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Functional cartilage repair capacity of de-differentiated, chondrocyte- and mesenchymal stem cell-laden hydrogels *in vitro*

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SUMMARY

Objective: The long-term performance of cell-seeded matrix-based cartilage constructs depends on (1) the development of sufficient biomechanical properties, and (2) lateral integration with host tissues, both of which require cartilage-specific matrix deposition within the scaffold. In this study, we have examined the potential of tissue-engineered cartilage analogs developed using different cell types, i.e., mesenchymal stem cells (MSCs) vs chondrocytes and de-differentiated chondrocytes, in an established "construct in cartilage ring" model.

Design: Cell-laden constructs of differentiated chondrocytes, de-differentiated chondrocytes after two, five or eight population doublings, and MSCs were either implanted into a native cartilage ring immediately after fabrication (immature group) or pre-treated for 21 days in a transforming growth factor-β3 (TGF-β3) containing medium prior to implantation. After additional culture for 28 days in a serum-free, chemically defined medium, the extent of lateral integration, and biochemical and biomechanical characteristics of the implants as hybrid constructs were assessed.

Results: The quality of integration, the amount of accumulated cartilage-specific matrix components and associated biomechanical properties were found to be highest when using differentiated chondrocytes. De-differentiation of chondrocytes negatively impacted the properties of the implants, as even two population doublings of the chondrocytes in culture significantly lowered cartilage repair capacity. In contrast, MSCs showed chondrogenic differentiation with TGF-β3 pre-treatment and superior integrational behavior.

Conclusions: Chondrocyte expansion and de-differentiation impaired the cell response, resulting in inferior cartilage repair *in vitro*. With TGF-β3 pre-treatment, MSCs were able to undergo sustained chondrogenic differentiation and exhibited superior matrix deposition and integration compared to de-differentiated chondrocytes.

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Introduction

The surgical goal of repairing focal articular cartilage lesions is to achieve a functional and viable joint surface in the long-term and to prevent progression into an osteoarthritic joint state[1,2]. To date, matrix-based autologous chondrocyte implantation marks the latest generation of cartilage repair strategies for the treatment of isolated articular cartilage defects[3,4]. While promising clinical

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results have been published, *restitutio ad integrum* has not been achieved by any of these methods[5,6]. In addition to the fact that the newly synthesized tissue largely consists of fibrocartilage and exhibits inferior mechanical properties[7–9], the utilization of autologous chondrocytes entails additional disadvantages. Specifically, limited availability of healthy chondrocytes and chondrocyte dedifferentiation upon culture expansion[10,11] underscore the need for an alternative cell source.

By virtue of their easy availability from various tissues[12–15], extensive self-renewal capacity and chondrogenic differentiation potential, adult mesenchymal stem cells (MSCs) have been considered a promising cell source[3,16–19]. A large number of reports over the last decade have demonstrated the chondrogenic differentiation of MSCs employing various biomaterials, and the pro-chondrogenic, anabolic effects of growth factors, such as transforming growth factor-β3 (TGF-β3), strongly suggesting the applicability of MSCs for cartilage repair[3,18,20–22].

In principle, successful matrix-based cartilage repair *in vivo* relies on sustained cartilage-specific matrix deposition within the cell-laden scaffold, the development of sufficient mechanical properties and, in particular, integration of the developing neocartilage into the surrounding native cartilage[18,23,24]. Inferior interface stability impairs mechanical integrity of the restored joint surface, and may lead to pathological load distribution in the joint and long-term failure of the cartilage repair procedure. The interaction of the opposing tissues at the interface site, which is dependent on appropriate cellular activity and matrix deposition, determines the fate of a functional junction[1,2,25].

In cartilage tissue engineering, it has been shown that matrix deposition and mechanical properties of the engineered constructs depend significantly on the utilized cell type, cell-to-volume ratio, and culture conditions[26–28]. Unfortunately, expansion of chondrocytes in monolayer culture is accompanied by their dedifferentiation and loss of cartilage-specific gene expression[10,11]. In practice, de-differentiated chondrocytes are often processed and used for re-differentiation and subsequent deposition within a matrix to simulate a controlled pro-chondrogenic, three-dimensional environment prior to implantation[29,30]. In this manner, tissue-engineered cartilage analogs matured *ex vivo* have been shown to show enhanced cartilage repair *in vivo*, with increased amount of newly synthesized hyaline cartilage[30–32].

Compared to *in vivo* animal cartilage repair models that are time-consuming, expensive, and more difficult to control, *in vitro* cartilage repair models may be utilized to evaluate certain aspects of joint repair and to optimize specific environmental variables[24,33,34]. The main aim of this study was to directly compare the *in vitro* cartilage repair potential of various cell types, as applicable in surgical cartilage repair procedures, in terms of their interaction with native articular cartilage and the development of a functional interface and matrix deposition. In addition, we sought to address the question of whether maturation of the cell-laden construct prior to implantation, using pro-chondrogenic growth factor treatment, influences subsequent cartilage–implant interactions.

Our experimental approach was based on an established bovine "implant in cartilage ring" model[24] to assess functional integration and biochemical/-mechanical properties of cell-laden agarose constructs *in vitro*, utilizing differentiated chondrocytes, de-differentiated chondrocytes after two, five or eight population doublings in monolayer-culture, and MSCs. In the first study, freshly prepared constructs of the stated cell types were applied and cultured inside the native cartilage ring for 28 days in a serum-free, chemically defined medium (CM−). In a second experiment, corresponding samples were first matured in CM− supplemented with 10 ng/mL TGF-β3, designated as CM+, for 21 days and implanted afterwards.

Materials and methods

Chondrocyte and MSC isolation

MSCs were obtained from femoral and tibial bone marrow of 6–8 month old calves (Fresh Farms Beef, Rutland, VT) as previously described[26]. Bone marrow was removed with a spatula and mixed with an equal volume of Dulbecco's Modified Eagle's Medium (DMEM, Gibco BRL) supplemented with antibiotics (50 μg/mL streptomycin, 50 IU/mL penicillin, Gibco BRL) and 300 U/mL heparin (Sigma). Fat and soft tissue parts were removed by centrifugation (5 min, 300 ×*g*). The resulting cell suspension was plated out onto tissue culture plates (⌀ 15 cm) and cell adherence was allowed for 48 h. After washing the cultures with phosphate buffered saline (PBS, Gibco BRL), cells were kept in Expansion Medium (EM; high glucose DMEM + 1× antibiotics + 10% fetal bovine serum (FBS)) until reaching 70–80% confluence. Further culturing was performed at a 1:3 expansion ratio, with cultures of passage 3 utilized for these studies.

