



RESEARCH ARTICLE

A system for engineering an osteochondral construct in the shape of an articular surface: Preliminary results

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Summary

A tissue-engineered articular condyle could provide a new alternative approach to joint replacement. This study describes progress made towards engineering an articular condyle *in vitro* using human bone marrow stromal cells (hBMSCs) in a biphasic matrix.

hBMSCs were transferred to a rat collagen-I hydrogel which was then pressed onto a bovine cancellous bone matrix. The gel/cell suspensions, each at a density of approximately 5×10^5 cells/ml containing fourth passage cells pressed into an adult human tibial condyle form using CT scan based moulds. The osteochondral constructs fabricated *in vitro* were stimulated in a bioreactor using cyclic compression and continuous perfusion.

Penetration and cell distribution were demonstrated as homogeneous and cells were found to be viable after gel compression. The filamentous structure of the collagen fibres was more dense and homogeneous using compression. Mechanical tests showed a significant enhancement of primary matrix stability after initial compression. Stiffness was not observed to increase significantly over 7 days under loading in a bioreactor.

The successful integration of mechanical stimulation in the tissue engineering process leads to an improvement in the structural and biomechanical properties of

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these tissues and offers new possibilities in the management of joint injuries and degenerative diseases. Remarkably, the stiffness was enhanced in our setting after initial compression of the construct in the glass cylinder without observing a negative influence on cell viability. Further studies need to clarify the influence of compression and various mechanical and hydrostatic stress patterns over different periods of time.

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Introduction

Osteochondral defects of weight bearing joints represent an elementary problem in orthopaedic surgery. Much research has been carried out in the field of bone and cartilage tissue engineering in recent years (Angele et al., 1999; Bancroft et al., 2002; Kaspar et al., 2000; Vunjak-Novakovic et al., 2005). Next to ongoing applications of cell and tissue-based therapies, such as microfracturing, osteochondral, and chondrocyte transplantation with or without matrices for the treatment of articular cartilage defects, prosthetic joint replacement continues to be the predominant practice (Evans et al., 2006). All current implants and grafting procedures share certain deficiencies, such as reduced biocompatibility, donor site limitation and morbidity, dislocation, wear, and potential pathogen transmission. An entire articular condyle created by methods of regenerative medicine should overcome most of the deficiencies associated with the current prosthetic and grafting procedures. In tissue engineering of osteochondral constructs, biomaterials composed of biological or synthetic matrices and bone marrow stromal cells (BMSC) are promising approaches. Autologous BMSC can be obtained in daily clinical practice using bone marrow aspiration and *in vitro* expansion (Caplan, 1991). The advantages of these adult, autologous stem cells are a standardized harvesting process combined with a new technique to manufacture the construct which could be certified following successful testing for application in clinical practice (e.g. GMP label). Homogeneous cell distribution and sufficient initial scaffold stability remain key issues in a successful strategy. Mechanical stimulation and perfusion play a significant role in tissue repair of cartilage and bone as well in the differentiation of BMSCs (Bancroft et al., 2002; Kaspar et al., 2000; Mauney et al., 2004). The application of mechanical stress has been proven to effectively enhance chondrogenic commitment when using progenitor cells (Angele et al., 1999). Scaffolds have been seeded with cells either statically or dynamically in stirred flasks, using hydrogel as a cell delivery vehicle or by perfusion of

medium. Bioreactors used for the cultivation are of multiple technical origins to try to simulate the superior bioreactor of the human being *in vitro* (Vunjak-Novakovic et al., 2005). The aim is to achieve a fast and tight contact between the carrier and the bone or the cartilage following transplantation. There have been numerous and varied attempts to repair osteochondral injuries. Biology, gene therapy, and tissue engineering may provide a breakthrough in their treatment (Sharma and Elisseeff, 2004).

The aim of this study has been to investigate the *in vitro* effects of the application of initial compression forces during the first 24h of cell culture followed by various stress patterns in a bioreactor for 7d on proliferation, differentiation, and biomechanical properties of BMSC in an osteochondral matrix.

