Journal of Bioscience and Bioengineering
VOL. xx No. xx, 1–11, 2016

www.elsevier.com/locate/jbiosc

Cell compaction influences the regenerative potential of passaged bovine articular chondrocytes in an *ex vivo* cartilage defect model

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Received 31 May 2016; accepted 5 November 2016
Available online xxx

The loss and degradation of articular cartilage tissue matrix play central roles in the process of osteoarthritis (OA). New models for evaluating cartilage repair/regeneration are thus of great value for transferring various culture systems into clinically relevant situations. The repair process can be better monitored in *ex vivo* systems than in *in vitro* cell cultures. I have therefore established an *ex vivo* defect model prepared from bovine femoral condyles for evaluating cartilage repair by the implantation of cells cultured in various ways, e.g., monolayer-cultured cells or suspension or pellet cultures of articular bovine chondrocytes representing different cell compactions with variable densities of chondrocytes. I report that the integrin subunit α10 was significantly upregulated in suspension-cultured bovine chondrocytes at passage P2 compared with monolayer-cultured cells at P1 ($p = 0.0083$) and P2 ($p < 0.05$). Suspension-cultured cells did not promote cartilage repair when compared with implanted monolayer-cultured chondrocytes and pellets: 24.0 ± 0.66% for suspension cells, 46.4 ± 2.9% for monolayer cells, and 127.64 ± 0.90% for pellets ($p < 0.0001$) of the original defect volume (percentage of defect). Additional cultivation with chondrogenesis-promoting growth factors TGF-β1 and BMP-2 revealed an enhancing effect on cartilage repair in all settings. The advantage and innovation of this system over *in vitro* differentiation (e.g., micromass, pellet) assays is the possibility of examining and evaluating cartilage regeneration in an environment in which implanted cells are embedded within native surrounding tissue at the defect site. Such *ex vivo* explants might serve as a better model system to mimic clinical situations.

[**Key words:** Chondrocytes; Cartilage repair; Growth factors; TGFbeta1; BMP2; *Ex vivo* defect model; Cell compaction; Passaging; Suspension culture; Pellets]

Damage and injuries to articular joints are frequent reasons for people seeking help from physicians and specialists. In USA, more than 6 million people each year are hospitalized because of articular joint pain/problems or injuries to such joints, e.g., knee, ankle, or hip joints (1). Osteoarthritis (OA), which includes the loss of the hyaline cartilage matrix with consecutive changes in the structure of the joint and the experience of pain, might arise post-traumatically following injuries or fractures, might be caused by age-dependent metabolic changes in the micro-milieu of the articular joint, or might have idiopathic reasons (2,3). Cartilage has a very low potential for self-healing because of its lack of blood vessels and nerve innervation. Many surgical and interventional techniques are available for the treatment of articular cartilage diseases. Some methods include interventional procedures such as lavage, arthroscopy, laser abrasion, or chondroplasty, but more invasive techniques such as chondrectomy or meniscectomy are also possible. Their positive pain-releasing effects only last for a short time and cause long-term cell apoptosis or necrosis and malalignment (4). Much more radical methods include joint distraction, arthrodesis, or osteotomy (4). The most commonly used strategies and techniques for the restoration of joint continuity are total endoprosthesis (TEP), autologous chondrocyte transplantation (ACT), or microfracturing/drilling in order to regenerate new cartilaginous tissue with chondrocytes or bone-marrow-derived human mesenchymal stem cells (hMSC) (4). Of course, our knowledge concerning cartilage regeneration is not complete, and many processes and cascades of signal transduction are not yet thoroughly understood. Chondrocytes, when cultured in monolayers to amplify cell numbers and as a source for implantation, dedifferentiate upon passaging and culturing *in vitro*. Cells lose their physiological *in vivo* morphology and their chondrogenic phenotype characteristic markers such as collagen II or integrin a10, while upregulating fibroblastic markers such as collagen 1 or integrin a11. Hence, attempts at delaying or blocking the dedifferentiating process are urgently needed in experimental cartilage repair and regeneration. Many cell culture settings support cartilage repair and regeneration, e.g., hypoxic cultures (5,6) or special cultures exerting an influence on cell compaction, such as suspension or pellet culture. Suspension culture is a setting in which cells do not adhere to the surface of plastic culture flasks but multiply while suspended in an agitated liquid medium. The formation of cell aggregates in suspension culture leads to the upregulation of the chondrogenic marker *Itga10* during adhesion processes (7,8) and may have further enhancing effects on cartilage repair in *ex vivo* cultures. Similar investigations and studies for promoting the chondrogenic phenotype have been reported with chondrocyte pellet culture being employed to encourage the