Articular cartilage was harvested from the carpometacarpal joints of the same group of animals. Cartilage pieces were rinsed with DMEM containing 2× antibiotics, and kept in a tissue culture incubator (37°C, 5% CO_2) overnight. Afterwards, cartilage pieces were digested sequentially with pronase and collagenase, as described previously[26]. The obtained chondrocyte suspension was filtered (40 μm cell strainer; BD Falcon, Bedford, MA), pelleted (300 ×*g*, 5 min), resuspended in DMEM, and viable cells counted. These cells were used for the fabrication of agarose implants as either differentiated cells or sub-cultured in monolayer at a seeding density of 1.0×10^4 cells/cm^2 and maintained for two, five or eight population doublings, as calculated by microscopic determination of the cell number, to obtain de-differentiated cell populations. After 5–6 population doublings chondrocytes reached 70% confluency and cells were passaged in a 1:3 ratio after trypsin detachment to obtain cells with eight population doublings.

All cell-laden hydrogels and cartilage rings were fabricated by pooling cells from the same preparation derived from at least six animals, two preparations were performed in total.

Cell-laden agarose implant preparation, cartilage construct assembly and culture

Differentiated chondrocytes (Ch0), de-differentiated chondrocytes after two (Ch2), five (Ch5) or eight (Ch8) population doublings or bone marrow derived MSCs of passage 3 were used to prepare cell-seeded agarose implants as follows. A suspension of each of the cell populations (2.0×10^7 cells/mL) was mixed homogeneously (1:1) with a sterile agarose solution (42°C, 4% w/v in PBS, type VII, Sigma) to obtain a final concentration of 1.0×10^7 cells/mL. The cell suspension was then cast between two parallel glass plates and gelatinized for 20 min at room temperature[30], and cylindrical, cell-seeded implants (Ø 4.1 or 6.0 × 2.25 mm thickness) were cored out using a custom-modified punch (Ø 4.1 mm) or a dermal punch (Ø 6.0 mm, Miltex, York, PA), respectively.

To obtain native cartilage rings, full-thickness cartilage cylinders (Ø 8 mm) were harvested from the femoral condyles of calves. The superficial and deep layer was removed to obtain parallel surfaces (2.25 mm thickness) and a central "defect" (4 mm) was cored out. Native cartilage rings were incubated for 24 h (37°C, 5% CO_2) in a serum-free, chemically defined medium containing DMEM, 50 μg/mL ascorbate, 0.1 μM dexamethasone, 40 μg/mL L-proline, 100 μg/mL sodium pyruvate, 50 μg/mL ITS-plus and antibiotics, designated as CM−, prior to implantation of cell-laden agarose constructs.

In the first set of experiments, cell-laden agarose constructs of each cell type (Ch0/2/5/8 and MSC) were press-fitted into native cartilage rings immediately after fabrication and cultured as hybrid construct for 28 days in CM− after implantation. Lateral integration, biochemical composition, biomechanical properties, and histological appearance of the interface region were assessed after 4 weeks. In a second set of experiments, corresponding cell-laden agarose constructs (Ø 6.0 mm × 2.25 mm thickness) of the same cell preparations as in experiment 1 were first pre-treated for 21 days in CM− supplemented with 10 ng/mL TGF-β3, designated as CM+, prior to implantation. After pre-treatment, the cell-laden agarose constructs (Ø 6 mm) were rinsed with DMEM, trimmed to a diameter of 4.1 mm and implanted by press-fitting into freshly prepared cartilage rings. Further incubation of the hybrid constructs and data assessment was performed as described above. All cultures were maintained in a humidified atmosphere (37°C, 5% CO_2) and medium was changed twice weekly.

Mechanical testing

The equilibrium compressive Young's modulus (Eγ) of the implanted cell-laden agarose constructs was measured using a custom-made apparatus as previously described[26,35]. The implants were removed from the native cartilage ring, their thickness and diameter measured, placed in PBS and tested in unconfined compression. A tare load of 0.02 N was applied and stress relaxation tests were carried out with a compressive deformation of 1 μm/s to 10%, after which samples were allowed to relax to equilibrium (1200 s).

Interface strength

The adhesive strength of the interface between the outer native cartilage ring and the inner engineered tissue was evaluated 28 days after implantation by mechanical push-out test using a custom-made device mounted on an ELF3200 system (EnduraTec, Minnetonka, MN). The hybrid construct was placed on a confined metal ring with a center hole (Ø 5 mm). The implant was then pushed out with a metal plunger (Ø 3.5 mm), while the applied force was recorded real time. Failure stress (kPa) was computed from the applied force that caused total disruption of the interface and the area of integration (height times circumference)[36,37].

Biochemical analyses

The contents of sulfated glycosaminoglycan (sGAG), DNA, and bulk collagen (*ortho*-hydroxyproline, OHP) were analyzed to assess the biochemical composition of the implanted cell-laden constructs. All samples were first digested for 24 h in papain at 60°C. Aliquots were analyzed for sGAG content using the 1,9-dimethylmethylene blue dye-binding assay[33], for DNA content using the PicoGreen dsDNA Quantification kit (Molecular Probes, Eugene, OR), and for OHP content (after acid hydrolysis) by reaction with chloramine T and dimethylaminobenzaldehyde[38]. Conversion of OHP content to collagen content was done using a 1:10 ratio of OHP:collagen[39]. Each constituent (sGAG, DNA, OHP) was calculated as the total amount (wet weight) per implant, DNA content was expressed as relative value compared to the day of implant fabrication.

Histology and immunochemistry

Hybrid constructs were rinsed with PBS, fixed in 4% buffered paraformaldehyde, dehydrated in a graded series of ethanol, paraffin-embedded (Tissue Prep, Fisher Scientific, Hampton, NH), and sectioned at 8 μm thickness. Sections were stained with Alcian Blue (pH 1.0) to detect sGAG.

For immunohistochemistry, sections were first digested with 1 mg/ml hyaluronidase (Sigma) in 10 mM Tris–HCl (pH 7.5) for 30 min at 37°C. The sections were then incubated overnight at room temperature with the antibody to collagen type II (Developmental Studies Hybridoma Bank, Iowa City, IA) in Tris-buffered saline containing 0.1% bovine serum albumin. Immunostaining was detected histochemically using the streptavidin-peroxidase Histostain SP Kit for DAB (Zymed Laboratories, San Francisco), and imaged with a color charge coupled device camera and an inverted microscope.

Gene expression analysis by real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA (1 μg) was reverse transcribed using the MultiScribe reverse transcriptase (Applied Biosystems, Foster City, CA). Real-time PCR reactions were performed as previously described[36]. Gene-specific primers (forward and reverse) were designed based on GenBank cDNA sequences (primer sequences available on request). Specific transcript levels were normalized by comparison to that of GAPDH, and expressed as the fold difference (using the formula $2^{-\Delta\Delta Ct}$) between levels at implant fabrication.

Statistical analyses

All data are reported as the arithmetic mean value (95% confidence interval lower limit, upper limit) of 10–15 samples per group, measure, and time point. Analysis of variance was performed with cell source (Ch0/2/5/8 or MSC) and pre-treatment (none or 21 days CM+) as independent variables and Young's modulus, failure stress, sGAG/implant, DNA/implant, and bulk collagen/implant as dependent variables. Statistical significance was determined by Student's *t* test with significance at $P < 0.05$.