Materials and methods

Isolation and *in vitro* cultivation of hBMSCs

hBMSCs were harvested from the iliac crest during routine trauma surgery, e.g. dorsal instrumentation and ventral fusion following vertebral fractures. The seven donors were otherwise healthy and their age ranged from 24 to 53 years. All procedures were approved by the institutional ethical committee, and informed consent was obtained from all donors. Cell isolation and cultivation were performed according to a previously published modified protocol (Jagodzinski et al., 2004). Briefly, density centrifugation was used to obtain a cell pellet that was resuspended in culture medium (DMEM/Ham's F12 1:1, Biochrom, Berlin, Germany) supplemented with 10% fetal calf serum, 200 U/ml penicillin/streptomycin (Gibco, Karlsruhe, Germany), 2.5 µg/ml amphotericin B (Biochrom), 2.5 µg/ml ascorbic acid (Loges, Winsen, Germany), supplemented with FGF-2 (3 ng/ml, Pepro Tech, Offenbach, Germany) buffered with Hepes buffer (Roth, Karlsruhe, Germany; pH 7.0), and subsequently plated in 75 cm² culture flasks

(Nunc, Berlin, Germany) and incubated at 37 °C and 5% CO₂ in humidified atmosphere. The medium was changed twice a week. After reaching confluence on days 14–21, the cells were released with 0.25% trypsin (Gibco), counted and subcultured in 75 cm² cell culture flasks. Cells of the fourth passage were used for the experiments (Figure 1; systematic workflow).

Fabrication of osteochondral constructs

CT scans of patients with a non-injured lateral tibial plateau were used to construct a three-dimensional (3D) image of the surface in the bovine spongiosa. CT scans of tibial condyles in DICOM format were used as the basic data source. The scanner was a CT Light Speed 16 (GE Medical, Solingen, Germany). Files were imported in AMIRA 3.0 (Mercury Computer Systems Inc., Chelmsford, MA, USA). The surface could be cut manually or using the SurfaceGen modul. The data were stored in the STL format, read out using CAD/CAM Software Alpha-CAM Ver. 5 (Licom Ltd., Coventry, UK) and transferred to a NC-Code to steer over a Heidenhain module TNC355, a three-axis CNC milling cutter FP3A (Deckel, Munich, Germany).

Then, 1×10^7 cells as described above were mixed with GNL medium (Arthrocinetics® AG, Esslingen, Germany) containing 20% FCS; the medium was mixed at a ratio of 1:1 with a liquid collagen I suspension (Arthrocinetics) at a temperature of 5 °C; the cell–collagen mixture was transferred to a xenogeneic acellularized cancellous bone cylinder (Tutobone®, Tutogen Medical GmbH, Neunkirchen a. Br., Germany). The condyle was used as the construct stock on which the gel was pressed using a negative form of the joint surface. The porosity and mineral density of Tutobone were guaranteed to be homogeneous by the manufacturer within a close range. Correspond-

ing samples from the same specimens were used for this study. In order to achieve cyclic compression, the cancellous bone matrix was partially demineralized (Decal®, Decal Corporation, Tallman, NY, USA). After a decalcification period of 40 min, the samples could be compressed $10 \pm 0.5\%$ when a load of 3.18 kPa (0.318 N/cm²) was applied. The matrix was rinsed in deionized distilled water until a pH of 7.4 was restored. A similar system was established to produce chips with a diameter of 22 mm and height of 11 mm.

The collagen gel was mixed prior to transfer into a glass cylinder compression apparatus on top of the partially demineralized bone matrix. After 60 min at 37 °C in a humidified chamber, glass cylinders were placed on top of the gel for 24 h (0.104 N/cm²) and a vacuum force of 50 mmHg (0.667 N/cm²) was applied from below the constructs. After 24 h, constructs were transferred either to the mechanobioreactor (Figure 2) or to the static cell culture system. During the initial compression period, the constructs were compressed approximately 10-fold and stable constructs were produced for transfer into the mechanoreactor (Figure 3).