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1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2016.11.002

Please cite this article in press as: Schmutzer, M., Cell compaction influences the regenerative potential of passaged bovine articular chondrocytes in an *ex vivo* cartilage defect model, J. Biosci. Bioeng., (2016), http://dx.doi.org/10.1016/j.jbiosc.2016.11.002

deposition of cartilage-specific proteoglycans and extracellular matrix (ECM) (5,9,10). In the current study, I established an *ex vivo* cartilage defect model of bovine knee joints and evaluated the effects of various experimental (cell culture) settings on cartilage regeneration. This cartilage defect model was based on results from previous investigations of cartilage formation and regeneration (11,12). Osteochondral cylinders were drilled from femoral condyles, with each cylinder receiving 3 chondral lesions. Bovine chondrocytes, suspension aggregates, or pellets were then implanted into these chondral lesions to evaluate cartilage regeneration/repair and tissue formation. The role of passaging and cell compaction on neocartilage formation was investigated. The cylinders were incubated under normoxia (21% O_2/5% CO_2) or hypoxia (2% O_2/5% CO_2) with or without the administration of growth factors such as TGF-β1 or BMP-2 or their combination for 4 weeks with the medium being changed every second day. The newly formed tissue was evaluated by histological methods with safranin orange staining of sulfated proteoglycans. Additionally, suspension culture conditions were investigated by expression analyses for certain marker genes: *Col2a1*, *Col1a2*, *Itga10*, *Itga11*, *Agc*, and *Sox9*.

MATERIALS AND METHODS

Isolation and culture of bovine articular chondrocytes The bovine knee joint (from 10- to 12-month-old cattle) was dissected, and all ligaments, tendons, and muscles and the joint capsule were removed. The hyaline cartilage plaques were abraded from the femoral condyles with a scalpel and cut with scissors. The smaller plaques were washed twice with 15 ml sterile phosphate-buffered saline (PBS) (PAA Laboratories, Pasching, Austria) in a Petri dish. PBS and detritus were aspirated with a pipette. To digest the cartilage plaques, a collagenase solution was added consisting of DMEM/F-12 (Biochrom, Berlin, Germany) supplemented with 2 mg/ml collagenase type II (Worthington), 2% fetal bovine serum (FBS) (PAA Laboratories), 1% penicillin/streptomycin (PAA Laboratories). Prior to the addition of the solution to the cartilage plaques, it was heated to T = 37°C. Samples were incubated with this solution in a Petri dish at T = 37°C overnight (ON) under normoxic conditions (21% O_2). Digested plaques were mixed, aspirated, and collected in a 50 ml Falcon tube by filtration through a filter with a pore size of 70 μm (BD Falcon, Franklin Lakes, NJ, USA). Subsequently, cells were centrifuged at 500 ×*g* twice for 5 min at T = 21°C. Harvested chondrocytes at passage P0 were used for further studies including monolayer, suspension, and pellet cultures.

Monolayer culture setting Passage P1 chondrocytes were grown as monolayer cultures in various types of culture dish from Nunc (Roskilde, Denmark) and BD Biosciences (San Jose, CA, USA). The culture medium was DMEM/F-12 (Biochrom) supplemented with 10% FBS (PAA Laboratories) and 1% penicillin/streptomycin (PAA Laboratories). Chondrocytes were cultured in a humidified cell culture incubator (Jouan, St. Herblain, France) under constant normoxic conditions at T = 37°C and 5% CO_2 or under hypoxic conditions (Multi-gas incubator MCO-5M, SANYO, Japan). Chondrocytes were seeded at a density of 10,000 cells/cm^2. The medium was changed every 3 days, and chondrocytes were grown to 80–90% confluency.