Results

Chondrocyte de-differentiation in monolayer culture

Chondrocytes seeded at a density of 1.0×10^4 cells/cm^2 in cell culture flask showed plastic adherence after 24 h. De-differentiation was monitored by Col2 and Col1 gene expression level and compared to those of freshly isolated chondrocytes (Ch0). After two population doublings, chondrocytes revealed no significant difference in Col2 expression (1.14-fold), whereas the level of Col1 expression increased 6.06-fold. With increasing number of population doublings, decrease in Col2 and increase in Col1 expression levels became more pronounced. Finally, after eight population doublings, Col2 level dropped to 0.12-fold, while a 13.6-fold increase in Col1 expression was seen, compared to freshly isolated chondrocytes (data not shown).

Maturation of the cell-laden agarose constructs

TGF-β3 pre-treatment of the different agarose disks in CM+ for 21 days prior to implantation led to increased ECM deposition within the scaffolds [Fig. 1(A, B)]. Specifically, differentiated chondrocytes exhibited significant higher sGAG and bulk collagen deposition within the agarose disks then any other cell type. As a function of their population doublings, de-differentiated chondrocytes exhibited impaired matrix deposition. Interestingly, MSC-laden constructs displayed significantly higher sGAG and bulk collagen contents after the pre-culture period in CM+ than chondrocytes after two population doublings [Fig. 1(A, B)].

Accompanying the CM+ pre-culture induced accumulation of ECM, which depended on the cell type employed for construct assembly, major differences in the development of mechanical properties were also apparent [Fig. 1(C)]. Ch0-laden implants ($E\gamma = 38.76$ kPa (46.15, 31.35 kPa)) exhibited the highest increase in the equilibrium compressive Young's modulus. MSC-laden (22.35 kPa (28.07, 16.62 kPa)) constructs showed significantly lower mechanical ($P = 0.019$) properties, but became significantly stiffer ($P = 0.004$) than Ch5- (4.95 kPa (6.82, 3.08 kPa)) and ($P = 0.003$) Ch8- (4.07 kPa (5.68, 2.46 kPa)) laden implants [Fig. 1(C)]. On the other hand, no significant difference ($P = 0.295$) in mechanical properties was obvious comparing MSC-laden constructs and constructs fabricated with de-differentiated chondrocytes after two population doublings (19.86 kPa (24.50, 15.21 kPa); Fig. 1(C)).

DNA content increased slightly in all cell-laden constructs, but was not significant higher compared to the day of fabrication (D1; Fig. 1(D)).

Biochemical properties of engineered cartilage and the effect of de-differentiation and pre-treatment

The different cell-laden agarose implants were press-fitted into native cartilage rings either immediately after fabrication (immature) or pre-cultured for 21 days in CM+ (mature) and kept as hybrid constructs in CM− for 28 days.

For the immature implants, by day 28 after implantation of culture in CM−, sGAG and bulk collagen contents (mg/implant) significantly increased for all cell types used [Fig. 2(A, C)], compared to the day of implantation (D1). However, significant differences were obvious among the different cell types utilized. Differentiated chondrocytes revealed the highest amount of cartilage-specific ECM accumulation, compared to de-differentiated chondrocytes (Ch2/5/8) and MSCs [Fig. 2(A, C)], with MSC-laden constructs exhibiting no significant difference to those seeded with Ch2.

For the mature implants, cultured in CM− for 28 days as hybrid constructs, analysis of the accumulated cartilage-specific ECM molecules revealed significant differences among the cell types used [Fig. 2(B, D)]. Again, differentiated chondrocytes produced the highest amounts of sGAG and bulk collagen. Interestingly, even after removal of TGF-β3 supplementation, MSCs showed prolonged ECM production and their sGAG and bulk collagen contents were significantly higher than those of Ch2, Ch5, or Ch8 implants [Fig. 2(B, D)].

Mechanical properties of engineered cartilage and the effect of de-differentiation and pre-treatment

In the immature implants, the mechanical properties after 28 days in CM− culture medium as hybrid constructs closely correlated with the amount of accumulated ECM components. Thus, the equilibrium compressive Youngs's modulus (E_Y) of Ch0-laden implants exhibited the highest increase (18.4 kPa (10.06, 16.74 kPa)), 2.5-fold stiffer than those of Ch2- (7.87 kPa (8.79, 6.94 kPa)) and MSC- (7.48 kPa (8.55, 6.4 kPa)) laden implants [Fig. 3(A)]. Ch5- (2.78 kPa (3.51, 2.05 kPa)) and Ch8- (1.96 kPa (2.61, 1.32 kPa)) laden agarose implants showed no significant increase ($P = 0.151$ and $P = 0.363$ respectively) in mechanical stability above the baseline of freshly prepared disks (2.15 kPa (2.72, 1.57 kPa)) after implantation and culture.

For TGF-β3 pre-treated mature implants, sustained matrix production during subsequent culture as hybrid constructs in CM− medium led to further increase of mechanical properties [Fig. 3(B)]. The highest equilibrium Young's modulus was detected in Ch0-laden implants (60.84 kPa (68.55, 53.12 kPa)). Interestingly, even after removal of TGF-β3 during subsequent culture,

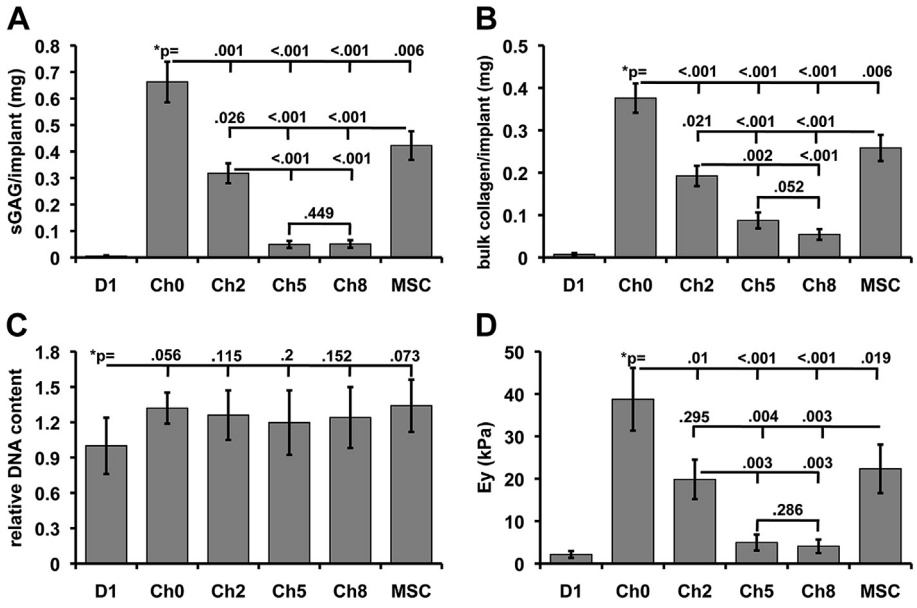

Fig. 1. Properties of cell-laden agarose constructs after pre-treatment in CM+ for 28 days. (A) sGAG content (mg/implant) and (B) bulk collagen content (mg/implant) of all cell-laden constructs were significantly increased compared to Day 1 (D1) freshly prepared constructs in a manner dependent on the cell type used. The highest accumulation of matrix components was found in differentiated chondrocytes (Ch0), while de-differentiated chondrocytes after two (Ch2), five (Ch5) and eight (Ch8) population doublings, as well as MSCs (MSC) showed significantly lower amounts of sGAG and bulk collagen. (C) Equilibrium Young's modulus ($E\gamma$; kPa). The mechanical property of the constructs appeared to be related to the accumulation of ECM after 28 days of CM+ pre-culture. Thus, differentiated chondrocyte-laden agarose disks showed the highest compressive Young's modulus. However, the higher ECM accumulation in MSC-laden constructs was not correlated with significant difference in mechanical properties compared to Ch2-laden constructs. (D) Relative DNA content compared to Day 1 (D1). All cell-laden agarose disks showed a slight but insignificant increase in cell number after the 28-day culture period. *p = *displays P-value vs D1.* Experiments represent culture replicates originating from pooled cells. Values are mean with 95% confidence interval upper limit and lower limit ($n = 10$).