Mechanoreactor

Of particular relevance for this study was the development of a mechanoreactor that combines the advantages of a perfusion system (Jasmund and Bader, 2002) with the capability of applying longitudinal mechanical strain.

Inside the mechanobioreactor, shown in Figure 2, the constructs could be perfused separately from both sides and simultaneously exposed to cyclic mechanical strain using a piston for compression. The load was constantly monitored by a load cell behind the constructs. Thus, the load-dependent cyclic compression strain of 10% at 0.5 Hz could be modulated using LabView. The perfusion rate

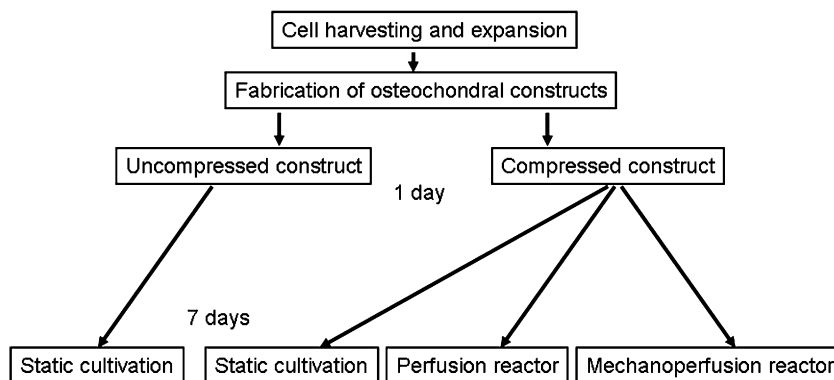


Figure 1. Experimental design and work flow: each level demonstrates a different point in time.

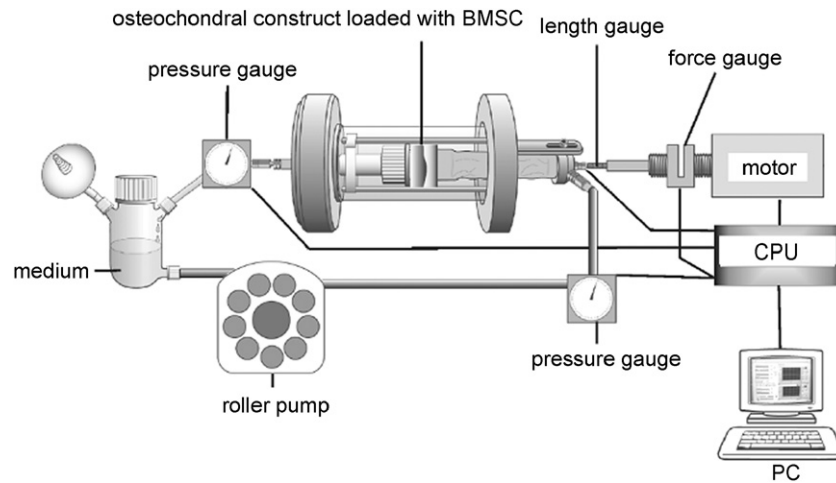


Figure 2. Mechanoperfusion bioreactor: the reactor contains a chamber which hosts the construct (human bone marrow stromal cells (hBMSCs) in a biphasic matrix). A pump steers the medium flow and an electric engine drives a gauge to provide cyclic mechanical loads. Every force is monitored and steered by a control panel on a personal computer (CPU).