Suspension culture setting For chondrocyte suspension culture, approximately 7.0–9.0 × 10^6 chondrocytes of passage P1 were placed together with DMEM/F-12 (Biochrom) supplemented with 10% FBS and 1% penicillin/streptomycin (PAA Laboratories) into a bacteria dish (d = 10 cm) covered with a thin layer of a mixture of warmed 2% agarose gel in 0.9% NaCl. Chondrocytes were cultivated ON in a humidified cell culture incubator (Jouan) under constant normoxic conditions at T = 37°C and 5% CO_2. For gene marker analysis, RNA was extracted for polymerase chain reaction (PCR) before (P1 bovine chondrocytes) and after suspension culture. As a comparison, one sample of the cells underwent passaging (P2 bovine chondrocytes), and RNA was extracted once again.

Pellet culture setting For chondrocyte pellet culture, bovine chondrocytes from passage P1 were used. One pellet consisted of 600,000 chondrocytes in polypropylene V-bottom 96-well plates (Corning, New York, NY, USA) and then centrifuged at 500 ×*g* for 5 min at T = 21°C. The culture medium was DMEM/high glucose (PAA Laboratories) supplemented with 1% penicillin/streptomycin (PAA), 10 μM dexamethasone (Sigma–Aldrich, St. Louis, MO, USA), 1 mM sodium pyruvate (Sigma–Aldrich), 0.195 mM L-ascorbate (Sigma–Aldrich), and ITS (insulin, transferrin, selenite) (Sigma–Aldrich). Chondrocyte pellets were cultivated for 28 days in a humidified cell culture incubator (Jouan) under constant normoxic conditions at T = 37°C and 5% CO_2. The medium was changed every 3 days.

Total RNA isolation and semi-quantitative RT-PCR analysis RNA was isolated with the Qiagen RNeasy Mini Kit (Qiagen, Germantown, MD, USA). Bovine chondrocytes were washed with PBS and disrupted by the addition of 1% β-mercaptoethanol (β-ME) (Sigma–Aldrich) and RLT buffer. Then, the lysates (700 μl) were placed on QIAshredder spin columns for filtration and mixed with 70% ethanol at a ratio of 1:1. Afterwards, samples were transferred to RNeasy spin columns and centrifuged for 15 s at 8000 ×*g*. Next, 350 μl of buffer RW1 was added to the RNeasy spin columns and centrifuged for 15 s at 8000 ×*g* to wash the spin column membrane. For the digestion of genomic DNA, a mixture of 10 μl 10 U DNase I and 70 μl buffer RDD was added and incubated for 15 min at T = 20–30°C to avoid contamination. Samples were washed and dried twice by the addition of buffer RPE and centrifugation for 15 s at 8000 ×*g*. RNA was eluted in RNase-free water and measured spectro-photometrically in order to analyze its concentration and purity. For purity measurements, the absorptions of RNA samples were determined at λ_1 = 260 nm (A_{260}) and λ_2 = 280 nm (A_{280}). The ratio of $A_{260}/A_{280} \geq 1.8$ was considered to represent a clear sample.