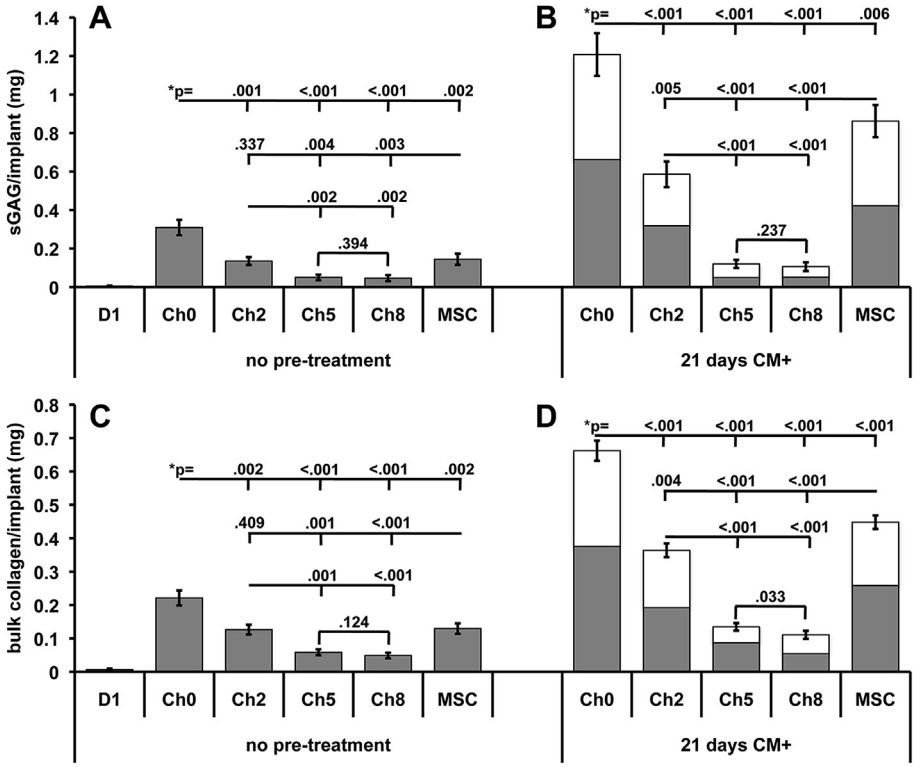

Fig. 2. sGAG and collagen contents of cell-laden agarose constructs after 28 days of culture in CM− as hybrid constructs. (A, B) sGAG content (mg/implant) and (C, D) bulk collagen content (mg/implant). (A, C) Immature constructs and (B, D) mature constructs. Chondrocyte expansion culture prior to construct fabrication led to a significant depletion in matrix accumulation for both immature and pre-treated implants, depending on the number of population doublings. Maturation of MSC-laden agarose constructs resulted in sustained matrix deposition that prolonged even after implantation and removal of TGF-β3. For each data bar, the amounts of (B) sGAG and (C) bulk collagen deposited during the 21-day pre-culture in CM+ is indicated in gray, and the amount synthesized during hybrid culture part in white. *p = *displays P-value* vs different cell type. Experiments represent culture replicates originating from pooled cells. Values are mean with 95% confidence interval upper limit and lower limit (n = 10–15).

MSC-laden implants (39.38 kPa (44.56, 34.19 kPa)) exhibited significantly higher (P = 0.027, P < 0.001 and P < 0.001 respectively) mechanical properties compared to those fabricated with Ch2 (29.46 kPa (33.09, 25.84 kPa)), Ch5 (6.79 kPa (7.63, 5.95 kPa)), or Ch8 (5.09 kPa (5.7, 4.48 kPa); Fig. 2(B)). In comparison, the surrounding native bovine cartilage exhibited a Young's modulus of 500–800 kPa.

Adhesive strength of the interface and the effect of de-differentiation and pre-treatment

Push-out tests after 28 days of culture as hybrid construct revealed the development of an adhesive interface between all implants and the native cartilage, but major differences were detected for both the immature and pre-treated groups [Fig. 4(A,

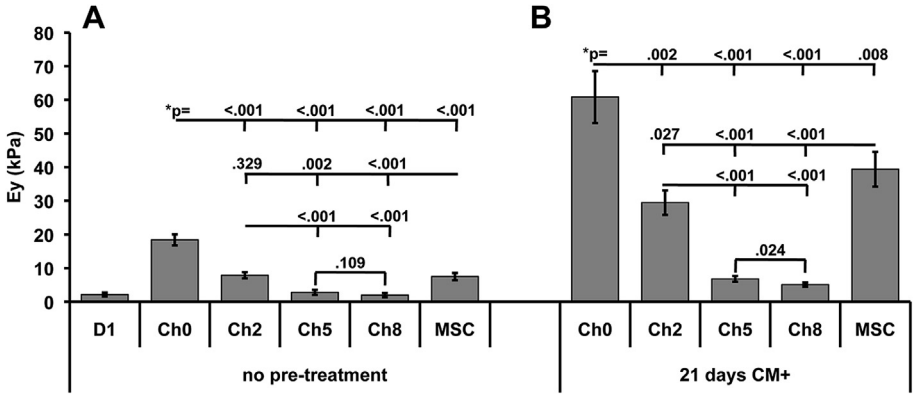

Fig. 3. Mechanical properties of (A) immature and (B) mature, TGF-β3 pre-treated cell-laden constructs 28 days after implantation into the native cartilage rings. The impairment in matrix synthesis described previously for de-differentiated chondrocytes after two (Ch2), five (Ch5) and eight (Ch8) population doublings was reflected by significantly lower mechanical properties compared to differentiated chondrocytes (Ch0) in the (B) mature as well as in the (A) immature group. In comparison to de-differentiated chondrocytes, MSC-laden constructs (MSC) exhibited superior mechanical properties only after pre-treatment with CM+. Differentiated chondrocytes (Ch0) outperformed all other cell types within the corresponding group. *p = *displays P-value* vs different cell type. Experiments represent culture replicates originating from pooled cells. Values are mean with 95% confidence interval upper limit and lower limit (n = 10–15).