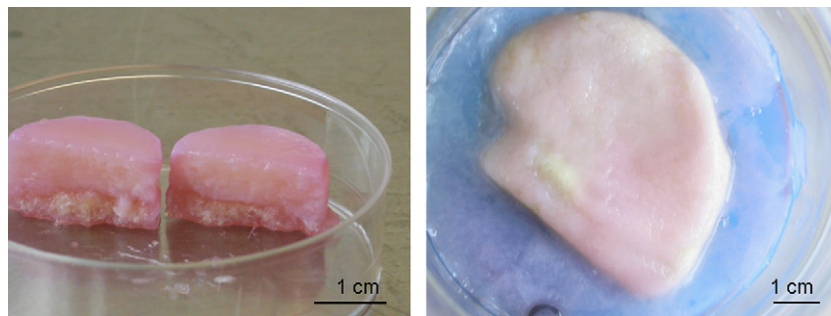


Figure 3. Compressed construct prior to transfer to the bioreactor system or static control. Left: Osteochondral cylinders. The lower phase of the split construct represents the cancellous Tutobone chip on which the cell containing collagen I-gel (upper phase) was pressed in a specially developed glass kit. Right: Three-dimensional joint surface from above, produced using the methods described.

of 12 ml/min resulted in low pressure differences; hence, quasi-hydrostatic conditions prevailed inside the bioreactor. The pressure was monitored during the entire procedure. Resected osteochondral fragments from young otherwise healthy patients with articular knee fractures as well as perfused, but initially compressed constructs, and uncompressed constructs served as controls.

All bioreactors were kept sterile and put in an incubator during the experiments. Cell culture medium (50 cc: 25% of the entire amount of medium) was changed twice a week. Culture media were the same as described above and FGF was replaced by 1.3^{-7} M dexamethasone (Mauney et al., 2004). After 7 d, the tissue samples were harvested and apportioned for histological, immunohistochemical, and biomechanical analysis.

Histological and immunohistochemical phenotyping

Results were evaluated using light microscopy after standard staining (HE, toluidine blue) to identify matrix penetration as well as collagen 2 (Acris, polyclonal – Biotin, R1038B), 10 (Sigma, Monoclonal Anti-Collagen Type X clone Col-10, C7974), and aggrecan (Acris Antibodies, Monoclonal Antibody to Human Aggrecan, clone HAG5G5) expression. All procedures were performed according to the manufacturer's guidelines after serum blocking and 1:100 dilutions. A live/dead assay (Molecular Probes, Leiden, the Netherlands) was performed according to the manufacturer's guidelines. The read out was supported by sigma scan (Systat Software Inc., San Jose, CA, USA). The mean intensity of cells and a background of one

image were used for standardization of colour intensity. A threshold was used to differentiate a positive reaction. The number of cells that stained red or green within three fields of view was recorded as a percentage.

GAG and DNA quantification

GAG (Blyscan Glycosaminoglycan, Biocolor, Newtonabbey, UK) and DNA (DNA Quantification Kit, Sigma-Aldrich, Taufkirchen, Germany) were quantified using standard kits according to the manufacturer's instructions for photometry. Controls were performed at every single step of the experiments to validate the methods (data not shown). Mean values of three read outs were quantified on 2 different days.

Biomechanics assessment

Special emphasis was put on changes in the biomechanical properties of the constructs. Preliminary biomechanical characterization was conducted using a confined compression quasi-static loading setup at 0.1 mm/s during which a complete push-out was performed. The size (length/diameter) of the harvested cylinders (nominal diameter 6 mm) was measured using a contact-less laser micrometer. The construct was placed in a cylindrical chamber with a diameter 6 mm. A press-fit technique using a special drilling chisel (OATS; Arthrex; Naples, FL, USA) was used to prepare cylindrical specimens from the raw construct taken from the bioreactor. This allowed an accurate and reproducible alignment of the construct with both the chamber plunger of 5.5 mm diameter, and a 4.5 mm hole centred below the support chamber. A computer activated micro-stepper motor controlled the displacement of the plunger (0.5 mm/min), while a load cell attached to the plunger was used to measure the outwardly directed (push-out) force. For each specimen, load measurements were recorded until the construct was fully displaced from the tube. Load take-up (toe-in region) and stiffness at 20% strain of the constructs were calculated from the maximum force measured during the failure process for the matrix constructs. Strain was computed based on the length of the entire construct under a nominal load of 0.1 N.

