results with those from similar experiments with human chondrocyte populations to exclude results that seem to be influenced by the donor species itself.

We found consistencies between adult human cartilage and pre-adult porcine cartilage (same porcine donors were used for auricular and articular chondrocyte isolation) in regard to type II collagen gene expression. However, the difference in sox9 expression between porcine articular and auricular chondrocytes could not be confirmed by the results of sox9 expression in human articular/auricular chondrocytes. It must be noted that the human auricular and articular chondrocytes investigated were derived from different donor

individuals. The proliferation rate is very important to acquire sufficient cells for a later 3D-cartilage construct preparation.

Supporting the study of van Osch *et al* (18) we found a higher proliferative capacity in porcine auricular compared with articular chondrocytes. Corresponding with the results of Malicev *et al* (14) elastin mRNA was expressed on a significantly higher level in auricular chondrocytes compared with articular chondrocytes but was also detectable in articular chondrocytes. Despite lubricin gene expression being higher in articular chondrocytes, it was also evident in auricular chondrocytes. Interestingly, Candrian *et al* (15) reported that lubricin expression could be induced by mechanostimulation in nasoseptal chondrocytes.

Moreover, Zhang and Spector (32) demonstrated lubricin expression also in 3D constructs seeded with auricular chondrocytes. Additionally, Johnson *et al* (16) indicated that heterotopic chondrocytes adhere on hyaline cartilage slices. These facts altogether encourage the use of heterotopic chondrocytes for articular cartilage repair suggesting some plasticity and possibly transdifferentiation potential of heterotopic chondrocytes, which might be influenced and further guided by the unique local biochemical and biomechanical conditions *in vivo*. However, co-cultures with both heterotopic chondrocyte species have not been described yet and should be further studied as an *in vitro* model for heterotopic cartilage repair.

In conclusion, articular and auricular chondrocytes can easily be co-cultured and survive in a mixed culture as shown by cell tracking co-culture experiments. They produce a similar type II collagen-containing extracellular cartilage matrix in 3D alginate culture as shown by confocal laser scanning micro-scopy. A partial adjustment of the ECM expression levels of both chondrocyte species was evident, suggesting a paracrinic interplay between heterotopic chondrocytes in co-cultures which requires further investigation. Based on these results, cartilage formation by co-cultured heterotopic chondrocytes in biomaterial-guided 3D cultures *in vitro* and *in vivo* followed by an analysis of biomechanical properties of the co-cultured chondrocytes would be mandatory.

Acknowledgements

We thank Hannah Gough for her support. This study was supported by a Kick-Off Grant from the Berlin Brandenburger Centrum of Regenerative Therapies by the Rahel Hirsh program of the Charité Medical School, Berlin, Germany, and by the Sonnenfeld Foundation, Berlin, Germany.

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