

Long-term effects of chondrospheres on cartilage lesions in an autologous chondrocyte implantation model as investigated in the SCID mouse model

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Abstract. Microtraumata often lead to articular cartilage lesions. Due to the bradytrophic character of hyaline cartilage, these lesions are hardly repaired by the organism. Autologous chondrocyte implantation (ACI) was established for restoring isolated structural cartilage defects in knee joints. However, results are not always convincing. Human chondrocytes from patients undergoing total knee arthroplasty were cultured in monolayer followed by condensing single chondrocytes to spheroids (chondrospheres®). The integrative capacity of chondrospheres was examined by implanting them into lesions in human articular cartilage specimens and co-implanting them into SCID mice. Mice were sacrificed after 4, 12 and 24 weeks. HE and safranin O staining as well as immunohistochemistry using anti-S100, anti-collagen I and II antibodies were performed and analyzed using semiquantitative scores. Integration of the chondrospheres with the (native) cartilage matrix was analyzed by determining the percentage of adhering surface. With respect to long-term stability, the chondrocytes within chondrospheres showed a typical chondrocytic morphology. Immunohistochemically, a high collagen II production was detected. Over a time period of 24 weeks, an increasing content of collagen type II, glycosaminoglycans and collagenous fibers were found. Importantly, the newly synthesized cartilaginous matrix integrated continuously with the native cartilage lesion border. In conclusion, the presented data demonstrate that chondrospheres are able to restore and conserve their phenotype for at least 24 weeks under *in vivo*

conditions. Moreover, chondrospheres adhere to full-thickness cartilage defects and appear to produce a cartilaginous extracellular matrix which fuses with native cartilage thus generating an autologous cartilage-like repair tissue.

Introduction

Cartilage lesions can be a consequence of etiologically and pathogenetically different primary and secondary disease entities of the joint. The lack of healing, both partial and full-thickness defects, is due to the insufficient regenerative capability of hyaline cartilage to synthesize cartilage-specific extracellular matrix molecules necessary for integration (1).

Different surgical procedures have been implemented for the treatment of hyaline cartilage defects. These methods are mainly directed towards accessing the subchondral vasculature and bone marrow (2) either by abrasion of the cartilage and subchondral bone plate with powered burrs (3-5), drilling of the subchondral bone (6), or microfracturing (7). However, the fibrocartilage-like reparative tissue that results from these techniques lacks the composition, structure, and mechanical properties of normal cartilage and the long-term clinical outcome is unpredictable (8). Patients treated by these techniques often experience only short-term pain relief but develop progressive osteoarthritic symptoms when the reparative tissue disintegrates (5). Alternatively, various surgical procedures were developed to deliver autologous chondrogenic precursor cells to the cartilage defect in the form of cell suspensions prepared by expanding cells originating from cartilage biopsies (9), precursor cells derived from the periosteum (10), or the perichondrium (11), with the latter eventually undergoing terminal differentiation to chondrocytes *in situ*.

Approaches to 'engineer' cartilage ('tissue engineering') were undertaken using cultivated de-differentiated autologous chondrocytes being implanted into the cartilage defect site (12). Though autologous chondrocyte implantation (ACI) has good clinical results in the long run (13), the questions of a sufficient attachment and integration of these cells in the cartilage lesion and the maintenance of their biomechanical properties for an extended period of time are unresolved. The implanted cells

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are insufficiently characterized with respect to viability, degree of differentiation, morphology, or production of extracellular matrix (14). Furthermore, covering the cartilage defect with a periosteal flap as effected in the original ACI procedure (9) represents a time- and skill-demanding invasive surgical procedure. To improve surgical treatment as a minimally invasive procedure, an *in vitro* engineered autologous chondrocytic tissue (chondrosphere[®]) was developed. In this study, the integration as well as cartilage remodeling and maturation under *in vivo* conditions were analyzed in a model analogous to ACI using the SCID mouse co-implantation model (15).