Statistics

Statistical significance was analysed using the unpaired *t*-test and Wilcoxon test. All results are shown as mean and standard deviation. Samples

were run at least in duplicate on 2 different days. Statistical analysis was performed using SPSS (Version 11.0, SPSS Inc., Chicago, IL, USA). The level of significance was predetermined at a *p*-value of 0.05.

Results

Histology

The macroscopic appearance of the osteochondral construct is shown in Figure 3. The lower phase of the biphasic construct represents the cancellous Tutobone chip on which the cell containing collagen I-gel (upper phase) was pressed in a specially developed glass kit. All sections have been cut vertically through the construct.

The response to the different strain protocols was studied using light and immunofluorescence microscopy. The variations in cell response were demonstrated by specific immunostainings. Surprisingly, it was not possible to demonstrate any expression of collagen II, X or aggrecan by immunostaining over the 7 days of investigation (sections not shown). The filamentous structure of the collagen fibres was denser and more homogeneous under the compression glass cylinder apparatus on day 1 (Figure 4). After 1 week of stimulation, it was possible to demonstrate a similar structure when comparing the compressed versus uncompressed construct with no significant differences regarding the stress protocol (Figure 5). On days 1 and 7, a similar finding was shown for all investigated groups (Figure 6). Only the compressive stimulus enhanced

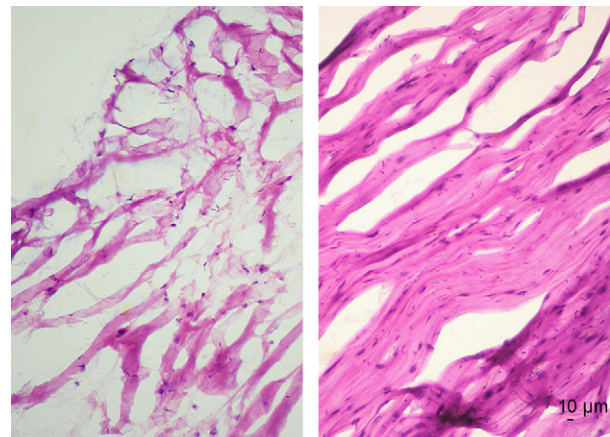


Figure 4. HE staining of the uncompressed construct (left side) and the compressed construct (right) demonstrating the denser filamentous structure of the collagen I scaffold on day 1 after application of pressure in the glass kit for 24 h.

the density of the structure of the collagen net in the hydrogel.

Penetration and cell distribution were demonstrated as homogeneous and cells were found to be viable after gel compression. Cell viability was examined by a live/dead assay combining ethidium

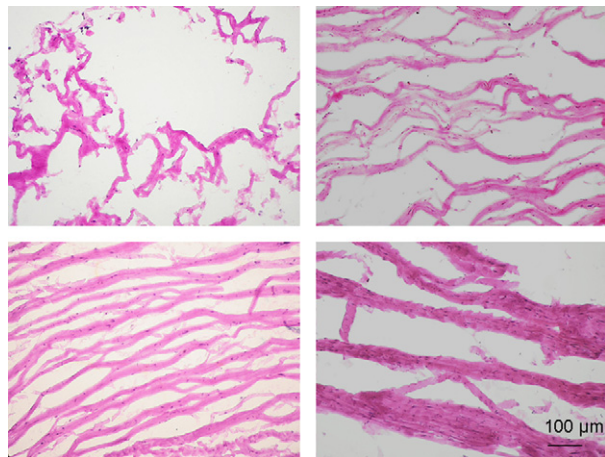


Figure 5. HE staining of the uncompressed construct (left upper side) and the compressed construct (right upper side) on day 7 and below the construct after 7 days perfusion (left) and mechanical stimulation (right).

bromide with acridine orange immunofluorescence staining. This live/dead assay showed $92.9 \pm 3.7\%$ vital cells on day 1 and $84.4 \pm 14.9\%$ on day 7 for the uncompressed control compared to compressed and stimulated constructs with $85.9 \pm 5.7\%$ on day 1 and $86.4 \pm 18.6\%$ on day 7. In regard to cell viability, there were no significant differences between compressed and uncompressed constructs.