B)]. In general, implants prepared using differentiated chondrocytes exhibited the strongest integration into the native cartilage rings in both groups, irrespective of TGF-β3 pre-treatment. Specifically, mature Ch0 implants showed significantly higher ($P < 0.001$) interface strength than immature Ch0 implants (82.67 kPa (90.32, 75.03 kPa) vs 25.5 kPa (28.81, 22.19 kPa)). In the immature group, the failure stress of Ch2 implants (12.9 kPa (14.97, 10.83 kPa)) was similar ($P = 0.16$) to of MSC implants (11.01 kPa (13.02, 9.01 kPa)). However, Ch5- (5.45 kPa (7.23, 3.67 kPa)) and Ch8-implants (4.94 kPa (6.02, 3.86 kPa)) integrated significantly less into the native cartilage [Fig. 4(A)]. Mature implants fabricated with Ch0, Ch2, Ch5 or MSCs showed significantly higher interface strength (82.67 kPa (90.32, 75.03 kPa, $P < 0.001$); 36.9 kPa (41.64, 32.15 kPa, $P < 0.001$); 12.04 kPa (15.99, 8.1 kPa, $P = 0.029$); and 51.88 kPa (58.3, 45.46 kPa, $P < 0.001$) respectively) compared to the corresponding immature agarose implants after 28 days in hybrid culture [Fig. 4(A, B)]. Notably, the integration of pre-treated MSC-laden implants was significantly stronger compared to that measured for de-differentiated chondrocytes after two, five or eight population doublings ($P = 0.016$, $P < 0.001$ and $P < 0.001$ respectively) in the mature implant group. In contrast, for Ch8 implants, TGF-β3 pre-treatment failed to increase interface strength significantly ($P = 0.064$) 28 days after implantation (mature Ch8 implants, 8.56 kPa (11.62, 5.48 kPa); immature Ch8 implants, 4.94 kPa (6.02, 3.86 kPa)).

Immunohistological analysis of the interface

The histological features of the interface region were consistent with ECM biochemical properties (Fig. 5). Collagen type II and alcian blue positive staining decreased with increased population doublings of chondrocytes. On the other hand, while comparable positive collagen type II staining was seen in TGF-β3 pre-treated Ch2 and MSC implants 28 days after implantation, alcian blue staining was stronger in the latter. Ch5 implants, even after TGF-β3 pre-treatment, exhibited very low staining intensity for cartilage-specific ECM and less visible cells. In addition, mature Ch5 implants revealed a more pronounced alcian blue staining in the inter-territorial region compared to the intensive pericellular staining of implants with Ch0/2 or MSCs (Fig. 5).

Discussion

Our data clearly demonstrate that monolayer expansion of chondrocytes severely impairs their maturation and the development of a sufficient interface between native articular cartilage and the implant. Reduced biosynthetic activity of the chondrocytes, and inferior mechanical properties, and tissue integration were evident after two population doublings. Our results are supported by the work of Darling and Athanasiou[40], showing rapid phenotypic changes in expanded goat articular chondrocytes. These changes in chondrocyte specific gene expression are not reversible, even in three-dimensional alginate culture[40]. In this assay system, the presence of living native cartilage after construct implantation provides additional environmental factors that influence the integration/maturation of the tissue-engineered implants. For example, freshly injured cartilage has been shown to secrete a number of growth factors, namely members of the TGF-β family[41], that are known for their pro-chondrogenic influence on chondrocytes and MSCs[20,27,42]. However, immature MSC-laden hydrogel constructs cultured in CM− after implantation exhibited only sporadic chondrogenic differentiation, as revealed by histological analysis, and their biosynthetic activity and the consequential mechanical properties and integration were comparable to chondrocytes after two population doublings, although significantly higher than that of chondrocytes that were further de-differentiated by additional population doubling.

The successful long-term restoration of isolated cartilage defects using matrix-based autologous chondrocyte implantation is dependent on the development of neo-cartilage with biochemical composition and mechanical properties comparable to those of native articular cartilage. Effective integration of the developing neo-tissue into the surrounding native cartilage is needed to optimize physiological load distribution within the joint and avoid

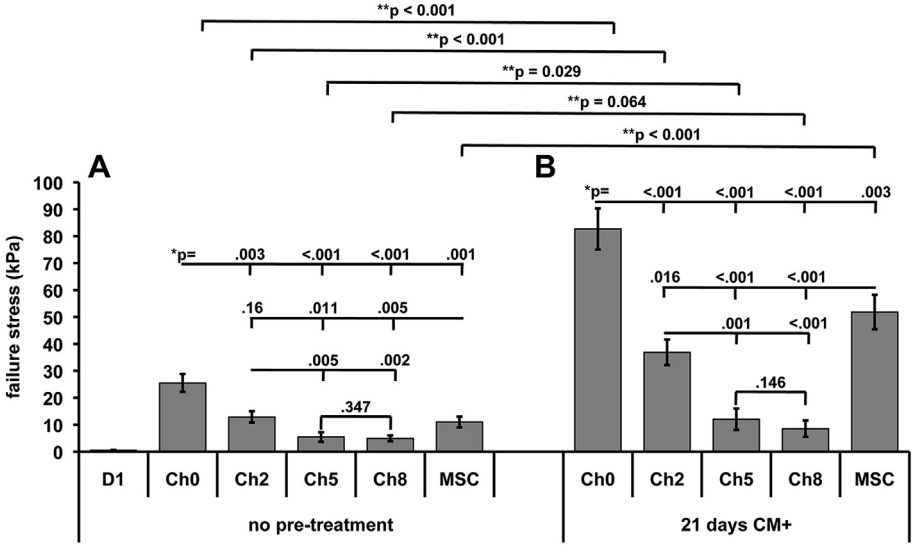

Fig. 4. Interface strength (kPa) developed between the cell-laden implant and the opposing native cartilage for (A) immature and (B) mature constructs 28 days after implantation. For chondrocytes, in addition to the negatively affected gross matrix synthesis and lower mechanical properties, interface strength was also compromised in a manner related to the number of population doublings prior to construct fabrication. (A) Mature cell-laden constructs of differentiated chondrocytes (Ch0), de-differentiated chondrocytes after two (Ch2) or five (Ch5) population doublings, and MSCs (MSC) exhibited significantly higher interface strengths compared to the corresponding (A) immature implant. *p = *displays P-value* vs different cell type; **p = displays P-value vs corresponding cell-type of the immature group. Experiments represent culture replicates originating from pooled cells. Values are mean with 95% confidence interval upper limit and lower limit ($n = 10–15$).