Materials and methods

Chondrocyte culture and spheroid generation. Articular cartilage was obtained from macroscopically unworn human articular condyles from 5 voluntary patients undergoing total knee arthroplasty after written informed consent. Cartilage (60–100 mg) was minced and digested in a 50 ml Falcon tube using 20–25 U/mg collagenase type II (Biochrome, Germany) at 37°C for 8 h in a gyratory shaker (110 r/minute). Isolated cells were washed and resuspended in culture medium with the addition of pooled human serum from different human volunteers (n=6). No growth factors, cytokines or other supplements were added. Chondrocytes were seeded in culture flasks (Falcon, 75 cm²) and maintained at 37°C in a humidified atmosphere and 5% CO₂. Medium was changed twice per week. After reaching confluence the cells were trypsinized using trypsin-EDTA (PAA-Laboratories GmbH, Germany) and cultured in larger flasks (Falcon, 225 cm²). For generation of spheroids, 2x10⁵ cells of third passage chondrocytes were seeded in hydrogel-coated 96-well-plates (Fig. 1). After 1 week, four to twelve single spheroids were transferred into one well allowing coalescence of spheroids. After 2 weeks, fused spheroids were used for subcutaneous transplantation. Single spheroids were cultured for 3 weeks before transplantation.

Severe combined immunodeficiency (SCID) mouse model. The integrative capacity of the chondrospheres was investigated in the severe combined immunodeficiency (SCID) mouse co-implantation model (15). First, using a sharp spoon, small, nearly full-thickness, lesions of a 5 mm diameter and 3–4 mm depth were generated in fresh, healthy appearing human cartilage specimens which were obtained during total knee arthroplasty from 5 patients suffering osteoarthritis at the Department of Orthopedics, University of Regensburg, Germany. All the patients gave written, informed consent to the study. Several chondrospheres (5–12, according to the maximum number of chondrospheres available) were put into the defects, and these constructs (in total 37) were implanted subcutaneously into SCID mice. Work, with respect to SCID mice, was approved by the local ethics committee of the Medical faculty of the University of Regensburg. The 14-week old SCID mice were obtained from a germ-free breeding colony (Charles River, Sulzfeld, Germany) and examined for macroscopic anomalies before and during surgery as well as for macro- and histopathologic abnormalities after sacrifice. The mice were anesthetized and a small incision was made in the left and right flank. After preparation of an appropriate



Figure 1. Chondrosphere[®] spheroids were generated by culturing 2x10⁵ cells of third passage chondrocytes in hydrogel-coated 96-well plates.

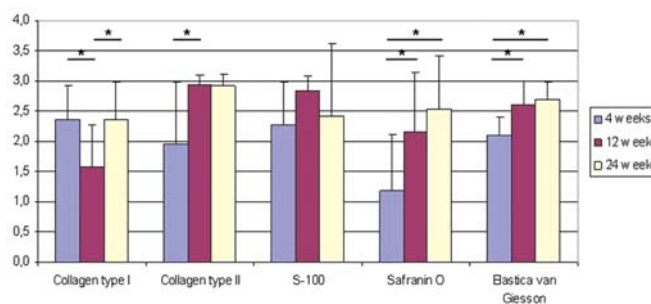


Figure 2. Production of extracellular matrix proteins by chondrospheres detected by immunohistochemistry using antibodies against collagen types I, II and S100, as well as histological staining with safranin O and Elastic van Gieson. The numbers of the experiments were 7, 10 and 8 for weeks 4, 12 and 24, respectively. *p<0.05.

cavum in the subcutaneous tissue, the constructs were implanted, and subsequently, the skin was sutured. After 4, 12 and 24 weeks, the mice were sacrificed and the implants removed. After decalcification in a saturated EDTA solution, the tissue was dehydrated, fixed in 4% buffered formalin and embedded in paraffin according to standard procedures.