GAG and DNA quantification

GAG quantification showed no significant differences within the groups (Table 1). DNA quantification showed a significant difference on day 1 in compressed versus uncompressed and on day 7 in uncompressed versus compressed stimulated probes. Cell density was higher in the compressed probes.

Biomechanical testing

Transmission of the loads during stimulation of the scaffolds with a predetermined length change was continuously monitored. Mechanical tests showed an enhancement of primary matrix stability

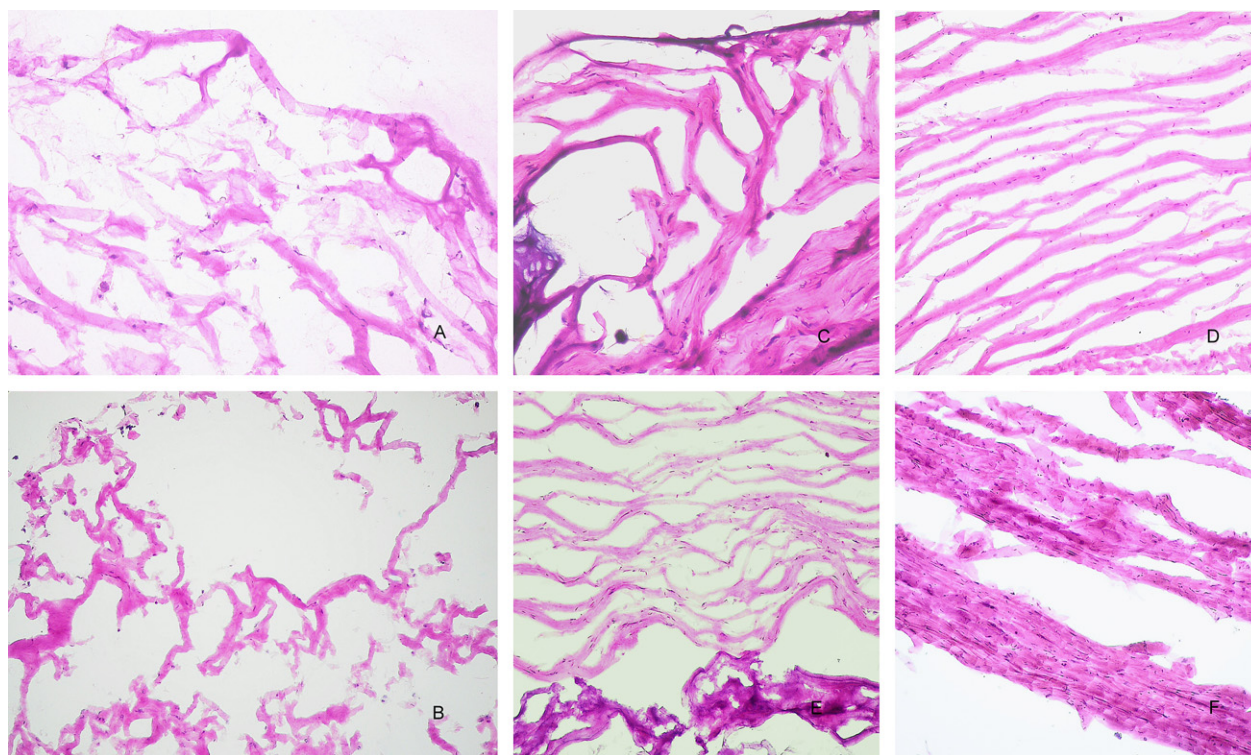


Figure 6. HE staining of the compressed and uncompressed gel matrix on day 1 (upper row) and day 7 (lower row): (A) uncompressed gel matrix day 1; (B) uncompressed gel matrix day 7; (C) compressed gel matrix day 1; (D) compressed gel matrix day 7 (static); (E) compressed gel matrix day 7 (hydrodynamic); (F) compressed gel matrix day 7 (mechanical). In the upper part of section C a lamella-like surface of the gel was detected and in the lower part of section E the cancellous bovine bone chip is shown.