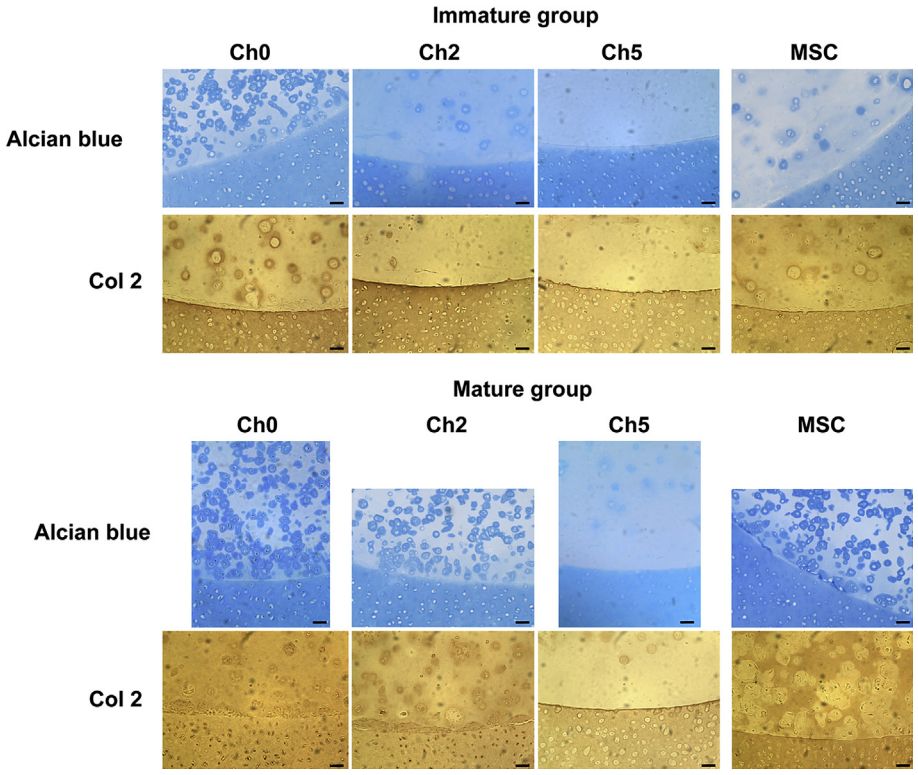

Fig. 5. Histological cartilage matrix deposition at the interface zone obtained 28 days after the implantation of immature and mature cell-laden agarose hydrogels. In each panel, cell-laden construct is on top and native cartilage is in the bottom. Alcian blue staining and collagen type II immunostaining both showed decreased cartilage matrix components with increased population doublings of chondrocytes for both the immature and the matured agarose disks. Comparable, positive collagen type II staining was seen in TGF-β3 pre-treated Ch2 and MSC implants, while alcian blue staining seemed slightly stronger in MSC-laden cartilage analogs. Bar = 50 μm.

further destruction of the cartilage[2,25,43,44]. *In vitro* maturation of tissue-engineered cartilage analogs prior to implantation has been reported to result in improved defect filling and a higher amount of hyaline cartilage in a canine cartilage defect model[31]. In addition, *in vitro* experimental data from studying the integration of opposing native cartilage pieces showed stronger fusion when cartilage pieces were of comparable developmental stage[45], with the integration processes dependent on cellular activity and collagen deposition[45–48].

Members of the TGF-β superfamily have been shown to induce chondrogenic differentiation in MSCs[6,20,26,27,42] and to promote a chondrogenic phenotype[26]. Our data showed that constructs of freshly isolated chondrocytes exhibited sustained ECM deposition after pre-culture in TGF-β3 containing medium. In comparison, matrix deposition by de-differentiated chondrocytes is significantly diminished, as a function of the number of prior population doublings. Remarkably, after pre-treatment with TGF-β3, the MSCs seeded in agarose hydrogel showed significantly higher matrix production with superior mechanical properties compared to those seeded with culture expanded chondrocytes. In addition, cell-laden constructs of Ch0, Ch2 and MSCs showed further significant increase of cartilage-specific ECM content and mechanical properties after implantation and maintenance as hybrid culture in CM−, accompanied by improved integration when compared to the corresponding immature implants culture under the same conditions.

These results expose the deficiency in chondrogenic cell response to TGF-β3-mediated cartilage maturation caused by increased population doubling of de-differentiated chondrocytes, resulting in subsequent limited cartilage repair *in vitro*. This is likely to be the underlying cause of the weak interface strength observed when there was disparity in developmental stage between the implant and the opposing native cartilage, with accompanying reduction in collagen deposition[23,24,34,45,47]. Nevertheless, it is noteworthy that differentiated chondrocytes outperformed all cell types tested here in terms of cartilage repair capacity, and should thus be the cell type of choice. However, other factors need to be considered as well, as matrix deposition and mechanical properties of the engineered tissue constructs depend not only on the cell type utilized, but also on cell viability, cell-to-volume ratio, culture conditions, and donor age and species[11,20,24,27,28,35,37]. To obtain a sufficient number of chondrocytes, monolayer expansion of chondrocytes is a routinely used approach in the treatment of cartilage defects utilizing autologous chondrocyte implantation. Our *in vitro* findings thus strongly suggest that the current limitations of autologous chondrocyte implantation and related approaches to cartilage repair is a consequence of impairment in cellular activity and responsiveness to anabolic factors as a result of culture expansion of the cells[49]. Recent publications have reported strategies to avoid or minimize the impact of the de-differentiation process on chondrocyte phenotype during culture expansion, by varying the initial seeding density[50], medium composition[51,52] and culture conditions[53–55].

Remarkably, pre-differentiated MSC-laden collagen hydrogels have been demonstrated to result in superior repair tissue compared to hydrogels seeded with either un-differentiated MSCs or chondrocytes in an ovine osteochondral defect model after 6 and 12 months[30,32]. In addition, initial clinical trials applying MSC-laden collagen type I-hydrogels into isolated cartilage defects in humans have shown promising results[56,57]. In contrast, hypertrophic differentiation and ectopic bone formation after *in vivo* implantation of MSCs have been reported in animal experiments[58,59]. However, we observed, without pre-treatment in CM+, only sporadic chondrogenic differentiation and inferior matrix accumulation in MSC-laden implants, resulting in weak integration between

the native cartilage and the construct. On the other hand, TGF-β3 pre-treatment of MSC-laden hydrogels resulted in an increased deposition of ECM components and cartilage integration after implantation, superior to de-differentiated chondrocytes. Interestingly, while significantly higher amount of collagen deposition in MSC-laden constructs was seen after implantation compared to those derived from de-differentiated chondrocytes (Ch5 and Ch8), their total collagen content as well as immunohistochemically detected collagen type II matrix were similar to those of Ch2 but not differentiated chondrocytes. These findings suggest incomplete chondrogenic differentiation and maturation of the MSCs in the engineered construct, and the need for further investigation to optimize the guided chondrogenic program of MSCs for improved cartilage-specific matrix deposition and integration[26].

Conclusion

Our findings clearly indicate that freshly isolated articular chondrocytes exhibit the highest quality of *in vitro* cartilage repair, such that mature implants derived from these cells show accelerated integration of the cell-laden implants into the opposing native articular cartilage. Culture-expanded, de-differentiated chondrocytes are compromised in their cartilage repair capacity with increasing number of population doublings. In addition, de-differentiated chondrocytes exhibit inferior cell response to chondrogenic anabolic factors, such as TGF-β3, accompanied by inferior mechanical properties and integration. In comparison, MSCs pre-treated with TGF-β3 CM+ conditions show favorable chondrogenic differentiation and superior accumulation of cartilage-specific ECM after implantation, supporting their applicability in matrix-based cartilage repair strategies.