Copy DNA (cDNA) synthesis was performed with a Transcriptor First Strand cDNA Synthesis Kit (Roche). Aliquots containing 1 μg total RNA, 60 μM Random Hexamer Primers, and PCR-grade water were heated for 10 min at T = 65°C for the denaturation of RNA. Afterwards, the denatured RNA was added to a mixture of 8 mM Transcriptor Reverse Transcriptase Buffer, 20 U Protector RNase Inhibitor, 10 mM dNTPs, and 10 U Transcriptor Reverse Transcriptase. This mixture was incubated for 10 min at T = 25°C, followed by 60 min at T = 50°C. To inactivate Transcriptor Reverse Transcriptase, the sample was heated to T = 85°C for 5 min. The synthesized cDNA was analyzed for the expression of the housekeeping gene GAPDH to evaluate the quality of the samples and to normalize them. For semi-quantitative RT-PCR analysis, primers for the various cartilage-specific genes were used as follows: *Col2a1*, *Col1a2*, *Itga10*, *Itga11*, *Agc*, and *Sox9*. The housekeeping gene was *GAPDH*. Depending on the expression level of GAPDH, a specific amount of cDNA was added to the PCR master mix, which consisted of 10× PCR Buffer, 25 mM $MgCl_2$, 10 mM each dNTP, forward (F′) and reverse (R′) gene-relevant primers, PCR-grade water, and 5 U/μl FastStart Taq DNA polymerase (Roche). The PCR tubes containing the mixture of a single PCR were placed on a Peltier Thermal Cycler DNA Engine (BioRad, Hercules, CA, USA). The PCRs consisted of 28–32 cycles depending on the gene. The amplified PCR products were separated on 2% agarose gels containing Tris–acetate–EDTA buffer and visualized and analyzed by 0.7% ethidium bromide following exposure to UV light. A 100-bp molecular-weight standard DNA marker (Invitrogen) was used as a reference for the correct size of the PCR product. Images of the products were obtained at various exposure times by using a gel imaging system (Vilber Lourmat, Germany). To quantify the results of the PCR, the expression of each gene was described relative to GAPDH.

Preparation of osteochondral defects for explant culture The preparation procedure is illustrated in Fig. 1, which shows the various steps of the dissection of the osteochondral explant cylinder. Following the cleaning of the condyles and the former femoral part of the joint-bearing area (Fig. 1A), osteochondral cylinders were drilled out of the condyles by using a trephine drill head (Hager & Meisinger GmbH) with a diameter of 8.0 mm and a Proxxon MICROMOT 50/E drill with a suitable supply unit (Fig. 1B). The number of rotations for this operation was between 7000 and 10,000 rpm. During the drilling, the trephine drill head and the working area on the femoral condyles had to be cooled continuously with sterile PBS (PAA Laboratories) supplied by a syringe. The osteochondral cylinder was released from the bone with a raspatory (Fig. 1C and D), which was inserted between the cylinder and the intact femoral condyle by means of a hammer and moved until it became loose (fracturing, Fig. 1E). The osteochondral cylinders underwent further preparation under microscopical observation (Typ 325400, Wild Heerbrugg, Switzerland). The cartilaginous part of the explant cylinders (thickness: 2.0 mm) was prepared with 3 chondral lesions by a sterile dermal biopsy punch (Fig. 1F) having a diameter of 1.5 mm and a depth of 1.0 mm (Kai Medical, Tokyo, Japan). The blanked cartilage tissue was removed with a scalpel, leaving 3 small holes on the cartilaginous side of the explants. The bony part of the osteochondral defects was almost completely removed by a circular diamond-saw driller head (Fig. 1G) with a diameter of 38.0 mm (Proxxon GmbH). This drill head could also be used in combination with the Proxxon MICROMOT 50/E drill and the suitable supply unit. About 2.0 mm of the bony part of the osteochondral cylinder was left to ensure the better stability of the remaining explant plaque. The final step of preparation involved the cleaning of the osteochondral explant with sterile PBS (Fig. 1H). The structure of this cartilage explant model is demonstrated schematically in Fig. 1I.

Explant culture settings To evaluate the effect of the various cells on cartilage regeneration and repair, monolayer cells of passage P2 were seeded into the lesions and cultivated under normoxia and hypoxia. For each chondral lesion, 200,000 cells in a volume of 1 μl were used for implantation. Chondrocyte pellets were also cultivated overnight prior to implantation into the chondral lesions. One pellet was implanted per chondral lesion. Pellets of P2 bovine chondrocytes were cultivated under normoxic and hypoxic conditions. Bovine chondrocytes at passage P2 were implanted into chondral lesions of explant plaques after cultivation in the suspension culture overnight. The exact number of cells used per chondral lesion could not be determined, because the aggregations of chondrocytes in suspension culture had different sizes. The chondral lesions were filled with chondrocyte aggregates and cultivated under normoxic and hypoxic conditions.

Chondrogenic medium for culture settings Osteochondral explant plaques were cultivated in chondrogenic growth medium (CGM), which consisted of

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