Table 1. GAG and DNA quantification in ng/mg probe as mean values including standard deviation

	Day 1	Day 7
DNA ng/mg probe		
Static	209 ± 44	173 ± 21
Perfusion		204 ± 33
Mechanical		214 ± 5
GAG ng/mg probe		
Static	366 ± 118	311 ± 57
Perfusion		442 ± 53
Mechanical		406 ± 53

There were no statistically significant differences for either assays when comparing the results.

Table 2. Mechanical loading at 20% compression as mean values and standard deviation ($n = 5$ per group)

mN	Day 1	Day 7
Uncompressed	33.9 ± 8.6	20.4 ± 3.9
Static	191 ± 17.9	59 ± 5.6
Perfusion		112 ± 42
Mechanical		414 ± 209

Significant differences were observed between the uncompressed group to all other groups on the same day ($p < 0.05$).

after initial compression (Table 2). Stiffness was observed to increase over 7 days under mechanical loading in the bioreactor. Interestingly, stiffness was enhanced after initial compression of the construct on day 1, to be reduced by day 7. On day 1, compressed constructs were stiffer than uncompressed constructs and all compressed constructs were stiffer than uncompressed on day 7. Comparison of perfusion and mechanical stimulation on day 7 showed an increase in load-resistance of the mechanically stimulated constructs.

Discussion

The aim of this project has been to investigate the response and cell distribution of hBMSCs seeded on a 3D biologic hybrid scaffold using hydrostatic compression and vacuum forces. The combination of cells, scaffolds, and bioactive factors represents a promising approach. Pluripotent cells from bone marrow could be isolated and cultured *ex vivo* and their histogenic differentiation could be induced by external factors (Sharma and Elisseeff, 2004). The selection of cell source was based on the fact that the advantages of hBMSCs in regenerative medicine

have often been outlined (Pittenger et al., 1999). Secondly, our matrix components were selected for practical reasons. We wanted to construct a hybrid matrix to mimic an osteochondral tissue and use clinically established products. With a view to a later clinical application, the matrix consisted of the commercially available products for treatment in humans: CaReS[®] (rat collagen I, Ars Arthro, Esslingen, Germany) and Tutobone[®] (bovine spongiosa, Tutogen Medical GmbH, Neunkirchen a. Br., Germany). Thirdly, the integration of mechanical stimulation in the tissue engineering process could lead to progress in the structural and biomechanical properties, and the successful transfer to and stimulation in our bioreactor system was demonstrated.

Designing osteochondral tissue needs a successful strategy. The natural contours of the articulating surface, the mechanical properties and the functional load-bearing ability have to be achieved to the highest standard possible. Last but not least integration in the host tissue must be viable. The use of mechanical stimulation in the tissue engineering process could lead to progress in the structural and biomechanical properties of these tissues and offers new possibilities. The effects of cyclic longitudinal stretching have been studied by Kaspar et al. (2000) in human osteoblasts. Their analysis indicated that a strain rate of 1% and a frequency of 1 Hz resulted in increased proliferation. Experiments investigating the influence of cyclic strain on 3D cell cultures (BMSC in a collagen gel (Altman et al., 2002)) showed that a strain rate of 10% and 1 Hz significantly fostered cell alignment and density and up regulated collagen I and III expression. In this setup, there was no up regulation of bone or cartilage specific differentiation markers. Aggregates of BMSC exposed to cyclic hydrodynamic pressure for 7 days reacted with a significant increase in collagen and proteoglycan content (Angele et al., 2003). Continuous perfusion has been proven to stimulate cell proliferation and differentiation of BMSC in a titanium mesh (Bancroft et al., 2002). The experiments indicated that 1 and 3 ml/min were more effective than 0.3 ml/min.