In conclusion, our study demonstrate the utility of an *in vitro* assay system that has functionally predictive read-outs in terms of tissue integration for the development and optimization of tissue engineering approaches to cartilage repair.

Author's contribution

LR designed the experiment, performed experimental work, analyzed and prepared the data and drafted the manuscript; FD performed experimental work, the analysis of the data and revised the manuscript for intellectual content; SJ helped in the collection and analysis of the data; UR and RST participated in experimental design and data analysis, revised the manuscript draft, gave the final approval, and supervised the project. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

References

1. Buckwalter JA, Mankin HJ. Articular cartilage repair and transplantation. Arthritis Rheum 1998;41(8):1331–42.
2. Buckwalter JA, Mankin HJ. Articular cartilage: degeneration and osteoarthritis, repair, regeneration, and transplantation. Instr Course Lect 1998;47:487–504.
3. Noth U, Rackwitz L, Steinert AF, Tuan RS. Cell delivery therapeutics for musculoskeletal regeneration. Adv Drug Deliv Rev 2010;62(7–8):765–83.
4. Brittberg M. Cell carriers as the next generation of cell therapy for cartilage repair: a review of the matrix-induced autologous chondrocyte implantation procedure. Am J Sports Med 2010;38(6):1259–71.
5. Saris DB, Vanlauwe J, Victor J, Haspl M, Bohnsack M, Fortems Y, *et al*. Characterized chondrocyte implantation results in better structural repair when treating symptomatic cartilage defects of the knee in a randomized controlled trial versus microfracture. Am J Sports Med 2008;36(2):235–46.
6. Schneider U, Rackwitz L, Andereya S, Siebenlist S, Fensky F, Reichert J, *et al*. A prospective multicenter study on the outcome of type I collagen hydrogel-based autologous chondrocyte implantation (CaReS) for the repair of articular cartilage defects in the knee. Am J Sports Med 2011;39(12):2558–65.
7. Peterson L, Brittberg M, Kiviranta I, Akerlund EL, Lindahl A. Autologous chondrocyte transplantation. Biomechanics and long-term durability. Am J Sports Med 2002;30(1):2–12.
8. Shapiro F, Koide S, Glimcher MJ. Cell origin and differentiation in the repair of full-thickness defects of articular cartilage. J Bone Joint Surg Am 1993;75(4):532–53.
9. Grigolo B, Roseti L, De Franceschi L, Piacentini A, Cattini L, Manfredini M, *et al*. Molecular and immunohistological characterization of human cartilage two years following autologous cell transplantation. J Bone Joint Surg Am 2005;87(1):46–57.
10. Schnabel M, Marlovits S, Eckhoff G, Fichtel I, Gotzen L, Vecsei V, *et al*. Dedifferentiation-associated changes in morphology and gene expression in primary human articular chondrocytes in cell culture. Osteoarthritis Cartilage 2002;10(1):62–70.
11. Benz K, Breit S, Lukoschek M, Mau H, Richter W. Molecular analysis of expansion, differentiation, and growth factor treatment of human chondrocytes identifies differentiation markers and growth-related genes. Biochem Biophys Res Commun 2002;293(1):284–92.
12. De Bari C, Dell'Accio F, Tylzanowski P, Luyten FP. Multipotent mesenchymal stem cells from adult human synovial membrane. Arthritis Rheum 2001;44(8):1928–42.
13. Friedenstein AJ, Chailakhjan RK, Lalykina KS. The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. Cell Tissue Kinet 1970;3(4):393–403.
14. Noth U, Osyczka AM, Tuli R, Hickok NJ, Danielson KG, Tuan RS. Multilineage mesenchymal differentiation potential of human trabecular bone-derived cells. J Orthop Res 2002;20(5):1060–9.
15. Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, *et al*. Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng 2001;7(2):211–28.
16. Barry F, Boynton RE, Liu B, Murphy JM. Chondrogenic differentiation of mesenchymal stem cells from bone marrow: differentiation-dependent gene expression of matrix components. Exp Cell Res 2001;268(2):189–200.
17. Johnstone B, Hering TM, Caplan AI, Goldberg VM, Yoo JU. In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. Exp Cell Res 1998;238(1):265–72.
18. Kuo CK, Li WJ, Mauck RL, Tuan RS. Cartilage tissue engineering: its potential and uses. Curr Opin Rheumatol 2006;18(1):64–73.
19. Chen FH, Rousche KT, Tuan RS. Technology insight: adult stem cells in cartilage regeneration and tissue engineering. Nat Clin Pract Rheumatol 2006;2(7):373–82.
20. Noth U, Rackwitz L, Heymer A, Weber M, Baumann B, Steinert A, *et al*. Chondrogenic differentiation of human mesenchymal stem cells in collagen type I hydrogels. J Biomed Mater Res A 2007;83(3):626–35.