Immunohistology and immunohistochemistry. Serial paraffin sections (3 to 5 µm thickness) were cut through the center of the cartilage defect, and were mounted on 'Superfrost Plus' slides, heated for 20 min at 72°C, deparaffinized and re-hydrated. Following antigen retrieval in a citrate buffer at pH 7.3 in a microwave for 30 min at 240 W, the slides were rinsed and endogenous peroxidase activity was blocked using methanolic peroxide. The slides were rinsed again and primary monoclonal antibodies against collagen I, II (MP Biomedicals, LLC, Aurora, Ohio, USA; both dilutions 10 µg/ml) and S100 (Dako, Hamburg, Germany, dilution 1:100) were applied. Slides for S100 immunohistochemical staining were incubated using a Ventana machine (Ventana Medical Systems, basic DAB detection kit, Ventana Medical Systems Inc., USA). The streptavidin-biotin-peroxidase method was used for visualization. The immunohistochemical staining procedures for collagen I and II were performed manually. After incubation with the primary antibody, the slides were rinsed and the biotinylated secondary antibody was applied at a dilution of 1:200. The streptavidin-biotin-peroxidase method was used for visualization resulting in brown staining of

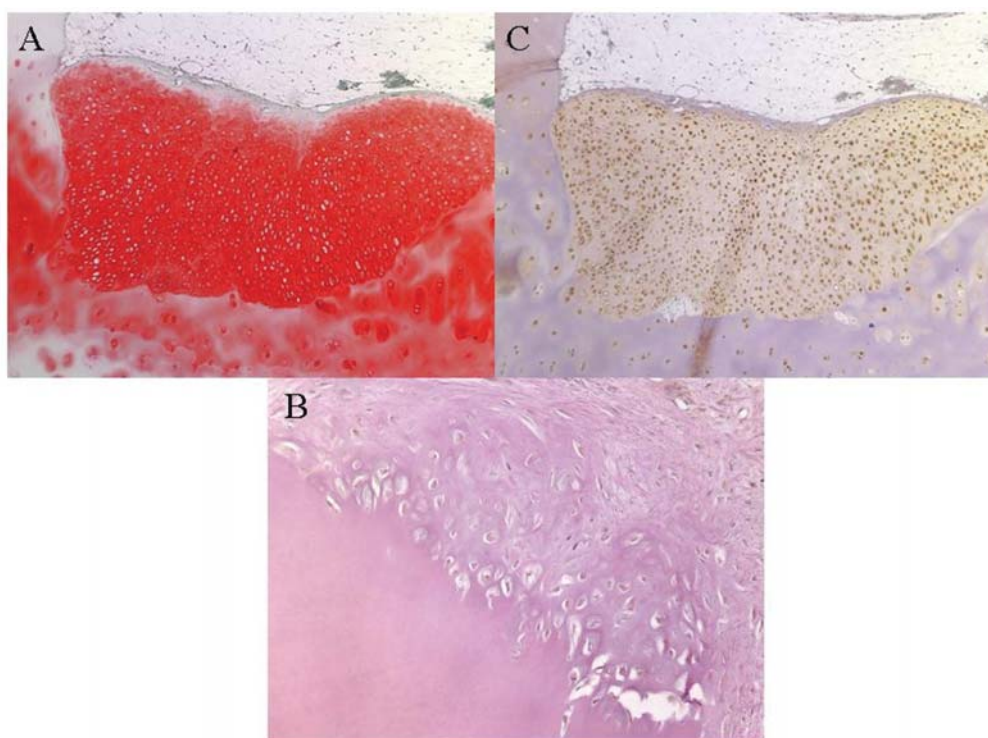


Figure 3. (A) Histological staining with safranin O, week 12 and (B) Elastic van Giesson, week 4 as well as (C) immunohistochemistry using antibodies against S100, week 12 in paraffin sections of chondrospheres implanted in cartilage lesions. Original magnifications A and C, x50; B, x200.

extracellular matrix, counterstaining was applied using hematoxylin. For negative controls, isotype-matched control antibodies were employed. Safranin O and Elastic van Giesson staining were performed according to standard procedures.