We used a fixed mechanical stimulation of 0.5 Hz and 10% strain. The long-term results are currently being investigated. Despite a significant increase of matrix stability after initial compression, it was not possible to demonstrate significant differences for the mechanical stimulation or perfusion. However, the results indicate that this could be expected for mechanical stimulation over time and with a larger series. A greater density (1 million cells/ml) and in addition a longer stimulation period

(up to 4 weeks) would probably lead to advanced tissue maturation.

The morphological appearance of the cells in the tissue-engineered osteochondral constructs was more fibroblast-like. Expression of cartilaginous markers such as collagen II or X was not demonstrated. As multiple factors influence the tissue generation, recombinant proteins, e.g. TGF and FGF were omitted from media during our experiments. Cell culture conditions for hBMSCs investigated earlier had already clarified the best proliferation rate using FGF which was added during the proliferation (data not shown). Within the extracellular matrix, the GAG contents were not significantly different. We expect a change in the chemical analysis over time. The cells were demonstrated to be vital and the DNA content did not change significantly over 1 week. Regarding cell activity, it has been demonstrated that DNA content was higher in the stimulated probes than in the static control. One major shortcoming of this study was the heterogeneity of hBMSC. These cells belong to a lineage hierarchy in which only some of the cells are multipotential stem cells or primitive progenitors whereas others are more restricted (Aubin and Triffitt, 2002). The findings of this study must be interpreted with care due to the small experimental series, the heterogeneity of the individual BMSC cultures, and the short period of observation. However, adherent BMSC growth in cell culture was shown to fulfil criteria to be considered pluripotential mesenchymal stem cells (Pittenger et al., 1999; Suva et al., 2004). These cells displayed a stable phenotype and remained as a monolayer *in vitro*. We focused on different stimulation processes to investigate a new method for clinical access. Therefore, control experiments with a different cell type were omitted, but controls without cells used (data not shown). The implant needs to copy the natural contours of the articulating surface, show adequate mechanical properties and must have early functional load-bearing abilities. During the last few years, several new implant materials have been investigated in order to combine bone and cartilage defects (Cao et al., 2003; Gao et al., 2002; Weinzweig et al., 2000). Typically the cartilage region is seeded with cells, either chondrocytes or BMSCs, while the bone region remains acellular or is seeded with osteogenic cells such as osteoblasts, periosteal cells, BMSCs, or bone marrow. The minimum requirement for a scaffold is to provide a temporary structure while cells seeded within synthesize a natural environment parallel to artificial matrix degradation. Different scaffold strategies for osteochondral defects such as replacement

without a cartilage layer, composite scaffolds, a heterogeneous or bilayered scaffold or a homogeneous, single-layered scaffold have been discussed in the literature (Martin et al., 2006). In most cases, biphasic scaffold materials were introduced in which two single materials (e.g. calcium phosphates for the bony and gels for the cartilage part) were manufactured separately and then bonded together, e.g. using fibrin glue.

The most challenging task appears to be functional tissue engineering of an articular condyle which is able to withstand the mechanical loads experienced by normal articular condyles. Various cell seeded constructs are commercially available to reconstruct cartilage defects (MACI[®], CaReS[®], AMIC[®], Carticel[®] II, etc.). The application is limited, however, to isolated defects of one side of the joint. Some areas of the joints are not accessible for these methods. Affection of the subchondral bone is a further limitation. Clinical results have been somewhat disappointing in terms of life activity scores (Knutsen et al., 2004). The application of mechanical stress has been proven to effectively enhance chondrogenic commitment (Angele et al., 1999). Until the present, there has been no clinical application of tissue engineering techniques using progenitor cells in the field of osteochondral transplants.

The integration of mechanical stimulation in the tissue engineering process may lead to progress in the structural and biomechanical properties of these tissues and offers new possibilities in the management of bone injuries and degenerative diseases. This treatment approach attempts to overcome the surgical difficulties and to determine whether these types of implants can be used to repair larger defects. Further studies over a longer period currently being performed need to clarify the influence of compression, and different mechanical and hydrostatic stress patterns over various periods of time prior to testing in an animal model.

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