21. Wakitani S, Mitsuoka T, Nakamura N, Toritsuka Y, Nakamura Y, Horibe S. Autologous bone marrow stromal cell transplantation for repair of full-thickness articular cartilage defects in human patellae: two case reports. Cell Transplant 2004;13(5):595–600.
22. O'Sullivan J, D'Arcy S, Barry FP, Murphy JM, Coleman CM. Mesenchymal chondroprogenitor cell origin and therapeutic potential. Stem Cell Res Ther 2011;2(1):8.
23. Moretti M, Wendt D, Schaefer D, Jakob M, Hunziker EB, Heberer M, *et al*. Structural characterization and reliable biomechanical assessment of integrative cartilage repair. J Biomech 2005;38(9):1846–54.
24. Djouad F, Rackwitz L, Song Y, Janjanin S, Tuan RS. ERK1/2 activation induced by inflammatory cytokines compromises effective host tissue integration of engineered cartilage. Tissue Eng Part A 2009;15(10):2825–35.
25. Ahsan T, Sah RL. Biomechanics of integrative cartilage repair. Osteoarthritis Cartilage 1999;7(1):29–40.
26. Mauck RL, Yuan X, Tuan RS. Chondrogenic differentiation and functional maturation of bovine mesenchymal stem cells in long-term agarose culture. Osteoarthritis Cartilage 2006;14(2):179–89.
27. Byers BA, Mauck RL, Chiang IE, Tuan RS. Transient exposure to transforming growth factor beta 3 under serum-free conditions enhances the biomechanical and biochemical maturation of tissue-engineered cartilage. Tissue Eng Part A 2008;14(11):1821–34.
28. Lima EG, Bian L, Ng KW, Mauck RL, Byers BA, Tuan RS, *et al*. The beneficial effect of delayed compressive loading on tissue-engineered cartilage constructs cultured with TGF-beta3. Osteoarthritis Cartilage 2007;15(9):1025–33.
29. Capito RM, Spector M. Effect of expansion medium on ex vivo gene transfer and chondrogenesis in type II collagen-glycosaminoglycan scaffolds in vitro. Osteoarthritis Cartilage 2006;14(12):1203–13.
30. Marquass B, Schulz R, Hepp P, Zscharnack M, Aigner T, Schmidt S, *et al*. Matrix-associated implantation of predifferentiated mesenchymal stem cells versus articular chondrocytes: in vivo results of cartilage repair after 1 year. Am J Sports Med 2011;39(7):1401–12.
31. Lee CR, Grodzinsky AJ, Hsu HP, Spector M. Effects of a cultured autologous chondrocyte-seeded type II collagen scaffold on the healing of a chondral defect in a canine model. J Orthop Res 2003;21(2):272–81.
32. Zscharnack M, Hepp P, Richter R, Aigner T, Schulz R, Somerson J, *et al*. Repair of chronic osteochondral defects using predifferentiated mesenchymal stem cells in an ovine model. Am J Sports Med 2010;38(9):1857–69.
33. Obradovic B, Martin I, Padera RF, Treppo S, Freed LE, Vunjak-Novakovic G. Integration of engineered cartilage. J Orthop Res 2001;19(6):1089–97.
34. Hunter CJ, Levenston ME. Maturation and integration of tissue-engineered cartilages within an in vitro defect repair model. Tissue Eng 2004;10(5–6):736–46.
35. Mauck RL, Soltz MA, Wang CC, Wong DD, Chao PH, Valhmu WB, *et al*. Functional tissue engineering of articular cartilage through dynamic loading of chondrocyte-seeded agarose gels. J Biomech Eng 2000;122(3):252–60.
36. Dhert WJ, Verheyen CC, Braak LH, de Wijn JR, Klein CP, de Groot K, *et al*. A finite element analysis of the push-out test: influence of test conditions. J Biomed Mater Res 1992;26(1):119–30.
37. Maher SA, Mauck RL, Rackwitz L, Tuan RS. A nanofibrous cell-seeded hydrogel promotes integration in a cartilage gap model. J Tissue Eng Regen Med 2010;4(1):25–9.
38. Stegemann H, Stalder K. Determination of hydroxyproline. Clin Chim Acta 1967;18(2):267–73.
39. Vunjak-Novakovic G, Martin I, Obradovic B, Treppo S, Grodzinsky AJ, Langer R, *et al*. Bioreactor cultivation conditions modulate the composition and mechanical properties of tissue-engineered cartilage. J Orthop Res 1999;17(1):130–8.
40. Darling EM, Athanasiou KA. Rapid phenotypic changes in passaged articular chondrocyte subpopulations. J Orthop Res 2005;23(2):425–32.
41. Bos PK, van Osch GJ, Frenz DA, Verhaar JA, Verwoerd-Verhoef HL. Growth factor expression in cartilage wound healing: temporal and spatial immunolocalization in a rabbit auricular cartilage wound model. Osteoarthritis Cartilage 2001;9(4):382–9.
42. Li WJ, Tuli R, Okafor C, Derfoul A, Danielson KG, Hall DJ, *et al*. A three-dimensional nanofibrous scaffold for cartilage tissue engineering using human mesenchymal stem cells. Biomaterials 2005;26(6):599–609.
43. Harris JD, Siston RA, Brophy RH, Lattermann C, Carey JL, Flanigan DC. Failures, re-operations, and complications after autologous chondrocyte implantation–a systematic review. Osteoarthritis Cartilage 2011;19(7):779–91.
44. Niemeyer P, Pestka JM, Kreuz PC, Erggelet C, Schmal H, Suedkamp NP, *et al*. Characteristic complications after autologous chondrocyte implantation for cartilage defects of the knee joint. Am J Sports Med 2008;36(11):2091–9.
45. DiMicco MA, Waters SN, Akeson WH, Sah RL. Integrative articular cartilage repair: dependence on developmental stage and collagen metabolism. Osteoarthritis Cartilage 2002;10(3):218–25.
46. McGowan KB, Sah RL. Treatment of cartilage with beta-aminopropionitrile accelerates subsequent collagen maturation and modulates integrative repair. J Orthop Res 2005;23(3):594–601.
47. DiMicco MA, Sah RL. Integrative cartilage repair: adhesive strength is correlated with collagen deposition. J Orthop Res 2001;19(6):1105–12.
48. Giurea A, DiMicco MA, Akeson WH, Sah RL. Development-associated differences in integrative cartilage repair: roles of biosynthesis and matrix. J Orthop Res 2002;20(6):1274–81.
49. Heywood HK, Lee DA. Monolayer expansion induces an oxidative metabolism and ROS in chondrocytes. Biochem Biophys Res Commun 2008;373(2):224–9.
50. Watt FM. Effect of seeding density on stability of the differentiated phenotype of pig articular chondrocytes in culture. J Cell Sci 1988;89(Pt 3):373–8.
51. Stewart MC, Saunders KM, Burton-Wurster N, Macleod JN. Phenotypic stability of articular chondrocytes in vitro: the effects of culture models, bone morphogenetic protein 2, and serum supplementation. J Bone Miner Res 2000;15(1):166–74.
52. Yang KG, Saris DB, Geuze RE, Helm YJ, Rijen MH, Verbout AJ, *et al*. Impact of expansion and redifferentiation conditions on chondrogenic capacity of cultured chondrocytes. Tissue Eng 2006;12(9):2435–47.
53. Heywood HK, Lee DA. Low oxygen reduces the modulation to an oxidative phenotype in monolayer-expanded chondrocytes. J Cell Physiol 2010;222(1):248–53.
54. Takahashi T, Ogasawara T, Asawa Y, Mori Y, Uchinuma E, Takato T, *et al*. Three-dimensional microenvironments retain chondrocyte phenotypes during proliferation culture. Tissue Eng 2007;13(7):1583–92.
55. Nawata M, Wakitani S, Nakaya H, Tanigami A, Seki T, Nakamura Y, *et al*. Use of bone morphogenetic protein 2 and diffusion chambers to engineer cartilage tissue for the repair of

defects in articular cartilage. Arthritis Rheum 2005;52(1):155–63.
56. Wakitani S, Okabe T, Horibe S, Mitsuoka T, Saito M, Koyama T, *et al*. Safety of autologous bone marrow-derived mesenchymal stem cell transplantation for cartilage repair in 41 patients with 45 joints followed for up to 11 years and 5 months. J Tissue Eng Regen Med 2011;5(2):146–50.
57. Wakitani S, Nawata M, Tensho K, Okabe T, Machida H, Ohgushi H. Repair of articular cartilage defects in the patello-femoral joint with autologous bone marrow mesenchymal cell transplantation: three case reports involving nine defects in five knees. J Tissue Eng Regen Med 2007;1(1):74–9.
58. Kolf CM, Cho E, Tuan RS. Mesenchymal stromal cells. Biology of adult mesenchymal stem cells: regulation of niche, self-renewal and differentiation. Arthritis Res Ther 2007;9:204.
59. Scott MA, Levi B, Askarinam A, Nguyen A, Rackohn T, Ting K, *et al*. Brief review of models of ectopic bone formation. Stem Cells Dev 2012;21(5):655–67.