Histological evaluation. The sections were evaluated by a semiquantitative score. In respect to reactivity with S100, the following grading was applied, 0, expression in 0-25% of cells; 1, 26-50%; 2, 51-75%; 3, 76-100%. In analogy, staining with safranin O and Elastic van Giesson as well as immunohistochemistry for collagen types I and II was scored semiquantitatively, 0, no; 1, weak; 2, medium; and 3, strong staining. As internal reference, the strength of the respective staining in the adjacent native cartilage or bone was quantified as 3 points representing the maximum score of expression. Similarly, chondrocyte morphology was evaluated using a score ranging from 0, fibrocytic to 3, chondrocytic appearance. Integration of the chondrospheres with the (native) cartilage matrix was defined as the percentage of the chondrosphere surface adhering to the cartilage lesion borders evaluated in 3 consecutive sections. As such, 100% integration would indicate coverage of the complete surface of the spheroid by the (native) cartilage borders which, however, was not feasible for geometrical reasons. The evaluation was performed by two independent investigators (T.S., J.S.), who were blinded to the point of time at which the mice were sacrificed.

Statistical evaluation. Statistical analysis of histological and immunohistological semiquantitative data was performed using SPSS 13.0. Using the non-parametrical Mann-Whitney U-test p-values <0.05 were regarded as significant.

Results

Histomorphology of constructs after SCID mouse sacrifice. After 4, 12 and 24 weeks, 7/12, 10/13 and 8/12, respectively, constructs comprising chondrospheres within the artificially generated cartilage lesion were evaluated.

Production of hyaline cartilage-specific markers by chondrospheres. Using routine histology, a significant increase in the staining intensity of safranin O as indicator of glycosaminoglycan (GAG) content of the newly synthesized cartilage matrix was shown after 24 weeks when compared to 4 weeks ($p=0.026$). The safranin O staining intensity resembled the intensity of the surrounding hyaline cartilage which served as internal control (Figs. 2 and 3A). The intensity of staining for Elastic van Giesson representing collagenous fibers (regardless of type) started from a higher level than safranin O expression and also increased significantly over time ($p=0.013$ after 24 weeks; Figs. 2 and 3B). S100 expression was detected at a medium level at 4 weeks after implantation and increased after 12 weeks ($p=0.58$). After 24 weeks no further up-regulation of S100 was observed (Figs. 2 and 3C).

Using immunohistochemistry, an intense collagen II deposition was detected after 4 weeks which increased for up to 12 weeks ($p=0.015$), and after 24 weeks collagen type II expression levels were similar to the surrounding cartilage (Figs. 2, 4A and B). Collagen type I expression, on the other hand, was slightly higher after 4 weeks when compared to collagen type II expression (Fig. 4C), however, decreased within 12 weeks ($p=0.029$) to again increase after 24 weeks ($p=0.028$; Figs. 2 and 4D). After 24 weeks collagen type II

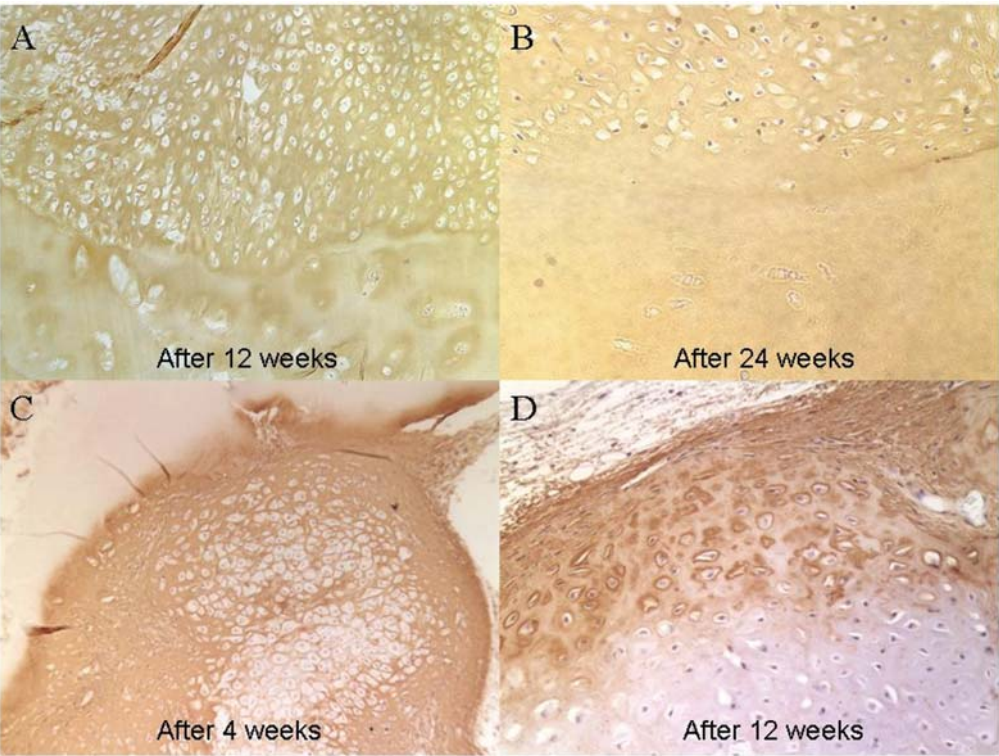


Figure 4. Immunohistochemistry using antibodies against collagen type II after 12 (A) and 24 (B) weeks, as well as against collagen type I after 4 (C) and 12 (D) weeks, respectively, in paraffin sections of chondrospheres implanted in cartilage lesions. Original magnifications A and C, x100; B and D, x200.

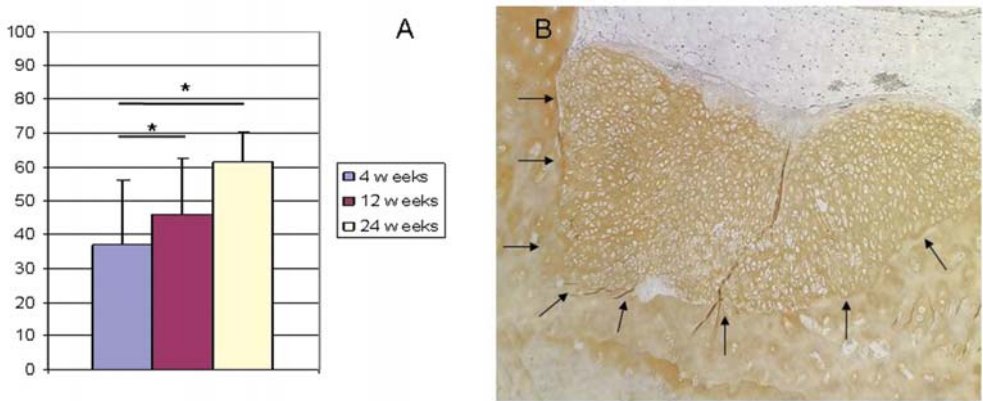


Figure 5. (A) Integration of newly synthesized extracellular matrix produced by chondrospheres with the cartilage lesion border. Integration was defined as the percentage of the chondrosphere surface adhering to the cartilage lesion borders. * $p<0.05$. (B) Integration as visualized by collagen type II production of chondrospheres. Arrows indicate the site of integration. Original magnification x50.

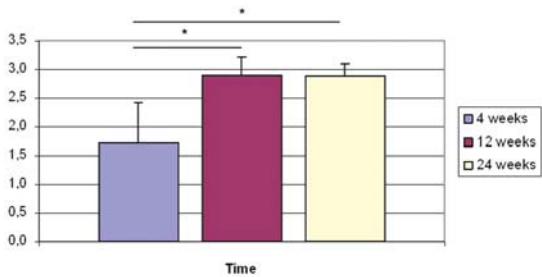


Figure 6. Change of chondrosphere morphology over time. 1, fibroblast-like phenotype; 3, chondrocytic phenotype. * $p<0.05$.

expression was stronger than collagen type I expression reflecting the predominating hyaline cartilage-specific properties of the neo-generated matrix.

Integration of matrix produced by chondrospheres into native cartilage. Integration was defined as the percentage of the surface of the chondrosphere adhering to the cartilaginous substrate. Extensive integration of the cartilaginous matrix newly synthesized by the chondrospheres with the cartilage lesion borders was found after 4 weeks. Importantly, after 12 and 24 weeks, this integrative process continued and after 24 weeks, was significantly higher than after 4 weeks ($p=0.016$; Fig. 5A and B).



SPANDIDOS PUBLICATIONS *cyte morphology within chondrospheres.* After 4 about half of the chondrocytes within transplanted chondrospheres showed a typical chondrocytic morphological appearance (round cell shape with small round nucleus), others exhibited a rather fibrocytic phenotype (elongated cell body, oval nucleus). After 12 and 24 weeks, however, almost all chondrospheres were of typical chondrocytic morphology (Fig. 6).

Discussion

Cartilage lacks the ability to regenerate adequately after structural damage. Moreover, small cartilage lesions initiate a progressive degradation that eventually may lead to osteoarthritis. Autologous chondrocyte implantation (ACI) is widely used to regenerate focal traumatic cartilage lesions (16-18). However, the method is technically demanding due to the necessity of using a chondrocyte suspension and suturing a periosteal graft or a synthetic membrane over the cartilage defect. Therefore, a less complicated operative procedure is desirable for the biological treatment of cartilage lesions. The transplantation of an *in vitro* engineered solid cartilage-like tissue may provide an alternative to conventional ACI. The engineering of chondrocyte spheroids is one way to obtain grafts suited to this purpose (19). However, little is known about the capacity of spheroids to integrate, proliferate, differentiate and maintain a chondrocytic phenotype following their implantation into an articular lesion in humans *in vivo*. In order to study the capacity of chondrocyte spheroids ('chondrospheres'®) to integrate into articular lesions *in vivo*, spheroid loaded human cartilage explants were investigated as an ACI analogon in the SCID mouse co-implantation model. We showed that chondrospheres were able to adhere and integrate into native hyaline cartilage. Proliferation and differentiation were paralleled by the production of cartilage-specific proteins which increased over time, and a preserved chondrocytic phenotype. These results support the finding of a study performed in which the authors used the same approach with chondrospheres which were transplanted into cartilage lesions in horse joints (20). In this study, newly synthesized extracellular matrix produced by ACI-grafted chondrospheres integrated sufficiently into the adjacent cartilage resulting in significantly better macroscopic and microscopic healing of the cartilage lesion when compared to untreated controls.

Since conclusions drawn from animal models are difficult to extrapolate for corresponding human conditions (21), in our study artificial defects were created within human cartilage explants which were used for subsequent chondrosphere adhesion. Co-implants of spheroids and human cartilage specimens were transplanted subcutaneously into the SCID mouse model which was originally designed for *in vivo* evaluation of the invasive properties of human synovial fibroblasts with respect to the co-implanted cartilage in rheumatoid arthritis (15). The approach to investigate the integrative capacity of chondrospheres in the *in vivo* SCID mouse co-implantation model is novel, and was chosen because *in vitro* experiments lack the respective specific, articular environment required for maintenance of chondrocyte metabolism. As an *in vivo* model, it combines the advantage of enabling an

evaluation under *in vivo* conditions for an extended period of time, and at the same time, ease of use. To the best of our knowledge, the model presented here is the first ACI analogon model investigating human chondrocytes under *in vivo* conditions in SCID mice.

Most importantly, over time, the transplanted chondrospheres exhibited a significantly closer attachment and integration into the surrounding native cartilage thus meeting the essential requirement for tissue reconstruction. This integrative process was paralleled by a time-dependent significant increase in cartilage-specific collagen type II protein and GAG. The process of matrix maturation was underscored by the increasing production of S100. Equally, the amount of fibers detected by Elastica van Gieson staining increased over 24 weeks, representing the combined content of collagen types I and II fibers. On the other hand, collagen type I protein, often seen in regenerating fibro-cartilage-like tissue, was expressed in lower amounts as was collagen type II after 24 weeks. These findings are in line with a study published in which monolayer-expanded adult human articular chondrocytes were seeded into disks and were subsequently implanted subcutaneously in nude mice for up to 8 weeks (22). In contrast to freshly seeded scaffolds, the effects described in this study were only achieved after preculturing the chondrocytes in low density and after addition of differentiation medium for 2 weeks. Importantly, in our study, no growth factors, cytokines or other supplements were added to the transplanted chondrospheres. Nevertheless, the transplanted cells developed a hyaline cartilage-specific phenotype over time. Although the effect of 'native' transplanted chondrocytes was not compared with growth factor stimulated chondrocytes in our study, our results are encouraging in view of a study in which improved integration was achieved only by using a mixture of primary and TGF- β producing chondrocytes (23).

Our results, therefore, suggest that a close contact with their (native) extracellular matrix is sufficient for chondrospheres to arrange themselves in three-dimensional cell aggregates in order to synthesize cartilage-specific proteins and matrix components and to deposit them in the intercellular space.

In ACI procedures periosteal grafts are thought as essential both in animal models and humans (24) for different reasons: periosteal flaps are able to cover the transplanted chondrocytes and retain them within the cartilaginous lesion (9). Furthermore, they support the vitality and differentiation of transplanted chondrocytes (20,25). The disadvantages of periosteal flaps are time-consuming technical efforts, the generation of additional cartilaginous lesions by suturing the graft to the cartilage, and the potential for secondary hypertrophy and/or delamination necessitating second-look arthroscopy (16). Moreover, the periosteum exerts potential immunogenic effects (26). Therefore, some attempts were made to replace the periosteum using different resorbable scaffolds (27-33). In our model, the chondrospheres presumably adhered to the cartilage surface border by surface tension forces. Moreover, very good integration results were demonstrated.

The integrative capacity of transplanted chondrospheres was accompanied by the development of an initially more fibroblast-like appearance to a predominating chondrocytic phenotype. This finding is consistent with observations showing a re-differentiation of de-differentiated chondrocytes in high-

density cultures after culture in monolayers (34) and provides evidence for the chondrogenic potential of chondrospheres.

One limiting factor when comparing our results with ACI in animal joint models is that the influence of mechanical load and kinetic effects on chondrocytes and cartilage in a physiological joint, which are essential for the maintenance of the physiological properties of chondrocytes (35), could not be evaluated. Nevertheless, cartilage-specific matrix proteins such as collagen type II were produced by the chondrospheres in high amounts despite the lack of physiological conditions. Further investigations will have to focus on biomechanical properties of chondrospheres after transplantation as seen under physiological conditions.

In conclusion, these data suggest that human chondrocytes cultured as chondrospheres are able to restore and maintain their phenotype for at least 24 weeks under *in vivo* conditions in an ACI analogon model. Moreover, chondrospheres adhering to and integrating with superficial cartilage defect surfaces show remarkable remodeling capacity *in vivo* as shown by an ongoing hyaline-specific maturation of chondrosphere matrix potentially leading to cartilage repair.

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